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Myoglobin production in emperor penguins

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SUMMARY

Increased oxygen storage is essential to the diving capacities of marine mammals and seabirds. However, the molecular mechanisms underlying this adaptation are unknown. Myoglobin (Mb) and Mb mRNA concentrations were analyzed in emperor penguin (*Aptenodytes forsteri*) adults and chicks with spectrophotometric and RNase protection assays to evaluate production of their large Mb-bound O_2 stores. Mean pectoral Mb concentration and Mb mRNA content increased throughout the pre-fledging period and were 15-fold and 3-fold greater, respectively, in adults than in 3.5 month old chicks. Mean Mb concentration in 5.9 month old juveniles was 2.7±0.4 g 100 g^{-1} muscle (44% that of wild adults), and in adults that had been captive all their lives it was 3.7±0.1 g 100 g^{-1} muscle. The Mb and Mb mRNA data are consistent with regulation of Mb production at the level of transcription as in other animals. Significant Mb and Mb mRNA production occurred in chicks and young juveniles even without any diving activity. The further increase in adult Mb concentrations appears to require the exercise/hypoxia of diving because Mb concentration in captive, non-diving adults only reached 60% of that of wild adults. The much greater relative increase in Mb concentration than in Mb mRNA content between young chicks and adults suggests that there is not a simple 1:1 relationship between Mb mRNA content and Mb concentration. Nutritional limitation in young chicks and post-transcriptional regulation of Mb concentration may also be involved.

Key words: blood volume, emperor penguin, mRNA, myoglobin.

INTRODUCTION

Increased storage of oxygen (O_2) is one of the essential adaptations in diving mammals and penguins. The molecular mechanisms underlying increased O_2 storage are pertinent to the maturation of newborns into proficient adult divers, and to the regulatory mechanisms of gene transcription and protein production.

Although the magnitudes of the blood and muscle O_2 stores in many species have been quantified with hemoglobin (Hb), myoglobin (Mb) and blood volume (BV) measurements, the molecular mechanisms responsible for the elevated Hb and Mb concentrations in these animals are not known. To our knowledge, only one study has investigated the potential role of erythropoietin in Hb and hematocrit development in marine mammals (Richmond et al., 2005). Similarly, although transcriptional processes are considered the primary regulators of Mb production in muscle (Kanatous et al., 2009; Ordway and Garry, 2004; Underwood and Williams, 1987), the molecular mechanisms responsible for the exceptional elevations in Mb content in diving mammals and penguins also remain unknown.

In order to begin to address the molecular aspects of diving physiology, we chose to examine Mb production in emperor penguins (*Aptenodytes forsteri*). Several biochemical characteristics of this bird make it an excellent model for such investigations (Ponganis et al., 1997; Ponganis et al., 1999). First, the concentration of Mb (6.4 g 100 g^{-1} muscle) in pectoral muscle of adult emperor penguins is among the highest measured in any vertebrate. Second, this adult pectoral Mb concentration is approximately 3 times the concentration in the sartorius muscle of the leg, and it is also approximately 20 times greater than the lowest pectoral values of 3 month old emperor penguin chicks. Third, pectoral Mb concentrations progressively increase throughout the development of the chick and into adulthood. Emperor penguins, therefore, are an ideal model for studying both the differential and developmental regulation of Mb production.

Regulation of the production of Mb has been considered to occur primarily at the level of transcription (Chin et al., 1998; Kanatous et al., 2009; Ordway and Garry, 2004; Underwood and Williams, 1987). Mb mRNA content has been found to be proportional to Mb concentration in several species (Underwood and Williams, 1987; Weller et al., 1986). It has been proposed that increased Mb mRNA transcription is mediated *via* neural and muscular activities that result in elevated intracellular calcium concentrations, which, in turn, increase the expression of calcineurin. Calcineurin is able to dephosphorylate the transcription factor NFAT (nuclear factor of activated T cells), which translocates into the nucleus and coordinates Mb gene expression with other transcription factors. Hypoxia in combination with exercise accentuates this pathway and Mb production (Kanatous et al., 2009).

In this study, we examined the change in Mb concentration and Mb mRNA content of pectoral muscle as chicks developed from 3 months to 6 months of age in comparison to those of adults. We hypothesized simply that Mb concentration and Mb mRNA content would increase on a 1:1 basis throughout development and into adulthood. We also examined Mb concentration in emperor penguins that had been in captivity from a pre-fledging age in order to evaluate Mb concentration in birds that had never performed the long, deep dives which occur in the wild. Lastly, because the Mb concentration in juvenile emperor penguins was only a third that of adults while Hb concentrations were almost equivalent (Ponganis et al., 1999), we also compared blood

volumes of 6 month old juveniles and adults. We hypothesized that mass-specific blood volumes and blood O_2 stores would be equal in juveniles and adults, and that further development of the muscle O_2 store at sea was the primary determinant of the progressive increase in dive duration observed in juveniles during their first trip to sea (Ponganis et al., 1999).

MATERIALS AND METHODS

Muscle biopsies

Percutaneous needle muscle biopsies were conducted on emperor penguins (*Aptenodytes forsteri* Gray) as previously described (Ponganis et al., 1997). Duplicate biopsies at the same site were obtained for mRNA content (first biopsy) and Mb determination (second biopsy) when both were assayed. Pectoral muscle samples were obtained under local anesthesia [1% lidocaine in the skin (Ponganis et al., 1999)] from emperor penguin chicks at the Cape Washington colony in mid-November and at the end of the first week of December 2000. Assuming a hatch date of August 1 2000, approximate ages of these birds were 3.5 and 4.3 months, respectively. These age estimations are relative, and are provided to distinguish the time between sample collections. The absolute ages of the chicks are not known because the actual dates of hatching of the individual chicks are not known.

All other biopsies and blood volume determinations were conducted under general isoflurane anesthesia (Ponganis et al., 1997). Young juveniles from the Cape Washington colony (see below, approximate age 5.9 months) were biopsied at the end of January 2001. Adult birds were collected near the McMurdo Sound ice edge in October, and maintained at a sea ice camp for physiological studies at an experimental dive hole for 2 months as previously described (Ponganis et al., 2001; Ponganis et al., 2003). Biopsies of pectoral and sartorius muscles were obtained from nine adults. Blood volumes were determined in three other adult birds.

Pectoral muscle biopsies of four adult penguins were conducted at Sea World (San Diego, CA, USA). These captive adult birds had been transported to Sea World as chicks in 1988 (Kooyman and Ponganis, 1994). A 3 year old sub-adult, born in captivity, was also biopsied.

Chick sea ice camp

Ten fledgling chicks, as evidenced by molting (loss of down feathers on chest or abdomen, and/or visible appearance of yellow coloration in posterior auricular region of the head), were randomly collected from the Cape Washington colony in mid-December 2000, and transported by Twin Otter plane to the sea ice camp, located on the stable McMurdo Sound sea ice behind Scott Base and adjacent to the Ross Ice Shelf. Each bird was transported individually in a 1 m×0.7 m cylindrical, rubber container (a rubber garbage can) with 4–5 cm of packed snow in the base, and ventilation holes in the lid.

At the camp, a corral for the chicks was 10 m^2, and included a 1 m deep, 2 m×3 m pool. In addition, there was gated access to an adjacent 16 m long, 1 m wide, 1 m deep swim channel, which was also enclosed in a corral. The sea ice was 3 m thick, and sea water was pumped into the pool with a sump pump.

Chicks were hand fed about 10% of their body mass per day with Antarctic cod (*Dissostichus mawsoni* Norman) available from McMurdo fish biologists, and S. African pilchard (*Sardina ocellata* Jenyns). The birds were allowed access to the pool, and once they began to swim, they were given daily access to the 'swim channel'. Five cooperative birds, which were trainable with food reward, swam up to 10 underwater laps (<30 s submersion) per day in the channel for 10 days until the channel was taken over by Weddell seals (*Leptonychotes weddellii*). The latter reamed through the bottom ice and then used the channel as a breathing hole.

At the end of January 2001, muscle biopsies (eight birds) and blood volume determinations (five birds) were conducted under general isoflurane anesthesia. The birds were later released after helicopter transport to the McMurdo Sound ice edge (Kooyman and Ponganis, 2008).

Muscle assays

Myoglobin

Muscle biopsies were assayed for Mb concentration as previously described (Ponganis et al., 1993; Reynafarje, 1963); all such biopsies for Mb determinations were immediately frozen in liquid N_2 and maintained at –80°C until assayed.

Isolation of RNA

Total RNA was purified from muscle samples using the RNAwiz™ reagent (Ambion Inc., Austin, TX, USA) as described by the manufacturer. Muscle biopsies were homogenized in 2 ml of RNAwiz™ per 100 mg tissue immediately after biopsy at Cape Washington and at the McMurdo sea ice camp using a Tenbroeck tissue grinder. Homogenates were flash frozen in liquid nitrogen for transport to the US Antarctic research station, McMurdo Station, where RNA purification was completed. Electrophoretic analysis of RNA samples in 2% agarose revealed that this procedure yielded sufficient quantities of high quality RNA.

Cloning of the emperor penguin Mb cDNA

The complete emperor penguin Mb cDNA was amplified by RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) using the GeneRacer™ kit (Invitrogen, Carlsbad, CA, USA) utilizing the Mb-specific primers myo2 (5′-GCCACCAAGC-ACAAGATCCC-3′) and myo3 (5′-CGGAAGAGCTCCAGGG-CCTT-3′). These primers were specifically designed to amplify a portion of the Mb gene from the genomic DNA of diverse mammals, birds and reptiles (Slade et al., 1993). In this study these primers were used to amplify overlapping fragments containing the full-length 5′- and 3′-ends of the Mb cDNA. The RML-RACE procedure amplifies only full-length transcripts by elimination of uncapped messages from the reaction. Amplified fragments were subsequently cloned using the TA cloning procedure (Invitrogen), sequenced and assembled to yield a full-length cDNA. The sequence presented here has been deposited in GenBank under accession number GU735491.

Estimation of relative mRNA levels

Relative Mb mRNA levels were assessed by ribonuclease protection assay (RPA). The emperor penguin Mb-specific riboprobe template was generated by linking the T7 promoter sequence to a PCR-generated fragment of the Mb gene utilizing emperor penguin Mb-specific primers MyP-4 (5′-GCGACC-AAGCACAGAGTCCC-3′) and MyP-5 (5′-CGGAACAGCTC-CAGGGCCTT-3′) and the Lig-N-Scribe kit (Ambion). This resulted in a 137 bp antisense transcription template. An 18S antisense template was also used as an internal control for normalization (pTRI-18S, Ambion). Riboprobes were generated by *in vitro* transcription (Maxiscript kit, Ambion) in the presence of [α-^{32}P]UTP. RNase protection assays were carried out using the RPAIII kit (Ambion) as described by the manufacturer, and resolved by urea-PAGE (6%). Signal was quantified using a Molecular Dynamics 300/400× Phosphoimager.

Blood volume determinations

Plasma volume (PV) and hematocrit (Hct) were determined by Evan's blue dye dilution and microcentrifugation as previously described (Ponganis et al., 1997; Ponganis et al., 1993) in three adults and five juveniles. BV was calculated by the formula: BV=PV/(1–Hct).

Statistics

All results are expressed as means ± standard error unless otherwise specified. ANOVA and *t*-tests were conducted in Excel and SPSS (www.spss.com). Significance was assumed at $P<0.05$. Graphics were performed with Origin software (www.originlab.com).

RESULTS

The emperor penguin Mb cDNA was amplified by RML-RACE using Mb-specific primers. This approach yielded overlapping fragments containing the 5′- and 3′-ends of the Mb cDNA which were subsequently assembled to produce a full-length 722 nucleotide (nt) sequence. DNA sequence analysis revealed that the Mb cDNA consists of a 5′-untranslated region (UTR) of 60 bp followed by an open reading frame (ORF) encoding the deduced 154 amino acid Mb protein. The coding region is followed by a 3′-UTR of 247 nt. As expected, the predicted Mb amino acid sequence was highly conserved and greater than 97% similar and 86% identical to Mb proteins found in other birds (Miyazaki et al., 1998). Probes for the mRNA determinations described below utilized a central portion of the Mb coding sequence.

Mean pectoral Mb concentrations ranged from 0.4±0.1 g 100 g^{-1} muscle in five 3.5 month old chicks to 6.1±0.6 g 100 g^{-1} muscle in nine adults from McMurdo Sound. Values in the 4.3 and 5.9 month old birds were 0.9±0.2 (N=5) and 2.7±0.4 g 100 g^{-1} muscle (N=8), respectively. In captive Sea World birds, Mb concentrations were 3.7±0.1 g 100 g^{-1} muscle in adults and 3.4 g 100 g^{-1} muscle in the 3 year old sub-adult. Mean pectoral Mb concentrations were distinctly different among all the chick groups, the Sea World adults and the McMurdo adults (ANOVA, Scheffe, $P<0.05$). Mean sartorius muscle Mb concentration was 1.8±0.4 g 100 g^{-1} muscle in nine adult McMurdo penguins.

Relative Mb mRNA content was estimated by RNase protection as described in the Materials and methods section in five 3.5 month old chicks, five 4.3 month old chicks, eight 5.9 month old chicks and two of the adults. Mean pectoral Mb mRNA content (normalized to 18S RNA) ranged from 0.42 in 3.5 month old chicks to 1.1 in 5.9 month old juvenile birds (Table 1, Fig. 1). In the two adults, normalized pectoral Mb mRNA content was 1.78±0.14 (Table 1). Mean values for each age group were significantly different (ANOVA, Scheffe, $P<0.05$) except between the 3.5 and 4.3 month old birds. Sartorius normalized Mb mRNA content was 0.75, and Mb concentration 1.9 g 100 g^{-1} in one adult.

Table 1. Pectoral muscle myoglobin (Mb) mRNA content (normalized to 18S RNA) and Mb concentration in emperor penguin chicks and wild adults

Age	Mb mRNA (normalized)	Mb (g 100 g^{-1})	*N*
3.5 months	0.42±0.16	0.4±0.1	5
4.3 months	1.00±0.23	0.9±0.2	5
5.9 months	1.10±0.21	2.7±0.4	8
Adult	1.78±0.14	6.2±0.0	2

Data are means ± s.e.m.

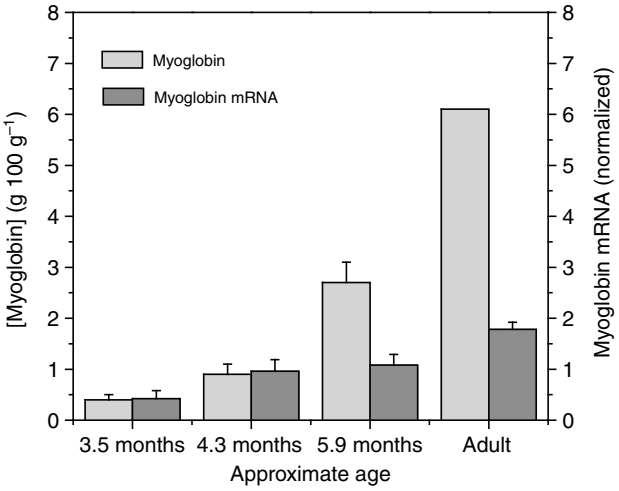

Fig. 1. Pectoral muscle myoglobin concentration and myoglobin mRNA content (normalized to 18S RNA) in emperor penguin chicks and wild adults.

In general, Mb mRNA content and Mb concentration of individuals increased proportionately on a 1:1 basis until a Mb concentration of about 1.5 g 100 g^{-1} muscle (Fig. 2). Mean Mb content and Mb mRNA of age groups appeared to increase on a 1:1 basis only for the 3.5 and 4.3 month old age groups (Fig. 1).

Mass-specific BV in three of the McMurdo adults (107±15 ml kg^{-1}) was not significantly different (t-test, $P<0.05$) from that in five of the 5.9 month old juveniles (93±12 ml kg^{-1}).

Body masses of the 3.5 and 4.3 month old Cape Washington chicks were 10.7±2.1 and 15.0±3.6 kg, respectively. The McMurdo adults weighed 24.7±2.6 kg. Captive chicks at sea ice camp initially weighed 14.8±1.8 kg; prior to release (at the time of muscle biopsies and blood volume determinations), mean body mass of all the juveniles was 18.5±1.9 kg. Individual birds gained 1.9–6.8 kg during captivity. The four Sea World adults were 22.8±1.4 kg; the sub-adult was 23.0 kg.

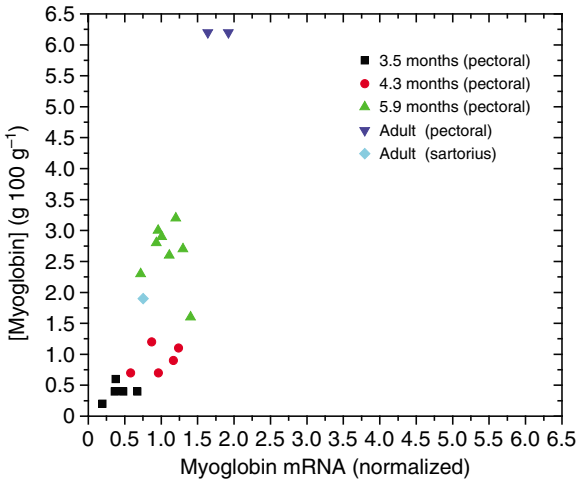

Fig. 2. Myoglobin concentration *vs* myoglobin mRNA content (normalized to 18S RNA) in pectoral muscle of individual emperor penguin chicks and juveniles (3.5, 4.8 and 5.9 months approximate age), and wild adults. A sample from the sartorius muscle of a wild adult is also included.

DISCUSSION

Pectoral muscle Mb concentrations in developing chicks and wild adults, and the sartorius muscle concentrations confirm previously reported values in emperor penguins. The adult Sea World penguins, which had never performed long, deep dives, had Mb concentrations that were only slightly greater than the 3.4 g 100 g^{-1} value of a captive sub-adult, and 60% of the mean value of adults in the wild. As previously suggested (Noren et al., 2001), the further increase in Mb beyond the juvenile stage into adulthood may require the muscular work and tissue hypoxia associated with the deep, long dives performed in the wild (Kooyman and Kooyman, 1995). Such an increase to adult Mb levels may be regulated through altered calcium transients, increased calcineurin activity and induction of Mb gene expression secondary to both hypoxia and activity as has been demonstrated in mammalian muscle (Chin et al., 1998; Kanatous et al., 2009). Indeed, increased Mb mRNA content has been demonstrated with chronic electrical stimulation of muscle and with training under hypobaric, hypoxic conditions (Underwood and Williams, 1987; Vogt et al., 2001). Some seasonal changes in Mb concentrations, presumably associated with longer dives under ice during winter, have also been reported in skeletal muscles and hearts of muskrats (*Ondatra zibethicus*) and in skeletal muscles of the Baikal seal (*Phoca sibirica*) (MacArthur, 1990; Petrov and Shoshenko, 1987).

However, the increase in Mb content to 2.7 g 100 g^{-1} muscle in 5.9 month old juveniles, and to 3.7 g 100 g^{-1} muscle in captive adult emperor penguins suggests that the swim activity and hypoxia of deep, long dives are not required to achieve a concentration that would be considered significant in most animals (i.e. 2–4 g 100 g^{-1} muscle). Prior to fledging, emperor penguin chicks do not swim. The only pectoral muscle activity during this time period in the colony is occasional wing flapping. Mb concentrations also increase during development in young elephant seals (*Mirounga angustirostris*), but during this time period the seals increase swimming and breath-hold activity in shallow water (Thorson, 1993; Thorson and Le Boeuf, 1994). Although Mb concentrations in other phocid seal pups also do not increase significantly prior to swimming and breath-hold activity, it is notable than Mb content is already high, near 2 g 100 g^{-1} muscle, in the neonatal period of these seals (Burns et al., 2005; Burns et al., 2007; Lestyk et al., 2009; Neshumova et al., 1983; Noren et al., 2005; Petrov, 1985). The mechanisms responsible for this elevation in fetal Mb are unclear because moderate hypoxia alone without exercise did not result in Mb elevation (Kanatous et al., 2009). Perhaps, however, a much more severe tissue hypoxia *in utero* during diving activity of the mother may elicit the elevations in Mb content in these newborn pinnipeds. The lowest arterial P_{O2} and hemoglobin saturations of free-diving elephant seals are less than even those of humans breathing ambient air near the top of Mount Everest (Meir et al., 2009).

In contrast to pinnipeds, the pre-fledge emperor penguin and probably chicks of other penguin species (Noren et al., 2001; Weber et al., 1974) represent models for the production of elevated Mb concentrations in the absence of any diving activity (i.e. without exercise and hypoxia). Shivering, which could serve as a contractile stimulus for activation of the calcineurin pathway for Mb production, is the primary form of thermogenesis in 1 month old king penguin chicks (*A. patagonicus*) as they first become thermally independent and leave the relative warmth of the parent's brood patch (Duchamp et al., 2002). In addition, non-shivering thermogenesis, as proposed by Noren and co-workers, may also promote Mb production in king penguin chicks (Noren et al., 2001). Non-shivering thermogenesis appears to occur in experimentally cold-adapted king penguin chicks (Duchamp et al., 1991; Duchamp et al., 1993). The increase in ryanodine receptors and Ca^{2+}-ATPase activity in the sarcoplasmic reticululm of experimentally cold-adapted birds provides evidence that intracellular calcium recycling may stimulate non-shivering thermogenesis in avian muscle (Bicudo et al., 2001; Dumonteil et al., 1993; Meis et al., 2005; Mozo et al., 2005). These calcium-linked processes may contribute to activation of the calcineurin-based pathway for Mb production. Notably, cold adaptation also caused a 2-fold elevation in Mb concentration in house sparrows (*Passer domesticus*) (Chaffee et al., 1965). It thus appears that shivering, non-shivering thermogenesis and the wing flapping of chicks are the most likely stimuli to elevate sacroplasmic calcium concentrations and promote Mb production *via* the calcineurin pathway in emperor penguin chicks. These mechanisms for increased Mb production may also apply to other birds. In the pigeon guillemot (*Cepphus columbia*), which has a pectoral Mb concentration of 2.2 g 100 g^{-1} in the adult, Mb content increases from non-detectable levels in the chick to 0.5 g 100 g^{-1} in the fledgling (Haggblom et al., 1988).

The increase in pectoral Mb concentration in developing emperor penguin chicks was associated with an increase in Mb mRNA, consistent with regulation of Mb content at the level of transcription. And, although limited in sample size, the approximately 3-fold difference in both Mb concentration and Mb mRNA content between adult pectoral and sartorius muscles would also support primary transcriptional regulation of Mb concentration (Fig. 2). However, while mean Mb concentration of adults was approximately 15 times that of the 3.5 month old chicks, the corresponding Mb mRNA content varied by only a factor of three (Fig. 1). This lack of a 1:1 correlation was also evident in the relationship between individual Mb and Mb mRNA content values (Fig. 2). These findings certainly do not support our hypothesis that Mb concentration and Mb mRNA content would simply increase in a 1:1 fashion. Thus, the regulation of Mb concentration in emperor penguins appears to be more complex than we had hypothesized. In young chicks, it is possible that nutritional limitations restrict the synthesis of Mb despite adequate Mb mRNA (i.e. low iron intake, amino acid requirements in other tissues of the developing chick including new feathers during the fledgling molt). Other regulatory mechanisms in older chicks and adults may include more efficient translation leading to increased production of Mb from the same amount of mRNA, and decreased breakdown of Mb, leading to an increased half-life and net accumulation of Mb. A good example of such post-transcriptional regulation is the role of iron regulatory proteins in mRNA stabilization and control of mRNA translation (Wallander et al., 2006).

Post-transcriptional regulation in addition to transcriptional regulation of Mb concentration has been proposed for the mouse (Weller et al., 1986). In that study, although seal and human muscle were reported to have about a 7-fold difference in both Mb concentration and Mb mRNA content, a similar 1:1 relationship for the differences in Mb and Mb mRNA between seal and mouse muscles was not found. The lack of a proportionate difference between Mb concentration and Mb mRNA content has also been reported in cardiac muscles of notothenoid fishes; this observation again led those authors to suggest that Mb content was not dependent on the magnitude of the Mb mRNA pool (Moylan and Sidell, 2000). Post-transcriptional and even post-translational regulation of protein function and/or degradation secondary to hypoxia have previously been demonstrated for other proteins (Kumar and Klein, 2004; Semenza, 2004). For instance, O_2-dependent hydroxylation of

specific proline residues of HIF 1α (hypoxia inducible factor) leads to ubiquitination, proteasomal degradation, and a decreased concentration of that protein. Hypoxia, on the other hand, decreases the hydroxylation and degradation rate of HIF 1α and results in its accumulation. Thus, although different mechanisms may be involved, it remains possible that changes in degradation rate of message and/or protein may promote the accumulation of Mb in emperor penguin muscle.

Lastly, the importance of the continued regulation of Mb production from the fledgling stage into adulthood is supported by the lack of a significant difference between the blood volume of 5.9 month old juveniles and adults in this study. The blood volume data, combined with previous findings for Hb concentration (Ponganis et al., 1999), demonstrate that it is only the muscle O_2 store that is not fully developed as fledgling chicks depart for their first trip to sea. Both the mass-specific blood O_2 store and body mass are nearly equivalent to those of small adults, while the muscle O_2 store and pectoral Mb content are only about one-third the adult value. It is also possible that prioritization of globin precursors (iron, amino acids) for Hb synthesis in the developing chick may contribute to the relatively slow increase in Mb concentration prior to fledging.

CONCLUSIONS

Although this research does not thoroughly define the regulatory mechanisms of Mb production in emperor penguins, it does support a primary role for transcriptional regulation of Mb mRNA in the production of the striking Mb concentrations in penguin muscle. The lack of a 1:1 relationship between Mb and Mb mRNA content in chicks and adults suggests that nutritional limitations or post-transcriptional regulation may also contribute to the Mb concentrations observed in given age groups. In addition, the increase in Mb and Mb mRNA content in chicks prior to fledging demonstrates that swimming and diving are not required to initiate increased Mb mRNA production and achieve a Mb concentration of at least 2 g 100 g^{-1} muscle. The further increase in Mb from the juvenile to adult stage, however, is likely dependent on the muscle activity and hypoxia incurred during long, deep dives because Mb concentration in adults which have been captive all their lives is only 60% of that in adults in the wild.

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