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# GENETIC AND PHENOTYPIC DIVERGENCE BETWEEN LOW- AND HIGH-ALTITUDE POPULATIONS OF TWO RECENTLY DIVERGED CINNAMON TEAL SUBSPECIES

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**Spatial variation in the environment can lead to divergent selection between populations occupying different parts of a species' range, and ultimately lead to population divergence. The colonization of new areas can thus facilitate divergence in beneficial traits, yet with little differentiation at neutral genetic markers. We investigated genetic and phenotypic patterns of divergence between low- and high-altitude populations of cinnamon teal inhabiting normoxic and hypoxic regions in the Andes and adjacent lowlands of South America. Cinnamon teal showed strong divergence in body size (PC1; $P_{ST} = 0.56$) and exhibited significant frequency differences in a single nonsynonymous α-hemoglobin amino acid polymorphism (Asn/Ser-α9; $F_{ST} = 0.60$) between environmental extremes, despite considerable admixture of mtDNA and intron loci ($F_{ST} = 0.004$–$0.168$). Inferences of strong population segregation were further supported by the observation of few mismatched individuals in either environmental extreme. Coalescent analyses indicated that the highlands were most likely colonized from lowland regions but following divergence, gene flow has been asymmetric from the highlands into the lowlands. Multiple selection pressures associated with high-altitude habitats, including cold and hypoxia, have likely shaped morphological and genetic divergence within South American cinnamon teal populations.**

**KEY WORDS:** **Andes, gene flow, hemoglobin, hypoxia, morphology, waterfowl.**

Populations distributed across ecologically different environments often comprise locally adapted species. As populations colonize new environments, divergent selection can modify traits that enable individuals to exploit differences in habitat or to gain advantages in resource competition (Mayr 1963; West-Eberhard 1983; Endler 1986; Schluter 2001). In heterogeneous landscapes, selection can also restrict gene flow because beneficial traits in one environment may have reduced fitness in another environment (Nosil et al. 2008; Milá et al. 2009). However, selection is not uniform across the genome, and neutral alleles not linked to loci under selection can move between environments (McKay and Latta 2002; Emelianov et al. 2004; Garant et al. 2007; Via 2009). Similarly, adaptive differentiation can occur even in the face of gene flow if the strength of selection is greater than the force of migration (Slatkin 1987; McKay and Latta 2002; McCracken et al. 2009a). Recent colonization of new environments can thus facilitate divergence in advantageous traits with rapid changes at key loci while the rest of the genome remains undifferentiated (Wu 2001; Via 2009).

Under parapatric models of divergence, biological differences that are genetically based and result from environmental differences can present barriers to gene flow and promote the evolution of subsequent isolating barriers (e.g., Cadena et al. 2012). For example, individual preferences for specific environments






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may reduce the likelihood of encounters between individuals from different populations, facilitating prezygotic isolation when mating occurs in or near preferred habitat (Rundle and Nosil 2005; Hendry et al. 2007). Furthermore, limited gene flow between populations adapted to spatially segregated environments may promote population divergence, and lead to genome-wide differentiation through genetic drift (Wright 1931). If gene flow is sharply restricted (e.g., $4Nm < 2$) and isolating mechanisms are reinforced by selection, speciation may occur (Servedio and Noor 2003). Alternatively, gene flow may persist in selection-migration equilibrium, leading to population differentiation but inhibiting or preventing complete speciation.

A comparative approach contrasting functional genes and phenotypic traits with independent neutral markers can help determine how populations respond to contrasting environmental selection in heterogeneous landscapes (e.g., Ribeiro et al. 2011). This approach may be especially effective in the early stages of population divergence, when neutral markers reflect a combination of unsorted ancestral polymorphisms (stochastic lineage sorting) and recent gene flow (Maddison 1997; Via 2009). The premise is that selection has locus-specific effects, whereas demographic processes (nonadaptive processes) have genomic effects (Lewontin and Krakauer 1973; Beaumont 2005).

High-altitude regions provide an excellent opportunity to investigate the molecular and morphological bases of local adaptation. The low temperatures, increased desiccation, higher atmospheric radiation, and especially hypoxia (oxygen ($O_2$) partial pressure is 40% lower at 4000 m than at sea level) that prevail at high altitudes can be debilitating for lowland individuals (Tucker 1968; Scott et al. 2009). The selective forces imposed by high-altitude habitats are thus relatively well understood, and numerous genes under selection have been identified (Jessen et al. 1991; Cheviron and Brumfield 2009; Storz 2010; Storz et al. 2010; Yi et al. 2010; Peng et al. 2011; Scott et al. 2011). Hemoglobin (Hb) in particular exhibits an important evolutionary response to hypoxia (e.g., Jessen et al. 1991; Weber et al. 1993; León-Velarde et al. 1996; Weber et al. 2002; Storz et al. 2010), and often only one or a few key amino acid changes are found in the Hb protein of high-altitude species relative to their low-altitude counterparts (Heibl et al. 1987; Braunitzer and Hiebl 1988; McCracken et al. 2009b). Likewise, body size is often correlated with hemoglobin $O_2$ affinity, with larger animals tending to have higher $O_2$ affinity (Schmidt-Nielson and Larimer 1958; Hopkins and Powell 2001), which is likely a thermoregulatory adaptation to the colder climates at high altitude. Functional changes in hemoglobin structure and increases in body size thus provide two important mechanisms for coping with high-altitude conditions.

The cinnamon teal (*Anas cyanoptera*; family Anatidae) is an excellent species for studying the molecular and morphological basis of adaptation to high altitude. Two subspecies of cinnamon teal inhabit the Andes of southern South America. A small-bodied subspecies *A. c. cyanoptera* is widespread in lowland habitats (<1000 m) from the Pacific coast of Peru to southern Argentina, whereas a larger bodied subspecies *A. c. orinomus* occupies elevations of 3500–4600 m in the central Andes (Wilson et al. 2010). The two subspecies exhibit significant frequency differences in a single amino acid change in the major hemoglobin protein. In high-altitude habitats, the $\alpha^A$-globin gene of *A. c. orinomus* is nearly fixed for Ser-α9, whereas the lowland population of *A. c. cyanoptera* is nearly fixed for Asn-α9, which is the ancestral state in waterfowl (McCracken et al. 2009b,c).

Using a series of vouchered specimens collected from low- and high-altitude habitats in South America, we compared patterns of divergence in the $\alpha^A$ and $\beta^A$ hemoglobin genes and phenotypic characteristics to patterns of divergence in five nuclear introns and the mitochondrial DNA (mtDNA) control region. Specifically, we aim to assess which evolutionary mechanism, genetic drift following recent isolation or divergent selection, better explains the observed patterns in genetic and morphologic variation between highland and lowland populations. Observing that functional traits have diverged greater than neutral loci would suggest that those traits have diversified more than expected by genetic drift alone, supporting a role of selection. In addition, we assessed how adaptations to high-altitude influence population genetic structure and gene flow between environments.

## Material and Methods

### SPECIMEN COLLECTION AND DNA EXTRACTION

We collected 52 *A. c. cyanoptera* and 50 *A. c. orinomus* from low- and high-altitude regions of Argentina, Bolivia, and Peru between 2001 and 2005 (Fig. 1). We used published subspecific morphological characters to classify each specimen to subspecies (Snyder and Lumsden 1951; Blake 1977; Wilson et al. 2010). Six small-bodied individuals were collected in highland localities (>2100 meters) at both the northern and southern limits of the *orinomus* distribution. Based on morphological characters, these individuals were assigned to the lowland *cyanoptera* population and therefore treated as part of the lowland population (Wilson et al. 2010). Including these individuals in the *cyanoptera* sample did not affect the results when comparing analyses with and without these specimens. In addition, preliminary analysis showed no significant structure among lowland localities in Argentina and the west slope of the Andes in Peru (mtDNA: $F_{ST} = 0.03$, $P = 0.07$; introns: $F_{ST} = 0.00$); therefore all lowland individuals were treated as a single population. Vouchered specimens and frozen tissues are archived at the University of Alaska Museum (Fairbanks, Alaska), Museo de Historia Natural de la Universidad de San Marcos (Lima, Peru), and Colección Boliviana de Fauna

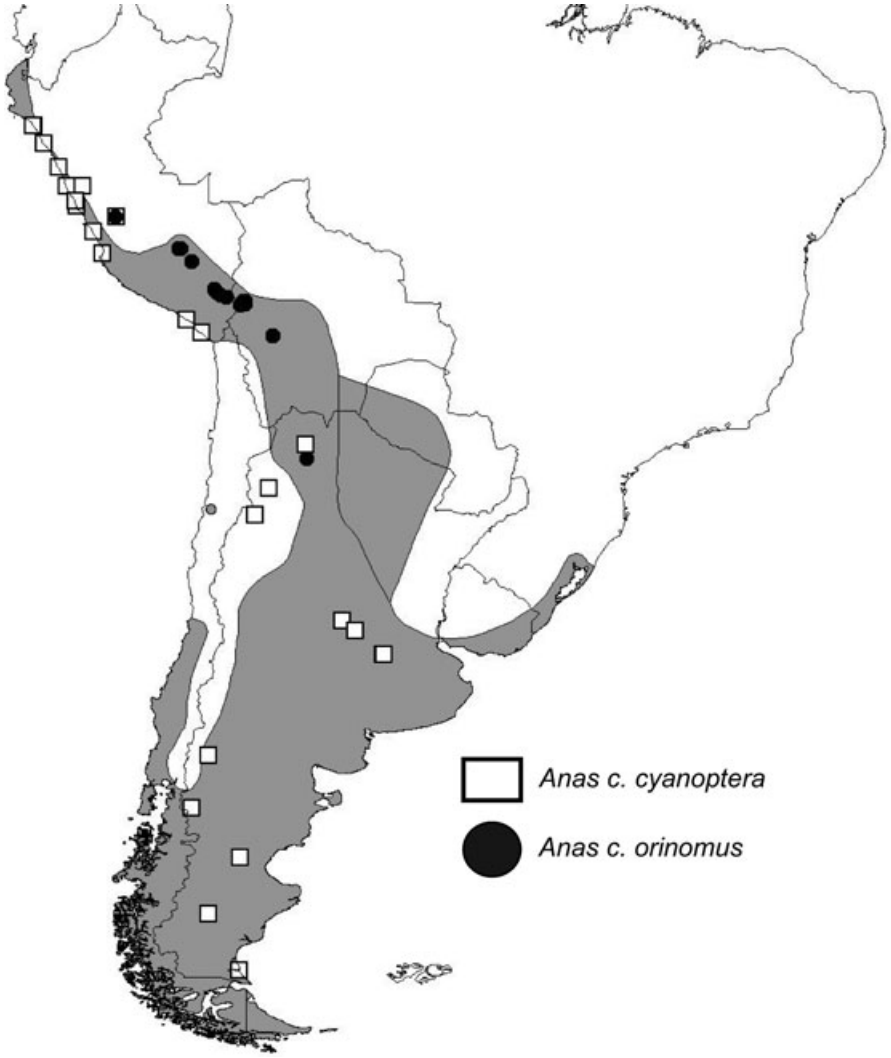


**Figure 1.** Sampling localities and geographic range (shaded gray) for cinnamon teal (*Anas cyanoptera*) in this study (Ridgely et al. 2003).

(La Paz, Bolivia). Genomic DNA was extracted from muscle tissue using standard protocols and a QIAGEN DNeasy Tissue Kit (QIAGEN, Valencia, CA).

## DNA SEQUENCING

We sequenced the two hemoglobin genes (α*A*; 677 bp and β*A*; 1582 bp) that comprise the major hemoglobin isoform (HbA) of adult birds. Five introns that map to different locations in the chicken genome were also sequenced to be compared to hemoglobin genes (Table 1): ornithine decarboxylase intron 5 (ODC1; 351 bp), α-enolase intron 8 (ENO1; 312 bp), beta fibrinogen intron 7 (FGB; 245 bp), N-methyl D aspartate receptor type I intron 11 (GRIN1; 330 bp), and phosphoenolpyruvate carboxykinase intron 9 (PCK1; 345 bp). Polymerase chain reaction (PCR), sequencing protocols and primers are described by McCracken et al. (2009a). We also sequenced 1272 bp of the mtDNA control region and adjacent phenylalanine tRNA and 12S rRNA gene using the overlapping primer pairs L78−H774 and L736−H1530 (Sorenson and Fleischer 1996; Sorenson et al. 1999), and two additional primers (L627: 5′–TAAGCCTGGACACACCTGCGTTATCG–3′; H693: 5′–CAGTGTCAAGGTGATTCCC–3′) designed specifically for cinnamon teal.

Sequences from opposite strands were reconciled using Sequencher 4.1.2 (Gene Codes Corporation, Ann Arbor, MI). Sequences that contained double peaks, indicating the presence of two alleles, were coded with IUPAC degeneracy codes and treated as polymorphisms. Indels were resolved by comparing the unambiguous 5′-ends of sequences to the 3′-ambiguous ends between forward and reverse strands (Peters et al. 2007). Gaps resulting in shifted peaks in the chromatograms, thus, enabled us to resolve length polymorphisms within sequences. All sequences were aligned by eye using the sequence alignment editor SeAl 2.0a11 (Rambaut 2007). Sequences and voucher information

**Table 1.** Genes sequenced and their chromosomal positions in the chicken genome.

| Locus | Base pairs sequenced | Chicken chromosome[1] |
|---|---|---|
| mtDNA control region (mtDNA) | 1270–1272 | mtDNA |
| Ornithine decarboxylase intron 5 (ODC1) | 351 | 3 |
| α enolase intron 8 (ENO1) | 312 | 21 |
| β fibrinogen intron 7 (FGB), partial | 245 | 4 |
| N-methyl D aspartate 1 glutamate receptor intron 11 (GRIN1), partial | 330 | 17 |
| Phosphoenolpyruvate carboxykinase intron 9 (PCK1), partial | 345 | 20 |
| α*A* hemoglobin subunit (HBA1) | 678 | 14 |
| β*A* hemoglobin subunit (HBB) | 1578–1582 | 1 |

[1]Location in the chicken genome as defined by Hillier et al. (2004).

including georeferenced localities are deposited in GenBank (accession numbers GQ269364–GQ269772, GQ271146–GQ271246, GQ271884–GQ271985, JF914653–JF919754).

## GAMETIC PHASE OF NUCLEAR ALLELE SEQUENCES

The allelic phase of each sequence that was heterozygous at two or more nucleotide positions was determined using allele-specific priming and the software PHASE 2.1 (Stephens et al. 2001). PHASE uses a Bayesian method to infer haplotypes from diploid genotypic data with recombination and the decay of linkage disequilibrium (LD) with distance. Each dataset was analyzed using the default values (100 main iterations, 1 thinning interval, 100 burn-in) followed by 1000 main iterations and 1000 burn-in ($-\times 10$ option) for the final iteration. The PHASE algorithm was run five times automatically ($-\times 5$ option) from different starting points, selecting the result with the best overall goodness of fit. We next selected individuals with allele pair probabilities <80% and designed allele-specific primers to preferentially amplify one allele (Bottema et al. 1993). The resulting haploid allele sequence was then subtracted from the diploid consensus sequence to obtain the gametic phase of the second haplotype. Each dataset was then analyzed five more times using PHASE and the additional known allele sequences (-k option). The gametic phases of 97.1% ($n = 692$) of the 713 individual sequences that we analyzed were identified experimentally or with >95% posterior probability, and 98.0% ($n = 699$) were identified with >90% posterior probability.

## ESTIMATION OF GENETIC DIVERSITY

Nucleotide diversity ($\pi$), expected and observed heterozygosities, and LD between nuclear loci were calculated in ARLEQUIN 3.11 (Excoffier et al. 2005). Allelic networks were constructed in NETWORK 4.5.1 (Fluxus Technology Ltd. 2004) using the reduced median algorithm (Bandelt et al. 1995), to illustrate possible reticulations in the gene trees due to homoplasy or recombination. Gaps were treated as a fifth character state, and indels were treated as a single insertion/deletion event.

To test for departures from neutrality in scenarios characterized by an excess of rare alleles, we calculated Fu's $F_s$ (Fu 1997) and Tajima's $D$ (Tajima 1989). Significantly negative values for these test statistics may indicate a population evolving under nonrandom processes such as directional selection or demographic expansion.

## ESTIMATION OF POPULATION SUBDIVISION

To assess levels of population subdivision between highland (*orinomus*) and lowland (*cyanoptera*) populations, we calculated pairwise $\Phi_{ST}$ and $F_{ST}$ in ARLEQUIN using the best-fit nucleotide substitution model, as identified in MODELTEST 3.06 (Posada and Crandall 1998) under the Akaike Information Criterion (AIC; Akaike 1974). *P*-values were adjusted for multiple comparisons using permutations (3000) or Bonferroni corrections ($\alpha = 0.05$).

We used STRUCTURE 2.2 (Pritchard et al. 2000) to identify and assign individuals to populations. STRUCTURE uses a Bayesian method to assign individuals to populations by maximizing Hardy–Weinberg equilibrium and minimizing LD. Data were analyzed using an admixture model and assuming correlated frequencies with a burn-in of 100,000 iterations and 1,000,000 Markov chain Monte Carlo iterations. Four analyses were performed, one including only the five introns and three additional analyses with five introns and mtDNA plus either the α*A* subunit or β*A* subunit. No prior population information was used, and analyses were performed for one and two population models ($K = 1$ or 2) to compute the probability of assignment to the lowland or highland population and to identify individuals with admixed genotypes. We performed 10 independent runs for each $K$ to ensure consistency across runs.

## ESTIMATION OF GENE FLOW AND TIMING OF DIVERGENCE

We estimated gene flow between highland and lowland populations using two methodologies; IM (Hey and Nielsen 2004; Hey 2005) and BayesAss 1.3 (Wilson and Rannala 2003). IM uses the isolation-with-migration coalescent model, which treats divergence $t$ ($T\mu$) and population splitting ($s$) as independently estimated parameters in addition to the effective population size parameter ($\theta = 4N_e\mu$) and gene flow ($M = m/\mu$; Hey and Nielsen 2004; Hey 2005). BayesAss uses an assignment

methodology, which does not incorporate genealogy (Wilson and Rannala 2003). Estimates of the gene flow rate can thus be interpreted at different temporal scales with IM estimating gene flow since population divergence, whereas BayesAss reflects gene flow that occurred in the past several generations.

For the IM analyses, we simultaneously estimated the following parameters scaled to the mutation rate per locus: effective population sizes ($\theta$), immigration rates ($M$), and time since population divergence ($t$). We also estimated the splitting parameter ($s$) to test for the direction of colonization (Hey 2005; Peters et al. 2008). IM assumes that the loci are free from intralocus recombination. We tested for recombination within each locus using the four-gamete test in DNAsp version 4.10 (Rozas et al. 2003) and included the largest independently segregating block of sequence consistent with no recombination. Only ENO1 and GRIN1 showed evidence of recombination and were truncated to the 5′ end positions 15–312 and 60–206, respectively. For the hemoglobin genes, the longest fragment with no recombination that included all nonsynonymous amino acid replacements was 1–338 for the $\alpha A$ subunit and 118–587 for the $\beta A$ subunit. The remaining loci had no detectable recombination; therefore the full sequences were used in the analysis. Additionally, we verified the four-gamete tests with an independent estimate of the overall recombination rate ($r$) for each locus using LAMARC 2.1.6 (Kuhner 2006) with the lower and upper limits for $r$ set to 0 and 10, respectively.

We defined inheritance scalars in IM for mtDNA as 0.25 (maternally inherited) and for hemoglobins and introns as 1.0 (biparentally inherited) to reflect differences in effective population sizes. We used the HKY model of mutation for mtDNA and infinite sites model for the nuclear loci. We initially ran IM using large, flat priors for each parameter. Based on the results of these runs, we defined narrower upper bounds for each parameter that encompassed the full posterior distributions from each initial run. Using those priors, we then used a burn-in of 500,000 steps and recorded results every 50 steps for more than 2 million steps. Effective sample sizes for each parameter exceeded 100. We repeated the analyses three times using a different random number seed to verify that independent runs converged on the same values.

To convert IM estimates to biologically informative values, we estimated the mutation rate ($\mu$ per locus) using a mutation rate of $4.8 \times 10^{-8}$ substitutions/site/year (s/s/y) for the mtDNA control region (range: $3.1 \times 10^{-8}$–$6.9 \times 10^{-8}$ s/s/y; Peters et al. 2005) and calibrated mutation rates for introns on the goose-duck split following methods outlined by Peters et al. (2007, 2008). Using the geometric mean of substitution rates averaged for mtDNA and introns ($7.57 \times 10^{-7}$ s/locus/y), we converted $t$ to years before present ($T$) using $t = T\mu$.

For the BayesAss analysis genotypic allelic data were grouped as follows: (1) five introns, (2) five introns and $\beta A$ subunit, and (3) five introns, $\beta A$ subunit, and $\alpha A$ subunit. BayesAss was initially run with the default delta values for allelic frequency ($P$), migration rate ($m$), and inbreeding ($F$). Subsequent runs incorporated modified delta values so that proposed changes between chains at the end of the run were between 40% and 60% of the total chain length to maximize log likelihood values and ensure the most accurate estimates (Wilson and Rannala 2003). Final delta values used were $\Delta P = 0.06$, $\Delta m = 0.03$, and $\Delta F = 0.09$. We performed five independent runs (50 million iterations, 5 million burn-in, and sampling frequency of 2000) with different random seeds to ensure convergence across runs.

## SIMULATED NEUTRAL GENETIC DIVERSITY

Using the parameters inferred from the isolation-with-migration coalescent model, we simulated genetic data under a model of selective neutrality in the program ms (Hudson 2002). To account for uncertainty in population history, we randomly selected 1000 "histories" visited during the IM run (see Peters et al. 2012). We included locus-specific recombination rates ($\rho$), calculated as $\rho = 4N_e r$ or $\theta r$, where $r$ is the recombination rate estimated in LAMARC (see above); we randomly selected 1000 values of $r$ from the posterior distributions. We also included estimates of relative mutation rates estimated from the five introns and mtDNA as described above. For each simulated dataset, we adjusted $\theta$ by calculating $\theta\mu_R$, where $\mu_R$ is the mutation rate for locus $i$ divided by the average rate among all loci included in IM (Peters et al. 2012). We simulated a total of 1000 datasets for each locus, each with slightly different parameter values, and we calculated the expected distribution of $\Phi_{ST}$ between *cyanoptera* and *orinomus* based on the inferred population history and selective neutrality in the program ms.output (Peters et al. 2012). We then compared values of $\Phi_{ST}$ between the empirical and simulated data. We also compared measures of morphometric divergence ($P_{ST}$) to these simulated values to determine if a neutral trait could generate the observed differences.

## ESTIMATION OF MORPHOMETRIC DIVERGENCE

Phenotypic differentiation was assessed for seven previously published body size measurements taken from a recent analysis of cinnamon teal morphology (Wilson et al. 2010): wing chord length (carpal joint to longest primary feather unflattened, $\pm 1$ mm), tail length (base of the uropygial gland on back to tip of the center tail feather, $\pm 1$ mm), exposed culmen length, bill length at nares (anterior edge of nares to tip of nail, $\pm 0.1$ mm), tarsus bone length (tarsometatarsus, $\pm 0.1$ mm), bill height (height of upper mandible at anterior edge of nares, $\pm 0.1$ mm), and bill width (width of upper mandible at anterior edge of nares, $\pm 0.1$ mm). Measurements were taken from 48 individuals from *cyanoptera* (14 females, 34 males) and 47 *orinomus* (15 females, 32 males).

Due to low female sample size and equality of variances between the sexes confirmed by a Bartlett's equal variance test, female values were adjusted to male equivalents by adding the mean difference between the sexes following Merilä (1997). A principal components analysis was performed on $\log_{10}$-transformed data excluding body mass for which normality was confirmed using a correlation matrix to extract an overall body size index. A Pearson correlation analysis was used to test if the first principal component represents an accurate representation of overall size as PC1 scores and body mass should be positively correlated (Rising and Somers 1989). In addition, a discriminant analysis was performed to estimate the probability of assignment to highland or lowland subspecies using seven noncorrelated measurements. All statistical analyses were performed with MINITAB Statistical Software (Minitab, State College, PA).

To assess the degree of differentiation in phenotypic traits, we partitioned the morphological variation between subspecies by calculating a phenotypic-$Q_{ST}$ ($P_{ST}$; Sæther et al. 2007; Whitlock 2008). Phenotypic-$Q_{ST}$ can be interpreted as an $F_{ST}$ analogue for quantitative traits provided that within- and between-population variance in trait values is exclusively attributable to additive genetic effects (Wright 1951; Rogers and Harpending 1992). Otherwise, $P_{ST}$ estimates may be biased if within- and between-population components of environmental variance are not proportional (Merilä and Crnokrak 2001). Comparisons of $P_{ST}$ and $F_{ST}$ are typically interpreted as follows: $P_{ST} > F_{ST}$, the trait(s) that $P_{ST}$ was calculated from has diversified more than expected based on genetic drift alone; $P_{ST} < F_{ST}$, the trait(s) are under stabilizing selection that maintained the same value across the heterogeneous landscape in spite of genetic drift; and $P_{ST} = F_{ST}$, there is insufficient evidence to suggest that selection is acting differentially or uniformly across the landscape and genetic drift cannot be ruled out as a driving force in diversification (Whitlock 1999; Merilä and Crnokrak 2001; McKay and Latta 2002).

$P_{ST}$ variance measures and sensitivity analysis were calculated as described in Storz (2002) and Sæther et al. (2007). Phenotypic variation was partitioned into within- and between-group components using a variance component model (Model II ANOVA) on PC1 and PC2 scores and individual measurements from which $P_{ST}$ was calculated using the equation in Storz (2002). As the data are solely phenotypic, assumptions about the heritability of the traits were made as outlined in Storz (2002) and Merilä (1997), where morphological traits were assumed to have a narrow-sense heritability ($h^2$) of 0.5 based on heritabilities of quantitative traits in avian and mammalian taxa (e.g., Larsson 1993; Merilä and Gustafsson 1993). In addition, morphological trait differences (e.g., wing chord) have been shown to be genetically controlled along an elevational gradient in passerines (*Junco hyemalis*; Rasner et al. 2004; Bears et al. 2008). Moreover, some species of high Andean waterfowl (e.g., *Anas puna* and *Lophonetta specularioides*) bred in captivity in the lowlands have similar morphologies to wild individuals that differ from their lowland counterparts (R. E. Wilson, pers. obs.), suggesting a strong genetic component. A sensitivity analysis was performed simulating different values of $h^2$ (0.25–1.0) and the assumed additive genetic proportion of differences between populations ($g$; 0.1–1.0) as outlined in Sæther et al. (2007) to determine if conclusions are sensitive to varying parameters.

Finally, it is important to note that comparisons of $P_{ST}$ and $F_{ST}$ provide an initial starting point to identify potential characters that may be under selection as reciprocal transplant experiments, physiological studies, and correlation of morphology with environmental conditions are needed to confirm findings of these types of comparisons (Whitlock 2008). However, the selection pressures imposed by high altitude are well defined, and two main environmental factors (temperature and hypoxia) are known to facilitate morphological variation (Bergmann 1847; James 1970; Hopkins and Powell 2001). Therefore, trait(s) showing elevated phenotypic divergence ($P_{ST}$) would be predicted to be under directional selection associated with high elevation.

# Results

## GENETIC DIVERSITY

Five to 22 alleles were identified in the five introns, with three to 18 polymorphic sites per locus. Observed heterozygosity was moderate to high (0.48–0.92) for introns and similar between subspecies (Table 2). Allelic diversity and π/site were similar for the hemoglobin subunits and introns (Table 2). Twenty-seven mtDNA haplotypes characterized by 18 variable sites were identified within cinnamon teal (Fig. 2).

Heterozygosity at the α*A* and β*A* subunits was similar to levels observed for introns for lowland *cyanoptera* ($H_O = 0.58$ and 0.77, respectively; Table 2). In contrast, heterozygosity for the α*A* subunit was considerably lower than levels observed for the five introns for highland *orinomus*; most individuals in the highlands were homozygous for a single α*A* subunit allele ($H_O = 0.06$; Table 2, Fig. 2). All introns were in Hardy–Weinberg equilibrium except for FGB; *cyanoptera* exhibited a heterozygote deficiency (Table 2). In contrast to the five introns, neither hemoglobin subunit was in Hardy–Weinberg equilibrium. Heterozygote deficiency was observed within the α*A* subunit when highland and lowland populations were pooled ($H_o = 42\%$ vs. $H_e = 68\%$; $P < 0.001$); however, *orinomus* and *cyanoptera* were in Hardy–Weinberg equilibrium when analyzed separately ($P$s > 0.5). In addition, the lowland population was found to be heterozygote deficient ($H_o = 58\%$ vs. $H_e = 78\%$) for the β*A* subunit,

**Table 2.** Number of alleles, nucleotide diversity, heterozygosity (observed *Ho* and expected *He*), $F_{ST}$, and $\Phi_{ST}$ for five unlinked introns, mtDNA control region, and the αA and βA hemoglobin subunits from *A. c. cyanoptera* (lowland) and *A. c. orimonus* (highland) subspecies of cinnamon teal.

| | Population | No. polymorphic sites | No. alleles | Nucleotide diversity (π/site) | Tajima's *D* | Fu's $F_s$ | *Ho*/*He*[1] | $F_{ST}$[2] | $\Phi_{ST}$[2] |
|---|---|---|---|---|---|---|---|---|---|
| Ornithine decarboxylase | *cyanoptera* | 9 | 7 | 0.0078 | 1.86 | 2.95 | 0.81/0.79 | **0.060** | 0.011 |
| | *orinomus* | 9 | 7 | 0.0079 | 1.71 | 2.97 | 0.74/0.81 | | |
| α enolase | *cyanoptera* | 14 | 9 | 0.0129 | −0.11 | 3.20 | 0.77/0.86 | **0.035** | **0.087** |
| | *orinomus* | 7 | 7 | 0.0077 | −0.05 | 1.92 | 0.74/0.78 | | |
| β fibrinogen | *cyanoptera* | 5 | 4 | 0.0078 | 2.05 | 4.14 | **0.65/0.70** | **0.103** | **0.043** |
| | *orinomus* | 6 | 5 | 0.0056 | 0.38 | 1.48 | 0.50/0.50 | | |
| N-methyl D aspartate receptor type I intron 13 | *cyanoptera* | 18 | 18 | 0.0161 | 1.97 | −0.59 | 0.75/0.80 | 0.015 | 0.019 |
| | *orinomus* | 12 | 12 | 0.0146 | 2.77 | 1.97 | 0.92/0.83 | | |
| Phosphoenolpyruvate carboxykinase | *cyanoptera* | 4 | 5 | 0.0019 | −0.33 | −0.76 | 0.48/0.60 | 0.009 | 0.007 |
| | *orinomus* | 3 | 4 | 0.0022 | 0.59 | 0.70 | 0.60/0.63 | | |
| Averaged introns | *cyanoptera* | – | – | 0.0092 | – | – | – | 0.004 | **0.036** |
| | *orinomus* | – | – | 0.0075 | – | – | – | | |
| mtDNA control region | *cyanoptera* | 29 | 16 | 0.0034 | −0.75 | −3.43 | – | **0.168** | **0.072** |
| | *orinomus* | 21 | 13 | 0.0019 | −1.47 | −3.33 | – | | |
| α*A* hemoglobin | *cyanoptera* | 12 | 16 | 0.0025 | −0.75 | **−7.15** | 0.77/0.71 | **0.602** | **0.551** |
| | *orinomus* | 7 | 4 | 0.0002 | **−1.94** | **−2.84** | 0.06/0.06 | | |
| β*A* hemoglobin | *cyanoptera* | 59 | 15 | 0.0076 | 0.40 | 9.64 | **0.58/0.78** | **0.082** | **0.061** |
| | *orinomus* | 54 | 11 | 0.0042 | −1.07 | 5.99 | 0.48/0.46 | | |

[1]Populations not in Hardy–Weinberg equilibrium are shown in bold.
[2]Significant values ($P < 0.05$) are shown in bold.

whereas the highland population was in Hardy–Weinberg equilibrium (Table 2). The five introns were in linkage equilibrium ($P$s $> 0.05$).

Tajima's $D$ and Fu's $F_s$ were not significant for any comparisons involving the five introns, β*A* subunit, or mtDNA ($P$s $>$ 0.50; Table 2). However, *orinomus* exhibited a significantly negative Tajima's $D$ and Fu's $F_s$ and *cyanoptera* exhibited a significant Fu's $F_s$, indicating a significant excess of rare alleles for the α*A* subunit (Table 2, Fig. 2).

## POPULATION SUBDIVISION

Significant variance in allelic and haplotypic frequencies was observed across loci within cinnamon teal. Low to moderate levels of genetic structure were observed across the five introns ($\Phi_{ST} =$ 0.007–0.087; Table 2, Fig. 2). In contrast, very high differentiation was observed for the α*A* subunit between the lowland *cyanoptera* and the highland *orinomus* population ($\Phi_{ST} = 0.551$, Table 2, Fig. 2). Despite high levels of subdivision for the α*A* subunit, the β*A* subunit showed levels of structure similar to the introns ($\Phi_{ST} = 0.061$; Table 2, Fig. 2).

Little evidence of population structure was detected within cinnamon teal using STRUCTURE and a two-population model ($K = 2$) for the mtDNA and intron dataset (posterior assignment probability averaged 50.7%; Fig. 3) with introns only giving similar results (posterior assignment probability = 50.8%). Results from the combined analysis of five introns and mtDNA with the β*A* subunit were similar (posterior assignment probabilities = 51.4 ± 3.2% SD; Fig. 3). However, with the inclusion of the α*A* subunit, lowland *cyanoptera* individuals and highland *orinomus* individuals were assigned to two clusters with high posterior probabilities (94.5 ± 9.4% SD; Fig. 3).

## HEMOGLOBIN AMINO ACID SUBSTITUTIONS

Two nonsynonymous substitutions were observed on the α*A* subunit. One substitution resulted in an amino acid replacement (Asn → Ser-α9), which showed highly significant allele frequency differences between *cyanoptera* and *orinomus* ($F_{ST} =$ 0.94) and has not been recorded in any other waterfowl species (McCracken et al. 2009b,c). Ninety-four percent of individual *cyanoptera* were homozygous for Asn, whereas 94% of individual

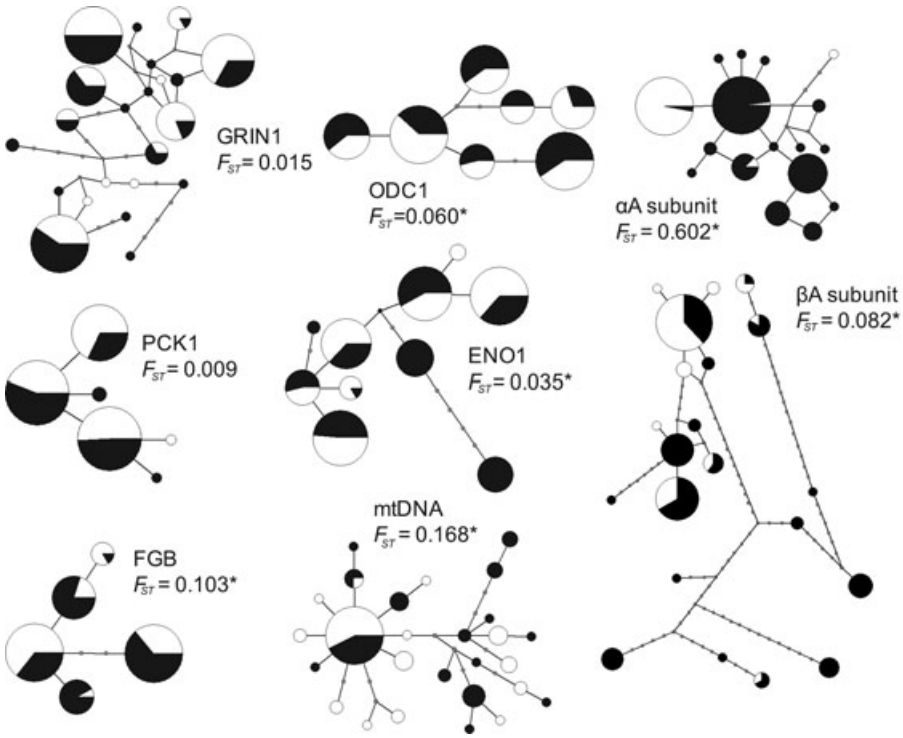


**Figure 2.** Allelic networks for eight loci. Alleles for *A. c. cyanoptera* are shown in black, and alleles for *A. c. orinomus* are shown in white. Significant $F_{ST}$ ($P < 0.05$) is indicated by an asterisk. Circle area is proportional to the number of each allele found and small gray circles indicate intermediate alleles not sampled.

*orinomus* were homozygous for Ser (Table S1). Three (6%) heterozygous individuals were found in *cyanoptera* and three (6%) in *orinomus*, but no individuals were homozygous for the rarer allele found within their respective subspecies. All six *cyanoptera* individuals collected above 2000 m and at the northern and southern borders of Altiplano were homozygous for the allele that occurred at high frequency in the lowland population, Asn-α09. The second substitution we observed was Thr-α28. One *orinomus* (KGM499) collected on Lake Titicaca and four *cyanoptera* in Patagonia were heterozygous (Ala/Thr-α28) with all remaining individuals homozygous (Ala/Ala-α28). Ala is a synapomorphy for cinnamon teal and other members of the blue-winged duck group, whereas Thr-α28 is the ancestral state found in all other dabbling ducks (McCracken 2009b,c). The β*A* subunit possessed only silent (synonymous) polymorphisms.

## TIME SINCE DIVERGENCE

Based on joint estimate for mtDNA and five introns, time since divergence peaked at 0.13 (90% highest posterior density [HPD] = 0.03–0.43; Fig. 4), suggesting that lowland and highland subspecies began diverging about 170,000 years before present (ybp; range = 40,000–570,000). Assuming an exponential growth model, the posterior distribution of the splitting parameter, *s*, peaked at 94.95% (90% HPD = 0.15–99.75) as the percent of the ancestral population that contributed to *cyanoptera*, supporting a highland colonization from the lowlands.

## GENE FLOW

IM estimated the joint gene flow rate for mtDNA and introns into the lowlands ($M_c$) to be approximately 29.19 times greater than the mutation rate (90% HPD = 8.31–66.31), which exceeded the migration rate into the highlands ($M_o$ = 1.94; 90% HPD = ∼0.00–39.47; Fig. 4). There was considerable overlap in estimates; however, we could not reject a hypothesis of no gene flow into the highlands. Multiplying through by θ, the joint estimate indicated that there were, on average, 26.9 migrants/generation ($4N_em$) into the lowlands and effectively no migrants into the highlands following divergence.

Consistent with the pattern observed for the IM model, an asymmetrical downslope migration rate for the five introns was also observed under the BayesAss model. There was restricted upslope migration, with approximately 0.8% (0.0–3.1%) of the highland population comprised of migrant origin and 27.2% (16.1–32.5%) of the lowland population having a highland origin. Similar results were obtained with introns and β*A* subunit combined. However, there was restricted gene flow in both directions

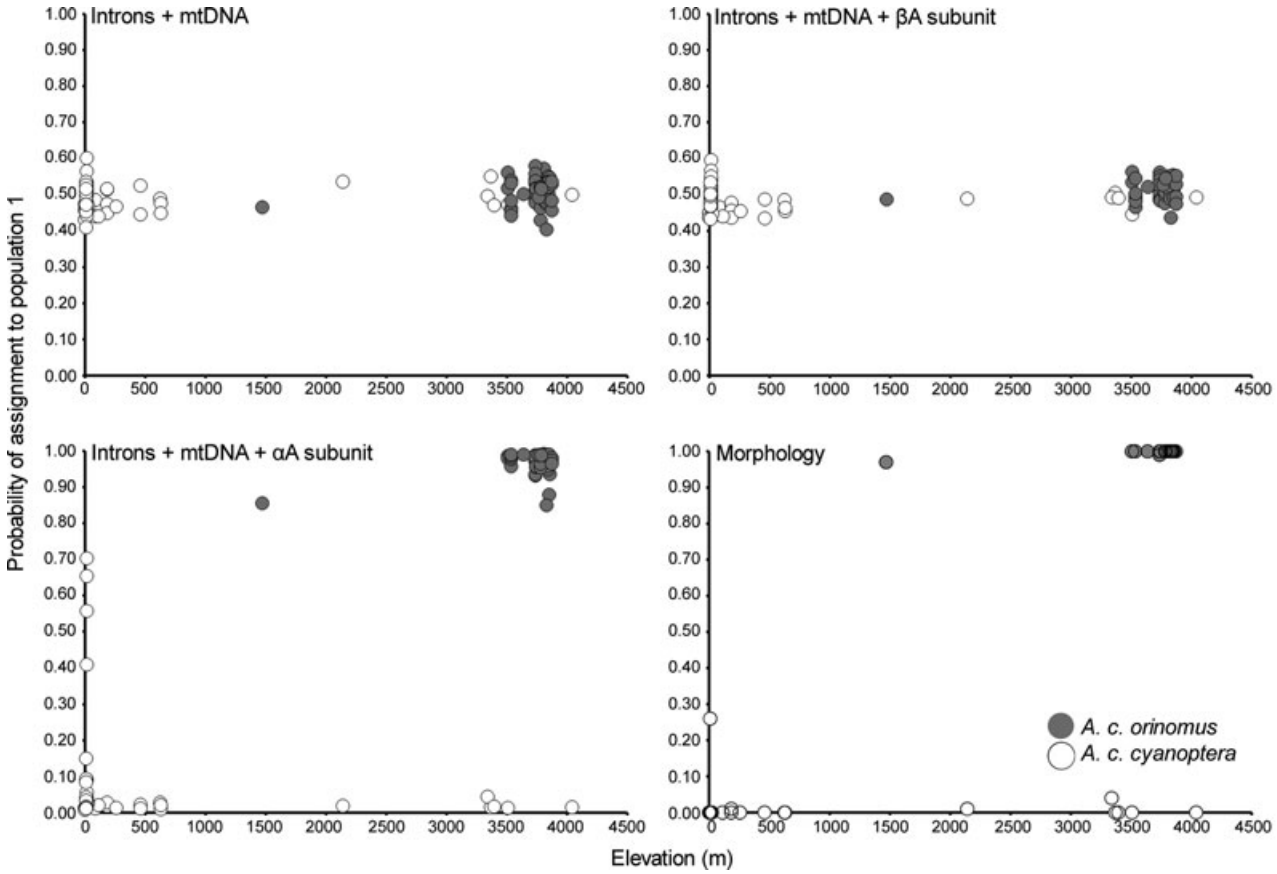


**Figure 3.** Posterior probability of assignment to the highland population versus elevations for genetic data using STRUCTURE 2.2 and morphological data using a discriminant analysis.

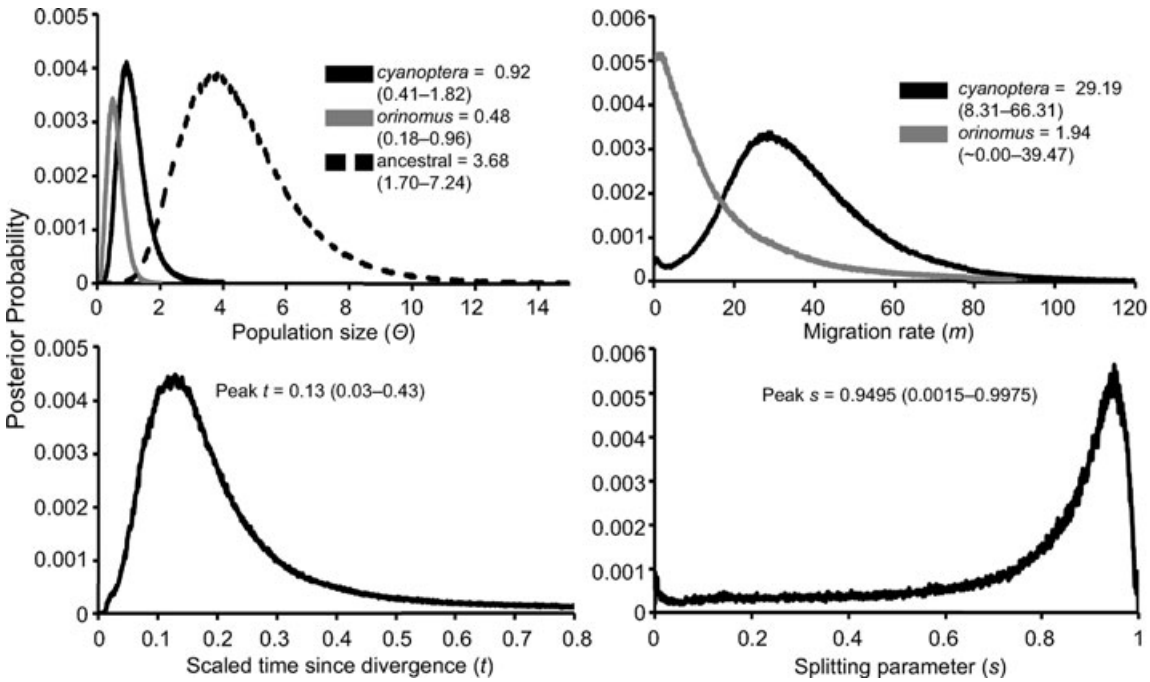


**Figure 4.** Posterior distributions of estimated migration rates (*m*), effective population size (θ), time since divergence (*t*), and splitting parameter (*s*) calculated using IM (scaled to the neutral mutation rate, μ). Peak estimates for each parameter are given and the 90% highest posterior distribution is shown in parentheses.

when the αA subunit was included, with 0.7% (0.0–2.6%) of the highland population and 1.5% (0.1–4.4%) of the lowland population showing a migrant origin.

### POPULATION SIZE

The population size parameter, θ, estimated in IM for mtDNA and introns combined was higher for *cyanoptera* than *orinomus* (Fig. 4). The effective population sizes ($N_e$) for *cyanoptera* and *orinomus* were estimated to be 98,000 (42,000–190,000) and 50,000 (19,000–99,000), respectively. Contemporary population sizes were smaller than the ancestral size (400,000; 180,000–750,000), suggesting population contractions following divergence. The effective population size and census size were in close agreement, as current population estimates for *cyanoptera* range from 25,000 to 100,000 (Rose and Scott 1997) and 10,000 to 100,000 for *orinomus* (Wetlands International 2002).

### MORPHOMETRIC DIFFERENTIATION

The first principal component (PC1) accounted for 65.9% of the variance in morphology (eigenvalue = 4.61; Table S2) and represented an overall body size vector, as factor loadings for all measurements were uniformly high and positive and PC1 scores were positively correlated with body mass ($r = 0.755$, $P < 0.001$). The second principal component (PC2) accounted for 11.4% of the variance and represented a bill shape difference, as bill measurements were the most influential variables. Highland (*orinomus*) individuals were significantly larger in size than lowland (*cyanoptera*) individuals (ANOVA: $F_{1,93} = 89.31$, $P < 0.001$). In addition, there were significant added variance components ($P$s $< 0.05$) except for bill length at nares, indicating that individuals from different environments differ more from each other than do individuals from the same environment. Discriminant analysis showed a high overall probability of subspecific assignment (Fig. 3). For males, only one *cyanoptera* (REW 316; 75%) had an assignment probability of less than 95% to subspecies. All females had 100% probability of assignment.

### COALESCENT SIMULATIONS—NEUTRAL EVOLUTION VERSUS SELECTION

The observed $\Phi_{ST}$ value for the αA subunit fell outside the 95% confidence limit of the simulated data based on inferred population history and selective neutrality indicating that selection has influenced the observed divergence (Fig. 5). In contrast, the $\Phi_{ST}$ values for all five introns were within the simulated data suggesting that the observed population divergence is consistent with the inferred population history and are selectively neutral. In addition, the $\Phi_{ST}$ value of the βA subunit, which did not possess any amino acid substitutions, fell within the simulated dataset; therefore we cannot reject neutral evolution for this hemoglobin gene.

Body size (PC1) divergence between subspecies was approximately five times larger ($P_{ST} = 0.482$) than the highest $F_{ST}$ value (0.103, FGB) from the five introns. Among individual measurements, $P_{ST}$ estimates for wing chord (0.822), tarsus (0.470), and tail length (0.472) showed a similar pattern as PC1 (body size), with $P_{ST}$ falling far outside the 95% confidence limit of simulated neutral genetic data (Fig. 5). Differences in bill shape were roughly similar to $F_{ST}$ estimates falling within the simulated data: bill length at nares ($P_{ST} = 0.000$), culmen length (0.217), bill width (0.127), and bill height (0.135). All $P_{ST}$ values were similar when males and females were analyzed separately (data not shown). Sensitivity analysis where $P_{ST}$ was recalculated under different assumptions about $h^2$ and $g$ showed that these results are not sensitive to varying parameters. Only an extremely small additive genetic proportion of the variance between populations ($g$) would affect the estimation of $P_{ST}$ and lead to accepting the null hypothesis of neutral phenotypic divergence for PC1, wing chord, tarsus, and tail length (Fig. S1). This result is in agreement with other studies (Merilä 1997; Storz 2002; Sæther et al. 2007).

## Discussion

### HEMOGLOBIN GENETIC AND BODY PHENOTYPE DIVERGENCES

Cinnamon teal showed strong divergence in hemoglobin and body size despite considerable admixture of mtDNA and introns between environmental extremes. The lack of significant allelic frequency differences among introns (average $F_{ST} = 0.004$) and STRUCTURE analysis indicated little evidence for genetic structuring among sampled sites. However, a single nonsynonymous αA hemoglobin substitution (Asn → Ser-α9; $F_{ST} = 0.94$) and large body size ($P_{ST} = 0.482$) were two characteristics of highland individuals, whereas lowland individuals mostly lacked this allele and possessed a smaller body size. The observed amino acid substitution (α9) is located on an exterior, solvent-accessible position on the A helix (McCracken et al. 2009c), which undergoes an important conformational change during the transition from the deoxy to the oxy state suggesting this amino acid may confer a functional response to hypoxia (Perutz 1990).

If genetic drift has played a major role in shaping traits between low- and high-altitude populations, then differences in predicted functional traits should be similar to divergence in neutral markers. Indicators of overall body size and the αA hemoglobin showed elevated divergence in comparison to mtDNA and introns. Divergence estimates for these traits were outside the distribution values simulated under neutrality indicating that genetic drift alone would be unlikely to generate the observed levels of divergence. Therefore, adaptation to environmental conditions associated with high altitude, notably low temperatures and hypoxia, likely influenced body size and hemoglobin structure. Indeed,

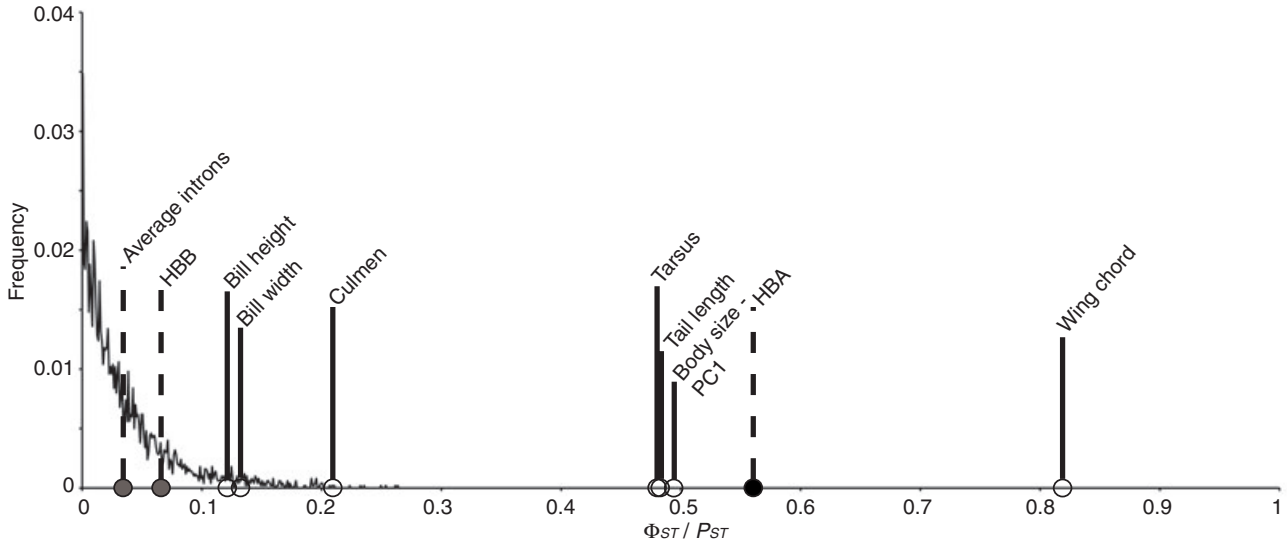


**Figure 5.** Simulated values of $\Phi_{ST}$ for 1000 simulated datasets for neutral loci (black line) and empirical values for the $\alpha A$ hemoglobin (HBA; black filled circle and dashed line), average across five introns and $\beta A$ hemoglobin (gray filled circles and dashed line), and phenotypic-$Q_{ST}$ ($P_{ST}$, open circles and solid black line) for morphological traits.

the increase in body size observed with elevation is consistent with Bergmann's rule—individuals in colder arid environments tend to be larger in size (Bergmann 1847; Wilson et al. 2010). In addition, the synergistic effects of multiple environmental pressures at high altitudes (Monge and Leon-Velarde 1991), may pose a greater challenge than each factor alone, which might explain why larger animals also tend to have higher hemoglobin-$O_2$ affinity (Schmidt-Nielsen and Larimer 1958; Hopkins and Powell 2001).

Pre-zygotic isolation can occur when populations are separated in space or when genetically based adaptations to contrasting environments (habitat segregation) reduce the likelihood of heterospecific encounters (Johnson et al. 1996; Rundle and Nosil 2005). The spatial distributions of hemoglobin alleles and body types suggest for a tendency for individuals to remain within their native elevation, as there were only a few individuals with genotypes or phenotypes mismatched to their environment. Specifically, six individuals in the highlands possessed the lowland $\alpha A$ hemoglobin genotype and small body sizes indicative of lowland *cyanoptera*. On the basis of gonad size, at least five of these individuals were not in breeding condition. Furthermore, two of these individuals were found in sympatry with *orinomus* at Laguna de Paca (Junin, Peru) where *orinomus* is not known to breed, and the remaining four individuals were collected at the periphery of *orinomus's* range. Lowland *cyanoptera* were never encountered within the main breeding range of *orinomus*. Thus, although encounters between highland and lowland subspecies do occur, such encounters appear to be infrequent and probably result in few successful breeding attempts between subspecies (see below).

## UPSLOPE AND DOWNSLOPE GENE FLOW IN THE ANDES

Although gene flow estimates were restricted between highland and lowland populations, dispersal is potentially much higher than the rate of gene flow (Garant et al. 2007). Transplant experiments demonstrate that lowland birds have difficulty breeding at high altitude (Monge and León-Velarde 1991) and that selection imposed by hypoxia causes low hatchability of eggs (Visschedijk et al. 1980). There is a shift in physiological mechanisms regulating gas exchange from conservation of water and $CO_2$ at middle elevation to improving $O_2$ diffusion above 4000 m (Carey 1994). Adult hemoglobin appears by day 6 during embryonic development (León-Velarde and Monge 2004), and an amino acid substitution that confers a higher oxygen affinity would likely ensure higher $O_2$ content required for embryonic growth and development at high elevations. Individuals possessing mismatched genotypes were found in cinnamon teal (this study) and in yellow-billed pintail (*A. georgica*, McCracken et al. 2009a), indicating that individuals can disperse into the highlands, perhaps because they can acclimatize to hypoxia via multiple physiological pathways. However, the susceptibility of the avian embryo to hypoxia might limit reproduction of the lowland subspecies in the highlands.

Reference loci (mtDNA and introns) suggested asymmetric gene flow, with higher immigration into the lowlands from the highlands, whereas immigration into the highlands was indistinguishable from zero. Highland species have been successfully bred in captivity in lowland environments from wild stock without detectable difficulties (Delacour 1956), and these Andean

waterfowl possessed the hemoglobin and morphological traits of wild populations (*Chloephaga melanoptera* Hiebl et al. 1987; *Anser indicus* Scott et al. 2011; *A. puna* R. E. Wilson, pers. obs.). Thus, it seems unlikely that high-altitude adaptations restrict highland birds from breeding in the lowlands. However, we found only one highland individual at approximately 1500 m (corresponding to lowland habitat), suggesting that movement into the lowlands is uncommon despite populations being separated by $<100$ km in some parts of their range. Factors other than high-altitude adaptations might restrict movements to the lowlands (e.g., migratory behavior, availability of foraging habitat, etc.) although gene flow might have been more common historically.

### TIMING AND DIRECTION OF COLONIZATION AND ADAPTIVE TRAIT CHANGE

High-altitude resident species often develop long-term, genetic adaptations, such as observed in hemoglobins, to reduce physiological stress imposed by hypoxia (León-Velarde et al. 1996; Weber 2007; McCracken et al. 2009b,c). These adaptations can accumulate over a long period of time or can occur rapidly. For example, high-altitude populations of chickens in the Andes have likely acquired higher Hb-$O_2$ affinity following their introduction by Spaniards within the last 500 years (León-Velarde et al. 1991). Coalescent analyses suggest that lowland *cyanoptera* and highland *orinomus* have been diverging for at least 40,000 years. Compared to other waterfowl species in the high Andes, cinnamon teal have a shallow divergence (e.g., Johnson and Sorenson 1999; McCracken et al. 2009b; Bulgarella et al. 2012), suggesting that cinnamon teal are a more recent Andean resident. This conclusion is consistent with the observation that a single, derived amino acid polymorphism (Asn/Ser-α9) distinguished the lowlands from the highlands, whereas other species of Andean waterfowl possess multiple polymorphisms segregating between lowland and highland habitats (McCracken et al. 2009b,c).

The splitting parameter provided evidence that the highlands were likely colonized from the lowlands as less than 5% of the ancestral population contributed to *orinomus* and approximately 95% contributed to *cyanoptera*. This direction of colonization is consistent with patterns suggested for other Andean avifauna (Fjeldså 1985; Vuilleumier 1986; McCracken et al. 2009b). Assuming a lowland origin of *orinomus*, we can infer that small body size and Asn-α9 are the ancestral states. Other Andean waterfowl show similar patterns of body size (Blake 1977; Bulgarella et al. 2007) and derived hemoglobin substitutions (McCracken et al. 2009b,c) between lowland and highland counterparts. Because many closely related species differ in elevational distributions and their time of divergence, habitat change likely preceded the evolution of those traits (e.g., Diamond 1986; Richmond and Price 1992) rather than being ancestral conditions.

## Conclusion

Our conclusions about the adaptive function of morphological and hemoglobin divergence rely on three assumptions about the underlying evolutionary mechanisms: (1) divergent selection in different habitats has caused the differences in traits, (2) hemoglobin amino acid polymorphism alters hemoglobin function, and (3) morphological traits are heritable and not subject to substantial phenotypic plasticity. The concordant body size changes (Blake 1977) and parallel patterns of hemoglobin amino acid substitution across Andean waterfowl are consistent with functional predictions to deal with a hypoxic, cold habitat and suggest that these differences resulted from directional selection within the highlands (Bulgarella et al. 2007, 2012; McCracken et al. 2009b,c; Wilson et al. 2010). However, the structural and functional effect of the observed Asn $\rightarrow$ Ser-α9 is still unknown, and further research is required to determine its role in the oxygen transport system. For example, the Ser-α9 substitution could be effectively neutral and linked to another positively selected polymorphism in the same α-globin linkage group, or if it does not influence Hb-$O_2$ affinity, affect some other functional property of hemoglobins such as their solubility in red blood cells. Although the structural and functional effects of the observed amino acid change are yet to be determined, neutral evolution is unlikely to have caused the divergence, supporting the conclusion that selection imposed by the high-altitude environment has contributed to the divergence of high-altitude cinnamon teals from their low-altitude, ancestral counterparts.

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## *Supporting Information*

The following supporting information is available for this article:

**Figure S1.** $P_{ST}$ sensitivity plots with varying the additive genetic proportion of variance between population ($g$) and heritability ($h^2$).

**Table S1.** Catalog numbers, locality, elevation, and α*A* hemoglobin subunit amino acid genotypes of lowland (*A. c. cyanoptera*) and highland (*A. c. orinomus*).

**Table S2.** Principal components (PC1 and PC2), eigenvectors, eigenvalues, and percent of variance.

Supporting Information may be found in the online version of this article.

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