Molecular Ecology (2008) **17**, 4556–4569 doi: 10.1111/j.1365-294X.2008.03942.x

Transcriptomic variation and plasticity in rufous-collared sparrows (*Zonotrichia capensis*) along an altitudinal gradient

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Abstract

As modern genomic tools are developed for ecologically compelling models, field manipulation experiments will become important for establishing the role of functional genomic variation in physiological acclimation and evolutionary adaptation along environmental clines. High-altitude habitats expose individuals to hypoxic and thermal stress, necessitating physiological acclimation, which may result in evolutionary adaptation. We assayed skeletal muscle transcriptomic profiles of rufous-collared sparrows (*Zonotrichia capensis*) distributed along an altitudinal gradient on the Pacific slope of the Peruvian Andes. Nearly 200 unique transcripts were differentially expressed between high-altitude [4100 m above sea level (a.s.l.)] and low-altitude (2000 m a.s.l.) populations in their native habitats. Gene ontology and network analyses revealed that these transcripts are primarily involved in oxidative phosphorylation, protein biosynthesis, signal transduction and oxidative stress response pathways. To assess the plasticity in gene expression differences between populations, we performed a 'common garden' experiment in which high- and low-altitude individuals were transferred to a common low-altitude site (~150 m). None of the genes that were differentially expressed between populations at the native altitudes remained significantly different between populations in the common garden. The role of gene expression variation in adaptation and acclimation to environmental stress is largely unexplored in natural populations of birds. These results demonstrate substantial plasticity in the biochemical pathways that underpin cold and hypoxia compensation in *Z. capensis*, which may mechanistically contribute to enabling the broad altitudinal distribution of the species.

Keywords: adaptation, cross-species hybridization, ecological genomics, high altitude, microarray, phenotypic plasticity

Received 2 June 2008; revision received 4 August 2008; accepted 20 August 2008

Introduction

Identifying the genomic basis of adaptive traits is a major ambition of evolutionary biology research (Orr 2005). Vertebrate adaptations to high-elevation environments are classic examples of natural selection (Monge & Monge 1968; Frisancho 1975; Perutz 1983; Bouverot 1985). The environmental stressors on endothermic vertebrates at high altitude are well understood: cold, hypoxic conditions interact to create a metabolically challenging environment within which constant energy production must be maintained in the face of reduced oxygen availability and increased thermal stress.

Numerous studies have uncovered polymorphisms that underlie physiological adaptation to high altitude. Haemoglobin polymorphisms have provided well-known examples of adaptive divergence among high- and low-altitude taxa (Snyder 1978, 1981; Snyder *et al*. 1982, 1988; Perutz 1983; Chappell & Snyder 1984; Jessen *et al*. 1991; Monge & Leonvelarde 1991; Leonvelarde *et al*. 1996; Ramirez *et al*. 1999; Ostojic *et al*. 2000, 2002; Storz 2007; Storz *et al*. 2007), but recent studies of other metabolic pathways have also documented putative cases of adaptive divergence at the molecular level (Ehinger *et al*. 2002; Coskun *et al*. 2003; Mishmar *et al*. 2003; Ruiz-Pesini *et al*. 2004). This work has

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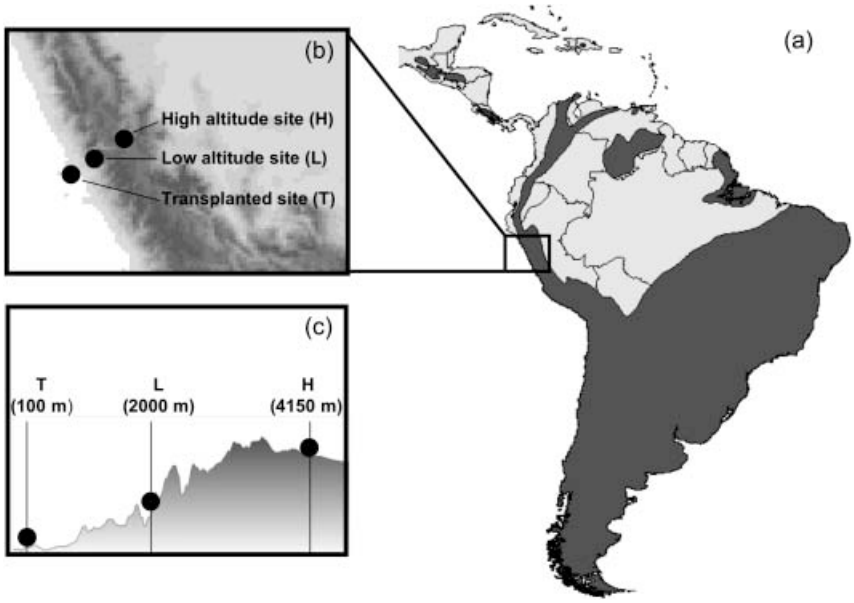

Fig. 1 Sampling localities. (a) Distribution of *Zonotrichia capensis* is illustrated in grey. (b) Sampling localities. Altitude is indicated by shading with higher altitudes being indicated as darker colours. (c) Cross-section of sampling transect. T, L, and H refer to the transplant, low altitude, and high altitude sampling sites, respectively. Altitudinal profile was generated using georeferenced sampling localities and SimpleDEM viewer. Digital range map created by Ridgely *et al*. 2003 and downloaded from InfoNatura (2005).

clearly shown that structural changes within the coding regions of candidate genes can be important for adaptation to high altitude. However, adaptive evolution may also proceed through genetic changes that alter transcript abundance, and the adaptive role of transcriptional variation in high-altitude environments remains largely unexplored.

Several lines of evidence suggest that divergence in gene expression patterns may be especially important in the early stages of adaptive divergence (King & Wilson 1975; Abzhanov *et al*. 2004, 2006; Whitehead & Crawford 2006a, b; Ortiz-Barrientos *et al*. 2007). In particular, physiological acclimation to stressful environments, such as high altitude, is often associated with up-regulation of hormones and proteins that is correlated with changes at the transcription level. Thus, on physiological timescales, variation in protein expression accounts for many acclimation mechanisms, and similar regulatory changes may also contribute to adaptation over evolutionary timescales.

Studies of the transcriptomic response to environmental stressors can provide key insights into the genes and biochemical pathways involved in physiological acclimation and evolutionary adaptation, but until recently, the genomic tools needed for large-scale transcriptome analyses were only available for a handful of model organisms. Although functional genomic studies within a select suite of model organisms have revolutionized the biological sciences, these organisms offer few opportunities to study the genomic underpinnings of acclimation and adaptation in nature, where the ecological context of adaptive evolution is better understood. Recent advances in the development of genomic tools and databases provide opportunities to study adaptation in natural populations of nonmodel organisms in unprecedented detail (Gracey *et al*. 2001, 2004; Lexar *et al*. 2004; Stolz *et al*. 2004; Butlin & Roper 2005; Rogers & Bernatchez 2005; Derome & Bernatchez 2006; Hoekstra *et al*. 2006; Lai *et al*. 2006; Wang *et al*. 2006; Whitehead & Crawford 2006a). Such studies have already provided key insights into outstanding questions regarding the process of adaptive evolution and speciation, but much work remains. In particular, field manipulation experiments offer the power to distinguish transcriptional variation that is plastic over physiological timescales, from that which is fixed over such timescales. The former is likely to be important in phenotypically plastic acclimation responses, whereas the latter may be due to heritable variation that is important in evolutionary adaptation. To our knowledge, the transcriptomics of high-altitude acclimation and adaptation are unexplored in natural populations of any organism. Wild bird populations offer a powerful model system for such studies.

Here, we tested for differential gene expression among populations of a widespread Neotropical passerine, the rufous-collared sparrow (*Zonotrichia capensis*), that are distributed along an altitudinal gradient on the Pacific slope of the Peruvian Andes. Gene expression levels were profiled from birds sampled in their native habitats, and also from birds transplanted to a common altitudinal environment. The primary goal was to test whether altitude-specific expression patterns were plastic or fixed, thus offering insights into the role of regulatory variation in physiological acclimation and evolutionary adaptation to high altitude.

The rufous-collared sparrow has one of the largest distributions of any Neotropical passerine, occurring from southern Mexico, south through Central America and several Caribbean Islands, and across nearly the entire continent of South America (Chapman 1940; Ridgely &

Tudor 1989; Fig. 1). Due to its broad geographic distribution that spans heterogeneous landscapes, there is potential for local adaptation. Along the Pacific slope of the Peruvian Andes, the rufous-collared sparrow is continuously distributed from sea level to over 4600 m above sea level (a.s.l.), but gene flow is substantially reduced along altitudinal gradients (Cheviron and Brumfield, in preparation). Moreover, populations occurring at different altitudes differ in physiological parameters that are likely to be adaptive. Individuals collected at 4500 m a.s.l. have significantly lower critical temperatures (the temperature at which metabolic resources must be used to maintain constant body temperature) than those collected at sea level, consistent with adaptation to cold, high-altitude habitats (Castro 1983; Castro *et al*. 1985; Castro & Wunder 1990).

In this study, we extended a heterospecific genomic tool, zebra finch (*Taeniopygia guttata*) cDNA microarrays, to the study of variation in transcriptomic patterns between two rufous-collared sparrow populations that are distributed along an altitudinal gradient. This study had two main objectives. First, we tested for evidence of differential expression between populations sampled in their native high- and low-altitude environments to identify genes and biochemical pathways that may be important for high-altitude stress compensation. Second, we performed a 'common garden' experiment, wherein high- and low-altitude individuals were transplanted to a single low-altitude site, to distinguish expression differences that are plastic in response to the environment, fixed between populations, or interact between population and environment.

Materials and methods

Sample collection and experimental design

Eight adult *Zonotrichia capensis* individuals were sampled using mist nets from each of two study sites, one high altitude and one low altitude, on the Pacific slope of the central Andes in Peru (Fig. 1). The high-altitude site was located approximately 4150 m a.s.l. near the town of Ondores (11 05′S, 76 09′W). The low-altitude site was located at about 2000 m a.s.l., approximately 3.5 km N of the town of Huinco (11 44′S, 76 36′W) below the altitude at which many physiological acclimation mechanisms begin to be manifested (Bouverot 1985). The high- and low-altitude populations are genetically differentiated (mtDNA $\Phi_{ST} = 0.16$; $P = 0.01$), and the population sampled at 2000 m is genetically indistinguishable from populations sampled near sea level (400 m a.s.l.; mtDNA $\Phi_{ST} = 0.0$; Cheviron and Brumfield, in preparation). A phylogeographic break between the high- and low-altitude populations occurs at approximately 3800 m a.s.l., approximately 1800 m above the low-altitude site (Cheviron and Brumfield, in preparation).

Expression profiles were measured in four treatment groups: high-altitude native, high-altitude transplant, low-altitude native, and low-altitude transplant. Within sites, individuals were randomly assigned to one of two experimental groups: native or transplant. Individuals in the native high-altitude and native low-altitude treatments were captured, housed in cages at ambient temperature for 1 to 4 days, and tissues were sampled at their native altitude. Individuals in the transplanted high-altitude group were mist-netted at 4150 m a.s.l. and transplanted to Lima, Peru (~150 m a.s.l.), housed in cages at ambient temperature for 7 days and sacrificed. This treatment allowed us to determine the degree of plasticity in gene expression patterns by testing whether expression differences between the native high- and low-altitude groups persisted during a 1-week acclimation period near sea level. Although physiological acclimation to high altitude can take several weeks to months, in this study, we transplanted birds from high to low altitudes. Quantitative polymerase chain reaction (qPCR) analyses have shown that although several genes are up-regulated at high altitude in humans, transcript abundance of all of these genes reaches low-altitude levels within a day of arrival at sea level (Appenzeller *et al*. 2006). Although a reciprocal transplant experiment, wherein low-altitude individuals are transplanted to a high-altitude site, would be a logical extension of these experiments, this was not logistically feasible for this study.

We sampled approximately 400 mg of pectoral muscle tissue from each individual. Tissue samples were surgically removed immediately after sacrifice and flash-frozen in liquid nitrogen. All individuals included in the study were sampled in February 2007 within a 1-week period in order to minimize seasonal variation in expression profiles. Voucher specimens for the tissue samples are housed in the ornithological collections of the Louisiana State University Museum of Zoology and Centro de Biodiversidad y Ornitologia in Lima, Peru (Appendix).

Sample preparation

We extracted total RNA from approximately 40 mg of pectoral muscle tissue using TRIzol (Invitrogen) in conjunction with an RNeasy mini kit (QIAGEN) according to the manufacturers' protocols. Purified total RNA was quantified using a Nanodrop ND-1000 spectrophotometer, and RNA integrity was assessed via gel electrophoresis on an Experion Automated Electrophoresis Station (Bio-Rad). All RNA extracts were stored at –80 C until further processing.

To prepare samples for cDNA microarray hybridizations, 10 g of total RNA was reverse-transcribed in 30 L reaction volumes containing 2 L of dT_{18} primer (1 mg/mL), 1 L of random hexamer primer (3 mg/mL; Life Technologies), 6 L of 5× First Strand Buffer (Invitrogen), 0.3 L 0.1 M DTT, 0.6 L 50× aminoallyl dNTP mix (25 mM dATP, 25 mM

dCTP, 25 mM dGTP, 15 mM dTTP, 10 mM aminoallyl-dUTP), 2 L (400 U) of SuperScript III Reverse Transcriptase (Invitrogen) and 0.5 L (20 U) of RNase Inhibitor (New England Biolabs). Reactions were incubated overnight at 46 C. Template RNA was then hydrolyzed by the addition of 10 L of 1 M NaOH and 10 L 0.5 EDTA and incubated at 65 C for 15 min, neutralized by adding 10 L 1 M HCl, and cDNA was purified using a QIAGEN QIAquick PCR purification kit. Cyanine 3 (Cy3) and Cyanine 5 (Cy5) coupling volumes were performed in 9 L reactions by resuspending Cy-dyes in 9 L of 0.1 M Na_2CO_3 and adding them to the dried cDNA samples, incubating the mixture at room temperature for 2 h in the dark, and purified using a QIAGEN QIAquick PCR purification kit.

Microarray hybridization

We used a spotted cDNA microarray developed for zebra finch (*Taeniopygia guttata*) (Wada *et al*. 2006) to characterize transcriptomic profiles for each individual in the study. This microarray contains 19 928 clones that were isolated from zebra finch brain cDNA libraries (Wada *et al*. 2006). Cross-species microarray hybridization is a relatively novel means of extending genomic tools to nonmodel species (Bar-Or *et al*. 2007). The utility of this approach depends on the degree of sequence divergence between the target species (the species of interest) and the reference species (the species for which the microarray was designed). Competitive hybridization between rufous-collared sparrow and zebra finch transcripts failed to reveal any substantial reduction in the hybridization efficiency of sparrow transcripts compared to those of zebra finch (Fig. S1, Supporting information), and we were able to conservatively detect hybridization to nearly 6000 cDNA spots (see Results). Although direct comparisons between *Z. capensis* and *T. guttata* could be problematic because of heterologous hybridization bias, our comparisons should not be compromised by such biases because the recently diverged (~10 000 years ago) high- and low-altitude populations of rufous-collared sparrow are equally phylogenetically distant from *T. guttata*.

Microarray analysis was applied to the four individuals from each of the four experimental groups. Each sample was hybridized twice, labelled once with Cy3 and once with Cy5 to eliminate dye bias, resulting in a total of 32 hybridizations. The Cy3 and Cy5 hybridizations were balanced (although incompletely) in a loop design.

Immediately prior to hybridization, arrays were incubated at 42 C in prehybridization buffer (5× SSC, 0.1% SDS, 0.5% BSA) for 1 h, then rinsed in de-ionized water, and spun dry by centrifugation at 150 × *g* for 3 min. Labelled cDNA samples were resuspended in 40 L of SlideHyb hybridization buffer (Ambion), paired Cy3 and Cy5 labelled samples were mixed together, and incubated at 70 C for 10 min before hybridization to the arrays. Hybridized arrays were placed in Corning Microarray hybridization chambers and incubated 12–18 h in a 42 C waterbath. Following hybridization, slides were washed in 0.1× SSC with 0.2% SDS at 42 C for 5 min. Two successive washes were carried out at room temperature. The first was in 0.1× SSC with 0.2% SDS, and the second was in 0.1× SSC. Slides were spun dry by centrifugation at 150 × *g* for 3 min and scanned using a Packard Bioscience ScanArray Express microarray scanner. The software package ImaGene (Biodiscovery) was used to quantify spot signals as fluorescence intensities for each dye channel.

Data processing and statistical analyses

Raw fluorescence intensities were normalized using the general intensity-dependent model implemented in the program eCADS (Dabeny & Storey 2007). Normalized $\log_2$ values of Cy3 and Cy5 fluorescence intensities were treated as technical replicates and averaged for each individual. We tested for evidence of differential gene expression among experimental groups using a linear model with two factors representing population of origin (high altitude – 4100 m vs. low altitude – 2000 m) and location of sampling (native altitude – 4100 or 2000 m vs. transplanted altitude – 150 m). For a given gene, this model can be written as:

$$I_{abj} = \quad + \alpha_a + \beta_b + \alpha\beta_{ab} + \varepsilon_{abj},$$

where I is the $\log_2$ fluorescence intensity of gene j, and $a = b = j = 1, 2, \ldots, j$ genes. In this two-way factorial model, is the parametric mean, α and β are the main effects of population of origin and sampling location, respectively, and $\alpha\beta$ is a two-way interaction between the two main effects. The random error term ε is assumed to have a mean of zero and constant variance σ^2 pooled across slides. Genes with significant main effects of α or β are differentially expressed between populations or transplant treatments, respectively. Each cDNA spot was treated independently, and this model was fit to each spot on the array using the *lmfit* function in the R program limma (Smyth 2005). Pooled variance was estimated using empirical Bayes standard error shrinkage using the *ebayes* function in limma. Multiple testing was corrected by calculating the false discovery rate (FDR) using the program QVALUE (Storey & Tibshirani 2004). Unequal variance among treatment groups was tested for genes with significant main effects using a Levene's test in the program JMP 5.0.1.2 (SAS Institute). Finally, hierarchical clustering was performed for all genes showing significant main effects using JMP.

To examine the degree of plasticity in gene expression profiles, we used *post hoc* Tukey HSD tests for each gene exhibiting significant population main effects to determine which of the group means were significantly different

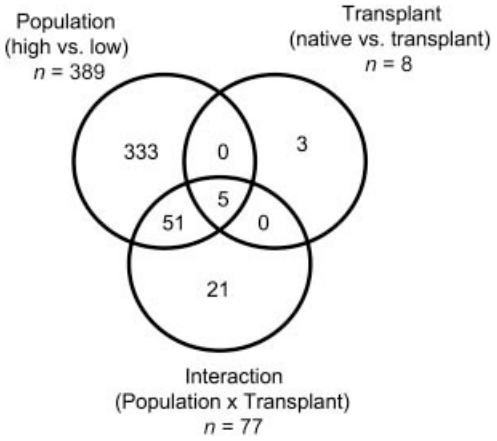

Fig. 2 Venn diagram of cDNAs with significant linear model main effects.

($P < 0.05$). Gene expression levels were considered plastic if mean expression levels differed in comparisons between native altitude groups, but were not significantly different in the common environment. *Post hoc* Tukey HSD tests were performed using JMP.

For logistical reasons, total cage time varied among individuals in the experimental treatments. Specifically, individuals were collected over a 1-week period, which resulted in some individuals being kept in captivity longer than others. All of the individuals in the transplanted treatments were kept in the common garden for 1 week, but the variance in total cage time resulted from the time it took to catch individuals at the native elevations before transplantation to the common garden. To test whether cage time affected expression variation, we tested for correlations between cage time and expression levels for every gene that exhibited a significant linear model main effect using linear regression analysis; only three genes (0.9%) exhibited a significant ($P < 0.05$) correlation between time in captivity and expression level. Likewise, because the sexes of *Z. capensis* are morphologically indistinguishable by non-invasive examination, random assignment of individuals to treatment groups resulted in unbalanced sex ratios among groups. We evaluated the effect of sex (determined by dissection) on gene expression using *t*-tests; expression levels did not differ significantly between males and females for any of the genes with significant linear model main effects. Linear regressions and *t*-tests were performed using JMP. These tests indicate that main treatment effects are not influenced by altered sex ratios or differences in cage time between the experimental groups.

Gene ontology analyses and pathway analyses

We used several databases to determine gene ontology terms for each of the transcripts that showed significant linear model main effects. These databases included AmiGO (http://amigo.geneontology.org), the NCBI Gene database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene), and the Ingenuity Knowledge Database tool in the Ingenuity Pathways Analysis (IPA) software package (Ingenuity Systems). Pathways were generated using IPA.

Results

Microarray performance

We used several quality control criteria to parse the original data set of 19 928 spot fluorescence intensities. First, we only considered spots with intensities greater than twice the local background fluorescence. Second, any spot flagged for shape or position irregularities during the Imagene analysis on any of the 16 arrays was discarded. A total of 5980 spots (27.3%) met these criteria, and the proportion of included spots is comparable to other studies utilizing heterologous hybridization (Derome & Bernatchez 2006 = 33.2%; Kennerly *et al*. 2008 = 12.0%; Saint-Cyr *et al*. 2008 = 24.0%). The criteria implemented here are conservative and undoubtedly resulted in the exclusion of 'good' spots. However, they did ensure that only the highest quality spots were included in subsequent analyses. MIAME (minimum information about a microarray experiment)-compliant data (Brazma *et al*. 2001) are publicly available at: http://www.museum.lsu.edu/brumfield.html, or by request from Z.A.C.

Differential gene expression among treatment groups

A total of 333 of 5980 cDNAs exhibited significant main effects of population of origin (high altitude – 4100 m vs. low altitude – 2000 m) without an interaction effect ($P < 0.01$, FDR = 1.7%; Fig. 2). We considered this set of cDNAs to be differentially expressed between groups living at different altitudes. For clarity, in the following discussion, we refer to spots on the array with significant population of origin effects as cDNAs, which are distinguished from unique annotated transcripts, which we refer to as transcripts.

Seventy-four of the 333 cDNAs with significant population of origin effects were either unannotated or did not generate significant sequence matches in BLAST searches (Wada *et al*. 2006). The remaining 259 cDNAs represent 188 unique annotated transcripts. Of these, 187 were up-regulated in high-altitude individuals relative to low-altitude individuals, whereas only a single transcript, metallothionein 2, was up-regulated in low-altitude individuals. Among the 333 cDNAs with significant population of origin effects, 27 (8.1%) exhibited unequal variance among the experimental groups (Levene's test, $P < 0.05$). Of these 27 cDNAs, the transplanted groups exhibited

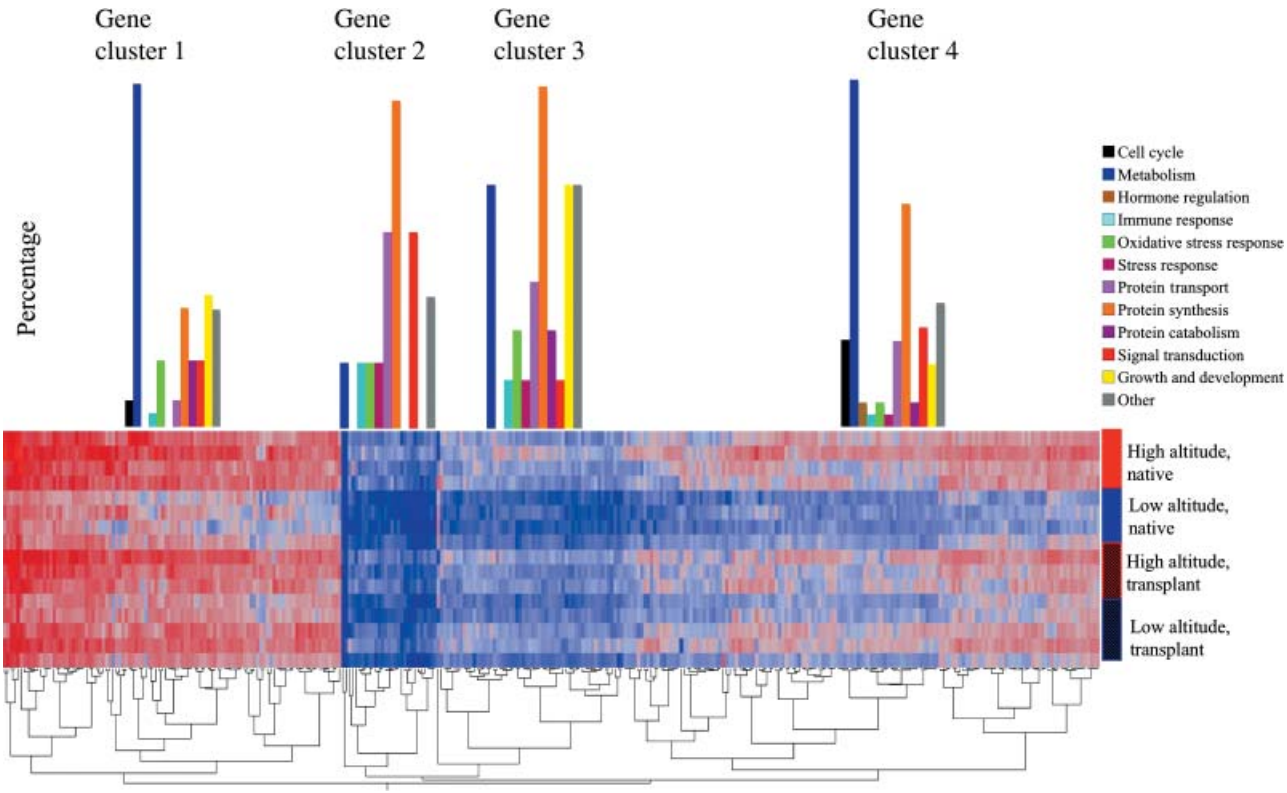

Fig. 3 Hierarchical clustering analysis of transcripts that had significant main effects of population of origin (333 genes; $P < 0.01$, FDR = 1.7%). Warmer colours indicate higher transcription. Bar charts indicate the proportion of transcripts classified into a given gene ontology (GO) category. GO categories were determined using AmiGO and NCBI Gene databases (see Methods).

elevated variance for 23 (85.2%), indicating a trend toward higher variance among transplanted birds for a small fraction of the genes.

Hierarchical clustering analysis of the 333 cDNAs with significant main effects of population of origin revealed that cDNAs clustered into four main groups based on their transcription patterns, and these groups differed in their distribution of gene ontology categories (Fig. 3). Gene clusters 1 and 4 best distinguished the high- and low-altitude sample clusters and primarily consisted of genes involved in metabolic processes, particularly those involved in oxidative phosphorylation. Gene clusters 2 and 3 consisted of genes that were relatively under-transcribed and primarily involved in protein synthesis.

We used Ingenuity Pathway Analysis to explore the degree of connectivity among the differentially expressed transcripts with population effects. This analysis revealed that many of these transcripts directly interact in several biological pathways. This result was most striking for genes involved in metabolism (Fig. 4a) and signal transduction (Fig. 4b). Nearly all of the genes included in these networks (metabolism – 89.8%, signal transduction – 88.6%) were up-regulated in high-altitude individuals, suggesting large-scale up-regulation of these pathways at high altitude in rufous-collared sparrows.

To assess the degree of plasticity in expression of cDNAs with population effects, we used *post hoc* Tukey HSD tests to test for differences in expression level among the four experimental groups. After correction for multiple comparisons, 81.1% of the cDNAs with significant main effects of population of origin were differentially expressed in comparisons between individuals sampled at their native altitude, but none were differentially expressed in the common garden, suggesting that plasticity largely governs variation in transcriptomic profiles among populations native to different altitudes. The relatively high variance in expression levels among individuals in the common garden coupled with the small sample sizes may have obscured persistent differences in gene expression in the common garden. However, the variances for most of the cDNAs (91.9%) that were differentially expressed at native altitudes were not significantly different among treatments, and sample sizes were identical for all groups.

We divided cDNAs into groups based on their Tukey HSD significance patterns, hereafter referred to as plasticity patterns, to test for commonalities among genes within a plasticity category (Table 1). Because the critical value for

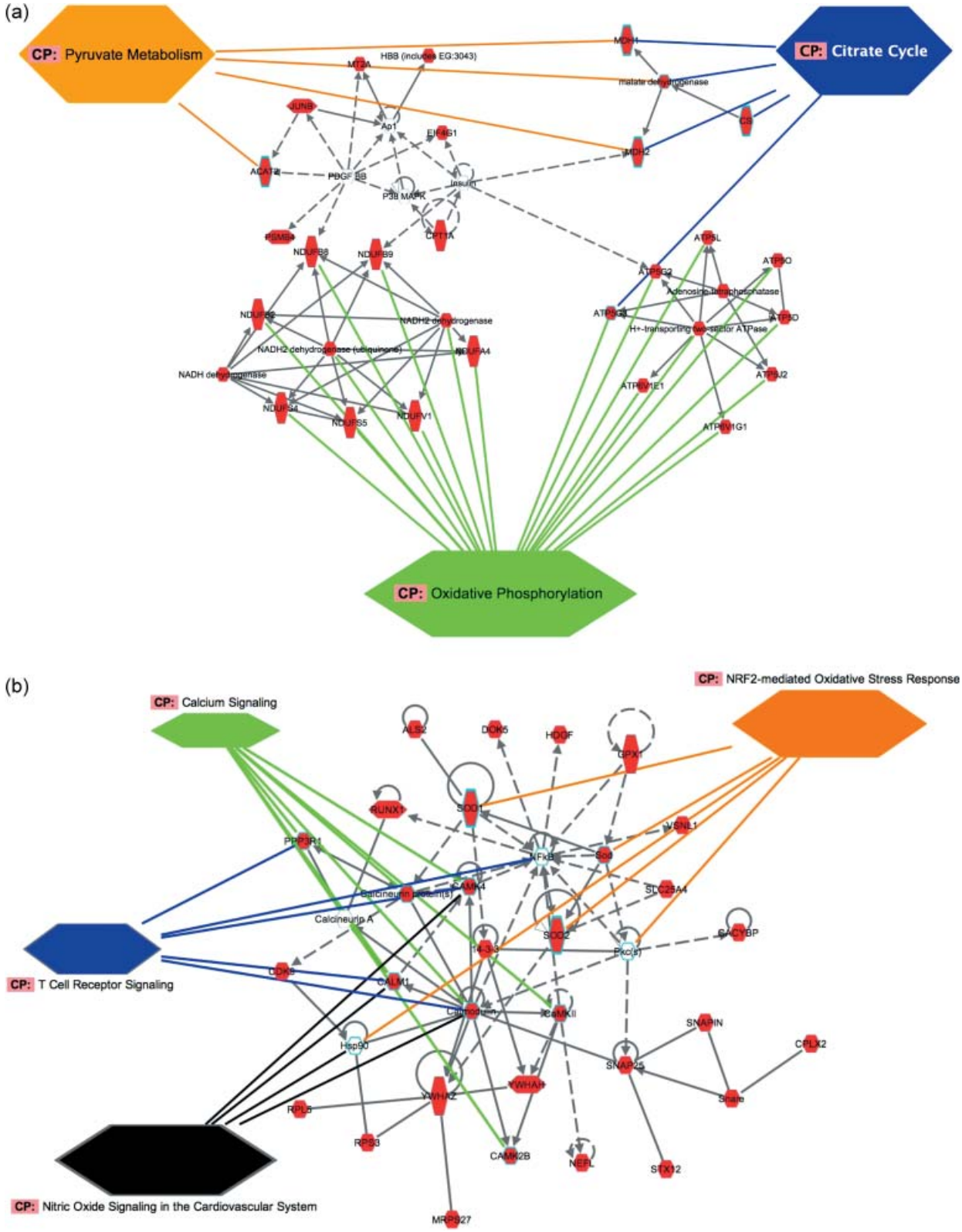

Fig. 4 Pathway analysis of differentially expressed genes. Large coloured polygons represent biological pathways, and genes involved in a given pathway are indicated by colour-coded solid lines. Smaller red polygons represent genes that are up-regulated in high altitude sparrows. Gray lines represent interactions among genes, with solid lines represent direct interactions and dashed lines indicate indirect interactions. (a) Network of genes involved in metabolic pathways. (b) Network of genes involved in signal transduction pathways.

Table 1 Plasticity patterns in gene expression for cDNAs with significant population of origin effects without interactions

Plasticity pattern	No. of cDNAs	Percentage
Group means not significant after multiple test correction	61	18.3
1. Convergence towards intermediate expression	184	55.3
2. Convergence towards native high altitude	86	25.8
3. Convergence towards native low altitude	2	0.6

the Tukey HSD test is adjusted for the number of means being compared to control for multiple comparisons (Sokal & Rolf 1995), mean fluorescence intensities for 61 cDNAs (18.3%) with population of origin effects did not differ significantly among the experimental groups. Of the remaining 272 cDNAs, 270 (99.2%) were significantly different between the native high- and native low-altitude groups, whereas none were significantly different in the common garden (between the transplanted groups). The most common plasticity pattern (pattern 1, Table 1) was one in which the native high- and low-altitude individuals were significantly different, but the transplanted birds were not different from each other or either native group (Table 1), indicating convergence towards an intermediate expression level. This result was unexpected and is difficult to interpret. A total of 184 cDNAs fell into this category, representing 112 unique annotated transcripts. Gene ontology analyses revealed that this group consisted of genes primarily involved in metabolic processes, particularly oxidative phosphorylation, the citrate cycle, and pyruvate metabolism.

Eighty-six cDNAs exhibited degrees of convergence toward native high-altitude expression levels in the transplanted birds (plasticity pattern 2, Table 1). Six of the 27 cDNAs with unequal variance among treatment groups were classified into this category, but all three transcripts that exhibited significant correlations between florescence intensity and cage time are included. Similarly, gene ontology analyses revealed that this group contained several transcripts involved in immune response signalling pathways. The combined evidence of these analyses suggests that convergence toward the high-altitude expression levels (plasticity pattern 2) may be due to transplant-induced stress response.

Finally, expression levels of two unique transcripts, NDUFB9 and YWHAH, converged towards the low-altitude expression level (plasticity pattern 3, Table 1). There was no evidence of unequal variance among experimental groups for either of these genes and transcript abundance was not correlated with cage time. Gene ontology analysis revealed that this group contains genes involved in ubiquinone biosynthesis and PTEN-signalling.

Expression levels of eight cDNAs exhibited significant main effects of sampling locality (native altitude vs. transplanted altitude) (Fig. 2) ($P < 0.01$, FDR = 99.1%). For three of these cDNAs, the effect of sampling locality was independent of population of origin. These cDNAs represent two unique annotated transcripts, cystatin and zinc finger protein 216. However, given the high FDR for these genes, it is likely that they are false positives.

Seventy-seven cDNAs had significant interactions between population of origin and sampling locality ($P < 0.01$, FDR = 11.0%). Of these 77 cDNAs, 51 had significant population main effects (Fig. 2) and fell into each of the main plasticity categories. Twenty-three exhibited convergence toward an intermediate expression level in the common garden (plasticity pattern 1). Eighteen converged on the high-altitude expression level (plasticity pattern 2), and two converged on the low-altitude expression level (plasticity pattern 3). Given that all of the plasticity patterns that we recovered represent a degree of interaction, it is surprising that a higher proportion of spots did not have significant interactions. This is probably indicative of a low statistical power to detect such interactions.

Discussion

We identified a panel of 188 unique annotated transcripts that are differentially expressed between populations of rufous-collared sparrows at high and low altitude, representing 8.6% of the unique annotated transcripts assayed; 187 of these genes were up-regulated in the high-altitude birds. The differentially expressed genes belong to relatively few gene ontology categories, with genes involved in oxidative phosphorylation, oxidative stress response, protein biosynthesis and signal transduction being overrepresented in the list of differentially expressed transcripts (Fig. 3, Table 2). A number of these differentially expressed genes are known to be involved in response to cold and hypoxic stress in a wide range of vertebrate taxa, suggesting that response systems to these stressors are also important at high altitude in rufous-collared sparrows. Expression levels of these genes, however, were highly plastic. None of the transcripts identified as being differentially expressed between individuals sampled at their native altitudes remained different in the common environment, which is remarkable given the relatively short 1-week acclimation period that was investigated. To our knowledge, this is the first study to apply transcriptomic profiling to the study of high-altitude adaptation in natural populations of any species. As with many microarray studies, these expression data are largely exploratory. The genes and biological pathways identified here are good candidates for more

Table 2 Gene ontology (GO) categories represented in the list of transcripts differentially expressed between high- and low-altitude populations. GO categories determined using the Amigo and NCBI gene databases

Gene ontology category	Percentage of differentially expressed transcripts
Metabolism	27.7
Protein synthesis	20.6
Signal transduction	9.4
Growth	7.3
Protein transport	6.1
Cell cycle	5.6
Oxidative stress response	3.9
Immune response	2.8
Protein catabolism	2.9
Hormone regulation	1.1
Stress response	1.1
Other	11.5

targeted studies of the role of gene expression variation at high altitude.

Differential gene expression between high- and low-altitude populations

Nearly 200 unique annotated transcripts were differentially expressed between high- and low-altitude populations. These genes are primarily involved in metabolism and protein biosynthesis, although a number of other gene ontology categories, including signal transduction, growth, and oxidative stress response, were also well represented (Table 2). These biological pathways are known to be the targets of natural selection at high altitude in other vertebrates, and many of the genes that are up-regulated in the high-altitude individuals are induced by exposure to cold and hypoxia in other taxa. A full list of the unique transcripts that are differentially expressed between high- and low-altitude birds is presented in Table S1, Supporting information.

When challenged with cold stress, endotherms must increase metabolic heat production to maintain a constant body temperature (MacMillen & Hinds 1992). This response is often mediated by an increase in metabolic rate, and thermogenic capacity has been shown to be under natural selection in high-altitude deer mice (*Peromyscus maniculatus*) (Hayes & O'Connor 1999). High-altitude rufous-collared sparrows have significantly greater cold tolerance than those from coastal populations (Castro 1983; Castro *et al*. 1985; Castro & Wunder 1990), suggesting cold adaptation that could be mediated through increased metabolic thermogenic capacity.

All of the individuals in this study were collected during early February 2007. During this time of year, the mean minimum temperature at the high-altitude site is approximately 1 C, approximately 10 C colder than the low-altitude site with a mean minimum temperature of 11 C (www.worldclim.org). A similar difference in temperature (13 C) causes the up-regulation of a suite of metabolic genes in carp (Gracey *et al*. 2004), many of which were also up-regulated in the high-altitude birds. Several genes involved in ATP production (ADP, ATP translocases and ATP synthases) and the citric acid cycle (malate dehydrogenase and isocitrate dehydrogenase) that are up-regulated in carp upon cold exposure were also up-regulated in the high-altitude birds (Fig. 4a). Additionally, transcription levels of several other genes involved in oxidative phosphorylation were significantly greater in the high-altitude individuals. These included genes in all five of the major complexes of the electron transport chain (e.g. Complex I – NADH dehydrogenase α 4, β 2, β 8, Fe-S; Complex II – succinate dehydrogenase; Complex III – cytochrome *c*; Complex IV – cytochrome *c* oxidase V1a; and Complex 5 – F_0 ATP synthase subunits d, f, and o, and F_1 ATP synthase δ). This result is consistent with the up-regulation of mitochondrial transcripts in cold-exposed fish (Itoi *et al*. 2003; Gracey *et al*. 2004), genes showing adaptive patterns of expression in cold-distributed killifish (Whitehead & Crawford 2006a), the increased mitochondrial densities in skeletal muscle of high-altitude birds (Hepple *et al*. 1998; Mathieu-Costello *et al*. 1998; Mathieu-Costello 2001), the adaptive significance of increased metabolic capacity in high-altitude rodents (Hayes & O'Connor 1999; Sears *et al*. 2006), and the increased cold tolerance in high-altitude populations of rufous-collared sparrows (Castro *et al*. 1985; Castro & Wunder 1990).

High-altitude habitats are unique in that the partial pressure of oxygen covaries with temperature. As a result, cold-stressed endothermic vertebrates are forced to up-regulate metabolic pathways to maintain thermal homeostasis in the face of reduced oxygen availability at high altitude. Hypoxia is known to cause changes in expression of metabolic genes by inducing expression of genes involved in glycolytic pathways (Gracey *et al*. 2001). Two glycolytic enzymes, enolase and lactate dehydrogenase B, were up-regulated in high-altitude rufous-collared sparrows, consistent with up-regulation of these genes in the livers of long-jaw mudsuckers (*Gillichthys mirabilis*) exposed to hypoxia (Gracey *et al*. 2001), although up-regulation of lactate dehydrogenase B is a well-known example of cold adaptation in killifish (*Fundulus heteroclitus*) (Crawford & Powers 1989, 1992; Crawford *et al*. 1990; Powers *et al*. 1991; Segal & Crawford 1999).

Because reactive oxygen species (ROS) are a by-product of oxidative metabolism, the up-regulation of metabolic pathways at high altitude likely increases the production of ROS (Finkel & Holbrook 2000). Correspondingly, we also found that a number of genes involved in oxidative stress

response and antioxidant protection were up-regulated in high-altitude birds. Perioxiredoxin 6, glycoxylase 1, glutathione peroxidase, superoxide dismutase 2, Cu/Zn superoxide dismutase 1, and heme oxygenase are all enzymes involved in minimizing ROS oxidative damage that were up-regulated in high-altitude birds. These enzymes are known to be important players in the oxidative stress response (Scandiolios 2002) and many are up-regulated in taxa exposed to both cold and hypoxia (Gracey *et al*. 2001, 2004; Whitehead & Crawford 2006a).

Another GO category that was highly represented in the list of differentially expressed genes was protein synthesis (37 transcripts). This result was somewhat surprising given that genes in this GO category are often down-regulated with exposure to acute hypoxia (Gracey *et al*. 2001; Hoshikawa *et al*. 2003; Simon *et al*. 2008), but is consistent with up-regulation of protein synthesis genes in cold exposed carp (Gracey *et al*. 2004). Up-regulation of these genes at high altitude likely represents a transcriptional response to cold rather than hypoxia. Protein catabolism (related to increased protein synthesis) was another GO category represented in the list of differentially expressed transcripts. Several genes involved in ubiquitin-dependant protein catabolism were up-regulated in high-altitude birds including proteasome subunits and ornithine decarboxylase antienzyme, which are also up-regulated in cold exposed carp (Gracey *et al*. 2004).

Finally, a group of genes that are known to be involved in hypoxic stress response were also among the genes with significant population effects. Included in this group were β-globin (α-globin was also significantly differentially expressed, but not at the $P < 0.01$ cutoff; $P = 0.015$) and preprocathepsin-D. Additionally, several other genes involved in nitric oxide signalling were up-regulated in the high-altitude birds (Fig. 4b).

In sum, the transcriptomic profiles of high-altitude birds seem to be more similar to those observed in response to cold exposure than to hypoxia, suggesting that for rufous-collared sparrows, cold may impose a more severe physiological demand than hypoxia at high altitude. One striking result of this study was that nearly all (187 of 188) of the differentially expressed genes were up-regulated in the high-altitude individuals. This transcriptomic signature is similar to that found in cold-exposed carp where 97% of the differentially expressed genes were up-regulated in response to decreased temperature (Gracey *et al*. 2004), but differs from that of fish exposed to hypoxic stress where approximately equal proportions of genes were up- and down-regulated (Gracey *et al*. 2001; Hoshikawa *et al*. 2003). These gene expression results are consistent with physiological studies suggesting increased cold resistance but not hypoxia resistance in high-altitude *Zonotrichia capensis* (Castro *et al*. 1985). However, a number of genes that are known to be involved in hypoxia response were differentially expressed between high- and low-altitude individuals, suggesting compensation for hypoxic stress as well. Controlled experiments are needed to separate the transcriptional effects of hypoxia from those of cold and to evaluate the relative physiological demands of these environmental stressors.

Plasticity in transcriptomic patterns

Gene expression is inherently plastic. Nonetheless, several studies have demonstrated heritable variation in gene expression among populations that persists under common-garden conditions (reviewed in Whitehead & Crawford 2006b), and some of these expression differences may be adaptive. Our results suggest a great deal of plasticity in the transcriptomic profiles of *Z. capensis*. Nearly 200 transcripts were differentially expressed between high- and low-altitude populations when they were sampled at their native altitudes, but none of these genes were significantly differentially expressed in the common environment. The high degree of plasticity in gene expression patterns over the relatively short acclimation period is striking. Although it is possible that such a short acclimation period may not have allowed the transplanted individuals to fully acclimate to the common garden, which could mask persistent expression differences, these results are consistent with human studies of plasticity in gene expression at high altitude. Humans native to high altitude in the Andes (~4300 m) exhibit elevated expression of several genes known to be involved in hypoxia response compared to low-altitude (~1500 m) populations, but expression of these genes drops to low-altitude levels within hours after arrival at sea level (Appenzeller *et al*. 2006). Likewise, myoglobin protein levels in indigenous Tibetans (Sherpas) at high altitude are nearly twice those of lowland Nepali controls, but this difference is reduced by 70% when Sherpas and Nepalis are compared at low altitude (Gelfi *et al*. 2004). Transplant experiments with an extended acclimation period are needed to determine the nature and extent of phenotypic plasticity in gene expression patterns, but these results suggest that the flexibility of the transcriptomic response to environmental stressors in *Z. capensis* may play a role in explaining its exceptionally broad altitudinal distribution.

Acknowledgements

We wish to thank INRENA and the Peruvian government for issuing scientific collecting permits (INRENA Permit no. 004-2007). Erich Jarvis, Jason Howard, Pankaj Agarwal, and Laura-Leigh Rowlette provided the cDNA microarrays and bioinformatics support. David Clayton, Kirsten Replogle, Stacy Carling, and Jen Roach provided technical assistance and advice on the laboratory procedures. Matt Carling and Terry Chesser assisted with the fieldwork. Louis Bernatchez, Curt Burney, Matt Carling, James Maley, and

two anonymous reviewers provided useful comments on an earlier version of the manuscript. This work was funded by NSF grants DEB-0543262 to R.T.B., EF-0723771 and BES-0652006 to A.W., and grants from the American Ornithologists' Union, American Museum of Natural History, Explorers' Club, LSUMNS Birdathon Funds, LSU T. Vinton Holmes Award, Society of Integrative and Comparative Biology, and Sigma Xi to Z.A.C. Experimental procedures were in accordance with Louisiana State University IACUC standards (protocol no. 06-124). The map data were provided by NatureServe in collaboration with Robert Ridgely, James Zook, The Nature Conservancy-Migratory Bird Program, Conservation International — CABS, World Wildlife fund — US, and Environment Canada — WILDSPACE.

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Supporting Information

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

Fig. S1 Competitive hybridization of rufous-collared sparrow and zebra finch muscle tissue mRNA transcripts to the zebra finch brain cDNA microarray used in this study. Zebra finch transcripts are labelled with Cy3 and rufous-collared sparrow transcripts are labelled with Cy5. The yellow colour of most spots on the array indicates little reduction in the hybridization efficiency of rufous-collared sparrow transcripts relative to zebra finch transcripts.

Table S1 List of unique transcripts that are significantly differentially expressed between high- and low-altitude individuals. All of the transcripts that are included in the list have significant main effects of population of origin ($P < 0.01$, FDR = 1.7%), but do not exhibit a significant interaction between population of origin and transplant status. Unknown cDNAs (i.e. those transcripts that were either unannotated or did not generate significant sequence matches in BLAST searches) are excluded from the list. Transcripts are listed alphabetically. Annotations are from Songbird Brain Transcriptome Database (http://songbirdtranscriptome.net/).

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Appendix

Tissue voucher numbers, experimental group assignment, sex, and cage time for individual samples. Voucher number refers to the tissue voucher housed in the Louisiana State University Museum of Zoology Collection of Genetic Resources

Voucher no.	Experimental group	Sex	Time in captivity (days)	Date collected
B-56622	High altitude, native	F	2	11 February 2007
B-56623	High altitude, native	M	4	9 February 2007
B-56624	High altitude, native	M	1	13 February 2007
B-56625	High altitude, native	M	1	13 February 2007
B-56626	High altitude, transplant	F	10	13 February 2007
B-56631	High altitude, transplant	M	14	9 February 2007
B-56632	High altitude, transplant	M	10	13 February 2007
B-56633	High altitude, transplant	M	10	13 February 2007
B-56627	Low altitude, native	M	1	15 February 2007
B-56628	Low altitude, native	F	1	15 February 2007
B-56629	Low altitude, native	F	1	15 February 2007
B-56630	Low altitude, native	M	1	15 February 2007
B-56634	Low altitude, transplant	F	8	15 February 2007
B-56635	Low altitude, transplant	F	8	15 February 2007
B-56636	Low altitude, transplant	F	8	15 February 2007
B-56637	Low altitude, transplant	M	8	15 February 2007