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Blood oxygen-binding characteristics of bigeye tuna (*Thunnus obesus*), a high-energy-demand teleost that is tolerant of low ambient oxygen

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Abstract We found blood from bigeye tuna (*Thunnus obesus*) to have a significantly higher O_2 affinity than blood from other tunas. Its P_{50} (partial pressure of oxygen, P_{O_2} required to reach 50% saturation) was 1.6 to 2.0 kPa (12 to 15 mmHg) when equilibrated with 0.5% CO_2. Previous studies employing similar methodologies found blood from yellowfin tuna (*T. albacares*), skipjack tuna (*Katsuwonus pelamis*), and kawakawa (*Euthynnus affinis*) to have a P_{50} of 2.8 to 3.1 kPa (21 to 23 mmHg). These observations suggest that bigeye tuna are more tolerant of low ambient oxygen than other tuna species, and support similar conclusions derived from laboratory whole-animal studies, depth-of-capture data, and directly-recorded vertical movements of fish in the open ocean. We also found the O_2 affinity of bigeye tuna blood to be essentially unaffected by a 10 C° open-system temperature change (as is the blood of all tuna species studied to date). The O_2 affinity of bigeye tuna blood was, however, more affected by a 10 C° closed-system temperature change than the blood of any tuna species yet examined. In other words, bigeye tuna blood displayed a significantly enhanced Bohr effect (change in log P_{50} per unit change in plasma pH at P_{50}) when subjected to the inevitable changes in partial pressure of carbon dioxide (P_{CO_2}) and plasma pH that accompany closed-system temperature shifts, than when subjected to changes in plasma pH accomplished by changing P_{CO_2} alone. In vivo, the resultant large decrease in O_2 affinity (i.e. the increase in P_{50}) that occurs as the blood of bigeye tuna is warmed during its passage through the vascular counter-current heat exchangers ensures adequate rates of O_2 off-loading in the swimming muscles of this high-energy-demand teleost.

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Introduction

Although their geographic ranges overlap (Sund et al. 1981; Joseph et al. 1988), the behavior, depth distribution, and thermal ecology of bigeye tuna (*Thunnus obesus*) are distinctly different from those of yellowfin tuna (*T. albacares*) and skipjack tuna (*Katsuwonus pelamis*). The latter two species spend the majority of their time within the warm (≃20 to 28 °C over their range), uniform-temperature surface layer (typically < 100 m) (Dizon et al. 1978; Mohri et al. 1996; Block et al. 1997; Brill et al. 1999), whereas the daytime depth distribution of juvenile (≃3 to 5 kg body mass) bigeye tuna is deeper (> 200 m) and appears to be set by the depth of the 15 °C isotherm (Sharp 1978; Holland et al. 1990). Adult bigeye tuna (> 25 kg) routinely occupy depths down to 500 m during the daytime, where water temperature can be ≃7 °C, and appear to follow the daily vertical migrations of the small nektonic organisms (crustaceans, cephalopods, and fishes) of the deep sound-scattering layer (Boggs 1992; Josse et al. 1998; Dagorn et al. 2000; C. Boggs, M. Musyl, R. Brill, NMFS, Honolulu Laboratory, unpublished observations). They are thus able to exploit these organisms as prey more effectively than other tunas (Grudinin 1989).

Bigeye tuna are also tolerant of low ambient oxygen (hypoxia), and routinely inhabit water with an O_2 content of ≃1 ml O_2 l^{-1}(Hanamoto 1987; C. Boggs, M. Musyl, R. Brill, NMFS, Honolulu Laboratory, unpublished observations). In contrast, a reduction of O_2 content to only 3.5 ml O_2 l^{-1} limits the depth distribution of yellowfin and skipjack tunas (Ingham et al. 1977;

Barkely et al. 1978; Gooding et al. 1981; Cayré 1991; Cayré and Marsac 1993; Brill 1994). In laboratory studies, the onset of cardio-respiratory adjustments during acute hypoxia occurs at lower ambient O_2 levels in bigeye tuna than in skipjack or yellowfin tuna (Bushnell et al. 1990). The extreme hypoxia tolerance of bigeye tuna seems incongruous in the light of their elevated metabolic rates (Bushnell et al. 1990), in that physiological adaptations needed to tolerate hypoxia can compromise rates of oxygen delivery to the tissues (Yang et al. 1992).

As a result of their almost continuous vertical movements, bigeye, yellowfin, skipjack and bluefin tunas are subject to ambient temperature changes of ≥10 C° over time periods of tens of minutes or less (e.g. Dizon et al. 1978; Holland et al. 1990; Lutcavage et al. 2000). Blood passing through their gills must, therefore, maintain its functional properties whilst subjected to open-system temperature changes; in other words, when subjected to temperature changes under conditions where transfer of gases and proton equivalents between the blood and another medium is able to occur. In this instance, the "other medium" is the water passing over the gills.

Tunas (family Scombridae, tribe Thunnini) also possess vascular counter-current heat exchangers, that enable them to sustain deep red-muscle temperatures significantly above ambient temperature (Carey and Teal 1966; Stevens and Neill 1978; Brill et al. 1994). Vascular counter-current heat exchangers also reduce rates of heat transfer to or from the environment following abrupt changes in ambient temperature (Carey 1973; Neill et al. 1976). As a result of the latter, changes in muscle temperature lag significantly behind abrupt changes in water temperature, and deep red muscle temperature of even relatively small (≃3 to 5 kg) bigeye tuna routinely differ from the water temperature by ≥10 C° or more (Holland et al. 1992). The blood of tunas can, therefore, be subjected to significant closed-system temperature changes (whereby it is unable to exchange gases or proton equivalents with another medium) as it passes from the gills (where blood is at ambient temperature) through the vascular counter-current heat exchangers. Under such circumstances, the blood O_2 content ($[O_2]$) remains constant, whereas the partial pressures of O_2 and CO_2 (P_{O_2} and P_{CO_2}, respectively) are variable. Moreover, whereas open-system temperature changes occur at a constant P_{CO_2}, and are thus accompanied by relatively minor changes in plasma pH (pH_e) (≃0.004 $\Delta pH_e/C°$), closed-system temperature changes are accompanied by substantial changes in P_{CO_2} and pH_e (≃0.016 $\Delta pH_e/C°$) because of changes in CO_2 solubility in the plasma and the reciprocal titration of plasma proteins and plasma bicarbonate (Cech et al. 1984; Perry et al. 1985; Truchot 1987; Brill and Bushnell 1991a; Brill et al. 1992).

Blood from all tuna species studied to date has shown little or no shift in O_2 affinity when subjected to open-system temperature changes in vitro (Carey and Gibson 1983; Cech et al. 1984; Brill and Bushnell 1991a). There is, however, species-specific variation when blood is subjected to closed-system temperature changes. Responses range from blood O_2 affinity unaffected in skipjack tuna, through a normal temperature effect (i.e. a decrease in O_2 affinity with increasing temperature) in yellowfin tuna and kawakawa, to an unusual reverse temperature effect (i.e. an increase in O_2 affinity with increasing temperature) in albacore (*Thunnus alalunga*) and northern bluefin tuna (*T. thynnus thynnus*) (Carey and Gibson 1983; Cech et al. 1984; Jones et al. 1986; Brill and Bushnell 1991a). A reversed closed-system temperature effect has been hypothesized to be necessary to prevent premature off-loading of O_2 as blood is warmed in the vascular counter-current heat exchangers (Carey and Gibson 1983; Cech et al. 1984; Hochachka and Somero 1984). As described by Bushnell and Jones (1994), however, this unusual reverse temperature response would reduce the P_{O_2} gradient from the blood to the mitochondria of the swimming muscles (and thus possibly reduce rates of O_2 delivery). It could also compromise O_2 loading at the gills. The latter occurs because the P_{O_2} of venous blood would increase as it is cooled back towards ambient temperature, and thus reduce the O_2-driving gradient across the gills from the water to the venous blood. Indeed, a reverse closed-system temperature effect is not seen in blood from kawakawa, yellowfin tuna, or skipjack tuna and is, therefore, not necessarily correlated with the presence of vascular counter-current heat exchangers and the ability to maintain elevated muscle temperatures (Jones et al. 1986; Brill and Bushnell 1991a).

Given the behavioral differences between bigeye, yellowfin, and skipjack tunas, and the data clearly indicating a greater tolerance of hypoxia by bigeye tuna, we speculate that these differences should be reflected not only in blood O_2 affinity but also in the effects of open- and closed-system temperature changes on blood O_2 affinity. Specifically, we hypothesize that bigeye tuna would have blood with an elevated O_2 affinity (i.e. low P_{50}), as has been shown for hypoxia-tolerant freshwater and marine fishes (Lykkeboe and Weber 1978; Powers 1980, 1985; Yang et al. 1992; Jensen et al. 1993), and fishes specifically acclimated to hypoxia (Wood and Johansen 1972, 1973; Wood et al. 1975). To test our hypothesis, we investigated the O_2 affinity and the effects of open- and closed-system temperature changes on the O_2 affinity of bigeye tuna blood in vitro using methods essentially identical to those employed previously with yellowfin and skipjack tuna blood (Brill and Bushnell 1991a).

As with whole blood, the O_2 affinity of hemoglobin (Hb) solutions derived from various tuna display species-specific changes in O_2 affinity when subjected to temperature change (Sharp 1975). Moreover, the O_2 affinity of Hb solutions derived from just northern bluefin tuna blood have been reported to be independent of temperature, to increase with increasing temperature, and to decrease with increasing temperature above P_{50} while simultaneously decreasing below P_{50}

(Rossi-Fanelli and Antonini 1960; Carey and Gibson 1977; Ikeda-Saito et al. 1983). Therefore, in a separate set of experiments using Hb solutions derived from blood of bigeye, yellowfin, and skipjack tuna, we briefly re-examined the effect of closed-system temperature change on O_2 affinity. Our immediate goal was to determine if data obtained using Hb solutions subjected to a closed-system temperature change match those obtained using whole blood. Our overall objective was to ascertain if the observed species-specific consequences of closed-system temperature change to tuna blood-O_2 affinity resides in the hemoglobin(s) themselves, or involves changes in intracellular ligands effected by the red blood cells.

Materials and methods

Fish handling, surgical and initial blood-handling procedures

Juvenile bigeye tuna (*Thunnus obesus*) were purchased from local commercial fishermen and maintained in outdoor tanks supplied with running sea water (25 °C ± 2 C°) at the Kewalo Research Facility (National Marine Fisheries Service, Southwest Fisheries Science Center, Honolulu Laboratory). Fish were held in captivity for only a few days before use. Food was presented daily, but was withheld for ≃20 h prior to use in an experiment to allow sufficient time for gut clearance (Magnuson 1969). A total of six fish (mean ± SEM body mass = 2.25 ± 0.29 kg) were used for the experiments on whole blood.

We constructed O_2 dissociation curves under conditions mimicking those experienced by blood in vivo. Equilibration temperatures (15 and 25 °C) approximated the minimum and maximum water temperatures occupied by juvenile bigeye tuna near the main Hawaiian Islands (Holland et al. 1990, 1992). Likewise, the two CO_2 levels employed cover the range of arterial and venous blood P_{CO_2} (≃0.4 to 1.3 kPa, 3 to 10 mmHg) measured in tunas (Jones et al. 1986; Bushnell and Brill 1992; Korsmeyer et al. 1997).

To obtain blood with normal acid-base status, hematocrit, and lactate concentrations, we employed procedures described by Brill and Bushnell (1991a) and Brill et al. (1992). Fish were dip-netted from their holding tank, anesthetized by placing in a plastic bag containing 5 liters oxygenated seawater with 1 g l^{-1} benzocaine (Sigma Chemical Co, St. Louis, Missouri), and then immediately moved into the laboratory and placed ventral side up on a surgical table. Anesthesia was maintained by pumping seawater containing 0.1 g l^{-1} benzocaine over the gills. A 20 gauge, 3.2 cm catheter (Insyte Vialon, Becton Dickinson Vascular Access, Sandy, Utah) was introduced into the ventral aorta under manometric guide and connected to a 20 cm length of polyethylene tubing (PE 160). The catheter hub was sutured to the skin immediately anterior to the pelvic fins. Fish were then turned upright, spinally blocked with 2% lidocaine, and allowed to recover (≃1 to 2 h) under sedative doses of the steroid anesthetic Saffan (Glaxovet, Harefield, Uxbridge, England), a highly effective, injectable fish anesthetic (Oswald 1978), until blood pH stabilized at ≥7.6. Following this, procedure, ≃20 to 40 ml of blood were withdrawn as quickly as possible, and with minimal disturbance. Fish were then immediately killed with an overdose of sodium pentobarbital. To prevent clotting, sodium heparin (10 000 IU ml^{-1}) was added to the blood to a final nominal concentration of 100 IU ml^{-1} blood.

As catecholamines were most probably released during anesthesia, surgery, and blood withdrawal, we took steps to minimize their effects on blood-O_2 affinity. Catecholamines were degraded by exposing blood samples to bright light for 1 h at room temperature (≃22 to 25 °C) whilst being gently swirled (Gilmour et al. 1994). The blood was then stored overnight at 4 °C to ensure that the red blood cells were in steady state with respect to ion levels, water content, and intracellular pH prior to use in an experiment (Bourne and Cossins 1982; Gallardo Romero et al. 1996; Kaloyianni and Rasidaki 1996; Roig et al. 1997). The blood was allowed to warm to room temperature for ≃1 to 2 h (whilst being gently swirled) before 1.5 ml samples were placed in glass tonometers.

Gas-mixing, tonometry, and measurement of Hb and plasma bicarbonate concentrations

Six glass tonometers were placed in a water bath maintained at 15 °C (±0.5 C°), and six in a water bath maintained at 25 °C (±0.5 C°). The tonometers were flushed with water-vapor-saturated gas mixtures of six different nominal P_{O_2}s (0.66, 1.33, 2.67, 5.33, 10.7, 20.0 kPa; 5, 10, 20, 40, 80, 150 mmHg) and either 0.5 or 1.5% CO_2 for at least 1 h before the addition of blood. Gas mixtures were produced immediately prior to each experiment using three precision gas-flow controllers connected to a digital display (MKS Instruments, Andover, Massachusetts), and stored in automobile tire inner tubes. During mixing, P_{CO_2} was continuously monitored with a Hewlett-Packard 47210A Capnometer (Hewlett-Packard Germany, 7030, Böblingen, Germany).

Whole-blood Hb concentrations ([Hb]) were measured using the cyanmethemoglobin method (Dacie and Lewis 1984). Plasma bicarbonate concentrations ($[HCO_3^-]$, mmol l^{-1}) were quantified in samples taken from the blood equilibrated to a P_{O_2} of 20 kPa (150 mmHg) and 0.5 or 1.5% CO_2 following the procedures described by Cameron (1971). Measurements were corrected for CO_2 in solution using CO_2 solubility values for yellowfin tuna (*Thunnus albacares*) plasma from Brill et al. (1992).

Construction of O_2 dissociation curves in blood subjected to open- and closed-system temperature change at two CO_2 levels

To construct blood-O_2 dissociation curves, we measured both the O_2 content ($[O_2]$) and P_{O_2} of samples removed from the tonometers, the former as described by Tucker (1967) and the latter using Radiometer P_{O_2} electrodes installed in two separate MKS Mark 2 blood-gas analyzers (Radiometer America, Westlake, Ohio). The pH of all blood samples (pH_e, also commonly referred to as "plasma pH", "true plasma pH" or "extracellular pH") was assessed with the capillary pH electrodes of the Radiometer blood-gas analyzers. The water jacket surrounding the P_{O_2} and capillary pH electrodes of one instrument was maintained at 15 °C, the other at 25 °C.

The effects of open-system temperature change, closed-system temperature change, and P_{CO_2} on blood-O_2 affinity were quantified by constructing a total of eight blood-O_2 dissociation curves using blood taken from any one fish, as described by Brill and Bushnell (1991a). To measure the effects of open-system temperature change, we constructed two blood-O_2 dissociation curves using samples taken from tonometers immersed in the water baths maintained at 15 and 25 °C. In this instance, the P_{O_2} and pH_e were measured at the same temperature as that to which the blood had been equilibrated. To measure the effects of closed-system temperature change, we constructed two additional blood-O_2 dissociation curves. To construct the first, we injected blood that had been equilibrated to the various gas mixtures at 25 °C into the P_{O_2} and pH electrodes maintained at 15 °C. For the second, we did the opposite. We injected blood samples that had been equilibrated at 15 °C into the P_{O_2} and pH electrodes maintained at 25 °C. Once injected, blood samples are essentially sealed, thus effecting rapid closed-system temperature changes that closely mimic those occurring in vivo (Cech et al. 1984; Jones et al. 1986).

To measure the effects of changes in P_{CO_2}, after measurements were obtained at one CO_2 level, we changed the gas mixtures to the other CO_2 level. Six fresh tonometers were placed in each of the two water baths and again flushed with the various gas mixtures for at least 1 h before addition of blood samples. Blood samples were given 1 h to equilibrate before a second set of four blood-O_2 dissociation curves were obtained as described above.

Blood-O_2 dissociation curves were constructed by fitting the P_{O_2} and $[O_2]$ data to a logistics function by least-squares regression (Sigmaplot, SPSS Inc., Chicago, Illinois). We calculated maximum $[O_2]$ (i.e. 100% saturation) based on measured [Hb] and a maximum Hb O_2-carrying capacity corrected for temperature (1.19 ml O_2 g^{-1} Hb at 15 °C and 1.25 ml O_2 g^{-1} Hb at 25 °C: Ganong 1973). We then determined the P_{O_2} at which the blood would be 50% saturated (P_{50}) using the regression parameters. We used this calculation method to make our data directly comparable to those of previous studies. However, as explained by Nikinmaa (1990), this procedure disregards any influence of the Root effect (i.e. the reduction in maximum $[O_2]$ with increasing P_{CO_2}) on P_{50}.

Calculation of apparent heat of oxygenation, pH_{50}, Bohr effect, Hill numbers, and Root effect

To quantify the temperature sensitivity of bigeye tuna blood, and to make our data readily comparable to those presented in other studies (e.g. Grigg 1969; Powers et al. 1979; Wood 1980; Cech et al. 1984; Brill and Bushnell 1991a), we calculated the apparent heat of oxygenation (kcal mol^{-1}) = $2.303 \cdot R \cdot (\Delta \log P_{50}/\Delta 1/T)$, where R = universal gas constant (1.987 cal $°K^{-1}$ mol^{-1}), and T = equilibration or measurement temperature in °K. Although a convenient measure for comparing the temperature sensitivity of the blood from various species, the apparent heats of oxygenation are not equivalent when calculated using data from whole blood to those based on data from Hb solutions. As explained in Powers et al. (1979), Wood (1980), and Truchot (1987), when whole blood is subjected to temperature changes, there are several additional effects (e.g. the exchange of proton equivalents between the red cells and plasma) that change the intracellular environment and influence Hb conformation and O_2 affinity that do not occur when Hb solutions are subjected to temperature changes.

The pH_e at P_{50} (pH_{50}) was estimated by regressing pH_e against the log of % saturation and then interpolating. The Bohr effect was calculated as $\Delta \log P_{50}$:ΔpH_e. Note that we achieved changes in pH_e in two different ways: by equilibration of the blood with two levels of CO_2 (0.5 and 1.5%), and by subjecting the blood to closed-system temperature changes. We also constructed Hill plots (a linear regression of log P_{O_2} vs log [% saturation/(100−% saturation)], where % saturation = [(measured blood $[O_2]$/maximum $[O_2]$) · 100]). When data from 0 to 100% saturation are included, Hill plots are nonlinear (Ikeda-Saito et al. 1983). We therefore included only midrange data (≃20 to 80% saturation). To calculate the Root effect, we expressed the $[O_2]$ of blood equilibrated to a P_{O_2} of 20 kPa (150 mmHg) as a percent of maximum possible $[O_2]$ based on [Hb].

Determination of effects of closed-system temperature change on O_2 affinity of tuna Hb

In a second set of experiments, we measured spectrophotometrically the effect of closed-system temperature change on the O_2 affinity of Hb solutions derived from blood of bigeye, yellowfin, and skipjack (*Katsuwonus pelamis*) tuna. Our methods were based on those of Carey and Gibson (1983). Hb solutions were prepared by adding frozen blood to a phosphate buffer (pH 7.3 at room temperature) then centrifuging to remove cellular debris. Hemoglobin concentrations were adjusted until the absorbance was ≃1.2 to 1.3 at 410 nm, then equilibrated with air + 1% CO_2 in 50 ml glass tonometers (maintained in a 25 °C water bath) following the procedures employed for whole blood. The hemoglobin solutions were transferred (without exposure to air) into sealed spectrophotometer cuvettes that had been flushed with the same gas mixture to which the hemoglobin solutions were equilibrated. The cuvettes were completely filled to allow no gas to remain above the hemoglobin solution, placed in a water-jacketed cuvette holder maintained at 25 °C by a circulating water bath, and allowed to equilibrate to this temperature. Absorbance was then measured using a BioSpec-1601 (Shimadzu Corp., Tokyo, Japan) as the wavelength was scanned from 600 to 360 nm. The temperature of the water bath was decreased to 15 °C, the hemoglobin solution in the cuvette was allowed to equilibrate to the lower temperature for approximately 2 h, and the absorbance was then re-measured.

Statistical procedures

All results in the text, tables, and graphs are means ± standard error of the mean (SEM). Significant differences were determined from the 95% confidence intervals (i.e. two times the SEM).

Results

Whole blood

Hematocrit [Hb], mean cell [Hb] (i.e. [Hb]/hematocrit) $[HCO_3^-]$, and pH_{50} are listed in Table 1. Representative blood-O_2 dissociation curves are shown in Fig. 1. Approximately half (46%) of all blood-O_2 dissociation curves were sigmoid (Hill number > 1.6); the remainder were intermediary between sigmoid and hyperbolic (Hill number between 1.2 and 1.6). Bigeye tuna (*Thunnus obesus*) blood displayed a significant Root effect, in that the % maximum O_2 saturation fell when CO_2 increased. The mean (±SEM) change was −6.0 ± 1.6% and −8.4 ± 1.3% at 15 and 25 °C, respectively.

The O_2 affinity of bigeye tuna blood was higher than that of blood from other tuna species measured under similar conditions (Table 1). The P_{50} values of yellowfin tuna (*Thunnus albacares*), skipjack tuna (*Katsuwonus pelamis*), and kawakawa (*Euthynnus affinis*) blood between 20 and 30 °C were 2.8 to 3.1 kPa (21 to 23 mmHg) with 0.5% CO_2, and 4.3 to 6.5 kPa (32 to 49 mmHg) with 1.5% CO_2 (Jones et al. 1986; Brill and Bushnell 1991a). The P_{50} of bigeye tuna blood subjected to closed-system temperature changes increased directly with increasing temperature (Table 1). In other words, there was a normal temperature response, in that O_2 affinity decreased as temperature increased. The corresponding changes in pH_{50} are listed in Table 2.

Changes in blood-O_2 affinity during open- and closed-system temperature changes, expressed as the apparent heat of oxygenation, are shown in Fig. 2. During an open-system temperature change, the value for bigeye tuna blood was statistically different from zero at 0.5% CO_2, but not at 1.5% CO_2. The values for bigeye tuna blood were statistically different from zero at both levels of CO_2 during closed-system temperature change, and were clearly different from the responses of yellowfin and skipjack tuna blood recorded previously (Brill and Bushnell 1991a).

The Bohr effect in bigeye tuna blood caused by changing P_{CO_2} alone was large (Fig. 3), but not different from those previously recorded for yellowfin and skipjack tuna blood (Brill and Brill 1991a). When changes in pH_e and P_{CO_2} were brought about by a 10 °C closed-system temperature change, however, bigeye tuna blood showed an enhanced Bohr effect. When treated under nearly identical conditions, yellowfin tuna blood displayed an equal Bohr effect under the two

Table 1 *Thunnus obesus* (means ± SE, $n = 6$) Blood parameters, bicarbonate concentration ($[HCO_3^-]$), maximum $[O_2]$ saturation, pH_{50} (pH at P_{50}), and O_2 affinity (expressed as P_{50}) of bigeye tuna blood. Data for blood equilibrated to 0.5% CO_2 and 1.5% CO_2 show effects of changing those variables alone (15 °C, 25 °C effects of open-system temperature changes; *15 → 25 °C, 25 → 15 °C* effects of closed-system temperature changes; – not applicable)

	Mean	0.5% CO_2				1.5% CO_2			
		15 °C	25 °C	15 → 25 °C	25 → 15 °C	15 °C	25 °C	15 → 25 °C	25 → 15 °C
Hematocrit (%)	34.5 ± 2.2	–	–	–	–	–	–	–	–
[Hb] (g dl^{-1})	12.3 ± 0.9	–	–	–	–	–	–	–	–
Mean cell [Hb] (g dl^{-1})	34.5 ± 0.35	–	–	–	–	–	–	–	–
$[HCO_3^-]$ (mmol l^{-1})	–	14.6 ± 0.9	12.8 ± 1.1	–	–	18.5 ± 0.9	17.0 ± 1.3	–	–
% $[O_2]$ saturation	–	95.6 ± 1.0	93.7 ± 2.6	–	–	89.6 ± 2.0	85.3 ± 4.3	–	–
pH_{50}	–	8.05 ± 0.03	8.08 ± 0.02	7.91 ± 0.03	8.20 ± 0.03	7.71 ± 0.03	7.78 ± 0.06	7.59 ± 0.02	7.87 ± 0.03
P_{50} (kPa)	–	1.67 ± 0.09	2.12 ± 0.21	2.77 ± 0.09	1.00 ± 0.12	3.56 ± 0.68	3.96 ± 0.25	4.54 ± 0.15	1.87 ± 0.08
(mm Hg)	–	(12.5 ± 0.7)	(15.9 ± 1.6)	(20.8 ± 0.7)	(7.5 ± 0.9)	(26.7 ± 5.1)	(29.7 ± 1.9)	(34.1 ± 1.1)	(14.0 ± 0.6)

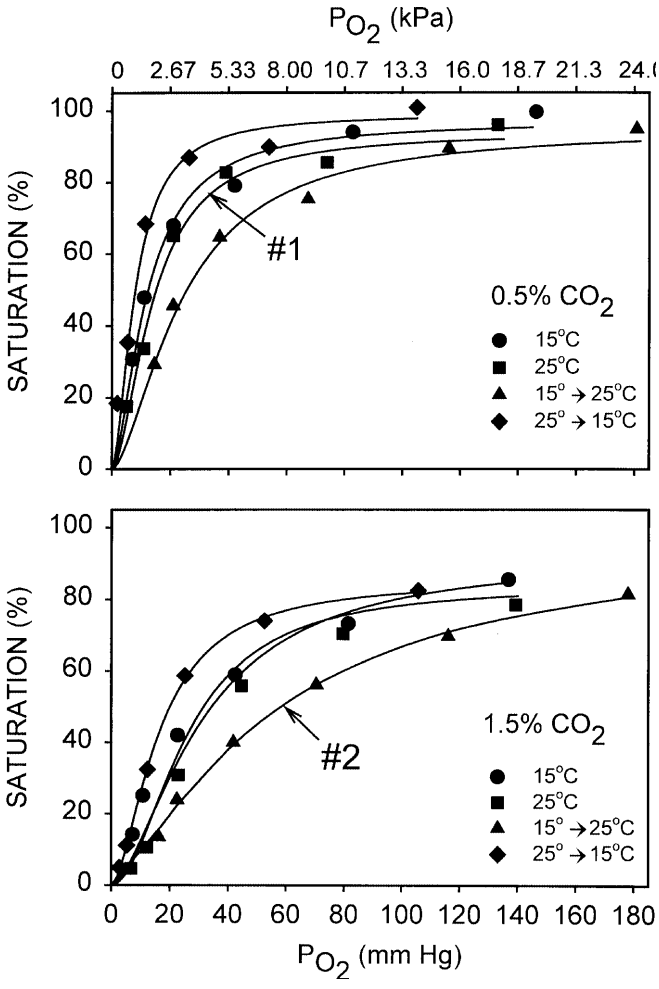

Fig. 1 *Thunnus obesus*. Representative O_2 dissociation curves from bigeye tuna blood equilibrated with CO_2 levels mimicking those of arterial and venous blood (*0.5%*, *1.5% CO_2*, respectively) at 15 and 25 °C, and subjected to closed-system (*15 → 25 °C*, *25 → 15 °C*) temperature changes [*Curves #1 and #2* changes in partial pressure of O_2 (P_{O_2}) and O_2 content taking place during passage through gills and capillary beds of deep red swimming muscles, respectively]

circumstances, whereas skipjack tuna blood showed the exact opposite response from that of bigeye tuna blood, a reduced Bohr effect. In other words, during closed-system temperature shifts, in bigeye and skipjack tuna blood, there are interactive (but opposite) effects of the simultaneous changes temperature, P_{CO_2}, and pH_e on blood oxygen affinity, whereas in yellowfin tuna blood there are not.

Hb solutions

The effects of temperature change on the O_2 affinity of bigeye, yellowfin, and skipjack tuna Hb solutions are shown in Fig. 4. [The data for bluefin tuna Hb were taken from Carey and Gibson (1983).] There was an apparent increase in oxygenation state (i.e. an increase in O_2 affinity) in skipjack and bluefin tuna Hb when the temperature increased, whereas the O_2 affinity of bigeye

Table 2 *Thunnus obesus*. Changes (means ± SEM; $n = 6$) in pH_{50} (pH at P_{50}) of bigeye tuna blood subjected to open- and closed-system temperature shifts

System	0.5% CO_2	1.5% CO_2
Open	0.0030 ± 0.0012	0.0062 ± 0.0018
Closed	−0.0123 ± 0.0015	−0.0102 ± 0.0006

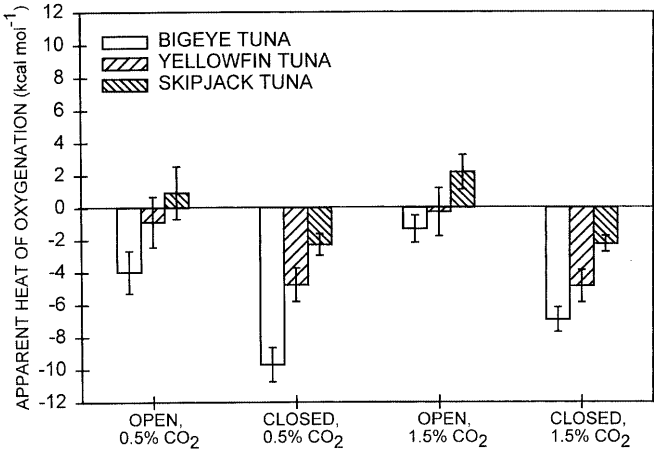

Fig. 2 *Thunnus obesus*, *T. albacares*, *Katsuwonus pelamis*. Temperature sensitivity of blood from bigeye tuna, yellowfin tuna, and skipjack tuna expressed as apparent heat of oxygenation (in all cases $n = 6$). [Data for yellowfin and skipjack tuna blood were taken from Brill and Bushnell (1991a): measurement temperatures were 20 and 30 °C]

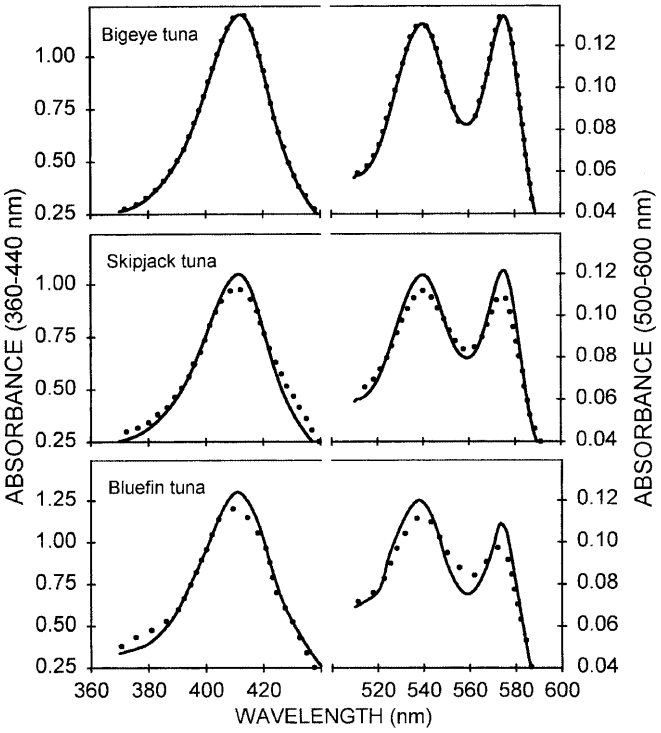

Fig. 4 *Thunnus obesus*, *Katsuwonus pelamis*, *T. thynnus*. Absorbance spectra showing changes in O_2 affinity of Hb solutions subjected to closed-system temperature change (25 → 15 °C). (*Continuous lines* higher temperatures; *dotted lines* lower temperature) Data for bluefin tuna from Fig. 2 in Carey and Gibson (1983)

and yellowfin tuna Hb (data for yellowfin tuna not shown) were unaffected; i.e., as explained by Carey and Gibson, the data imply that a reverse temperature effect occurred in Hb from skipjack tuna and bluefin tuna subjected to a closed-system temperature change, whereas the O_2 affinity of Hb from bigeye tuna and yellowfin tuna was temperature-independent.

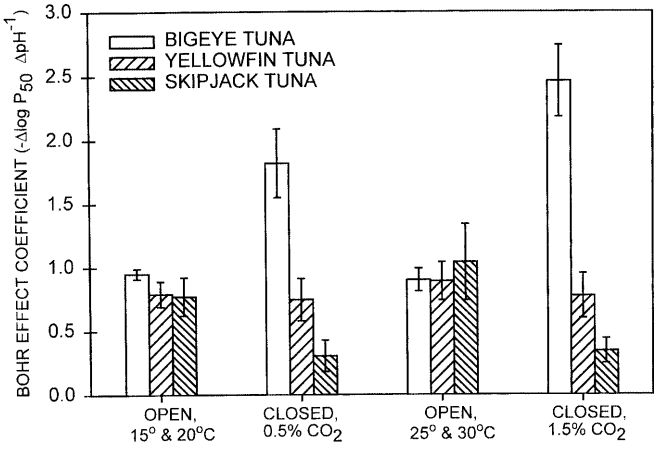

Fig. 3 *Thunnus obesus*, *T. albacares*, *Katsuwonus pelamis*. Bohr effect coefficients measured in bigeye, yellowfin, and skipjack tuna blood (in all cases $n = 6$). Extracellular pH (pH_e) changes were created either by equilibration of blood to gas mixtures containing 0.5 and 1.5% CO_2, or subjection to closed-system temperature changes (*OPEN* and *CLOSED*, respectively). Data for yellowfin and skipjack tuna blood were taken from Brill and Bushnell (1991a). Bigeye tuna blood was equilibrated at 15 and 25 °C; yellowfin and skipjack tuna blood at 20 and 30 °C

Discussion and conclusions

All values listed in Table 1 for *Thunnus obesus* are within ranges considered normal for tunas in vivo (Jones et al. 1986; Brill and Bushnell 1991a; Bushnell and Brill 1992; Brill et al. 1992; Korsmeyer et al. 1997). Our procedures for obtaining and handling bigeye tuna (*T. obesus*) blood were, moreover, identical to those used in a separate study that measured the effects of catecholamines on O_2 affinity in blood of yellowfin (*T. albacares*) and skipjack (*Katsuwonus pelamis*) tuna (Lowe et al. 1998). The P_{50}s for both yellowfin and skipjack tuna blood found prior to treatment with catecholamines were identical to those found by Brill and Bushnell (1991a). In the latter study, samples were taken directly from the fish and immediately placed in tonometers or stored overnight at 4 °C, warmed to room temperature, and then placed in tonometers. From these data we conclude that our collection and handling procedures have no detrimental effects on intracellular ligands which influence P_{50} (e.g. ATP, GTP). Our techniques are, therefore, the best currently available for obtaining tuna red blood cells not stimulated by catecholamines and that respond normally in vitro. We

did not use the alternative approach of washing and suspending the red blood cells in Ringers or buffered saline, as the composition of the media can significantly influence blood O_2 affinity (Dalessio et al. 1991).

Bigeye tuna blood had a higher O_2 affinity (P_{50} = 1.6 to 2.0 kPa or 12 to 16 mmHg at 0.5% CO_2) than the blood of yellowfin tuna, skipjack tuna, and kawakawa blood (P_{50} = 2.8 to 3.1 kPa or 21 to 23 mmHg at 0.5% CO_2: Jones et al. 1986; Brill and Bushnell 1991a). These data agree with our hypothesis that bigeye tuna are more hypoxia-tolerant than the latter three species. Blood O_2 carrying capacity and rates of transport in tunas exceed those of other fishes and approach those of mammals (Brill and Bushnell 1991b; Bushnell and Brill 1991, 1992; Brill and Jones 1994; Bushnell and Jones 1994; Brill 1996; Dickson 1996; Korsmeyer et al. 1996). Our data conformed with this generalization; bigeye tuna blood had a high hemoglobin concentration and a high mean cell hemoglobin concentration (Table 1). The shapes of the O_2 dissociation curves for bigeye tuna ranged from sigmoid to intermediary between sigmoid and hyperbolic, and differed significantly from the hyperbolic O_2 dissociation curve reported for albacore, the only other tuna species reported to have high O_2-affinity blood (Cech et al. 1984). A sigmoid O_2 dissociation curve is expected in tunas because of their high metabolic rates (Riggs 1970; Jones et al. 1986), and albacore remains the only tuna species reported so far to have a hyperbolic blood O_2 dissociation curve (Cech et al. 1984).

The Root effect of bigeye tuna blood (−6.0 ± 1.6% at 15 °C and −8.4 ± 1.3% at 25 °C) was within the range of values reported for yellowfin tuna (−10.5 ± 1.5% at 20 °C and −5.0 ± 1.4% at 30 °C) and skipjack tuna (−17.1 ± 4.2% at 15 °C and −6.1 ± 2.5% at 25 °C) blood (Brill and Bushnell 1991a). In contrast, albacore blood has been reported to have a negligible Root effect (Cech et al. 1984). Bigeye tuna, yellowfin tuna and albacore all have swim bladders, whereas skipjack tuna do not (Godsil and Byers 1944). Therefore, there is no correlation between the presence of blood with a significant Root effect and the presence of a swim bladder; i.e. in tunas as in other teleosts (Brittain 1987), blood with a significant Root effect does not appear to be necessary to excrete O_2 into a swim bladder. The reasons for the differences in the Root effect among tuna species remain to be elucidated. The functional significance of the Root effect in fish blood in general, however, appears to be to enhance the O_2 gradient from the blood to the mitochondria, and thus ensure adequate rates of O_2 delivery despite the relatively low capillary density in fish muscle (Mathieu-Costello et al. 1992; Brauner and Randall 1996).

Temperature sensitivity of blood-O_2 affinity and regional endothermy

During an open-system temperature change at the 1.5% CO_2, the O_2 affinity of bigeye tuna blood was clearly temperature-independent (i.e. apparent heat of oxygenation not significantly different from zero: Fig. 2). At 0.5% CO_2, the apparent heat of oxygenation was significantly different from zero, although it was less than that recorded for the blood of other teleosts (Grigg 1969; Powers et al. 1979). Similar results have been reported for the blood from albacore, bluefin, yellowfin and skipjack tunas (Carey and Gibson 1983; Cech et al. 1984; Brill and Bushnell 1991a). Rossi-Fanelli and Antonini (1960) were the first to propose that this temperature insensitivity "... would enable the animal to live in waters of very different temperatures without modification of the functional properties of its respiratory pigment"; this is most probably the reason for its commonality among all five tuna species.

In contrast, during a closed-system temperature change, at both levels of CO_2 bigeye tuna blood showed changes in O_2 affinity (i.e. apparent heat of oxygenation) significantly greater than those seen in yellowfin or skipjack tuna blood (Fig. 2). The different behaviors of yellowfin, skipjack, and bigeye tuna blood during closed-system temperature changes are also clearly illustrated in Fig. 5, which shows the changes in P_{O_2} resulting from a closed-system temperature change. The elevated temperature sensitivity of bigeye tuna blood results in larger changes in P_{O_2} (especially at higher initial P_{O_2} levels), whereas the reduced temperature sensitivity of skipjack blood results in smaller changes in P_{O_2}, than those in yellowfin tuna blood.

During closed-system temperature changes (Table 2), changes in the pH_{50} of bigeye tuna blood were in the expected direction, but slightly below the expected magnitude ($\simeq -0.016\ \Delta pH\ °C^{-1}$). In contrast, during open-system temperature changes, changes in pH_{50} were of the expected (smaller) magnitude, but opposite to the expected direction (Cech et al. 1984; Perry et al. 1985; Jones et al. 1986; Truchot 1987; Brill and Bushnell 1991a; Brill et al. 1992).

More important than the changes in pH_e per se, are the interactive effects of changes in pH_e and temperature on blood O_2 affinity during closed-system temperature change. The identical Bohr effects recorded in yellowfin tuna blood during open- and closed-system temperature changes (Fig. 3) indicate that the consequences of changing pH_e were the same whether the change was effected by changing the P_{CO_2} alone, or by the inevitable changes of P_{CO_2} and pH_e that accompany closed-system temperature shifts. Alterations in the P_{50} of yellowfin tuna blood during closed-system temperature change were, therefore, due to changes in P_{CO_2} and pH_e and not the change in temperature per se. In contrast, skipjack tuna blood showed a reduced temperature effect, and bigeye tuna blood an enhanced temperature effect, during closed-system temperature change (Figs. 2, 3, 5). Therefore, in bigeye and skipjack tuna blood there are interactions among the changes in P_{CO_2}, pH_e, and temperature occurring during closed-system temperature shifts. These interactions reduce the change in O_2 affinity of skipjack tuna blood, but increase the change in O_2

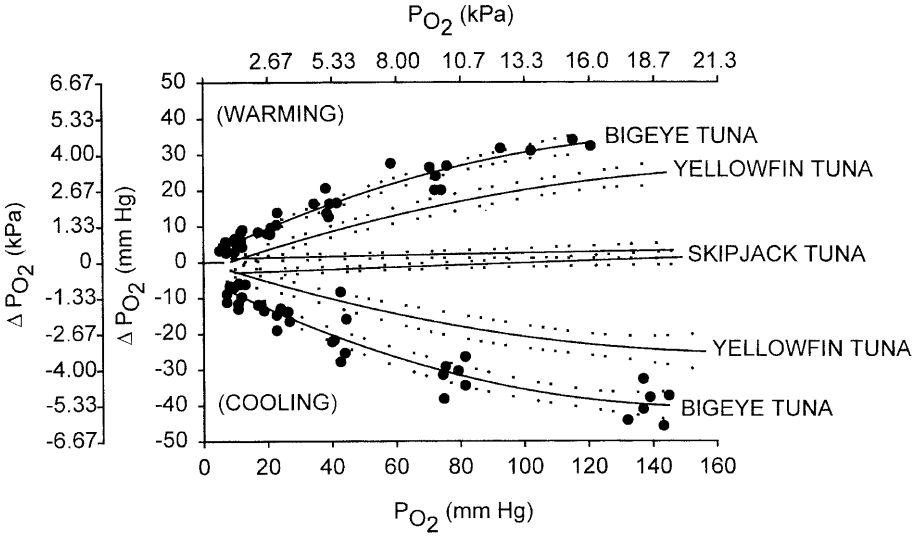

Fig. 5 *Thunnus obesus*, *T. albacares*, *Katsuwonus pelamis*. Effect of closed-system 10 C° temperature change on P_{O_2} of blood from bigeye (●), yellowfin, and skipjack tuna. Data for latter two species from Brill and Bushnell (1991a): only regression lines (*continuous lines*) and 95% confidence intervals (*dotted lines*) are shown. Blood from bigeye tuna was subjected to temperature change between 15 and 25 °C, blood from yellowfin and skipjack tuna to temperature change between 20 and 30 °C (*Abscissa* P_{O_2} to which blood was equilibrated prior to closed-system temperature change

affinity of bigeye tuna blood. Note that skipjack tuna blood displayed a smaller change in O_2 affinity even though the pH_e change (−0.0179 and −0.0178 $\Delta pH_e/C°$ in blood equilibrated to 0.5 and 1.5% CO_2, respectively; Brill and Cousins unpublished observations) is larger than in bigeye tuna blood (Table 2). An increased temperature sensitivity during closed-system temperature change has not been previously reported for any tuna species. Elevated CO_2 levels have, however, been reported to reduce the temperature effects on O_2 affinity of blood for several fish species (Powers et al. 1979).

Our data, and those presented by Jones et al. (1986) and Brill and Bushnell (1991a) do not support the hypothesis that fish with counter-current exchangers require blood with a reversed temperature effect (i.e. increased O_2 affinity when subjected to closed-system temperature changes) to prevent O_2 offloading as blood is warmed during its passage through vascular counter-current heat exchangers (Carey and Gibson 1983; Cech et al. 1984; Hochachka and Somero 1984). Rather, blood from various tunas species shows a broad range of responses to closed-system temperature change. All tuna species studied to date have multiple hemoglobins (2 in skipjack tuna, 4 in bigeye tuna and bluefin tuna, 6 in yellowfin tuna, 6 to 8 in albacore, Sharp 1969, 1973). Data on the O_2-binding properties of hemolysates made from the blood of various tuna tunas species are available (Sharp 1975; Carey and Gibson 1977, 1983; Ikeda-Saito et al. 1983), but do not appear to reflect the interspecific differences observed with whole blood. No data are available on the properties of specific hemoglobin components, and investigations into how the various hemoglobins of tunas relate to their whole-blood O_2-binding characteristics are clearly warranted. Moreover, cranial endothermy is present in bat rays (Elasmobranchii, Batoidea, Myliobatiformes; Alexander 1995) and billfishes (Istiophoridae and Xiphiidae; Carey 1982; Block 1986), and swimming muscle endothermy in several species of lamnid sharks (Carey et al. 1985). The billfishes, like the tunas, also exhibit multiple hemoglobins (Sharp 1973). We therefore argue that measuring the changes O_2-affinity during open- and closed-system temperature changes in blood and component hemoglobins from these widely divergent species is also needed. The data could help elucidate the reasons for the widely different responses to temperature change seen in tuna blood, and perhaps the underlying physiological mechanisms.

Blood-O_2 affinity and hypoxia tolerance

Marine and freshwater fishes tolerant of hypoxia typically have blood and/or hemoglobin with a higher O_2 affinity (i.e. with a lower P_{50}) than less hypoxia-tolerant species (Wood et al. 1975; Weber and Lykkeboe 1978; Powers 1980, 1985; Yang et al. 1992; Jensen et al. 1993). Moreover, short-term (i.e. days) acclimation of fish to hypoxia causes a decrease in the intracellular ATP concentration of red blood cells and an increase in blood O_2 affinity, thus improving O_2 extraction from the water (Wood and Johansen 1972, 1973; Wood et al. 1975; Weber et al. 1976; Lykkeboe and Weber 1978). The higher blood-O_2 affinity of bigeye tuna blood found in our study supports our hypothesis that adaptations for hypoxia tolerance in this species include blood with a high O_2 affinity. In turn, this enhanced tolerance of low ambient O_2, allows bigeye tunas a greater degree of vertical mobility than yellowfin and skipjack tunas. The latter two species have blood with lower O_2 affinities and typically inhabit only the warmer and well-oxygenated surface layer (Sharp 1978; Sund et al. 1981; Hanamoto 1987; Holland et al. 1992; Cayré and Marsac 1993; Brill 1994; Mohri et al. 1996; Block et al. 1997).

We further hypothesize that bigeye tuna have a distinct advantage for off-loading O_2 in muscle capillaries compared to hypoxia-tolerant ectothermic teleosts. The large decrease in blood O_2 affinity and the resultant increase in P_{O_2} during a closed-system temperature change (Fig. 5) means that O_2 off-loading in

the warmed swimming muscles is not compromised. As shown in Fig. 1, bigeye tuna blood would operate on O_2 dissociation curve "A" during its passage through the gills, and on the right-shifted curve "B" during passage through capillaries of the warmed deep red muscle. The high O_2 affinity (Table 1) and enhanced Bohr effect during closed-system temperature changes (Fig. 3) seen in bigeye tuna blood thus provide an effective system for extracting O_2 at high rates from the ventilatory water stream even during environmental hypoxia, but do so without compromising rates of O_2 delivery and off-loading in the muscle capillaries.

Our observations imply that, although bigeye tuna are tolerant of hypoxia, they are still able to sustain high metabolic rates, and thus benefit from the selective advantages provided by such rates (Brill 1996). The standard metabolic rate of bigeye and yellowfin tuna are $\simeq$300 to 400 mg O_2 kg^{-1} h^{-1} (Brill 1987; Bushnell et al. 1990), and routine metabolic rates are $\simeq$500 to 800 mg O_2 kg^{-1} h^{-1} (Bushnell and Brill 1991; Dewar and Graham 1994). In contrast, the routine metabolic rate of other equal-sized, active teleosts (measured under similar temperature conditions) is $\simeq$100 mg O_2 kg^{-1} h^{-1} (Yamamoto 1991; Bushnell and Jones 1994). The maximum metabolic rates have not been measured in any tuna species, although yellowfin and skipjack tuna can achieve metabolic rates as high as $\simeq$2500 mg O_2 kg^{-1} h^{-1} (Gooding et al. 1981; Dewar 1993). It seems reasonable to assume bigeye reach the same levels. The maximum metabolic rate measured in other teleosts is $\simeq 1000 \pm 200$ mg O_2 kg^{-1} h^{-1} (Brett 1972).

This situation is in direct contrast to that found for the scorpaenid fishes *Sebastolobus alascanus* and *S. guttata* studied by Yang et al. (1992). The former species occurs predominantly within the oxygen minimum zone on the upper continental slope (400 to 1200 m), and can regulate oxygen uptake rates down to 0.3 ml O_2 l^{-1}. The latter species, however, lives in shallow water ($<$180 m), and shows decreases in oxygen uptake rates when O_2 concentrations fall below 1 ml O_2 l^{-1}. As with tuna species, the P_{50} of *S. guttata* blood (3.23 kPa, 24.2 mmHg at 20 °C) is significantly above that of *S. alascanus* blood (1.85 kPa, 13.9 mmHg at 20 °C). In other words, the more hypoxia-tolerant species has blood with a higher oxygen affinity. However, the metabolic rate of *S. alascanus* is approximately one-half that of *S. guttata* when measured at the same temperature, implying that blood with a high O_2 affinity can compromise rates of O_2 delivery to the tissues.

Phylogeny of the genus *Thunnus*

The exact phylogenetic relationships of bigeye and yellowfin tunas to other members of the genus *Thunnus* remain controversial and depend on which anatomical, allozyme, or genomic data are considered (Gibbs and Collette 1967; Collette 1978; Sharp and Pirages 1978; Block et al. 1993; Chow and Kishino 1995; Elliott and Ward 1995; Finnerty and Block 1995; Bremer et al. 1997). Based on anatomical data, Collette (1978) divided the genus *Thunnus* into two subgenera: the temperate subgenus *Thunnus* containing bigeye tuna, and the tropical subgenus *Neothunnus*, containing the yellowfin tuna. Subsequent allozyme (Elliott and Ward 1995) and genomic (Block et al. 1993; Chow and Kishino 1995) data have failed to support this division. Some authors (e.g. Chow and Kishino 1995) declare bigeye tuna to be an early offshoot of the genus *Thunnus*, whereas others (e.g. Bremer et al. 1997) continue to use Collette's original subgeneric nomenclature and consider bigeye tuna to be more closely related to the tropical subgenus *Neothunnus* than the temperate subgenus *Thunnus*.

We consider our data for bigeye tuna blood, and those presented by Brill and Bushnell (1991a) for the blood of yellowfin and skipjack tuna, to be most congruent with the phylogenies proposed by Bremer et al. (1997) and Sharp and Pirages (1978), i.e. that bigeye tuna are derived from a tropical tuna ancestor that expanded its vertical range. It is our opinion that, by the evolution of the unique blood-O_2 affinity characteristics described herein (and most probably other physiological characteristics that remain to be discovered), bigeye tuna have become better able to exploit food resources below the thermocline and in hypoxic waters than yellowfin or skipjack tunas. Indeed, support for our assertion is suggested by the comparative analysis of gut contents provided by Kornilova (1981), Pelczarski (1988), and Grudinin (1989). All three investigators concluded that bigeye tuna feed at deeper depths than yellowfin tuna.

Temperature sensitivity of Hb solutions vs whole blood

We reexamined the effects of temperature on O_2 affinity of Hb solutions derived from bigeye, yellowfin, and skipjack tuna blood because the effects of closed-system temperature changes on the O_2 affinity of whole blood from these species are dramatically different. When subjected to a closed-system temperature change, a Hb solution from skipjack tuna blood displayed a reversed temperature effect (increasing temperature caused an increase in Hb O_2 affinity) identical to that seen in Hb solutions derived from bluefin tuna (Fig. 4). The O_2 affinity of Hb solutions derived from bigeye and yellowfin tuna blood displayed almost no response. In direct contrast, bigeye tuna blood showed the largest response to a closed-system temperature change of any tuna species examined so far, whereas skipjack tuna blood showed the smallest. Our results, therefore agree with the conclusion of Powers et al. (1979) that the effect of temperature on Hb O_2 affinity does not necessarily reflect the influence of temperature on blood-O_2 affinity. This difference between the influence of temperature on the O_2 affinity of Hb solutions and whole blood probably occurs because intact erythrocytes are able to regulate one or more ligands (e.g. ATP, GTP, intracellular

pH, etc.) that effect Hb O_2 affinity (Lykkeboe and Weber1978; Powers 1980). However, studies dealing with the effects of temperature on intracellular ligands usually involve either seasonal temperature acclimation, whereby temperature changes usually take months to occur, or experiments where fish are acclimated to various temperatures and O_2 conditions for at least tens of hours and up to several months (Jensen et al. 1998; Lykkeboe and Weber 1978). The temperature changes that tuna blood experiences during its passage through vascular counter-current heat exchangers occur within a few seconds, and the 10 C° or more changes in ambient temperature to which bigeye tuna are repeatedly subjected on a daily basis occur over tens of minutes. These time periods may be too short for any active adjustments in red blood cell intracellular organic phosphates (e.g. ATP) to occur.

The temperature sensitivity of bluefin tuna blood remains to be evaluated using techniques similar to ours. If it is important that the off-loading of O_2 in the exercising muscle of tunas be at a high P_{50}, then we predict that a normal temperature effect during closed-system temperature change will also occur in bluefin tuna blood.

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