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HEMOGLOBIN TRANSCRIPT ABUNDANCE IN A cDNA LIBRARY FROM BONE MARROW OF CRESTED DUCKS (*LOPHONETTA SPECULARIOIDES*) IN THE PERUVIAN HIGH ANDES

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Abstract.—Hemoglobins play a key role in oxygen transport. High-oxygen-affinity hemoglobins are adaptive in hypoxic environments. To better understand adaptation to high-altitude hypoxia, we extracted RNA from the bone marrow of six Crested Ducks (*Lophonetta specularioides*) inhabiting the central high Andes of Peru (4,218–4,605 m elevation) and sequenced >2,000 expressed sequence tags (EST) from a non-normalized complementary-DNA (cDNA) library. Overall, we identified 1,692 ESTs in the expression profile representing 462 different genes. Among those, the ESTs that occurred at the highest frequency were the αA (major) hemoglobin subunit (HBA2; 22.7%), leukocyte cell-derived chemotaxin 2 (LECT2; 10%), αD (minor) hemoglobin subunit (HBA1; 9.6%), beta defensins (DEFB; 6.3%), and the βA hemoglobin subunit (HBB; 3.7%). These results provide the first quantitative identification of gene expression in bone marrow of individuals inhabiting high-altitude regions and are in agreement with the known hemopoietic and immune function of this tissue. The EST sequences identified here will be useful for a variety of studies focusing on other nontraditional model organisms. Further studies of Crested Ducks and other highland taxa will be required to determine whether the αA and αD hemoglobin subunits, which contribute to distinct isoforms with potentially different oxygen-binding properties, are differentially expressed in lowland and highland populations. *Received 28 October 2008, accepted 31 March 2009.*

Key words: cDNA library, Crested Duck, EST, gene expression, hemopoiesis, high altitude, hypoxia, *Lophonetta specularioides*.

Biblioteca de ADN Complementario Refleja la Abundancia de Transcriptos de Hemoglobina en la Médula Ósea de *Lophonetta specularioides* de los Andes Centrales de Perú

Resumen.—La molécula de hemoglobina juega un papel clave en el transporte de oxígeno. Hemoglobinas con gran afinidad por el oxígeno son adaptativas en ambientes hipóxicos. Para ampliar nuestro conocimiento acerca de la adaptación a la hipoxia de altura, se extrajo ARN de la médula ósea de seis espécimenes de *Lophonetta specularioides* de los Andes centrales de Perú (4218–4605 m) y se secuenciaron >2000 etiquetas de secuencias expresadas (EST por su sigla en inglés) de una biblioteca de cADN. En total se identificaron 1692 ESTs en el perfil de expresión las que representan 462 genes distintos. Entre éstos, las ESTs más frecuentes fueron la subunidad αA (mayor) de hemoglobina (HBA2; 22,7%), la quemotaxina 2 de los leucocitos derivada de células (LECT2; 10%), la subunidad αD (menor) de hemoglobina (HBA1; 9,6%), beta defensinas (DEFB; 6,3%) y la subunidad βA de hemoglobina (HBB; 3,7%). Estos resultados constituyen la primera identificación cuantitativa de expresión génica en la médula ósea de individuos que habitan regiones de alta montaña y coinciden con la función hematopoyética e inmune de este tejido. Las secuencias aquí identificadas pueden ser útiles para una variedad de estudios que se centren en organismos no tradicionales. Se necesitan más estudios de *L. specularioides* y otros taxones de regiones de alta montaña para determinar si hay expresión diferencial de las subunidades αA y αD de la hemoglobina entre las tierras bajas y las altiplanicies, ya que estas subunidades dan lugar a diferentes isoformas de la molécula que pueden tener distintas propiedades de unión de oxígeno.

Tolerance to high-altitude hypoxia varies widely, and some organisms have evolved extraordinary capabilities to survive under limited oxygen availability (Hochachka and Lutz 2001). Hypoxia is among the most important ecological factors affecting survival in high-altitude regions, which typically are defined as elevations above 2,000 m (Hornbein and Schoene 2001), and highland organisms have developed a variety of physiological and molecular mechanisms to cope with life at high altitude (Hochachka and Somero 2002, Weber 2007, Storz and Moriyama 2008).

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Two species of waterfowl, Bar-headed Goose (*Anser indicus*) and Andean Goose (*Chloephaga melanoptera*), have featured prominently in studies of high-altitude adaptation. Amino acid substitutions in two hemoglobin subunit genes (Pro→Ala-α119 and Leu→Ser-β55) produce similarly large effects by eliminating Van der Waals interactions on the same intersubunit contact, destabilizing the deoxy (T-state) structure and increasing the affinity of the major hemoglobin for oxygen (Perutz 1983, Jessen et al. 1991, Weber et al. 1993).

Most vertebrate hemoglobins (Hb) are tetrameric proteins composed of two α subunits and two β subunits. Birds express seven globin genes during development, in a variety of combinations. These include four embryonic genes (απ, βε, βρ, and βH) and three adult genes (αA, αD, and βA; Rowley and Ratcliffe 1988). Adult birds possess two hemoglobin components, one major isoform HbA (85% αA/βA) and one minor isoform HbD (15% αD/βA) (Borgese and Bertles 1965, Saha and Ghosh 1965). The major and minor isoforms differ only in composition of the α chains (αA vs. αD); the β chains are identical (Weber et al. 1993). The mature erythrocytes of adult birds thus contain a mixture of functionally distinct hemoglobin isoforms with different biochemical properties and potentially different oxygen-binding properties (Hoffmann and Storz 2007). Several species of birds, however, have been shown to lack a minor hemoglobin isoform in blood assays (Godovac-Zimmermann and Braunitzer 1984, 1985; Oberthür et al. 1986). Recently, Hoffmann and Storz (2007) concluded that the αD subunit of amniote vertebrates arose via duplication of a gene with a larval–embryonic function in the ancestor of all tetrapods, which suggests that the αD subunit of the HbD isoform was pre-adapted for oxygen loading at low partial pressures of oxygen. HbD has higher oxygen affinity and cooperativity of oxygen binding than HbA (Cirotto and Geraci 1975, Baumann et al. 1984, Riggs 1998, Knapp et al. 1999). Therefore, regulatory adjustments altering the stoichiometric ratio of these two isoforms may modulate rates of oxygen flux in response to changes in metabolic demand (Hiebl et al. 1987, 1988).

Several recent studies have examined gene expression in humans native to high-altitude regions. Appenzeller et al. (2006) studied gene expression induced by hypoxia in Andean highlanders in Peru, and Rajput et al. (2006) studied expression of endothelin 1 in Himalayan natives. Fewer studies of gene expression have been performed on high-altitude non-human animals, and most such studies have focused on traditional model organisms. For example, Dolt et al. (2007) investigated hypoxia-induced transcriptional changes in the liver of mice, Zhang et al. (2007) reported expression patterns of hypoxia-inducible factor 1α (HIF1α) in Tibetan Chicken (*Gallus gallus*) embryos, and D. P. Wang et al. (2006) measured expression levels of HIF1α in Tibetan Yak (*Bos grunniens*). Cheviron et al. (2008) applied transcriptomic profiling to the study of high-altitude adaptation in a natural population of non-model organisms. They studied differential gene expression in Rufous-collared Sparrows (*Zonotrichia capensis*) distributed along an altitudinal gradient in the Andes of Peru, where they observed substantial plasticity in the biochemical pathways associated with cold temperature and hypoxia compensation.

Expressed sequence tag (EST) analysis has been used extensively in large-scale complementary-DNA (cDNA) sequencing projects to identify differentially regulated genes (Brauch et al. 2005, Liu and Yang 2005). In addition, it provides quantitative information on the abundance of transcripts, as well as the possibility of identifying novel genes (Velculescu et al. 1995), and contributes to the development of polymerase chain reaction (PCR) primers for new loci for phylogenetic and phylogeographic studies (Backström et al. 2008). Several cDNA libraries have already been made for a small number of bird species, such as Wild Turkey (*Meleagris gallopavo*; Smith et al. 2000, Dranchak et al. 2003, Chaves et al. 2005), Zebra Finch (*Taeniopygia guttata*; Z. Wang et al. 2006b), House Finch (*Carpodacus mexicanus*; Z. Wang et al. 2006a), and Mallard (*Anas platyrhynchos*; Xia et al. 2007; reviewed by Bonneaud et al. 2008). Numerous other studies have analyzed gene-expression profiles in the Zebra Finch (Li et al. 2007, Slate et al. 2007). However, to our knowledge, this is the first cDNA library of a highland organism created from bone marrow, which is the principal site of hemopoiesis in adult birds. We quantified levels of gene expression in bone marrow of six Crested Ducks (*Lophonetta specularioides*) collected at 4,218–4,605 m elevation in the central high Andes of Peru, using >2,000 ESTs from a non-normalized cDNA library. The present study is an important first step for studies of avian transcription profiling, which ultimately may be used to determine whether tissue-specific gene expression of highland taxa differs from that of lowland taxa, with higher expression of the high-oxygen-affinity hemoglobin isoforms predicted to occur in the highlands.

METHODS

Sample collection.—We collected bone marrow from the epiphyses and diaphyses of leg and wing bones of six adult Crested Ducks from the high Andes of Peru in July 2006 at elevations of 4,218–4,605 m (Appendix). Tissues were harvested within 5 min of collection and stored in RNAlater, a stabilization reagent (Qiagen, Valencia, California) that allowed us to preserve the integrity of the RNA. After letting the buffer impregnate the tissues for 2–4 h, samples were stored in liquid nitrogen tanks in the field and −80°C laboratory freezers for long-term storage.

RNA isolation.—RNA was extracted from bone marrow using the RNeasy Mini Kit (Qiagen). Bone marrow was disrupted in liquid nitrogen and ground with a mortar and pestle into a fine powder. We added buffer RLT and homogenized the lysate by passing it at least five times through a blunt 20-gauge needle fitted to a syringe. Ethanol was then added to the lysate, promoting selective binding of RNA to the RNeasy membrane. The sample was applied to the RNeasy Mini spin column. Total RNA was bound to the membrane, whereas contaminants were washed away, and RNA was eluted in RNase-free water. All bind, wash, and elution steps were performed in a microcentrifuge.

cDNA library construction.—A non-normalized directional cDNA library was created from the bone marrow of highland Crested Ducks using the Creator SMART cDNA Library Construction Kit (Clontech, Mountain View, California). The library was generated from wing and leg bone marrow from four males and two females. First-strand cDNA synthesis was performed using PowerScript Reverse Transcriptase. Second-strand cDNA synthesis was performed by primer extension. cDNA was digested by Sfi I enzyme. The cDNA molecules were fractioned by column

chromatography to reduce the chance of cloning smaller inserts (<400 base pairs). cDNA was ligated to the Sfi I-digested, dephosphorylated pDNR-LIB vector provided with the kit. Recombinant plasmids were transformed into *Escherichia coli* after removing 1 μL of the ligation samples to mix with 20 μL ElectroMAX DH10B cells (Invitrogen, Carlsbad, California), and the mixture was electroporated by a Micropulser (BIO-RAD, Hercules, California). Products were resuspended and cultured at 37°C for 1 h, in a shacking incubator at 225 rpm. An aliquot of the library was incubated on Luria Bertani (LB) agar plates in the presence of 30 μg mL^{-1} of chloramphenicol overnight.

Template preparation and sequencing.—cDNA clones were propagated for sequencing by transferring recombinant *E. coli* colonies to 96-well plates containing 200 μL LB + 20 μg mL^{-1} chloramphenicol per well. Bacteria were grown at 37°C overnight. PCR was performed directly on this cell suspension (1 μL cell suspension template, 25 μL PCR volume) by using universal M13 primers. Amplified cDNA inserts were sequenced from the 5′ end with the forward M13 primer by Agencourt Bioscience (Beverly, Massachusetts).

EST annotation.—Raw sequences were first screened by visual inspection, and the pDNR-LIB vector was trimmed. Each cDNA sequence was subjected to the BLASTn local alignment program (v.2.2.18; Altschul et al. 1990) against the nonredundant nucleotide database. cDNA identifications were assigned, based on high sequence identity to previously characterized genes submitted to the database; only assignment with a BLASTn score >150 and an expectation value $<10^{-5}$ were considered significant annotations, and cDNA sequences not meeting these criteria were omitted from the expression profiles and statistical analysis, as well as all sequences shorter than 120 base pairs (Brauch et al. 2005). ESTs that exceeded these criteria were tabulated to determine the total number of each gene expressed in the bone marrow of highland Crested Ducks. Percent gene expression was computed by summing the number of ESTs matching the particular gene and dividing the sum by the total number of ESTs that matched known genes. We considered an EST a novel gene if it did not match any genes of known function in GenBank but matched numerous ESTs, which provided evidence that it represented a real transcript (Velculescu et al. 1995). One representative EST from every gene identified was submitted to the GenBank dbEST (accession numbers GO307587–GO308024).

RESULTS

The total number of cDNAs sequenced was 2,128. Among these, 234 yielded poor sequence read, no insert, or insufficient BLASTn alignment; 202 sequences were not identified in GenBank, potentially representing novel genes. Overall, we identified 462 different genes in the expression profile. Because the library was neither subtracted nor normalized, the number of clones from an individual gene may approximate the expression level of these genes in Crested Duck bone marrow (see also Xia et al. 2007). High redundancy of a specific cDNA sequence among ESTs is likely correlated with a higher expression level of that gene; therefore, the ESTs appearing frequently in the expression profile may be regarded as "highly expressed genes" (Liu and Yang 2005). In total, 1,692 unique GenBank identifications were made from a total of 1,963 high-quality sequences. A summary of the distributions of all cDNA data is shown in Table 1.

TABLE 1. Composition of Crested Duck bone marrow cDNA.

Total sequenced cDNA	2,128	Percentage
No insert, poor sequence read, insufficient BLASTn alignment	234	
No match in GenBank	202	
Mitochondrial DNA, rRNA	165	9.8
ESTs representing hemoglobins (total)	611	36.1
αA hemoglobin subunit (HBA2)	385	
αD hemoglobin subunit (HBA1)	163	
βA hemoglobin subunit (HBB)	63	
ESTs representing other proteins	916	54.1

The complete digital expression profile from this Crested Duck cDNA library is available upon request and at our website (see Acknowledgments). To establish a baseline for gene expression in duck bone marrow, we identified the 20 most abundant ESTs (Table 2). Our analysis shows that mRNA coding for the three subunits (αA, αD, βA) of the adult hemoglobin protein isoforms were among the five most common ESTs in the profile. The transcripts included hemoglobins (36%), together with leukocyte cell-derived chemotaxin 2 (LECT2; 10%), beta defensins (DEFB; 6.3%), mitochondrial cytochrome oxidase I (MT-CO1; 2.4%), immunoglobulin J (IGJ; 2.3%), ubiquitin C (UBC; 1.2%), and mitochondrial ATP synthase 6 (MT-ATP6) and cytochrome-*c* oxidase III (MT-CO3; 1%), among others. Considering only hemoglobins, the most frequently expressed subunit was αA (HBA2; 63%), the αD (HBA1) subunit constituted 26.7%, and the βA (HBB) subunit represented 10.3% of

TABLE 2. The 20 most frequent transcripts in Crested Duck bone marrow.

Gene	Counts	Percentage
αA hemoglobin subunit (HBA2)	385	22.75
Leukocyte cell-derived chemotaxin 2 (LECT2)	171	10.11
αD hemoglobin subunit (HBA1)	163	9.63
Beta defensin (DEFB)	76	4.49
βA hemoglobin subunit (HBB)	63	3.72
Cytochrome oxidase I (MT-CO1)	41	2.42
Immunoglobulin J (IGJ)	39	2.30
Ubiquitin C (UBC)	22	1.30
Beta defensin 3 (DEFB3)	20	1.18
ATP synthase 6 and cytochrome-*c* oxidase III (MT-CO3)	18	1.06
mRNA for hypothetical proteins	18	1.06
Cytochrome-*c* oxidase II (MT-CO2)	17	1.00
Predicted: hypothetical loci mRNA	13	0.77
Beta-defensin 1 (DEFB1)	12	0.71
Heat shock protein 90 alpha (HSP90A)	9	0.53
Predicted: similar to cysteine proteinase inhibitor	8	0.47
Predicted: similar to transglutaminase 3 (TGM3)	8	0.47
Lymphocyte antigen 86 (LY86)	7	0.41
H5 histone	7	0.41
BAC clone from chromosome Z	7	0.41

TABLE 3. Classification from the Gene Ontology (see Acknowledgments) for the most common protein-coding transcripts found in bone marrow of Crested Ducks.

Gene name	Biological process	Molecular function	Cellular component
Hemoglobins	Hemopoiesis Response to hypoxia Oxygen transport	Oxygen transport activity Oxygen binding	Hemoglobin complex
Leukocyte cell-derived chemotaxin 2	Chemotaxis Skeletal development	—	Cytoplasm Extracellular space
Beta defensins	Chemotaxis G protein coupled receptor protein signaling pathway Innate immune response	—	—
Immunoglobulin J	Immune response	Antigen binding	Extracellular region
Ubiquitin C	Regulation of transcription Response to heat, stress ATP-dependent proteolysis Phagocytosis, engulfment	Protein binding	Cytoplasm Nucleus
Cytochrome oxidases	Transfer of electrons from cytochrome-*c* to oxygen	Cytochrome-*c* oxidase activity	Mitochondrial inner membrane
Heat shock protein 90 alpha	Muscle thick filament assembly Myofibril assembly	Protein binding	Z disc
Lymphocyte antigen 86	Apoptosis Cell proliferation Immune response	Protein binding	Plasma membrane

the total hemoglobin expressed. We identified a total of 1,692 ESTs coding for 462 different genes in the profile. However, the gene number represented by these ESTs is actually <462. This is attributable to the fact that the ESTs corresponding to different regions of the same gene or paralogous subunits of a gene that belongs to duplicated gene families do not assemble together in the same contig. For example, in the chicken genome, 13 beta defensin genes are densely clustered within 86 Kb distance on chromosome 3q3.5–q3.7 (Xiao et al. 2004). Despite belonging to the same gene cluster, the beta defensin, beta defensin 1, and beta defensin 3 genes shown in Table 2 do not form a contig. Furthermore, alternatively spliced forms of a single gene can group in a different contig (Liu and Yang 2005). Following the Gene Ontology (GO) website (see Acknowledgments), we classified the most redundant transcripts according to their function and biological process (Table 3). GO associations organize genes into their respective biological niches and greatly reduce data complexity, revealing the most distinguishing characteristics of each group (Xia et al. 2007).

DISCUSSION

Red bone marrow produces red blood cells, white blood cells, and platelets through a process called hemopoiesis (Tortora and Derrickson 2006). It consists of developing blood cells, adipocytes, fibroblasts, and macrophages within a network of reticular fibers. It is present in developing bones of the fetus and in some adult bones, such as the pelvis, ribs, breastbone, vertebrae, skull, and ends of the bones of the arm and thigh. Yellow bone marrow consists mainly of adipose cells, which store triglycerides. Red bone marrow becomes the primary site of hemopoiesis in the last three months before birth and continues as the main source of blood cells after birth and throughout life (Tortora and Derrickson 2006). Red blood cells or erythrocytes contain the oxygen-carrying protein hemoglobin. Normal human blood has a hemoglobin concentration of 15 g per 100 mL (Hardison 1998). In birds of many species, hemoglobin occupies one-third of the red-blood-cell volume (Campbell 1995). Furthermore, hemoglobins are found in virtually all kingdoms, including eubacteria, unicellular eukaryotes, plants, and animals (Hardison 1998). Leukocyte cell-derived chemotaxin 2 functions in bone development and chemotaxis; genes encoding leukocyte markers can be expressed for generation of antibodies to discriminate between immune cell types (Xia et al. 2007). Beta defensins are cysteine-rich, cationic peptides with *in vitro* bactericidal effects for both avian and human bacterial pathogens (Ganz 2003). There are seven beta defensin genes predominantly expressed in bone marrow and the respiratory tract in chickens. However, the functional significance of these genes during inflammation and infection remains unknown (Xiao et al. 2004). Cytochrome oxidases are involved in aerobic respiration and oxidative phosphorylation. Immunoglobulins are glycoproteins with an antibody activity found in the blood, lymph, and vascularized tissues of all the jawed vertebrates (Davison et al. 2008).

Our EST data provide both sequence information and a general summary of the gene-expression profile in highland Crested Duck bone marrow. As expected, our results revealed ESTs for many genes related to oxygen transport and immunological function, therefore faithfully reflecting the role of bone marrow as a hemopoietic and immunological tissue.

Furthermore, 70% of the total level of α hemoglobin expressed corresponded to the αA subunit, and 30% to the αD subunit, in accordance with the known major and minor HbA and HbD components of bird blood (Borgese and Bertles 1965, Saha and Ghosh 1965). The low levels of βA globin subunit may be attributable to differential temporal hemoglobin synthesis. In the crustacean *Daphnia magna*, the strategy to optimize oxygen transport to the tissues when exposed to hypoxia includes the

differential synthesis of hemoglobin subunits of increased oxygen affinity (Zeis et al. 2003). Hemoglobin genes are thus regulated independently, allowing for differential expression. In vertebrates, hypoxic induction is regulated by a hypoxia-inducible transcription factor binding to oxygen-responsive elements on the DNA (for a review, see Zhu et al. 2002). Zhang et al. (2007) analyzed the expression pattern of hypoxia-inducible transcription factor 1α in Tibetan Chickens. Furthermore, Cheviron et al. (2008) reported a high degree of plasticity in the gene-expression patterns of Rufous-collared Sparrows inhabiting a steep elevational gradient in the Andes.

We do not know whether differential expression of the major (HbA) and minor hemoglobin (HbD) isoforms plays an important role in highland adaptation in species like the Crested Duck. Cirotto and Geraci (1975), Baumann et al. (1984), Riggs (1998), and Knapp et al. (1999) reported that HbD has higher oxygen affinity than HbA, but data are not yet available for Crested Ducks.

Crested Ducks comprise two subspecies: *Lophonetta s. specularioides* is endemic to Patagonia and the Falkland Islands; *L. s. alticola* inhabits the high Andes from central Peru to southern Mendoza, Argentina, and Talca, Chile (Navas and Bo 1998, Bulgarella et al. 2007). The two subspecies inhabit different elevational environments, ranging from sea level in Patagonia (*specularioides*) to 5,000 m in the central high Andes (*alticola*). Differential expression of major and minor hemoglobins may exist between highland and lowland populations of Crested Ducks if the αA and αD subunits differ in their intrinsic oxygen affinity or if individual αA or αD subunits coded by different alleles differ in their oxygen affinity because of specific amino acid mutations. Highland Crested Duck, in particular, would be expected to have higher expression levels of the minor HbD isoform if it possesses higher oxygen affinity. Physiological and quantitative molecular studies of gene expression in the lowlands and highlands should be performed to further explore adaptations to high-altitude hypoxia in birds.

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Appendix. Localities for Crested Ducks included in the present study.

Field catalogue number[a]	Date	Locality	Longitude	Latitude	Elevation (m)
KGM 1278	14 June 2006	Junín, 35 km SE Huaros	76.26123 (°W)	11.20559 (°S)	4,602
KGM 1288, 1290, 1292, 1293	15 June 2006	Junín, Marcapomacocha	76.17382 (°W)	11.24202 (°S)	4,218
KGM 1310	19 June 2006	Junín, Huarimarcán	76.23129 (°W)	11.05242 (°S)	4,605

[a]University of Alaska Museum.