# Signatures of High-Altitude Adaptation in the Major Hemoglobin of Five Species of Andean Dabbling Ducks

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ABSTRACT: Hypoxia is one of the most important factors affecting survival at high altitude, and the major hemoglobin protein is a likely target of selection. We compared population genetic structure in the $\alpha$A and $\beta$A hemoglobin subunits (HBA2 and HBB) of five paired lowland and highland populations of Andean dabbling ducks to unlinked reference loci. In the hemoglobin genes, parallel amino acid replacements were overrepresented in highland lineages, and one to five derived substitutions occurred at external solvent-accessible positions on the $\alpha$ and $\beta$ subunits, at $\alpha^1\beta^1$ intersubunit contacts, or in close proximity to inositolpentaphosphate (IPP) binding sites. Coalescent analyses incorporating the stochasticity of drift and mutation indicated that hemoglobin alleles were less likely to be transferred between highland and lowland populations than unlinked alleles at five other loci. Amino acid replacements that were overrepresented in the highlands were rarely found within lowland populations, suggesting that alleles segregating at high frequency in the highlands may be maladaptive in the lowlands and vice versa. Most highland populations are probably nonmigratory and locally adapted to the Altiplano, but gene flow for several species may be sufficiently high to retard divergence at unlinked loci. Heterozygosity was elevated in the $\alpha$A or $\beta$A subunits of highland populations exhibiting high gene flow between the southern lowlands and the highlands and in highland species that disperse seasonally downslope to midelevation environments from the central Andean plateau. However, elevated heterozygosity occurred more frequently in the $\alpha$A subunit but not simultaneously in both subunits, suggesting that selection may be more constrained by epistasis in the $\beta$A subunit. Concordant patterns among multiple species with different evolutionary histories and depths of historical divergence and gene flow suggest that the major hemoglobin genes of these five dabbling duck species have evolved adaptively in response to high-altitude hypoxia in the Andes.

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## Introduction

Hypoxia is one of the most important factors affecting survival in high-altitude regions. At high elevations such as those encountered in the Andes of South America, the low partial pressure of oxygen ($Po_2$) can result in a precipitous reduction in the $O_2$ saturation of arterial blood (Hornbein and Schoene 2001). At 4,000 m elevation, for example, the $Po_2$ of inspired air is approximately 60% of that at sea level. In the absence of specific adaptations or compensatory physiological mechanisms, $O_2$ transport to the tissues can be severely compromised, thus influencing an animal's metabolism and capacity for sustained physical activity such as flight. Several recent studies have shown that genetically based adaptations that increase the $O_2$ affinity of vertebrate hemoglobins play an important role in high-altitude-adapted populations (Storz et al. 2007; Weber 2007; Storz and Moriyama 2008).

Two species of highland waterfowl, in particular, have featured prominently in studies of high-altitude adaptation. Substitutions in two genes (Pro → Ala-$\alpha^{(A)}$119, Leu → Ser-$\beta^{(A)}$55) located on different chromosomes produce similarly large effects on the $O_2$ affinity of the major hemoglobin of bar-headed goose (*Anser indicus*) and Andean goose *(Chloephaga melanoptera*) by eliminating van der Waals interactions between the R groups of two amino acid residues at the same $\alpha^1\beta^1$ intersubunit contact (Perutz 1983; Jessen et al. 1991; Weber et al. 1993). Bar-headed and Andean geese obtain 50% saturation of the hemoglobin at lower $Po_2$ than do lowland species such as the greylag

goose (*Anser anser*; Hall et al. 1936; Petschow et al. 1977). The Andean goose is not a true goose, however, but belongs to a clade of ecologically convergent, gooselike ducks called sheldgeese (Livezey 1986). Parallel phenotypic evolution in these two distantly related waterfowl thus has arisen from two different genetic solutions resulting in the same basic phenotypic effect caused by substitutions in the same region of the hemoglobin protein. Studies of the crystal structures of the oxy- and deoxyhemoglobins of bar-headed goose and greylag goose further confirm that these substitutions are likely adaptive in high-altitude environments (Zhang et al. 1996; Wang et al. 2000; Liang et al. 2001*a*, 2001*b*; Liu et al. 2001).

In addition to the Andean goose, five species of dabbling ducks inhabit the wetlands and puna grasslands of the Altiplano and inter-Andean valleys up to approximately 5,000 m elevation (table 1). The same species or closely related sibling species are also common in the lowland (<1,500 m) Patagonian region of southern South America. Waterfowl are uncommon at intermediate elevations in the central Andean plateau because of the scarcity of wetlands on midelevation slopes, and most dabbling duck populations in the high Andes are allopatric, with populations inhabiting the southern lowlands. None of the five population pairs are closely related to the others or to any other highland species (Johnson and Sorenson 1999). The five lineages thus serve as evolutionary replicates, because they share the strong environmental pressure of high-altitude hypoxia. They also exhibit varying levels of population divergence; average pairwise uncorrected mtDNA control region sequence divergence between lowland and highland populations ranges from 0.3% to 2.7% (table 1). In one case, lowland and highland populations of yellow-billed pintail (*Anas georgica*) are not differentiated enough to be considered subspecies. In three other cases, lowland and highland populations of crested duck (*Lophonetta specularioides*), cinnamon teal (*Anas cyanoptera*), and speckled teal (*Anas flavirostris*) are classified as subspecies. Silver teal (*Anas versicolor*) and puna teal (*Anas puna*) are classified as sibling species (Fjeldså and Krabbe 1990). These dabbling duck species thus also represent a series of replicates with contrasting depths of evolutionary separation.

In comparisons across all major waterfowl lineages, the αA and βA hemoglobin subunit genes (HBA2 and HBB), which code the major hemoglobin protein isoform, display a striking pattern of parallel evolution in these highland waterfowl (McCracken et al. 2009*b*). More identical nonsynonymous codon substitutions occurred than expected by chance under a simulated neutral model of evolution, and recurrent substitutions with similar biochemical properties were observed at external solvent-accessible positions on the α and β subunits, at $\alpha^1\beta^1$ intersubunit contacts, or in close proximity to inositolpentaphosphate (IPP) binding sites. This widespread parallel evolution suggests that adaptation to high-altitude hypoxia has resulted from unique but overlapping sets of one to five substitutions in the major hemoglobin of each lineage.

**Table 1:** South American dabbling ducks collected from five paired lowland and highland populations in the Andes

| Species, population | mtDNA divergence (%) | No. specimens | Elevation (m) |
|---|---|---|---|
| Crested duck (*Lophonetta specularioides*): | | | |
| *specularioides* | 1.4 | 23 | 0–934 |
| ***alticola*** | | 57 | 1,522–4,611 |
| Cinnamon teal (*Anas cyanoptera*): | | | |
| *cyanoptera* | .3 | 52 | 0–4,039 |
| ***orinomus*** | | 50 | 1,468–3,871 |
| Silver/puna teal (*Anas versicolor*/*puna*): | | | |
| *versicolor* | 2.5 | 34 | 5–687 |
| ***puna*** | | 43 | 3,338–4,426 |
| Yellow-billed pintail (*Anas georgica*): | | | |
| Lowland | .4 | 65 | 8–1,809 |
| **Highland** | | 51 | 3,063–4,124 |
| Speckled teal (*Anas flavirostris*): | | | |
| *flavirostris* | 2.7 | 71 | 3–2,093 |
| ***oxyptera*** | | 70 | 2,367–4,405 |

Note: Highland subspecies or populations are shown in bold text. Six *Anas cyanoptera cyanoptera* were collected at 2,141–4,039 m, and one *Anas cyanoptera orinomus* was collected at 1,468 m. Uncorrected mtDNA control region divergence (%) is shown between lowland and highland population samples (K. G. McCracken, M. Bulgarella, and R. E. Wilson, unpublished data).

The goal of this article is to evaluate whether the predicted signature of divergent selection can be detected within the five species of Andean dabbling ducks. Comparative population genetic studies of closely related species experiencing similar contrasting forces of selection across the same environmental gradient offer unique opportunities to reveal shared evolutionary patterns that might not be apparent in single-species studies (e.g., Malhotra and Thorpe 1994; Watt et al. 1996; Katz and Harrison 1997). For example, strong differentiation at one locus despite evidence of low differentiation at other loci might be explained by adaptive divergence (Beaumont 2005; Storz 2005), but the stochastic variance of genetic drift and mutation can also generate this pattern. Observing concordant patterns for the same set of interlocus contrasts among multiple species with different evolutionary histories, however, would argue more strongly for the influence of selection, because stochastic, neutral forces should result in idiosyncratic patterns among species.

Interlocus contrast can also be used to strengthen the inference of adaptation by comparing the rates of gene flow among putatively neutral and nonneutral loci. When gene flow occurs between two populations locally adapted to contrasting environments, immigrant alleles may be quickly eliminated in populations where they are less fit, so that gene flow is partly restricted (Wu and Ting 2004). The stronger the selection, the more rapidly immigrant alleles of lower fitness will be eliminated (Maynard Smith and Haigh 1974), thereby reducing effective migration rates (Charlesworth et al. 1997). Quantifying differences in migration rates between candidate and unlinked reference loci using coalescent genealogy samplers (Kuhner 2008) can identify restricted migration resulting from differential selection while incorporating the stochastic variance of drift, mutation, and recombination. Coalescent methods can also detect assymetrical migration patterns that result when some alleles are transferred between environments more successfully than others.

We sequenced the $\alpha$A and $\beta$A subunits of five lineages of Andean dabbling ducks collected from sea level to 4,600 m elevation at widespread localities along a 6,000-km transect of the central and southern Andes (fig. 1). We predicted that the $\alpha$A and $\beta$A subunits of Andean highland populations would exhibit more genetic structure between the lowlands and highlands than unlinked reference loci. Furthermore, if there is a difference in the selective disadvantage of highland alleles in the lowlands or vice versa, we expected to see that difference reflected in levels of gene flow for the hemoglobin subunits compared to those for reference loci. We also predicted that the $\alpha$A and $\beta$A hemoglobin subunits would exhibit elevated recombination rates across species because new beneficial mutations may be more likely to fix or increase in frequency if they are unlinked to other selected loci (Hill and Robertson 1966; Barton 1995; Otto and Barton 1997). Each species also showed elevated heterozygosity on only one hemoglobin subunit, more frequently on the $\alpha$A subunit, and

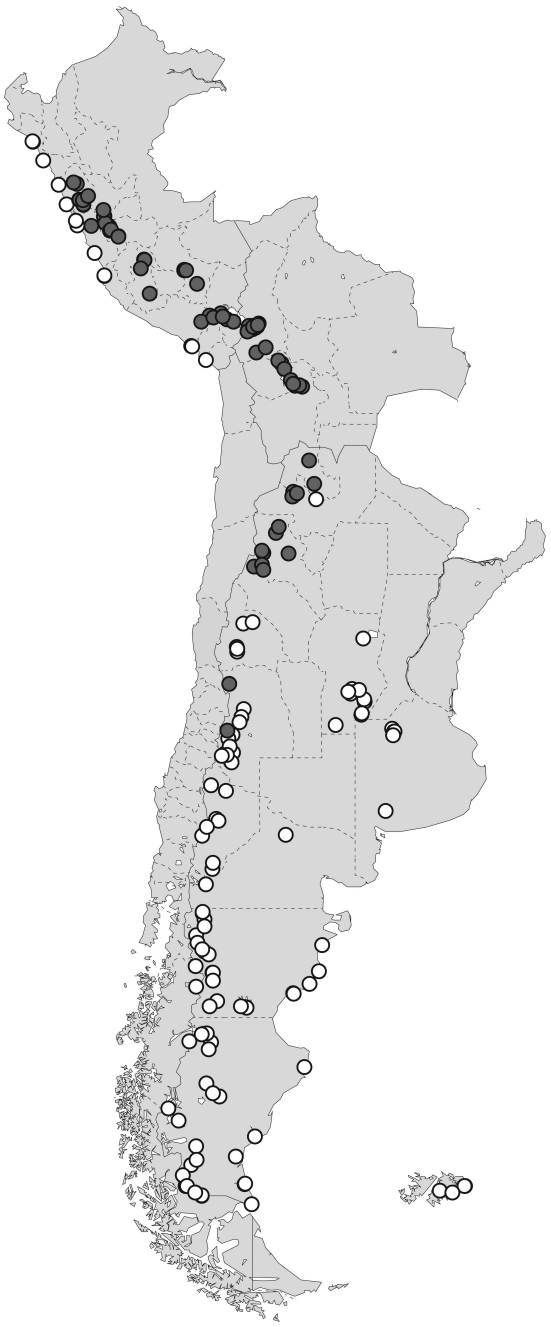

**Figure 1:** Localities for 516 dabbling ducks sampled from paired lowland and highland populations in Peru, Bolivia, Argentina, and the Falkland Islands. Gray circles indicate localities >2,000 m elevation; white circles indicate localities <2,000 m.

**Table 2:** Genes sequenced for the five paired lowland and highland populations of Andean dabbling ducks and chromosomal positions in the chicken genome

| Locus | Base pairs sequenced | Chicken chromosome |
|---|---|---|
| αA hemoglobin | 676–678 | 14 |
| βA hemoglobin | 1,578–1,584 | 1 |
| Ornithine decarboxylase intron 5 (ODC1) | 351–353 | 3 |
| α enolase intron 8 (ENO1) | 314 | 21 |
| β fibrinogen intron 7 (FGB) | 246 | 4 |
| N-methyl D aspartate 1 glutamate receptor intron 11 (GRIN1) | 330–744 | 17 |
| Phosphoenolpyruvate carboxykinase intron 9 (PCK1) | 345–351 | 20 |

Note: Chicken chromosome position is based on Hillier et al. (2004). A 414-bp short interspersed repeat in the 3′ end of the GRIN1 intron was overrepresented in the highland speckled teal population: 47.1% ($n = 33$) were heterozygous for the insertion, and 12.9% ($n = 9$) were homozygous. In contrast, only 8.5% ($n = 6$) of lowland individuals were heterozygous (see Zhu et al. 1991 for characterization in other waterfowl).

we hypothesize that these patterns may result from epistatic interactions between the two hemoglobin polypeptide subunits. Finally, we consider what other evolutionary processes, such as ancestral polymorphism and introgression, might have contributed to the parallel amino acid replacements we observed among these species.

## Methods

### *Specimen Collection*

We collected ducks ($n = 516$) in the Andes and adjacent lowlands of Argentina, Bolivia, Peru, and the Falkland Islands. We targeted the five lineages of dabbling ducks that are common both in the Andes and in the lowlands (table 1). These species were sampled widely from northern Peru south to the Strait of Magellan (fig. 1). Specimens were thus collected across two different elevational regions that also occur at different latitudes: (1) the central high Andes, which extends from Catamarca, Argentina, north to Cajamarca, Peru; and (2) the lowlands of Patagonia and central Argentina north to Mendoza and San Juan (fig. 1; table A1 in the online edition of the *American Naturalist*). Ducks were also collected at sea level on the Pacific coast of Peru, the Atlantic coast of Argentina, and the Falkland Islands.

### *Categorizing Individuals as Lowland or Highland*

Most ducks from the central high Andes were collected at elevations >2,000 m and were classified as "highland," whereas most ducks collected in Patagonia and central Argentina were collected at elevations <2,000 m and classified as "lowland," following criteria used in studies of human high-altitude adaptation (Hornbein and Schoene 2001). Ten individuals were considered exceptions to this classification. Three crested ducks collected at 1,522–1,891 m in Mendoza, Argentina, were categorized as highland based on plumage, body size measurements, and their mtDNA haplotypes (Bulgarella et al. 2007; K. G. McCracken, M. Bulgarella, and R. E. Wilson, unpublished data). On the basis of morphology, six cinnamon teal collected at 2,141–3,369 m in Argentina (KGM 442, 1110, 1142) and at 3,393–4,039 m in Peru (REW 118, 122, 164) were categorized as the lowland subspecies *cyanoptera* (Wilson et al. 2010). Likewise, one individual collected at 1,468 m in Salta, Argentina (REW 441), was categorized as the highland subspecies *orinomus*. Vouchered specimens were classified to subspecies (or species for silver/puna teal) using established phenotypic criteria.

### *DNA Sequencing*

Methods describing DNA sequencing for the αA and βA subunits and five autosomal introns (table 2) have been published by McCracken et al. (2009*a*). Primers were developed specifically for ducks (table A2 in the online edition of the *American Naturalist*), and reference loci were chosen blindly to levels of polymorphism so as not to bias parameter estimates. The gametic phases of sequences that were heterozygous at two or more nucleotide positions were determined using the software PHASE 2.1 (Stephens et al. 2001) and allele-specific primers (see McCracken et al. 2009*a*). For the βA subunit, we used PHASE to infer the gametic phase of each allele by excluding the introns and analyzing only the three exons. We chose this approach because the βA subunit was 1,578–1,584 bp and was sequenced with too many overlapping PCR fragments to

allow allele-specific priming for the entire gene. The gametic phases of 96.2% ($n = 3{,}469$) of the 3,608 individual sequences were identified experimentally or with >95% posterior probability. Sequences are deposited in GenBank ($\alpha$A hemoglobin subunit: GQ271065-GQ271144, GQ271146-GQ271246, GQ271248-GQ271324, FJ617587-FJ617702, GQ271325-GQ271465; $\beta$A hemoglobin subunit: GQ271804-GQ271883, GQ271884-GQ271985, GQ271986-GQ272062, FJ617703-FJ617816, GQ272063-GQ272202; five reference loci: GQ268964-GQ269363, GQ269364-GQ269873, GQ270617-GQ271001, FJ617817-FJ618396, GQ269874-GQ270616).

### *Population Genetic Analysis*

To measure population differentiation, we calculated the overall $F_{ST}$ and $\Phi_{ST}$ between each paired lowland and highland population for each locus. We calculated $F_{ST}$ for each segregating site in the $\alpha$A and $\beta$A subunits, noting the positions of amino acid replacements compared to synonymous polymorphisms. Hardy-Weinberg equilibrium was tested for each locus to determine whether there was a significant excess or deficit of heterozygotes, which might be associated with spatially divergent selection on different classes of alleles that were overrepresented in the highlands or lowlands. The $F_{ST}$ and Hardy-Weinberg tests were calculated for the complete sequences of the $\alpha$A and $\beta$A subunits and for only the amino acid sequences of the exons, with the two introns excluded from the analysis. Lowland and highland populations were analyzed both separately and together. Nonrandom associations resulting from linkage disequilibrium were evaluated for all seven loci combined to verify that they were unlinked.

These and other genetic diversity estimates were calculated using Arlequin 3.11 (Excoffier et al. 2005). The nonsynonymous/synonymous nucleotide diversity ratio ($\pi_N/\pi_S$), which if elevated may be indicative of divergent selection (Yang and Bielawski 2000), was calculated for the $\alpha$A and $\beta$A subunits using DnaSP 4.10 (Rozas et al. 2003). Allelic richness was standardized to the smallest sample size ($n = 46$ alleles). Allelic networks were illustrated using the median-joining algorithm in NETWORK 4.201 (Bandelt et al. 1999; Fluxus Technology).

Finally, we predicted that the $\alpha$A and $\beta$A subunits would disproportionately contribute to population structure between the lowlands and the highlands (compared to the five reference loci) if they have experienced a history of divergent selection. We used the software STRUCTURE 2.2 (Pritchard et al. 2000) to compare levels of population differentiation using two separate multilocus analyses, one including only the five autosomal introns and a second including the five introns and the $\alpha$A and $\beta$A coding sequence alleles. Because STRUCTURE does not accommodate sequence data, we treated each within-locus haplotype as a separate allele. STRUCTURE assigns individuals to populations by maximizing the Hardy-Weinberg equilibrium and minimizing linkage disequilibrium. We calculated the probability of assignment to a lowland or highland population ($K = 2$ populations) using the same software settings described by McCracken et al. (2009*a*). No prior population information was used. Finally, we explored the sensitivity of the STRUCTURE analyses to the total number of loci by withholding and then re-adding two intron loci.

### *Coalescent Analyses*

We used a coalescent model incorporating the effects of drift, mutation, and recombination to compare upslope and downslope allelic migration rates between the $\alpha$A and $\beta$A subunits and the five autosomal introns. We predicted that alleles experiencing divergent selection would be less likely to be transferred between the highlands and the lowlands than unlinked loci and that loci showing evidence of divergent selection would exhibit consistently elevated levels of recombination.

The population size parameter theta ($\Theta = 4N_e\mu$), migration rates ($M = m/\mu$) between lowland and highland populations, and the recombination rate ($r = \rho/\mu$) were estimated for each locus using LAMARC 2.1 (Kuhner 2006). We used Bayesian analyses with 1 million recorded genealogies sampled every 50 steps, with a burn-in of 100,000 (10%) genealogies. Priors were flat, with the upper limits for $\Theta$, migration, and recombination set to 0.1, 10,000, and 10, respectively. We used the Felsenstein (1984) substitution model with empirical transition/transversion ratios and base frequencies calculated from the data. Analyses were repeated three times to verify that parameter estimates converged within and among runs. The migration rate $M$ was multiplied by $\Theta$ for each recipient population to calculate $4N_em$, the average number of effective migrants dispersing into each population per generation. Coalescent analyses were conducted separately for the $\alpha$A and $\beta$A subunits; the five autosomal introns were combined under the assumption that they were unlinked by multiplying the likelihoods to obtain joint estimates.

LAMARC's migration rate estimates ($M$) are scaled to the mutation rate ($m/\mu$), where $m$ is the probability that a lineage immigrates per generation and $\mu$ is the mutation rate per site per generation. To calculate $m$ for each locus, independently of the mutation rate, and compare values among loci ($\alpha$A and $\beta$A subunits vs. autosomal introns), we multiplied $M$ for each locus by the per-site substitution rate $\mu$ for that locus. Substitution rates were calibrated separately for each locus using the duck-goose split (Peters

et al. 2007, 2008). The mean genetic distance between the snow goose (*Anser caerulescens*) sequence and the five dabbling ducks ($n = 516$ individuals) was divided by the midpoint of the Oligocene ($2 \times 30.5$ million years) and multiplied by a generation time for *Anas* ducks of 3.2 years (Peters et al. 2008). The averaged substitution rate for the autosomal introns was calculated as the mean.

## Results

### *Parallel Amino Acid Replacements Were Overrepresented in the Highlands*

If amino acid replacements in hemoglobin genes are adaptive, we would expect that they would exhibit parallel patterns across multiple independent lineages experiencing the same selection pressures. The five Andean dabbling ducks possessed segregating amino acid polymorphisms at multiple positions on the αA and βA subunits (fig. 2). Cinnamon teal had one amino acid replacement on the αA subunit segregating by elevation with elevated $F_{ST}$ but no βA subunit replacements. Yellow-billed pintails had no replacements on the αA subunit but had four segregating positions on the βA subunit. Crested duck, puna teal, and speckled teal each had three to five amino acid replacements on both subunits.

Parallel amino acid substitutions occurred in the αA subunit of two species (Thr-α77; fig. 2), and 10 such substitutions were observed in two species each at five different positions on the βA subunit (Ser-β4, Ser-β13, Glu-β94, Ser-β116, and Met-β133; fig. 2). Each of these parallel amino acid replacements resulted from identical codon substitutions. Thr-α77, in particular, was observed in three additional highland species belonging to three different waterfowl genera not analyzed here, and Ala-α8, Ser-β13, and Ser-β116 each were observed in one additional highland species from a different genus (fig. 4 in McCracken et al. 2009*b*).

The vast majority of ducks collected from the five lowland populations possessed the same amino acids as other lowland waterfowl (McCracken et al. 2009*b*) and lacked the amino acids observed at high frequency in the respective highland population (fig. 2). Moreover, 83.1%–100% of lowland individuals were homozygous for the αA subunit, and 95.7%–100% were homozygous for the βA subunit. The nonsynonymous/synonymous nucleotide diversity ratio ($\pi_N/\pi_S$) for one or both hemoglobin subunits thus was greater in the highlands for all five Andean dabbling duck species (table A3 in the online edition of the *American Naturalist*).

### *Hemoglobins Exhibited Greater Structure between the Lowlands and Highlands than Reference Loci*

If local adaptation is the cause of divergence in hemoglobin alleles, then we should see more divergence between populations in hemoglobin genes than in unlinked reference loci. Overall $F_{ST}$ and $\Phi_{ST}$ were significant for most reference loci for most population pairs (table 3). This is expected, given that these populations are largely allopatric. Silver/puna teal had the highest overall $\Phi_{ST}$ for the five introns. Crested duck and speckled teal had intermediate $\Phi_{ST}$ values but were widely variable, depending on the locus. Cinnamon teal and yellow-billed pintail had the lowest overall $\Phi_{ST}$ values. These five species thus represent a continuum of lowland/highland pairs ranging from high to low historical divergence, as also is indicated by their mtDNA divergence (table 1) and by the STRUCTURE analysis (fig. 3).

Despite this range of divergence, the αA or βA subunit sequences were more divergent than other loci in each of the five pairs. Overall $F_{ST}$ and $\Phi_{ST}$ for the αA subunit of cinnamon teal and speckled teal exceeded all other reference loci by large factors (table 3; fig. A2, in the online edition of the *American Naturalist*). Likewise, the overall $F_{ST}$ and $\Phi_{ST}$ for the βA subunit of crested duck, yellow-billed pintail, and speckled teal greatly exceeded the values for all reference loci. Yellow-billed pintail showed the most striking pattern; $\Phi_{ST}$ for the βA subunit was 0.65, whereas the maximum $\Phi_{ST}$ for any other single locus was 0.03 (McCracken et al. 2009*a*). The βA subunit of silver/puna teal also exhibited higher $\Phi_{ST}$ than all other reference loci ($\Phi_{ST} = 0.70$ vs. 0.28–0.58), despite the fact that silver/puna teal were the most differentiated population pair in our study (table 3).

As expected for strongly differentiated loci, the αA or βA subunit sequences of all five population pairs possessed fewer heterozygotes and more homozygotes than expected when pooled ($P$ values $\leq .03715$; fig. A1 in the online edition of the *American Naturalist*). Highland crested ducks also had fewer heterozygotes than expected for the βA subunit ($P = .01604$), as did lowland and highland yellow-billed pintails ($P$ values $\leq .00794$; fig. A1). No linkage disequilibrium was observed between the αA and βA subunits and the five reference loci, confirming that these loci are unlinked ($P$ values $> .99$).

This pattern of differentiation was confirmed when we added the αA and βA subunits to the STRUCTURE analyses that previously were conducted for only the five autosomal introns. Adding the αA and βA subunits increased the average probability of assigning most individuals to lowland or highland populations (fig. 3). Results were most striking for the species that lacked divergence at other loci, cinnamon teal (92.6% vs. 53.6%) and yellow-billed pintail

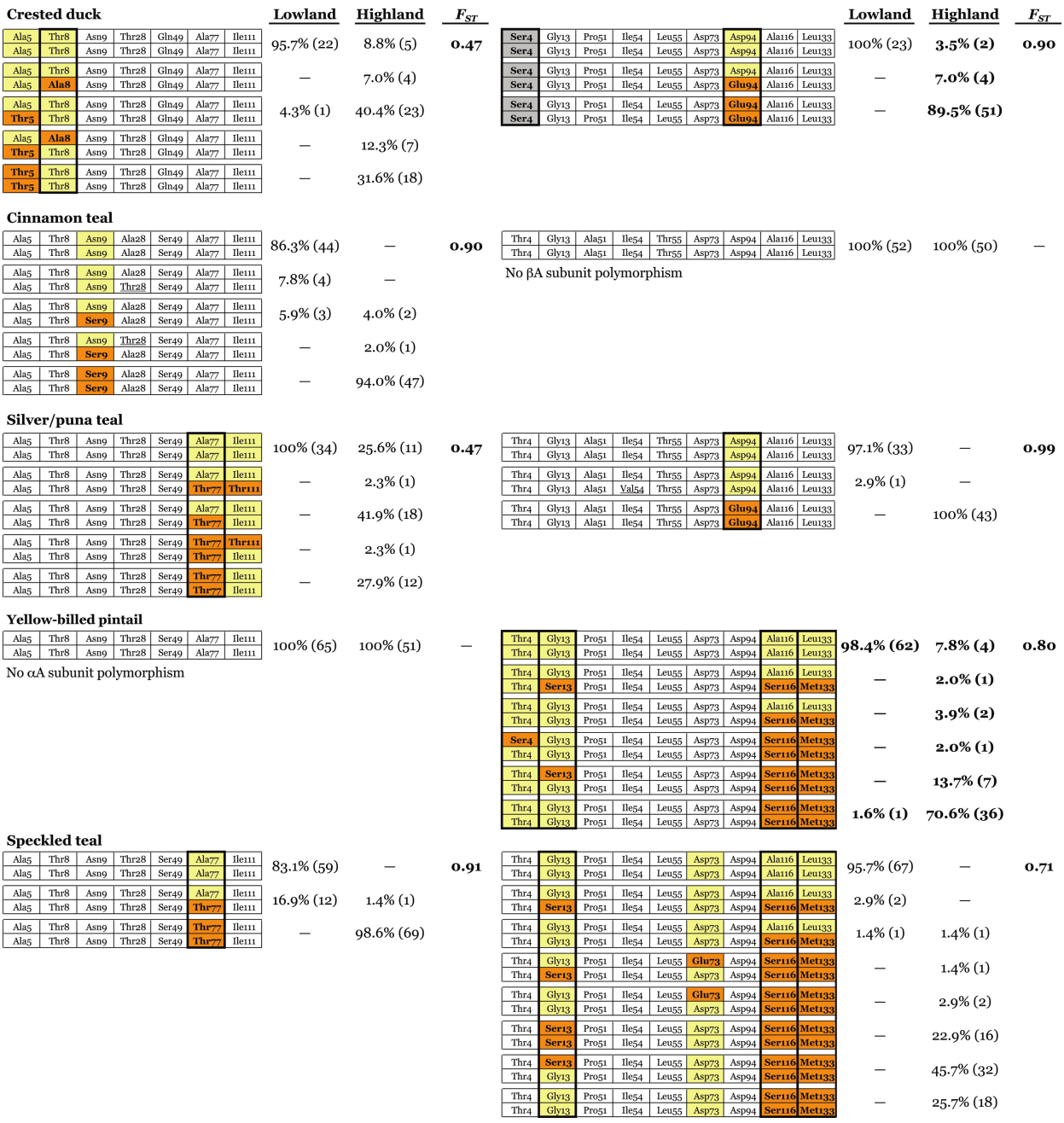


**Figure 2:** Genotypic frequencies for the αA and βA hemoglobin subunits and $F_{ST}$ for five paired lowland and highland populations of Andean dabbling ducks. Each line of consecutive amino acids represents an allele, and each pair of lines represents a genotype. Amino acids observed at higher frequency in highland populations are shown in orange, amino acids observed at higher frequency in lowland populations are shown in yellow, and positions that are not polymorphic within a single population pair are shown in white. Ser-β4, which is fixed for the same derived amino acid in lowland and highland populations of crested ducks, is shown in gray. Two rare amino acids segregating in lowland populations of cinnamon teal (Thr-α28) and silver teal (Val-β54) are underlined; Ala-α28 is a synapomorphy for cinnamon teal and shovelers, whereas Thr-α28 and Ile-β54 are the ancestral states for dabbling ducks (McCracken et al. 2009*b*). Bold black boxes indicate additional amino acid replacements that evolved in parallel in these taxa and/or the larger sample of highland waterfowl species (McCracken et al. 2009*b*). The numbers of individuals with each genotype are shown in parentheses. Significant departures from Hardy-Weinberg equilibrium and $F_{ST}$ ($P < .00001$) are shown in bold text. Hardy-Weinberg equilibrium tests are shown in figure A1 in the online edition of the *American Naturalist*.

**Table 3:** Number of polymorphic positions, alleles, standardized allelic richness, $F_{ST}$, and $\Phi_{ST}$ for the αA and βA hemoglobin subunits and five unlinked autosomal introns from five paired lowland and highland populations of Andean dabbling ducks

| Locus, species, population | No. polymorphic positions | No. alleles | Allelic richness (±SD) | $F_{ST}$ | $\Phi_{ST}$ |
|---|---|---|---|---|---|
| αA hemoglobin: | | | | | |
| Yellow-billed pintail: | | | | | |
| Lowland | 27 | 40 | 23.0 ± 2.2 | .01 | .01 |
| **Highland** | 19 | 25 | 17.4 ± 1.8 | | |
| Cinnamon teal: | | | | | |
| *cyanoptera* | 12 | 16 | 10.7 ± 1.5 | **.60** | **.55** |
| ***orinomus*** | 7 | 4 | 2.4 ± .9 | | |
| Crested duck: | | | | | |
| *specularioides* | 17 | 14 | 14 | **.12** | **.21** |
| ***alticola*** | 20 | 16 | 11.3 ± 1.4 | | |
| Speckled teal: | | | | | |
| *flavirostris* | 22 | 48 | 25.8 ± 2.3 | **.41** | **.54** |
| ***oxyptera*** | 5 | 6 | 3.7 ± 1.0 | | |
| Silver/puna teal: | | | | | |
| *versicolor* | 24 | 28 | 23.0 ± 1.6 | **.24** | **.66** |
| ***puna*** | 4 | 5 | 4.3 ± .7 | | |
| αA hemoglobin (cds): | | | | | |
| Yellow-billed pintail: | | | | | |
| Lowland | 11 | 14 | 8.9 ± 1.4 | .01 | .00 |
| **Highland** | 5 | 7 | 5.6 ± .8 | | |
| Cinnamon teal: | | | | | |
| *cyanoptera* | 4 | 6 | 5.2 ± .7 | **.69** | **.72** |
| ***orinomus*** | 2 | 3 | 2.2 ± .7 | | |
| Crested duck: | | | | | |
| *specularioides* | 6 | 6 | 6 | **.27** | **.28** |
| ***alticola*** | 7 | 9 | 7.2 ± 1.0 | | |
| Speckled teal: | | | | | |
| *flavirostris* | 4 | 7 | 5.4 ± .8 | **.65** | **.73** |
| ***oxyptera*** | 2 | 3 | 1.7 ± .7 | | |
| Silver/puna teal: | | | | | |
| *versicolor* | 4 | 5 | 4.5 ± .6 | **.27** | **.36** |
| ***puna*** | 3 | 4 | 3.3 ± .6 | | |
| βA hemoglobin (cds): | | | | | |
| Yellow-billed pintail: | | | | | |
| Lowland | 14 | 21 | 11.9 ± 1.8 | **.37** | **.65** |
| **Highland** | 9 | 10 | 7.2 ± 1.2 | | |
| Cinnamon teal: | | | | | |
| *cyanoptera* | 8 | 8 | 7.2 ± .7 | **.11** | **.10** |
| ***orinomus*** | 7 | 7 | 5.5 ± .9 | | |
| Crested duck: | | | | | |
| *specularioides* | 5 | 6 | 6 | **.68** | **.83** |
| ***alticola*** | 6 | 5 | 3.8 ± .7 | | |
| Speckled teal: | | | | | |
| *flavirostris* | 12 | 12 | 8.9 ± 1.1 | **.27** | **.68** |
| ***oxyptera*** | 10 | 7 | 4.9 ± .9 | | |
| Silver/puna teal: | | | | | |
| *versicolor* | 13 | 17 | 15.0 ± 1.1 | **.54** | **.70** |
| ***puna*** | 1 | 2 | 1.9 ± .3 | | |
| Ornithine decarboxylase: | | | | | |
| Yellow-billed pintail: | | | | | |
| Lowland | 24 | 16 | 11.0 ± 1.4 | .01 | **.02** |
| **Highland** | 16 | 13 | 10.2 ± 1.2 | | |

**Table 3** (*Continued*)

| Locus, species, population | No. polymorphic positions | No. alleles | Allelic richness (±SD) | $F_{ST}$ | $\Phi_{ST}$ |
|---|---|---|---|---|---|
| Cinnamon teal: | | | | | |
| *cyanoptera* | 9 | 7 | 6.9 ± .3 | .01 | .01 |
| ***orinomus*** | 9 | 7 | 7.0 ± .2 | | |
| Crested duck: | | | | | |
| *specularioides* | 2 | 2 | 2 | **.35** | **.36** |
| ***alticola*** | 4 | 5 | 4.0 ± .7 | | |
| Speckled teal: | | | | | |
| *flavirostris* | 14 | 7 | 5.5 ± .9 | **.08** | **.04** |
| ***oxyptera*** | 7 | 7 | 6.1 ± .8 | | |
| Silver/puna teal: | | | | | |
| *versicolor* | 28 | 13 | 11.4 ± 1.0 | **.48** | **.58** |
| ***puna*** | 18 | 4 | 3.4 ± .6 | | |
| $\alpha$ enolase: | | | | | |
| Yellow-billed pintail: | | | | | |
| Lowland | 12 | 13 | 10.5 ± 1.2 | .01 | .01 |
| **Highland** | 11 | 13 | 9.9 ± 1.2 | | |
| Cinnamon teal: | | | | | |
| *cyanoptera* | 14 | 9 | 8.1 ± .7 | **.03** | **.09** |
| ***orinomus*** | 7 | 7 | 6.8 ± .4 | | |
| Crested duck: | | | | | |
| *specularioides* | 14 | 6 | 6 | **.26** | −.02 |
| ***alticola*** | 15 | 5 | 4.6 ± .6 | | |
| Speckled teal: | | | | | |
| *flavirostris* | 7 | 7 | 4.3 ± 1.1 | **.07** | **.05** |
| ***oxyptera*** | 7 | 7 | 5.7 ± .8 | | |
| Silver/puna teal: | | | | | |
| *versicolor* | 18 | 11 | 10.2 ± .7 | **.21** | **.34** |
| ***puna*** | 12 | 2 | 2.0 ± .0 | | |
| $\beta$ fibrinogen: | | | | | |
| Yellow-billed pintail: | | | | | |
| Lowland | 6 | 6 | 5.4 ± .6 | .00 | −.01 |
| **Highland** | 5 | 5 | 4.7 ± .5 | | |
| Cinnamon teal: | | | | | |
| *cyanoptera* | 5 | 4 | 4.0 ± .0 | **.10** | **.04** |
| ***orinomus*** | 6 | 5 | 4.4 ± .6 | | |
| Crested duck: | | | | | |
| *specularioides* | 1 | 2 | 2 | **.13** | **.15** |
| ***alticola*** | 1 | 2 | 1.6 ± .5 | | |
| Speckled teal: | | | | | |
| *flavirostris* | 5 | 6 | 5.1 ± .8 | **.05** | **.09** |
| ***oxyptera*** | 2 | 3 | 2.6 ± .5 | | |
| Silver/puna teal: | | | | | |
| *versicolor* | 5 | 5 | 4.8 ± .4 | **.36** | **.28** |
| ***puna*** | 2 | 3 | 2.5 ± .5 | | |
| N-methyl D aspartate 1 glutamate receptor: | | | | | |
| Yellow-billed pintail: | | | | | |
| Lowland | 31 | 34 | 21.1 ± 2.1 | **.02** | **.02** |
| **Highland** | 22 | 23 | 16.1 ± 1.7 | | |
| Cinnamon teal: | | | | | |
| *cyanoptera* | 18 | 18 | 11.9 ± 1.6 | **.01** | **.02** |
| ***orinomus*** | 12 | 12 | 9.3 ± 1.1 | | |
| Crested duck: | | | | | |
| *specularioides* | 9 | 11 | 11 | **.06** | −.02 |
| ***alticola*** | 11 | 12 | 9.7 ± 1.0 | | |

**Table 3** (*Continued*)

| Locus, species, population | No. polymorphic positions | No. alleles | Allelic richness (±SD) | $F_{ST}$ | $\Phi_{ST}$ |
|---|---|---|---|---|---|
| Speckled teal: | | | | | |
| *flavirostris* | 28 | 35 | 20.8 ± 2.1 | **.04** | **.06** |
| ***oxyptera*** | 26 | 32 | 21.3 ± 2.0 | | |
| Silver/puna teal: | | | | | |
| *versicolor* | 6 | 7 | 6.1 ± .8 | **.14** | **.28** |
| ***puna*** | 2 | 3 | 2.5 ± .5 | | |
| Phosphoenolpyruvate carboxykinase: | | | | | |
| Yellow-billed pintail: | | | | | |
| Lowland | 14 | 9 | 5.9 ± 1.1 | **.03** | **.03** |
| **Highland** | 10 | 8 | 6.0 ± 1.0 | | |
| Cinnamon teal: | | | | | |
| *cyanoptera* | 4 | 5 | 4.1 ± .7 | .01 | .01 |
| ***orinomus*** | 3 | 4 | 3.5 ± .5 | | |
| Crested duck: | | | | | |
| *specularioides* | 1 | 2 | 2 | .01 | .02 |
| ***alticola*** | 1 | 2 | 2.0 ± .2 | | |
| Speckled teal: | | | | | |
| *flavirostris* | 9 | 10 | 7.2 ± 1.2 | **.06** | **.11** |
| ***oxyptera*** | 5 | 5 | 4.9 ± .3 | | |
| Silver/puna teal: | | | | | |
| *versicolor* | 6 | 7 | 6.4 ± .6 | **.40** | **.46** |
| ***puna*** | 3 | 4 | 3.5 ± .5 | | |

Note: Highland populations are shown in bold text. Bold numerals indicate a significant *P* value. Nonsignificant $\Phi_{ST}$ *P* values for α enolase and N-methyl D aspartate 1 glutamate receptor in crested duck result from indels being treated as "missing data" in the calculation. cds = coding sequence.

(73.1% vs. 50.4%), but were also plainly evident for crested duck (94.0% vs. 69.1%) and speckled teal (98.2% vs. 86.2%). Sensitivity analysis suggests that these data patterns did not result from adding more loci but were driven by greater divergence in the hemoglobin loci (see also McCracken et al. 2009*a*).

### *Hemoglobins Exhibited Other Signatures of Balancing Selection*

The allelic networks for the αA and βA subunits showed other patterns consistent with the effects of balancing selection or a selective sweep acting on amino acids segregating at high frequency in the highlands. The highland cinnamon teal subspecies *Anas cyanoptera orinomus* possessed only a single Ser-α9 allele, whereas the lowland subspecies *Anas cyanoptera cyanoptera* possessed 15 Asn-α9 alleles, (fig. A3 in the online edition of the *American Naturalist*). Thr-α77 occurred in five of the six different highland speckled teal alleles, but the lowland population had 48 alleles and only five of these 48 possessed Thr-α77; all such lowland individuals were heterozygous (fig. A3). Tajima's (1989) *D* was significant, indicating that there was a significant excess of low-frequency polymorphisms in highland populations of both species (table A3).

The βA subunit also showed evidence of divergent selection. Asp-β94 forms a salt bridge with the imidazole ring of the N-terminal His-β146 in human hemoglobin, which stabilizes the deoxy (T-state) structure and decreases $O_2$ affinity and contributes to the Bohr effect (McCracken et al. 2009*b*). Crested duck and silver/puna teal from the Altiplano and Patagonia were fixed for Glu-β94, whereas populations in Patagonia were fixed for Asp-β94 (fig. A3). The ancestral residue Asp-β94 was found in highland crested ducks only in Mendoza.

### *Interspecific Allele Sharing*

Parallel amino acid replacements such as those observed here may result from historically independent substitutions, shared ancestral polymorphisms, or introgression. Transspecific polymorphisms that are identical by descent within two or more populations are more likely to occur at central haplotype positions within a network than at peripheral positions.

The αA subunit alleles of crested duck were monophyletic, but the αA subunit alleles of the four *Anas* species

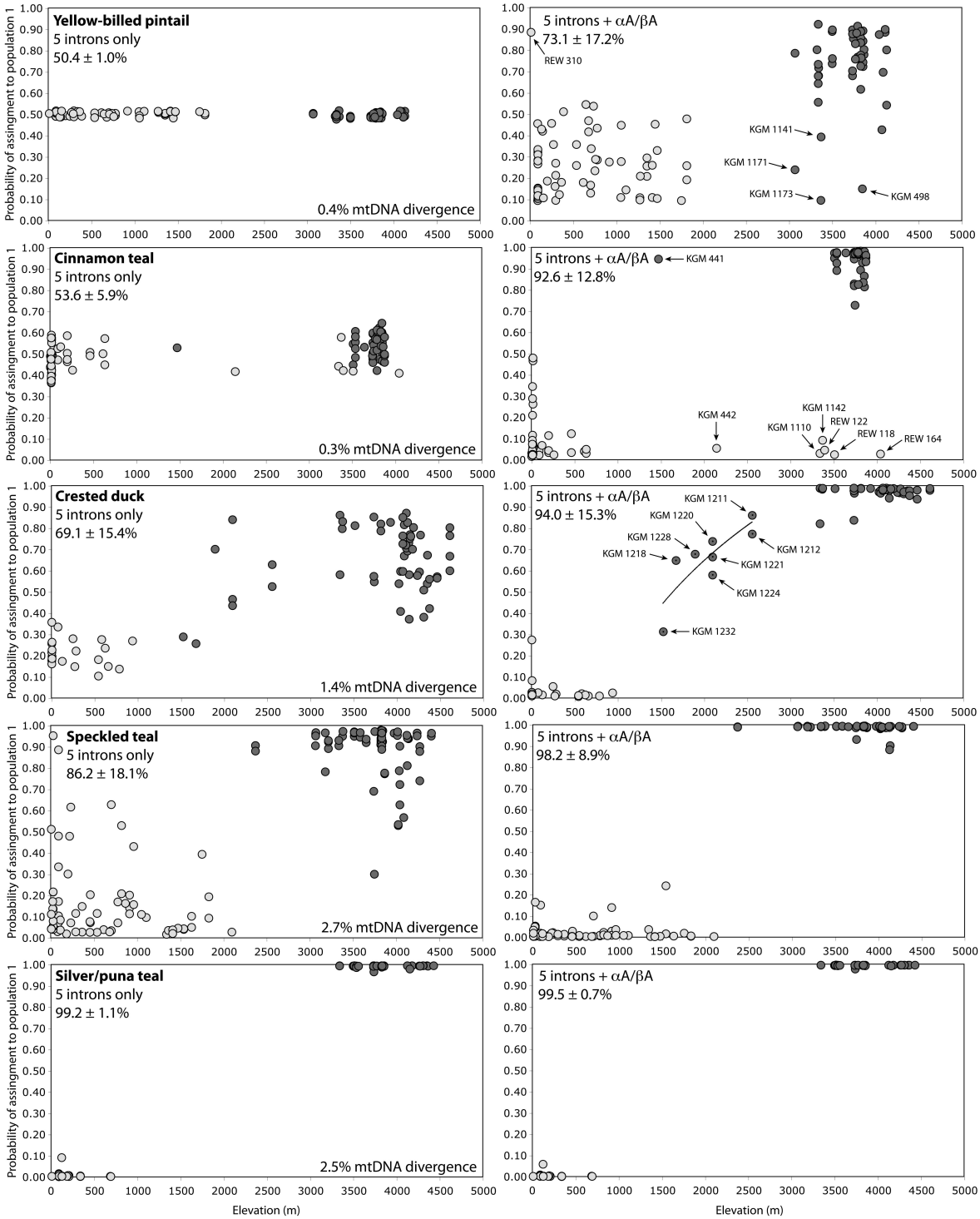


**Figure 3:** STRUCTURE analyses showing probability of assignment versus elevation for the five autosomal introns (*left column*) and the five autosomal introns and αA and βA hemoglobin subunit coding sequences combined (*right column*). Individuals collected in the highlands or classified as highland based on their morphology are shown in gray, and individuals classified as lowland are shown in white. Yellow-billed pintail, cinnamon teal, and crested duck specimens discussed in the text are labeled with their University of Alaska Museum (UAM) field catalog numbers. The five species are arranged top to bottom in order of increasing depth of divergence; uncorrected %mtDNA control region divergence (K. G. McCracken, M. Bulgarella, and R. E. Wilson, unpublished data) is shown for comparison. The logarithmic regression between posterior probability of assignment to the highlands and elevation ($y = 0.7402 \times \ln(x) - 4.9791$; $R^2 = 0.69$) is shown for eight individual crested ducks collected at intermediate elevations (1,522–2,552 m) in Mendoza, Argentina.

were not reciprocally monophyletic (fig. 4). Despite this lack of reciprocal monophyly, which is to be expected among closely related species in the same genus, only two αA subunit alleles were shared between species (fig. 4). One lowland yellow-billed pintail in Patagonia possessed an allele shared with speckled teal, and two speckled teal in the Falkland Islands possessed alleles shared with yellow-billed pintail. No two species collected in the highlands possessed shared αA subunit alleles.

Each αA subunit amino acid replacement that was overrepresented in the highlands (fig. 2) also occurred at the edge of the network (fig. 4), suggesting that these alleles were derived from historically independent codon substitutions. Alleles Thr-α5 and Ala-α8, in particular, do not occur on the same haplotype groups in crested ducks and probably evolved from divergent ancestral sequences (figs. 2, A3).

We were not able to fully evaluate allele sharing for the βA subunit because we analyzed only the coding region sequence (fig. A3). However, one particularly intriguing pattern was observed. In the highlands, the most common βA subunit alleles in yellow-billed pintail shared the same amino acid sequence as the most common speckled teal alleles. Alleles Ser-β116 and Met-β133, in particular, reside on the same exon and occur together on the same alleles in complete linkage disequilibrium in both species (fig. 2). This finding raises the possibility that the βA subunit alleles of yellow-billed pintail and speckled teal did not evolve independently but were inherited from an ancestral polymorphism that predates the species split or were transferred between species by hybridization (see "Discussion").

### *Hemoglobins Experienced Elevated Recombination Rates*

The αA and βA hemoglobin subunits showed consistently elevated recombination ($r > 1$) across taxa (fig. A4 in the online edition of the *American Naturalist*). Most introns, in contrast, had either low recombination rates ($r < 1$) or too little polymorphism to allow recombinations to be detected (fig. A4), except for GRIN1, which contained a transposable element (table 2) and had recombination rates >1.0 in four of the five sampled species (fig. A4). A recombination rate equal to 1.0 means that recombination is equally likely to occur as mutation.

### *Elevated Heterozygosity Occurred on Only One Polypeptide Subunit*

Because the αA and βA subunits occur on two different chromosomes but combine to form a single tetrameric protein, they might exhibit epistatic interactions that influence their levels of heterozygosity. Overall heterozygosities as measured by allelic composition of the amino acid sequences were greater for the αA subunit than for the βA subunit, but the pattern varied among species (fig. 2). Highland crested ducks and puna teal had high αA subunit heterozygosities (59.6% and 46.5%, respectively) relative to βA subunit heterozygosities (7.0% and 0.0%,

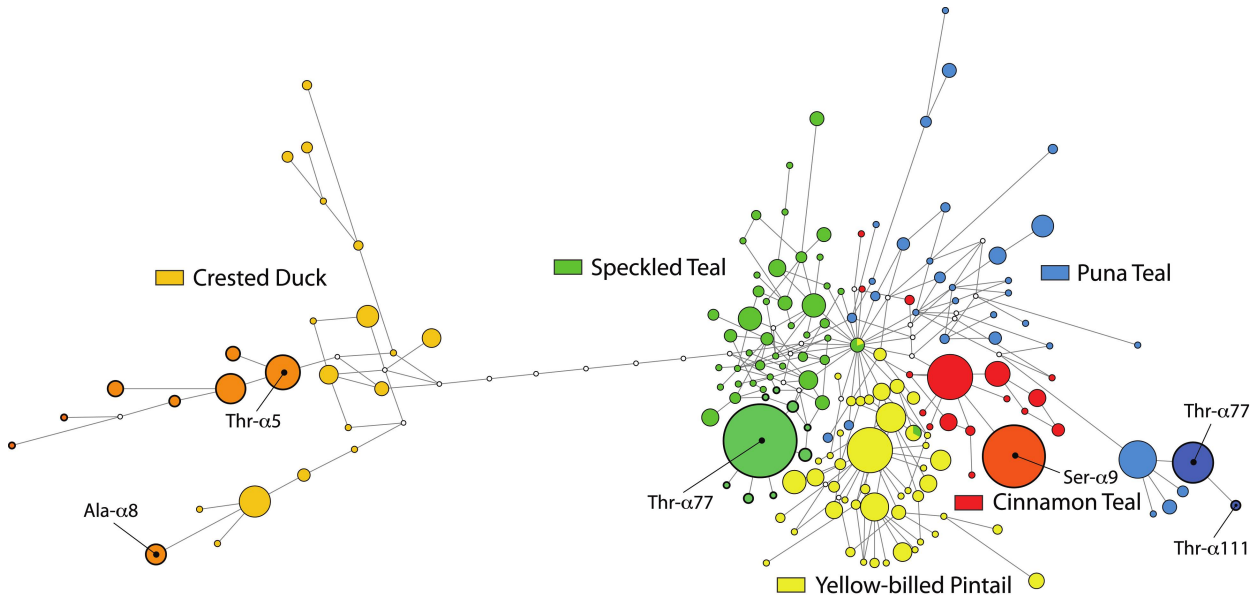


**Figure 4:** Allelic network for αA hemoglobin subunit for the five dabbling duck species combined. Alleles with amino acid replacements segregating in the highlands are shown with a different shade of color and bold outlines.

respectively). Highland cinnamon teal also had higher αA subunit heterozygosity (6.0%) than βA subunit heterozygosity (0.0%), although heterozygosity was relatively low for both subunits. In contrast, highland speckled teal and yellow-billed pintail had low αA subunit heterozygosities (1.4% and 0.0%, respectively) relative to βA subunit heterozygosity (51.4% and 21.6%, respectively). Thus, individual species that had elevated heterozygosities on the αA subunit were mostly homozygous on the βA subunit, whereas species that had elevated heterozygosities on the βA subunit were mostly homozygous or lacked amino acid replacements on the αA subunit. This intriguing pattern was also consistent in three other species of Andean ducks examined by McCracken et al. (2009*b*).

### *Effective Population Sizes Were Smaller in the Highlands*

The joint Θ ($4N_e\mu$) estimates for the five introns, as well as other genetic diversity estimates such as allelic richness, were smaller in the highlands than in the lowlands for all five population pairs (tables 3, A3; fig. A5). The Θ estimates for the αA and βA subunits also were smaller in the highlands, except for the βA subunit of crested duck, which had slightly higher Θ in the highlands (tables 3, A3; fig. A5). Estimates for the hemoglobin subunits may reflect retention of selectively favored mutations; the five introns, which are putatively neutral, offer more reliable information about relative population sizes.

### *Migration Was Greater Upslope than Downslope and More Restricted for Hemoglobin Alleles than for Reference Loci*

Loci under selection are expected to show more restricted migration rates than neutral loci. In addition, effective population size or the effects of selection on mismatched alleles may influence the directionality of inferred allelic migration rates. Upslope migration rates for the five autosomal introns overlapped LAMARC's programmatic upper limit of $M = 10{,}000$ for yellow-billed pintail and cinnamon teal (fig. 5). True values of $M$ in these species may therefore be even higher. High levels of gene flow from the lowlands to the highlands were observed overall, with upslope $4N_em$ exceeding 43.2 and 10.1 effective migrants per generation, respectively. Silver/puna teal, in contrast showed little evidence of migration either upslope or downslope, with all $4N_em < 1.0$ (fig. 5). Crested duck and speckled teal had intermediate values, with $4N_em$ ranging from 2.2 to 6.3 effective migrants (fig. 5). Upslope and downslope 95% joint estimates of $M$ for the introns overlapped widely. However, the most probable estimates were greater upslope than downslope for all species except crested duck.

The most probable estimates of $M$ for the αA and βA subunits of the four *Anas* species were 2.0–13.0 times greater upslope than downslope, despite overlap in 95% estimates (fig. 5). The αA and βA subunit alleles of most dabbling duck species that possessed amino acid replacements segregating by elevation thus were more likely to be transferred from the lowlands to the highlands than vice versa. Crested duck was unusual in this aspect of its biology; the $M$ estimate for the αA subunit of crested duck was 1.3 times greater upslope, but the $M$ estimate for the βA subunit was 2.8 times greater downslope.

The downslope estimates of $M$ for the αA and βA subunits of all five species were uniformly smaller than the joint estimates for the five autosomal introns (fig. 5). No overlap in 95% estimates was observed for cinnamon teal, yellow-billed pintail, or speckled teal. Crested duck and silver/puna teal also had smaller downslope $M$ estimates, but with overlapping 95% estimates. Importantly, the most probable estimates of downslope $4N_em$ for the αA and βA subunits did not exceed 1.8 for any species with segregating amino acid replacements, whereas $4N_em$ for the five introns ranged from 3.8 to 25.6 for all but silver/puna teal. Downslope $M$ for the yellow-billed pintail αA subunit and the cinnamon teal βA subunit, which lacked amino acid replacements, overlapped the joint estimates for the five introns. In summary, the αA and βA subunit alleles of species showing amino acid replacements segregating by elevation were much less likely to be transferred from the highlands to the lowlands than other unlinked alleles.

Upslope estimates of $M$ for the hemoglobin subunits were also diminished compared to those for the five introns, albeit less consistently among species (fig. 5). However, upslope $4N_em$ did not exceed 1.4 for any αA or βA subunit with amino acid replacements, whereas upslope $M$ for αA and βA subunits that lacked amino acid replacements fell close to the upper boundary of the prior. Upslope $M$ also was smaller for the βA subunit than for the αA subunit for the three species possessing amino acid replacements on both hemoglobin subunits.

Substitution rates for the averaged introns ($3.72 \times 10^{-9}$) were 2.0–2.5-fold greater than rates for the αA or βA subunit coding regions, but this did not change the overall results. The most probable estimates of $m$ varied in the same direction as $M$ ($m/\mu$). The observed differences in $M$ among loci were therefore not due to differences in substitution rates.

## Discussion

Hypoxia is among the most important factors affecting survival at high altitude (Hornbein and Schoene 2001; Hochachka and Somero 2002; Beall 2006), and the αA and βA hemoglobin subunits provide a simple mechanism for

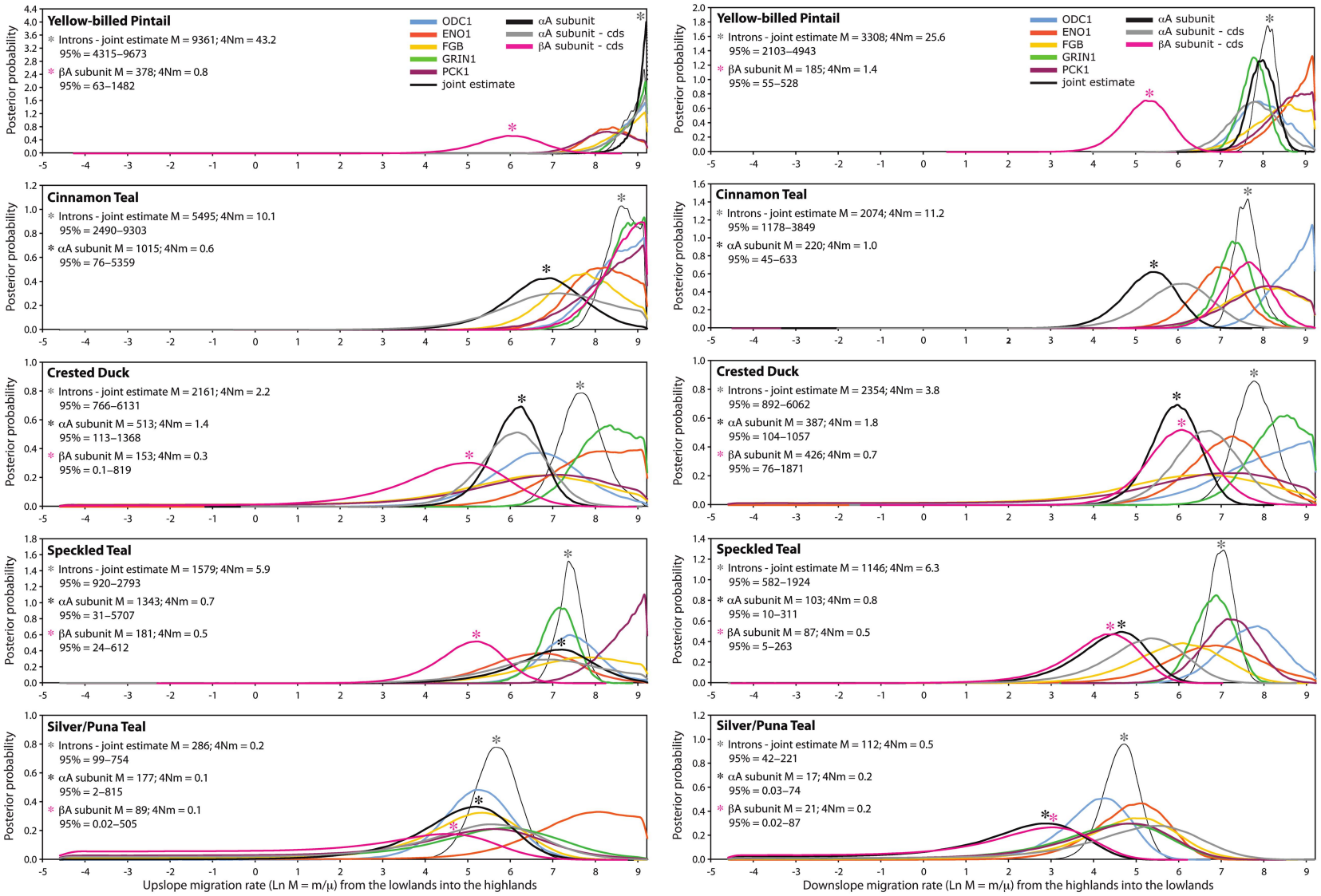


**Figure 5:** LAMARC analyses illustrating upslope (lowland to highland, *left column*) and downslope (highland to lowland, *right column*) migration rate ($M$) estimates and most probable estimates of $4N_em$ for the αA and βA hemoglobin subunits and five autosomal introns. See table 2 for intron abbreviations.

local adaptation involving two codominant loci (Storz and Moriyama 2008). Our basic predictions about the population genetic characteristics of these major hemoglobin loci were corroborated among the five independent lowland and highland lineages examined here. In the absence of selection, population history is expected to affect all loci similarly, except for the influence of stochastic processes. Indeed, we found that parameters estimated from the five introns had overlapping 95% confidence intervals within species, consistent with those loci having experienced similar population histories, despite differing historical divergences among species. In contrast, the αA and βA subunits consistently deviated for each species in the same direction and by similar degrees of magnitude, especially in estimates of migration rates and divergence, as indicated by $F_{ST}$. Such patterns are unlikely to result from stochastic forces such as genetic drift and mutation because these should result in idiosyncratic patterns among species with different evolutionary histories. Regardless of possible deviations between the inferred model and the actual population history, our results strongly support a different evolutionary history for the αA and βA subunits.

If the highland populations of these ducks are mostly nonmigratory or infrequently disperse to the lowlands, then amino acid substitutions that increase hemoglobin $O_2$ affinity would be advantageous. In principle, this can be accomplished by amino acid replacements that decrease the intrinsic stability of the deoxyhemoglobin (T-state) and shift $O_2$ equilibrium in favor of the oxy (R-state) structure or by changing the affinity for allosteric effectors that modulate hemoglobin $O_2$ affinity (Perutz 1989). Substitutions that decrease sensitivity to protons, chloride, and organic phosphate, for example, can increase $O_2$ affinity (Storz et al. 2009). Species with high-affinity hemoglobins typically possess a left-shifted $O_2$ equilibrium curve with lower $P_{50}$ (the $Po_2$ at which the hemoglobin is half-saturated with oxygen), and this characteristic has been shown to be common among endemic highland populations (Hall et al. 1936; Monge and León-Velarde 1991; Weber 2007). However, the same trait, while beneficial in the highlands, may be maladaptive in lowland environments if $O_2$ equilibrium fails to fall within the optimal range of $O_2$ tension in the tissues. Several studies have shown that high-affinity hemoglobins can be tolerated at low elevations, particularly in animals with low tissue oxygen tension (Monge and León-Velarde 1991; León-Velarde et al. 1996). However, duck muscle is known to exhibit higher venous oxygen tension than mammalian red muscle tissue (Grubb 1981), and high-altitude flight may impose more stringent selection pressure on $O_2$ binding and delivery. A variety of disorders associated with mutations that increase hemoglobin $O_2$ affinity also are known to occur in humans inhabiting lowland regions (David et al. 2002; Hardison et al. 2002; Wajcman and Galacteros 2005). In summary, there are numerous possible mechanisms that might cause the hemoglobins of these highland populations to be less fit in the lowlands or vice versa. Substitutions such as we observed at $\alpha^1\beta^1$ intersubunit contacts, in close proximity to IPP binding sites, and at external helical positions on the α and β subunits would implicitly be expected to be among the most important in mediating an adaptive response to high-altitude hypoxia.

### *Biological Interpretation of the Allelic Migration Patterns*

Our data indicated that migration estimates for the αA and βA subunits were sharply restricted compared to those for the five autosomal introns for most population pairs. This finding is consistent with the conclusion that most of these populations are experiencing gene flow balanced by countervailing selection on the major hemoglobin loci (see also McCracken et al. 2009*a*).

Fjeldså (1985) suggested that most dabbling ducks colonized the Andes from the southern lowlands. Colonization could cause estimates of migration rates from the source to the derived populations to be higher than in the opposite direction. We found that the αA and βA subunit alleles were less likely to be transferred downslope than upslope, with the exception of the crested duck βA subunit. In most cases the estimated number of effective immigrant αA and βA subunit alleles ($4N_em$) was greater downslope because Θ estimates for the lowland populations were larger, but $M$ was 2.0- to 10.0-fold greater upslope than downslope for seven of the 10 pairwise comparisons. Incorporating estimates of the locus-specific mutation rates did not affect these conclusions, and αA and βA subunits that possessed only silent polymorphisms yielded different migration patterns.

Based on joint Θ estimates for the five introns, effective population sizes ($N_e$) of all five species are probably larger in the lowlands, assuming that lowland and highland populations of the same species experienced approximately similar substitution rates. Lowland populations in southern South America are at least partially migratory, whereas the highland populations are probably nonmigratory and locally adapted to the Altiplano. If larger population size and migratory behavior facilitate dispersal, then we might expect more lowland individuals to immigrate into the highlands than vice versa. Higher migration rates upslope than downslope might alternatively reflect differences in the fitness of lowland and highland individuals in mismatched environments, but these two hypotheses are not mutually exclusive, and historical migration rates inferred using the coalescent model do not necessarily equate to current migration rates.

### *Other Aspects of Balancing Selection in Andean Dabbling Duck Species*

Divergent selection between lowland and highland populations as described above would be predicted to result in a stable polymorphism maintained by balancing selection and recurrent migration (Levene 1953; Maynard Smith 1970; Hedrick et al. 1976). This type of balancing selection is generally consistent with the patterns we observed. All five species exhibited derived amino acid replacements segregating at high frequency in the highlands, which for most species exceeded $F_{ST}$ for unlinked reference loci. The vast majority of individual ducks sampled in the lowlands, in contrast, were homozygous for ancestral alleles shared with other lowland waterfowl species (McCracken et al. 2009*b*).

Speckled teal and yellow-billed pintail are the most volant of all South American dabbling ducks and likely undertake occasional long-distance dispersals. Gene flow for these species may be sufficiently high to retard divergence at unlinked loci (McCracken et al. 2009*a*), and as a result, lowland alleles may be periodically reintroduced into the highlands, thus contributing to heterozygosity in the highlands. However, this is probably not the case for silver teal and puna teal, which were the most deeply diverged pair of taxa in our study. Between these taxa, upslope $4N_em$ was $\leq 0.2$ effective migrants per generation, suggesting that these two populations do not interbreed and are good biological species. Outlier detection in these two sibling species is thus confounded by historical linkage disequilibrium, but it also important to point out that the Thr-$\alpha$77 substitution found in puna teal evolved in five other highland waterfowl species, did not occur in other lowland waterfowl sampled worldwide, and likely has a simple adaptive basis related to IPP binding (McCracken et al. 2009*b*).

Balancing selection can also occur in a single panmictic population when some aspect of the environment is variable or fluctuating, or if individuals seasonally occupy a range of different environments subject to divergent selection pressures. In such cases, overdominance can lead to elevated heterozygosity. Heterozygous individuals that descend seasonally or periodically to lower elevations in the central high Andes, for example, might experience increased average fitness if they possess codominant mixtures of isoforms with variable oxygen affinities. Individuals that are heterozygous at a single position on the $\alpha$A subunit that modifies $O_2$ affinity, for example, could have three distinct major hemoglobin isoforms: low-affinity tetramers ($\alpha^{1L}\beta^1/\alpha^{2L}\beta^2$), hybrid tetramers ($\alpha^{1L}\beta^1/\alpha^{2H}\beta^2$), and high-affinity tetramers ($\alpha^{1H}\beta^1/\alpha^{2H}\beta^2$). Heterozygous individuals may thus have a dispersal advantage if their hemoglobin has a wider range of function. Such functional differentiation of hemoglobin isoforms has been shown to provide the basis for cascade mechanisms of blood $O_2$ transport, whereby circulating red blood cells contain a mixture of hemoglobin isoforms with different $O_2$-binding properties (Hiebl et al. 1988; Weber et al. 1988).

Two species in our study group exhibit these types of seasonal elevational movements. Crested ducks regularly descend from the Altiplano to 2,000 m in the central high Andes (and lower in Mendoza and Neuquén), as do puna teal, which can occasionally be found on the Pacific coast of Peru (Pearson and Plenge 1974; Fjeldså and Krabbe 1990). High levels of $\alpha$A subunit heterozygosity in these two species, 59.6% and 46.5% respectively, may thus be associated with seasonal movements and less influenced by gene flow with the southern lowland populations, which are distantly allopatric. Evaluating this hypothesis would require functional tests.

The highland cinnamon teal subspecies *Anas cyanoptera orinomus*, in contrast, appears to be more sedentary. The specimen we collected at 1,468 m in Salta, Argentina, is the first report we are aware of for this subspecies below the Altiplano (Fjeldså and Krabbe 1990; Hennessey et al. 2003). However, coexistence of two morphologically and genetically distinct subspecies of cinnamon teal in the highlands raises some interesting questions about their dispersal behavior and interbreeding (Wilson et al. 2010).

Finally, it is useful to examine the total number of amino acid substitutions on each allele, given that the total number of amino acid replacements for any single species was no greater than five (mean = 3.4; fig. 2). The maximum for any single $\alpha$A or $\beta$A subunit allele was three, but most alleles possessed only a single replacement. Crested duck ($n = 4$) and speckled teal ($n = 5$) possessed replacements on both subunits, exceeded the mean, and are the only dabbling duck species in the Andes that are widespread and abundant above 4,000 m, where hypobaric hypoxia is most severe. The only other waterfowl species abundant above 4,000 m is Andean goose, and it also possesses four amino acid replacements that are fixed on two subunits (McCracken et al. 2009*b*). Cinnamon teal and yellow-billed pintail, in contrast, possessed amino acid replacements on only one subunit. These two species are the least diverged at other loci and are not commonly encountered at the uppermost elevations in the Andes.

### *Did Ancestral Polymorphism or Introgression Contribute to Parallel Evolution?*

Transspecific ancestral polymorphism does not appear to have contributed to parallel $\alpha$A subunit substitutions. It is possible that replacements we observed at peripheral positions in the $\alpha$A subunit network (fig. 4) occurred at central haplotype positions historically and were incor-

porated into other sequences by recombination. However, this seems unlikely, given that all of the substitutions we observed occurred at peripheral positions and none of the same amino acid residues occurred in 115 other waterfowl species, including a series of 70 cinnamon teal from North America (*Anas cyanoptera septentrionalium*) and 24 white-cheeked pintails *(Anas bahamensis*) from South America (McCracken et al. 2009*b*).

Evaluating the $\beta$A subunit is more difficult, because analysis of the intervening intron sequences is not yet possible. At first glance, transspecific ancestral polymorphism seems like a tenable explanation for parallelism at some sites. Ser-$\beta$4, Glu-$\beta$94, or Ser-$\beta$116, in particular, were found in distantly related waterfowl genera such as *Dendrocygna*, *Cygnus*, *Anser*, *Stictonetta*, and *Tadorna* (McCracken et al. 2009*b*). However, these species possess highly divergent $\beta$A subunit sequences with various other nonsynonymous substitutions; homoplasy may thus be a more likely explanation because of their taxonomic distance.

The more pertinent question is whether amino acid replacements that were overrepresented in two or more Andean highland populations were observed in other dabbling duck species. No such replacements were found in 29 $\beta$A subunit sequences from 15 other *Anas* species (McCracken et al. 2009*b*). This finding does not preclude the possibility that Ser-$\beta$4, Ser-$\beta$116, or Met-$\beta$133 were present in the standing variation of a common ancestor that predated the split of the teal and pintail lineages. However, alleles possessing these amino acids exhibited sharply restricted migration and may experience lower fitness in lowland environments (where the common ancestors of these species likely existed). Selection may thus act to partially eliminate them from the standing variation in lowland environments. The possibility that Ser-$\beta$4, Ser-$\beta$116, or Met-$\beta$133 alleles were positively selected in the highland population of either yellow-billed pintail or speckled teal and introgressed into the highland population of the other species is perhaps more tenable, as these two species hybridize and routinely occur in mixed flocks in the highlands. Quantitatively evaluating these competing hypotheses would require a four-population coalescent model of divergence and asymmetrical migration that cannot yet be implemented in any coalescent genealogy sampler (Kuhner 2008).

### *Elevated Recombination Rates in the $\alpha$A and $\beta$A Subunits*

The elevated recombination rates we observed in the $\alpha$A and $\beta$A hemoglobin subunits of Andean dabbling ducks are consistent with studies showing that recombination is elevated in gene-dense regions subjected to strong balancing selection (Otto and Barton 1997, 2001; Barton and Charlesworth 1998; McVean et al. 2004) and that rates of recombination may increase in response to directional selection (Otto and Lenormand 2002; Fan et al. 2007). The $\alpha$ and $\beta$ subunits are coded on different chromosomes and include three linked $\alpha$ chain genes ($\alpha\pi$, $\alpha$D, $\alpha$A; Flint et al. 2001) and four linked $\beta$ chain genes ($\beta\rho$, $\beta$H, $\beta$A, $\beta\epsilon$; Reitman et al. 1993). The $\beta$ globin gene cluster experiences higher-than-average recombination (Chakravarti et al. 1984; Wall et al. 2003; Wood et al. 2005), as does the $\alpha$ globin cluster (Fodde et al. 1991). Storz et al. (2007) found elevated recombination in the duplicated $\alpha$ globins of deer mice inhabiting an elevational gradient (*Peromyscus maniculatus*).

### *Why Did Elevated Heterozygosity Occur on Only One Polypeptide Subunit?*

Each species showed elevated heterozygosity on only one hemoglobin subunit, more frequently on the $\alpha$A subunit (fig. 2). Of three additional Andean waterfowl species surveyed by McCracken et al. (2009*b*), Andean ruddy duck (*Oxyura jamaicensis ferruginea*) and Andean goose lack heterozygosity on either subunit, and torrent duck (*Merganetta armata*) shows segregating amino acid substitutions only on the $\alpha$A subunit.

Cooperative substrate binding is achieved in allosteric proteins by two or more polypeptide subunits, arranged symmetrically and existing in equilibrium between tense and relaxed states (Monod et al. 1965). Intersubunit contacts in quaternary structure proteins may thus constrain variability in amino acid sequences, and inverse relationships between heterozygosity and number of protein polypeptide subunits have been documented (Harris et al. 1977; Ward 1977). Heterozygosity of quaternary structure proteins has also been correlated with chromosomal linkage, with polypeptide subunits coded on the same locus having greater heterozygosity than subunits on different chromosomes (Harris et al. 1977).

No comparable data sets are yet available to determine whether the pattern we observed is a general feature of quaternary structure proteins, specific to hemoglobin, or a sampling artifact. One possibility is that elevated heterozygosities on two paired $\alpha$ and $\beta$ subunits might increase negative epistasis, with some combinations of alleles resulting in less-fit pairings of amino acid substitutions at different parts of the protein tetramer. Epistasis would potentially be unbalanced if one of the two subunits contributes disproportionately to more than one protein isoform. We found elevated $\alpha$A subunit heterozygosity in three out of four taxa with amino acid replacements on both subunits. The major ($\alpha A^1\beta A^1/\alpha A^2\beta A^2$) and minor ($\alpha D^1\beta A^1/\alpha D^2\beta A^2$) hemoglobin isoforms of birds possess identical $\beta$A subunits but differ in their oxygen-binding

properties because they possess different $\alpha$ subunits (Cirotto and Geraci 1975; Baumann et al. 1984; Riggs 1998; Knapp et al. 1999). The HbD isoform has higher oxygen affinity and cooperativity than HbA (Hoffman and Storz 2007; Bulgarella et al. 2009). Selection on the $\beta$A subunit may thus be more constrained than for either the $\alpha$A or $\alpha$D subunits because the $\beta$A subunit occurs in two distinct isoforms with different biochemical properties.

## Conclusion

In summary, our basic predictions about the population genetic characteristics of the major hemoglobin loci were corroborated among the five lowland and highland lineages. Interpretation of data for any single species may be equivocal in the absence of functional tests, but concordant patterns among multiple species, as we have demonstrated here, argue strongly for the influence of deterministic processes such as selection. Hypotheses presented here could be tested decisively by using biochemical assays of whole blood and purified hemolysates, followed by site-directed mutagensis to determine the intrinsic effects of the various substitutions encoding the alleles and genotypes we identified. Foremost among questions to be answered is whether the substitutions we observed increase hemoglobin affinity, and if so, are the mechanisms by which they might do so similar or dissimilar among different dabbling duck species? Many other diverse lineages inhabit the high Andes. Birds such as furnariids, tyrant flycatchers, finches, and hummingbirds notably are the most taxonomically diverse (Fjeldså and Krabbe 1990), and numerous species of mammals occur in this region (Mares et al. 1989). It is likely that most highland endemic taxa possess genetically based adaptations that confer tolerance to hypoxia and the other harsh conditions of the Altiplano. Parallel evolution such as we observed in ducks is likely more taxonomically widespread, and comparative studies far more extensive than those undertaken here could be used to test this hypothesis.

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