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Effect of temperature acclimation on red blood cell oxygen affinity in Pacific bluefin tuna (*Thunnus orientalis*) and yellowfin tuna (*Thunnus albacares*)

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ABSTRACT

Hemoglobin–oxygen (Hb–O_2) binding properties are central to aerobic physiology, and must be optimized for an animal's aerobic requirements and environmental conditions, both of which can vary widely with seasonal changes or acutely with diving. In the case of tunas, the matter is further complicated by large regional temperature differences between tissues within the same animal. This study investigates the effects of thermal acclimation on red blood cell Hb–O_2 binding in Pacific bluefin tuna (*T. orientalis*) and yellowfin tuna (*T. albacares*) maintained in captive tanks at acclimation temperatures of 17°, 20° and 24 °C. Oxygen binding properties of acclimated tuna isolated red blood cells were examined under varying experimental temperatures (15°–35 °C) and CO_2 levels (0%, 0.5% and 1.5%). Results for Pacific bluefin tuna produced temperature-independence at 17 °C- and 20 °C-acclimation temperatures and significant reverse temperature-dependence at 24 °C-acclimation in the absence of CO_2, with instances of reverse temperature-dependence in 17 °C- and 24 °C-acclimations at 0.5% and 1.5% CO_2. In contrast, yellowfin tuna produced normal temperature-dependence at each acclimation temperature at 0% CO_2, temperature-independence at 0.5% and 1.5% CO_2, and significant reverse temperature-dependence at 17 °C-acclimation and 0.5% CO_2. Thermal acclimation of Pacific bluefin tuna increased O_2 binding affinity of the 17 °C-acclimation group, and produced a significantly steeper oxygen equilibrium curve slope (n_H) at 24 °C-acclimation compared to the other acclimation temperatures. We discuss the potential implications of these findings below.

1. Introduction

Many species depend upon oxygen (O_2) transport proteins, such as hemoglobin (Hb) or hemocyanins, to effectively transport O_2 from the respiratory surfaces to tissues elsewhere in the body (Antonini and Brunori, 1970). Regardless of the specific transport protein or respiratory organ, O_2 transport must be optimized for an animal's aerobic requirements and environmental conditions, both of which can vary widely with seasonal changes, or acutely with deep foraging dives through the thermocline.

Warming oceans due to climate change will create significant challenges for fish species, and test physiological limits to temperature and carbon dioxide (CO_2) levels (Portner and Knust, 2007; Portner and Farrell, 2008). Understanding how species respond to warming ocean temperatures and increasing CO_2 levels is critical for predicting future species distributions and impacts of changing ocean environments. For fish species that provide an important source for human consumption, this information may be vital to our own species as well, because fisheries productivity may be impacted by shifting population distributions.

Tunas are highly migratory fishes of the family Scombridae that inhabit a wide range of oceanic niches. The speciation of the *Thunnus* genera has led to specialists of the tropics, subtropics, temperate and subpolar waters. However, in all cases, tunas return to spawn in warmer waters (tropics and subtropics), or, in the case of Atlantic bluefin tuna, warm temperate (Mediterranean) seas (Block et al., 2001). Thus the physiology of a temperate tuna (e.g. bluefin and albacore) must be able to respond to the thermal challenges of cooler temperatures during bouts of foraging at high latitudes in colder seas, and warmer temperatures at lower-latitude spawning grounds. Such broad ecological ranges would potentially select for a eurythermal physiology with Hb–O_2 binding that responds across a broad thermal range, a common situation in ectotherms occupying wide thermal niches.

However, the fact that bluefin tunas, as well as some other tuna species, possess regional endothermy, the ability to maintain tissue temperatures above those of the surrounding waters, complicates their O_2 transport requirements (Carey and Teal, 1966; Carey et al., 1971;

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Carey and Gibson, 1983; Cech et al., 1984; Clark et al., 2008). These regionally endothermic traits, shared with billfish and opah (cranial endothermy), sharks of the family Lamnidae (white, porbeagle, salmon and mako sharks), and some thresher sharks, rely on a system of countercurrent heat exchangers, or retia mirabilia. Retia, densely bundled arteries and veins, facilitate the transfer of heat from blood warmed by the metabolically active tissues (e.g. muscle, viscera, brain and eyes) to cold, well-oxygenated blood entering from the gills, allowing tunas to maintain body temperatures up to 20 °C greater than ambient water temperatures (Carey and Teal, 1966; Carey et al., 1971; Carey and Lawson, 1973; Dizon and Brill, 1979; Block et al., 1993, 2001; Lawson et al., 2010; Weber et al., 2010; Patterson et al., 2011).

Warming of muscle, viscera, brain and eye temperatures can increase physiological performance (Carey et al., 1971; Block et al., 1993; Block and Finnerty, 1994; Brill, 1996; Altringham and Block, 1997; Dickson and Graham, 2004), but importantly, at an organismal level, having internal warmth in metabolically active tissues coupled with a heart exposed to ambient temperatures (due to close proximity to the external environment and a coronary circulation at the temperature of ambient water) poses unique O_2 transport challenges in these fish. In most animals that rely on Hb as an O_2 transport protein, O_2 affinity decreases with increasing temperature, decreasing pH and increasing CO_2 levels (Bohr et al., 1904; Christiansen et al., 1914; Jensen, 2004). These shifts favor Hb offloading of O_2 to warmer, acidic and O_2-depleted tissues, such as working muscle (Barcroft and King, 1909; Weber and Campbell, 2011).

For bluefin tunas, which have the largest ecological niches of all tunas and the highest degree of endothermic characteristics (Carey et al., 1971; Block et al., 2001; Boustany et al., 2010; Block et al., 2011), one would predict Hb–O_2 binding properties that produce optimal O_2 transport in varying environments (i.e. the ability to extract O_2 from both warm and cold environments, and to unload O_2 to both warm and cold tissues). Numerous studies have explored Hb–O_2 binding characteristics of tunas (Rossi-Fanelli and Antonini, 1960; Carey and Gibson, 1977; Cech et al., 1984; Lowe et al., 2000; Clark et al., 2008). Studies of Atlantic bluefin tuna (*T. thynnus*) have demonstrated temperature-independent Hb–O_2 binding (Rossi-Fanelli and Antonini, 1960) and reverse temperature-dependence above 20% Hb–O_2 saturation (Carey and Gibson, 1977, 1983). Blood studies from albacore tuna (*Thunnus alalunga*), a temperate species that generally inhabits waters of 9–16 °C, also showed reverse temperature-dependence (Laurs et al., 1980; Cech et al., 1984). Southern bluefin tuna (*Thunnus maccoyii*) demonstrated reverse temperature-dependence between 10° and 23 °C, and temperature-independence above 23 °C (Clark et al., 2008). Some have postulated that reverse temperature responses may reduce premature O_2 dissociation as well-oxygenated blood travels from the gills and is warmed by the retia, while also facilitating O_2 delivery to the ambient temperature-equilibrated heart (Carey and Gibson, 1983; Clark et al., 2008). Existing data do not support a consensus explanation as to why temperature-independence and reverse temperature-dependence may have evolved in these organisms.

Prolonged cold exposure has been shown to elicit both physiologic and genomic responses in numerous fish species (Vanstone et al., 1964; Lucassen et al., 2006). For example, cold acclimation elicits a protective response that improves cardiac calcium handling in ventricular myocytes (Shiels et al., 2011), as well as enhanced performance at colder temperatures (e.g. 14 °C) and changes in gene expression (Jayasundara et al., 2013). Similar transcriptomic activation effects due to long-term temperature acclimation may occur in O_2 delivery systems, though such effects have not been examined.

In the present study, we characterize the oxygen equilibrium curves (OECs) of two tuna species, Pacific bluefin tuna (*Thunnus orientalis*) and yellowfin tuna (*Thunnus albacares*), and examine the effects of acute and chronic temperature acclimation on Hb–O_2 binding properties. Pacific bluefin tuna has not been previously analyzed for OECs, and neither species has been studied for acclimation effects on Hb–O_2 binding.

2. Materials and methods

2.1. Fish capture and long-term acclimation procedures

Pacific bluefin tuna (*T. orientalis*) and yellowfin tuna (*T. albacares*) were captured by hook and line off San Diego, CA, and kept live in seawater-filled wells onboard F/V "Shogun." Tunas were transferred by a water-filled tank aboard a specially-designed transport truck to the Stanford University Tuna Research and Conservation Center (TRCC) in Pacific Grove, CA, USA. The two tuna species were maintained in joint captivity in 109 · m^3 circular tanks for five months at the TRCC. Tanks were held at 20 ± 1.5 °C upon initial introduction of the fish. Tank temperatures were then changed to the appropriate acclimation temperature, at a rate of 1 °C maximum per week. Fish were fed a diet of squid, fish and vitamin-enriched gelatin, and were held under conditions similar to those described in Blank et al. (2004). All procedures were in accordance with Stanford University Institutional Animal Care and Use Committee (IACUC) protocols.

Three 20 °C-acclimated Pacific bluefin tuna and three 20 °C-acclimated yellowfin tuna were sampled over a period of two weeks. All individuals sampled for 20 °C-acclimation tests, considered baseline controls, were euthanized for additional experiments and not exposed to further acclimation. The tank temperature was then raised to 24 °C, as described above, and remaining fish in the tank were acclimated to 24 °C for four weeks. Five Pacific bluefin tuna and three yellowfin tuna were sampled and sacrificed over a period of two weeks, using the exact same protocols utilized at 20 °C acclimation. The process was then repeated at 17 °C-acclimation for remaining fish, although some 17 °C-acclimated fish were released back into the tank for future experiments. Acclimation sample sizes, mean mass and length, and mean hematocrit (%Hct) are reported in Table 1, although we note the limitations of stress-induced hematocrit sampling associated with pithing methods (Gallaugher and Farrell, 1998).

2.2. Sample extraction and preparation

Tunas were isolated for blood sampling by experienced fish handlers utilizing a water-filled vinyl sling. Fish were always captured live, and, once in the sling, were placed in supine position, which has a calming effect (tonic immobility) on the tuna. Fish were sampled using one of two techniques: the first method involved sampling fish live in the sling, using a series of visible targets along the ventral surface of the fish, where opercle bones meet, to insert a needle into the bulbus arteriosis within 60 s of immobilization in the sling. Fish sampled live in the sling were then either released back into the tank, without impact, to be used for future experiments, or were euthanized for additional cardiac and other sampling studies. The second method involved euthanizing fish by lifting out of the tank with the sling and rapidly pithing, then sampling from the caudal vein or the bulbus arteriosis. Comparisons of the two sampling methods (live in the tank vs. post-mortem) in the same fish did not yield significant differences in Hb–O_2 binding properties (Supp. Fig. 1).

Blood was extracted using a 21 G · 1 1/2 inch needle and 3 ml syringe, both of which were pre-rinsed with marine fish Ringer's solution (which contained (in mM): 150 NaCl, 5.4 KCl, 1.5 $MgCl_2$, 3.2 $CaCl_2$, 10 glucose and 10 HEPES) mixed with 0.05 M ethylenediaminetetraacetic acid (EDTA). All chemicals were purchased from Sigma (St. Louis, MO). After sampling, blood was immediately transferred to an EDTA-coated BD Vacutainer (10.8 mg), and additional samples were taken using 75 mm heparinized hematocrit tubes, in order to establish sample hematocrit values (Table 1). The blood sample (2–3 ml) was transferred to a 15 ml Falcon tube containing 10 ml fresh marine fish Ringer's solution, with pH adjusted to 7.80 with NaOH at 20 °C. The sample was centrifuged to rinse and pack RBCs and remove supernatant. RBC rinses were repeated three times, after which supernatant was discarded and RBCs were resuspended in aliquots of fresh Ringer's solution at

Table 1
Mean mass, straight fork length (SFL), hematocrit (Hct) and sample size for each species and individual acclimation treatment. Superscript letters represent statistical similarities between acclimation Hct values within one species.

	Pacific bluefin tuna (*T. orientalis*)			Yellowfin tuna (*T. albacares*)		
Acclimation	17 °C	20 °C	24 °C	17 °C	20 °C	24 °C
Mass (kg)	17.3 ± 1.0	11.8 ± 1.5	16.3 ± 2.7	9.7 ± 0.9	5.8 ± 0.6	9.9 ± 0.9
SFL (cm)	93.4 ± 3.0	81.9 ± 1.4	88.2 ± 4.2	78.4 ± 2.8	68.0 ± 1.5	78.7 ± 2.9
Hct (%)	33.1 (±6.5)	42.5 (±1.3)^{A}	43.4 (±1.1)^{A}	35.3 (±2.0)	45.3 (±0.4)^{B}	42.3 (±2.6)^{B}
n (fish)	4	3	5	3	3	3

concentration 100 μl RBCs:600 μl Ringer's. The RBC solution was measured to ensure pH 7.80, and additional washes were repeated as necessary to correct pH.

2.3. Oxygen equilibrium curve preparation and analysis

Samples for OEC analyses were prepared using a custom microplate-based, parallel-assay, multi-cuvette tonometry cell designed and refined at the Duke University Marine Lab, as previously described (Lilly et al., 2013). This method uses thin-film sample holders in a gas-sealed tonometry chamber, and allows for multiple samples to be tested simultaneously. Individual OEC samples were prepared by pipetting 10 μl of prepared blood sample onto a 1 μm thick gas-permeable membrane (Saran Wrap™) secured with an O-ring over a black plastic hollow stand-ring. A second membrane layer was lowered onto the sample and secured, to produce a thin sample layer. Assembled microcuvettes were placed into a clear plastic insert secured in the multi-cuvette tonometer. Three replicate samples were made for each individual fish and acclimation condition. Measurements for OECs were conducted sequentially by temperature, from 15° to 35 °C, within each CO_2 treatment. One set of samples was made for each set of temperatures at each CO_2 value (e.g. one set for all temperatures at 0% CO_2). All measurements were conducted within 48 h after sample extraction from the fish. Any samples not used immediately after extraction were placed on ice until processed.

RBC suspensions were analyzed at 15°, 20°, 25°, 30° and 35 °C at 0% CO_2, and 20°, 25° and 30 °C at 0.5% and 1.5% CO_2. Gas mixing was accomplished via a computer-controlled gas mixing apparatus (MCQ Gas Mixer, Italy). Before each new set of sampling tests, 100% pure N_2 was flowed continuously through the sample-holding chamber for 30 min to establish complete deoxygenation, verified by visual analysis of spectra. Oxygen was added in incremental steps, at 0.5, 1, 2, 3, 4, 5, 7, 10 and 21% O_2. Values for pO_2 were calculated by multiplying the decimal value of $\%O_2$ by 760 mm Hg, the average atmospheric pressure at sea level, where all experiments were conducted. A three-minute wait period was included between each addition and its corresponding sample run, to ensure complete O_2 equilibrium at each partial pressure, as previously validated for Hb samples using this method. For runs involving CO_2, a mixture of N_2 and the appropriate CO_2 value (e.g. 99.5% N_2 and 0.5% CO_2) was flowed continuously through the chamber for 30 min prior to sampling. A constant experimental $\%CO_2$ level (e.g. 0.5% or 1.5%) was flowed continuously through the cell during each CO_2 experiment, with only the N_2:O_2 ratio changing with incremental O_2 additions. An additional step of pure O_2–CO_2 was added for measurements involving CO_2 (e.g. 99.5% O_2 − 0.5% CO_2 for the 0.5% CO_2 treatments), in order to ensure that potential Hb saturation above 21% O_2 was accounted for. We chose to sample blood at physiologically-relevant CO_2 levels of 0.5% and 1.5% (3.8 and 11.4 mm Hg, respectively), according to previous studies of tuna species (Jones et al., 1986; Bushnell, 1988; Brill and Bushnell, 1991; Clark et al., 2008).

2.4. Data processing and statistics

Three replicate blood samples from each fish were analyzed under each set of experimental conditions, and were averaged to ensure reproducibility. The standard error of measurement (s.e.m.) between percent Hb–O_2 saturation values of replicate samples for one fish was always <0.02. Data from individual fish in the same treatment (same species and acclimation temperature) were averaged to produce one OEC for each set of conditions. All data were plotted using SigmaPlot Software (Version 10.0, Systat Software, CA).

Slight variations between individual OEC measurements occasionally produced lower saturation values for 0.5% and 1% O_2 concentrations than for 0% O_2. For consistency between calculations, we always used 0% O_2 as our assumed lowest saturation point in calculations, leading to occasional negative saturation values. We present these slight variations as a natural byproduct of our sampling process.

Analysis of variance (ANOVA) tests were run for data value comparisons between the three long-term acclimation temperatures for each species. Analyses of intra-species comparisons at a single acclimation and blood sample temperature were made using independent t-tests. All values are listed as mean (±s.e.m.). Statistical differences were considered significant at $p < 0.05$.

Oxygen equilibrium curve P_{50} values signify the O_2 pressure (pO_2) in mm Hg at 50% saturation. Hill Plot slope (n_H) values were calculated as $((\log(y)/(1-y))/(\log(pO_2))$ at 50% saturation, where y is the percent Hb–O_2 saturation. Hill Plot and P_{50} values were calculated using mid-range (20–80%) saturation values. Temperature sensitivity of OECs was determined in part by the van't Hoff Equation for the apparent heat of oxygenation for the Hb–O_2 reaction ($\Delta H' = 2.303 * R * ((\Delta \log P_{50})/(\Delta 1/T))$ kJ mol^{-1}, where R = universal gas constant (0.008314 kJ K^{-1} mol^{-1}), and T = measurement temperature in K).

3. Results

All OECs for both Pacific bluefin tuna and yellowfin tuna at 0% CO_2 displayed sigmoidal curves corroborated by Hill values (n_H) >1.8 (Fig. 1, Supp. Fig. 2, Table 2). In contrast, OECs for both species in the presence of CO_2 (0.5% or 1.5%) displayed decreased cooperativity, indicated by n_H values <1.7 (Figs. 3, 4, Table 3). Hematocrit values for both Pacific bluefin tuna and yellowfin tuna were significantly lower for the 17 °C-acclimation group than for the other acclimations (Table 1).

3.1. Temperature effect on Pacific bluefin tuna O_2 binding at 0% CO_2

Differences in Hb–O_2 affinity of Pacific bluefin tuna between measurement temperatures only reached statistical significance for the 24 °C-acclimation group between 15° and 35 °C, with individual significant differences at 24 °C-acclimation between 15 °C and both 30° and 35 °C (Fig. 2, Table 2). Although P_{50} values were higher at 35° than at 30 °C for all acclimations, differences were not significant. Apparent heat of oxygenation ($\Delta H'$) values, calculated from the van't Hoff Equation, were greatest for 17 °C-acclimation and 24 °C-acclimation groups at 15°–20 °C, and for 17 °C- and 20 °C-acclimation groups at 30°–35 °C, but were otherwise low (Table 4).

Comparisons of blood from the three Pacific bluefin tuna acclimations produced increasing O_2 affinity with colder acclimation. Blood from the 17 °C-acclimation group produced highest O_2 affinity of the

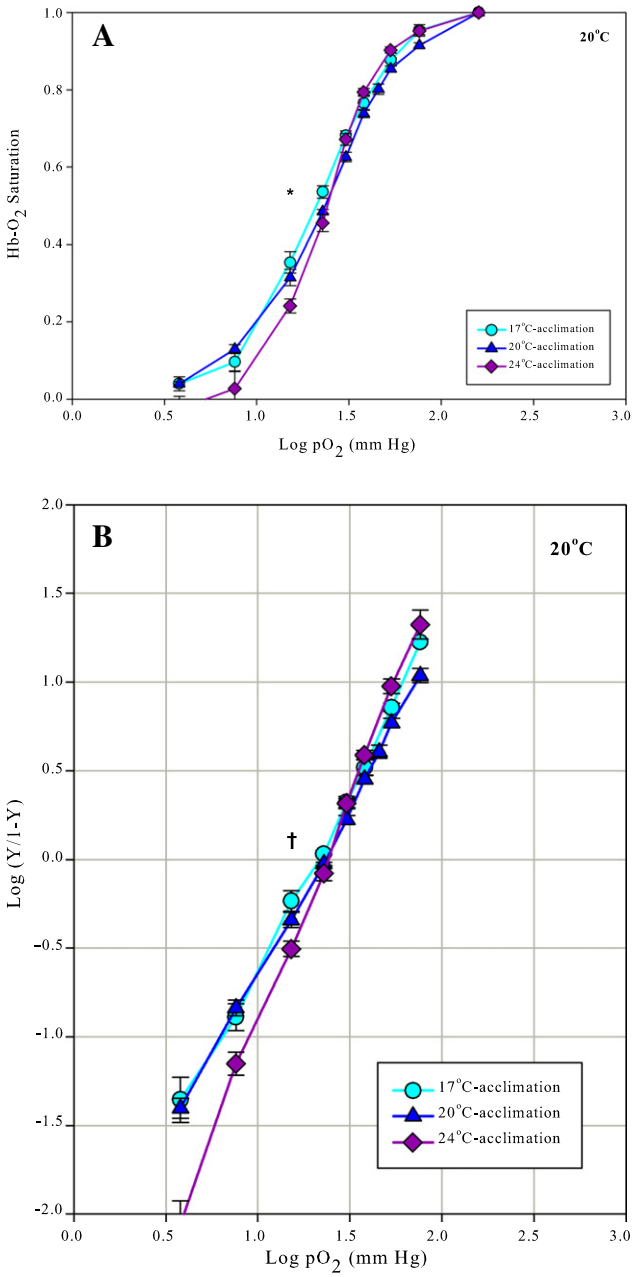

Fig. 1. (A) Oxygen equilibrium curve and (B) Hill Plot for Pacific bluefin tuna acclimation experiments (17°, 20° and 24 °C) measured at 20° at 0% CO_2 and pH 7.8. Symbols denote significant difference ($p < 0.05$) of P_{50} value (*) or n_H value (†) between 24 °C-acclimation experiment and other acclimations. Comparisons of n_H slope values yielded a significantly steeper slope for 24 °C-acclimated Pacific bluefin tuna than for the other acclimations at every measurement temperature.

three acclimations at all measurement temperatures. However, P_{50} value comparisons only yielded statistically significant differences when measured at 20 °C, between the 17 °C- and 24 °C-acclimations (Fig. 2, Table 2).

Comparisons of OEC binding curve slopes for the three Pacific bluefin tuna acclimations indicated a significantly steeper OEC slope for the 24 °C-acclimation group compared to 17°- and 20 °C-acclimations at all measurement temperatures, resulting in a crossover point between the 24 °C-acclimation curve and the other curves (Fig. 1A). Corresponding Hill Plots yielded significant differences between n_H (slope) values for 24 °C-acclimation versus 17°- and 20 °C-acclimations (Fig. 1B, Table 2). Measured at 20 °C, n_H was 2.04 (±0.15), 1.94 (±0.10), and 2.73 (±0.14) for 17°, 20° and 24 °C-acclimated fish, respectively. Comparisons of n_H values between 17 °C- and 20 °C-acclimations only produced significant differences at 25 °C.

3.2. CO_2 effect on Pacific bluefin tuna O_2 binding

The mix of temperature-independent and reverse temperature-dependent results observed in each Pacific bluefin tuna acclimation group at 0% CO_2 was also observed at physiological CO_2 concentrations (0.5% or 1.5% CO_2) (Fig. 3, Table 3). Reverse temperature-dependence between measurement temperatures was significant at both 0.5% and 1.5% CO_2 for the 17 °C-acclimation group, and at 1.5% CO_2 for the 24 °C-acclimation group.

Increasing CO_2 concentration within one measurement temperature resulted in a significant Bohr Effect, shifting OECs right and decreasing n_H slope values. For example, 17 °C-acclimated Pacific bluefin tuna n_H values measured at 20 °C were 2.04 (±0.15), 1.37 (±0.04), and 1.02 (±0.18) at 0%, 0.5% and 1.5% CO_2, respectively (Table 3).

3.3. Temperature effect on yellowfin tuna O_2 binding at 0% CO_2

Each yellowfin tuna acclimation produced overall significantly different P_{50} values when measured between 20° and 35 °C (Table 2, Supp. Fig. 3). Increasing measurement temperatures corresponded with increasing P_{50} values for both 20 °C- and 24 °C-acclimated yellowfin between 20 and 35 °C, and for 17 °C-acclimated yellowfin between 25 and 35 °C. Individual P_{50} value comparisons were significantly different for 17 °C-acclimation between 25 °C and both 20° and 35 °C, for 20 °C-acclimation between 35 °C and both 20° and 25 °C, and for 24 °C-acclimation between 35 °C and all other measurement temperatures.

In contrast to the Pacific bluefin tuna acclimation experiments, comparisons between the three yellowfin tuna acclimation groups at 0% CO_2 did not reveal a clear acclimation-related trend (Supp. Fig. 3). In fact, comparisons of 17 °C- and 24 °C-acclimated yellowfin tuna measured at 25° and 30 °C yielded nearly identical P_{50} values (Table 2). Comparisons of n_H values did not reveal significant slope differences between the three yellowfin tuna acclimations, except at 30 °C between 20 °C- and 24 °C-acclimations.

3.4. CO_2 effect on yellowfin tuna O_2 binding

Similar to Pacific bluefin tuna, the yellowfin tuna 17 °C-acclimation experiment at 0.5% CO_2 produced significant reverse temperature-dependence between 20° and 25 °C, with a notably highly endothermic $\Delta H'$ (Fig. 4A, Table 4). The other comparisons within the 17 °C-acclimation and 24 °C-acclimation groups in the presence of CO_2 yielded non-significant, temperature-independent results across measurement temperatures (Fig. 4B, Table 3). Insufficient CO_2 sampling data were collected at 20 °C-acclimation, so effects of this acclimation could not be determined.

As with Pacific bluefin tuna, yellowfin tuna at each acclimation demonstrated a significant Bohr Effect, shifting OECs right and significantly decreasing OEC slope. For 17 °C-acclimated yellowfin tuna, n_H values were 2.27 (±0.04), 1.28 (±0.01), and 1.02 (±0.17) at 0%, 0.5% and 1.5% CO_2, respectively. At each acclimation except 17 °C- and 24 °C-acclimations measured at 20 °C, comparisons between CO_2 concentration P_{50} values produced significantly different results (Table 3).

4. Discussion

Most vertebrates and invertebrates that have been examined for Hb–O_2 binding properties display a normal temperature effect of decreasing Hb–O_2 affinity with increasing temperature, facilitating O_2 unloading and delivery to metabolically active tissues. Certain tunas are among the few species that exhibit a reverse temperature Hb–O_2 dissociation curve, binding O_2 more tightly as temperature increases

Table 2
Oxygen equilibrium curve P_{50} (half-saturation, in mm Hg) and n_H (slope at half-saturation) values for Pacific bluefin tuna and yellowfin tuna at 0% CO_2 and pH 7.8. Superscript letters represent statistical similarities across acclimations within one species and measurement temperature. The presence of both upper case and lower case letters indicates that a value is similar to both other acclimations at that measurement temperature, but the other acclimations are significantly different from each other. Symbols (* and †) represent individual pairwise similarities between measurement temperatures within one acclimation.

Acclimation (°C)	Measurement temperature (°C)	Pacific bluefin tuna P_{50}	Pacific bluefin tuna n_H	Yellowfin tuna P_{50}	Yellowfin tuna n_H
17 °C-acclimation	15	22.71 (±1.13)^{A,*}	2.30 (±0.09)*	–	2.19 (±0.24)*
	20	20.51 (±0.92)^{B,*}	2.04 (±0.15)^{B,*}	27.22 (±1.37)^{B,†}	2.27 (±0.04)^{B,*}
	25	20.16 (±0.32)^{C,*}	2.29 (±0.09)*	23.48 (±0.63)^{C,*}	2.12 (±0.15)^{C,*}
	30	20.34 (±0.67)^{D,*}	2.10 (±0.14)^{D,*}	24.63 (±0.51)^{D,†,*}	1.99 (±0.03)^{D,d,*,†}
	35	22.17 (±0.28)^{E,*}	1.91 (±0.01)^{E,*}	27.37 (±0.39)^{E,†}	1.70 (±0.02)^{E,†}
20 °C-acclimation	15	–	–	–	–
	20	22.27 (±0.89)^{B,b,*}	1.94 (±0.10)^{B,*}	25.86 (±0.75)^{B,b,†}	2.40 (±0.04)^{B,*}
	25	21.47 (±0.88)^{C,*}	1.96 (±0.03)*	25.72 (±0.45)^{C,†}	1.97 (±0.02)^{C,†}
	30	20.94 (±0.84)^{D,*}	1.96 (±0.10)^{D,*}	27.65 (±0.73)^{D,†,*}	2.05 (±0.07)^{D,†}
	35	22.32 (±0.66)^{E,*}	1.92 (±0.08)^{E,v}	28.71 (±0.36)^{E,*}	2.02 (±0.06)^{E,†}
24 °C-acclimation	15	25.74 (±0.65)^{A,†}	2.60 (±0.10)*	–	–
	20	23.73 (±0.69)^{b,†,*}	2.73 (±0.14)*	22.65 (±0.98)^{b,†}	1.95 (±0.16)^{B,*}
	25	23.02 (±1.08)^{C,†,*}	2.73 (±0.09)*	24.09 (±0.80)^{C,†}	1.80 (±0.15)^{C,*}
	30	21.98 (±0.38)^{D,*}	2.84 (±0.16)*	24.14 (±1.32)^{D,†}	1.81 (±0.04)^{d,*}
	35	22.30 (±0.35)^{E,*}	2.47 (±0.09)*	30.48 (±1.10)^{E}	1.95 (±0.14)^{E,*}

(Carey and Gibson, 1977; Cech et al., 1984; Clark et al., 2008). Hypotheses exist as to whether or not these binding properties may be related to tunas' regional endothermy or to exposure to wide thermal niches.

This study characterized the O_2 binding properties of isolated RBCs from Pacific bluefin tuna and yellowfin tuna, and tested the hypothesis that temperature acclimation may alter Hb–O_2 binding properties at the cellular and molecular levels. We discuss these findings in the context of prior studies and ongoing discussions regarding the physiological significance of reverse temperature-dependent Hb–O_2 binding in tunas.

4.1. Pacific bluefin tuna Hb–O_2 binding

The data presented here for Pacific bluefin tuna demonstrate temperature-independent Hb–O_2 binding, with statistically significant reverse temperature-dependence at 0.5% and 1.5% CO_2 for 17 °C-acclimated fish, and at 0% and 1.5% CO_2 for 24 °C-acclimated fish. These findings corroborate previous findings of temperature-independent and reverse temperature-dependent Hb–O_2 binding in Atlantic bluefin tuna (*T. thynnus*), southern bluefin tuna (*T. maccoyii*) and albacore tuna (*T. alalunga*) (Carey and Gibson, 1977; Cech et al., 1984; Clark et al., 2008). Thus, all three bluefin tuna species possess these temperature-independent and reverse temperature-dependent traits.

Both Cech et al. (1984) and Clark et al. (2008) observed a switch from significant reverse temperature-dependence at low temperatures to temperature-independence at higher temperatures in tunas (above 30 °C for albacore tuna, and 23 °C for southern bluefin tuna). While our results did not reveal significant differences between 30° and 35 °C at any acclimation, we note that P_{50} values for all three Pacific bluefin tuna acclimations were higher at 35° than at 30 °C, possibly indicating a change in temperature-dependence of binding mechanisms above 30 °C. This reversal could be a strategy to help bluefin tunas offload O_2 at warm, metabolically-depleted muscles, while still taking advantage of reverse and temperature-independent effects at colder ambient temperatures to supply O_2 to the heart. Future studies with increased sample sizes may provide additional information on the nuances of Hb–O_2 binding across tunas' range of acute environmental and body temperatures.

4.2. Yellowfin tuna Hb–O_2 binding

In contrast to Pacific bluefin tuna, yellowfin tuna exhibited normal temperature sensitivity at all acclimations at 0% CO_2, similar to previous reports by Brill and Bushnell (1991). However, we observed non-significant differences, suggesting temperature-independence, in yellowfin tuna at several acclimation-CO_2 treatments. Notably, 17 °C-acclimated yellowfin tuna produced significant reverse temperature-dependence at 0.5% CO_2. These findings were unexpected given that, in an environmental context, yellowfin tuna in the California Current generally range in waters of 22–29 °C (Block et al., 1997; Graham and Dickson, 2004; Block et al., 2011).

Table 3
Oxygen equilibrium curve P_{50} values (half-saturation, in mm Hg) and Hill Plot n_H slope values for Pacific bluefin tuna and yellowfin tuna at 17 °C-, 20 °C- and 24 °C-acclimations and 0.5% and 1.5% CO_2. Top number within each cell is P_{50} value; bottom number is n_H. # indicates fish sample size of n = 1; all other sample sizes correspond to those listed in Table 1. Superscript letters represent statistical similarities between measurement temperatures within one acclimation and CO_2 value. The presence of both upper case and lower case letters indicates that a value was similar to both other acclimations at that measurement temperature, but the other acclimations were statistically different from each other. Symbols represents individual pairwise similarities between CO_2 values for P_{50} (*) or n_H (†) values within one acclimation and measurement temperature.

		Pacific bluefin tuna			Yellowfin tuna		
Acclimation (°C)	%CO_2	20 °C	25 °C	30 °C	20 °C	25 °C	30 °C
17 °C	0.5	42.72 (±0.63)^{A}	38.89 (±3.09)^{A,a}	33.98 (±0.88)^{a}	67.10 (±8.29)*	49.24 (±2.19)^{A}	47.54 (±0.79)^{A}
		1.37 (±0.04)†	1.57 (±0.02)	1.75 (±0.03)	1.28 (±0.01)^{A,†}	1.42 (±0.08)^{A,†}	1.62 (±0.15)^{A,†}
	1.5	74.01 (±2.53)	60.90 (±2.63)^{B}	52.60 (±1.52)^{B}	76.03 (±1.23)^{B,*}	79.60 (±2.43)^{B}	73.56 (±1.24)^{B}
		1.02 (±0.18)^{B,†}	1.11 (±0.05)^{B}	1.38 (±0.04)	1.02 (±0.17)^{B,†}	1.15 (±0.04)^{B,b,†}	1.52 (±0.07)^{b,†}
20 °C	0.5	43.72 (±1.57)^{A,*}	–	36.42 (±0.95)^{A}	59.54 (n/a)$^{\#}$	–	64.86 (n/a)$^{\#}$
		1.53 (±0.22)^{A}		1.54 (±0.18)^{A,†}	–		–
	1.5	55.74 (±6.40)^{B,*}	–	51.62 (±2.96)^{B}	–	–	91.62 (n/a)$^{\#}$
		0.83 (±0.12)		1.29 (±0.06)†			–
24 °C	0.5	36.74 (±1.64)^{A}	40.09 (±2.56)^{A}	32.07 (±2.47)^{A}	57.07 (±2.57)^{A,*}	–	53.54 (±3.86)^{A}
		1.45 (±0.08)	1.82 (±0.15)^{A}	2.09 (±0.15)^{A}	1.24 (±0.07)^{A,†}		1.52 (±0.11)^{A,†}
	1.5	60.93 (±1.46)^{B,b}	61.12 (±0.60)^{B}	47.16 (±3.65)^{b}	76.91 (±3.17)^{B,*}	–	83.98 (±4.92)^{B}
		1.05 (±0.08)	1.33 (±0.08)^{B}	1.56 (±0.06)^{B}	1.01 (±0.17)†		1.26 (±0.02)†

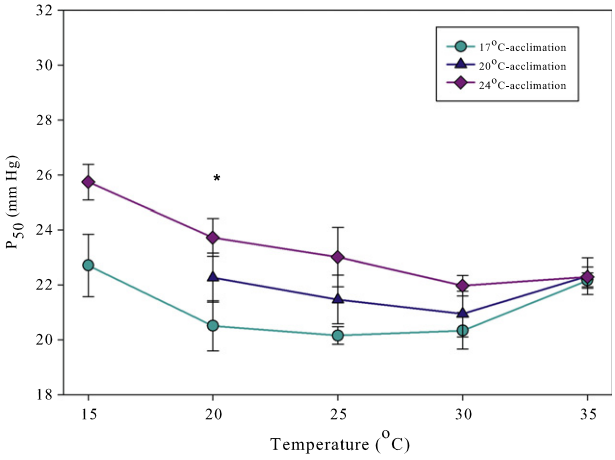

Fig. 2. Comparison of P_{50} values for Pacific bluefin tuna acclimation experiments (17°, 20° and 24 °C) between 15° and 35 °C measurement temperatures at 0% CO_2 and pH 7.8. Asterisk denotes significant difference ($p < 0.05$) of P_{50} value between 24 °C-acclimation experiment and other acclimations. Significant differences were also observed between P_{50} values within the 24 °C-acclimation overall and in individual comparisons between 15 °C and both 30° and 35 °C.

Phylogenetic analyses in recent years have placed yellowfin tuna as an advanced, derived branch of the scombrid lineage, and one of the species most closely related to the bluefins (Collette et al., 2001). The instances of reverse temperature-dependence and temperature-independence in 17 °C-acclimated yellowfin tuna found here may be an ancestral characteristic shared with bluefin and albacore tunas, and may support the theory that reverse temperature strategies help cold-inhabiting species facilitate O_2 delivery to the heart and viscera. The movement of the yellowfin tuna niche into subtropical seas may have reduced or eliminated their need for reverse temperature-dependence in Hb–O_2 binding.

Prior studies have theorized that reverse temperature-dependence evolved to facilitate efficient O_2 delivery in regional endotherms, such as tunas, though numerous hypotheses and debate exist about the importance of this trait. The first hypothesis, presented by Rossi-Fanelli and Antonini (1960), postulated that reverse temperature-dependence allows tunas to exploit waters of differing temperatures without compromising O_2 binding or delivery. The second theory suggested that reverse temperature-dependence evolved to prevent O_2 loss between the arteries and veins of retia mirabilia (Graham, 1973; Carey and Gibson, 1983). The most recent theory hypothesizes that reverse temperature-dependence evolved to prevent excessive O_2 offloading in regionally warm tissues, while still facilitating O_2 delivery to the ambient temperature-equilibrated heart and viscera (Clark et al., 2008). This strategy would be similar to reduced temperature-dependence in peripheral tissues of Arctic mammalian ungulates, such as reindeer, relative to human and other mammalian Hb. In cold environmental conditions, Arctic ungulates have large differences between core body temperature and peripheral tissue temperatures, yet their Hb appears not to be impaired in its O_2 delivery to the cold peripheries (Giardina et al., 1989).

A recent study by Clark et al. (2009) has challenged many of the prior theories. The study observed reverse temperature-dependence in other eurythermal scombrids such as the Pacific mackerel (*Scomber japonicas*), an ectothermic species considered an ancestral lineage within the scombrids. Pacific mackerel have a wide thermal tolerance and geographic distribution but lack countercurrent heat exchangers (Schaefer, 1986; Hernández and Ortega, 2000; Clark et al., 2009). As discussed by Clark et al. (2009), the presence of this reverse temperature effect supports the hypothesis that reverse temperature-dependence evolved in cold-tolerant scombrids prior to countercurrent heat exchangers and regional endothermy. Atlantic bluefin tuna and southern bluefin tuna pass through the widest temperature ranges of any tuna species (Carey et al., 1971; Block et al., 2001; Graham and Dickson, 2004), and Pacific bluefin tuna has also been shown to inhabit wide environmental temperature niches (Kitagawa et al., 2000; Boustany et al., 2010; Block et al., 2011). Thus, all three bluefin species possess cold-tolerant characteristics in common with their ancestral mackerel lineage, supporting the theory that these traits evolved to benefit eurythermal niches. Additionally, yellowfin tuna have reduced regional endothermy compared to the bluefins (Dickson and Graham, 2004), yet also displayed reverse and temperature-independent effects at 17 °C-acclimation in this study. These commonalities may suggest the importance of temperature-independent and reverse temperature-dependent binding in organisms with large ambient and internal thermal ranges, rather than strict correlation with the presence of regional endothermy.

4.3. Effects of thermal acclimation on Hb–O_2 binding in Pacific bluefin and yellowfin tuna

Acclimation to different environmental temperatures produced increased Hb–O_2 affinity with colder temperatures in Pacific bluefin tuna, although differences were not significant. In addition, the 24 °C-acclimated Pacific bluefin tuna experiment produced a significantly steeper OEC slope at 0% CO_2 than did colder acclimations. This finding may support the hypothesis that changes at the cellular or molecular level, such as the synthesis of different ratios of isohemoglobins or the production of allosteric regulators (e.g. GTP and ATP), may occur with temperature acclimation. Previous studies in fishes have observed

Table 4
Apparent heats of oxygenation ($\Delta H'$, kJ mol^{-1}) of RBC suspensions from Pacific bluefin tuna and yellowfin tuna at 17 °C-, 20 °C- and 24 °C-acclimations, calculated using the van't Hoff equation. Negative and positive values represent normal and reverse temperature effects, respectively. # Indicates $\Delta H'$ values calculated between 20°–30 °C. Superscript letters represent statistical similarities between measurement temperatures within one acclimation and CO_2 value. The presence of both upper case and lower case letters indicates that a value was similar to both other acclimations at that measurement temperature, but the other acclimations were statistically different from each other. * Indicates individual pair-wise similarities between CO_2 values within one acclimation and measurement temperature.

		Pacific bluefin tuna			Yellowfin tuna		
Acclimation (°C)	ΔTemp (°C)	0% CO_2	0.5% CO_2	1.5% CO_2	0% CO_2	0.5% CO_2	1.5% CO_2
17 °C	15–20	14.15 (±4.85)	–	–	−13.18 (±3.49)^{A}	–	–
	20–25	2.16 (±2.22)^{A}	14.59 (±5.09)^{B}	29.37 (±1.66)	19.96 (±1.10)	43.07 (±7.50)	−4.84 (±2.84)
	25–30	−1.52 (±3.61)^{A}	12.65 (±4.80)^{B,*}	16.40 (±5.69)*	−5.39 (±2.24)	6.90 (±2.52)	15.89 (±3.69)
	30–35	−13.40 (±1.79)	–	–	−16.44 (±3.72)^{A}	–	–
20 °C	20–25	5.27 (±1.24)^{A}	13.47 (±0.72)$^{\#}$	5.30 (±6.37)$^{\#}$	0.67 (±1.77)^{A}	−6.32 (n.a.)$^{\#}$	−18.05 (n.a.)$^{\#}$
	25–30	3.73 (±0.91)^{A}	–	–	−10.79 (±2.52)^{a}	–	–
	30–35	−10.06 (±1.16)	–	–	−5.93 (±3.99)^{A,a}	–	–
24 °C	15–20	13.14 (±2.22)	–	–	–	–	–
	20–25	4.71 (±2.82)^{A,*}	14.67 (±4.60)	4.92 (±3.25)*	−9.09 (±3.03)	−22.04 (±2.46)$^{\#}$	−33.34 (±6.78)$^{\#}$
	25–30	6.54 (±4.30)^{A,*}	9.28 (±2.04)*	10.09 (±4.57)*	−0.07 (±3.25)	–	–
	30–35	−2.25 (±2.54)	–	–	−36.52 (±6.59)	–	–

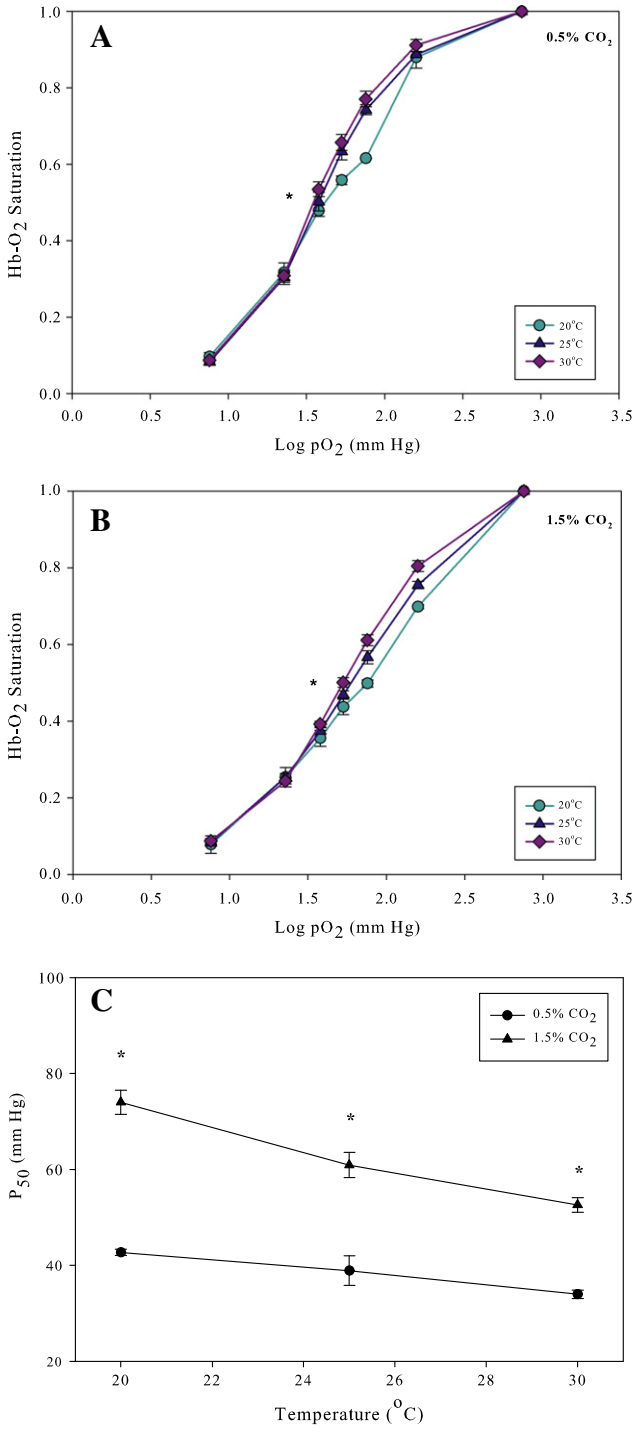

Fig. 3. Oxygen equilibrium curves for 17 °C-acclimated Pacific bluefin tuna at (A) 0.5% CO_2 and (B) 1.5% CO_2. Colors and symbols represent RBC measurement temperatures. (C) Graph of P_{50} values for the 0.5% and 1.5% CO_2 experiments. Asterisk denotes significant difference ($p < 0.05$) between P_{50} values of individual measurement temperatures within each CO_2 concentration.

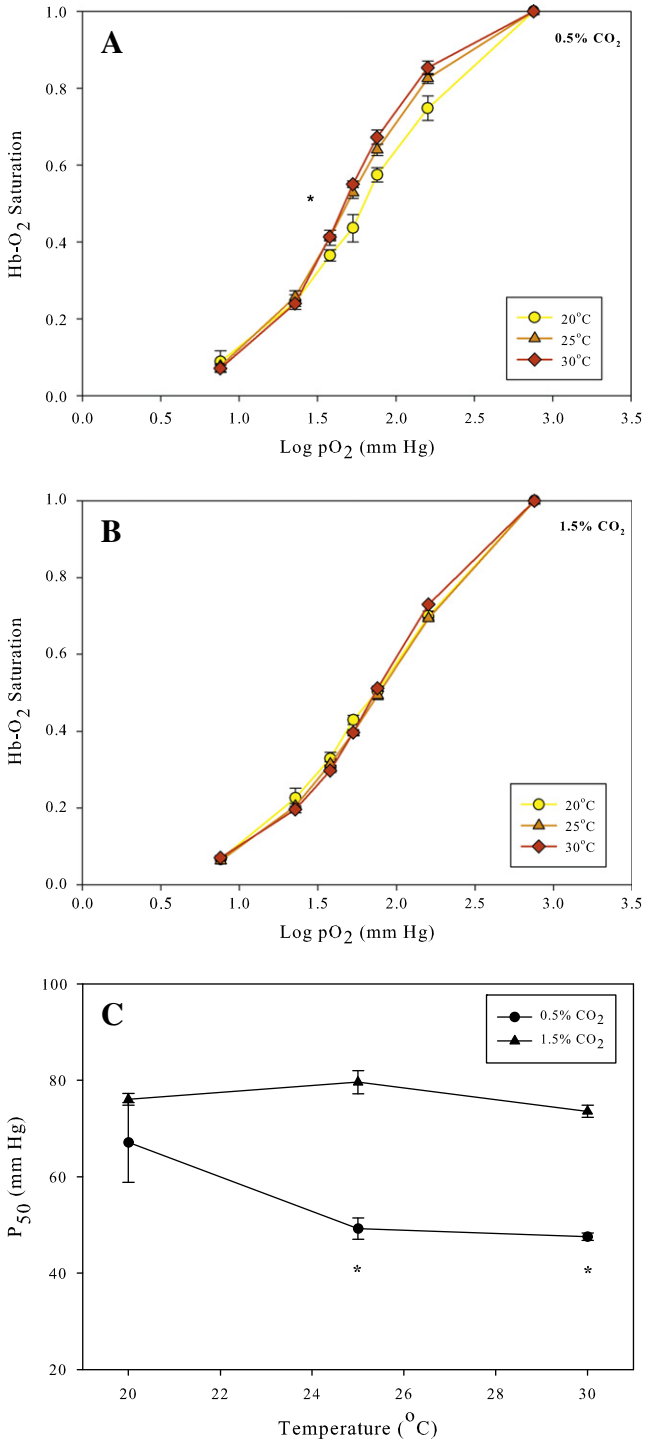

Fig. 4. Oxygen equilibrium curves for 17 °C-acclimated yellowfin tuna at (A) 0.5% CO_2 and (B) 1.5% CO_2. Colors and symbols represent RBC measurement temperatures. (C) Graph of P_{50} values for the 0.5% and 1.5% CO_2 experiments. Asterisk denotes significant difference ($p < 0.05$) between P_{50} values of individual measurement temperatures within each CO_2 concentration.

rapid changes of isohemoglobins under weeks-long thermal acclimation (Houston and Cyr, 1974; Brunori, 1975; Houston and Rupert, 1976), and changes to ATP/GTP levels on a diurnal cycle (Val et al., 1992). Regardless of the exact mechanism, our observation of increased cooperativity in 24 °C-acclimated Pacific bluefin tuna may be one physiological response to increasing temperatures.

In contrast, yellowfin tuna did not display a clear trend of acclimation-related Hb–O_2 binding affinity. Similar P_{50} values for the 17 °C- and 24 °C-acclimations were unexpected, as yellowfin tuna only occasionally pass through waters below 20 °C during foraging dives (Block et al., 1997, 2011). As noted above, however, the different binding signatures observed at 17 °C-acclimation compared to 20 °C- and 24 °C-acclimations may indicate that, at colder temperatures, yellowfin employ binding strategies similar to those used by bluefin tunas.

Both Pacific bluefin and yellowfin tunas produced significantly decreased hematocrits at 17 °C-acclimation compared to warmer acclimations, aligning with previous findings of reduced hematocrit levels at lower temperatures (Sun et al., 1995; Chen et al., 1996). Possible explanations could include increased metabolic demand with warmer temperature, leading to higher Hct; increased Hct to maximize overall O_2 uptake at warmer acclimations in response to decreased O_2 solubility in warmer water; decreased hematopoiesis caused by colder acclimation temperatures; or increased blood viscosity at colder temperatures, which may be countered by decreased Hct, reducing the work requirement for the heart (Gallaugher and Farrell, 1998).

The observed significant decreases in Hill Plot n_H values (<1.7) in the presence of CO_2 may be explained by the Root effect, which suggests possible intermediary Hb–O_2 binding site inactivation under the influence of CO_2 (Root, 1931). Intermediary binding site inactivation would reduce or eliminate the cooperativity of the four Hb binding sites, reducing the sigmoidal shape of OECs. In contrast, at conditions of 0% CO_2, both Pacific bluefin tuna and yellowfin tuna showed sigmoidal OECs and high cooperativity ($n_H > 1.8$).

We note here several limitations of our study. While our method allows for rapid, semi-automated measurement of OECs on multiple samples simultaneously, at varying temperatures, it does not at present allow for direct, concurrent measurements of sample pH. We recognize this as a potentially significant limitation of our results, and in light of our lack of direct measurements, we cannot conclude what role the Bohr Effect had in producing the observed P_{50} differences among measurement temperatures and CO_2 concentrations. Results highlighted by similar studies of tuna blood preparations in the presence of CO_2 indicate a large Bohr Effect (Yokoyama et al., 2004; Clark et al., 2008). We expect that a similar effect may play a role here.

Our study used washed RBCs in physiological Ringer's solution, rather than whole blood or isolated hemolysate, to evaluate cellular-level changes. A next step would be to analyze whole blood and hemolysate preparations, to determine whether acclimation effects occur at the extracellular or protein level. In our attempt to obtain fresh blood from live fish, we were limited in our sample size and thus the overall power of this study. Several trends were observed that did not reach statistical significance, and warrant further examination.

4.4. Conclusions

In summary, we tested Pacific bluefin tuna and yellowfin tuna for O_2 binding properties, and found a mix of temperature-independence and reverse temperature-dependence, and increased O_2 binding with colder acclimation temperatures, in Pacific bluefin tuna. In contrast, we observed normal temperature-dependent O_2 binding in yellowfin tuna at 0% CO_2, and temperature-independence at 0.5% and 1.5% CO_2, with significant reverse temperature-dependence at 17 °C-acclimation and 0.5% CO_2. In terms of acclimation effect, the significantly steeper OEC slope in 24 °C-acclimated Pacific bluefin tuna compared to colder acclimations may indicate that Pacific bluefin tuna and yellowfin tuna RBCs undergo molecular-to-cellular-level changes, such as to isohemoglobin ratios, with long-term temperature acclimation. The data presented here provide insight into the Hb–O_2 binding strategies of Pacific bluefin tuna and yellowfin tuna, but further work is needed to understand the relationship between reverse and independent temperature effects and the presence of cold-tolerance and regional endothermy.

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Author contributions

B.A.B. and J.B. designed the studies; L.E.L., J.B. and B.A.B. completed the experiments; and L.E.L., M.S.L., B.A.B. and J.B. contributed to the interpretation of experiment results and drafting and revising of the manuscript.

Author competing interests

The authors declare no competing financial interests.

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