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–O2) 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–O2 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 CO2 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 CO2, with instances of reverse temperature-dependence in 17 °C- and 24 °C-acclimations at 0% and 1.5% CO2. In contrast, yellowfin tuna produced normal temperature-dependence at each acclimation temperature at 0% CO2, temperature-independence at 0.5% and 1.5% CO2, and significant reverse temperature-dependence at 17 °C-acclimation and 0.5% CO2. Thermal acclimation of Pacific bluefin tuna increased O2 binding affinity of the 17 °C-acclimation group, and produced a significantly steeper oxygen equilibrium curve slope (nh) at 24 °C-acclimation compared to the other acclimation temperatures. We discuss the potential implications of these findings below.

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1. Introduction

Many species depend upon oxygen (O2) transport proteins, such as hemoglobin (Hb) or hemocyanins, to effectively transport O2 from the respiratory surfaces to tissues elsewhere in the body (Antonini and Brunori, 1970). Regardless of the specific transport protein or respiratory organ, O2 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 (CO2) levels (Portner and Knust, 2007; Portner and Farrell, 2008). Understanding how species respond to warming ocean temperatures and increasing CO2 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* genus has led to specialists of the tropics, subtropics, temperate and subpolar waters. However, in all cases, tunas return to spawn in warmer 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–O2 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 O2 transport requirements (Carey and Teal, 1966; Carey et al., 1971;
warm and cold tissues). Numerous studies have explored Hb
2004). These shifts favor Hb of
9
(43x312) -
ity decreases with increasing temperature, decreasing pH and increas-
238) –
ative response that improves cardiac calcium handling in ventricular
1964; Lucassen et al., 2006). For example, cold acclimation elicits a pro-
colder temperatures (e.g. 14 °C) and changes in gene expression
186) –
mako sharks), and some thresher sharks, rely on a system of counter-
30
water temperatures (Carey and Teal, 1966; Carey et al., 1971; Carey
249) –
O2 dissociation as well-oxygenated blood travels from the gills and is
176) –
Thunnus alalunga
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 - m² 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 condi-
tions 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-accli-
ated yellowfin tuna were sampled over a period of two weeks. All in-
dividuals 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 exper-
iments. Acclimation sample sizes, mean mass and length, and mean he-
matocrit (%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
arteriosus within 60 s of immobilization in the sling. Fish sampled live
in the sling were then either released back into the tank, without im-
pact, to be used for future experiments, or were euthanized for addition-
al 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 arteriosus.
Comparisons of the two sampling methods (live in the tank vs. post-
mortem) in the same fish did not yield significant differences in Hb–
O₂ binding properties (Supp. Fig. 1).
Blood was extracted using a 21 G · 1 1/2 inch needle and 3 ml sy-
ringe, both of which were pre-rinsed with marine fish Ringer’s solution
(which contained (in mM): 150 NaCl, 5.4 KCl, 1.5 MgCl₂, 3.2 CaCl₂, 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 solu-
tion, with pH adjusted to 7.80 with NaOH at 20 °C. The sample was cen-
trifuged 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


Cech et al., 1984; Clark et al., 2008). These re-
gionally endothermic traits, shared with billfish and opah (cranial endo-
thermy), sharks of the family Lamnidae (white, porbeagle, salmon and
mako sharks), and some thresher sharks, rely on a system of counter-
current heat exchangers, or retia mirabilia. Retia, densely bundled arter-
ies 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 in-
crease 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 tempera-
ture of ambient water) poses unique O₂ transport challenges in these fish. In most animals that rely on Hb as an O₂ transport protein, O₂ affin-
ity decreases with increasing temperature, decreasing pH and increas-
ing CO₂ levels (Bohr et al., 1904; Christiansen et al., 1914; Jensen,
2004). These shifts favor Hb offloading of O₂ to warmer, acidic and O₂-
depleted tissues, such as working muscle (Barrcroft and King, 1909;
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–O₂ binding properties that produce optim-
al O₂ transport in varying environments (i.e. the ability to extract O₂
from both warm and cold environments, and to unload O₂ to both warm and cold tissues). Numerous studies have explored Hb–O₂ bind-
ing 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–O₂ binding (Rossi-Fanelli and Antonini,
1960) and reverse temperature-dependence above 20% Hb–O₂ satu-
ration (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 (Laur et al.,
1980; Cech et al., 1984), Southern bluefin tuna (Thunnus maccoyii) demon-
strated 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
O₂ dissociation as well-oxygenated blood travels from the gills and is
warmed by the retia, while also facilitating O₂ 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 pro-
tective 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
(Jayashundara et al., 2013). Similar transcriptomic activation effects due
to long-term temperature acclimation may occur in O₂ delivery sys-
tems, 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–O₂ binding properties. Pa-
cific bluefin tuna has not been previously analyzed for OECs, and neither
species has been studied for acclimation effects on Hb–O₂ binding.
in order to ensure that potential Hb saturation above 21% O2 was accounted for. Oxygen was added in incremental steps, at 0.5, 1, 2, 3, 4, 5, 7, 10 and 15 L/kg. Values for pO2 were calculated by multiplying the decimal value of %O2 by 760 mm Hg, the average atmospheric pressure at sea level, to ensure complete O2 equilibrium at each partial pressure, as previously validated for Hb samples using this method. For runs involving CO2 (e.g. 99.5% O2−1.5% CO2) was added for measurements of %O2 by 760 mm Hg, the average atmospheric pressure at sea level, and were averaged to ensure reproducibility. The standard error of measurement (s.e.m.) between percent Hb−O2 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% O2 concentrations than for 0% O2. For consistency between calculations, we always used 0% O2 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 P50 values signify the O2 pressure (pO2) in mm Hg at 50% saturation. Hill Plot slope (nH) values were calculated as Hill Plot slope (nH) = \((\log(\Delta H'))/((\log(pO2)) at 50% saturation, where y is the percent Hb−O2 saturation. Hill Plot and P50 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−O2 reaction (Δ'H' = 2.303 * R * ((ΔlogP50)/(Δ1/T)), where R = universal gas constant (0.008314 J K^-1 mol^-1), and T = measurement temperature in K).

### 3. Results

All OECs for both Pacific bluefin tuna and yellowfin tuna at 0% CO2 displayed sigmoidal curves corroborated by Hill values (nH) >1.8 (Fig. 1, Supp. Fig. 2, Table 2). In contrast, OECs for both species in the presence of CO2 (0.5% or 1.5%) displayed decreased cooperativity, indicated by nH 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 O2 binding at 0% CO2

Differences in Hb−O2 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 P50 values were higher at 35° than at 30 °C for all acclimations, differences were not significant. Apparent heat of oxygenation (Δ'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 O2 affinity with colder acclimation. Blood from the 17 °C-acclimation group produced highest O2 affinity of the

### Table 1

<table>
<thead>
<tr>
<th>Acclimation</th>
<th>Pacific bluefin tuna (T. orientalis)</th>
<th>Yellowfin tuna (T. albacares)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>17 °C</td>
<td>20 °C</td>
</tr>
<tr>
<td>Mass (kg)</td>
<td>17.3 ± 1.0</td>
<td>11.8 ± 1.5</td>
</tr>
<tr>
<td>SFL (cm)</td>
<td>93.4 ± 3.0</td>
<td>81.9 ± 1.4</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>33.1 (± 6.5)</td>
<td>42.5 (± 1.3)</td>
</tr>
<tr>
<td>n (fish)</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

### 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
Hill plots yielded significant measurement temperature differences between the 24 °C-acclimation group compared to 17°- and 20 °C-acclimations at all measurement temperatures, except at 0% CO2, respectively. Comparisons of nH values between 17 °C- and 20 °C-acclimations only produced significant differences at 25 °C.

3.2. CO2 effect on Pacific bluefin tuna O2 binding

The mix of temperature-independent and reverse temperature-dependent results observed in each Pacific bluefin tuna acclimation group at 0% CO2 was also observed at physiological CO2 concentrations (0.5% or 1.5% CO2) (Fig. 3, Table 3). Reverse temperature-dependence between measurement temperatures was significant at both 0.5% and 1.5% CO2 for the 17 °C-acclimation group, and at 1.5% CO2 for the 24 °C-acclimation group.

Increasing CO2 concentration within one measurement temperature resulted in a significant Bohr Effect, shifting OECs right and decreasing nH slope values. For example, 17 °C-acclimated Pacific bluefin tuna nH 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% CO2, respectively (Table 3).

3.3. Temperature effect on yellowfin tuna O2 binding at 0% CO2

Each yellowfin tuna acclimation produced overall significantly different P50 values when measured between 20° and 35 °C (Table 2, Supp. Fig. 3). Increasing measurement temperatures corresponded with increasing P50 values for both 20 °C- and 24 °C-acclimated yellowfin yellow between 20 and 35 °C, and for 17 °C-acclimated yellowfin between 25 and 35 °C. Individual P50 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% CO2 did not reveal a clear acclimation-related trend (Supp. Fig. 3). In fact, comparisons of 17 °C- and 24 °C-acclimated yellowfin measured at 25° and 30 °C yielded nearly identical P50 values (Table 2). Comparisons of nH 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. CO2 effect on yellowfin tuna O2 binding

Similar to Pacific bluefin tuna, the yellowfin tuna 17 °C-acclimation experiment at 0.5% CO2 produced significant reverse temperature-dependence between 20° and 25 °C, with a notably highly endothermic ΔH (Fig. 4A, Table 4). The other comparisons within the 17 °C-acclimation and 24 °C-acclimation groups in the presence of CO2 yielded non-significant, temperature-independent results across measurement temperatures (Fig. 4B, Table 3). Insufficient CO2 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, nH values were 2.27 (±0.04), 1.28 (±0.01), and 1.02 (±0.17) at 0%, 0.5% and 1.5% CO2, respectively. At each acclimation except 17 °C- and 24 °C-acclimations measured at 20 °C, comparisons between CO2 concentrations produced significantly different results (Table 3).

4. Discussion

Most vertebrates and invertebrates that have been examined for Hb–O2 binding properties display a normal temperature effect of decreasing Hb–O2 affinity with increasing temperature, facilitating O2 unloading and delivery to metabolically active tissues. Certain tunas are among the few species that exhibit a reverse temperature Hb–O2 dissociation curve, binding O2 more tightly as temperature increases.
4.1. Pacific bluefin tuna Hb–O₂ binding

The data presented here for Pacific bluefin tuna demonstrate temperature-independent Hb–O₂ binding, with statistically significant reverse temperature-dependence at 0.5% and 1.5% CO₂ for 17 °C-acclimated fish, and at 0% and 1.5% CO₂ for 24 °C-acclimated fish. These findings corroborate previous findings of temperature-independent and reverse temperature-dependent Hb–O₂ 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

4.2. Yellowfin tuna Hb–O₂ binding

In contrast to Pacific bluefin tuna, yellowfin tuna exhibited normal temperature sensitivity at all acclimations at 0% CO₂, similar to previous reports by Brill and Bushnell (1991). However, we observed non-significant differences, suggesting temperature-independence, in yellowfin tuna at several acclimation-CO₂ treatments. Notably, 17 °C-acclimated yellowfin tuna produced significant reverse temperature-dependence at 0.5% CO₂. 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 2

Oxygen equilibrium curve P₅₀ (half-saturation, in mm Hg) and Hill Plot nₜ values for Pacific bluefin tuna and yellowfin tuna at 0% CO₂ 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.

<table>
<thead>
<tr>
<th>Acclimation (°C)</th>
<th>Measurement temperature (°C)</th>
<th>Pacific bluefin tuna P₅₀</th>
<th>Pacific bluefin tuna nₜ</th>
<th>Yellowfin tuna P₅₀</th>
<th>Yellowfin tuna nₜ</th>
</tr>
</thead>
<tbody>
<tr>
<td>17 °C-acclimation</td>
<td>15 °C</td>
<td>22.71 (±1.13)⁴, ⁴D</td>
<td>3.90 (±0.09)⁴ †</td>
<td>52.60 (±1.51)⁴ A</td>
<td>2.60 (±0.07)⁴ B</td>
</tr>
<tr>
<td></td>
<td>20 °C</td>
<td>20.51 (±0.92)⁴ B,A</td>
<td>4.04 (±0.15)⁴ B,A</td>
<td>52.60 (±1.51)⁴ B,A</td>
<td>2.60 (±0.07)⁴ B,A</td>
</tr>
<tr>
<td></td>
<td>25 °C</td>
<td>20.16 (±0.32)⁴ B,A</td>
<td>4.01 (±0.09)⁴ B,A</td>
<td>52.60 (±1.51)⁴ B,A</td>
<td>2.60 (±0.07)⁴ B,A</td>
</tr>
<tr>
<td></td>
<td>30 °C</td>
<td>20.34 (±0.67)⁴ B,A</td>
<td>4.10 (±0.14)⁴ B,A</td>
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<td>–</td>
</tr>
<tr>
<td></td>
<td>35 °C</td>
<td>22.17 (±0.28)⁴, ⁴E</td>
<td>1.91 (±0.01)⁴ E</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>20 °C-acclimation</td>
<td>15 °C</td>
<td>–</td>
<td>–</td>
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</tr>
<tr>
<td></td>
<td>20 °C</td>
<td>22.27 (±0.89)⁴, ⁴B,A</td>
<td>1.94 (±0.10)⁴ B,A</td>
<td>52.60 (±1.51)⁴ B,A</td>
<td>2.60 (±0.07)⁴ B,A</td>
</tr>
<tr>
<td></td>
<td>25 °C</td>
<td>21.47 (±0.98)⁴, ⁴E</td>
<td>1.96 (±0.03)⁴ E</td>
<td>52.60 (±1.51)⁴ E</td>
<td>2.60 (±0.07)⁴ E</td>
</tr>
<tr>
<td></td>
<td>30 °C</td>
<td>20.94 (±0.84)⁴ B,A</td>
<td>1.96 (±0.10)⁴ B,A</td>
<td>52.60 (±1.51)⁴ B,A</td>
<td>2.60 (±0.07)⁴ B,A</td>
</tr>
<tr>
<td></td>
<td>35 °C</td>
<td>22.32 (±0.66)⁴ B,A</td>
<td>1.92 (±0.08)⁴ E</td>
<td>52.60 (±1.51)⁴ E</td>
<td>2.60 (±0.07)⁴ E</td>
</tr>
<tr>
<td>24 °C-acclimation</td>
<td>15 °C</td>
<td>25.74 (±0.65)⁴ A, ⁴E</td>
<td>2.60 (±0.10)⁴ E</td>
<td>52.60 (±1.51)⁴ A</td>
<td>2.60 (±0.07)⁴ E</td>
</tr>
<tr>
<td></td>
<td>20 °C</td>
<td>23.73 (±0.69)⁴ B,A</td>
<td>2.73 (±0.14)⁴ B,A</td>
<td>52.60 (±1.51)⁴ B,A</td>
<td>2.60 (±0.07)⁴ B,A</td>
</tr>
<tr>
<td></td>
<td>25 °C</td>
<td>23.02 (±1.08)⁴ B,A</td>
<td>2.73 (±0.16)⁴ B,A</td>
<td>52.60 (±1.51)⁴ B,A</td>
<td>2.60 (±0.07)⁴ B,A</td>
</tr>
<tr>
<td></td>
<td>30 °C</td>
<td>21.98 (±0.38)⁴ B,A</td>
<td>2.84 (±0.16)⁴ B,A</td>
<td>52.60 (±1.51)⁴ B,A</td>
<td>2.60 (±0.07)⁴ B,A</td>
</tr>
<tr>
<td></td>
<td>35 °C</td>
<td>22.30 (±0.35)⁴ E</td>
<td>2.47 (±0.09)⁴ E</td>
<td>52.60 (±1.51)⁴ E</td>
<td>2.60 (±0.07)⁴ E</td>
</tr>
</tbody>
</table>

Table 3

Oxygen equilibrium curve P₅₀ (half-saturation, in mm Hg) and Hill Plot nₜ values for Pacific bluefin tuna and yellowfin tuna at 17 °C, 20 °C and 24 °C acclimations and 0.5% and 1.5% CO₂. Top number within each cell is P₅₀ value; bottom number is nₜ value. 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 CO₂ value. 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 were statistically different from each other. Symbols (*) and † represent individual pairwise similarities between measurement temperatures within one acclimation and CO₂ value.

<table>
<thead>
<tr>
<th>Acclimation (°C)</th>
<th>CO₂</th>
<th>Pacific bluefin tuna</th>
<th>Yellowfin tuna</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0%</td>
<td>20 °C</td>
<td>25 °C</td>
</tr>
<tr>
<td>17 °C</td>
<td>0.5</td>
<td>42.72 (±0.63)⁴</td>
<td>38.89 (±3.09)⁴</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>74.01 (±2.53)⁴</td>
<td>60.90 (±2.63)⁴</td>
</tr>
<tr>
<td>20 °C</td>
<td>0.5</td>
<td>43.72 (±1.57)⁴ A</td>
<td>36.42 (±0.95)⁴ B</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>55.74 (±6.40)⁴ B</td>
<td>51.62 (±2.96)⁴ B</td>
</tr>
<tr>
<td>24 °C</td>
<td>0.5</td>
<td>36.74 (±1.64)⁴ A</td>
<td>40.09 (±2.56)⁴ A</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>60.93 (±1.46)⁴ B,A</td>
<td>51.81 (±0.80)⁴ B,A</td>
</tr>
</tbody>
</table>

(With citations and references as appropriate for the points made in the text.)
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 bluefin (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 O2 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–O2 binding.

Prior studies have theorized that reverse temperature-dependence evolved to facilitate efficient O2 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 O2 binding or delivery. The second theory suggested that reverse temperature-dependence evolved to prevent O2 loss between the arteries and veins of retia mirabila (Graham, 1973; Carey and Gibson, 1983). The most recent theory hypothesizes that reverse temperature-dependence evolved to prevent excessive O2 offloading in regionally warm tissues, while still facilitating O2 delivery to the ambiant 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 O2 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 (Schafer, 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; Boustan 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 bluefin (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–O2 binding in Pacific bluefin and yellowfin tuna

Acclimation to different environmental temperatures produced increased Hb–O2 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% CO2 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
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–O2 binding affinity. Similar P50 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.

**Fig. 3.** Oxygen equilibrium curves for 17 °C-acclimated Pacific bluefin tuna at (A) 0.5% CO2 and (B) 1.5% CO2. Colors and symbols represent RBC measurement temperatures. (C) Graph of P50 values for the 0.5% and 1.5% CO2 experiments. Asterisk denotes significant difference (p < 0.05) between P50 values of individual measurement temperatures within each CO2 concentration.

**Fig. 4.** Oxygen equilibrium curves for 17 °C-acclimated yellowfin tuna at (A) 0.5% CO2 and (B) 1.5% CO2. Colors and symbols represent RBC measurement temperatures. (C) Graph of P50 values for the 0.5% and 1.5% CO2 experiments. Asterisk denotes significant difference (p < 0.05) between P50 values of individual measurement temperatures within each CO2 concentration.
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 O2 uptake at warmer acclimations in response to decreased O2 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 (Gallagher and Farrell, 1998).

The observed significant decreases in Hill Plot nH values (>-1.7) in the presence of CO2 may be explained by the Root effect, which suggests possible intermediary Hb–O2 binding site inactivation under the influence of CO2 (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% CO2, both Pacific bluefin tuna and yellowfin tuna showed sigmoidal OECs and high cooperativity (nH > 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 P50 differences among measurement temperatures and CO2 concentrations. Results highlighted by similar studies of tuna blood preparations in the presence of CO2 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 oxygen dissociation curves. 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 O2 binding properties, and found a mix of temperature-independence and reverse temperature-dependence, and increased O2 binding with colder acclimation temperatures, in Pacific bluefin tuna. In contrast, we observed normal temperature-dependent O2 binding in yellowfin tuna at 0% CO2, and temperature-independence at 0.5% and 1.5% CO2, with significant reverse temperature-dependence at 17°C-acclimation and 0.5% CO2. 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–O2 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.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpa.2014.11.014.

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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