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Alyssa M. Andres
University of South Florida

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The Effects of Temperature and Oxygen Availability on Aerobic Performance in Three Coastal Shark Species; *Squalus acanthias*, *Carcharhinus limbatus*, and *Carcharhinus leucas*

by

Alyssa M. Andres

A Dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy Marine Science
with a concentration in Biological Oceanography
Department of Biological Oceanography
College of Marine Science
University of South Florida

Major Professor: Brad A. Seibel, Ph.D.
Christopher Stallings, Ph.D.
Cameron Ainsworth, Ph.D.
Jay Dean, Ph.D.
Dean Grubbs, Ph.D.

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Keywords: aerobic scope, oxygen supply, ram-ventilation, hypoxia, thermal sensitivity

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DEDICATION

For my family. Across miles, oceans, continents, and decades, I have had your unending support in chasing my dreams. You have celebrated in all of my successes, help me navigate life’s challenges, and have given of yourselves, always, to see my success and happiness. There are no words to express the love and gratitude I have for all you have done. Thank you.

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For our *S. acanthias* research

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ABSTRACT

Anthropogenically driven climate changes are altering marine habitats globally. Rising sea surface temperatures and coastal eutrophication, arising from global warming and coastal nutrient loading, have resulted in progressive ocean deoxygenation. This may restrict available habitat of marine organisms as studies suggest that the balance between metabolic oxygen demand and environmental supply plays an important role in limiting viable habitat and species fitness. As ectothermic predators, with temperature-dependent metabolism and high metabolic demands, coastal shark species may be susceptible to shifts in ocean temperature and oxygen. Such environmental changes may alter metabolic performance and ultimately success and survival within shark habitat. However, our understanding of species-specific physiological responses to environmental shifts in temperature and oxygen is lacking for the vast majority of shark species. This dissertation explores the effects of temperature and declining oxygen availability on energetic performance in three ecologically and economically valuable shark species of the US Atlantic and Gulf of Mexico coasts; the spiny dogfish shark, *Squalus acanthias,* the blacktip shark, *Carcharhinus limbatus,* and the bull shark, *Carcharhinus leucas.*

These species represent coastal “apex” and meso-predators found across temperate, tropical, and subtropical waters. Across their ranges, these highly migratory species likely experience steep oxygen and temperature gradients but differ in habitat and lifestyle. *Squalus acanthias* is a much slower, smaller, benthopelagic species found in waters 7-24˚C. These cold-temperate sharks spend much of the year in deep, offshore locations but routinely enter shallow coastal waters. *Carcharhinus limbatus*, and *C. leucas* are more active, large coastal species, considered tropical-subtropical in distribution known to occupy waters 20-34˚C and 20-37˚C respectively. *Carcharhinus limbatus*, and *C. leucas* use confined estuarine and

riverine nursery habitats for their young where long-term residency in these shallow coastal habitats is important to species success and survival. In addition, *C limbatus* and *C. leucas* are obligate ram ventilators, thought to require forward motion to maintain oxygen supply, whereas *S. acanthias* have the ability to decrease activity and forcibly pump water over their gills while at rest (buccal pumping). Differences between species habitat, ecology, and activity level (metabolic demand) mean they likely exhibit varied environmental tolerances, responses to elevated temperature and hypoxia, and may display varied shifts in viable habitat with climate change. To better predict habitat suitability and species responses to climate change, we must first understand how the physiology that underpins animal behavior is fundamentally altered by changes in temperature and oxygen for each species.

Several environmentally sensitive physiological performance metrics have been used to identify viable species habitat, based on the balance of organism energetic demands and environmental oxygen supply. As animals require oxygen to derive cellular energy for all life activities, the amount of oxygen available in a given environment may dictate an organism's energetic potential. In addition, while temperature influences the rate of biochemical reactions in the body, it also causes elevated metabolic demands in ectothermic species. As temperatures rise, ectotherms require more oxygen to support elevated metabolic demands at high temperature. At a certain thermal or hypoxic threshold, the balance of animal oxygen demand to ambient supply may fall below sustainable levels. Environments that do not confer enough energetic potential to support population energetic needs are considered non-viable species habitat. Increased presence of such environments within occupied species range has consequences for animal distribution and abundance, and the potential for metabolic tradeoffs in growth, reproduction, movement, foraging, etc. My research aimed to establish the thermal and hypoxic sensitivity of metabolic demands and performance in marine sharks, quantify thermal and hypoxic limitation of aerobic energetic performance, and to test the species-specific utility of metabolic performance metrics such as aerobic scope and oxygen supply capacity as indicators of viable species habitat.

Aerobic scope, defined as an organism's aerobic energetic potential, provides a measure of the energy available for sharks to perform all life activities within a given environment. This metric is derived from two components that can be directly measured in aquatic species: 1) maximum metabolic rate (MMR), the highest rate of organism energy use, vital for predator evasion and prey capture, and 2) resting or standard metabolic rate (SMR), i.e., the lowest energy required to maintain the body's basal energetic needs while at rest. The difference between maximum and standard metabolic demand provides a measure of aerobic scope. This scope may be expressed in both absolute (absolute aerobic scope = MMR-SMR) and factorial terms (factorial aerobic scope = MMR/SMR), with each expression of aerobic scope providing distinct interpretations of species thermal optimality and or sublethal thermal limits. Factorial aerobic scope (FAS) is defined as the factorial change in aerobic energetic potential, or the factorial change in oxygen above what is needed at rest. This metric typically declines as temperatures rise and has been used to identify temperature and oxygen thresholds that limit viable habitat for species at the population level. At a limiting upper temperature, FAS reaches a minimum scope needed to support population energetic needs, and generally occurs between 2-5x what is needed to support basic resting metabolic demands of an individual.

Further, the temperature at which absolute aerobic scope (AAS) peaks, has been traditionally used to identify a species thermal "optimum" at which aerobic energetic potential is maximized and abundance and habitat use are expected to be highest the wild. Temperatures at which AAS declines significantly from its peak, are thought to represent sublethal limiting temperatures for the species, constraining wild distribution. A breakdown in the ability to supply oxygen to tissues (oxygen supply capacity) at high temperatures is thought to cause such decline in AAS by limiting maximum performance. As such, oxygen supply capacity, α, has been indicated as the primary driver of thermal tolerance in aquatic species, and represents another potential performance indicator of viable/evolved habitat.

Deficits in aerobic scope and oxygen supply capacity have been linked to shifts in organism habitat use, movement and migration, energetic tradeoffs in foraging, reproduction, growth and development. However, the aforementioned interpretations of thermal trends in metabolic performance and aerobic scope have not been tested mechanistically across a large number of aquatic species. In these studies, aerobic scope was evaluated as a predictive metric of "optimal" and limiting temperature for these three shark species. In addition, measured metabolic performance under declining ambient oxygen allowed for identification of critical oxygen limits for metabolic rates (critical oxygen partial pressure; Pc), and how aerobic scope is limited by environmental oxygen deficits in each species.

Within this dissertation, temperature-controlled intermittent respirometry was used to explore aerobic metabolic performance under two distinct metabolic challenges: environmental hypoxia and maximum aerobic exercise. Adult spiny dogfish sharks (TL=71-94.5cm) off the US Northeast Shelf (US NES) were tested at five experimental temperatures (10, 13, 17, 21, and 23˚C). Neonate/young-of-the-year (YOY) blacktip sharks (53-63 cm STL) and bull sharks (69-81cm STL) of the Eastern Gulf of Mexico were tested at 3-4 temperatures (22, 26, and 30 for bull sharks, 22, 26, 30 and 34°C for blacktips), within or bracketing species natural range.

My first study measured metabolic traits of the spiny dogfish shark, *S. acanthias,* across temperature and oxygen. The goal of this study was ultimately to assess the potential for rapidly rising temperature, and its interaction with oxygen, to influence viable habitat for members of a subpopulation of *S. acanthias* on the US NES. SMR for *S. acanthias* increased exponentially with temperature, from 10 to 23°C. MMR increased logistically with no decrement in maximum performance within measured thermal range. The α for *S. acanthias* followed the logistic trend in MMR in support of elevated metabolic demands across temperature, and with no decline in oxygen supply capacity within thermal range, oxygen supply appeared not to be the primary driver of thermal tolerance for this species. Behavioral observation revealed that sharks at rest began to lose equilibrium at 21°C, even when ambient oxygen exceeded levels that critically limit resting metabolic demands. Thus, internal systems other than physiological oxygen supply begin to fail for *S. acanthias* at 21°C. The peak in AAS ≥20.5°C, rather than indicating optimal temperature, marks the beginning of physiological failure in dogfish. Behavioral and metabolic results indicate that *S. acanthias* are likely living near their upper critical temperature (≈24°C) in the warmest seasons, reaching a FAS of 4.31 at 23˚C, within range of theoretical population thresholds (FAS=2-5). Declining ambient oxygen will additionally cause predictable limitation of maximum metabolic performance in *S. acanthias*,

and cause limiting FAS thresholds to be reached at lower temperatures, further limiting viable habitat if oxygen is below air saturation. This study identified environmental thresholds for spiny dogfish that may be used to predict integrative constraints on suitable habitat in the face of climate change.

The purpose of my second study was to identify the effect of temperature on aerobic performance within the natural range of blacktip sharks (*C. limbatus*) and bull sharks (*C. leucas*) of the Eastern Gulf of Mexico. The results stipulate that routine metabolic demand (an estimate of minimum demands for ram ventilators inclusive of locomotion to support adequate ventilation) increased significantly with temperature in neonate/YOY of both species. This indicated that the oxygen needed to support lowest routine swimming in these species increases as temperatures rise. Blacktip and bull sharks demonstrated marked differences in aerobic performance. Specifically, blacktip sharks exhibited higher metabolic rates than bull sharks at all active levels and demonstrated no significant change in both MMR and α as temperatures rose. For blacktip sharks this may indicate an evolved need to maintain MMR across a large thermal range. Bull sharks showed significant increase in both MMR and α within the range of temperatures tested, with particular temperature sensitivity of maximum performance to cold temperatures. For bull sharks this may indicate a lack of need to maintain MMR across a large thermal range, which may be related to distinct ecological and physiological strategies of salinity use in this species. Maximum metabolic rates for both species in this study are also among the highest recorded for shark species to date. Differing thermal sensitivities for all metrics between species, lead to opposite thermal trends in aerobic scope between bull and blacktip sharks. Aerobic scope in blacktip sharks, was driven primarily by minimum routine metabolic rate, whereas for bull sharks, aerobic scope was driven by both maximum metabolic rate and to a lesser degree, minimum routine metabolic rate. Neither MMR, α, nor aerobic scope identified limiting thermal thresholds for these species within the experimental temperature range (22-30°C bull sharks; 22-34°C blacktip sharks), and as such, critical population thresholds for temperature in these species are likely to lie below the minimum factorial aerobic scope of ~1.4 found in this study. This research will be used as a basis

for future studies to identify the bounds of thermal tolerance for these species and for comparison to historical distribution and habitat use to ground truth thermal relationships.

Finally, my third study served to identify the effect of ambient oxygen availability and ventilation mode on aerobic performance in obligate ram ventilators. Obligate ram-ventilating species, such as bull and blacktip sharks, are thought to have diminished capacity to reduce activity and use active ventilation (buccal pumping) to support metabolic demands. Instead, support of oxygen delivery to respiring tissues is inherently tied to locomotion. The costs of locomotion are also inherently greater than metabolic costs at rest for shark species. Thus, high oxygen demands of continuous locomotion to support ram ventilation may lead these species to be particularly vulnerable to diminished oxygen availability during hypoxic events within their small nursery range. In this study, neonate/YOY blacktip sharks demonstrated no ability to sustainably use active ventilation (buccal pumping mechanisms) and rest under normoxic or hypoxic conditions. Bull sharks by contrast demonstrated continuous swimming as well as some resting behavior under normoxia and hypoxia, allowing for opportunistic measurement of demands and oxygen supply capacity across various modes of activity. Metabolic rate at rest for bull sharks was ~57% of minimum routine metabolic rate across temperature, and though resting reduced oxygen demands significantly, oxygen supply capacity was significantly diminished when bull shark activity was reduced. The oxygen supply capacity at rest was not enough to support energetic demands at rest, and for this reason, resting metabolism ultimately declined unsustainably after brief periods of rest in bull sharks. The use of buccal pumping (active ventilation) was not a sustainable or likely hypoxic coping strategy for either species, as a higher oxygen partial pressure was required to meet metabolic needs at rest, relative to swimming under hypoxia. Although a few individuals of each species increased swim speed as environmental oxygen declined, increased swim speed to improve ventilation volume was not universally observed. Though increases in swim speed may be used in an attempt to maximize ventilation under mild-moderate hypoxia, the critical oxygen level below which locomotion for ventilation and routine metabolic demands can no longer be supported provides a limit to this hypoxic ventilatory strategy. Although more data (both lab derived thresholds and field based thermal affinity) are needed to say with certainty where oxygen thresholds limiting population success and causing avoidance responses occur, a limiting oxygen partial pressure likely falls between ~8 and 12 kPa, between the critical oxygen limit for lowest locomotion supporting ram ventilation and the critical oxygen limit for routine swimming. As environmental hypoxia becomes more extensive within their natal habitat under climate change, we believe young bull sharks and blacktips will be vulnerable to habitat loss. More data are needed to identify current and phenotypically plastic limits to hypoxia for these species.

These studies represent some of the first physical measurements of their kind for these species, allowing us to quantify energetic needs and identify limits to aerobic performance across different environmental scenarios and species diversity. We have tested the application of several metabolic metrics as indicators of optimal or limiting habitats for these species and have applied new frameworks from which to interpret dynamics of metabolic performance, energetic scope, and hypoxic limits to activity. Through this work we have derived several physiological baselines from which these species may be further investigated, with results that help inform evolved species habitat suitability.

CHAPTER 1

THE OXYGEN- AND TEMPERATURE-SENSITIVITY OF METABOLIC TRAITS IN THE SPINY DOGFISH SHARK, *SQUALUS ACANTHIAS*

Introduction

Habitat use by aquatic species is strongly influenced by ambient water temperature and oxygen concentration (Fry, 1947; Deutsch et al., 2020). On the US Northeast Continental Shelf (US NES), temperatures are rising faster than the global average as a result of atmospheric warming, northward shifts in the Gulf stream, as well as changes in Atlantic Multidecadal Oscillation and Pacific Decadal Oscillation (Perishing et al 2015; Saba et al., 2016; Ceasar et al., 2018), elevating concern for resident species (Friedland and Hare, 2007; Hazen et al. 2013; Kavanaugh et al., 2017). Many key local species in this region have exhibited poleward shifts in distribution in step with local climate (Nye et al., 2009; Morley et al., 2018), largely regarded as a response to rising ocean temperatures (Nye et al., 2009; Pinsky et al., 2013; Rogers et al., 2020). However, for other species such as the spiny dogfish, *Squalus acanthias*, responses to warming conditions have been less clear. With temperatures along the US NES projected to rise an additional 4-5°C in the next ~80 years (Saba et al., 2016; Kleisner et al., 2017), it is vital to understand how underlying physiology, influenced by changes in oxygen and temperature, dictate behavioral responses of marine species and how these responses are altered under different environmental scenarios.

Temperature influences aquatic organism physiology in a number of ways including dictating the activity and efficacy of metabolic enzymes (Daniel et al., 2008) used to activate and chaperone biochemical reactions, and facilitating exponential increases in the rate of biochemical reactions as temperatures rise, up to a thermal limit (Gillooly et al., 2001; Schnell and Turner, 2004; Dill et al., 2011). Temperature also facilitates increases in the energetic demands of ectotherms, requiring more oxygen and energy to satisfy metabolic demands at high temperatures. Lowest metabolic rates in ectothermic organisms typically increase by 2-3-fold for every 10˚C rise in temperature (Hochachka and Somero 2002), while active metabolic rates are typically less sensitive to temperature, and scale more slowly as temperatures rise. The temperature sensitivity of maximum metabolic performance is related to species-specific ecology, as maximum performance is thought to evolve across temperatures to satisfy the maximum needs of an organism within their evolved thermal range (Seibel and Deutsch, 2020). The difference in temperature sensitivities between metabolic demands of an organism at rest and at maximum performance contributes to changes in the energy available for an organism to perform all life functions. In addition, animals use oxygen to derive energy within their cells. Oxygen is the final electron receptor in the chain of biochemical reactions used to derive ATP and power all life activities. Deficits in environmental oxygen or a breakdown in an animal's ability to supply oxygen to tissues induced by thermal stress are known to cause further limits to energetic performance that may alter species ecology. As such, the balance of thermally sensitive metabolic demands of marine organisms to environmental oxygen supply may play a large role in dictating viable species habitat.

Evaluating the sensitivity of metabolic parameters across thermal ranges allows us to identify changes in performance and energetic needs across a species' thermal envelope. In particular, aerobic metabolic scope (AS) is thought to indicate the energy available, beyond basic maintenance metabolism, for growth, reproduction and activity (foraging, predator evasion, etc.) (Pörtner et al., 2010). Aerobic scope is defined as the difference between 2 metabolic components: 1) standard metabolic rate (SMR), representing metabolic demands of cellular and whole organism homeostasis (Brett, 1962; Frappell and Butler, 2004), (measured at rest in fasted animals), and 2) the maximum metabolic rate (MMR) of an organism, representing the maximum energetic demands of an animal in support of predator evasion and prey capture, (measured under maximum exercise). Aerobic scope can be expressed in absolute terms (MMR – SMR) or factorially (MMR/SMR), with each expression providing distinct interpretations (Fry 1971; Clark et al. 2013; Killen, et al., 2007; Halsey et al., 2018; Seibel and Deutsch, 2020; Deutsch et al., 2020). Factorial aerobic scope (FAS) is defined as the factorial change in aerobic energetic potential, or the factorial change in oxygen above what is needed at rest. This metric typically declines as temperatures rise and has been used to calibrate indices of available habitat for species based on based on a favorable ratio of oxygen supply to organismal demand, (Deutsch et al., 2015; 2020). Populations of marine organisms do not generally subsist below a minimum FAS threshold of 2-5. Environments that cause FAS to decline below the critical threshold do not confer enough energy to support activities necessary for population success and survival, including foraging, growth and reproduction (Deutsch et al., 2015; 2020). In addition, absolute aerobic scope (AAS) has been used to predict "thermal optimality" and sub-lethal limiting temperatures for marine organisms (Pörtner, 2010; Fry, 1971). For several species AAS has demonstrated increases with temperature along a bell-shaped curve (Pörtner, 2010; Fry, 1971), the peak of which is often interpreted as an "optimal" temperature. The decline in aerobic scope on either side of the peak is often attributed to a breakdown in the supply of oxygen, or oxygen limitation due to an inability of physiological mechanisms to adequately meet oxygen demand. These trends in AAS however, are not universal and their interpretations are seldom tested mechanistically (Lefevre, 2016, Slesinger et al., 2019, Jutfelt et al., 2018).

Measurement of aerobic scope in conjunction with direct measures of oxygen supply provides an opportunity to test the cause of declining performance at temperature extremes (Seibel and Deutsch, 2020). Oxygen supply capacity (α) encompasses all physiological traits and processes involved in the supply of oxygen to respiring tissues in the body (e.g., ventilation, circulation), and can be directly determined from metabolic measurements across oxygen and temperature. From the α metric, critical oxygen thresholds or the minimum ambient oxygen partial pressure that supports a given metabolic rate (Pc), can be calculated across a full activity spectrum (Seibel and Deutsch, 2020) and identify species-specific decline of maximum performance across oxygen availability. A significant decline in this metric alongside a decline in MMR at high temperatures would indicate oxygen limitation, and that a limiting upper temperature has likely been reached. Together, ascertaining such measures of aerobic performance may help us to better understand behavioral responses and shifts in viable habitat for economically and ecologically relevant species in ocean regions that are rapidly being altered by climate change.

Squalus acanthias is a small benthopelagic shark (110-130cm) found in North American continental shelf waters from Florida to Labrador, most commonly from Cape Hatteras, NC, to Nova Scotia (Burgess, 2002). This species has been detected most frequently at depths between 50-150m (Stehlik, 2007; Castro and Peebles, 2011) though reports detail utilization of waters from the surface to >500m (Stehlik, 2007; Castro and Peebles, 2011). *Squalus acanthias* is the most abundant elasmobranch within the Northwest Atlantic, and has been regarded as a keystone species, as prey for larger teleosts, elasmobranchs, and marine mammals (Castro 1983, Compagno 1984), an important intermediate predator that feeds at a high trophic position (just under large sharks) (Morgan and Sulikowski, 2015), and as ecosystem modifiers in the region (Morgan and Sulikowski, 2015). Together with endemic skate species, these small sharks have effectively replaced overfished cod and haddock as the dominant piscivores on the US NES (Link & Garrison, 2002; Rafferty et al., 2012; Morgan and Sulikowski 2015). In addition, *S. acanthias* is a valuable commercial resource in the region, with landings in the Western North Atlantic totaling more than 16.7 million lbs in 2020 (Unted States, 2020), valued at ~$3.2 million (USD). As such, there is a vested interest in the dynamics of this species in the region and in predicting potential habitat shifts in the face of climate change on the US NES.

To date, environmental tolerances are poorly understood for *S. acanthias*, particularly in the Atlantic. Due to the fact that this species demonstrates a large distributional range in the region and has been found at temperatures ranging from 6-24˚C, one might assume that *S. acanthias* is eurythermal, robust to environmental change and may exhibit similar thermal tolerances across the species range. However, there is evidence to suggest that there may be differences in thermal tolerance between (groups) of *S. acanthias* across their range. Although US populations of *S. acanthias* that span the Gulf of Maine to Cape Hatteras are managed as a single stock (ASMFC, 2002), *S. acanthias* exhibit variation in occupied environmental range (temp, oxygen) and habitat use (thermal, depth, horizonal, length of use etc.) between subpopulations of *S. acanthias* along a latitudinal gradient (Carlson et al., 2014). Furthermore, *S. acanthias* is known to school by size prior to sexual maturity, and by sex as well as size after maturity (Ford, 1921; Shepherd et al., 2002; Stehlik, 2007; Dell'Apa et al., 2014). Schooling groups have exhibited limited differences in horizonal, vertical and thermal habitat use depending on life stage, reproductive status, size and sex (Nammack et al., 1985). As such, schooling fish at different life stages and sizes may also exhibit differences in habitat viability and use that are likely tied to differences in energetic demands.

Squalus acanthias, most frequently captured at low temperatures ~7-15°C (Compagno, 1984) are well known to migrate latitudinally and offshore on a seasonal basis, generally avoiding temperatures >23°C (Carlson et al., 2004). In fact, temperatures ≥21°C are known to induce a heat-shock response in *S. acanthias* (Bockus et al., 2020). However, little is known of *S. acanthias* metabolic response to shifting temperature or oxygen, or how closely populations reside to their environmental limits. In addition, oxygen on the US NES ranges between 75 and 100% air saturation (~15-21kPa) but it is currently unknown how oxygen levels below air saturation influence energetic use, metabolic performance and thermal tolerance in this species. Understanding *S. acanthias* energetic physiology and performance under different thermal and oxygen scenarios can help us to identify how this species may be limited by abiotic factors within their occupied habitat and provide insight into how this species may respond to projected climate shifts.

In this study I investigated physiological traits of adult *S. acanthias* inhabiting the mid-Atlantic region of the US NES. I quantified the thermal and hypoxic sensitivity of key metabolic traits, including oxygen supply capacity (α) and aerobic scope in adult *S. acanthias*. I put to test several hypotheses: 1) Energetic demands of *S. acanthias* are indicative of an active, benthopelagic species; 2) Schooling *S. acanthias* of similar size and sex have similar metabolic demands, with larger adult females having lower mass-specific metabolic rates than male counterparts. I also assessed the utility of metabolic performance metrics, in predicting “optimal” and viable habitat for *S. acanthias*. I predicted that for *S. acanthias*: 3) Oxygen supply capacity limits MMR and AAS at high temperatures, and significantly declines alongside MMR at a sublethal upper thermal limit, >21˚C; 4) AAS exhibits a bell-shaped curve across temperature, and peaks at an optimal temperature between 10 and 21°C that coincides with peak thermal use for wild *S. acanthias*; 5) A significant decline in AAS beyond its peak will coincide with at an upper limiting temperature for S. acanthias, >21˚C; 6) Critical (lethal) thermal limits may be reached above 23°C for this population; 7) FAS demonstrates an inverse relationship with temperature, declining to a population limiting threshold between 2-5 at 23˚C for this population. 8) maximum performance declines in a 1:1 % ratio with decline in ambient oxygen and causes limiting FAS to occur at lower temperatures when oxygen is below full air saturation.

Results from this study will contribute to our understanding of how environmental factors limit energetic use and performance and dictate viable habitat for *S. acanthias* on the US NES.

Methods

Animal collection and care: Adult *S. acanthias* (n=90) were collected via gillnet (depth=35-50ft, temperature=11.7°C), off Long Beach Island, NJ, in November and December of 2016 and 2017 (Captain Kevin Wark, FV Dana Christine, Barnegat, New Jersey). Animals were transported to the James J. Howard Sandy Hook Marine Laboratory in Highlands, NJ, and held in designated circular holding tanks (diameter=1.83 - 2.44 m, 1601- 2846L), at ambient temperature and salinity (26). Holding tanks were connected to a flow-through system that filtered and UV treated water drawn from the bayside of Sandy Hook. Subjects were held in captivity at these conditions for a minimum of 2 weeks prior to experimentation, maintained on a diet of herring (*Clupea harengus*), butterfish (*Peprilus triacanthus*), and silversides (*Menidia menidia*), and fed *ad libitum* every other day to satiation. Sharks were measured for length (population mean ± SE: precaudal length (PCL) = 68.11 ± 0.54 cm; fork length (FL) = 74.74 ± 0.57 cm; stretch total length (STL)= 84.16 ± 0.61 cm; Table 1) and tagged for identification via color-coded T-bar tags (second dorsal fin).

Ethics statement: Captive animal husbandry was conducted under veterinary guidelines and procedures approved by the Institutional Animal Care and Use Committee at Rutgers University (protocol #15054). Health assessments (overall body condition, color, behavior, feeding, presence of abrasions/lesions) were conducted daily on captive animals. Individuals that displayed signs of illness or damage, upon arrival or over the duration of captivity, were isolated and treated according to IACUC protocols. If condition persisted or worsened after isolation and treatment, the individual was humanely euthanized using an overdose of MS-222 in excess of anesthetic concentrations (≥400mg/L; 25g in 60L seawater = 0.417g/L), in accordance with AVMA Guidelines for the Euthanasia of Animals. (2013 edition, *pg. 32*, 69-67*).*

Trial group selection and acclimation to experimental conditions: Acclimated *S. acanthias* individuals in "good condition", (i.e., feeding regularly, good appetite, swimming and resting normally, no overt illness) were selected for inclusion in physiological measurements. Eligible *S. acanthias* were sorted by sex and selected at random from each group. The ratio of females to males in experimental groups (Table 1) was ultimately dependent on the availability of healthy, acclimated males at the time of trials. Sharks (12 at a time assigned to a temperature trial group) were moved to holding tanks (d=1.83 m, 1601L) within a closed experimental system (see "Experimental Set-up", below) where temperature and salinity were incrementally changed at a rate of no more than 3°C and 2 ppt per day, until the designated trial temperature and salinity were reached 48 hours prior to experimentation. Subjects were fasted 72 hours prior to trials to achieve a post absorptive state (Chabot, McKenzie and Craig, 2016) for respirometry procedures. Following trials, sharks were allowed to recover in holding tanks. Due to subject attrition (≈31.11% total) and limited access to further collection, "recovered" sharks (in good condition) were also included in additional trial groups after a minimum of 6 days recovery and resumed feeding behavior. Sharks were used for experimentation once per temperature bin, and individuals were tested at 1-3 target temperatures. Metabolic performance metrics were evaluated at five experimental target temperatures (10, 13, 17, 21, and 23°C; Table 2), approximating the natural temperature range of *S. acanthias*.

Experimental set-up: Temperature-controlled intermittent respirometry (Clark et al., 2013; Svendsen et al., 2016) was used to measure metabolic performance under two distinct metabolic challenges: maximum aerobic exercise (exercise trials) and environmental hypoxia (hypoxia trials). The three-phase intermittent respirometry cycle included in sequence 1) a closed-chamber "measure" period where the decline in dissolved oxygen (DO) within the chamber was recorded; 2) an open-chamber "flush" period that allowed for refreshment of oxygen and removal of accumulated metabolic waste; and 3) a closed-chamber "wait" period that allowed uniform mixing and equilibration of oxygen before the next cycle. Duration of cycle phases (Table 1) was temperature-dependent and calibrated to reach starting oxygen levels by the end of a flush period, although the PO_2 (partial pressure of oxygen dissolved in the water) at the start of the measure period was often not fully saturated. Intermittent respirometry phases were automated using a DAQ-M instrument (Loligo Systems; Viborg, Denmark) during in exercise trials but were controlled manually during hypoxia trials.

Experiments were conducted in twin 8000L recirculating aquaculture systems. Each of these systems consisted of a sand filter (50 µm pore size, Arias 8000 sand filter), UV filtration (Twin Smart UV-High-Output Sterilizer), activated carbon (Clean & Clear Cartridge Filter), and biologic filtration using a bioreactor (Sweetwater Low-Space Bioreactor). The system was filled and regularly topped off (through water changes) with a combination of Sandy Hook Bay water supplied by the facility's seawater system, and Aquavitro's certified sea salt. The experimental set-up (Fig. 1) allowed for 4 sharks to be tested simultaneously in a single trial. For respirometry measurements, sharks were enclosed individually in each of four acrylic respirometry chambers (100 cm x 32.5 cm x 32.5 cm, 106.69 l; volume inclusive of tubing), submerged in two, 1.83 m diameter temperature baths (1200.96 l) of aerated seawater (2 chambers/temperature bath; Figure 1A-B). A recirculation pump (Eheim Universal 2400 l/s) was affixed to each chamber in a closed loop (via gas impermeable Tygon®tubing) and provided continuous mixing of the water in each chamber. A second pump (i.e., flush pump; Eheim Universal 1200 L/s) was affixed to each chamber, used in an open circuit to supply aerated seawater from the surrounding temperature bath during flush periods. A one-way valve attached to the posterior end of each chamber permitted outflow of water during flushes. Dissolved oxygen concentration within each chamber was measured using an oxygen dipping probe optical mini sensor (PreSens PSt3) placed within the closed recirculation loop and connected to a multichannel oxygen meter (Witrox-4 instrument, Loligo Systems). A temperature probe (Loligo Systems Pt1000) within the seawater bath allowed for continuous monitoring of temperature throughout trials. Oxygen and temperature were sampled at a rate of 1 Hz for the duration of each trial using Loligo Systems AutoResp Software. Temperature was maintained ± 1°C of starting temperature using in-line combination heater/chillers (Aqua Logic Delta Star) and tanks insulated with closed cell foam (5.08 cm). Experimental salinity was maintained at 31.98 ± 0.07 (mean±SE) for experimental trials. To minimize disturbance to the sharks during trials, experimental tanks were covered with insulated foam lids, with a small built-in window that maintained normal photoperiod.

For determination of hypoxic limits to performance, additional trials were conducted under hypoxia. In the set up for hypoxia trials, water supplied to the experimental chambers during flush periods was sourced from an external reservoir (Figure 1) in order to decrease experimental ambient oxygen. This reservoir was filled with experimental system water but contained four ceramic plate micro-oxygen diffusers (Point Four TM) used to disperse N_2 gas and displace dissolved oxygen to designated levels (Rogers et al., 2016; Snyder et al., 2016). DO within the reservoir was monitored using an additional oxygen dipping probe optical mini sensor (PreSens PSt3) connected to the Witrox-4 instrument, (Loligo Systems), with manual control of N_2 gas.

Exercise trial protocol: Immediately prior to respirometry, sharks were weighed and individually placed in a separate "chase" tank (d=1.83 m, 1,200 l). Sharks were then exercised to exhaustion via tactile stimulation, inducing burst swimming and c-start contractions, to achieve maximum sustained metabolic rate (MMR) (Clark et al., 2013; Rummer et al., 2016; Killen et al., 2017). Exhaustion was quantified by the subject's lack of response to both tactile stimulation and brief air exposure (≈1min). The sharks were then sealed within the experimental chambers for immediate respirometry measurement.

The rate of shark oxygen consumption (MO_2) was measured continuously over a 22-hour period, which encompassed the MMR after exercise (Norin and Clark, 2016) and subsequent recovery to resting metabolic rates. A 22-hour trial also accommodated a 2-hour post-trial background respiration measurement and a similar start time for the next trial. Rate of oxygen consumption over time was determined via the AutoResp software (Loligo Systems) for each closed-chamber measure period in the trial. At the end of the 22-hour trial, sharks were either removed from the chamber to recover at experimental temperature and salinity or remained within the chamber for further hypoxia testing.

Hypoxia trial protocol: A subset of sharks from year 2 of the study (total n=8 tested at each temperature bin) remained in the respirometry chamber following completion of the 22-hour exercise trial. This effectively served as an acclimation period for testing oxygen limitation of metabolic performance.

Intermittent respirometry continued as in the preceding exercise trial, but during alternating flush periods, dissolved oxygen within the chamber was manually reduced by ~10% air saturation throughout the trial, until the critical oxygen partial pressure (P_c) for standard metabolic rate (P_{cSMR}) was reached. This threshold represents the last oxygen level where resting metabolic rate may be maintained, the point at which survival is time limited (Job, 1957; Smit, 1965; Priede, 1985), and oxygen supply capacity is known to be maximized (Seibel et al., 2021). P_{cSMR} manifests in the real time MO_2 trace of the trial (AutoResp software, Loligo systems) as a switch to a continuous decline in resting metabolic rate (Figure 2A-C), indicating insufficient O_2 supply to maintain standard metabolic rate (SMR) (Claireaux and Chabot, 2016). After P_{cSMR} was reliably reached, sharks were removed from the respirometer and returned to holding tanks to allow for recovery at normoxia, experimental temperature and salinity.

Background respiration: Background oxygen consumption was determined over a continuous 2-hour closed chamber period following the removal of the animal, to account for microbial respiration within each chamber. Mean background MO_2 was then calculated from all measure periods within in the 2-hour background trial. To minimize microbial growth, respirometers were scrubbed and cleaned with fresh water once a week and allowed to air dry before reassembly.

Behavioral observations: Abnormal/sporadic swimming or loss of equilibrium have previously been used as identifiers of physiological stress and critical thermal limits for fish species (e.g., Elliott and Elliott, 2010; Brauner and Richards, 2020). These abnormal behaviors were noted during routine observation of *S. acanthias* in holding and during opportunistic observation in trials. This behavior served to help identify conditions that induced overt stress in *S. acanthias*, potential suboptimal temperatures, and potential critical lethal temperatures within/just outside of experimental thermal range.

Data analysis: All data and statistical analysis were performed in R version 3.4.4 and 3.6.3 (R Core team, 2017; 2020). For all statistical analysis, p-values less than 0.05 were considered statistically significant ($\alpha<0.05$), except in the case of manual Holm correction, where alpha levels were adjusted and specified (HB αlpha). For exercise trials, 24 *S. acanthias* individuals were tested at each experimental temperature (total 120 trials). Trials where female sharks gave birth (pupped) in the chamber and introduced increased respiration from shark pups (and unknown mass and respiratory effects on the mother), as well as trials for which pump malfunctions occurred and interfered with measurement, were excluded from analysis. At each temperature, 8 individuals (total 40 trials) were subsequently subjected to hypoxia trials. For trials in which individuals exhibited behavioral indicators of stress, such as continuously declining metabolic rate beyond sustainable standard rates, cessation of buccal pumping ventilation, sometimes paired with loss of equilibrium and blotchy coloration, regardless of $PO_{2,}$ individuals were removed early and these trials were omitted from analysis.

A summary list of metabolic performance metrics measured, acronyms, source trials from which each metric was derived, and equations for specific metric calculation may be used as a reference and are found in Table 2.

Metabolic rate (MO_2) and metabolic trait calculations: MO_2 was calculated for each respirometry measure period using Eq 1,

$$Eq\ 1.\ \ MO_2 = \frac{(S * V) - br}{M}$$

where MO_2 is the mass-specific rate of oxygen consumption (mg O_2 $kg^{-1}hr^{-1}$), *S* is the slope of the linear regression of MO_2 vs time, indicating the change in oxygen concentration within the chamber over the time frame of the measure period, *V* is the total volume of the respirometry chamber, *br* is the mean background microbial O_2 consumption rate (mgO_2hr^{-1}), and *M* is the mass of the individual *S. acanthias* (kg). Measure periods that showed a linear regression with an $R^2 < 0.75$ were excluded from the datasets (Killen et al., 2021).

In this study, MMR was defined as the maximum mass-specific MO_2 within the 22-hour exercise trial (Figure 2). Measures of SMR were determined for both exercise and hypoxia trials, from a subset of the lowest metabolic rates within each respirometry trial. For exercise trails, SMR was determined from a truncated dataset excluding the first 5 hours of the trial to eliminate elevated metabolic rates during recovery from exhaustive exercise. SMR from exercise trials represented lowest metabolic demands at <22 hours in the chamber. For hypoxia trials, SMR was calculated from all MO_2 measures prior to oxygen limitation and represented estimates of lowest metabolic demands at >22hours in the chamber. For both datasets, a technique was used for SMR estimation that is appropriate for when activity in experimental chambers cannot be directly observed or quantified (Chabot et al., 2016). MO_2 values were assumed to vary around true SMR with about half of the points in the truncated dataset falling below true SMR and half falling above true SMR, interspersed with MO_2 values above SMR due to uncontrollable spontaneous shark activity during trials (Chabot et al., 2016). Under this assumption Chabot et al., (2016) stated that the concept of a quantile may be appropriately used, that splits the truncated dataset into smallest (*p*) and largest (1-*p*) proportions (Chabot et al., 2016). The quantile at p=0.2, ($q_{0.2}$), was used to represent SMR in accordance with Chabot et al. (2016). The $q_{0.2}$ appropriately represented an estimate of the lowest resting metabolic rate of *S. acanthias*, while minimizing the effect of outliers and metabolic changes due to spontaneous activity of *S. acanthias* within trials.

SMR values at <22 hours (exercise trials) were then compared to SMR at >22 hours (hypoxia trials) for individuals that completed both trial types. Paired t-tests with Holm-correction for the number of t-tests run across temperature (accounting for error introduced by multiple t-tests) were used to identify significant differences between SMR values. If SMR <22 hours was significantly higher than SMR >22 hours, than SMR at >22 hours (hypoxia trials) was chosen to represent the population.

Oxygen supply and metabolic capacity: Oxygen supply capacity was determined following the methods in Seibel et al. (2021). First, instantaneous oxygen supply was calculated for each measure period as $\alpha_0 = MO_2/PO_2$. Oxygen supply capacity, α, was then defined as the highest α_0 value achieved during respirometry trials (hypoxia or exercise trial). The critical oxygen pressure (P_c) was then calculated for a given metabolic rate as P_c = Metabolic rate/α. Using that equation, an "α-line" was generated to describe the metabolic rate dependence of critical oxygen partial pressure (P_c) (Seibel and Deutsch, 2020). Because oxygen supply capacity and metabolic performance are both maximized at P_c, regardless of metabolic rate (Figure 2; Seibel and Deutsch, 2020), the α-line predicts the maximum metabolic rate that can be achieved across PO_2, from P_{cSMR}, the critical oxygen level for resting metabolic rate, to P_{cMAX}. P_{cMAX} approximates the highest prevailing PO_2 in a species environment (Seibel and Deutsch, 2020), which for most coastal species is near air-saturation (21 kPa; Figure 2). This value represents the limiting oxygen level for maximum metabolism at a given temperature. Increases in PO_2 beyond P_{cMAX} have no effect on performance.

S. acanthias frequent the upper 50m of the water column (Stehlik, 2007; Castro and Peebles, 2011; Carlson et al., 2014), encountering air saturated waters with regularity, so I assumed P_{cMAX} = 21 kPa, (Seibel and Deutsch, 2020) for metabolic calculations. Oxygen on the US NES typically ranges between 75 and 100% saturation (15-21 kPa). In this study, the starting PO_2 was always less than air-saturation, ranging from 14.88 ± 0.05 to 16.58 ± 0.04 kPa (Table 1). Thus, direct measures made in this study represent the realistic lower end of potential performance across wild oxygen range. To then predict performance across the full activity spectrum for *S. acanthias*, I also predicted MMR at full air saturation (MMR at 21 kPa) using $P_{cMAX,}$ the measured α and Equation 2 (Seibel et al., 2021).

$$\text{Eq 2. } \alpha = \left(\frac{MMR}{P_{cMAX}}\right) = \left(\frac{SMR}{P_{cSMR}}\right)$$

Mass correction of metabolic metrics: Mass-dependent metabolic metrics, (i.e., SMR, MMR, α), were mass corrected to control for the effect of body mass on metabolism using Equation 3.

$$\text{Eq 3. } MM = b_0 * M^b$$

Where *MM* is the metabolic metric in question for an individual (SMR, MMR, α), b_0 is a normalization constant, *M* is the mass of the animal, and *b* is a mass scaling coefficient. Mass scaling coefficient values (*b*) were obtained for all relevant metrics using *calc_b* ("Respirometry" package v 1.3.0 Birk, 2021). These values (Table A1) were then used to correct individual performance metrics values to the study's average *S. acanthias* mass of 2.4 kg using the *scale_MO₂* function (Respirometry v 1.3.0 R package; Birk, 2021) within each temperature. Spearman correlation coefficients were also generated as descriptors of the correlation of mass and each metabolic metric (Table A1).

Thermal scaling of metabolic metrics: In order to determine the shape of metabolic thermal trends within the experimental range and identify any optimality or deficits in metabolic performance across temperature thermal performance curves were generated for all metabolic metrics. First, a permutational multifactor analysis of covariance ("PermANCOVA") was used to assess the effect of temperature and other factors, inclusive of sex and field season, on the variation in each metabolic performance metric, and to identify factors that should be accounted for in modeling thermal performance. Preliminary analysis suggested temperature was the only factor that significantly impacted our measures. In addition, the effect of random error due to individual variation and the use of some individuals at more than one temperature was also assessed (see Appendix A; Figure A1). These effects were deemed negligible for all metabolic metrics and were excluded from further exploration of thermal metabolic performance.

Thermal performance curves were then generated for each metabolic performance metric, (SMR, MMR, α, AAS, FAS, P_{cSMR}) across average trial temperature. SMR and P_{cSMR} are known to exponentially increase with temperature in most ectothermic species (Ege & Krogh 1914, Clarke & Johnston 1999; Clarke and Frasier, 2004), therefore SMR and P_{cSMR} were modeled using an exponential curve where log SMR and log P_{cSMR} values were regressed against trial temperature in °C (non-linear version of Arrhenius relationship using average trial temperature in °C). 95% confidence bands were calculated by bootstrapping using the *bootMer* function (iterations = 5000) from the *lme4* package (Bates et al., 2015).

For MMR, previous studies (e.g., Slesinger et al., 2019, Farrell et al., 2008) have observed variation in the relationship between MMR and temperature, resulting in this metric being modeled differently across previous studies and species. As such, Cullen and Frey graphs, comparing normal, uniform, exponential, logistic, beta, lognormal, Weibull, and gamma distributions were implemented with bootstrap sampling (n=1000 iterations) to determine the closest approximations of error distribution for MMR and α measures in this study. Using the *fitdistrplus* package (Delignette-Muller and Dutang C, 2015) the top 4 most closely matching distributions (Weibull lognormal, gamma, logistic) were then compared to MMR and α data via visual inspection of Quantile-Quantile (qq) plots, histogram and theoretical density plots, cumulative distribution frequency (CDF) plots and Probability-Probability (PP) plots. Weibull, logistic and lognormal distributions were then selected for further comparison of "goodness of fit" using maximum likelihood (fitdistrplus package). A logistic distribution yielded the best fit according to Akaike Information Criteria (lowest AIC score) and was ultimately selected to represent the relationship between maximum performance metrics (MMR and α) and temperature within the experimental range. Since MMR predicted at air saturation (MMR at 21 kPa) was derived from α*21, alpha and MMR at 21kPa demonstrated the same distributions.

The logistic function for maximum performance metrics was defined by a three-parameter logistic equation (Eq 4):

$$\text{Eq 4. } y = \frac{A}{1 + exp^{\frac{(B-x)}{C}}}$$

Where *y* represents the metabolic metric (MMR or α), *x* represents the individual's average trial temperature (continuous), *A* represents the theoretical upper horizontal asymptote of the thermal curve, *B* is the temperature value at the inflection point of the logistic curve, and *C* represents a scaling parameter indicating the steepness of the increase in metabolic rate with temperature. The self-starting function *SSlogis* (stats v3.6.3 package, R Core Team, 2020) was used to determine each parameter of the thermal performance curve. Thermal performance curves for MMR, α, and MMR at 21 kPa were then fit using a non-linear (weighted) least squares regression via the *minpack.lm* package (Elzhov et al., 2016). Confidence bands were calculated through bootstrap sampling (iterations = 5000) using the *nlstools* package (Baty et al., 2015). The variance and 95% confidence intervals (2.5% and 97.5% quantiles) were calculated from the bootstrapped model predictions at each temperature.

Thermal performance curves for AAS were generated by subtracting modeled SMR from modeled MMR at each temperature (AAS=MMR-SMR). As AAS is a simple difference of random normally distributed variables, the variance of AAS at each temperature was calculated as the difference of the variance (σ) observed in the SMR and the MMR model predictions at each temperature. The 95% confidence intervals were thus calculated using Eq 5. The "t" subscript denotes AAS, SMR, and MMR at each given temperature.

$$\text{Eq 5. } CI = AAS_t \pm 1.96\sqrt{\sigma^2_{SMR_t} + \sigma^2_{MMR_t}}$$

Factorial aerobic scope (FAS) was calculated as the ratio of the modeled MMR to the modeled SMR at each temperature (FAS = MMR/SMR). However, the expected variance of the *ratio* of random variables is difficult to compute and does not follow a normal distribution. Thus, we used a Monte Carlo simulation to estimate the 95 % confidence interval of the FAS at each temperature. At each temperature, we calculated FAS as the ratio of randomly simulated MMR and SMR values based on each variable's predicted mean and variance. This process was repeated 10,000 times and the 2.5% and 97.5% quantiles of the simulated FAS were used to estimate the confidence bands for FAS at each temperature.

Two distinct methods were then used to quantify the temperature sensitivity of each metabolic metric (SMR, MMR, α, AAS, FAS, and P_{cSMR}). The Q_{10}, is a measure of the factorial change in metabolic metric for a 10°C change in temperature. A Q_{10} was calculated between all target temperatures (Table A2) in accordance with Eq 6.

$$\text{Eq 6. Q10} = \left(\frac{R_2}{R_1}\right)^{\left(\frac{10}{T_2 - T_1}\right)}$$

Where R_1 is the measured variable at a given lower temperature (T_1), and R_2 is that variable measured at some higher temperature (T_2). The 'activation energy of metabolism' (Gillooly et al., 2001; Clarke, 2006) *E*, measured in electron volts (eV), represents another measure of temperature sensitivity. *E*-values were derived from Boltzmann-Arrhenius plots. In these plots the natural logarithm of each mass-corrected metabolic metric was plotted against inverse temperature, $1/k_BT$, where k_B is Boltzmann's constant (relating thermodynamics and relative kinetic energy of gas particles) and *T* is the absolute temperature of the trial (K). In these Arrhenius plots, *E* values were defined by the slope of the linear regression for each metric.

Results

Of the total 90 *S. acanthias* obtained, 71 individuals successfully completed trials and were used in analyses (males=22, females= 49; 1.2–4.38 kg, mean = 2.4 kg; STL=71-94.5 cm, mean= 84.156 ± 0.62 cm; Table 1). Of the total 120 exercise trials conducted across temperature, 16 trials were unusable, of which 13 trials were excluded due to female sharks birthing in the chamber, and 3 trials excluded due to pump malfunctions. Of the total 40 hypoxia trials conducted across temperature, 5 trials were unusable due to early removal of individuals that exhibited behavioral indicators of stress regardless of PO_2. Metabolic results are reported as modeled means ± standard deviation (SD) unless otherwise specified.

Metabolic traits: SMR within the 22-hour exercise trials was consistently higher than during the subsequent hypoxia trials at all temperatures, yielding a 53.6-66.8% reduction in $q_{0.2}$ between exercise trials and hypoxia trials (Figure A2). Therefore, we defined the $q_{0.2}$ within hypoxia trials as SMR for the population. SMR increased exponentially with temperature from 26.86 ± 1.07 $mgO_2kg^{-1}h^{-1}$ to 77.09 ± 1.07 $mgO_2kg^{-1}h^{-1}$ between 10 and 23°C (Table 3; Figure 2A) with a temperature sensitivity of E = 0.61 eV and Q_{10} = 2.25 (Table 3; Figure 3) across the measured range.

Maximum metabolic rate, measured at trial PO_2 of 14.88± 0.05 SE – 16.58 ± 0.04 SE kPa, was modeled using the equation: y = 246.15/(1+ exp((10.328-x)/4.853)). Measured MMR increased from a minimum of 118.92 ± 7.79 mgO_2 $kg^{-1}h^{-1}$ to a maximum of 229.31 ± 6.18 mgO_2 $kg^{-1}h^{-1}$ between 10 and 23°C (Table 3; Figure 2A). Temperature sensitivities for measured MMR were calculated as E=0.36 eV and Q_{10}=1.66. As predicted, MMR increased at a slower rate than SMR as temperatures rose (Table 3). MMR was also more readily achieved with shorter chase times as temperatures increased (chase time: E=0.73 eV between 10 and 23°C). MMR at full air saturation (MMR at 21 kPa) demonstrated similar temperature sensitivity as the measured MMR (E=0.38 eV; Q_{10} = 1.73) (Table 3). MMR at 21 kPa was modeled by the equation: y = 389.318/(1+ exp((12.036-x)/6.207)), and increased from 163.02 ± 12.67 $mgO_2kg^{-1}h^{-1}$ to 332.48 ± 10.78 $mgO_2kg^{-1}h^{-1}$ between 10 and 23°C. Modeled MMR at 21 kPa scaled faster (c=6.207; Eq. 4) than measured MMR (c=4.853; Eq. 4), with both MMR measures reaching a theoretical vertical asymptote in the logistic curve at temperatures in excess of 30°C. Although MMR was lower than predicted using α and Eq.2 at the assumed P_{cMAX} of 21 kPa but was well with values predicted at the PO_2 of measurement, using α independently derived from SMR/P_{cSMR} (Eq 2). This suggests that MMR that could be achieved in trials was oxygen-limited and that our measurements following exhaustive exercise were likely near the maximum achievable rate at the given measurement PO_2 . If true, our predictions of MMR and aerobic scope in air-saturated waters (21 kPa) are still reasonable.

Oxygen supply capacity (α, $mgO_2kg^{-1}h^{-1}kPa^{-1}$) reflected the trends in MMR across temperature and was modeled by the equation: $y = 18.539/(1+ \exp((12.036-x)/6.207))$ (Figure 2B). The α metric increased from 7.76 ± 0.6 $mgO_2kg^{-1}h^{-1}kPa^{-1}$ to 16.18 ± 0.51 $mgO_2kg^{-1}h^{-1}kPa^{-1}$ between 10 and 23°C and showed the same thermal sensitivity as MMR at 21 kPa (Table 3; Figure 3). This is not surprising given that the predicted MMR at 21 kPa is a simple multiple of α. Alpha lines, (α-lines), dictating critical oxygen limits from rest to maximum metabolism, were generated for each individual (e.g., Figure 4) as well as for the population average at each temperature (Figure 5) to understand how maximum performance declines with ambient oxygen across temperature. The highest rates of oxygen supply derived from hypoxia trials closely matched values oxygen supply values following maximum exercise (Figure 4), meaning that maximum performance does decline in the manor predicted by the α-line (Seibel and Deutsch, 2020; Seibel et al., 2021), declining 1% for every 1% decline in ambient PO_2 across the thermal range.

The critical oxygen partial pressure for SMR (P_{cSMR}) ranged from 3.39 ± 1.08 kPa at 10°C to 4.72 ± 1.08 kPa at 23°C. Though this metric increased with temperature, indicating basal metabolic demands increase in cost as temperatures rise, this metric and showed relatively low sensitivity to temperature with $E = 0.21$ eV and $Q_{10} = 1.29$ (Table 3; Figure 3). Additionally, a comparison of P_{cSMR} obtained through the methods of Seibel et al., (2021) used in this study, and more traditional methods of P_{cSMR} estimation are described in Appendix B.

Measured absolute aerobic scope, AAS, increased with temperature ($E = 0.27$ eV, $Q_{10} = 1.47$) from 92.06 ± 8.01 $mgO_2kg^{-1}h^{-1}$ at 10°C to a maximum of 156.03 ± 5.97 $mgO_2kg^{-1}h^{-1}$ at 20.5°C , with virtually no change between 20.5°C and 23°C (152.21 ± 8.17 $mgO_2kg^{-1}h^{-1}$) (Fig. 2C). AAS at 21kPa increased continuously with temperature from 136.16 ± 12.81 $mgO_2kg^{-1}h^{-1}$ at 10°C to 255.39 ± 12.03 $mgO_2kg^{-1}h^{-1}$ at 23°C with thermal sensitivities of E =0.35 and $Q_{10} = 1.61$ (Tables 3; Figure 2C).

Factorial aerobic slope (FAS) scaled inversely with temperature, ($E = -0.23$ eV and Q_{10}=0.74), reached a modeled maximum ratio of MMR to SMR of 4.56 ± 0.3 at 12.5°C then declined to a minimum of 2.97± 0.23 at 23°C within the thermal range tested (Table 3: Figure 2D). FAS at 21 kPa declined from a maximum of 6.14 ± 0.47 at 12.0°C to a minimum of 4.31 ± 0.34 at 23°C within the thermal range tested. and demonstrated temperature sensitivities of E =-0.16 and Q_{10} = 0.77 between 10 and 23˚C (Table 3: Figure 2D).

Sex differences: *S. acanthias* experimental groups at each temperature ranged from 19 to 47% male (Table 2). Of the individuals that completed hypoxia trials, 33.3-50% were male. There was a significant difference in mass between the sexes (Welch's T-test *t*(101.31)=14.33, *p-value* = < 2.2e-16), with the females on average 1.07 kg larger (1.73x mass of males), and 10.05 cm longer than their male counterparts (Table 2). Prior to mass correction, larger female sharks had lower mass-specific metabolic rates than smaller sharks (Figure A3). The smaller sample size for hypoxia trials (Table 2) precluded reliable statistical comparisons between sexes for SMR and $P_{cSMR,}$ however, after eliminating the effect of mass on metabolism (Eq. 2, Table A1), there was no significant effect of sex on remaining metabolic traits (Permutational ANCOVA: p >0.05).

Behavioral observations across temperature: At temperatures greater than 21°C, all sharks at rest (and periodically during swimming in holding) exhibited a loss of equilibrium (Fig. 6), regardless of ambient PO_2. Equilibrium loss was remedied when sharks were returned to 18°C with no overt residual issues. It is also important to note that, while at *rest* all sharks exposed to ≥21°C struggled with equilibrium, swimming during chase protocols appeared unaffected. However, attempts to acclimate sharks to 25°C, resulted in 41.6% mortality (n=5 of 12 individuals in the trial group) within the first 36 hours of exposure to water ≥24˚C, with 33.33% of that mortality occurring within 12-24 hours of exposure.

Discussion

Absolute aerobic scope as an indicator of thermal optimality: Measured absolute aerobic scope in *Squalus acanthias* increased with temperature to 20.5°C but did not significantly decline between 20.5-23°C, while AAS at 21 kPa did not reach a peak or plateau within the measured temperature range. According to Pörtner et al., (2010), these results should indicate that 20-21°C is optimal for *S. acanthias*, as the energy available to support activity is maximized. Or if AAS at air saturation is used as a predictor of optimality, then the continuous increase in AAS at 21 kPa through the experimental temperature range should indicate that the optimal temperature for this species lies in excess of 23°C. However, neither our observed behavioral data, nor reported habitat usage for *S. acanthias* are consistent with such interpretations. At ~21°C, sharks at rest exhibited a loss of equilibrium, sometimes accompanied by sporadic swimming behavior, indicating that physiological stress was induced at the supposed "optimal" temperature, for this species. Loss of equilibrium (LOE) is commonly used in as an indicator of behavioral impairment and is used as one of several reflex indicators of mortality following capture and release (Davis et al., 2010, Raby et al., 2012; Whitney et al., 2021; Holder et al., 2020). If pervasive during locomotion, LOE has even been used to indicate critical thermal maximum (CT_{MAX}) on different time scales (Elliott and Elliott, 2010; Brauner and Richards, 2020). In this study, oxygen limitation did not appear to be the cause of LOE as the aberrant behavior was observed both in holding tanks at 70-100% air-saturation (well above determined levels of P_{cSMR} for this species) and during respirometry, regardless of oxygen level. Equilibrium was restored when sharks were returned to 18°C, all else being held constant, which suggests that this behavioral impairment is a result of thermal stress.

Additionally, Bockus et al., (2020) found that heat shock proteins, vital in the protection of protein structure and function against thermal stress, were significantly elevated (3-fold) at 21°C relative to controls maintained at 15°C for *S. acanthias*. Although *S. acanthias* has been captured at temperatures higher than 22°C, their presence at such higher temperatures is unusual across their range in the North Atlantic and catch frequency for this species is generally highest between 6-15°C (Jensen, 1966; Castro, 1983; McMillan and Morse, 1999; Stehlik, 2007). Selected temperature ranges are reported to be even narrower when groups are categorized by ontogeny (7-11°C for adults; Castro and Peebles, 2011; Stehlik, 2007 and 6-14°C for juveniles Stehlik, 2007). In addition, a 3-year study of *S. acanthias* movement within the US NES conducted by Carlson et al., (2014), revealed that despite a large thermal range occupied by *S. acanthias*, selected temperatures were skewed to colder values (mean±SE of overall temperature used: 9.2±0.1 °C, northern tagged individuals; and 12.7±0.1 °C, southern tagged individuals; Carlson et al 2014). Such mounting physiological and ecological evidence of the onset of thermal stress at 21°C and increased thermal habitat use at much lower temperatures, make it difficult to reconcile our measured peak in AAS at 20.5-23°C with thermal "optimality".

Although AAS was highest at the warmest temperatures, it is important to remember that SMR, representing the cost of basic maintenance and activity, was also exponentially higher at such warm temperatures. In other words, it costs more (~2.57x more calorically) to live at "peak AAS temperature" compared to 10°C, a temperature that does not confer as much "energetic potential". Greater effort is then required at higher temperatures for foraging activities to support elevated caloric demand (caloric calculations from Schmidt-Nielsen, 1972). These additional energetic costs must be weighed against any potential advantages in growth rate, scope for predator evasion, or potential reproductive output at higher temperatures. Our study revealed AAS to be lowest within experimental thermal range at 10°C (at about 60% of maximum AAS), much closer to the temperature where wild *S. acanthias* appear to spend the majority of their time (Jensen, 1966; Scott, 1982; Castro, 1983; Compagno, 1984; Grace and Henwood, 1997; McMillan and Morse, 1999; Shepherd et al., 2002; Stehlik, 2007; Lucey and Nye, 2010; Carlson et al 2014; Bockus et al., 2020). While more active pelagic species such as Mahi-mahi (*Coryphaena hippurus*) have been shown to benefit from residing in the tight thermal range that provides their greatest aerobic capacity (Heuer et al., 2021), for *S. acanthias*, a relatively less active benthopelagic species, it appears as though maximized aerobic scope at higher temperatures is not worth the trade-off of residing at temperatures with continuously elevated metabolic demands. AAS is then a poor indicator of thermal "optimality" and selected thermal habitat for this species.

Oxygen supply limitation and absolute aerobic scope as an indicator of thermal tolerance: Diminished aerobic scope at sublethal thermal limits has been attributed to oxygen supply limitation in several species (e.g., Eliason et al. 2011). However, failure of oxygen supply and limitation of aerobic scope do not universally explain species thermal limits (Lefevre 2016; Ern et al., 2016; Jutfelt et al., 2018; Slesinger et al., 2019; Seibel and Deutsch, 2020). In this study there were no significant decrements in maximum metabolic performance, aerobic scope, or oxygen supply capacity for *S. acanthias* that might indicate a sublethal thermal limit. If thermal tolerance were limited by oxygen supply capacity and aerobic scope, there should be a clear decline in these metrics as they approached critical thermal levels, according to Pörtner et al., (2010), but this was not the case in my study. Metabolic performance was maintained through the tested range (Lefevre, 2016; Norin et al., 2014, Nati et al., 2016; Jutfelt et al., 2018; Slesinger et al., 2019), despite clear thermal stress (LOE) at the two highest temperatures tested and subject mortality just beyond experimental thermal range. In this study, 24°C represented a likely critical thermal maximum for this species subpopulation. The observation of mortality during acclimation to temperatures >24˚C was similar to studies that identified incremental thermal maximum (critical upper temperature resulting in death after low rates of temperature increase e.g., 0.25 °C*day^{-1}; Lutterschmidt and Hutchison, 1997; Zanuzzo et al., 2019; Bartlett et al., 2022) and the incremental thermal maximum-like stress that has resulted in morbidity in aquaculture scenarios (Brauner and Richards, 2020). Such lack of decline in AAS as thermally stressful and lethal temperatures were approached suggests that AAS is a poor indicator of sublethal thermal limits for this species.

The ability of an organism to supply oxygen to vital processes within the body depends on both, ambient environmental oxygen levels, and oxygen supply capacity, which includes all physiological processes in the cascade of oxygen delivery, (i.e., oxygen diffusion at the gills, blood oxygen binding and transport, extracellular and intracellular O_2 diffusion rates, cardiac activity, etc.; Weibel et al., 1991). These factors are affected by environmental changes in oxygen and temperature (Hochachka and Somero 2002; Fry 1947; Fry and Hart 1948). Measures of α for *S. acanthias* in this study increased with similar temperature sensitivity as MMR, in support of the increased metabolic demands at high temperature as expected. This indicates that physiological adjustments are made at various places in the oxygen supply chain to accommodate elevated maximum metabolic demand, within the evolved thermal range of *S. acanthias* (Seibel and Deutsch, 2020). The lack of decline in α at the highest temperatures suggests the physiological mechanisms involved in oxygen supply do not fail in *S. acanthias*, even as critical thermal limits are approached. This indicates that 23˚C is likely still within evolved thermal range for this species, but that we may see a decline in any of these performance metrics at critical temperatures outside of the organism’s evolved thermal range (beyond 24˚C). Outside the evolved thermal envelope, any number of processes including oxygen supply capacity may fail (e.g., protein unfolding, cytoskeleton network collapse, errant localization of organelles, reduced mitochondrial function, morphological changes in membrane, aberrant RNA splicing etc.; Richter et al., 2010) as there is reduced selective pressure to preserve function at temperatures outside the natural range. Reduced aerobic scope at high temperature may also result from a failure of the metabolic machinery to *use* oxygen (e.g., muscle oxidative performance) or an inability of the muscles to *produce equivalent work* rather than an inability to *provide* sufficient oxygen. Protein degradation or the integration of neuromuscular function may also be compromised at high temperature as suggested by accumulation of heat-shock proteins (Bockus et al., 2020) and the loss of equilibrium at rest in *S. acanthias* in this study. Based on the evidence in this study, oxygen supply capacity is not the limiting factor for AAS, thermal tolerance, or habitat suitability for this species.

Factorial aerobic scope and viable habitat for *S. acanthias:* Although absolute aerobic scope does not appear to provide a clear understanding of acute thermal limits or thermal optimum in *S. acanthias*, recent work suggests that a minimum factorial aerobic scope (i.e., *Metabolic Index;* Deutsch et al., 2015; 2020) is required to sustain a population. A survey of diverse marine species demonstrated that the warm end of species natural distribution is coincident with a factorial aerobic scope generally between 2 and 5 (mean of 3.3) (Deutsch et al., 2015; Deutsch et al., 2020). This population threshold was based upon the thermal sensitivity of species’ metabolic needs, historical distribution of species populations, and chemo-physical dynamics of a given habitat. A population FAS threshold of between 2 and 5 appears to hold true for *S. acanthias*, which reaches an FAS of 4.31 at 23°C (Table 2, Figure 2D) in air-saturated waters, near critical thermal thresholds the species (~24°C). FAS trends in this study demonstrated agreement with previous literature, declining across the thermal range of the species (Deutsch et al 2015; 2020), with the highest values of FAS occurring at 12-12.5°C, around the upper end of reported selected thermal habitat of adult *S. acanthias* (Burgess, 2002). FAS is particularly robust to variation in metabolic rate measures, especially MMR. In cases where variation in MMR is greater than variation in SMR, as is the case in this study, FAS has been regarded as a more stable estimate of aerobic scope (Halsey et al., 2018). In the case of *S. acanthias*, FAS appears to be a more useful measure of aerobic scope in predicting population level thermal limits, in agreement with field studies of *S. acanthias* habitat use on the U.S. Northeast shelf.

FAS in this study suggests this subpopulation of the *S. acanthias* on the US NES is approaching upper thermal limits. Surface and bottom temperatures in some locations within their native range already exceed 24°C seasonally. Declining ambient oxygen levels complicate thermal tolerance, reducing the scope for activity and maximal performance across $PO_{2.}$ With dissolved oxygen levels in the region typically ranging between 75 to 100% air saturation (15-21 kPa), declining oxygen can cause a shift in MMR and FAS by 25% and up to as much as 50% at the lowest oxygen values *S. acanthias* is likely to experience. As waters warm and O_2 fluctuates, FAS may drop below critical values as a result of decreasing oxygen and/or increasing temperature (Figure 2D). Thus, the measured and predicted FAS values in this study (between ~15-21 kPa) are ecologically relevant and suggest changes in FAS may contribute habitat shifts with ocean warming and deoxygenation for *S. acanthias*.

Considerations for *S. acanthias* long-term success and survival in the era of climate change: Ultimately, the ability of ectothermic species to survive and thrive as climate change intensifies (Scheffers et al., 2016; Pecl et al., 2017; IPCC, 2019) will depend on myriad factors. These include current physiological capabilities, capacity for acclimation and/or adaptation to new conditions, generation time, the rate of environmental change (Nguyn et al., 2011; Pinsky et al., 2013), as well as other ecological factors such as how heavily species rely on certain prey items to name a few. These factors remain largely unexplored in *S. acanthias*. *Squalus acanthias* have been observed to vertically migrate in pursuit of abundant prey resources such as longfin squid (Stehlik, 2007, Sagarese et al., 2014), and trends in the distribution of various size classes have been correlated with prey abundance (Sagarese et al., 2014). However, *S. acanthias* are considered generalist predators, demonstrating highly opportunistic feeding subject to changes in time and geographic location (Stehlik, 2007). These animals have also been highly successful in filling vacated trophic niches (Link and Garrison, 2002) and dominate as piscivores in their ecosystems. Bearing that in mind, their abilities to tolerate, acclimate, and/or adapt to environmental change will likely be at the forefront of their distributional responses to climate change.

This study demonstrated metabolic changes of *S. acanthias* across a thermal envelope with sharks incrementally acclimated to target temperatures on the timescale of *days*. Changes on this timeframe reflect *S. acanthias* abilities to cope with "stints" at elevated temperatures, relevant in the face of increasing mean temperatures and increasing intensity/frequency of heat events (Easterling et al., 2000; Meehl and Tebaldi, 2004; Rahmstorf and Coumou, 2011; Stocker et al., 2013). Though identification of metabolic responses to thermal acute change is relevant, the capacity for thermal acclamation (phenotypic plasticity) in *S. acanthias* will be equally important in identifying thermal limits on longer time scales. Acclimation has been known to increase thermal tolerance by reducing metabolism relative to the initial acute thermal change, modulating SMR and aerobic scope, and allowing for compensation of the direct thermodynamic effects on metabolism (Precht 1958; Johnston and Dunn, 1987; Angiletta, 2009; Schulte et al., 2011; Seebacher and Franklin, 2012; Sandblom et al., 2013). In addition, though thermal adaptation is possible across generations, for many marine species climate change is occurring faster than adaptation through natural selection can keep up (Gienapp et al., 2008; Reusch, 2014; Fox et al., 2019). Given the rapid rate of environmental change on the US NES, and slow generational turnover for this species, (*S. acanthias* being long lived, late to mature: females ~12 years, males ~6 years; Nammack et al., 1985; Burgess, 2002; Castro and Peebles, 2011), with low fecundity (1-15 pups per reproductive cycle in the NW Atlantic; Colvocoressesa and Musick 1980; Burgess, 2002) and long gestational times (18-24 months; NEFSC, 2006), rapid thermal adaptation seems unlikely. Current adaptation and acclimation capabilities, however, remain undetermined for *S. acanthias*.

For highly mobile species like *S. acanthias*, behavioral thermal regulation is a much more readily available means of coping with climate change. Although historically *S. acanthias* is reported as "strongly associated with the benthos" (Stehlik, 2007; Lucey and Nye 2010), the higher metabolic rates of the species suggests that they are active and highly mobile, in agreement with several recent movement studies (Sulikowski 2010; Carlson et al 2014). The metabolic rates measured in this study were found to be comparable to elasmobranchs with this type of highly migratory benthopelagic lifestyle (Brett and Blackburn, 1978; Hanson and Johansen, 1970; Scharold et al., 1989; Scharold and Gruber; Pritchard et al, 1958; Lear et al., 2018; Lenfant and Johansen, 1966), but were higher than metabolic demands of benthic skates and rays in the region (Schwieterman et al., 2019), and other benthic associated elasmobranchs (Hughes and Umezawa, 1968; Short et al., 1979, Ferry-Graham and Gibb, 2001; Whitney et al., 2016; Luongo and Lowe, 2018).

Squalus acanthias are known to undergo seasonal migrations (both on-offshore, and latitudinal) coincident with seasonal bottom temperature changes (Stehlik, 2007, Ulrich et al., 2007; Sagarese et al., 2014; Templeman, 1984). Resident *S. acanthias* in Rhode Island, have also been noted to selectively avoid bottom temperatures in Narragansett Bay above 22-23°C Bockus et al., (2020) Additionally, as reported in Carlson et al, (2014), mean daily temperatures for either northern or southern tagged *S. acanthias,* did not exceed 23°C. For both the northern and southern parts of the stock (Carlson et al 2014) the highest average daily temperatures occurred in months when they used the shallowest habitat (summer/autumn for northern sharks, autumn/winter for southern sharks) and in that time, mean daily depth did not exceed 50m for either portion of the stock (Carlson et al 2014). However, sharks in that study were recorded to have used depths from the surface to 481.5 m (northern tags) and 214.5m depth (southern tags) across the full timeframe of tag deployments and used a range of depths in the water column throughout the day. Additionally, studies have recorded maximum depths of between 500-900m (Compagno, 1984; Stehlik, 2007) suggesting that vertical displacement to deeper waters in pursuit of thermal refuge may be a viable strategy for this species in times of thermal stress.

As their current occupied range continues to warm, exhibiting greater frequency and severity of thermal fluctuation (Easterling et al., 2000; Meehl and Tebaldi, 2004; Rahmstorf and Coumou, 2011; Stocker et al., 2013), likely first responses to changes outside their evolved thermal envelope include poleward shifting distributions, longitudinal shifts to more frequent use of offshore habitats and/or vertical displacement, decreased residency times or shifts in core habitat used etc. While poleward distributional shifts have not been reported for this population to date, studies reported that the extensive range of the population on the US NES outside the bounds of study surveys, and growing numbers due to the rebuilding of *S. acanthias* stocks overfished through the 1990's, complicated assessment and potential distributional shifts warrant further investigation as conditions continue to change (Nye et al., 2009). In addition, the degree to which this species is likely to shift distributionally on the US NES with latitude and/or depth will depend not only on the rate of environmental change, but on the specific thermal tolerance of subgroups within the region. Due to their propensity for long migrations and wide species range along the North American Eastern seaboard, *S. acanthias* is managed as a single stock on the U. S. Atlantic coast (ASMFC, 2002). However, there is evidence to suggest there are important subgroups/subpopulation within the stock that exhibit more limited movement and distribution within the full species range (Campana et al., 2008; 2009; Carlson et al., 2014) and may exhibit regional differences in thermal habitat utilization and tolerance for this species. Some southern studies have reported maximum temperatures at capture of between 24-29.1°C (Ulrich et al., 2007; Bangley and Rulifson, 2014), higher than the critical temperature found in this study. Tagging studies show the southern portion of the stock, captured off of Cape Hatteras, appear to spend more time at elevated temperatures (21+°C) and tend to occur in shallower water closer to shore (Rulifson & Moore, 2009; Rulifson et al., 2012) relative to the northern portion of the stock (mid-Atlantic Bight and higher latitudes). The proportion of time that *S. acanthias* spend near their thermal limits is not known, nor is the regularity with which they move through potentially challenging thermal environments. The presence of active *S. acanthias* at temperatures >24°C contrasts evidence for critical limits found in this study and may suggest that phenotypic plasticity, acclimation, or local adaptation may have resulted in different thermal limits across *S. acanthias* latitudinal range in the Atlantic. However, it is at present unclear what the differences in thermal tolerance are between subpopulations and if differences may be due to phenotypic plasticity in acclimation, local adaptation, or that the southern portions of the stock may just be living chronically closer to their thermal limits.

Sex and size related metabolic traits and potential effects on thermal tolerance: Although adults in this study spanned less than 1 order of magnitude in mass, our work confirmed the extensive previous inter- and intraspecific evidence that MO_2 scales with body mass (Clarke and Johnston, 1999; Glazier, 2005; Killen et al., 2010; Norin and Gamperl, 2017) (Table A1). Smaller animals had a higher per kg tissue (mass-specific) metabolic rate than larger conspecifics (Schmidt-Nielsen, 1972), despite the greater overall (non-mass-specific) metabolic demand in larger animals (Figure A3). However, when the effect of mass was removed, *S. acanthias* did not demonstrate significant differences in metrics between the sexes indicating that metabolic differences between the sexes can be largely attributed to size differences. In situ measurements of temperature in tagged adult wild *S. acanthias* (Carlson et al., 2014) revealed that mean seasonal temperature differences did not exceed 1°C between the sexes (see also: Shepherd et al., 2002). Though differences in metabolic demands between sizes (and thus by sex for this species) could confer differences in thermal tolerance especially across ontogenetic stages (Sagarese et al., 2014), it appears that size differences between adults of this population (<1 order of magnitude) are not enough to drive differential thermal tolerance between the sexes.

There may however be additional metabolic costs associated with pregnancy in *S. acanthias*. In our study there were several trials where large pregnant *S. acanthias* gave birth inside our chamber to multiple pups, and disallowed full trial comparison for these individuals. However, initial data analyzed from parts of the trial where the pups were not present, indicate that metabolic rates of pregnant females just before giving birth, and after pups had been removed from the trial chamber, were elevated compared to males and female individuals that were not pregnant or were post-partum (determined via ultrasound, and visual confirmation of birth). The observation of potentially elevated metabolic costs associated with pregnancy is relevant, given that mature *S. acanthias* females spend much of their time in gamete investment and gestation. *Squalus acanthias* has one of the longest gestational periods of any vertebrate species and can have simultaneous pregnancies of various stages (observed in this study via ultrasound and postmortem dissection). Differential metabolic demands between reproductive stages may confer differential thermal sensitivity. If pregnant females have higher metabolic demands, these stages could demonstrate heightened vulnerability to climate shifts than conspecifics (Dahlke et al., 2020b). Upper thermal tolerance may decline as a result of reproductive input in aquatic species (Pörtner and Knust, 2007; Pörtner and Farrell, 2008; Pörtner and Peck, 2010) based on the increase in oxygen demand for gamete production (Masonjones, 2001; Kraffe et al., 2008; Madeira et al., 2012). Additionally, for live-bearing species like *S. acanthias*, the increasing oxygen demands of developing embryos within the uteri add to metabolic burden and may impose further constraints on upper thermal tolerance in carrying females (Dygert and Gunderson, 1991; Baeza and Ferńandez, 2002; Timmerman and Chapman, 2003; Green and McCormick, 2005; Auer et al., 2021). Future warming may limit viable habitat for sharks in these stages more readily, especially if they already experience greater frequency of exposure to thermal limits by entering warmer coastal waters during parturition (Moore, 1998; Shepherd et al., 2002) or to accelerate embryo development (Harris 1952; Pistevos et al., 2015) and later stages of gestation in live bearing species (Hight and Lowe 2007; Jirik and Lowe 2012, Nosal et al. 2014). Comparison of pregnant vs non-pregnant metabolic demands in *S. acanthias* was outside the core objectives of the current study but future controlled analysis of the metabolic costs of pregnancy is warranted.

Future studies should focus on thermal acclimation potential including seasonal and chronic acclimation, multigenerational common garden experiments (if captive rearing for this species can be improved to allow for such studies) across multiple latitudes to identify local adaptation, in addition to measuring *current* physiological performance of the southern portion of the stock. We also recommend that future studies investigate the differential thermal vulnerability across a greater number of sizes and life stages, and stages of female reproduction of these organisms. These investigations can start to tease apart the current and long-term ability of these sharks of the US. Northeast shelf to cope with a changing thermal environment across their extensive distribution. We can also begin to identify true differences in thermal tolerance between local populations that may contribute to enhanced or diminished thermal tolerance between portions of this stock.

Conclusions

Our metabolic data support previous studies indicating *S. acanthias* is a very active benthopelagic species, with implications for this species being less associated with the benthos than previously thought. Oxygen supply capacity supports maximum metabolic demand for *S. acanthias* from 10°C to 23°C, with absolute aerobic scope for this species increasing with temperature across the measured range. Oxygen supply limitation does not adequately explain thermal limitation in this species, nor does it appear that absolute aerobic scope can be used to identify “thermal optimality” or sublethal thermal limits for this species. Factorial aerobic scope (highest at 10°C) appears to be a likely predictor of viable habitat on a population level, with temperatures where *S. acanthias* spend most time in the wild corresponding to the temperatures with the greater values of FAS in this study (10-17°C). For *S. acanthias* of the US NES, 23°C may represent a functional tolerance limit, with a critical thermal limit of 24°C (on time scales >12 hr) for *S. acanthias* of the mid-Atlantic. This subpopulation appears to be living near upper thermal limits in the warmest seasons and exhibits a critical FAS near 4.31 for population level success. Finally, oxygen deficits in the region may cause shifts in FAS that further limit thermal tolerance and cause predictable limits to maximum performance across temperature for *S. acanthias*. Changes in FAS may be used to fine tune predictions of viable habitat based on energetic balance of aerobic demands and scope. With the projected climate change in the region, it is likely that these animals may exhibit poleward or horizontal shifts in their distribution and/or a change in biomass depth utilization as temperatures continue to rise.

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Table 1. *S. acanthias demographics and trial summaries*. Measures are reported as means ± SE.

Temperature Bin (°C)	*10*	*13*	*17*	*21*	*23*
Intermittent Respirometry Cycle (cycle phase lengths in min) – range and (mode)					
Flush	3-6 (6)	6-8 (6)	7-11 (7)	10-12 (12)	12-14 (12)
Wait	1	1	1	1	1
Measure	8-12 (10)	6-8:30 (7.5 and 8.5)	6-7:30 (7.5)	5-6 (5)	4-5 (5)
Maximum Metabolic Rate Trials					
n	22	17	21	23	21
n (for MMR at 21 kPa and for α)	22	17	21	27	21
Sex ratio (F:M)	14:8	9:8	16:5	15:8	17:4
Mean temp (°C)	10.14 ± 0.02	13.35 ± 0.02	17.11 ± 0.05	21.24 ± 0.02	23.12 ± 0.02
Precaudal length (PCL)	67.1 ± 1.1	66.59 ± 1.30	68.15 ± 1.27	68.43 ± 1.07	69.98 ± 1.4
Fork length (FL)	73.85 ± 1.23	72.59 ± 1.21	74.70 ± 1.38	75.19 ± 1.25	76.89 ± 1.22
Stretch total length (STL)	83.28 ± 1.34	81.89 ± 1.36	84.47 ± 1.48	84.36 ± 1.19	86.3 ± 1.27
Mass (kg)	2.3 ± 0.04	2.13 ± 0.04	2.21 ± 0.03	2.26 ± 0.03	2.32 ± 0.03
PO_2 (kPa)	16.58 ± 0.04	15.89 ± 0.07	15.72 ± 0.05	14.88 ± 0.05	15 ± 0.06
Hypoxia Trials					
n	8	7	6	8	6
Sex ratio (F:M)	4:4	3:4	4:2	4:4	4:2
Mean temp (°C)	9.64 ± 0.01	13.50 ± 0.002	16.90 ± 0.12	20.44 ± 0.07	22.19 ± 0.0004
Precaudal length (PCL)	64.19 ± 0.99	64.92 ± 1.5	70.8 ± 2.46	65.25 ± 0.93	67.1 ± 1.85
Fork length (FL)	70.5 ± 1.14	71.25 ± 1.56	77.4 ± 2.71	71.69 ± 0.97	73.67 ± 2.10
Stretch total length (STL)	79.91 ± 1.32	80.58 ± 1.77	87.8 ± 3.03	81.23 ± 1.09	83.12 ± 2.25
Mass (kg)	1.89 ± 0.04	1.83 ± 0.08	2.15 ± 0.11	1.86 ± 0.03	2 ± 0.09

Table 2. *Metabolic trait definitions, methods and associated equations for S. acanthias*

Metric	*Name/definition of Metric*	*Measured and/or Modeled*	*Defined By:*	*Source Trial*	*Associated equation*
SMR	Standard metabolic rate; lowest metabolic rate supporting basal metabolism of conscious shark at rest	Measured (limited), Modeled	$q_{0.2}$ of MO_2 values prior to oxygen limitation	Hypoxia	NA
P_c	Critical (limiting) O_2 partial pressure for a given metabolic rate across the activity spectrum. (e.g., ***PcSMR*** = critical O2 for SMR; ***PcMAX*** = critical O2 for MMR at 21kPa)	Measured (limited), Modeled	α, MO_2, PO_2 (a-method; Seibel et al., 2020)	Both	$P_c = MO_2/\alpha$ e.g., P_{cSMR}=SMR/α
MMR (measured)	Highest metabolic rate achieved in trials	Measured, Modeled	Highest MO_2 value in trial	Maximum Metabolic Rate	NA
MMR *(at 21kPa)*	Predicted maximum metabolic rate at air saturation (MMR = α* PcMAX of 21kPa)	Modeled	SMR, P_{cSMR}, and/or α, P_{cMAX} =21kPa	Both	MMR=α* P_{cMAX} (MMR= α*21)
AAS	Absolute aerobic scope (modeled and calculated using population mean MMR and SMR values)	Measured, Modeled	MMR(at 21kPa), MMR, SMR	Both	AAS=MMR - SMR
FAS	Factorial aerobic scope (modeled and calculated using population mean MMR and SMR values)	Measured, Modeled	MMR(predicted at 21kPa), MMR, SMR	Both	FAS=MMR/SMR
α	Physiological oxygen supply capacity (Maximum measured value of physiological oxygen supply; α_0)	Measured, Modeled	Maximum value of α_0 (instantaneous oxygen supply) in trial	Both	Highest value of $\alpha_0=MO_2/PO_2$
α -Line	Linear relationship of critical O_2 partial pressure across activity	Modeled	α = slope of α-line (α= MO_2/P_c for given MO_2), intercept= (0,0), P_{cMAX},	Both	y = α*x MO_2= α*PO_2
Chase time	Length of chase exercise to "exhaustion"	Measured	Time to exhaustion (s)	Maximum Metabolic Rate	NA

Table 3. *Modeled metabolic traits and temperature sensitivities for S. acanthias.* Measures are reported as means ± SD for an average 2.4 kg animal (mass corrected to population mean) with the exception of P_{cSMR}.

Temperature	*10*	*13*	*17*	*21*	*23*	*E (eV)*	*Q_{10}*
Standard (SMR) Metabolic Rate (mg O_2 kg^{-1}h^{-1})							
SMR	26.86 ± 1.07	34.26 ± 1.05	47.39 ± 1.04	65.55 ± 1.06	77.09 ± 1.07	0.61	2.25
Maximum Metabolic Rate (MMR; mg O_2 kg^{-1}h^{-1})							
MMR	118.92 ± 7.79	156.13 ± 5.86	196.47 ± 6.28	221.58 ± 4.73	229.31 ± 6.18	0.35	1.66
MMR (at 21 kPa)	163.02 ± 12.67	209.74 ± 9.52	268.6 ± 10.72	315 ± 7.87	332.48 ± 10.78	0.38	1.73
Absolute Aerobic Scope (AAS; mg O_2 kg^{-1}h^{-1})							
AAS	92.06 ± 8.01	121.87 ± 6.12	149.08 ± 6.55	156.03 ± 5.97	152.21 ± 8.17	0.27	1.47
AAS (at 21kPa)	136.16 ± 12.81	175.49 ± 9.67	221.22 ± 10.88	249.46 ± 8.67	255.39 ± 12.03	0.35	1.62
Factorial Aerobic Scope (FAS; mg O_2 kg^{-1}h^{-1})							
FAS	4.43 ± 0.43	4.56 ± 0.29	4.15 ± 0.21	3.38 ± 0.2	2.97 ± 0.23	2	0.74
FAS (at 21kPa)	6.07 ± 0.65	6.12 ± 0.42	5.67 ± 0.32	4.81 ± 0.29	4.31 ± 0.34	0.16	0.77
Oxygen supply capacity (**α**; mg O_2 kg^{-1}h^{-1}kPa^{-1})							
α	7.76 ± 0.6	9.99 ± 0.44	12.79 ± 0.5	15 ± 0.37	16.18 ± 0.51	0.38	1.76
Critical Oxygen Partial Pressure (P_{cSMR} and $P_{cmax;}$ kPa)							
P_{cSMR}	3.39 ± 1.08	3.66 ± 1.06	4.05 ± 1.04	4.49 ± 1.07	4.72 ± 1.08	0.21	1.29

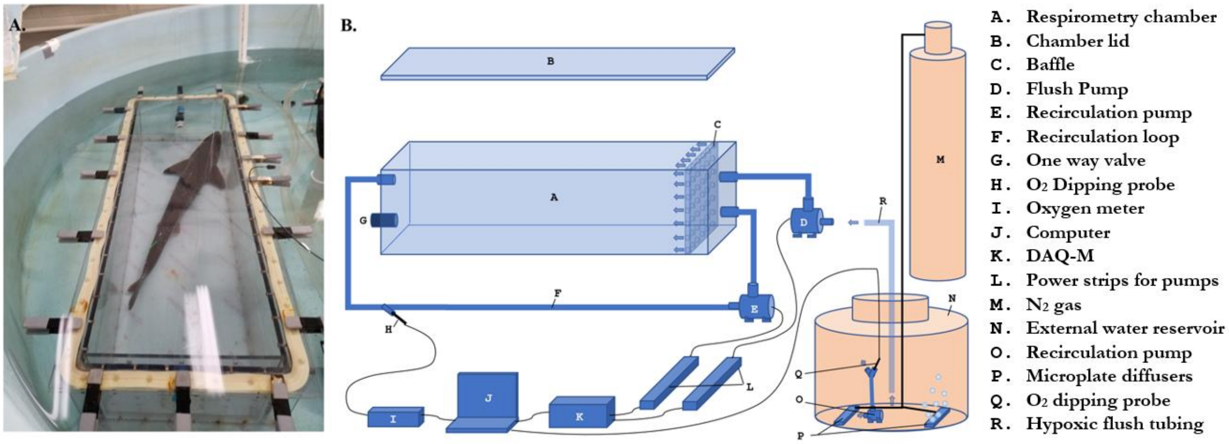

Figure 1. *Experimental set-up for S. acanthias*. **(A)** *S. acanthias* individual sealed within the intermittent respirometry chamber and surrounding temperature bath during metabolic trials. **(B)** Experimental set up with labels corresponding to item letters located to the right of the figure. Items A-H were fully submerged within the temperature bath for the duration of trials. Items M-R were used in hypoxic trials only. Blue arrows indicate direction of water flow.

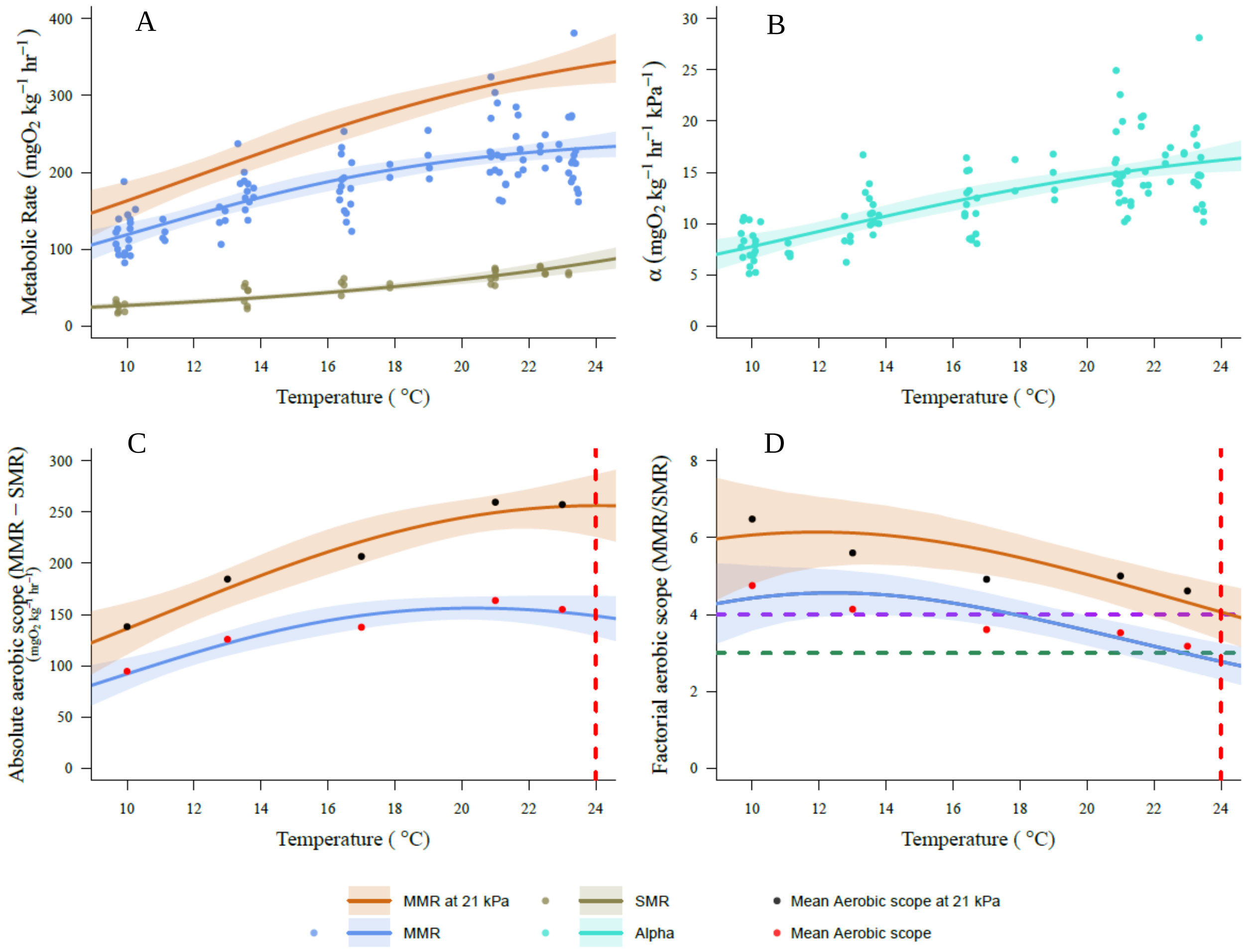

Figure 2. *Modeled temperature effects on metabolic traits for S. acanthias.* A) MMR measured (blue) and predicted (orange), and SMR (gold). **(B)** oxygen supply capacity (α) C) Measured and predicted AAS, D) Measured and predicted FAS. Predicted values in each panel are calculated for air-saturation using MMR = 21* α. Colored bands around the models are 95% confidence intervals. The purple dashed line in (D) represents theoretical FAS population threshold for *S acanthias* based on critical temperature of 24°C (red dotted line) and the navy-blue dashed line represents the mean critical Metabolic Index (3.3), defined by the warm (equatorward) edge of a population (Deutsch et al., 2020). The reduced trial PO_2 causes a decrement in MMR and aerobic scope that may result in reduced temperature thresholds for population sustenance.

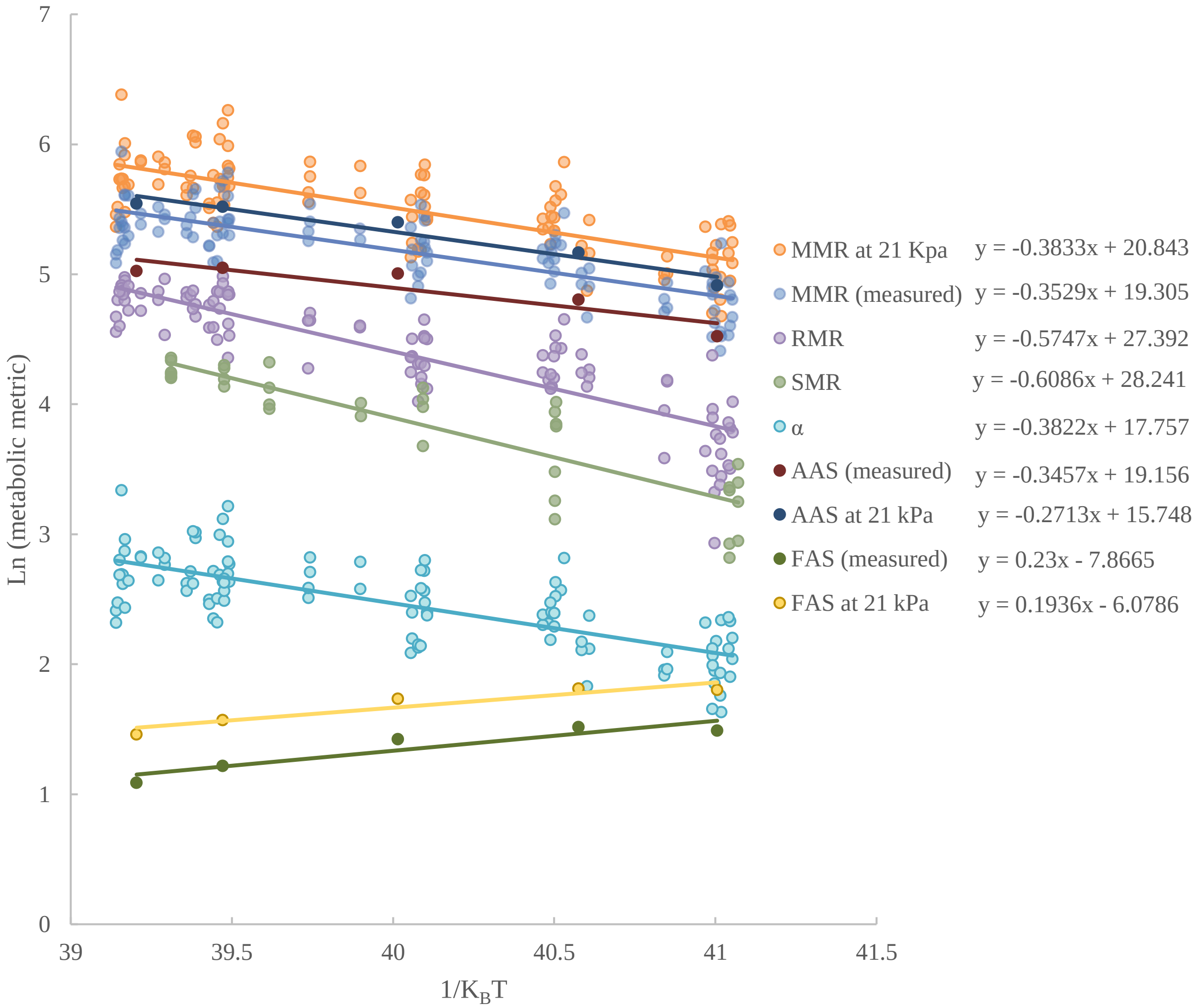

Figure 3. *Arrhenius relationships of metabolic metrics for S. acanthias*. The temperature coefficient, *E* (eV), is derived from the slope of the relationship (right), where k_B is Boltzmann's constant and T is temperature (K). Note that a negative slope indicates an increase with temperature (positive *E* value) despite a negative slope.

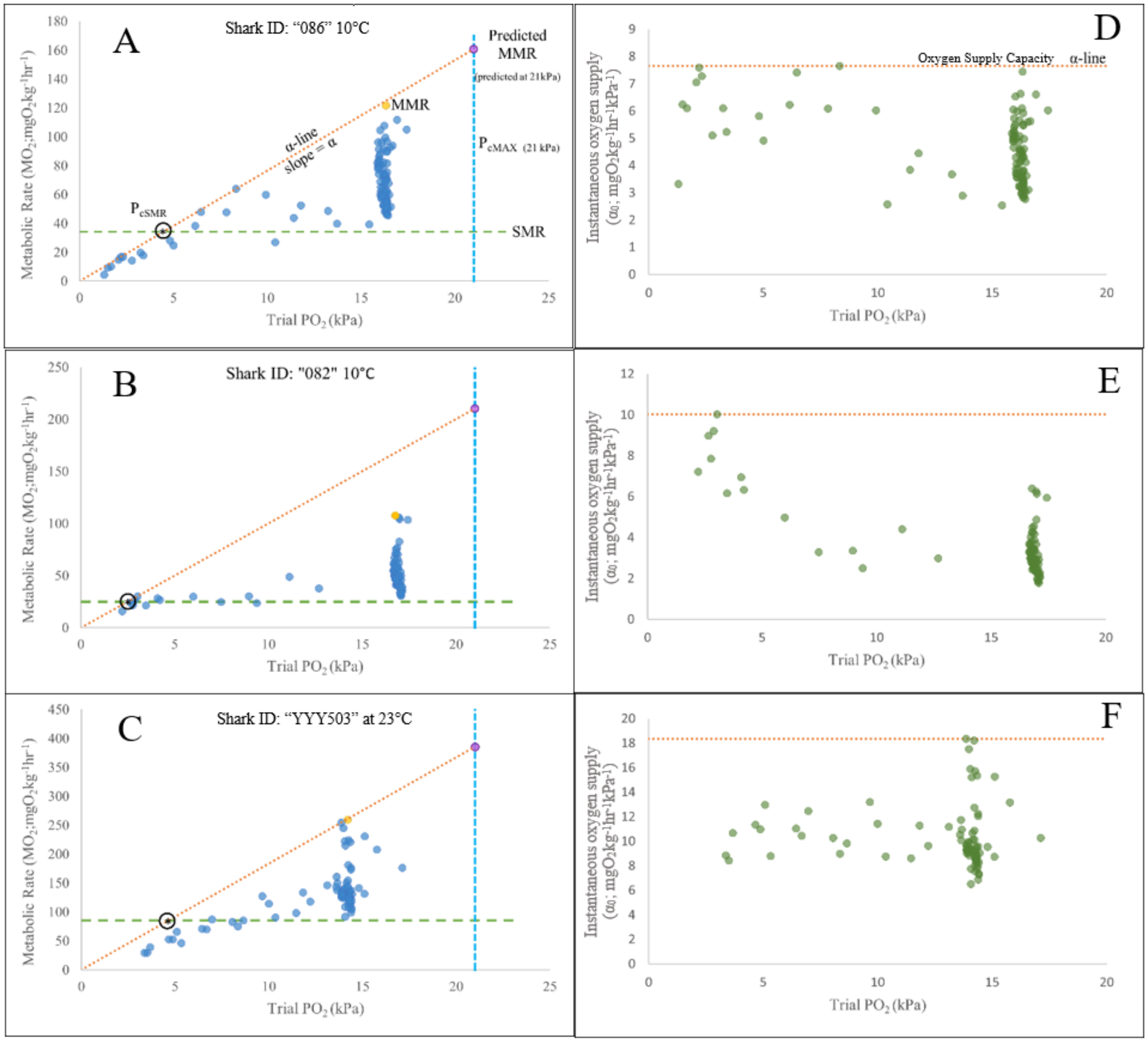

Figure 4. *Representative respirometry trials and oxygen supply capacity determination for S. acanthias individuals.* Panels **(A-C):** Oxygen consumption rates (MO_2) for each measure period across both MMR and hypoxia trials, for an individual shark. The α-line (orange dashed; MMR = α*PO_2) describes the oxygen dependency of MMR between P_{cmax} and P_{cSMR}. The P_{cSMR} (black circle) is the PO_2 at which SMR (green dashed) intersects the α-line. P_{cMAX} (dashed teal) is the PO_2 at which MMR becomes oxygen dependent, here assumed to be 21kPa. The highest measured (yellow) and estimated maximum (purple) metabolic rates are indicated at the trial PO_2 and P_{cmax}, respectively. Panels **(D-F)** demonstrate *α* determination. The physiological oxygen supplied per unit available pressure (α_0=MO_2/PO_2; green) increases as PO_2 declines during each measure period across both trial types, for each individual. The highest α_0 value is *α*, the oxygen supply capacity (yellow dashed line), which is reached at maximum exertion and/or at the critical PO_2. (**A, D)** an individual trial with very close agreement between MMR and hypoxia trial alpha values. **(B, E)** The *α* was reached only during the hypoxia trial. **(C, F)** The *α* was reached only during the MMR trial.

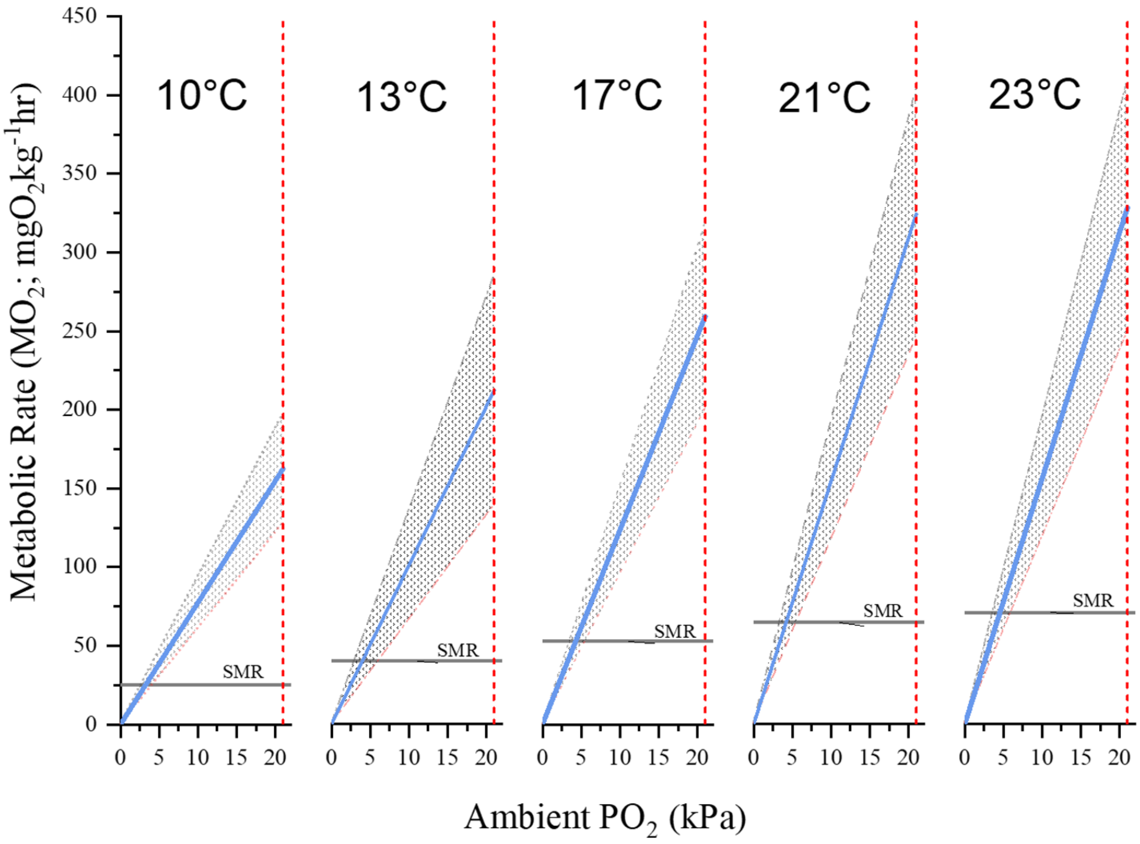

Figure 5. *Oxygen and temperature dependency of MMR for S. acanthias.* Blue lines represent the average α-line (MMR = α *PO_2) at each temperature bin extrapolated to a P_{cMAX} of 21kPa (air-saturation, red dashed-lines), while the grey shaded section represents a 95% confidence interval.

Figure 6. *S. acanthias displaying loss of equilibrium at rest, at ≥21°C.* Oxygen in holding tanks at higher temperatures ranged from 70-95% air saturation, substantially exceeding P_c for SMR (P_{cSMR}), indicating that oxygen limitation is not the cause of the aberrant behavior. The righting reflex was restored when sharks were returned to cooler temperatures (18°C).

CHAPTER 2

THERMAL SENSITIVITY OF AEROBIC PERFORMANCE IN THE BULL SHARK, *CARCHARHINUS LEUCAS*, AND BLACKTIP SHARK, *CARCHARHINUS LIMBATUS*

Introduction

Global warming has led to the elevation of ocean temperatures (IPCC, 2022), threatening the success and viability of marine populations around the world. Changes in temperature may disrupt the balance of oxygen supply and energetic demand in marine ectotherms, altering organism ability to derive energy in support of movement, foraging, growth, etc. Temperature influences the metabolic process in ectotherms (Kieffer, 2000), from protein conformation, thermodynamics of metabolic processes, activation energies, efficacy of metabolic enzymes, to increases in overall metabolic demands (Hochachka & Somero, 2002). As such, ectothermic shark species, whose energy use is inherently tied to environmental temperature, are susceptible to energetic changes under thermal shifts. However, our understanding of the elasmobranch energetic response to environmental pressures is extremely limited, and a mechanistic understanding of how metabolic performance and environmental tolerance change across temperature is lacking for many shark species.

Coastal species such as bull sharks (*Carcharhinus leucas*) and blacktip sharks (*Carcharhinus limbatus*), may be vulnerable to these climate changes due to highly active lifestyles, life history and environmental requirements that necessitate proximity to inshore regions (Castro, 2010). Studies show 71% of the worlds coastlines are experiencing significant warming at variable rates (Hansen et al., 2006; Rayner et al., 2006; Burrows et al., 2011; Lima & Wethey, 2012). Coastal estuaries and river habitat play a large role in the life histories of bull and blacktip sharks. These habitats are used as nursery grounds, where parturition occurs and young remain resident for several months (Hueter & Tyminski, 2007). Shark nurseries have been identified as areas of higher neonate-juvenile abundance, where high site fidelity is exhibited, over extended periods of time (Beck et al., 2001; Heupel et al., 2007). These locations are thought to convey shelter from predation and or increased foraging opportunities to reduce mortality in the most vulnerable life stages (Heupel et al., 2007).

Habitat use by marine species is strongly influenced by ambient water temperature (Fry, 1947; Deutsch et al., 2020). Daily and seasonal shifts in temperature occur within nurseries (Simpfendorfer et al., 2005) and contribute to the patterns of shark occurrence on short-seasonal time scales (Heupel & Simpfendorfer, 2008; Yeiser et al., 2008; Heithaus et al., 2009). Individuals may respond to such shifts by compensating with metabolic adjustments or behavioral thermoregulation; making local or long-distance migrations or changing behavior in their habitat (Heupel & Hueter, 2001; Klimley et al., 2002; Speed et al., 2010). Adult bull and blacktip sharks use coastal and offshore locations and undergo latitudinal migration largely driven by seasonal temperature changes (Castro, 2010). As such, adults have greater abilities to behaviorally thermoregulate and remain within suitable thermal range. Nursery habitat by contrast may range from ~1-5 km^2 in the first year of life for these species. Temperatures within these coastal nursery habitats vary widely, and with limited distributional capabilities relative to adults, young sharks must find ways to cope with thermal change within the confines of their natal range, or else face habitat displacement, potential increased predation risk, and limitation of access to vital foraging habitat.

Studies of movement patterns for these species have suggested that habitat use and movement may "optimize energy allocation" and individuals may select habitats that favor a reduction in energetic demand (Heupel & Simpfendorfer, 2008). But for both species, we lack the understanding of what these energetic demands are and how exactly they change across environmental factors. Using whole organism energetic performance metrics such as maximum metabolic rate, MMR, (the maximum cost of aerobic performance), and minimum routine metabolic rate, mRMR, (the metabolic cost of basic maintenance at minimum swimming activity) we can calculate a functional aerobic scope. This scope represents the total energy available at a given temperature for an animal to perform all life activities beyond basic maintence. The absolute difference in aerobic scope has variably been used to indicate both non-lethal liming thermal thresholds and thermal “optimality”, but this interpretation has not been universally true. The factorial difference in aerobic scope however may provide information about the minimum energetic scope needed to support populations in the wild. In addition, oxygen supply capacity (α), the maximum ability of an organism to supply oxygen to tissues in support of metabolic demands, provide insight as to potential causes of thermal limitation of metabolic performance. This metric encompasses all physiological processes involved in the supply of oxygen from the environment to respiring tissues and is thought to have evolved to support an organism’s maximum demand required in a given environment (Seibel and Deutsch, 2020). A significant decline in α at high temperatures may indicate a failure of oxygen supply to support metabolic needs, and likely, that evolved thermal limits of a species range have been reached. Measurement of these metabolic performance metrics across temperature will allow us to quantify the temperature sensitivity of metabolic costs and identify if performance is limited within a thermal range.

In the Gulf of Mexico at large, coastal sea surface temperature has been increasing between 0.1 and 0.2˚C per decade (Lima & Wethey, 2012), with the potential for temperatures to rise at this latitude 1-3°C before end of century (IPCC, 2022). In addition, the frequency, intensity, and spatial coverage of “marine heat waves”, constituting abnormally high temperatures that last days (5 days min) to months (Hobday et al., 2016; Frölicher et al., 2018) are projected to rise within the Gulf of Mexico (Ranasinghe et al., 2021). Temperatures outside of the range of tolerance for animals threaten loss of viable habitat as conditions become energetically unsustainable and can lead to alterations in habitat and resource use (Frederiksen et al., 2008; Lea et al., 2009), population structure (Matich & Heithaus, 2012) and downstream effects on ecosystem interactions (Dee et al., 2020). To maximize habitat use in a highly variable nursery environment, early life stages for these bull and blacktip sharks may operate more like ‘thermal generalists’ (Gilchrist, 1995; Lear et al., 2019; Bouyoucos et al., 2022), that may benefit from having decreased metabolic thermal sensitivity until individuals have greater dispersal capabilities as they grow. Evaluating the thermal sensitivity of metabolic performance metrics in neonate and young-of-the-year (YOY) blacktip and bull sharks will allow us to better understand how energetic needs change within occupied thermal range, and the degree to which shark species within an ecosystem vary in their responses to environmental change.

In this study I tested several hypotheses for neonate/YOY blacktip and bull sharks. 1) Differences in metabolic performance (SMR, MMR, α), can largely be attributed to size differences between species; smaller blacktip sharks have higher mass-specific metabolic rates and α in support of those elevated demands at all comparable temperatures. However, when mass is corrected for, species at these same life stages demonstrate similar metabolic demands. 2) The thermal sensitivities of metabolic rates are comparable between the two species, as they occupy similar thermal habitat, generally 22-34˚C for blacktip sharks, and 22-37˚C for bull sharks. 3) The thermal sensitivity of metabolic performance for both species is comparable to thermal sensitivities of other nursery occupying shark species of the same stage, ($E<0.5$ eV) indicative of a thermal generalist. In addition, I predicted that, 4) critical and sublethal thermal limits lie outside the bounds of experimental range (22-34˚C), indicated by a lack of significant decline in maximum metabolic performance (MMR and a) at the warmest tested temperatures. 5) Similar thermal sensitivity of metabolic rates between species should lead to similar thermal sensitivity of aerobic scope. Absolute aerobic scope will increase continuously with temperature within the experimental range for both species. 6) AAS may indicate thermal optimality in these species with a peak likely around 30˚C. 7) FAS, as in many ectothermic species, will demonstrate an inverse relationship with temperature, but within the experimental thermal range, does not reach limiting thresholds for population sustenance for these species

Methods

Collection, acclimation, and captive husbandry: Neonate/YOY blacktip (53-63cm STL) and bull sharks (69-81cm STL) were collected in May-July from Terra Ceia Bay FL (2018), and the mouth of the Alafia River, FL (2019) respectively by species. Sharks were captured on rod and reel (blacktip sharks), and on bottom set longline (bull sharks) consisting of a 1 cm braided nylon mainline (183m), anchored on both ends with 25-30 evenly spaced monofilament gangions (1.83m), fitted with 11/0-12/0 Mustad offset tuna circle hooks and soaked for 40-45 min. All gear was baited with frozen mullet/threadfin herring.

Sharks were transported to Mote Marine Laboratory, Sarasota, FL where blacktips were acclimated to captive conditions in a 151,000-l recirculation-tank (6.096 m (w) x 12.192 m (l) x 1.83 m (h)) designated holding tank and maintained at 27.92±0.12˚C (Aquacal, SQ145) and 34.62±0.22 salinity for 8 days. Bull sharks were acclimated in 10667.98L (d=3.66m or 12') and 66,695.35L (d=9.14m or 30') designated holding tanks at 28±1°C (Aqulogic Delta Star Chiller, DS2; Aquacal, SQ145) and 17±1 salinity for 7 days prior to acclimation to trial temperatures. For both species, feeding resumed within 1-2 days of capture, and sharks were maintained on a diet of threadfin herring and mullet, fed *ad libitum*. Individuals exhibiting signs of illness or decline in captivity were excluded from experimental trials. 48 hours prior to trials, sharks were measured for length (precaudal length=PCL, fork length=FL, and stretch total length=STL), ID tagged (color T-bar tags), and fasted to eliminate the effect of post prandial metabolism (Chabot, McKenzie and Craig, 2016). Acclimation to experimental temperatures took place at a rate of no more than ±2°C per day until the desired temperature was reached at least 24 hours prior to testing, constituting "acute" exposure to trial temperature. Experiments were conducted at 3-4 temperatures within the known thermal range for neonate/YOY bull sharks (22, 26, and 30˚C), and blacktips *(*22, 26, 30 and 34˚C). Sharks were maintained at a natural photoperiod through captivity and testing. At the conclusion of the project sharks in good condition were released at the location of capture.

Ethics statement and permissions: Animals were collected under SAL-19-2143-SRP and captive animal husbandry was conducted under veterinary guidelines and procedures approved by the Institutional Animal Care and Use Committee at the University of South Florida (protocol IS00004975). Health assessments were conducted daily on captive animals. Individuals that displayed signs of illness or damage, upon arrival or over the duration of captivity, were isolated and treated according to IACUC protocols. If condition persisted or worsened after isolation and treatment, the individual was humanely euthanized using MS-222 in excess of anesthetic concentrations (≥400mg/L; 25g in 60L seawater = 0.417g/L), in accordance with AVMA Guidelines for the Euthanasia of Animals (2013 edition).

Experimental set up: Research was conducted in a closed life support system that sourced water from Sarasota Bay, filtered via biological and mechanical (sand) filtration, adjusted for pH, salinity and temperature, and maintained ±1˚C of designated temperature (Aquacal, SQ145). Experiments took place within two custom fiberglass annular respirometers plumbed for intermittent flushing (Figure 8) and submerged within their own temperature baths (d=2.44m). Baths were continually circulated with aerated system water, to maintain experimental temperature.

Each ring-shaped respirometer (Figure 7) was composed of two inner compartments: 1) a working section, where sharks were allowed to free swim during trials, (38.1 cm (w) x 40.64 cm (h), V= 704.27 l) , and, 2) a mixing section (7.62 cm (w) x 40.64 cm (h), V= 96.37 l) where new system water and recirculated water entered the respirometer. Compartments were separated by a coated fiberglass "baffle" column (1 cm (w) x 40.64 cm (h)) bearing 1000 evenly spaced holes (d=0.7 cm) to control and evenly disperse water from the mixing section into the working section. A 9.5 mm thick custom clear acrylic lid sealed the respirometers and allowed for behavioral observation of shark subjects during trials. A 1/2 hp pump (Hayward Tristar, 113.562-132.489 l/min) was used in conjunction with ball valve to control water flow and intermittently flush oxygenated system water into the experimental chambers during trials, and to expel metabolic by-products (e.g., CO_2, urea, etc.) via the large one-way valve plumbed into each chamber. These respirometers were also plumbed for closed recirculation of water within the respirometry chamber (Iwaki Mag Drive pump) that allowed for continuous uniform mixing and water flow across the oxygen measurement probe in the recirculation loop during periods of closed measurement. Dissolved oxygen and temperature within the chamber were sampled continuously at a rate of 1 Hz via an oxygen dipping probe optical mini sensor (PreSens PSt3) and a temperature dipping probe (Loligo Systems Pt1000) connected to a multichannel oxygen meter (Witrox-4 Instrument, Loligo Systems). Oxygen and temperature were recorded via Autoresp Software (Loligo systems). This software calculated mass specific oxygen consumption (MO_2; Equation 1) for each individual measure period in a trial in real time.

Experimental protocol: *Experimental Temperature:* Metabolic metrics (Table 4) were evaluated at 3-4 experimental target temperatures (22, 26, and 30 for bull sharks, 22, 26, 30 and 34°C for blacktips) within the natural temperature range of species. Trials were "binned" by target temperatures, referred to hereafter as "temperature bins".

Exercise trials: Immediately prior to trials, sharks were weighed and chased in a separate "condition" tank (4270 L, d=2.44m; Figure 1) to induce exhaustive exercise and elicit maximum metabolic rate (MMR) via tactile stimulation (Kieffer, 2000). Chase was concluded when sharks were deemed "exhausted", i.e., reaction to handling and brief air exposure (~5s) was minimized. Care was taken not to "over-chase" individuals. In preliminary testing, "over-chase" lead to a state of extreme exhaustion/stress where the animal no longer responded to handling or air exposure and demonstrated an inability to recover if left within the experimental chamber, (i.e. immediate buccal pumping and lethargy, extreme "patchiness" of color, rapid decline in rate of buccal pumping (buccal pumps/min), and failure to complete the trial resulting in death if not removed). Sharks were then placed within the respirometer and the chamber was immediately sealed. A three-phase continuous intermediate respirometry cycle began that consisted of a 15 min closed measure period, where the rate of dissolved oxygen (DO) decline was measured, a 5 min flush period, and a 2 min wait period where the chamber DO was allowed to equilibrate before the next closed measure period.

Exercise trials were concluded after 24 hours, or at any point that individual sharks demonstrated all behavioral indicators of "decline": 1) patchiness in coloration, 2) lethargy, sometimes coupled with inability to maintain equilibrium, and loss of ability to locomote, 3) reliance upon buccal pumping as sole ventilation method (continuous resting), 4) decline of buccal pumping below 27 pumps/min (for bull sharks), 5) extended gill flaring and/or "coughing" (Roberts, 1978), 6) rapid and continuous decline of mass specific oxygen consumption (MO_2) below lowest routine levels observed in trial. Individuals that demonstrated indicators of decline were removed from the chamber and allowed to recover overnight in the system condition tank. Individuals that did not demonstrate such indicators of decline during exercise trials were allowed to remain in the chamber for further testing in critical oxygen trials.

Hypoxia trials: 24 hours after the start of aerobic scope trials, sharks underwent critical oxygen testing. Sharks that had been removed from exercise trials and allowed to recover in the system condition tank, were placed back in their designated experimental chamber, taking care to minimize handling time and stress to keep metabolism from spiking. Sharks were allowed a 2-hour acclimation period at normoxia, during which intermittent respirometry cycles remained the same as exercise trials. Once the acclimation period was completed, a new measurement cycle began consisting of 4-8 (temperature dependent) consecutive 15-minute closed measure periods, followed by a 90-100 second flush with water sourced from external hypoxia reservoirs at a lower DO. For hypoxia trials, a "flush pump" used to supply chambers with new water was connected to four external water reservoirs (Figure 8) where N_2 gas was bubbled via four microbubble ceramic plate oxygen diffusers (Point Four^{TM}) and displaced DO supplied to respirometers during flushes. This allowed for manual control of experimental PO_{2w}. Flushes decreased the DO within each chamber by approximately 5-10% air saturation, followed by a 12 min wait for DO equilibration before the start of the next closed measure. Trials were concluded once individuals demonstrated the aforementioned indicators of decline. Sharks were removed from the chamber upon conclusion of the trials and placed within the condition tank for 5-15 min observation to assess recovery (strengthening locomotive abilities and resumed normal ventilation). Those that were demonstrating signs of strong recovery were left to in the condition tank overnight before being transferred back to holding. Those that were struggling to recover within the condition tank were immediately transferred back to the larger holding tanks for improved forward ram ventilation leading to successful recovery. Sharks were allowed to continue recovery in holding for a minimum of 6 days. Once this period expired and sharks resumed feeding for at least 5 days, sharks could be prepared for fasting and experimentation at another temperature.

Behavioral observations: Behavioral observations were conducted throughout exercise and hypoxia trials. For each measure shark behavior was broadly categorized by comments noting whether the shark was swimming continuously, exhibiting a mix of swimming and resting behavior throughout the measure period, or were continuously resting, lasting the duration of the measure.

For sharks that exhibited intermittent or continuous resting behavior during trials, active ventilation was quantified to monitor decline by sampling the number of buccal pumps the animal demonstrated in a one-minute period (Barreto & Volpato, 2004). Ventilation was sampled three times per applicable measure period and averaged.

Background microbial respiration: To eliminate the effect of background microbial respiration, once sharks were removed from trials, the respirometers were re-sealed (empty), and oxygen was measured continuously over a 4-hour closed chamber timeframe. The rate of oxygen decline within each chamber was used to determine background MO_2 (mgO_2hr^{-1}) for each closed measure period within the 4-hour background trial. Microbial MO_2 was averaged across all measure periods of the background trial and was subtracted from the individual shark's MO_2 measures (Equation 1).

Calculation of metabolic rate: A mass-specific metabolic rate of oxygen consumption, MO_2 (mg O_2 $kg^{-1}hr^{-1}$), was calculated for each respirometry measure period and corrected for background respiration using Equation 1.

$$Eq\ 1.\ MO_2 = \frac{(S * V) - br}{M}$$

S is the slope of the linear regression of MO_2 vs time, or the change in oxygen concentration within the chamber over the time frame of the measure period. *V* is the total volume of the annular respirometry chamber and associated recirculation tubing (~800.36 l), *br* is the background microbial O_2 consumption rate (mgO_2hr^{-1}), and *M* is the mass of the individual shark (kg).

Data validation: Failed trials, where the animal demonstrated strong decline and removal close to the start of the trial, (<6 usable measures within the trial), were omitted from analysis. A multi-step data validation processes was then used to verify MO_2 values from remaining trials. Measure periods with linear regression fits, $R^2 < 0.7$ were excluded from datasets, in addition to negative (-) MO_2 values. Any measure periods containing a >0.3°C ± shift in temperature (~0.31-1.25 ℃) were also eliminated (periods where the heater/chiller turned on to maintain system water within ±1˚C) as they were not comparable to the majority of other MO_2 measures within trials (≤0.3°C), and such thermal drift caused oxygen probe drift. After calculation of metabolic metrics (see below), metrics across all temperatures underwent visual outlier analysis. Outlier measures underwent analysis of the raw DO trace to identify any remaining systemic errors present (e.g., a plateau in DO at the beginning of a measure period, accounting for more than 30% of the measure, indicating a delayed closing of the flush valves). If systemic error was present, the measure period was eliminated, but those that did not show clear systemic error were included with analysis.

Calculating metabolic metrics: A summary list of metabolic performance metrics measured, acronyms, and source trials from which each metric was derived, may be used as a reference and are found in Table 4.

MMR, the maximum metabolic rate achieved in trials, was defined as the highest MO_2 value observed for an individual across both exercise and hypoxia trials. mRMR is defined as the minimum routine metabolic rate, adapted from (Crear et al., 2019) and refers to the lowest metabolic rate of animals continuously swimming under normoxic conditions. For the calculation of lowest metabolic rates, the first 3 hours of measure periods within exercise trials were omitted from analysis for blacktip sharks (Chabot et al., 2016), and a minimum routine metabolic rate (mRMR) (Crear et al., 2019) was calculated as the lowest 10% of MO_2 measures using the R package *Mclust* (Fraley & Raftery, 2002; Fraley et al., 2014).

Due to the shorter nature of the bull shark exercise trials (Table 5) we did not remove the first 3 hours of the trials, but instead used the lowest 10th quantile (*Mclust;* Fraley &Raftery, 2002; Fraley *et al.*, 2014) of continuous swimming measures at ambient oxygen partial pressure, PO2, >15 kPa to represent mRMR within a trial. For each individual bull shark, the lowest mRMR across trial types represented mRMR for the given individual shark. Individuals who only swam during the measure period designated as MMR were eliminated from mRMR analysis. Standard, or resting metabolic rate (SMR) was determined from opportunistically sampled measures of bull sharks at rest. The lowest 10th quantile of continuously resting measures at PO_{2W} >15 kPa was calculated within an individual trial and the lowest SMR across trial types represented SMR for a given individual.

Physiological oxygen supply capacity calculations: For each respirometry measure period an "instantaneous" measure of physiological oxygen supply (α_0) was calculated using Equation 2a. Oxygen supply capacity (α) was designated as the highest value of instantaneous oxygen supply (α_0) observed for an individual across trial types.

$$\text{Eq 2a. } \alpha_0 = \left(\frac{MO_2}{PO_{2W}}\right)$$

$$\text{Eq 2b. } \alpha = \left(\frac{MMR}{P_{cMAX}}\right) = \left(\frac{SMR}{P_{cSMR}}\right) = \left(\frac{mRMR}{P_{cmRMR}}\right) = highest\ \alpha_0$$

Where α_0 is the instantaneous oxygen supply of a given measure, MO_2 and PO_{2W} are the metabolic rate and ambient partial pressure of oxygen in the chamber for a given measure respectively. α represents oxygen supply capacity and P_{cMAX}, P_{cSMR}, P_{cmRMR} are the critical oxygen thresholds for maximum metabolic rate, standard (or resting) metabolic rate, and minimum routine metabolic rate respectively, representing the point at which the given metabolic rate can no longer be maintained independent of PO_2 and below which the given metabolic rate cannot be achieved.

Predicting MMR at air saturation: The average experimental PO_2 achieved in exercise studies was 19.27±0.35 - 19.92±0.1 kPa for blacktips and 19.24±0.15 - 20.09±0.17 kPa for bull sharks. To predict MMR at 21 kPa (full air saturation), the likely evolved upper critical oxygen threshold for MMR (a.k.a. $P_{cMAX,}$) I used Equation 2b according to (Seibel and Deutsch, 2020) using a P_{cMAX} of 21 kPa and measured α.

Calculating aerobic scope: In this study, aerobic scope is defined as the "functional aerobic scope", the absolute or factorial difference between maximum aerobic demand and minimum routine swimming metabolic rate (absolute: MMR-mRMR; factorial: MMR/mRMR), as these species are obligate ram ventilators. Functional aerobic scope was calculated for both blacktip and bull sharks for each individual that demonstrated MMR and mRMR. Traditional aerobic scope (MMR-SMR or MMR/SMR) could only be calculated for a subset of bull sharks individuals that exhibited continuous resting behavior for comparison.

Pooled aerobic scope was also calculated from mean MMR values and/or mean mRMR/SMR values for comparison. This metric allowed for potentially greater population estimation, (e.g., measures of

MMR to be included for any individuals that may have had a MMR measurement, but not an mRMR or SMR measure, if they quit a trial early, etc.). Comparisons of metrics across species were completed using Welch's t-test with Bonferroni correction of p-values across temperature.

Thermal scaling of metabolic metrics: Temperature coefficients (*E*, measured in eV) were derived from Arrhenius relationships of metabolic metrics, modeled using linear mixed effects regression (lmerTest Package, Kuznetsova et al., 2017). The Arrhenius relationship was modified and modeled by the equation, log(metabolic metric) ~ $1/k_BT$ + (1 | ID), where the natural log of each metabolic metric was regressed against $1/k_BT$, k_B is Boltzmann's constant, (a physical constant relating particle kinetic energy to temperature), T is the absolute temperature of the individual trial in kelvin (K), with individual ID incorporated as a random factor to account for individual variation and any lack of temporal independence between temperature samples. Temperature coefficients were derived from the negative slope of the regressions (negative slope of the fixed factor, $1/k_BT$). Significance of the thermal relationship of each metric was determined through the Kenward-Roger approximation of each mixed model at significance threshold of $\alpha=0.05$. Temperature coefficients were also calculated for blacktips across the 22-30˚C range for comparison with bull shark coefficients in the same range.

Elimination of mass effects on metabolism between species: Bull sharks in this study were larger on average than blacktips. In order to identify if differences in metabolic rates for blacktips were due to size differentials alone, we used Kleiber's Law and assumed a quarter power metabolic scaling coefficient (Hochachka & Somero, 2002; Banavar et al., 2010) to normalize average bull shark mRMR and MMR to the average mass of blacktips at each temperature. Comparisons of normalized metabolic metrics across species were completed using Welch's t-test with Bonferroni correction of p-values across temperature.

Results

Metabolic rates were measured for neonate/-young-of-the-year blacktip and bull sharks across 3-4 temperatures within the known thermal range of these species in the Gulf of Mexico (Table 5, 6). A total of 14 blacktips and 16 bull sharks successfully completed respirometry trials. Individual sharks underwent experimentation for exercise and hypoxia trials, no more than once at each temperature, with a maximum of 3 temperatures tested for an individual, (blacktips: n=2 individuals tested at one temperature, n=8 individuals tested at two temperatures, n=3 individuals tested at 3 temperatures; bull sharks: n=9 individuals tested at one temperature, n=6 individuals tested at 2 temperatures, n=0 individuals tested at 3 temperatures). Experimental temperature, salinity, sample sizes, and trial durations for each species across temperature are reported (Table 5). Results are reported as mean ±SE unless otherwise specified.

Metabolic measures of bull and blacktip sharks across temperature: Bull sharks as a whole demonstrated all behavioral categories of activity (continuous swimming, continuous resting, and mixed behavior) in exercise and/or hypoxia trials. Individual variation in behavioral expression/metabolic rate was high for bull sharks, and significantly impacted all metrics except aerobic scope and SMR (alpha >0.05; Table 7). For blacktips, individual variation was significant across all metrics except α, and absolute aerobic scope (alpha >0.05) (Table 7). Individual variation for both species was accounted for within linear mixed models and used to determine thermal sensitivity of metabolic metrics.

The mRMR measured for blacktip sharks increased with temperature from 295.89±15.81 to 437.78±14.96 $mgO_2kg^{-1}hr^{-1}$between 22-34°C (Table 6; Figure 8), with a significant temperature sensitivity of E=0.34eV (alpha <0.05; Table 7). mRMR for blacktips increased faster with temperature between 22-30°C (Figure 9) exhibiting significant temperature sensitivity of E=0.45eV (alpha <0.05; Table 7). Within this same range, mRMR for bull sharks increased from 279.77±16.48 $mgO_2kg^{-1}hr^{-1}$ to 348.16±21.73 $mgO_2kg^{-1}hr^{-1}$ (Figure 8) with a slightly lower slope of E=0.22eV (alpha <0.05; Table 7). The mRMR for bull sharks was within the SE of the mean for blacktips at 22 and 26˚C but was below the estimate for blacktips at 30˚C (Table 8).

Bull sharks that were measured at rest across trials demonstrated SMR between 149.61±25.18 $mgO_2kg^{-1}hr^{-1}$ and 185.68±32.64 $mgO_2kg^{-1}hr^{-1}$ at 26 and 30°C respectively (Figure 8) but showed no significant thermal sensitivity across temperature for this metric (E=0.1eV; alpha >0.05; Table 7). SMR was significantly less than mRMR at all temperatures for bull sharks (alpha <0.05; Table 8), on average ~56.85% of individual mRMR across temperature. SMR estimates were not obtained for blacktips as resting for this species led to rapid decline of MO_2, animal condition (see methods), and an inability to recover if sharks remained resting over a full measure period. Consequently, blacktips were removed from trials within roughly 3 minutes of the onset of resting behavior if they could no longer voluntarily locomote.

Oxygen supply capacity, α, (*E*=0.01 eV) and MMR (*E*=0.05 eV) did not change significantly across temperature for blacktip sharks between 22-34°C or between 22-30°C (alpha >0.05, Table 7; Figures 8.9). However, for bull sharks, both α and MMR significantly increased with temperature (alpha <0.05, Table 7). MMR for bull sharks increased from 396.3±21.02 $mgO_2kg^{-1}hr^{-1}$ at 22°C to 541.22±31.41 $mgO_2kg^{-1}hr^{-1}$ at 30°C (Figure 8) with a temperature sensitivity of *E*=0.31 eV (Figure 9). For bull sharks, α increased in support of elevated metabolic demands across temperature from 20.37±1.28 $mgO_2kg^{-1}hr^{-1}kPa^{-1}$ at 22°C to 28.16±1.67 $mgO_2kg^{-1}hr^{-1}kPa^{-1}$ at 30°C (Figure 8) with a temperature sensitivity similar to MMR (*E*=0.336 eV). MMR and α were higher for blacktip sharks than for bull sharks at all shared temperatures of measurement (Table 6). In addition, the α measured in exercise trials was not significantly different from that measured in hypoxia trials for either species under continuous locomotion. MMR values were achieved during exercise trials for most individuals across temperature (85.71% for bull sharks, and 93% for blacktips) and for the remaining individuals, MMR was achieved during the start of hypoxia trials.

Mass corrected values of mRMR and MMR for bull sharks (to the average mass of blacktips in the study), were within the SE of the mean of blacktip measurements at 26 and 30°C, and within 24.72 $mgO_2kg^{-1}hr^{-1}$ of SE of the mean mRMR for blacktips at 22°C (Figure 10). Mass normalized bull shark MMR at 22°C was ~88.03 $mgO_2kg^{-1}hr^{-1}$ below SE of the mean for blacktip MMR.

Aerobic scope across temperature: Blacktip sharks and bull sharks demonstrated opposite trends in absolute aerobic scope (AAS) (Figure 11). Blacktip shark AAS showed a non-significant decrease (*E*= -0.47 eV) in in the range of 22-30°C (alpha >0.05, Table 7) but a significant decline in AAS between 22-34°C (*E*= -0.3 eV; alpha <0.05, Table 7). Bull sharks demonstrated a non-significant increase in AAS between 22-30°C, (*E*=0.49 eV) increasing from 141.2±34.1 $mgO_2kg^{-1}hr^{-1}$ to 227.48±49.73 $mgO_2kg^{-1}hr^{-1}$ between 22 and 26°C but no change between 26 and 30°C (226.76±35.76 $mgO_2kg^{-1}hr^{-1}$) (Figure 11). Pooled AAS (calculated as the difference between mean MMR and mean mRMR at each temperature) demonstrated similar values for blacktips but slightly lower estimates for bull sharks. AAS for blacktips was significantly higher than for bull sharks at all temperatures except 30°C (alpha <0.05, Table 8).

Factorial aerobic scope (FAS) declined across all temperature ranges for blacktips, decreasing significantly from 2.27±0.31 $mgO_2kg^{-1}hr^{-1}$ at 22°C, to 1.51±0.09 and 1.53±0.05 $mgO_2kg^{-1}hr^{-1}$at 30 and 34C respectively. Temperature sensitivities for FAS in blacktip sharks were E= -0.38 eV, within the 22-30°C range and E= -0.27 eV in the 22-34°C range(Table 7). Pooled FAS for blacktips demonstrated similar values and thermal trends. Pooled FAS demonstrated no appreciable change across temperatures for bull sharks. FAS range across temperature was larger for blacktip sharks than for bull sharks and FAS for blacktip sharks was significantly higher than that of bull sharks only at 22°C (alpha <0.05, Table 8).

Discussion

Minimum routine metabolic demands of bull sharks and blacktip sharks: In this study, the significant increase in mRMR with temperature in neonate/YOY bull and blacktip sharks indicated that the oxygen needed to support lowest routine swimming and maintenance metabolism increased with temperature as predicted in these species. For bull sharks, measured mRMR in this study was higher than estimates of metabolic rate made in previous studies (51.6-73.52 $mgO_2kg^{-1}hr^{-1}$; Schmid & Murru, 1994, conversion via Lucas & Watson, 2002) using caloric conversion and bioenergetics modeling, rather than direct respirometry, for bull sharks of the same size range (~65cm TL). Routine field metabolic rates for juvenile bulls in the Fitzroy River, Western Australia, (RMR: 271 mg O_2 $kg^{-0.86}$ h^{-1}at 25°C; Lear et al., 2020) were within the range mRMR measured in this study, and fell closest to my measures of mRMR at 22˚C (279.77±16.48 $mgO_2kg^{-1}hr^{-1}$) but below measures at 26°C in this study (309.38±13.31 $mgO_2kg^{-1}hr^{-1}$). Measures from Lear et al (2020) however, demonstrated greater thermal sensitivity across a larger temperature range than the bull sharks within this study (187–506 mg O_2 $kg^{-0.86}$ h^{-1}; $E_A = 0.48$ eV; 3.0–7.0 kg ;18-34˚C).

Previous measures of RMR for blacktips of the same age form the same capture location (362±39 $mgO_2kg^{-1}hr^{-1}$; range = 282-448 $mgO_2kg^{-1}hr^{-1}$; 29.4˚C; Lear et al., 2017) were comparable to our measures of mRMR at similar temperatures, lending validity to my measurements of metabolic demands within the same population of blacktip sharks across sample years.

Maximum metabolic performance, species-specific thermal sensitivity, and implications for species ecology: This study presents the first direct measures of maximum metabolic rate and oxygen supply capacity for either species. Measured metabolic rates for both blacktips and bull sharks are comparable to measures of other ram-ventilating shark species (Lowe, 2001; Dowd, 2003; Carlson et al., 2004; Sepulveda et al., 2007; Bernal et al., 2012; Lear et al., 2017), with MMR measurements in this study among the highest measured for shark species to date (Graham et al., 1990; Sepulveda et al., 2007; Gleiss et al., 2010). However, the PO_2 during our exercise trials was slightly below air saturation (Table 6) and measured alpha values suggest a higher MMR is possible at air saturation (21 kPa) for both species. Additionally, though blacktip sharks demonstrated higher mass specific maximum metabolic rates than bull sharks, as expected due to their smaller size, once mass was corrected for in bull sharks I found that discrepancies in metabolic demands between these species can be largely attributed to the difference in mass between 26-30°C. However, the lower-than-expected mass-corrected bull shark MMR at 22˚C was either due to underestimation of MMR, or more likely due to differing species ecology and temperature sensitivity, particularly at cold temperature.

The lack of significant thermal sensitivity of MMR and α in blacktip sharks suggests that similar performance may be required across occupied habitats, regardless of temperature. Maximum performance is critical during predator evasion and prey capture, two incredibly important skills especially for neonate/YOY sharks. In these stages, animals have more potential predators owing to their small size, and in the first few weeks of life must rapidly obtain food resources and master prey acquisition in order to survive (Heupel & Simpfendorfer, 2011). Blacktips are generally smaller on average (size at birth: 55-60cm; (Castro, 2010) than bull sharks of the same age (size at birth: 60-80cm; (Snelson et al., 1984) and smaller animals have a higher predation risk. Lower thermal sensitivity of MMR may allow blacktip individuals maximum escape capacity regardless of thermal habitat. For blacktip sharks that occupy more open nursery habitats in moderate salinities of 20-36 (Bethea et al., 2006; Bethea et al., 2009; Froeschke et al., 2010), in areas in which larger predatory species have also been found, this could be an advantageous trait.

While neonate/YOY blacktip sharks generally prefer temperatures above 20˚C, these data may suggest that blacktips have likely evolved to maintain maximum performance across their thermal envelope. More data are needed, however, to understand how ecology changes with temperature or how metabolic demands change for this species under other interacting abiotic factors (e.g., salinity). In addition, in this study I have measured the cost of maximum metabolic performance across temperature, but it is possible that the lack of change in MMR across temperature for blacktips may not be a maintenance of the same swim speed and performance across thermal range, but that same metabolic rate may result from different swimming speeds at different temperatures in blacktip sharks. The design of this study precluded measurement of MMR at the precise swim speeds that elicited such rate, (MMR obtained after exhaustive chase) so I was not able to measure the true cost of transport for these species across the spectrum of blacktip shark activity. However, additional analyses of the interactions of swim speed, oxygen supply, and temperature within this study are being undertaken, and may help to inform the reason for the maintenance of MMR across temperature for blacktip sharks.

Bull sharks demonstrated higher thermal sensitivity of MMR, α, and aerobic scope than blacktips across experimental range (Table 7), with decreased oxygen supply and maximum performance at 22˚C relative to higher temperatures. Bull sharks avoid water temperatures of 18-21°C and below (Simpfendorfer et al., 2005; Blackburn et al., 2007; Wiley & Simpfendorfer, 2007; Froeschke et al., 2010) and have been reported in cold stunning and thermal fish kill events if young bull sharks lacked the capability to vacate affected nursery habitat as temperatures dropped (Snelson Jr & Bradley Jr, 1978; Curtis et al., 2011; Matich & Heithaus, 2012). However, the combination of larger size and greater osmoregulatory abilities relative to blacktip sharks, may allow bull sharks to compensate for their higher degree of cold sensitivity by affording greater capacity for behavioral thermoregulation. Bull sharks are euryhaline (Anderson et al., 2005; Pillans et al., 2005) generally preferring salinities from 7-17 (Heupel and Simpfendorfer, 2008). Bull shark movement within estuaries along salinity gradients (Heupel & Simpfendorfer, 2008) is thought to drive a ~50% reduction in mortality relative to blacktips in the same early life stages (Heupel & Simpfendorfer, 2011). Use of mesohaline habitats largely excludes interspecific predation and competition, as low salinities are beyond the tolerance of larger elasmobranchs and teleosts of different species (Heupel & Simpfendorfer, 2008, 2011). High osmoregulatory capacity and larger sizes may explain why bull sharks have not evolved, or have perhaps lost, the need to maintain maximum metabolic rate as blacktips do. More data are needed to further explore this potential association.

Discussion of bull shark and blacktip shark thermal limits and low relative thermal sensitivity: Critical or sublethal thermal limits were not reached for either species within the range of experimental temperatures. Oxygen supply capacity evolves to meet maximum aerobic demand within an organism's evolved thermal range (Seibel & Deutsch, 2020). Thus, the lack of significant decline in oxygen supply capacity and maximum performance at high temperatures (Figures 9, 10; Table 7) indicates that upper thermal limits of oxygen supply were not reached at 30°C for bull sharks or 34°C for blacktips. However, 34°C may be in close proximity to an upper thermal limit for blacktip sharks, as previous studies have identified 33-34°C as the upper occupied temperature for young blacktip sharks (occupied range: 20-34°C; (Bethea et al., 2009; Morgan & Carlson, 2010; Lear et al., 2019). Low thermal sensitivity of MMR close to maximum environmental temperatures has been identified in other nursery dwelling species (Bouyoucos et al., 2022) and suggests that minimum thermal sensitivity (E closer to 0) around upper thermal limits may allow sharks to better tolerate higher temperatures and remain within nursery habitat (Bouyoucos et al., 2020). For bull sharks, previous research indicates an upper occupied temperature of ~37-38°C, a limit exceeding 30°C, in agreement with our results (occupied range: 20-37°C; Simpfendorfer et al., 2005; Bethea et al., 2006; Parsons & Hoffmayer, 2007; Heupel & Simpfendorfer, 2008; Bethea et al., 2009; Ortega et al., 2009; Curtis et al., 2011; Matich & Heithaus, 2012; Lear et al., 2019). Further metabolic testing at >34°C is required to say what may limit upper thermal tolerance for both species and where physiological tolerances to temperature actually lie.

Neonate/YOY of these species have decreased capacity for behavioral thermoregulation compared to their adult counterparts and maintain tight home ranges generally within 1-5 km^2 range in the first several months of life. Though neonate/YOY blacktip sharks and bull sharks show varying degrees of thermoregulation across various spatiotemporal scales (Simpfendorfer et al., 2005; Hueter & Tyminski, 2007; Heupel & Simpfendorfer, 2008; Ortega et al., 2009; Curtis et al., 2011), and long-range, permanent emigration to other nurseries is possible under extreme thermal events (Wiley & Simpfendorfer, 2007), there is a strong benefit for these stages to maintain performance across a wider breath of temperatures relative to adult stages and remain within nursery habitat (Lear et al., 2019). Studies have demonstrated declining thermal performance breadth and increased thermal sensitivity in overall dynamic body acceleration (ODBA), a proxy for activity, as sharks age for both species (Lear et al., 2019). Thermal sensitivities of ODBA for young bulls and blacktips (juvenile bull sharks, 0.32 ± 0.01eV, 19-35°C; juvenile blacktip sharks, 0.13 ± 0.51eV, 28-32°C) were similar to temperature sensitivities of mRMR found in this study (Table 7), but were significantly less than adult thermal sensitivity of ODBA (adult bull sharks: 23-31°C, 0.69 ± 0.10eV; adult blacktip sharks: 18-31°C, 0.86 ± 0.09 eV; Lear et al., 2019). Increased thermal sensitivity in adult stages supports the notion that by necessity, nursery bound young of these species need to be more robust to thermal change than their highly mobile adult counterparts. In addition, it has been proposed that maintaining lower activation energies (thermal sensitivity) may be advantageous for faster start velocities (Dell et al., 2011) key for predator evasion in these vulnerable life stages. It is likely that this thermal trend across ontogeny for ODBA may be reflected in metabolism (Gillooly et al., 2001) especially as adults will have greater incentive to mitigate the effects of temperature on metabolic demands and greater capacity to select thermal habitat.

Aerobic scope of bull and blacktip sharks: Aerobic scope did not demonstrate a peak within the experimental thermal range and did not indicate a preferred temperature in neonate/YOY of either species. Both FAS and AAS demonstrated opposite thermal trends for blacktip and bull sharks (Table 7; Figure 11). The direction of the trend in aerobic scope across temperature relates to which metabolic metric is driving the changes in AAS and FAS. This is dictated by the thermal sensitivities of MMR and mRMR (Table 7). We found that changes in FAS and AAS in blacktips are driven by changes in mRMR across temperature, whereas bull shark aerobic scope was driven by both MMR and RMR, with a greater thermal sensitivity of

MMR for this species. The greater thermal sensitivity of MMR, relative to RMR, means that aerobic scope increases with temperature.

In bull sharks, we found that SMR was on average ~57% (from pooled estimates: ~53% range of 48-59%) of mRMR, which indicates that the energy needed for sustained locomotion and minimum ram ventilation (Roberts, 1978) takes up a large portion of "total" FAS (MMR/SMR) leaving only ~57% of total FAS to be used for all other life activities for this species. For ram ventilators, mRMR is encompassing of both baseline metabolic demands, and the locomotion necessary for ventilation in support of those demands (Andres Chapter 3). Thus, functional aerobic scope provides a more accurate measure of the amount of energy a ram-ventilating organism has to spend on activities beyond basic maintenance (foraging, growth, reproduction). This demonstrates how total FAS for these species may belie true energetic budgets for ram ventilators. While thermal optima cannot be reliably drawn from measures of aerobic scope (Clark et al., 2013; Gräns et al., 2014; Jutfelt et al., 2018), previous studies suggest a minimum factorial aerobic scope is needed to support wild populations (Deutsch et al., 2020). Critical FAS for both species likely lies below ~1.4 (Table 6) given that 30 and 34°C were not limiting for bull sharks and blacktip sharks respectively. It is difficult however to compare functional aerobic scope for these species to traditional population limiting thresholds of FAS= ~2-5 (mean=3.33) (Deutsch et al., 2015; Deutsch et al., 2020), as these thresholds were derived from species that do not require locomotion to supply oxygen to tissues. More metabolic data in conjunction with historical species distributional data are needed to say for certain where FAS population limits truly lie.

Conclusions

In this study, I ascertained the first estimates of maximum metabolic performance for bull sharks and blacktip sharks, among the highest measures of MMR for shark species to date. Though blacktip sharks demonstrate higher mass-specific metabolic demands than bull sharks, I found these differences to be largely mass-related. There were, however, differences in thermal sensitivity of aerobic performance between the species, likely as a result of divergent species ecology within nursery habitats. Blacktip sharks demonstrated thermal sensitivity of routine metabolic demands (mRMR), however, maximum performance for neonate/YOY was insensitive to temperature change from 22 to 34˚C. For blacktip sharks, this indicated that there is likely an evolved need to maintain performance and/or metabolic costs of operation across a wide breadth of temperatures, potentially related to their inability to escape interspecific predation and competition to the same degree as bull sharks. Bull sharks by contrast demonstrate significant thermal sensitivity of all metabolic demands, particularly in cold water (22˚C). Bull shark osmoregulatory capabilities and use of low saline habitats to largely eliminate interspecific predation and competition, may preclude the need to maintain maximum performance across a larger thermal range and may allow for greater behavioral thermoregulation within nursery habitat. Generally, thermal sensitivities of SMR are comparable to other nursery using shark species of the same life stage, which experience variable thermal habitat and are generally indicative of animals that implore a "thermal generalist" strategy. In this study, AAS did not indicate an optimal temperature within the range tested, and aerobic scope had opposing thermal trends for both species, due to different temperature sensitivities of each metric between species. Thermal limits for both blacktip sharks and bull sharks were found to lie outside of the tested temperature range (22-34˚C or 22-30˚C respectively), noted by a lack of decline in maximum metabolic rate and oxygen supply capacity. In addition, limiting thresholds of FAS for population energetic needs likely lie below 1.4 FAS for blacktip and bull sharks.

Identifying population limiting thresholds for FAS in these species requires further comparison of temperatures outside the range of this study, the incorporation of historical species distribution and movement data, and integration of the effects of other abiotic factors such as oxygen or salinity. Future physiological studies should examine the dynamics of metabolic performance at temperatures 30-38˚C and below 22˚C, to identify sublethal limiting thermal thresholds for these species. Measurement across a greater number of ontogenetic stages would also allow for confirmation of increased metabolic sensitivity with age in these species. Closer examination of the potential connection between low salinity uses and enhanced behavioral thermoregulatory capabilities in bull sharks is also warranted. In addition, thermal acclimation studies at either ends of the thermal envelope would better inform the limits of phenotypic plasticity in thermal tolerance for neonate/YOY bull sharks and blacktips, across different nursery locations as climate change progresses.

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Table 4. *Metabolic trait definitions, methods and associated equations for bull and blacktip sharks*

Metric	*Name/definition of Metric*	*Measured and/or Modeled*	*Defined By:*	*Source Trial*
SMR	Standard metabolic rate; lowest metabolic rate supporting basal metabolism of conscious shark at rest	Measured, Modeled	*Lowest 10th quantile of* MO_2 during measures of continuous rest (>15 kPa)	Both
mRMR	Minimum routine metabolic rate, lowest metabolic rate of animals continuously swimming under normoxic conditions.	Measured, Modeled	*Lowest 10% of* MO_2 during exercise trials for blacktips (>15 kPa) *Lowest 10th quantile of* MO_2 during measures of continuous swimming for bull sharks (>15 kPa)	Both
MMR (measured)	Highest metabolic rate achieved in trials	Measured	Highest MO_2 value in trial after data validation	Exercise (but sometimes Hypoxia)
MMR *(at 21kPa)*	Predicted maximum metabolic rate at air saturation (MMR = α* PcMAX of 21kPa)	Predicted	α, P_{cMAX} =21kPa	Both
α_0	Instantaneous physiological oxygen supply for a given measure	Measured	$\alpha_0 = MO_2/PO_2$	Both
α	Physiological oxygen supply capacity (Maximum measured value of physiological oxygen supply; α_0)	Measured	Maximum value of α_0 ($\alpha_0 = MO_2/PO_2$) in trial	Both
AAS	Absolute aerobic scope (modeled and calculated using population mean MMR and SMR values)	Measured	MMR, mRMR, SMR	Both
FAS	Factorial aerobic scope (modeled and calculated using population mean MMR and SMR values)	Measured	MMR, mRMR, SMR	Both
Chase time	Length of chase exercise to "exhaustion"	Measured	Time to exhaustion (s)	Exercise
P_{cMAX}	Critical oxygen level for highest metabolic rate at a given temperature, for normoxic species, this is generally air saturation (21kPa)	NA	P_{cMAX} =21kPa	NA

Table 5: *Bull and blacktip shark group demographics and trial summaries*. Measures are reported as means ± SE.

Species	**Blacktip sharks**				**Bull Sharks**		
Temperature Bin (°C)	*22*	*26*	*30*	*34*	*22*	*26*	*30*
Precaudal length (PCL)	43.25±0.79	42.69±0.74	42.86±0.7	42.56±0.82	54.2±1.44	56.08±1.05	53.78±0.87
Fork length (FL)	48.37±0.83	47.75±0.78	47.89±0.69	47.63±0.85	59±1.67	61±1.54	59.89±1.1
Stretch total length (STL)	59.78±1.06	59±1.02	59.39±1.02	58.81±1.1	74.3±1.91	76.33±1.5	74.83±1.41
Exercise Trials							
n	5	8	6	8	7	6	6
Mean trial temperature (°C)	22.18±0.01	25.87±0.12	30.09±0.13	33.74±0.02	21.91±0.02	25.42±0.14	29.63±0.17
Mean Salinity	32.32±0.21	32.61±0.47	34.02±0.36	30.9±0.59	17.2±0.08	17.36±0.08	17.29±0.1
Mass (kg)	1.13±0.04	1.08±0.05	1.17±0.05	1.29±0.08	2.81±0.23	2.73±0.09	2.75±0.13
Length of exercise trials (h)	22.93±1.81	22.84±0.9	11.6±3.24	20.41±2.24	8.8±1.43	9.96±2.01	10.46±2.68
Hypoxia Trials							
n	5	7	6	7	6	5	3
Mean trial temp (°C)	14.73±4.66	22.72±3.26	30.3±0.04	19.92±4.98	21.87±0.02	25.73±0.13	29.89±0.2
Mean salinity	32.18±0.19	32.7±0.52	34.02±0.36	30.9±0.59	17.8±0.15	17.16±0.12	17.48±0.08
Length of hypoxia trials (h)	3.73±053	4.37±0.46	3.05±0.4	3.17±0.62	6.87±2.1	7.98±0.31	5.83±0.36

Table 6. *Metabolic results by species and temperature for bull and blacktip sharks*. Results reported as means ± SE.

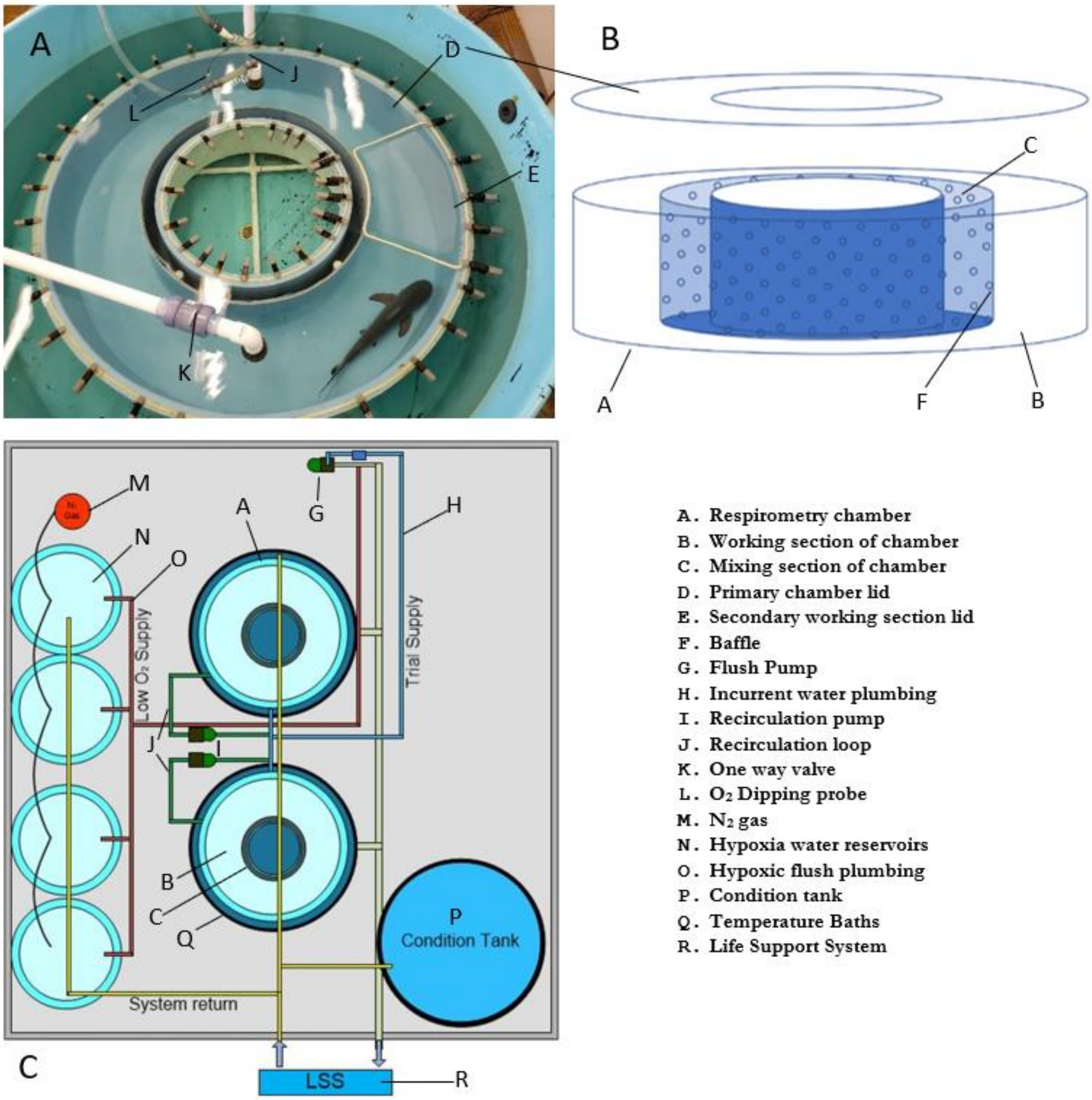
Species	**Blacktip Sharks**				**Bull Sharks**		
Temperature Bin (°C)	*22*	*26*	*30*	*34*	*22*	*26*	*30*
Maximum Metabolic Rate (mgO$_2$kg^{-1}hr^{-1})							
MMR	619±55.32	639.04±39.89	662.92±30.57	664.02±22.56	396.3±21.02	501.02±42.58	541.22±31.41
MMR at 21kPa	740.72±42.5	736.23±61.6	751.68±45.11	754.8±34.66	427.83±26.81	540.08±48.06	591.42±35.12
PO$_2$ at MMR (kPa)	19.85±0.75	19.92±0.1	19.27±0.35	19.88±0.25	20.09±0.17	19.59±0.18	19.24±0.15
Chase time	179.17±11.92	147.13±9.06	117.5±10.77	73.5±2.65	151.17±9.92	150.17±10.77	104.25±7.41
n (MMR, α, MMR at 21kPa)	6	8	7	8	6	6	9
Oxygen Supply Capacity (mgO$_2$kg^{-1}hr^{-1}kPa^{-1})							
α	35.27±2.02	35.06±2.93	35.79±2.15	35.94±1.65	20.37±1.28	25.72±2.29	28.16±1.67
Routine Swimming and Resting Metabolic Rates (>15 kPa; mgO$_2$kg^{-1}hr^{-1})							
mRMR	295.89±15.81	349.04±31.38	443±31.44	437.78±14.96	279.77±16.48	309.38±13.31	348.16±21.73
n (mRMR)	5	8	6	8	6	5	9
SMR (Exercise trials)	NA	NA	NA	NA	166.54±18.63	201.9±18.36	215.34±55.16
SMR (overall)	NA	NA	NA	NA	166.26±18.69	149.61±25.18	185.68±32.64
n (SMR) Top: overall Bottom: Exercise trials	NA	NA	NA	NA	4 4	4 4	6 4
Absolute Aerobic Scope (AAS; mgO$_2$kg^{-1}hr^{-1})							
AAS (MMR-mRMR)	357.2±68.57	290±38.32	217.96±25.9	226.24±20.6	141.2±34.1	227.48±49.73	226.76±35.76
Pooled AAS	323.11	290	219.92	226.24	116.23	191.64	193.06
Pooled AAS (SMR)	NA	NA	NA	NA	230.04	351.41	355.54
Factorial Aerobic Scope (FAS; ratio = unitless)							
FAS (MMR/mRMR)	2.27±0.31	1.91±0.18	1.51±0.09	1.53±0.05	1.43±0.11	1.74±0.17	1.83±0.15
Pooled FAS	2.09	1.83	1.5	1.51	1.42	1.62	1.55
Pooled FAS (SMR)	NA	NA	NA	NA	2.38	3.35	2.91

Table 7. *Thermal scaling coefficients of metabolic metrics for bull and blacktip sharks.* Significance of p-values were determined by alpha <0.05 form the Kenward-Roger approximation of each linear mixed model. Gray colored rows indicate thermal scaling in the range of 22-30°C.

Species	Metabolic Metric	Thermal Slope (E)	SE	df	t-value	p-value (Pr(>|t|))	Significant random effect (ID)	Temp range (°C)
Bull	MMR	0.31	0.09	9.78	3.44	***0.007***	yes	22-30
Bull	α	0.33	0.10	10.31	3.39	***0.007***	yes	22-30
Bull	mRMR	0.22	0.06	5.89	3.67	***0.01***	yes	22-30
Bull	SMR	0.1	0.26	10.56	0.4	0.07	no	22-30
Bull	AAS	0.49	0.27	10.29	1.82	0.09	no	22-30
Bull	FAS	0.1	0.1	10.22	1.02	0.33	no	22-30
Blacktip	MMR	0.05	0.05	20.52	1.098	0.28	yes	22-34
Blacktip	α	0.01	0.05	18.30	0.2	0.84	no	22-34
Blacktip	mRMR	0.34	0.05	14.31	6.999	***5.56E-06***	yes	22-34
Blacktip	AAS	-0.30	0.12	20.24	-2.419	***0.03***	no	22-34
Blacktip	FAS	-0.27	0.07	19.51	-3.744	***0.001***	yes	22-34
Blacktip	MMR	0.07	0.1	15.55	0.82	0.42	yes	22-30
Blacktip	α	0.2	0.12	06.06	.026	0.797	no	22-30
Blacktip	mRMR	0.45	0.08	5.48	5.71	***0.002***	yes	22-30
Blacktip	AAS	-0.47	0.23	13.01	2.06	0.06	no	22-30
Blacktip	FAS	-0.38	0.15	13.007	2.602	***0.02***	yes	22-30

Table 8. *Welch's t-test results*. Bull represents bull sharks, BT represents blacktip sharks. All P values adjusted via the Bonferroni method. Bold and italicized p values are significant (p<0.05)

Species	Comparison	Temperature	df	t statistic	Bonferroni adjusted p-value
Bull	mRMR v SMR	22	7	4.55	***0.0004***
Bull	mRMR v SMR	26	7	1.85	***0.018***
Bull	mRMR v SMR	30	9	4.14	***0.0004***
Bull v BT	MMR	22	6	3.76	***0.002***
Bull v BT	MMR	26	11	2.37	***0.006***
Bull v BT	MMR	30	14	2.78	***0.002***
Bull v BT	α	22	8	6.23	***0.00004***
Bull v BT	α	26	12	2.51	***0.005***
Bull v BT	α	30	12	2.80	***0.003***
Bull v BT	mRMR	22	9	0.71	0.08
Bull v BT	mRMR	26	9	1.16	0.05
Bull v BT	mRMR	30	10	2.48	***0.005***
Bull v BT	AAS	22	4	3.45	***0.004***
Bull v BT	AAS	26	9	1.85	***0.01***
Bull v BT	AAS	30	12	0.69	0.08
Bull v BT	FAS	22	4	2.69	***0.009***
Bull v BT	FAS	26	10	0.99	0.058
Bull v BT	FAS	30	12	-0.5	0.1



A. Respirometry chamber
B. Working section of chamber
C. Mixing section of chamber
D. Primary chamber lid
E. Secondary working section lid
F. Baffle
G. Flush Pump
H. Incurrent water plumbing
I. Recirculation pump
J. Recirculation loop
K. One way valve
L. O_2 Dipping probe
M. N_2 gas
N. Hypoxia water reservoirs
O. Hypoxic flush plumbing
P. Condition tank
Q. Temperature Baths
R. Life Support System

Figure 7. *Experimental set up of intermittent respirometry for bull sharks and blacktip sharks.* **(A)** Bull shark individual sealed within the intermittent respirometry chamber and surrounding temperature bath during metabolic trials. **(B-C)** Experimental set up with labels corresponding to item letters located to the right of the figure. Figure 7C made courtesy of coauthor John Morris.

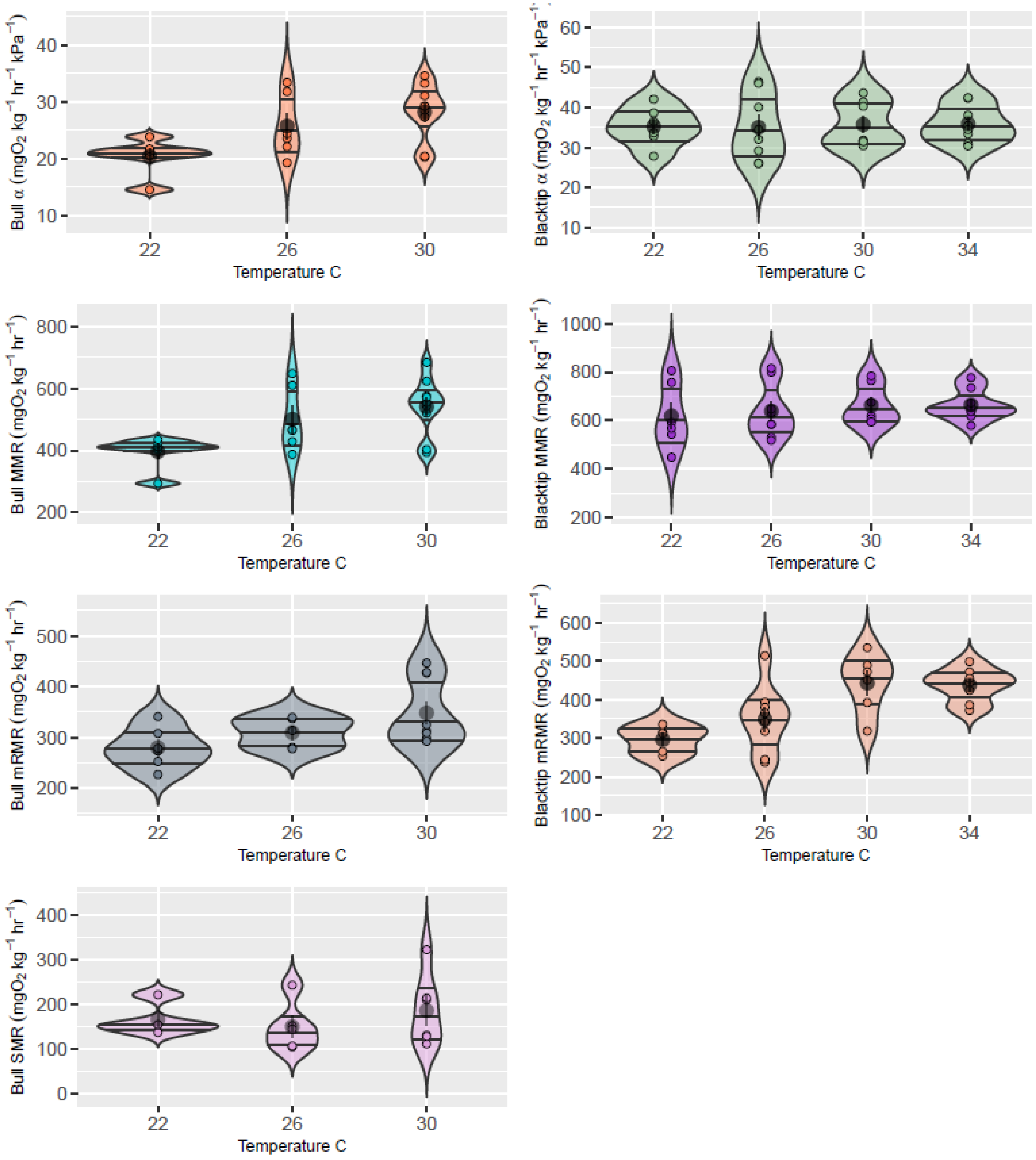


Figure 8. *Violin plots of thermally binned metabolic rates and oxygen supply capacity for bull and blacktip sharks.* Violins represent the kernel distribution of metabolic data. Points indicate individual metabolic measures. Different colors correspond to different metabolic metrics consistent with Figure 9. Lines in ascending order on each violin represent 0.25, 0.50, 0.75 quantiles. Black circles with vertical bars indicate means ± SE for each temperature bin.

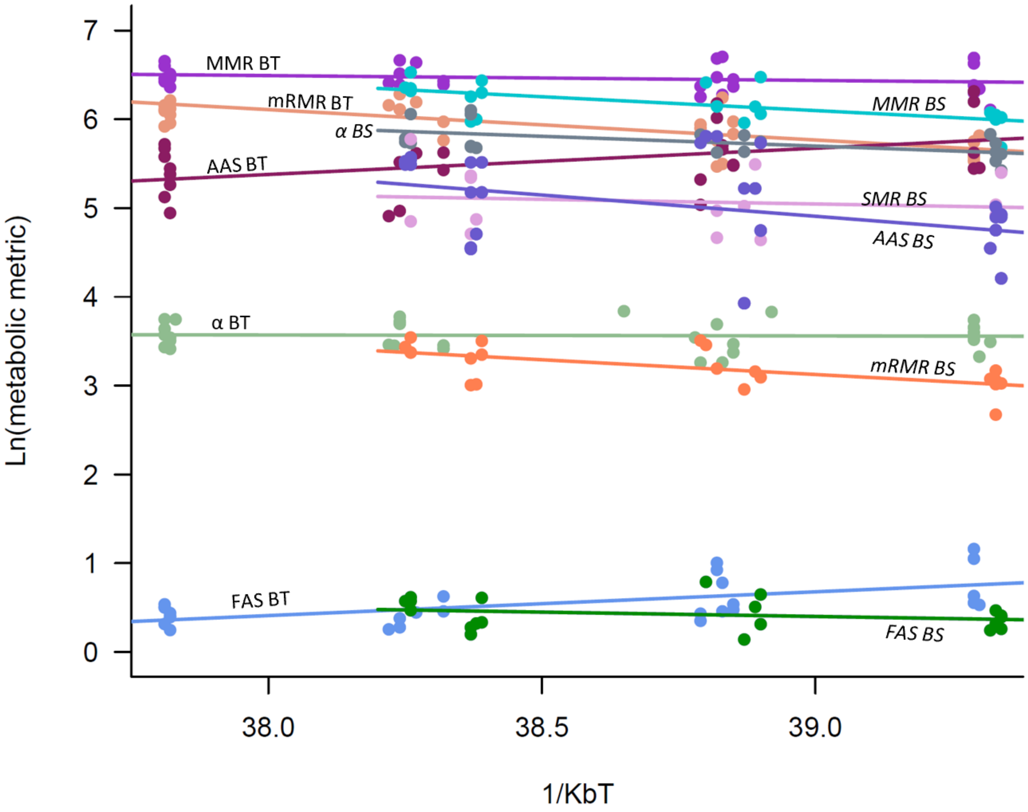


Figure 9. *Arrhenius relationships of metabolic metrics for bull and blacktip sharks*. Points indicate individual metabolic measurements. Different colors identify different metabolic metrics. Linear mixed effects models were used to visualize Arrhenius relationships of each metabolic metric and are represented by colored lines across temperature. The log of each metric is regressed on the inverse of Boltzmann's constant*average trial temperature in kelvin. The negative slope of the fixed factor, temperature (1/KbT) was used to represent thermal scaling coefficients of each metric (*E*) expressed in eV. BT stands for blacktip and BS stands for bull shark. Temperature in degrees Celsius increases from right to left along the x-axis.

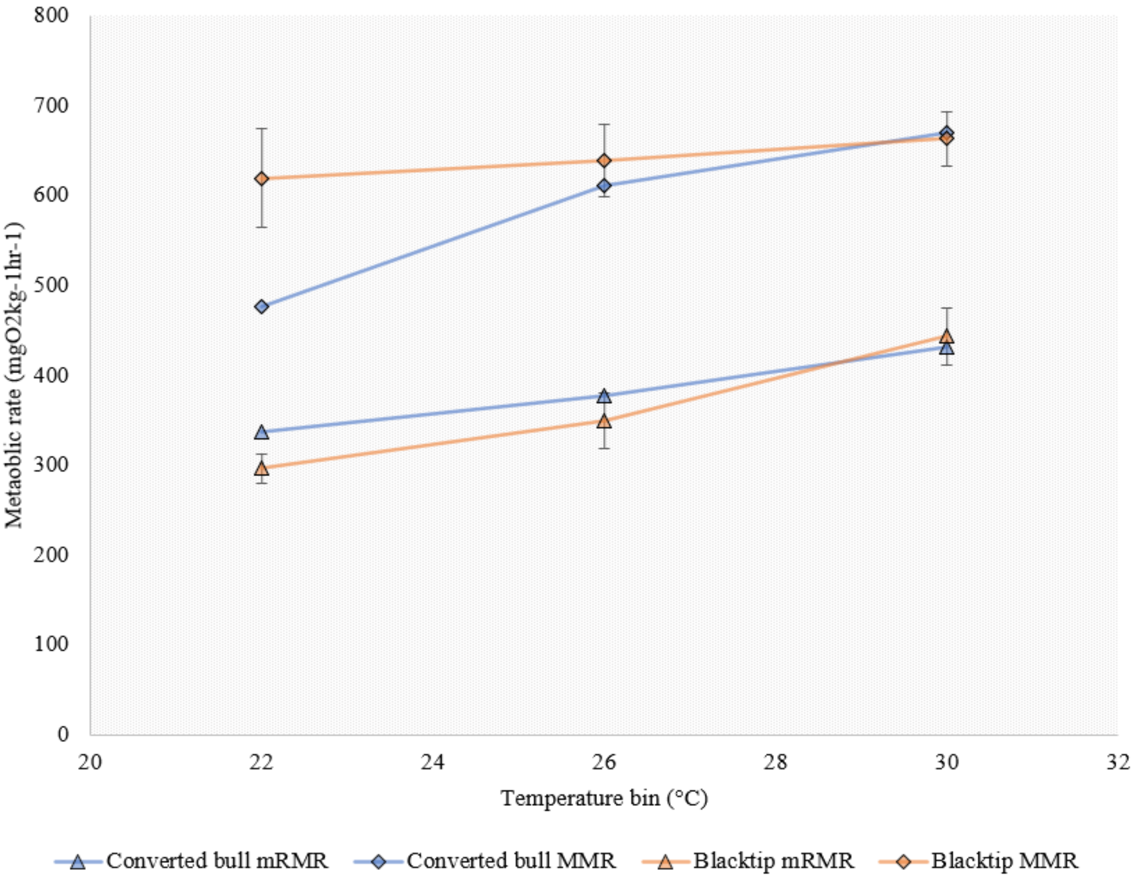


Figure 10. *Bull shark MMR and mRMR converted to the average mass of blacktips in each temperature bin.* A mass scaling coefficient of 0.25 was used to calculate rates at smaller masses. Blue points represent converted average MMR (diamonds), and mRMR (triangles) for bull sharks while orange points represent average measured MMR and mRMR for blacktips.

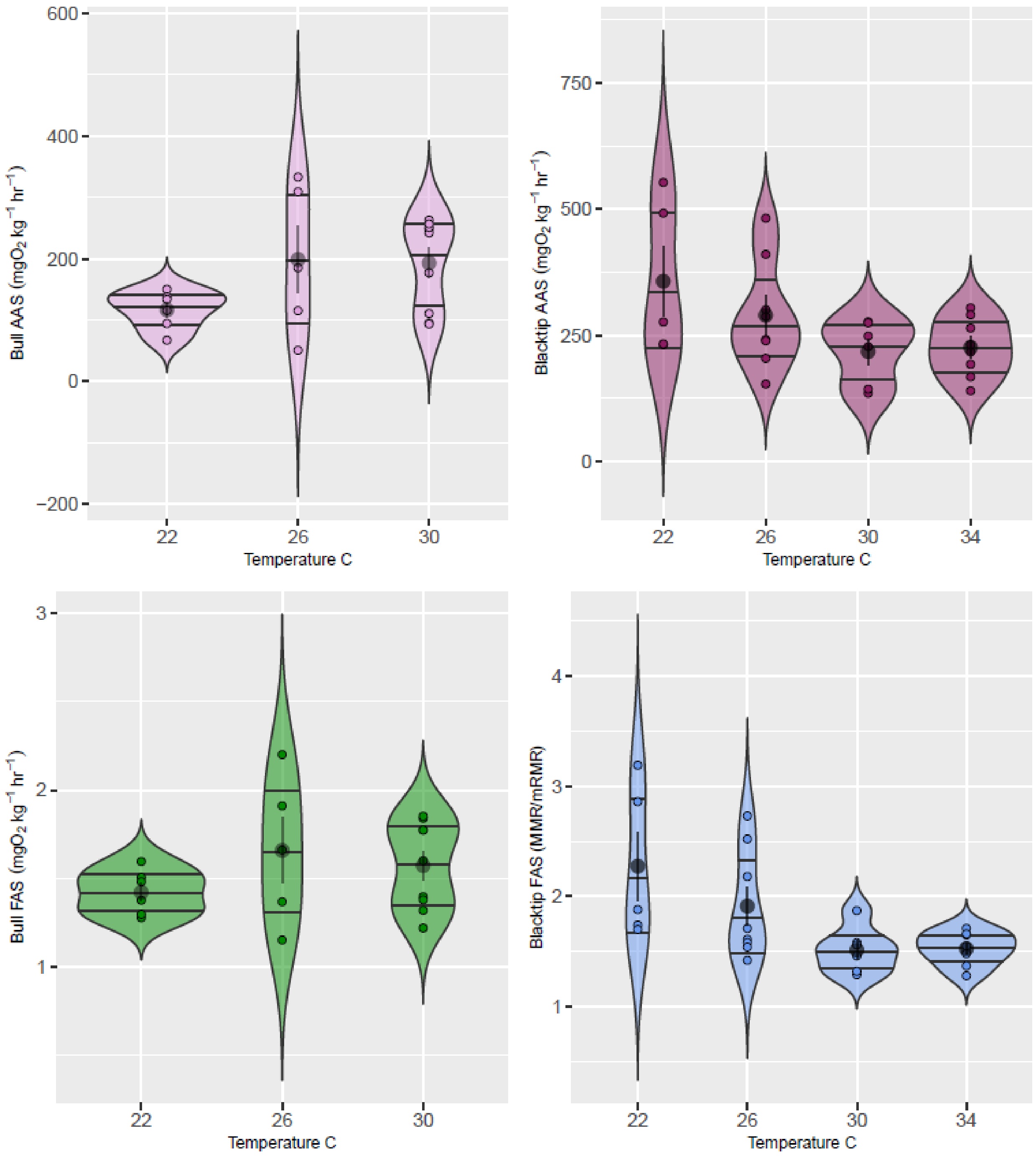


Figure 11. *Violin plots of thermally binned absolute and factorial aerobic scope for bull and blacktip sharks*. Violins represent the kernel distribution of aerobic scope. Points indicate individual metabolic measures. Different colors correspond to different metabolic metrics consistent with Figure 9. Lines in ascending order on each violin represent 0.25, 0.50, 0.75 quantiles. Black circles with vertical bars indicate means ± SE for each temperature bin.

CHAPTER 3

THE EFFECT OF HYPOXIA AND RAM-VENTILATION ON AEROBIC PERFORMANCE IN TWO COASTAL SHARKS SPECIES

Introduction

Ocean deoxygenation, driven by global warming and increased eutrophication in coastal waters, is causing alterations in marine habitats and costal populations (Breitburg et al., 2018). Both warming and deoxygenation impose metabolic constraints on marine organisms via elevated O_2 demand and reduced O_2 availability, respectively, with the potential to limit suitable habitat (Breitburg, 2002; Pörtner, 2010; Deutsch et al., 2015; Deutsch et al., 2020), influence reproduction and growth, and disrupt or alter marine species distributions (Zhang et al., 2009; Bertrand et al., 2011). However, an understanding of low oxygen tolerance is lacking for many coastal shark species. Environmental oxygen thresholds that trigger significant declines in performance, distributional shifts, or avoidance responses in elasmobranchs are largely unknown.

The physiological supply of oxygen to the body is influenced by the oxygen gradient from the environment to respiring tissues. Ventilation, the first step in this oxygen cascade, is no exception. Perry et al. (2009) stated that "the hypoxic ventilatory response is arguably the single most important physiological response accompanying the exposure of fish to lowered ambient PO_2". Behavioral mechanisms by which ventilation volume is increased (e.g. increased mouth gape or amplitude, ventilation frequency, or increased ventilation volume), increase the partial pressure of oxygen within systemic arteries and may allow for greater hypoxic tolerance (Perry et al., 2009). But the hypoxic ventilatory response is highly variable across species, and the efficacy of alterations in ventilation to maintain oxygen supply are dependent on many factors, including the severity and duration of exposure among others.

Animals are known to use different behavioral and ventilatory strategies to cope with hypoxia. Many fish have the ability to ventilate at rest, also known as buccal pumping or “active ventilation”. In such species, the reduction of metabolic demand at rest, paired with an increased ventilatory frequency and or amplitude, has proved an effective method for coping with hypoxia above a critical threshold (Randall, 1970; Metcalfe & Butler, 1984; Carlson & Parsons, 2001; Routley et al., 2002; Carlson & Parsons, 2003; Perry et al., 2009). Obligate ram ventilators however have evolutionarily reduced the branchiostegal systems that are required to sustain buccal pumping (Roberts, 1978; Carlson et al., 2004). Instead, they rely on continuous, open-mouth swimming to passively drive a flow of water across the gills (passive ventilation). In contrast to the metabolic rate reduction that can be achieved at rest during buccal pumping, ram ventilating fish species may increase swim speed and or mouth gape during hypoxic exposure (Bushnell & Brill, 1991, 1992; Carlson & Parsons, 2001, 2003). But locomotion is costly, and increased swim speeds and increased mouth gape to improve ventilation can only be sustained for so long under oxygen limited conditions (Roberts, 1978).

It may be possible that in confined habitats, ram ventilating species are capable of using active ventilation as an alternative strategy for surviving hypoxic stints. Some obligate ram ventilators, such as adult sandbar (*Carcharhinus plumbeus*), blacktip (*Carcharhinus limbatus*), and tiger sharks (*Galeocerdo cuvier*), have been observed in a resting state, buccal pumping for up to several hours in the wild to facilitate successful recovery from capture and inducted ventilatory stress (K. Lear, personal communication, data derived from unpublished data in Whitney et al., 2021). The viability or use of this strategy to specifically cope with environmental stress has not been explored. Whether buccal pumping is sufficient to support oxygen demand for ram ventilators over longer periods and under what environmental conditions this strategy might be effective is largely unknown. But ventilation method will influence the physiological oxygen supply capacity that can be achieved, in turn determining maximum metabolic performance and the aerobic scope available to organisms for activities such growth, reproduction and locomotion (Deutsch et al., 2020; Seibel & Deutsch, 2020).

Blacktips and bull sharks are considered obligate ram ventilating species, which have highly mobile lifestyles. As these species typically occupy the oxygenated upper waters of the ocean, they are not expected to be especially tolerant of low oxygen. My previous work predicted a critical oxygen pressure for maximum metabolic rate (PcMAX) near air-saturation (21 kPa; Andres Ch. 2). As such, the capacity to supply oxygen to tissues (α) in support of performance likely evolved for these species under air saturated conditions (Seibel and Deutsch, 2020). I the oxygen supply capacity is unaffected by activity level, then environmental oxygen below 21 kPa is directly proportional to the MMR that can be achieved at a given temperature (Seibel and Deutsch, 2020). If true, the decline in maximum performance and habitat suitability can be modeled across environmental oxygen levels (Seibel & Deutsch, 2020; Seibel et al., 2021). However, if oxygen supply capacity changes with activity (swimming vs resting), as supposed for ram ventilators, such modeling efforts cannot be reliably applied to obligate ram ventilating sharks at rest.

Species richness and abundance have been shown to decline at low dissolved oxygen concentrations, generally below 4 mgO_2l^{-1} (SWFWMD, 1999), as most marine fish actively avoid hypoxia below a level that limits growth or reproduction (Breitburg, 2002). This is likely the case for adult bull sharks (*Carcharhinus leucas*) and blacktip sharks (*Carcharhinus limbatus*) that have capabilities for large scale dispersal (Castro, 2010) as well as greater incentive to avoid hypoxic zones, given their higher oxygen demand at larger body size and high-performance lifestyles. The youngest stages of these species, however, have limited dispersal as they use estuarine and riverine habitat as nurseries to shelter from predators, take advantage of ample food supply, and in many cases improve survival in their most vulnerable life stages (Branstetter, 1990; Beck et al., 2001; Heupel et al., 2007; Curtis et al., 2011; Heupel & Simpfendorfer, 2011). Animals within more confined habitats experience greater exposure to environmental shifts, and abiotic factors have been shown to alter habitat selection for these species within their natal ranges (Heupel & Simpfendorfer, 2008; Heithaus et al., 2009; Curtis et al., 2011; Matich & Heithaus, 2012). For resident species within an estuary, hypoxia avoidance cannot always be achieved (Breitburg, 1992) and the energetic costs of hypoxic avoidance (Taylor & Miller, 2001), and perceived predation risk (Randle & Chapman, 2005; Shingles et al., 2005) must be accounted for in behavioral responses to hypoxia.

Nursery habitats for neonates/young-of-the-year (YOY) of these species within the Tampa Bay watershed experience hypoxia to varying degrees. In the summer season ocean temperatures are highest (Janicki et al., 2001), leading to decreased oxygen solubility and greater thermal stratification that may prevent oxygen replenishment in deeper waters. Simultaneously, high nutrient input to coastal waters due to anthropogenic influence increased community oxygen consumption (Breitburg et al., 2018). Together these factors may increase the spatial and temporal extent of hypoxia (Janicki et al., 2001; Diaz & Breitburg, 2009) at a time when temperature drives up metabolic demand in ectothermic fish. Parturition occurs for bull sharks and blacktips (Hueter & Tyminski, 2007) in the late spring-summer seasons, and neonate-juvenile bull and blacktip sharks need to be robust to these environmental changes during their summer residency in these habitats, which may occur over several years (Heupel & Hueter, 2001; Thorburn & Rowland, 2008; Curtis et al., 2011). But for the majority of shark species, it is unknown what levels of hypoxia may be tolerated, or cause shifts in habitat association, or the species-specific behavioral strategies that may be used to cope with hypoxia.

Increased severity and residency of hypoxic zones within their occupied nursery range may cause habitat compression for these species (Coutant, 1990; Diaz & Breitburg, 2009). It is therefore imperative for us to understand to what degree oxygen availability and oxygen supply capacity drives habitat suitability and alters performance and behavior within these species most critical life stages. In this study we evaluated neonate/young of the year bull and blacktip sharks of the Eastern Gulf of Mexico, to identify the effect of hypoxia and ram-ventilation on aerobic performance.

I put to test several new and prevailing hypotheses regarding hypoxia and ram ventilation for blacktip and bull sharks in this study. 1) If buccal pumping (active ventilation) is sustainable for this species and facilitates greater tolerance to hypoxia I expect a lower limiting oxygen level for resting than swimming, and greater endurance under hypoxia when blacktip and bull sharks are at rest. 2) If buccal pumping is unsustainable and does not confer greater hypoxic tolerance, than sharks will require a higher ambient oxygen level to support resting metabolism than swimming metabolism under hypoxia, and swimming would provide greater endurance to low O_2. I expect however that the latter is true, that 3) oxygen supply capacity is diminished at rest relative to swimming in obligate ram ventilators. 4) Though resting reduces oxygen consumption relative to routine swimming, buccal pumping at rest is not a sustainable means of ventilation or hypoxic tolerance for either species. I expect an inability of both species to maintain standard (resting) metabolic rate (SMR), indicated by a decline in SMR under both normoxic and hypoxic conditions due to oxygen supply limitation at rest. 5) I predict these two obligate ram ventilating species will instead increase overall swim speed to improve the hypoxic ventilatory response as other ram ventilators have done. 6) I predict that hypoxia causes declines in maximum metabolic rate (MMR) for this species in a 1:1% ratio as ambient oxygen declines, and that hypoxia causes predictable decreases in aerobic scope for both species. 7) Blacktip and bull sharks likely require a minimum partial pressure of oxygen (PO_2) to maintain delivery of O_2 to tissues, and this minimum value will fall around the critical oxygen level for minimum routine swimming metabolic rates (P_{cmRMR}). 8) I predict that potential population limiting thresholds for these species may lie close to the P_{cmRMR} threshold.

Through this work, we can determine whether or not blacktip sharks and bull sharks can maximize oxygen supply under specific behaviors, if certain behaviors provide increased tolerance under hypoxia, and what hypoxic limits to performance and population may be in the wild.

Methods

Detailed methods for capture, transport, acclimation, experimental set up and protocol for this research can be found in Andres, Chapter 2.

Additional behavioral observations: Behavioral observations were conducted throughout exercise and hypoxia trials. While sharks exhibited swimming behavior, the length of time needed for the shark to swim one full circle around the outer diameter of the working section, known as “time-to-circle”, was recorded. Time-to-circle was recorded three times over each closed measurement period, averaged, and used to calculate average swim speed in body lengths per second (BLs^{-1}), according to Equation 1.

$$\text{Eq 1.} \quad A = \frac{d}{t(b)}$$

Where *A* is average swim speed in body lengths per second (BLs^{-1}), *d* is the circumference of the outer edge of the working section within the respirometer (m), *t* is the time it took the shark to swim one full circle around *d* in seconds (s), and *b* is the individual shark's body length (STL) in meters (m).

For bull sharks that exhibited intermittent or continuous resting behavior during trials, active ventilation was quantified for these periods by sampling the number of buccal pumps the animal demonstrated in a one-minute period. Three measurements of buccal pumps/min were taken per applicable measure period and averaged to give mean buccal pumps/min per measure period. We were unable to opportunistically sample buccal pumps for blacktips however, as a switch to resting and facultative buccal pumping in these individuals led to rapid decline of MO_2 and animal condition and an inability to recover resulting in mortality if allowed to continue to rest and facultatively buccal pump over the full 15 min measure. Behavioral indicators of decline in animal condition are as follows: 1) patchiness in coloration, 2) lethargy, potentially coupled with inability to maintain equilibrium, and loss of ability to locomote, 3) reliance upon buccal pumping as sole ventilation method (continuous resting), 4) decline of buccal pumping below 27 pumps/min (for bull sharks), 5) extended gill flaring and/or "coughing" (Roberts, 1978), 6) rapid and continuous decline of mass specific oxygen consumption (MO_2) below lowest routine levels observed in trial. Consequently, blacktips were removed from trials within roughly 3 minutes of the onset of resting behavior if they could no longer be motivated to swim. This was necessary to reduce subject attrition and to preserve subjects for testing at other temperatures.

The onset of continuous burst swimming was also recorded. Continuous burst swimming was categorized by cycles of short, rapid, increases in swimming effort "bursts", followed by a "stall" or "glide" behavior (cessation of activity), generally, >15 "bursts" per measure. We acknowledge that mouth gape has been used in previous studies as an additional indicator of changes in ram ventilation along with changes in swim speed (Carlson & Parsons, 2001, 2003) under hypoxia. However, the set up in this study precluded quantifiable measures of individual changes in mouth gape as PO_2 declined.

Metabolic rate determination: Metabolic rates were determined in Andres Chapter 2 and have been reiterated here for reference. MMR, the maximum metabolic rate achieved in trials, was defined as the highest MO_2 value observed for an individual across exercise and hypoxia trials.

Minimum routine metabolic rate, mRMR, was defined as the minimum routine metabolic rate, adapted from (Crear et al., 2019) and refers to the lowest metabolic rate of animals continuously swimming under normoxic conditions. For the calculation of mRMR in blacktips, the first 3 hours of measure periods within exercise trials were omitted from analysis (Chabot et al., 2016), and a minimum routine metabolic rate (mRMR) was calculated as the lowest 10% of MO_2 measures using the R package *Mclust* (Fraley & Raftery, 2002; Fraley et al., 2014).

Due to the shorter nature of the bull shark exercise trials (Table 5) we did not remove the first 3 hours of the trials, but instead used the lowest 10th quantile (*Mclust;* Fraley & Raftery, 2002; Fraley et al., 2014) of continuous swimming measures at PO_2 >15 kPa to represent mRMR within a trial. For each individual bull shark, the lowest mRMR across trial types represented mRMR for the given individual shark. Individuals who only swam during the measure period of MMR were eliminated from mRMR analysis. Standard, or resting metabolic rate (SMR) was determined from opportunistically sampled measures of bull sharks at rest. The lowest 10th quantile of continuously resting measures at PO_2 >15 kPa was calculated within an individual trial and the lowest SMR across trial types represented SMR for a given individual.

In addition, the lowest metabolic rate we observed under continuous swimming in hypoxia (<15kPa) was designated as the minimum locomotive rate, the MO_2 of the lowest continuous swimming that will sustain ram ventilation.

Oxygen supply capacity and critical oxygen thresholds: Physiological oxygen supply capacities (α; Eq. 2) were determined using the method of Seibel et al., 2021 (Fig. 13). Briefly, the instantaneous oxygen supply ($\alpha_0 = MO_2/PO_2$) was determined for each individual trial measure period and the highest value was taken as the oxygen supply capacity, (α). This value was determined independently for hypoxia and exercise trials. The α is typically constant between maximum exercise and rest and describes the oxygen-dependency of MMR between $P_{cmRMR,}$ the lowest oxygen level where mRMR can be maintained, and P_{cmax} (MMR = α*PO_2; Seibel and Deutsch, 2020; Seibel et al., 2021) for each temperature. The resulting equations (α-lines) are plotted in Figs. 14 (Bull Sharks) and 15 (Blacktip sharks).

$$\text{Eq 2. } \alpha = \left(\frac{MMR}{P_{cMAX}}\right) = \left(\frac{SMR}{P_{cSMR}}\right) = \left(\frac{mRMR}{P_{cmRMR}}\right) = \ highest\ \alpha_0$$

Critical oxygen thresholds (Pc) for MMR, mRMR and SMR were calculated by dividing each metric by the individual's measured oxygen supply capacity (α), using Equation 2. P_{cmRMR} represents the critical oxygen partial pressure below which minimum routine activity (lowest continuous swimming) can no longer be maintained, and P_{cSMR}, the critical oxygen partial pressure below which SMR can no longer be maintained. For each completed hypoxia trial, the highest "instantaneous" measure of physiological oxygen supply (α_0) 16 kPa (Figure 13) was identified and categorized by the shark's continuous behavior during the measure (swimming, resting, or a mix of the two). These values were then compared to α, and the PO_2 of these measures (the last measures where the animal attempted to increase or regulate oxygen supply) was compared to predicted P_{cmRMR}, P_{cSMR}.

Results

The decline in maximum performance across PO_2 was determined for each individual of each species (e.g., Figure 12), and for the average population sample of each species (Figures 13, 14). For bull sharks, which demonstrated an increase in α across temperature, maximum performance appeared to decline at a faster rate as PO_2 declined at nigher temperatures (Figure 13). For blacktip sharks the rate of decline in maximum performance across PO_2 was not significantly different between temperatures within the range of 22-34°C (Figure 14) owing to the lack of significant change in oxygen supply capacity between 22-34˚C (Andres, chapter 2). However, in actuality, MMR declines ~1% per 1% decline in oxygen saturation regardless of temperature, for both species. Predicted P_{cmRMR} increased with temperature for both species within the range of 22-30˚C (Figures 13, 14) and, for blacktips, showed no change between 30-34°C.

For blacktip sharks, the oxygen supply achieved at minimum locomotive rate was well within SE of the mean α achieved in exercise trials, indicating that individuals were able to maximize oxygen supply under hypoxia. Bull sharks that continuously swam also achieved similar oxygen supply values under hypoxia and during exercise trials, although at 30°C, one individual quit the trial early and another bull shark had systemic error that caused elimination of a few MO_2 measures that may have skewed the average oxygen supply under hypoxia low (Table 9). Thus, the predicted critical oxygen pressures for bull sharks under locomotion fall on the α-lines. However, some bull shark individuals stopped swimming and engaged in buccal pumping during part or all of the trial. In those cases, oxygen supply was substantially lower than at capacity and the critical pressures for SMR were much higher than for locomotive rates despite lower metabolic demand (Figure 13). For individuals that continuously swam in hypoxia trials, Pc of minimum locomotive rate (lowest swimming metabolism) was lower than P_{cmRMR} (Table 9), though sample size was low. P_{cSMR} for bull sharks increased between 22-30°C (Table 9).

Oxygen supply capacity was only reached during swimming and similar values were achieved at maximum exertion and minimum locomotive rate. Bull shark individuals that rested continuously had a significantly lower mean oxygen supply at rest than under locomotion at 22-30°C (Table 9, 10; Figure 15). Some individuals displayed a continuous mix of swimming and resting behaviors within a measure period. Depending on the behavior exhibited and PO_2 of the measure period, oxygen supply for these "mixed behaviors" ranged from 16.70 to 22.43 $mgO_2kg^{-1}hr^{-1}kPa^{-1}$ (22-30˚C). In addition, there was no appreciable difference in oxygen supply at SMR for bull sharks between 22-30˚C (Table 9).

Oxygen supply capacity did not vary with chase time or length of exercise trials (Pearson correlation coefficients <0.3), indicating that results from hypoxia trials were not significantly impacted by the prior exhaustive exercise or recovery after exercise trials. Swim speed (measured in body lengths per second; BLs^{-1}) increased slightly with decreasing PO_2 for only 3-5 individuals of each species during hypoxia trials (bull sharks: 2 individuals at 30˚C, 2 individuals at 26˚C, 1 individual at 22˚C; slope range 0.003-0.006 $BLs^{-1}kPa^{-1}$; R^2= 0.09-0.6; blacktips: 2 individuals at 30˚C, 1 individual at 34˚C; slope range

0.002 and 0.2, $BLs^{-1}kPa^{-1}$; R^2=0.05-0.16). Blacktips did demonstrate increased burst swimming behavior at 8.51±1.23 kPa to 9.51±0.63 kPa from 22 to 34°C respectively. For bull sharks at rest, no increase in ventilation rate (buccal pumps per min) was observed, but rather a decline in buccal pumps as PO_2 progressed (~ 40 - <27 bp/min).

Discussion

In this study we hypothesized that, if sharks had the capacity to facultatively buccal pump, reducing activity and increasing the rate of ventilation could enhance hypoxia tolerance as may be required in their natal estuarine habitat. Alternatively, if sharks did not demonstrate the capacity for facultative buccal pumping, then, as ram ventilators, adjustments to improve hypoxia tolerance might include increases in swim speed to increase ventilation volume.

Blacktip sharks swam continuously during our trials until failure, precluding an estimate of the oxygen supply capacity at rest. Failure at the end of trials were marked by a decline in animal condition: 1) patchiness in coloration, 2) lethargy, sometimes coupled with inability to maintain equilibrium, and loss of ability to locomote, 3) reliance upon buccal pumping as sole ventilation method (continuous resting), 4) decline of buccal pumping below 27 pumps/min (for bull sharks), 5) extended gill flaring and/or “coughing” indicating ventilatory hypoxia (Roberts, 1978), 6) rapid and continuous decline of mass specific oxygen consumption (MO_2) below lowest routine levels observed in trial. Given that condition declined rapidly once blacktips stopped swimming, a reduction in activity and switch to active ventilation does not appear to be a sustainable means of hypoxic endurance for neonate/YOY of this species. However, bull shark individuals demonstrated varied ventilatory behavior and activity in our annular respirometer, which allowed us to opportunistically measure metabolic rate and oxygen supply capacity across different modes of ventilation (passive ram and active facultative buccal pumping). Some bull sharks did rest during trials, leading to a significant reduction in metabolic rate (~57%) (alpha <0.05; Table 8), ventilation rate (~ 40 to <27 buccal pumps/min), and oxygen supply capacity. In some species, (e.g., white sturgeon, *Acipenser transmontanus*) a reduction in activity paired with a decline in ventilation frequency increases tolerance to hypoxia by reducing the cost of ventilation per unit of oxygen extracted (Burggren & Randall, 1978; Perry et al., 2009). In that case, oxygen supply is maintained as the ventilation rate falls. If a reduction in activity and ventilation rate facilitated greater tolerance to low oxygen in bull sharks, then oxygen supply capacity should have also been maintained at rest, resulting in a decreased critical oxygen pressure for standard metabolic rate (P_{cSMR}). However, at rest, bull shark oxygen supply capacity declined dramatically and a much greater environmental PO_2 was required to sustain even the reduced, resting, rate of oxygen consumption. The reduced oxygen supply capacity results in a reduced factorial aerobic scope. This suggests that while individuals were able to actively ventilate for brief periods of time and demonstrated a greater capacity to reduce activity than blacktips, this is also an unlikely strategy for enduring hypoxia in bull sharks.

Obligate ram ventilators have been shown to attempt facultative pumping under duress. These scenarios include physical restriction (i.e. capture, transport, etc.), under environmental stressors such as low temperature (juvenile bull sharks; K. Lear personal communication from unpublished data from Lear et al., 2020), high temperature and low oxygen (juvenile sandbar sharks; Crear et al., 2019) and after capture induced exercise and ventilatory stress (adult sandbar and blacktip sharks; K. Lear personal communication, unpublished data from Whitney et al., 2021). All were instances where escape or unrestricted ventilation was not an option, or the energy and oxygen supply required for locomotion were likely compromised (e.g., physical exhaustion and fatigue after capture, or chase). These periods ranged from minutes to a few hours depending on the individual but in all cases, the ability to effectively pump was time limited and highly species-specific, thought to be influenced by morphology and metabolic demand (Roberts, 1978). Under supersaturated oxygen conditions however, buccal pumping for ram ventilators has been shown to be sustainable (Noxon et al.,1982). Several species of obligate ram ventilators (e.g., grey reef, tiger sharks, bull sharks, etc.) were previously observed voluntarily buccal pumping at rest in the wild for hours at a time in the low salinity, oxygen supersaturated waters of Isla Mujeres caves in the Yucatan peninsula and were documented by Dr. Eugene Clark in a National Geographic Documentary, (Noxon et al.,1982). As this behavior can apparently be sustained in DO greater than normoxia, this supports our findings that oxygen supply limitation is what prevents sustainable buccal pumping behavior in ram ventilating species, and only in conditions above normoxia is oxygen supply at rest enough to support basal metabolic needs.

While we could not quantify changes in individual mouth gape under hypoxia, it is likely that bull and blacktip sharks increase mouth gape as oxygen declines. Several observations during our hypoxia trials mentioned swimming sharks opened their mouths wider as trials progressed. This trend has previously been reported for bonnethead sharks, (*Sphyrna tiburo),* and blacknose sharks, (*Carcharhinus acronotus)* (Carlson & Parsons, 2001, 2003), that promotes higher ventilation volume. This strategy has also been connected to an increase in swim speed under low oxygen. In this study only a very slight increase in swim speed (BL/s) was observed for 5 bull sharks (slope of increase: 0.003-0.006), and 3 blacktips (slope of increase: 0.002-0.2) across $PO_{2.}$ This minor change in swim speed may have been used to increase ventilation volume for some, but this was not a universal strategy for individuals across the study, with most demonstrating no clear change or a decrease in swim speeds across PO_2. Though tailbeat amplitude was not impeded by the respirometers and sharks had ample space for locomotion, the use of annular respirometers does limit linear swimming and forces repetitive turning behavior thought to increase energetic demand (Boisclair & Tang, 1993). This could have played a role in limiting swim speeds for the rest of the sharks. It is possible that a greater number of individuals and/or a more pronounced increase in swim speed under hypoxia could be seen in less restrictive scenarios for these species. Bull sharks and blacktip sharks may benefit from increased swim speeds under moderate hypoxia, but this requires further investigation, as well as consideration of how long this strategy can be maintained.

For obligate ram ventilators, this strategy of increased mouth gape and increased swim speed makes sense to increase oxygen supply under acute and moderate hypoxic exposure, up to a point. Mouth gape is ultimately limited by mouth morphology and body size, and locomotion is limited by the amount of oxygen needed to support a particular swimming speed (Roberts, 1975, 1978). As PO_2 declines the maximum performance that can be achieved declines ~1% for every ~1% decline in PO_2 (Figures 13, 14). At a critical level, the PO_2 becomes too low to support continuous swimming or in some species, to maintain hydrostatic equilibrium (Carlson & Parsons, 2001). Peak ventilatory effort for many fish species corresponds to their critical oxygen threshold (see review, Perry et al., 2009), supporting the idea that the last PO_2 where oxygen supply capacity is reached under hypoxia, is the last place where ventilatory adjustments can be made to support maintenance (SMR) metabolism. For blacktip and bull sharks peak ventilatory effort occurs at the Pc for minimum locomotive rate (Figure 14 and 15) the point beyond which locomotion can't be sustained. Blacktips demonstrated cycles of burst swimming behavior within the range of Pc for minimum locomotive rate and predicted P_{cmRMR}. For bull sharks, the last attempt at either continuous or mixed swimming under hypoxia occurred either at Pc for minimum locomotive rate or within 0.3-2.58 kPa of that value. In addition for both species, "coughing" or extending gill flaring was mentioned at and below these thresholds, seen in other fish species as a known side effect of ventilatory hypoxia (Roberts, 1978).

Increased swim speed in ram ventilating teleosts such as skipjack (*Katsuwonus pelmis*; Bushnell & Brill, 1992), yellowfin (*Thunnus albacares*; Bushnell & Brill, 1991, 1992) and big eye tuna (*Thunnus obesus*; Bushnell and Brill, 1992) has been categorized as escape behavior rather than an attempt to improve ventilation volume in hypoxia. While we can't rule out increases in swim speed, burst swimming, or last "mixed" swimming attempts as escape responses, we do know that attempts at increased locomotion provided greater oxygen supply (Figure 14). It is likely that our sharks exhibited these behaviors in an attempt to maintain oxygen extraction (Carlson & Parsons, 2001, 2003) as in other ram ventilating shark species.

It is known that fish will respond to hypoxia in whatever manner is most energetically favorable for the species and given environment they are exposed to (see review, Perry et al., 2009). If ventilation at rest does not support base metabolic needs, then under hypoxia, sharks must make tradeoffs between expending energy on ventilatory adjustments while swimming to endure hypoxic conditions down to a limiting threshold for P_{cmRMR}, or to expend energy avoiding hypoxia (Taylor & Miller, 2001) and risk predation by vacating preferred nursery habitat (Heupel & Simpfendorfer, 2011). Hypoxic limitation and aerobic scope have been indicated as key physiological factors in forecasting marine species distributions (Pörtner, 2001; Deutsch et al., 2015). For both species, the oxygen supply capacity we measured under minimum routine swimming in hypoxia and following exhaustive exercise (at MMR) were very similar, suggesting that MMR and factorial aerobic scope (FAS) are directly dependent on oxygen pressure below 21 kPa (air-saturation).

As MMR and oxygen supply capacity are relatively insensitive to temperature in blacktip sharks (Andres, chapter 2), the P_{cMAX} is also independent of temperature and falls near air-saturation (21 kPa). Thus, FAS for blacktip sharks depends only on PO_2 and the temperature sensitivity of mRMR. For Bull Sharks, FAS depends on PO_2 and on relative temperature sensitivities of both MMR and mRMR.

During shark collection and in preliminary survey results from the Alafia River bull sharks were not found below a PO_2 of 9.23 kPa (3.4 mgO_2l^{-1}, at 28.1˚C, 12.9 salinity) with all but one individual captured above ~12 kPa (4.91-6.67 mgO_2l^{-1}; 27.7-31.9˚C; 0.98-23.6 salinity) (environmental ranges: 25.5-31.9˚C; 0.18-25 salinity; 0.39-6.67 mgO_2l^{-1}) (Table 11). No environmental data was available for blacktip shark collections (due to YSI malfunction). While these samples were opportunistic and constitute preliminary data on presence/absence of bull sharks in the Alafia river, other habitat use and movement studies on both blacktip sharks and bull sharks provide additional insight into hypoxic limitations of habitat. Surveys conducted by Heithaus et al. (2009) on YOY-Juvenile bull sharks in Shark River (Everglades, FL) reported a potential limiting threshold of <3.5mgO_2/l for juvenile bull sharks and a lowest oxygen threshold of 2.9 $mgO_2l^{-1}$for YOY. In Tampa Bay blacktip neonate and YOY have been reported at minimum DO of 3.2-3.7 mgO_2l^{-1} (Hueter & Tyminski, 2007) and in a long-term presence/absence and movement study by Gardner and Wiley (unpublished) in Lower Tampa/Sarasota Bay, YOY blacktip sharks were not found below 9.9 kPa (3.12 mgO_2l^{-1}; 29.7 C; 29.16 salinity), juveniles, 11.2 kPa (13.8 kPa (4.74 mgO_2l^{-1}; 27.2 C; 31.54 salinity), YOY bull sharks at 9.1 kPa (3.14 mgO_2l^{-1}; 31.2 C; 9.46 salinity), and for juveniles at 11.2 kPa (3.77 mgO_2l^{-1}; 27.4 C; 24.45 salinity). For a 30-year study on bulls in the Indian River lagoon, FL), minimum O_2 for shark presence was 3.2 mgO_2l^{-1} for YOY (27.9–33.5°C range; 1.6–34.2 salinity range) and 3.8 mgO_2l^{-1} (20.0–37.0°C range; 1.1–42.0 salinity range) for juveniles (Curtis et al., 2011). In the Caloosahatchee River reported min O_2 for bull sharks was 2.2 mgO_2l^{-1} bottom, 3.6 mgO_2l^{-1} surface (Ortega et al., 2009).

Due to the fact that lowest oxygen was reported in concentration alone from most previous studies (without the specific salinity and temperature associated with the minimum measurement), survey data are a bit ambiguous. It is difficult to say for sure without full presence/absence datasets how closely these oxygen minimums agree with limiting thresholds in this study (i.e. predicted P_{cmRMR}) as the pressure exerted by a given O_2 concentration changes with temperature and salinity (Figure 16) (Diaz & Breitburg, 2009). At a constant salinity of 30, a concentration of 3.5 mgO2/L is equal to ~12 kPa at 34°C, but ~9.5 kPa at 20°C. At a constant temperature of 30°C the concentration 3.5 mgO2/L is equal to ~10 kPa at a salinity of 7, but ~11 kPa at a salinity of 30. So, a single concentration threshold might be limiting at one temp or salinity, but not another.

While we require more comprehensive historical distributional data to know for sure how limits to oxygen supply capacity and performance translate to limiting population thresholds, what we can say is the apparent oxygen thresholds we found during collection for bull sharks, fall within the range of Pc for minimum locomotive rate for both bull and blacktip sharks across temperature (Figure 16) and are in general agreement with reported concentration limits from previous research on both species based on the information we have. We have shown that at Pc for minimum locomotive rate, and Pc_{mRMR}, lowest routine activity is supported, but leaves little aerobic scope for growth and digestion and increased activity. At lower PO_2, swimming is not supported and buccal pumping is insufficient to support even resting demand. Thus, the critical pressure for minimum locomotive rate is likely the absolute critical pressure for the species. The threshold of ~12 kPa fits well with predicted P_{cmRMR} for both species and may represent a threshold for cruising swimming speeds rather than a critical level for absolute lowest locomotion. This is consistent with studies of other ram-ventilating fishes, in that cruising speeds are generally in excess of the minimum speed needed to sustain baseline ram ventilation for fish (Brown & Muir, 1970; Roberts, 1975, 1978).

We have not measured metabolic rates across salinities and are uncertain how the cost of osmoregulation might change FAS dynamics for these species. However, oxygen supply capacity may change with salinity and alter critical oxygen thresholds. Sharks may reach limiting FAS for a given temp at a different salinity. Bull sharks are found within a preferred salinity range of 7-17.5 (Simpfendorfer et al., 2005). We do not know how hypoxic endurance would change across salinity, but this requires consideration given that the use of mesohaline habitats for young bull sharks has been shown to significantly reduce mortality (<50% of rates of other species at same ontogenetic stage) by substantially reducing the risk of interspecific predation and competition (Simpfendorfer et al., 2005; Heupel & Simpfendorfer, 2011).

It is likely that the neonate/YOY of these two species, are by necessity the most hypoxia tolerant stages of the species, but also the most likely to bear impacts of hypoxic exposure. Several metrics have bene used to establish differences in thermal sensitivities with ontogenetic stage that identify the youngest free-swimming stages as the most environmentally robust (Lear et al., 2019) Based on presence absence studies and reported oxygen concentration thresholds and habitat partitioning (Heupel & Hueter, 2001; Simpfendorfer et al., 2005; Heupel et al., 2007; Heupel & Simpfendorfer, 2008; Heithaus et al., 2009; Froeschke et al., 2011; Curtis et al., 2013) there are likely differences in environmental sensitivities to O_2 across ontogeny. In their neonate to juvenile stages, there is both a greater potential for exposure to hypoxia due to long-term nursery habitat use in affected coastal regions (Heupel & Hueter, 2001; Janicki et al., 2001; Thorburn & Rowland, 2008; Curtis et al., 2011), limited distribution capacity within these nursery habitats and high incentive to avoid predation in more open waters, as well as lower oxygen demand overall (not per kg tissue) compared to larger conspecifics. However, suitability of abiotic factors plays a significant role in habitat selection for these species, and bull and blacktip sharks show strong site fidelity for YOY-juvenile that may range from <1km^2 as neonates to ~5km^2 within the first several month of life (Heupel & Hueter, 2001; Heupel et al., 2004). After birth, bull and blacktip sharks must provide themselves with food enough to support not just maintenance demands, but growth demands in excess of basal needs. Natural mortality within these life stages, as a result of predation, and trial and error of YOY sharks learning to find and capture prey, occurs largely within the first 1-2 months (summer season) after birth (Heupel & Simpfendorfer, 2011). Factors that further limit access to ample food resources or shelter from predators such as habitat loss due to summer hypoxia will contribute to mortality within this critical phase. Given that neonate/YOY for neither species demonstrated strong markers of hypoxia tolerance as in other estuarine species (P_{cMAX}~50%; Seibel & Deutsch, 2020), and the occurrence and persistence of seasonal hypoxia is increasing (IPCC, 2022), hypoxia is likely to have strong habitat compression effects on these species life stages as climate change continues. Because survival of neonate- juvenile individuals is crucial to recruitment and maintenance of adult population (Beck et al., 2001; Gillanders et al., 2003), loss of habitat for these stages poses a bigger threat to the species populations at large.

Conclusions

Results from this study lead me to conclude that buccal pumping (active ventilation) is not a sustainable means of oxygen supply for either species. Though metabolic demands and oxygen consumption are significantly reduced at rest in bull sharks, so too is oxygen supply capacity, leading to an inability to maintain basal metabolic demands and body condition at rest for these species. I do not expect either species to reduce activity below lowest locomotion in support of ram ventilation, or to use buccal pumping under hypoxia as a viable means of tolerance under hypoxia. I identified that a higher oxygen partial pressure is required to sustain resting metabolism under hypoxia, decreasing hypoxic endurance at rest relative to swimming, with both species demonstrating behavioral signs of ventilatory stress below routine swimming. In addition, increased swim speed under hypoxia was not found to be a universal means of improving the hypoxic ventilatory response or hypoxic tolerance for either bull or blacktip sharks.

As such, sharks are likely to vacate portions of nursery habitat and alter distribution if oxygen levels fall below hypoxic limiting thresholds. Based on preliminary comparisons of field data for these species, these critical limiting oxygen thresholds for population substance appear to lie between the critical oxygen level for routine swimming, and the critical oxygen level for the lowest locomotion supporting ram ventilation, an oxygen partial pressure of 8-12 kPa. Though, more data are needed to validate these thresholds. I conclude that these species are not particularly evolved for life in a hypoxic environment, and I expect that as hypoxia within occupied nursery range is predicted increase in severity, frequency and persistence, in months where neonate/YOY of this species are relying on most of these nursery areas (summer), both species are vulnerable to habitat loss.

It is possible that acclimation to hypoxia in addition to temperature may provide greater tolerance to low oxygen (Routley et al., 2002) and allow for greater upregulation of oxygen supply mechanisms. In addition, differences in geological and hydrological makeup of the estuary, differences in history of hypoxic exposure at different nursery locations or latitudes may change the influence of hypoxia across population. Remote O_2 sensing in conjunction with presence/absence will help us to understand with what frequency sharks are moving through hypoxic environments, the length of time of hypoxic exposure, and to ground truth critical oxygen thresholds across activity we have calculated within this study. All require further exploration to better understand oxygen habitat association across the lives of these species.

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Table 9. *Oxygen supply and hypoxic results for bull and blacktip sharks*. All results reported as means ± SE unless otherwise specified.

Species	Blacktip sharks				Bull sharks		
Temperature Bin (°C)	*22*	*26*	*30*	*34*	*22*	*26*	*30*
Oxygen supply capacity (a; mgO$_2$kg^{-1}hr^{-1}kPa^{-1})							
α	35.27±2.02	35.06±2.93	35.79±2.15	35.94±1.65	20.37±1.28	25.72±2.29	28.16±1.67
Maximum oxygen supply achieved under “mixed” behavior or resting behavior (mgO$_2$kg^{-1}hr^{-1}kPa^{-1})							
mixed	NA	NA	NA	NA	16.70±10.01	17.49±3.84	22.43±3.04
rest	NA	NA	NA	NA	13.56±1.64	16.28±1.11	17.55±2.03
Predicted critical oxygen values for mRMR and SMR (Pc; kPa)							
P_{cmRMR}	8.52±1	10.32±1	12.86±0.66	12.36±0.67	13.86±0.69	12.5±1.55	12.53±0.7
P_{cSMR}	NA	NA	NA	NA	8.51±0.89	6.43±1.22	6.74±1.0
Metabolic rate of lowest continuous swimming in hypoxia (below 15kPa; mgO$_2$kg^{-1}hr^{-1})							
Swimming (n)	292.56±30.88	316.02±25.55	403.14±42.01	393.53±29.34	214.55 (1)	210.01±25.51 (2)	12.29±1.3 (4) *270.04±0.84 (2)
critical oxygen values (Pc) for lowest continuous swimming in hypoxia (measured PcmRMR) (below 15kPa; kPa)							
Swimming (n)	10.32±1.73 (5)	10.75±0.57 (7)	12.99±0.57 (6)	10.74±0.73 (7)	9.01 (1)	7.93±0.04 (2)	12.29±1.3 (4) *10.12±0.47 (2)
Highest oxygen supply reached in hypoxia (below 15kPa) across behavior (mgO$_2$kg^{-1}hr^{-1}kPa^{-1})							
Swimming (n)	32.08±3.73 (5)	31.02±4.1 (7)	32.13±2.73 (6)	36.89±2.36 (7)	23.80 (1)	26.62±3.34 (2)	23.57±1.9 (4) *26.75±1.17 (2)
Mixed (n)	NA	NA	NA	NA	12.90±1.57 (2)	13.61±0.18 (2)	13.71 (1)
Rest (n)	NA	NA	NA	NA	NA	8.58 (1)	8.98 (1)
Onset of burst swimming (kPa)							
Burst swim	8.51±1.23	8.52±0.51	9.43±0.64	9.51±0.63	NA	NA	NA

*mean±SE excluding individuals that either quit early before P_{cmRMR} could be reached, or critical measure periods are missing due to elimination of thermal error

Table 10. *Welch's t-test results for oxygen supply capacity during rest and swimming*. Bull represents bull sharks; BT represents blacktip sharks. All P values adjusted via the Bonferroni method. Bold and italicized p values are significant ($p<0.05$)

Species	Comparison	Temperature	df	t statistic	Bonferroni adjusted p-value
Bull	α swim v α rest	22	7	2.84	***0.004***
Bull	α swim v α rest	26	6	3.99	***0.001***
Bull	α swim v α rest	30	9	1.6	***0.049***

Table 11: *Environmental conditions sampled at time and location of C. leucas capture.*

Date	Latitude of Capture	Longitude of Capture	Bottom Type	Depth Range (m)	Surface Temp (˚C)	Bottom Temp (˚C)	Surface Salinity (ppt)	Bottom Salinity (ppt)	Surface DO (mgO2/L)	Surface DO (kPa)	Bottom DO (mgO2/L)	Bottom DO (kPa)
17-Jun-19	27.8563	-82.38894	mud	0.88-1.49	27.7	28.1	7.5	12.9	4.91	13.22	3.4	9.23
27-Jun-19	27.8563	-82.38894	mud	1.07-1.68	31.9	- *	17.1	- *	5.2	15.08	- *	- *
13-July-19	27.8563	-82.38894	mud	1.22-3.35	29.2	29.3	16.3	23.6	5.14	14.23	5.34	14.81
13-July-19	27.85687	-82.38822	mud	1.22-3.35	29.2	29.6	17.3	11.9	6.24	17.28	5.8	16.17
13-July-19	27.85687	-82.38822	mud	1.28	29.4	29.7	14.5	22.7	5.8	16.12	5.98	16.70
13-July-19	27.85687	-82.38822	mud	1.74-4.11	29.9	29.4	13.5	23.5	6.33	17.74	4.97	13.81
13-July-19	27.85687	-82.38822	mud	1.16-1.89	30.2	30.1	12.5	9.2	6.45	18.17	6.67	18.76
22-July-21	27.85803	-82.35353	mud	2.13-2.56	28.6	- *	0.98	- *	5.04	13.4	- *	- *

*YSI malfunction

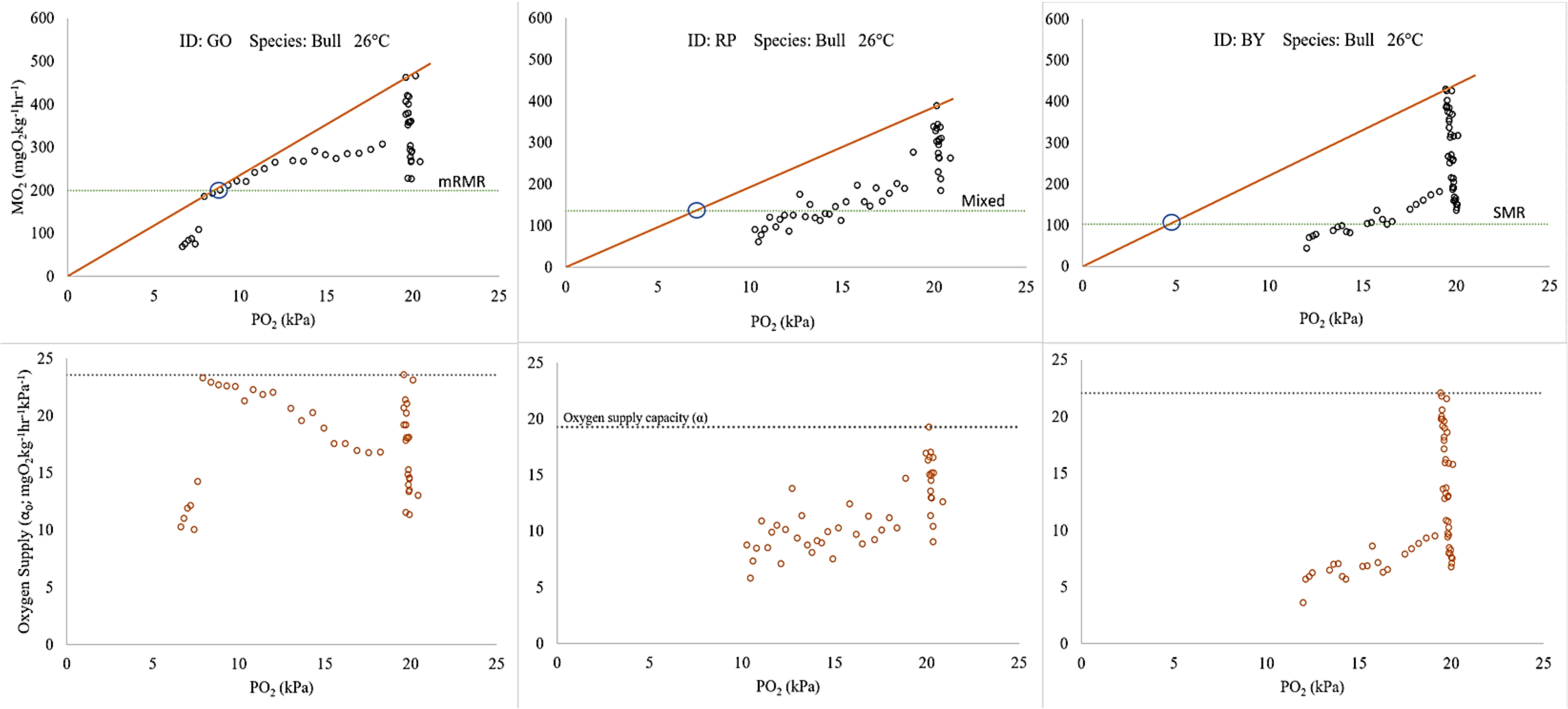

Figure 12. *Individual metabolic rate and oxygen supply across PO_2 for 3 individual bull sharks at 26°C.* Figures A-C depict metabolic rate vs oxygen partial pressure for 3 individual bull sharks at 26°C. Empty black circles represent individual MO_2 measurements from both exercise trials and hypoxia trials. Each individual pictured here exhibit different behavior within hypoxia trials. The individual in plot A swam continuously until failure. The individual in plot B demonstrated a continuous mix of swimming and resting before failure, and figure C shows an individual that rested for the majority of the hypoxia trial. Red lines describe the oxygen-dependency of MMR (MMR = α *PO_2; α-lines) and denote critical oxygen thresholds across the spectrum of activity for each individual. Measured mRMR, mixed and SMR values for each individual are represented by the horizontal green dashed line across PO_2. Blue circles where the α-line intersects the designated metabolic rate (green line) represent predicted critical oxygen threshold for the designated metabolic rate. Figures D-F depict instantaneous physiological oxygen supply (α_0) for each measure across trials for these same individuals in A-C. Each red circle represents MO_2/PO_2 of the measure period. The dashed gray line identifies the oxygen supply capacity, α, for the individual.

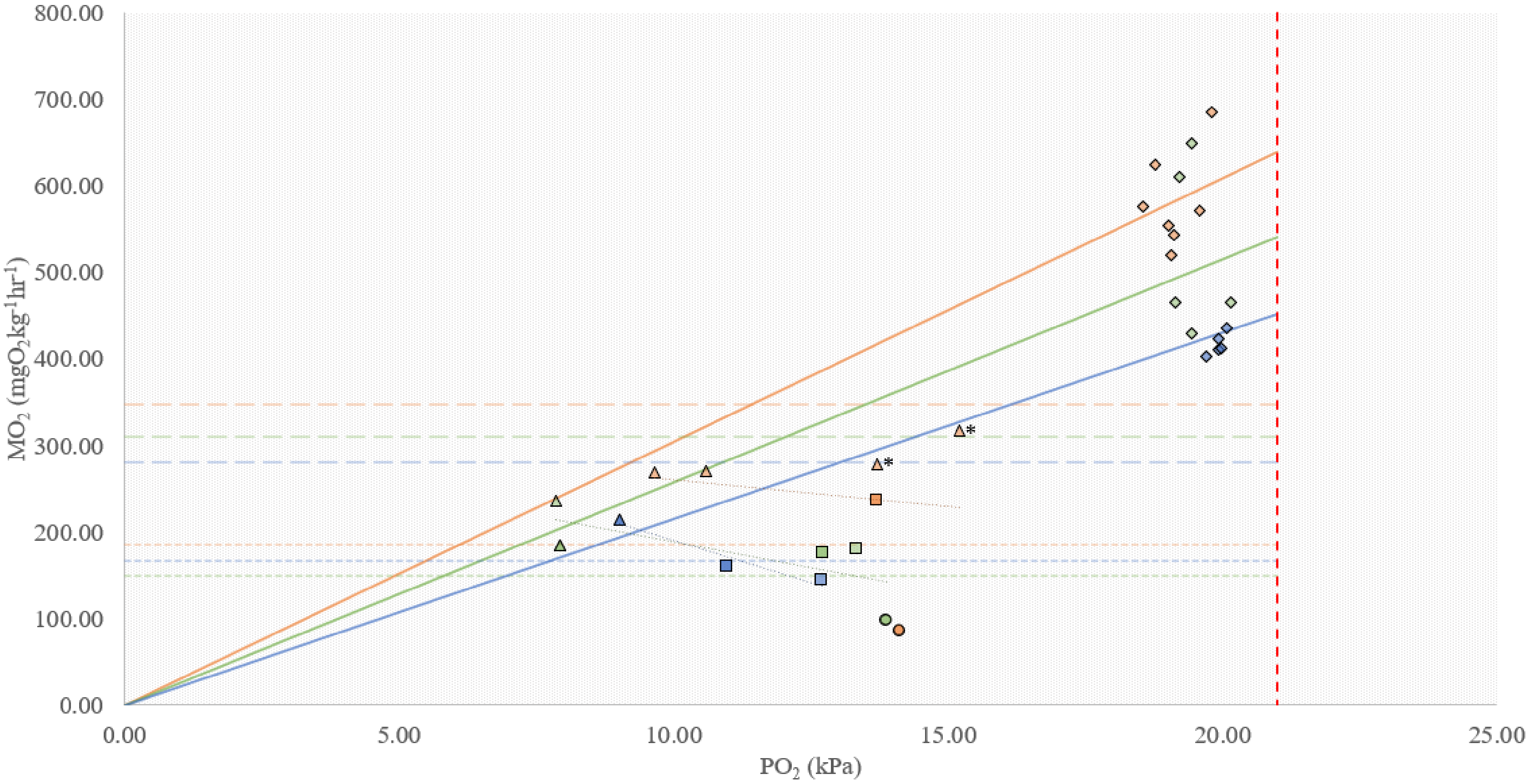

Figure 13. *Hypoxic limitation of metabolic rate in bull sharks.* Different colors represent different temperatures (blue, 22°C; green, 26°C; orange, 30°C), and each point represents a measure from one individual trial. Solid lines describe the oxygen-dependency of MMR (MMR = α *PO_2; α-lines). Triangle points represent continuous swimming measures, filled boxes represent measures where bull sharks demonstrated mixed (resting and swimming) behavior, and filled circles identify where sharks rested continuously. Triangle, square, and circle points represent the MO_2 and PO_2 of the measure period where the highest oxygen supply under hypoxia occurred. Triangular points also correspond to Pc of minimum locomotive rate, however, triangular points with an asterisk next to them indicate individuals that either quit early before P_{cmRMR} could be reached, or critical measure periods are missing due to elimination of thermal error. Diamond points indicate individual measures of MMR within each temperature bin. The PO_2 at which each mRMR (large dashes) or SMR line (small dashes) intersects the corresponding solid α-line of the same temperature, is the P_c for that rate.

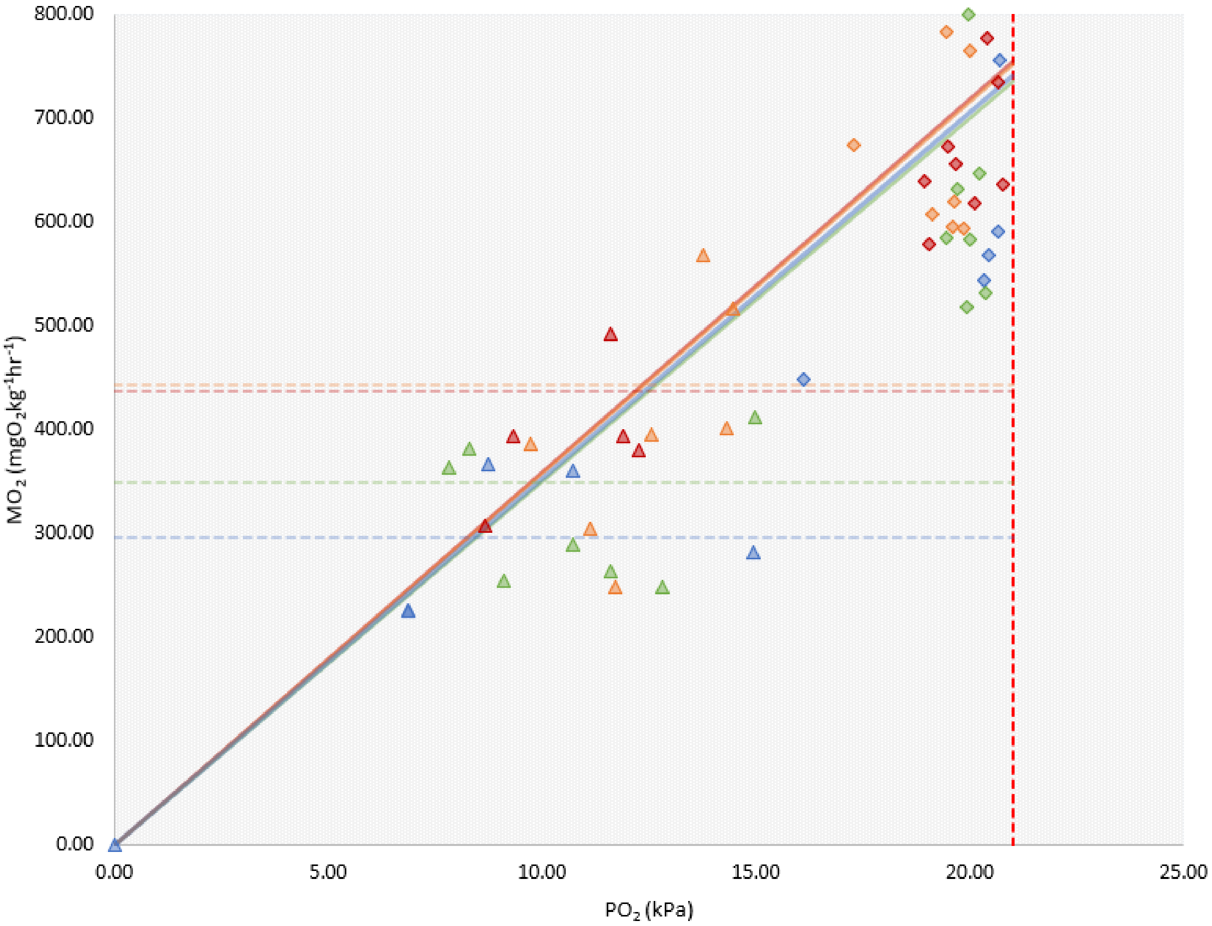

Figure 14. *Hypoxic limitation of metabolic rate in blacktip sharks.* Triangles represent the Pc of minimum locomotive rate measured in hypoxia (below 15 kPa). Diamonds indicate individual measures of MMR within each temperature bin. Horizontal dashed lines indicate the mRMR for each temperature. The vertical red line is the P_{cmax}. Solid lines describe the oxygen-dependency of MMR (MMR = α *PO_2; α-lines). Different colors represent different temperatures (blue, 22°C; green, 26°C; orange, 30°C; red, 34°C), and each point represents a measure from one individual trial. The PO_2 at which each mRMR line intersects the corresponding solid α-line of the same temperature, is the P_c for that rate.

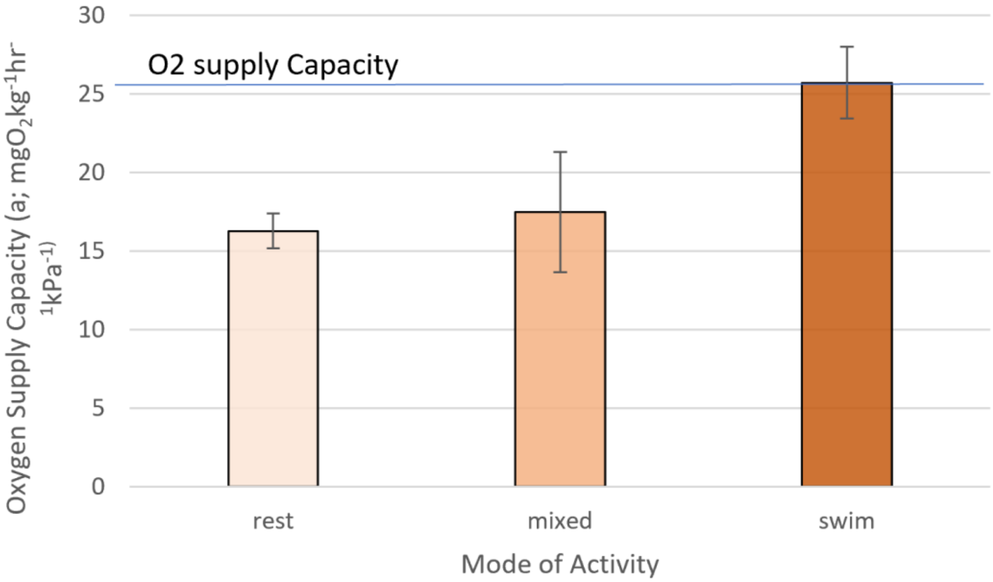

Figure 15. *Oxygen supply capacity by behavior in bull sharks*. Each bar graph represents the mean of the highest oxygen supply reached under a given behavior for bull sharks at 26˚C with error bars representing standard error.

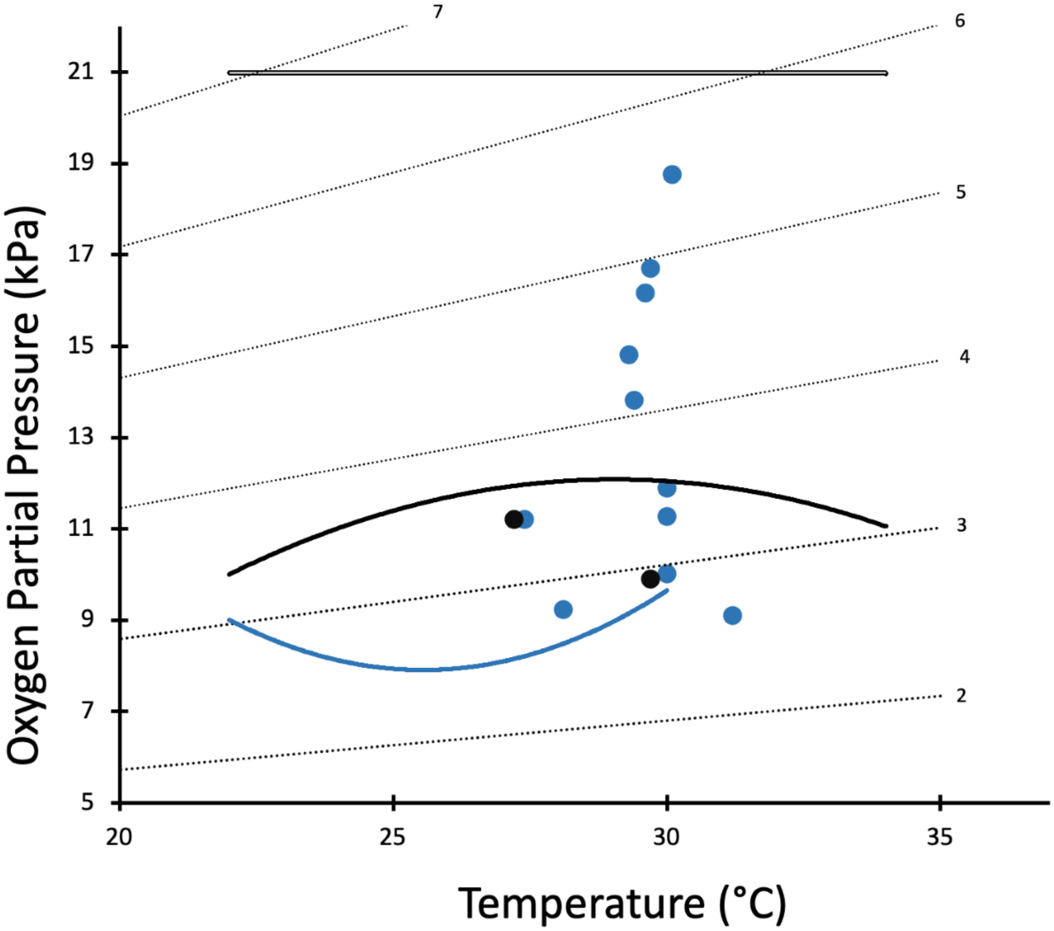

Figure 16. *Theorized population limiting oxygen thresholds*. Different colors indicate different species. blue represents bull sharks and black represents blacktip sharks. The blue curve indicates the calculated critical oxygen limit for lowest swimming in bulls , and the black line indicates calculated P_{cmRMR} in blacktips. The gray line at the top of the figure indicates a P_{cMAX} of 21 kPa (independent of temperature). The distance between the P_{cMAX} line and the curved lines represent functional aerobic scope, changing in opposite directions with temperature according to species. Circular points represent environmental data from individual fishing sets where sharks were captured. Blue points were sampled in 2019 and 2021 from the Alafia river. Black and gray points represent data from Gardiner and Wiley (unpublished) for YOY and juvenile representing the lowest oxygen level sharks were captured at within Terra Ceia Bay or Manatee river, FL. Black points represent blacktip lowest O_2 and gray points represent bull shark lowest O_2 from that study. Dashed gray lines represent the change in oxygen concentration across temperature.

APPENDICES

Appendix A: Pc methods

For comparison with more traditional methods of critical oxygen thresholds, P_{cSMR} was also calculated using a break-point method. Using SMR determined as described in the main text methods, we calculated P_{cSMR} using a breakpoint method via the *calo2crit* package (Claireaux and Chabot, 2016). A regression line ($MO_2 = mPO_2 + b$) was then fitted through all MO_2 values in the hypoxia trial that fell below the minimum MO_2 value contributing to SMR, (i.e., the oxygen conforming section of the curve). P_{cSMR} was then assigned to the PO_2 at which the "oxygen conforming" regression line intersected SMR (Claireaux and Chabot, 2016).

We then compared the resultant P_{cSMR} values from the α-method (Seibel et al, 2020; see methods section) and the breakpoint method of Pc determination. The α-method is inherently more conservative than other methods. Accordingly, in 83% of trials, P_{cSMR} was lower using the α-method than the breakpoint method, however, estimates of P_{cSMR} were still considered comparable between the two methods at all temperatures except 23°C (Welch's t-tests, Holm correction: 23°C, *t*(9.88) = 5.65, *HB alpha*= 0.001, *p-value* = 0.00022). As in the α-method, P_{cSMR} calculated using the break-point method also demonstrated a general increasing trend with temperature increasing from 4.04 ± 0.1 kPa to 6.24 ± 0.1 kPa between 10 and 21°C, with a non-significant decline to 6.05 ± 0.08 at 23°C, and a thermal sensitivity slightly lower than P_{cSMR} via the α-method ($E = 0.29$; $Q_{10} = 1.37$; ST 4). While the two methods produced comparable results, the α-method was used in our study because it is a direct measure of α, regardless of trial type it is measured in, which reveals the critical PO_2 for any metabolic rate, not just SMR .

Appendix B: The effect of pseudo replication

In this study, it was necessary to reuse animals in good health after the appropriate recovery period (see methods section), due to limited access to further *S. acanthias* collection, to minimize the number of individuals obtained for captive experiments, and due to some subject attrition in the lab. Individuals were only tested ("run") once per temperature bin, with no "repeat" measures within a target temperature (within-bin independence). No individual was run at more than 3 temperatures across the study. For individuals that completed both trial types, the MMR and hypoxia trial at that temperature was counted as a single "run" as trials were run consecutively and the animal was not removed between trials. Of the total 104 runs across temperature, 59.62% (n=62) trials were first runs, 37.5% (n=39) were second runs, and 2.88% (n=3) were tertiary runs. We acknowledge that some samples lack temporal independence across temperature bins. To identify if random error due to individual variation in metabolism, and using recovered animals (temporal non-independence), had a significant impact on the trends of metabolic traits across temperature, we ran several tests.

When modeling SMR and RMR, mixed effects models using the lme4 package (Bates, Maechler & Bolker 2012) were easily overparameterized and often resulted in singular fits due to uneven sampling and small effect sizes of "individual" and "run". Similarly non-linear mixed effects model using nlme (Pinheiro et al., 2013) for MMR and alpha also produced singular fits when random effects for individuals and run were included. To explicitly determine if there was an effect of "run" on SMR or RMR, ANCOVA models were run with temperature and "run"(factor), which resulted in insignificant effects of "run" (RMR: $F_{2,100}$=0.10, p=0.90; SMR: $F_{2,31}$=1.01, p = 0.37). "Run" also had an insignificant effect on alpha, MMR (ANOVA, alpha: $F_{3,11}$ = 0.85, p = 0.46, MMR: $F_{2,97}$=0.0787 p=0.92).

To affirm that pseudo replication due to repeated runs did not affect our results, we subset the data for each metabolic metric to only include the first chronological trial for each individual. We then modeled metabolic relationships (each metric according to the "Methods" section), using only the first-run data, which we refer to as the "first-run" model. However, this model was not appropriate for comparison alone, since most secondary and tertiary runs occurred at the higher temperatures as a result of stepwise acclimation (10C: 100% run 1; 13C: 82.35% run 1, 17.65% run 2; 17C: 76.19% run 1, 23.81% run 2; 21C: 34.78% run 1, 65.22% run 2; 23C: 9.52% run 1, 76.19% run 2, 14.29% run 3), skewing the "first run" data to lower temperatures, and decreasing or eliminating sample sizes for the higher temperatures.

To explicitly remove pseudo replication due to multiple runs within an individual, we subsampled the data so that each individual only contained a single measurement. For individuals with multiple runs, we randomly selected only a single run (run#: 1, 2, or 3) for inclusion and discarded its other runs. From this subsampled data we ran the same nonlinear regressions to generate a model output. This process was repeated 10,000 times allowing us to produce a 95% confidence interval if each individual only contained a single run. The "first run" model and the complete model, inclusive of all runs across all individuals and temperatures, were then graphically compared to this generated CI. The complete model (including measurements from all individuals) fell within the simulated CI. However, the "run 1" model(only individuals first run) deviated outside the confidence intervals at higher temperatures, likely due to decreased sample size in the "first run" data at high temperatures. Except for >22 C, the two models generated output values within 5% of each other. Thus, including individual subsequent runs increased sample size and thus precision especially at higher temperatures. We determined the amount of variation reused individuals introduced to the data was negligible, and proceeded with further analysis of metabolic traits, modeling without random effects.

Appendix B: The effect of pseudo replication

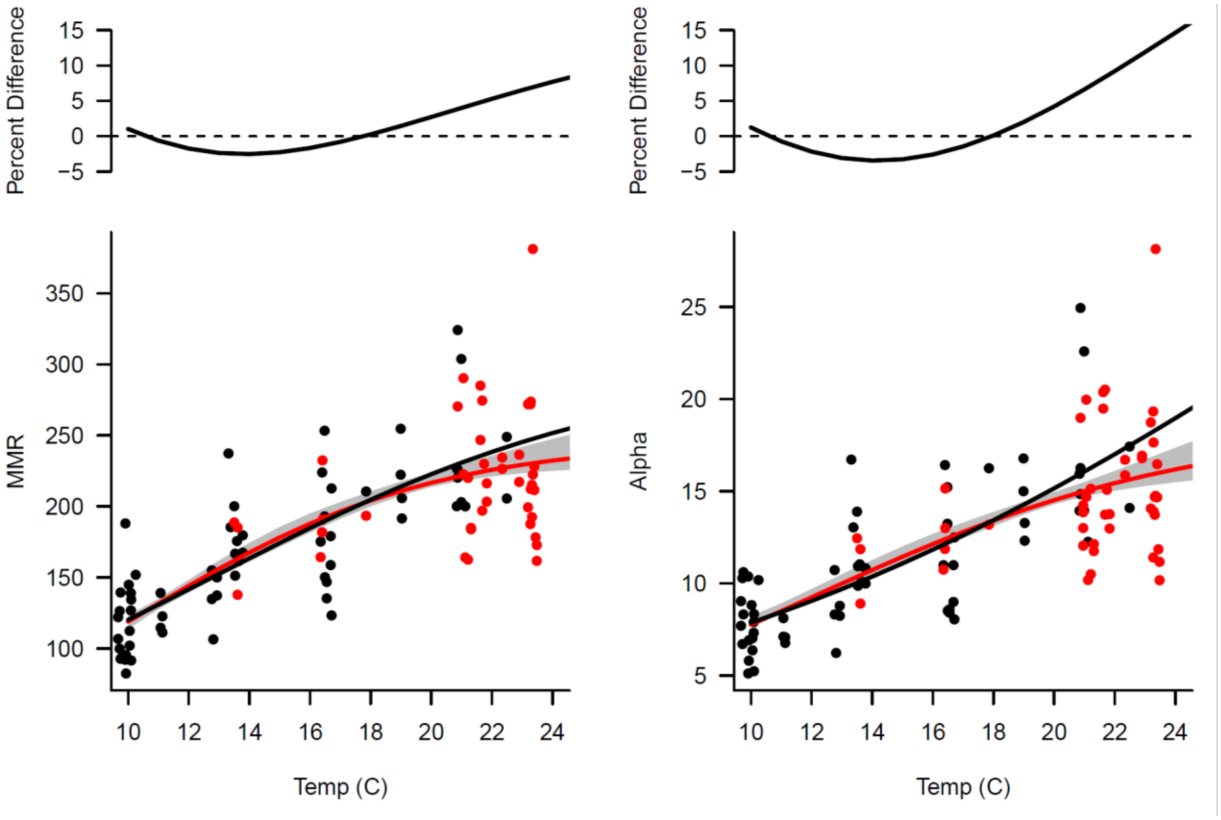

Figure A1. *Exploration of the effect of pseudo replication.* (B and D) Of the total 104 runs across temperature, 59.62% (n=62) trials were primary runs (trials) (individual points in black), 37.5% (n=39) were secondary runs, and 2.88% (n=3) were tertiary runs (second and tertiary runs represented by red points). The solid black line represents the "first-run" model, a nonlinear least squares regression (logistic) of MMR and alpha on temperature for the first chronological trials of individuals. The solid red line represents the "null" model, a nonlinear least squares regression (logistic) of MMR and alpha on temperature, inclusive of all trials regardless of run number. The 95% confidence interval (gray band) was calculated by completing 10,000 repetitions of 1) randomly selecting only a single run (run#: 1, 2, or 3) from each individual, and 2) running the same nonlinear regressions to generate a model output. The "first-run" model only deviated outside the confidence intervals at higher temperatures, likely due to decreased sample size in the "first run" data at high temperatures (10C: 100% run 1; 13C: 82.35% run 1, 17.65% run 2; 17C: 76.19% run 1, 23.81% run 2; 21C: 34.78% run 1, 65.22% run 2; 23C: 9.52% run 1, 76.19% run 2, 14.29% run 3). **(A and C)** Percent difference between the first run model and the null

Appendix C: Mass correction

Table A1. *Spearman mass correlation coefficients and calculated mass scaling coefficients (b) for relevant metabolic metrics. b*-values were calculated using the *calc_b respirometry package* (Birk 2017) using Equation 2, within each designated temperature bin. B-values were used to correct metabolic metrics to the

Temp Bin (°C)	*SMR*	*RMR*	*MMR (measured at trial PO2)*	*MMR (at 21kPa)*	*α*
Spearman Mass Correlation Coefficients					
10	-0.24	-0.36	-0.52	-0.75	-0.75
13	0.29	-0.63	-0.71	-0.61	-0.61
17	-0.83	-0.55	-0.33	-0.34	-0.34
21	-0.33	-0.33	-0.72	-0.59	-0.59
23	-0.49	-0.43	-0.63	-0.61	-0.61
Mass Scaling Coefficients (b)					
10	-0.81	-0.47	-0.46	-0.57	-0.57
13	0.04	-0.30	-0.58	-0.53	-0.53
17	-0.75	-0.53	-0.26	-0.34	-0.34
21	-0.46	-0.21	-0.53	-0.50	-0.50
23	-0.43	-0.26	-0.50	-0.47	-0.47

Appendix D: Q10 thermal sensitivities

Table A2. *Q_{10} values for metabolic metrics across all temperature comparisons*. Q_{10} values represent a measure of temperature sensitivity and describes for every 10°C rise in temperature, the concomitant rise in the metabolic metric. Q_{10} values were calculated using Eq 4, and modeled metric means at each given temperature. Values highlighted in dark grey represent the values calculated between modeled averages at 10°C and 23°C, i.e., the Q_{10} values chosen to represent temperature sensitivity across the full thermal range tested.

Metabolic Trait	***Q10 10-13℃***	***Q10 10-17℃***	***Q10 10-21℃***	***Q10 10-23℃***	***Q10 13-17℃***	***Q10 13-21℃***	***Q10 13-23℃***	***Q10 17-21℃***	***Q10 17-23℃***	***Q10 21-23℃***
SMR	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25	2.25
MMR	2.48	2.05	1.76	1.66	1.78	1.55	1.47	1.35	1.29	1.19
MMR (21kPa)	2.32	2.04	1.82	1.73	1.86	1.66	1.59	1.49	1.43	1.31
AAS	2.55	1.99	1.62	1.47	1.66	1.36	1.25	1.12	1.04	0.88
AAS (21 kPa)	2.33	2.00	1.73	1.62	1.78	1.55	1.46	1.35	1.27	1.12
FAS	1.10	0.91	0.78	0.74	0.79	0.69	0.65	0.60	0.57	0.52
FAS (21 kPa)	1.03	0.91	0.81	0.77	0.83	0.74	0.70	0.66	0.63	0.58
α	2.32	2.04	1.82	1.76	1.85	1.66	1.62	1.49	1.48	1.46
P_{cSMR}	1.29	1.29	1.29	1.29	1.29	1.29	1.29	1.29	1.29	1.28

Appendix E: Comparison of standard metabolic rates

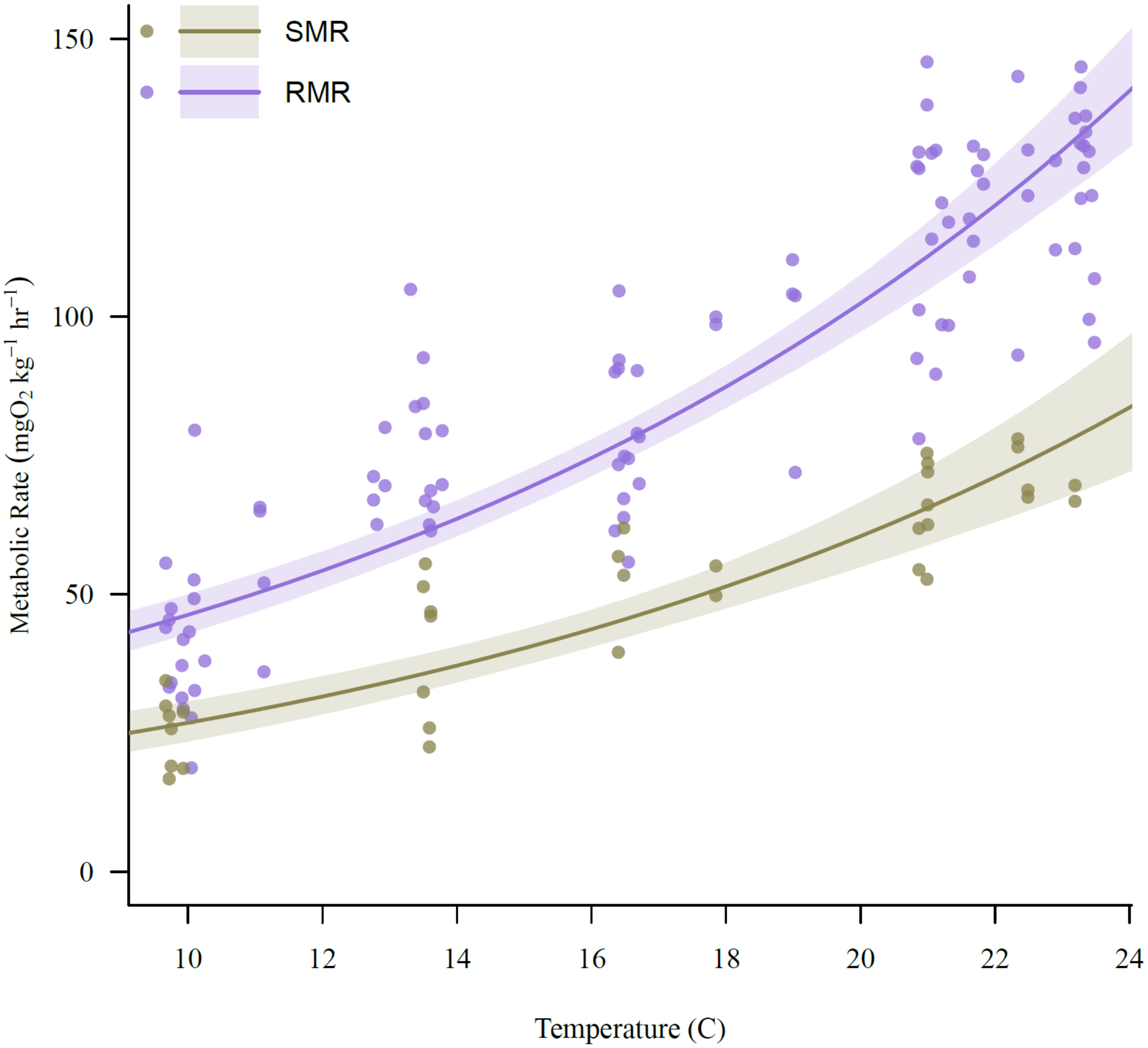

Figure A2. *Standard metabolic rate (SMR) and resting metabolic rate (RMR).* Resting metabolic rate (RMR), represented by the color purple, was deemed as the $q_{0.2}$ within the 22-hour exercise trial (SMR <22hours) and SMR, represented by the olive-green color, was deemed as the $q_{0.2}$ of points prior to oxygen limitation in hypoxia trials (SMR >22hours). Filled circles represent individual direct measures. Solid lines represent exponential models of metabolic metrics, and shaded regions surrounding models represent 95% confidence intervals. Temperature sensitivities of resting and standard metabolic rates: RMR: E = 0.57; Q_{10} = 2.23; SMR: E = 0.61; Q_{10} = 2.25. SMR was between 53.6-66.8% of RMR across temperature.

Appendix F: Mass scaling of MMR, RMR and SMR

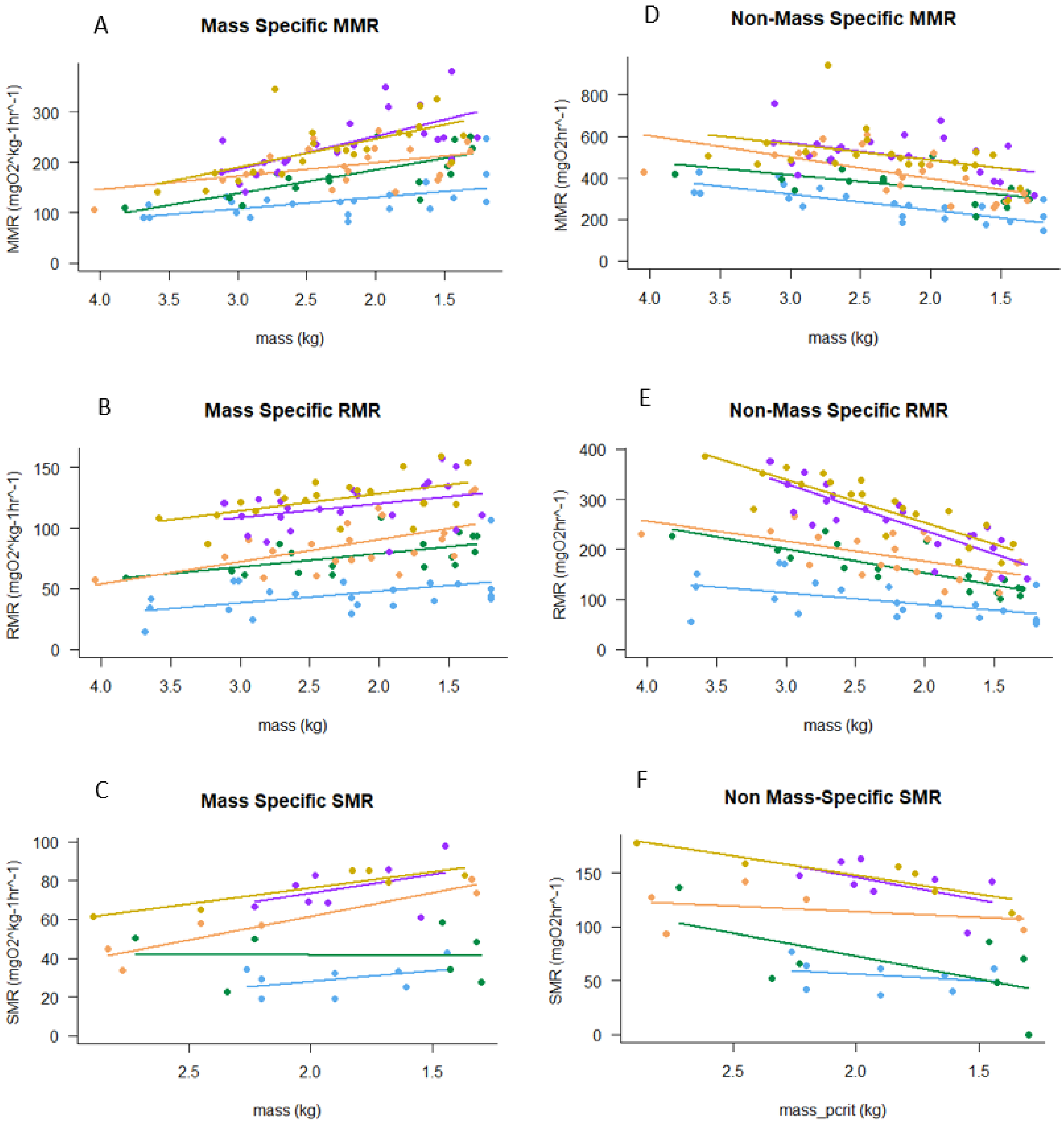

Figure A3. Mass scaling of MMR, RMR and SMR, both mass-specific (A-C), and non-mass-specific (D-F) prior to mass correction. Different colors represent different target temperature bins, and colors are consistent throughout the figure, with 10°C represented in blue, 13°C in green, 17°C in orange, 21°C in purple, and 23°C in gold.