Running, swimming and diving modifies neuroprotecting globins in the mammalian brain

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The vulnerability of the human brain to injury following just a few minutes of oxygen deprivation with submergence contrasts markedly with diving mammals, such as Weddell seals (Leptonychotes weddellii), which can remain underwater for more than 90 min while exhibiting no neurological or behavioural impairment. This response occurs despite exposure to blood oxygen levels concomitant with human unconsciousness. To determine whether such aquatic lifestyles result in unique adaptations for avoiding ischaemic–hypoxic neural damage, we measured the presence of circulating (haemoglobin) and resident (neuroglobin and cytoglobin) oxygen-carrying globins in the cerebral cortex of 16 mammalian species considered terrestrial, swimming or diving specialists. Here we report a striking difference in globin levels depending on activity lifestyle. A nearly 9.5-fold range in haemoglobin concentration (0.17–1.62 g Hb 100 g brain wet wt\(^{-1}\)) occurred between terrestrial and deep-diving mammals; a threefold range in resident globins was evident between terrestrial and swimming specialists. Together, these two globin groups provide complementary mechanisms for facilitating oxygen transfer into neural tissues and the potential for protection against reactive oxygen and nitrogen groups. This enables marine mammals to maintain sensory and locomotor neural functions during prolonged submergence, and suggests new avenues for averting oxygen-mediated neural injury in the mammalian brain.

**Keywords:** neuroglobin; marine mammal; cerebral cortex; haemoglobin; diving; brain

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1. INTRODUCTION

The mammalian response to acute oxygen deprivation as occurs during cerebrovascular accidents and drowning varies widely from rapid irreparable neural injury (Kooyman 1989; Neal 1997) and high incidence of mortality (Zuckerbraun & Saladino 2005; Heron & Smith 2007) among humans to apparent resistance to hypoxia exhibited by marine mammals (Kooyman 1989). With reports of several new classes of globins in neural tissues and their implied role in neuronal survival following ischaemic–hypoxic events (Burmester et al. 2000), we asked the question: does globin deposition in the brain and concomitant neuroprotection vary with routine aquatic activity by mammals? A potential explanation for these different responses is related to the deposition of globin proteins. Globins are complex oxygen-carrying proteins that are elevated in concentration in the blood and skeletal muscles of marine mammals (Kooyman 1989). With reports of several new classes of globins in neural tissues and their implied role in neuronal survival following ischaemic–hypoxic events (Burmester et al. 2000), we asked the question: does globin deposition in the brain and concomitant neuroprotection vary with routine aquatic activity by mammals?

Therefore, in this study, we assessed the localized presence and variability of globin proteins in marine and terrestrial mammals (table 1). The content of circulating globins (haemoglobin, Hb) and of a family of recently discovered (Burmester et al. 2000) resident neural globin...
Table 1. Mammalian species, general habitat classification and circumstance of death for the carcasses sampled in this study. (All animals with the exception of the mice were obtained from the wild following accidental death or euthanasia. Laboratory mice were euthanized by cervical dislocation (CD) or exposure to CO₂. Here n represents the number of individuals in each group.)

<table>
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<tr>
<th>mammal species</th>
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<th>preferred habitat</th>
<th>circumstance</th>
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<td>terrestrial</td>
<td>acute trauma</td>
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<td>red fox (Vulpes fulva)</td>
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<td>acute trauma</td>
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<tr>
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<tr>
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<td>euthanized (15 ea. CD, CO₂)</td>
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<td>stranded, n=2</td>
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<td>pinnipeds</td>
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<td>stranded</td>
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<td>pilot whale (Globicephala macrorhynchus)</td>
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<td>risso’s dolphin (Grampus griseus)</td>
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<td>Blainville’s beaked whale (Mesoplodon densirostris)</td>
<td>1</td>
<td>pelagic</td>
<td>stranded</td>
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2. MATERIAL AND METHODS

(a) Animals

Samples of brain were collected from 41 terrestrial mammals and 23 marine mammals representing 15 different wild species and 1 laboratory species (table 1). All animals except mice were acquired opportunistically. These included stranded animals, fisheries bycatch, road kills or animals purposely trapped and killed in state and federal programmes due to threats to other animals or humans. Mice were obtained from a laboratory vivarium following euthanization. Carcasses were immediately sampled in the field, refrigerated for necropsy within 24 hours or frozen at 0°C until examination. Only mature animals in fresh post-mortem condition, as evidenced by the presence of rigor mortis, minimal autolysis, and general integrity of internal and external tissues were used.

By categorizing the carcasses according to the manner of death, we were able to compare variations in globin content in the cerebral cortex with the relative duration of ante-mortem hypoxia. Because the brain responds to hypoxia by transient increases in cerebral blood flow (Hudak et al. 1986; Kanaan et al. 2006), samples from prolonged mortality events (i.e. trauma and live stranding) were considered representative of a maximum, acute globin response in this study. Conversely, samples obtained following relatively short mortality events (i.e. euthanization) were considered a minimum response. This paradigm was tested in mice by comparing globin concentrations in the cerebral cortex of animals euthanized by CO₂ exposure (hypoxic group) or by cervical dislocation (normoxic group). Similar comparisons were conducted for sea otters and California sea lions following accidental death or euthanization. Consistently, higher values were measured for the accidental group. Thus, to ensure comparable conditions, all values reported here are from samples obtained from carcasses following a presumed prolonged mortality (hypoxic) event unless noted.

All procedures involving tissues and animals followed NIH guidelines as approved under the UCSC Chancellor’s Animal Research Committee. The use of terrestrial and marine mammal tissues was approved by permits through the California Department of Fish and Game and National Marine Fisheries Service-Protected Resources Division, respectively.

(b) Tissue sample collection

Brain samples were taken from both fresh and frozen carcasses. Once the brain was isolated, samples of cerebral cortex were obtained. Total size of each sample was dependent on the animal but was minimally 6 g except for mice. For the latter, both cerebral hemispheres were isolated and used in entirety in the assays. All samples were placed in airtight containers and immediately frozen at 0 or −80°C until analyses. Prior to analysis, the dura mater and arachnoid containing the major meningeal blood vessels were dissected away in each sample. For consistency, samples from all tested species except mice used peripheral grey matter with the
underlying white matter tracts removed. Samples were subdivided into four replicates for simultaneous analysis.

(c) Circulating and resident globin protein contents

The content of globin proteins measured in g globin 100 g wet wt$^{-1}$ in brain samples was determined using a modification of spectrophotometric methods and calculations by Reynafarje (1963). Semi-frozen samples (approx. 1–2 g) were weighed, minced and placed in cold, low ionic strength buffer (40 mM phosphate at pH = 6.6). To ensure adequate globin concentration for spectrophotometric analyses, the buffer to tissue ratio was adjusted to 5.6–20.0 ml buffer g$^{-1}$ wet tissue depending on species. Paired tests using different buffer dilutions demonstrated no change in globin recovery over this range. Each sample was sonicated (Fisher Scientific Sonic Dismembrator 100) for 1–2 min on ice, and immediately centrifuged (Sorval RC-5B refrigerated Superspeed, DuPont Instruments) at 0°C and 28 000 g for 50 min. The clear supernatant was drawn and bubbled at room temperature with pure CO for 8 min. Approximately 0.02 g of sodium sulphite was added to ensure complete reduction. The absorbance of each sample was read at room temperature on a desktop spectrophotometer (UV-visible Bio spec-1601, Shimadzu Corp., Kyoto, Japan) over the wavelength range 416–568 nm encompassing peak absorbencies for Hb (Zijlstra & Buurmsa 1997), cytoglobin (Fago et al. 2004a) and neuroglobin (Burmeister et al. 2000; Dewilde et al. 2001; Fago et al. 2004a).

Samples from each animal were run in triplicate. During individual tests, replicates from one terrestrial and one marine species were assayed in tandem. In addition, in each assay a blank comprised of buffer alone was used as a zero reference; a corresponding skeletal muscle sample of known myoglobin content was used to calibrate for span globin values. By combining resident globin proteins, adjusting buffer levels and using samples following hypoxia associated with prolonged mortality events, we obtained globin concentrations within the range required for accurate spectrophotometric analysis. Using these techniques, the estimated detection limit was approximately 0.05 g of globin 100 g brain wet wt$^{-1}$.

(d) Calculations

Globin protein contents were calculated according to Beer’s law by eliminating resident globins (neuroglobin and cytoglobin combined) from Hb values in a modification of Reynafarje (1963). Hb concentration ($C_{Hb}$, mol l$^{-1}$) was determined from

\[
C_{Hb} = \frac{\text{Abs}_{538} - \text{Abs}_{561}}{1900},
\]

using an extinction coefficient ($\epsilon$) for carboxy-Hb of $13.8 \times 10^3$ and $11.9 \times 10^3$ at 538 and 561 nm, respectively (Reynafarje 1963; Antonini & Brunori 1971), and assumed equality in $\epsilon$ for resident globins at these wavelengths (Dewilde et al. 2001; Fago et al. 2004a). Although the reported difference for $\epsilon$ for these wavelengths varies by 2.6% (Fago et al. 2004a), the level of error in $C_{Hb}$ was minimal. This was determined by assaying whole dolphin blood of known Hb content, which resulted in a less than 1.0% error. $C_{Hb}$ from equation (2.1) was converted to g Hb 100 g brain wet wt$^{-1}$ by multiplying by the buffer dilution factor, a unit constant of 0.1, and the reported molecular weight for Hb of cetaceans (MW = 16 035 Da) for single polypeptide subunits), pinnipeds (MW = 15 920 Da) or mice (MW = 16 363 Da; http://www.expasy.org/sprot/) where appropriate.

Calculating the concentration of the remaining neural globin proteins in the tissues was complicated by the comparative lack of information regarding extinction coefficients or normative values. Cyroglobin concentrations for neural tissues are currently unavailable, and the estimated neuroglobin concentration in total mouse brain extracts and retina ranges from 1 to 100 μM (Burmeister et al. 2000; Schmidt et al. 2003). Burmeister & Hankeln (2004) also state that ‘local concentrations of neuroglobin are probably much higher’ than the estimated 1 μM in the brain. To avoid these uncertainties, we first determined a resident globin concentration ($C_{\text{RNG}}$) estimate for each sample from the difference in sample absorbency attributed to Hb (equation (2.1)) and the remaining combined neuroglobin–cytoglobin concentration. This estimate was then used to calculate the relative RNG level from the ratio of $C_{\text{RNG}}$ for each species and $C_{\text{RNG}}$ for mice, the only mammalian species for which neuroglobin concentrations have been reported. Patterns in relative globin levels were validated by mRNA expression analyses for the same tissues (described below). Using this ratio, we circumvented potential over- or underestimates in actual concentrations while preserving the relative magnitude in resident globin levels for the species examined. The use of relative levels also enabled us to account for potential background contributions from non-oxygen-binding haem proteins, such as cytochrome c. If present in brain tissue extracts, these proteins would be expected to absorb light at 538 and 561 nm, although with different extinction coefficients than used in the analyses.

\[
C_{\text{RNG}} = \frac{\text{Abs}_{561} \times C_{\text{RNG}}^1}{C_{\text{RNG}}^1 - \text{Abs}_{561}} + \frac{RNG \times C_{\text{RNG}} + C_{\text{RNG}}^1}{C_{\text{RNG}}},
\]

where $\epsilon_{561}^1$ is as above and $C_{\text{RNG}}^1$ is from equation (2.1). In the absence of available extinction coefficients for cyroglobin, we used an assumed $\epsilon_{561}^1$ set at the reported $\epsilon_{561}^1$ for neuroglobin of $11.2 \times 10^3$ (Fago et al. 2004a). The oxygen-binding kinetics of cyroglobin and its relatedness to myoglobin (Fago et al. 2004a) suggest that $\epsilon_{561}^1$ could vary by approximately 30% from this assumed value (the range of $\epsilon$ for neuroglobin, Hb and myoglobin at a wavelength of 561 nm). As this will not affect the proportional relationship of $C_{\text{RNG}}$ between mammalian groups, we used $\epsilon_{561}^1$ as an approximation of the combined $\epsilon_{561}^1$ to assess relative resident globin concentrations for terrestrial and marine species.

(e) Neuroglobin and cyroglobin mRNA expression analysis

To validate the patterns in RNG between species, we isolated total RNA from samples of cerebral cortex from eight representative species of terrestrial (mountain lion, bobcat and mouse) and marine (sea otter, harbour porpoise, common dolphin, pilot whale and melon-headed whale) mammals. For each sample, the Qiagen RNasey Mini Kit was used according to the manufacturer’s instructions as previously reported by Burmeister et al. (2000, 2002). Total RNA (2 μg) was reverse transcribed with a combination of oligo(dT) and random hexamer primers using standard conditions prescribed in the Qiagen Omniscript Reverse Transcription Kit. PCR was conducted using the Qiagen HotStarTaq protocol and one-tenth of the total reverse
transcription reaction. Previously published primers were used for cytoglobin (Burmester et al. 2002); primers for neuroglobin were 5′-ATGGAGCGCCCCGGAG-3′ and 5′-ACTGCGCATCCCCAGCCCTG-3′.

(f) Statistics
Differences in globin concentrations between terrestrial and marine mammal groups were determined by t-tests (SYSTAT v. 10, 1998, SPSS, Inc.) with Hb and RNG tested separately. To evaluate the effect of hypoxia events on Hb and RNG concentrations, we ran two-way ANOVAs on raw values for each globin type in mice, sea otters and sea lions. The differences in globin concentrations between hypoxia and normoxia, between species, and potential interactive effects were tested with the same statistical software using species and hypoxic status as factors. The relationship between RNG level and maximum dive duration was determined using a least-squares regression analysis for nonlinear functions (SIXMA, v. 3.5, 2005, SigmaStat, Inc.). All means for optical densities, Hb concentrations and RNG levels are reported as ±1 s.e.m. unless noted.

3. RESULTS

(a) Hb levels in terrestrial and marine mammal brains

Pigmentation of the cerebral cortex was strikingly different between terrestrial and marine mammals, and attributed primarily to relative Hb concentration (figure 1a). Cortical areas were substantially less pigmented for felids, canids and mice than for homologous sites in cetaceans, pinnipeds and sea otters. Spectrophotometric analyses of total pigmentation at the average absorbance peak wavelength of 558–568 nm for carboxy-Hb (Zijlstra & Buursma 1997) and deoxy-neuroglobin (Burmester et al. 2000) revealed a 2.4-fold higher optical density for samples from marine species (0.599 ± 0.078 OD, n = 10) compared with terrestrial species (0.245 ± 0.022 OD, n = 5). Admittedly, several factors may contribute to visible staining, and therefore pigmentation, of the tissues including the post-mortem interval, degree of erythrocyte disruption and blood vessel degradation. However, these post-mortem effects would not alter total tissue optical density.

Translating these absorbencies into Hb concentrations revealed wide variation for the cerebral cortices of mammals. A nearly 10-fold difference in Hb concentration revealed wide variation for the cerebral cortices of terrestrial species (0.599 ± 0.078 OD, n = 10) and mice, sea otters and sea lions (0.245 ± 0.022 OD, n = 5). Admittedly, several factors may contribute to visible staining, and therefore pigmentation, of the tissues including the post-mortem interval, degree of erythrocyte disruption and blood vessel degradation. However, these post-mortem effects would not alter total tissue optical density.

(b) RNG levels in swimmers, divers and terrestrial mammals

Like Hb, the level of RNGs in the form of neuroglobin and cytoglobin reached higher levels in the cerebral cortex and were statistically distinct (n = 15 species, t14 = 3.18, p = 0.002) for marine mammals compared with terrestrial mammals (figure 1b). Estimated RNG concentration for the mouse cerebral cortex was 5.40 ± 0.75 μM (n = 15 mice) and within the range of estimated values for neuroglobin in other neural tissues in mice (Burmester et al. 2000; Schmidt et al. 2003; Burmester & Hankeln 2004). We found a threefold range in RNG levels among the 16 mammalian species tested in the present study. Based on corresponding Hb level, preferred habitat and general activity pattern of each species, relative RNG levels clustered into three discrete groups, terrestrial mammals, swimming specialists and diving specialists (figures 1b and 2). Mean relative RNG levels based on the ratio of wild species to mouse values were 1.23 ± 0.29 (n = 4 species) for terrestrial mammals, 1.05 ± 0.12 (n = 4 species) for divers and 2.04 ± 0.11 (n = 6 species) for swimmers. Here ‘swimming specialist’ and ‘diving specialist’ encompasses more than performance capability. Rather, the designations follow those of Hedrick & Duffield (1991) for marine mammals in which swimmers generally reside in shallower waters, dive for shorter periods and demonstrate faster sustained aerobic swimming activities than deep-diving specialists. The pattern of mRNA expression for neuroglobin confirmed this distinction with comparatively greater globins levels evident for fast-swimming species (figure 1b).

(c) Variability in globin levels within the cerebral cortex

Because previous studies have demonstrated hypoxia-induced increases in cerebral blood flow (Hudak et al. 1986; Kanaan et al. 2006) and enhanced expression of neuroglobin (Sun et al. 2001; Roesner et al. 2006) and cytoglobin (Schmidt et al. 2004) in neural tissues, we examined globin levels in brain samples obtained from mice, sea otters and sea lions exposed to different durations of ante-mortem hypoxia. We found that the globin response to presumed hypoxic events differed between terrestrial and marine mammals (figure 3). For laboratory mice, hypoxia induced by CO2 exposure resulted in a 58.2% increase in Hb concentration within the cerebral cortex compared with a normoxic control group. The response was even greater between normoxic and hypoxic beach-stranded marine mammals (two-way ANOVA with species and hypoxic status as factors, where F2,16 = 30.57 for species, F1,16 = 118.07 for status and F2,16 = 17.50 for the interaction term at p < 0.001). Resident globins demonstrated smaller changes with short-term exposure to hypoxic events regardless of mammalian group (figure 3), although differences between species remained significant (two-way ANOVA, where F2,16 = 16.53, p < 0.001 for species; F1,16 = 0.01, p = 0.94 for hypoxic status and F2,16 = 2.11, p = 0.15 for the interaction term).

4. DISCUSSION

Our study indicates that both circulating and resident globin protein levels in the mammalian brain are modified...
by activity type, and are poised to provide complementary safety factors for defending against ischaemic–hypoxic injury. All marine species in this study showed elevated Hb concentrations when compared with terrestrial animals, with the largest deep-diving species representing the most extreme levels (figure 1). The second factor, an elevation in the concentration of RNG proteins, is demonstrated by comparing swimmers and divers (figures 1–4). On average, swimming specialists maintained RNG levels in the cerebral cortex that were 1.7–1.9 times higher than observed for most terrestrial or deep-diving specialists. Differences in cerebrovascular morphology and physiology probably contributed to the variation in globin concentrations observed for marine and terrestrial species. Kerem & Elsner (1973) reported higher capillary densities and lower mean capillary distances within the cerebral cortex of phocid seals compared with terrestrial mammals including man, a cardiovascular adjustment typical of exposure to chronic hypoxia (Kanaan et al. 2006). This morphological characteristic allows seals to achieve the same rate of oxygen supply to the brain as dogs but at lower blood oxygen gradients, thereby increasing cerebral

Figure 1. The range of (a) Hb and (b) RNG levels in the mammalian cerebral cortex. Both globins varied among mammals according to preferred habitat (subdivided by the vertical lines). Species within habitat types are ordered according to the rank in globin value. Points and error bars denote the mean ± 1 s.e.m. for terrestrial (open circles) and marine (closed circles) species. Numbers in parentheses indicate the number of individuals examined for each species. Inset photographs in (a) show the difference in pigmentation for a mid-coronal section of the cerebrum at the level of the longitudinal fissure for a representative terrestrial (coyote) and marine (sea lion) mammal. In (b), the inset shows a representative mRNA expression analysis for terrestrial (green) and swimming (light blue) and diving (dark blue) species, where total RNA was isolated and subjected to RT-PCR with primers specific for neuroglobin.
tolerance to hypoxia (Kerem & Elsner 1973). Based on figure 1, such a response appears graded among diving mammals, and was most evident for two pelagic cetaceans, the pilot whale and Pacific white-sided dolphin (*Lagenorhynchus obliquidens*). Furthermore, the response is inducible with marine species demonstrating comparatively larger increases in Hb concentrations with hypoxia (figure 3).

Among marine mammals, the variation in globin levels for swimmers and divers also reflects the haematological and rheological trade-offs for aquatic performance. Haematological characteristics of deep-diving specialists facilitate oxygen storage in the blood through increased haematocrits, which concomitantly restricts fast-sustained swimming behaviour due to elevated blood viscosity (Hedrick & Duffield 1991). Conversely, swimming specialists optimize oxygen transport through lower haematocrits but display comparatively limited diving ability. A similar mechanism appears to affect the deposition of globins in the cerebral cortex of marine
mammals; deep divers preferentially rely on circulating globins in the brain while faster swimming coastal species use enhanced RNG stores (figure 2), perhaps to overcome globins in the brain while faster swimming coastal species use enhanced RNG stores (figure 2), perhaps to overcome the haematocrit shortfall. Thus, we find that RNG concentration is inversely related to maximum dive duration in marine mammals (figure 4).

The effect of activity patterns on globin levels may also explain an interesting exception among terrestrial mammals. The bobcat (Lynx rufus) showed a mean relative RNG level of 2.07 ± 0.38 that was the highest among terrestrial mammals and comparable with those of swimming specialists (figure 1b). Admittedly a poor diver, the bobcat is an ambush predator that relies on sprinting activity to capture prey (Sunquist & Sunquist 2002). In comparison, highly aerobic terrestrial species exemplified by canids (Schmidt-Nielsen 2001) exhibited the lowest brain RNG levels, averaging half that of bobcats (figure 1b). Based on this, it is intriguing to consider possible interrelationships between routine activities (e.g. sprinting versus endurance and diving versus swimming), tissue-specific globin concentrations (circulating versus resident), and corresponding levels of neuroprotection of the brain.

What are the specific benefits provided by elevated globin proteins in the cerebral cortex? The advantage of Hb as a deliverer of oxygen to neural tissues is straightforward (Kerem & Elsner 1973). Conversely, the exact physiological roles of neuroglobin and cytoglobin have not been discerned, although several functional characteristics suggest advantages for highly active mammals. Rather than serving as an oxygen store per se, the oxygen-binding characteristics (Trent et al. 2001) and low tissue concentrations of neuroglobin indicate a function in scavenging reactive oxygen and nitrogen groups and subsequent defence against cellular damage during hypoxia (Fago et al. 2004b). Cytoglobin, through its ubiquitous presence (Burmeister et al. 2002) and high oxygen affinity, which mimics myoglobin (Fago et al. 2004a), indicates a role in facilitating oxygen transfer and storage (Fago et al. 2004b; Hankeln et al. 2005). Because hypoxia can upregulate the expression of RNGs (Sun et al. 2001; Schmidt et al. 2004; Roesner et al. 2006), activities associated with acute decreases in blood oxygen levels, such as sprinting by runners and swimmers or prolonged diving by marine mammals would also result in physiological conditions that improve the oxygen storage capacity of both globins (Fago et al. 2004b; Hankeln et al. 2005).

In view of these findings, we propose that the immediate, general response of marine mammals to reduced access to air upon submergence is enhanced delivery of oxygen through circulating globins in the intracranial vasculature. By maintaining (Zapol et al. 1979) or increasing (figure 3) an exceptionally rich haematocrit (Kooyman 1989; Hedrick & Duffield 1991; Neal 1997; Noren & Williams 2000),...
REFERENCES


