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Ecophysiological responses of fishes to increasing ocean acidification and warming

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ECOPHYSIOLOGICAL RESPONSES OF FISHES TO INCREASED OCEAN ACIDIFICATION AND WARMING

by

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ABSTRACT

A major goal in conservation biology is to understand the effects of short and long term environmental change on organisms. Fishes are the most valuable marine resource, however very little is known about the synergistic effect of current ocean warming and acidification, and the role of body size and local adaptation on their resilience.

There is growing evidence that increased environmental temperature correlates with a reduction in ectotherm body size, suggesting a universal response to warming. To investigate the potential advantage of small body size in fish resilience, I made intra- and inter-specific comparisons of dwarf- and normal-size cleaner gobies of the genus *Elacatinus*. I first tested the hypothesis that smaller body size would correlate with a wider thermal tolerance by using same-age but different-size gobies reared at 'common garden' conditions. By employing critical thermal methodology, I provided empirical evidence supporting thermal biology theories that predict wider thermal tolerance windows as body size shrinks. These results provided the motivation to examine the

effect of body mass on digestive performance, an indicator of fitness. Only smaller fish increased digestive metabolic scope at higher temperatures, thus suggesting that temperature increase caused by global warming will favor smaller individuals.

To investigate the role of local adaptation on resilience in climate change, I compared the responses to warming and acidification between latitudinally- and morphologically-distinct populations of the little skate *Leucoraja erinacea*, by focusing on the most vulnerable life stages, embryos and juveniles. Embryos maintained at common garden conditions showed countergradient variation in performance curves. In juvenile skates, post-exercise metabolic curves shifted performance optima, exhibiting thermal adaptation in the two populations examined. This suggests that as skates hatch and are able to thermoregulate, they can change their temperature optima to exploit local thermal environments. Lastly, temperature and acidification levels predicted by the end of the century may reduce fitness of the northern population of skates, thus increasing vulnerability to local extinction.

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List of Abbreviations

°C	degree Celsius
ANCOVA	analysis of covariance
ANOVA	analysis of variance
CH	Cape Hatteras
CO_2	carbon dioxide
CTM	critical thermal methodology
CTMax	critical thermal maximum
CTMin	critical thermal minima
D:L	dark:light
E. lobeli	*Elacatinus lobeli*
E. oceanops	*Elacatinus oceanops*
et al.	*et alii*
g	gram
GB	Georges Bank
GM	Gulf of Maine
GoM	Gulf of Maine
L	liter
L. erinacea	*Leucoraja erinacea*
LOE	loss of equilibrium

m	meter
MA	Mid-Atlantic
min	minute
mm	millimeter
MO_2	oxygen consumption rate
ppm	parts per million
ppt	parts per thousand
s.e.m	standard error
SD	standard deviation
SDA	specific dynamic action
STL	standard length
Tmax	maximum temperature
Tmin	minimum temperature
Topt	thermal optimum
Tpejus	thermal pejus
WWC	Wee Wee Caye
WWM	wet weight mass

Introduction

A. Ocean warming and acidification

Since the industrial revolution, global atmospheric carbon dioxide (CO_2) concentrations have increased from 280ppm to about 400ppm (in 2014), thus reaching levels that have not experienced in the past 800,000 years (Lüthi *et al.* 2008). Two major consequences of the increase in atmospheric CO_2 concentration are a rise in ocean temperature and acidification (i.e. lower pH). During the next century, global ocean temperatures are projected to increase about 6°C globally, although the highest change is predicted for the highest latitudes (IPCC 2013). In forecasting the consequences of global warming on marine fauna, researchers have analyzed the environmental changes that caused the mass extinction of benthic fauna during the Pliocene-Eocene Thermal Maximum (Gingerich 2006; Marsili 2008; Jaramillo *et al.* 2010; Speijer *et al.* 2012). During this period, global temperatures increased by about 5-6 ºC in 20,000 years (Jaramillo *et al.* 2010; Speijer *et al.* 2012). A similar magnitude of ocean temperature increase is projected to occur by year 2100 (IPCC 2013). As a result, the current anthropogenically-induced temperature increase is projected to be 2,000 times faster than the warming occurred during the Pliocene-Eocene Thermal Maximum.

In addition to increase warming, approximately 30% of CO_2 in the atmosphere is transferred to the oceans where it reacts with water to form carbonic acid which dissociates into carbonate and bicarbonate, thus releasing hydrogen ions that reduce water

pH. The increase in CO_2 has already reduced ocean pH by 0.1 units from pre-industrial values and it is expected to further decrease ocean pH by 0.4 units by the end of the century as atmospheric levels of CO_2 reach 1100ppm according to the model RCP 8.5 (Meinshausen, Raper & Wigley 2008; IPCC 2013). Although some fluctuation in pH is natural in upwelling areas, this may be exacerbated by anthropogenic increases in CO_2 (Melzner *et al.* 2009).

B. Effect of ocean warming on fishes

1. Temperature as an ecological resource

Temperature is a key component of a fish' habitat and it defines, with other resources such as food, its multidimensional niche (Magnuson, Crowder & Medvick 1979). Fish thermal niches are defined by tolerance limits as well as optima (Angilletta & others 2002), which can vary depending on ontogenetic and physiological processes (Beitinger *et al.* 1975; Magnuson *et al.* 1979). Changes in the preferred temperature or thermal tolerance limit during ontogeny or as a result of environmental conditions in some fishes can produce partitioning of thermal resources in time and space between age and size classes, and sexes. For instance, gravid elasmobranchs are known to prefer significantly warmer temperatures than non-gravid females and males (Wallman & Bennett 2006) thus creating segregation between females close to parturition and the rest of the population (Hight & Lowe 2007). In post-glacial lakes, dwarf and normal-size coregonoid fishes show niche separation based on temperature (Ohlberger *et al.* 2008).

Even individual fish are known to shift their diel preferred temperature to enhance a particular physiological process such as swimming or digestion (SIMS *et al.* 2006; Di Santo & Bennett 2011a; Pang, Cao & Fu 2011).

Competition for the preferred thermal resource is therefore comparable to that for food resources (Magnuson *et al.* 1979). In fact, larger dominant individuals often exploit the optimal temperature range, excluding subordinates. In a classic study, Beitinger and Magnuson (1975) showed that small bluegills placed in a thermal gradient actively chose to spend most time at 31°C if alone. However, if a dominant male was introduced in the aquarium, it would occupy the preferred temperature space (31°C), while the smaller males shifted to sub-optimal lower or higher temperatures (Beitinger & Magnuson 1975).

In a bioenergetics sense, temperature has a *quantitative* influence on physiological processes. A fish shuttling between temperatures can reduce or increase the amount of calories expended and can enhance specific processes as the body temperature reaches equilibrium with the ambient temperature (Fry 1971; Di Santo & Bennett 2011b). As ocean temperatures increase as a consequence of climate change, we might expect fish to adjust to the thermal environment through physiological acclimatization (Somero 2010; Donelson *et al.* 2011), adaptation (Angilletta, Oufiero, C. E & Sears, M. W 2004; Baumann & Conover 2011), or behavioral thermoregulation (Perry *et al.* 2005; Greenstein & Pandolfi 2008).

2. Responses of fishes to global warming

Ocean warming is already affecting demography, geographic range and biological timing of populations and species (Murawski 1993; Walther *et al.* 2002; Perry *et al.* 2005; Parmesan 2006; Dulvy *et al.* 2008; Chen *et al.* 2011). Demographic changes include shifts in recruitment, body size and survival (Pörtner & Farrell 2008; Genner *et al.* 2010; Gardner *et al.* 2011). Geographic shifts includes those toward the poles (Walther *et al.* 2002; Perry *et al.* 2005; Beaugrand *et al.* 2008; Chen *et al.* 2011), and phenological changes include anticipating the timing of spawning and migrations (Farrell *et al.* 2008; Martins *et al.* 2011a; Eliason *et al.* 2011), causing a mismatch between prey and predator timing (Raubenheimer, Simpson & Tait 2012).

Shifts in geographic ranges – Warming is thought to expand species ranges toward higher latitudes or deeper waters, to find suitable thermal refugia (Walther *et al.* 2002; Parmesan 2006; Burrows *et al.* 2011). However, whenever migration or dispersal capacities are limited, or surrounding habitats are unsuitable, it is expected to cause widespread extinctions (Dulvy *et al.* 2008). Because the response to temperature increase is likely to be species-specific, ocean warming may influence the distribution of species.

Local extirpation of ecologically important organisms such as keystone species have the potential to destabilize ecosystem functioning (Rossi *et al.* 2013). It has been suggested that species that exhibit faster generation times will be able to respond more rapidly to environmental changes either through adaptation or by shifting geographic ranges towards more suitable habitats (Somero 2010). In a large-scale study that looked

at the effect of long-term temperature increase (0.6°C over 40 years) on 90 species of demersal fishes, Perry and co-authors (2005) found that about 60% of the species taken under consideration in the North Sea had showed a directional shift towards cooler waters, either by moving poleward or to deeper water. Shifting species tended to be smaller in body size, and to reach maturity faster (Perry *et al.* 2005). Given these trends, it is perhaps not surprising that it has been hypothesized that smaller individuals may have an advantage in warming oceans (Daufresne, Lengfellner & Sommer 2009; Clark *et al.* 2012).

Changes in phenology – Phenology, the study of yearly cyclical life events such as the timing of migrations and spawning, have been useful to detect early responses of fishes to environmental change, in particular warming (Hughes 2000; Edwards & Richardson 2004). Because ecosystem functioning depends on synchronization of timing between functional groups at different trophic levels, and the response to warming is likely to be species-dependent, mismatch of phenological events has the potential to modify interactions at the ecosystem-level (Edwards & Richardson 2004).

Seasonal changes in temperature have important consequences for timing of migration and spawning in fishes. To synchronize food acquisition and reproduction with seasonal surge of food, many species use temperature and photoperiod as cues (Cushing 1990; Sims *et al.* 2004). Warming has already accounted for an earlier start of migration in fishes (Martins *et al.* 2011b; Eliason *et al.* 2011). If fishes start spawning or migrating sooner because of early warming, but their prey follow different cues (i.e. photoperiod),

there is a high risk that the fitness of many species will be reduced because of a mismatch between energy requirements and available supply. In fact, according to the "match-mismatch" hypothesis, fish fitness and survival depends on the precise offset timing between the peaks of abundance of food and spawning (Mertz & Myers 1994). Many ecologically important species such as the Pacific herring have a temperature-dependent gonadal development so they spawn and migrate earlier with warming (Ware & Tanasichuk 1989).

Reduced body size – The size of fishes is affected by a number of biotic and abiotic factors, such as dominance, prey size and availability, temperature, and oxygen (Beitinger & Magnuson 1975; Jobling 1981; Cheung *et al.* 2012). A correlation between increased temperatures and a reduction in body size has been observed across taxa, suggesting a 'third universal response to warming' (Gardner *et al.* 2011). Because body size affects energetic requirements for growth and reproduction, a widespread shift in mass is likely to affect the stability of marine ecosystems in projected climate change. Although rapid evolution of morphological traits in response to other climate-related stressors (i.e. drought) have been observed in terrestrial organisms (such as a Galapagos finch; Gardner *et al.* 2011), we still have no empirical evidence that a reduced body size is driven by an increase in temperature. It is possible that a decrease in body mass may represent a plastic response to climate related or indirect stressors such as food availability, habitat loss and chronic stress that can reduce growth, reproduction and resistance to diseases (Gienapp *et al.* 2008; Huusko *et al.* 2011). Although, until recently plastic responses were not considered a possible long-term solution to unidirectional

change in the environment, recent studies have proposed that transgenerational acclimation to warming may increase performance of offspring of fishes exposed to increased temperature (Ho & Burggren 2009; Donelson *et al.* 2011; Grossniklaus *et al.* 2013).

Cheung and co-authors (2012) modeled the effect of temperature increase expected by year 2050 on 610 species of demersal fishes around the world and found that even a small increase in temperature, accompanied by a minimal decrease in dissolved oxygen, will reduce assemblage size of fishes by 14-24%. The reduction in body size was produced by an individual decrease in body mass (physiological response) and a shift in fish assemblages (behavioral response) that are likely to destabilize current marine ecosystems (Cushing 1990; Perry *et al.* 2005; Cheung *et al.* 2012).

C. Effect of acidification on fishes

Most studies on ocean acidification have investigated the effects of increased CO_2 on calcifying marine invertebrates and just a few studies have analyzed the effect on fishes (Hofmann *et al.* 2010; Yu *et al.* 2011; Gaylord *et al.* 2011; Bignami *et al.* 2013; Chambers *et al.* 2013; Byrne & Przeslawski 2013). Most of the studies of fishes were obtained by exposing adults to unrealistically high levels of hypercapnia (blood CO_2 concentrations of about 10,000ppm or higher; Choe & Evans 2003) to induce a physiological response (Graham, Turner & Wood 1990; McKendry, Milsom & Perry 2001; Hayashi, Kita & Ishimatsu 2004; Brauner & Baker 2009) because adult fishes are

very efficient in regulating acid influxes in their body and are therefore considered to be able to counteract current rates of ocean acidification (Claiborne, Edwards & Morrison-Shetlar 2002).

On the other hand, ocean acidification has been shown to have a quantifiable negative effect on early life stages of fishes, such as embryos. Increased acidification decreased survival and growth in embryonic, larval and juvenile fishes (Baumann, Talmage & Gobler 2011; Miller *et al.* 2012). Beside these two studies, acidification has been shown to have a sub-lethal effect on the species examined, causing significant changes in physiological processes such as metabolism, aerobic performance and growth (Doney *et al.* 2009; Rummer *et al.* 2013), and in behavioral and sensory responses such as learning, vision and homing (Munday *et al.* 2009; Simpson *et al.* 2011; Ferrari *et al.* 2012; Nilsson *et al.* 2012). In addition, otolith size and asymmetry increase with increasing CO_2 showing a different response than marine invertebrates (Checkley *et al.* 2009; Bignami *et al.* 2013), but the consequences on fish performance (for example locomotion) are yet to be investigated (Munday *et al.* 2011; Munday, McCormick & Nilsson 2012). Additionally, acidification can alter tissue morphology, health (Chambers *et al.* 2013), and body condition (Franke & Clemmesen 2011; Miller *et al.* 2012). In some cases, acidification is even found to enhance growth, swimming and development in some fishes (Simpson *et al.* 2011; Rummer *et al.* 2013), suggesting that the response to increased CO_2 will likely be age and species-specific.

Studies that look at metabolic costs of ocean acidification aim to quantify the overall costs associated with maintaining homeostasis at low pH. As elevated CO_2 concentrations in blood reduce pH, fish synthesize bicarbonate and eliminate H ions to maintain homeostasis. Because ATP is required to pump out hydrogen ions, the fish incurs metabolic costs in maintaining acid/base balance (Claiborne *et al.* 2002; Pörtner, Langenbuch & Reipschläger 2004; Melzner *et al.* 2009). An increase in metabolic costs to maintain homeostasis may reduce individual fitness especially if the effects of changes in pH are chronic as expected in projected climate change. This may result in reduced energy for growth, with consequences for long-term survival (Baumann *et al.* 2011; Miller *et al.* 2012). If fishes grow more slowely and, as a consequence, mature later, they spend an extended time at a non-reproductive, vulnerable life stage (juvenile and sub-adult). This could ultimately slow down population growth due to a reduced number of reproducing adults.

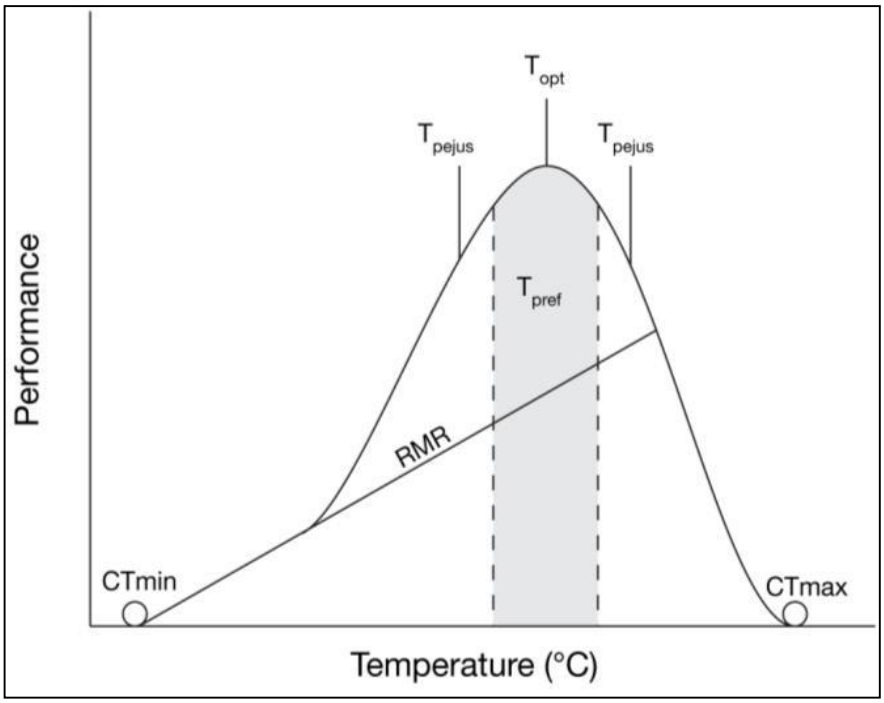
D. Reaction norms

Physiological responses to changes in the environment are best described by phenotypic plasticity through analysis of *reaction norms* (also known as *performance curves*; Angilletta *et al.* 2004; Pörtner & Farrell 2008). A reaction norm defines the relationship between a continuous environmental variable (most commonly temperature) and a performance trait (for example, growth, digestion, reproduction, locomotion) (Baumann & Conover 2011). Plasticity in this case is defined as the slope (or derivative)

of a reaction curve (Figure I1). If the slope of the curve is zero then the particular physiological process is "insensitive" to temperature (Angilletta 2009). A thermal performance curve is delimited by critical thermal maxima and minima (CTMax and CTMin), and a thermal optimum (peak of performance) (Figure I1). Although protein denaturation seems to represent the maximum temperature that can be tolerated by organisms at the biochemical level, the limit of performance (or lethal limit) at the organismal level occurs much earlier at lower temperatures, and seems to be imposed by oxygen limitation (oxygen-limited-thermal-tolerance hypothesis, or OLTT hp) (Pörtner 2001; Fields, Rudomin & Somero 2006). The OLTT hypothesis states that as temperature increase beyond an optimal range (thermal optimum) there is a mismatch between oxygen demand at the tissue level and the capacity for delivery of adequate oxygen supply by the cardio-respiratory system (this temperature range is defined as *thermal pejus*) (Pörtner 2001). At thermal pejus, organisms employ short term tactics such as anaerobic metabolism. If warming continues, metabolic demands to maintain cellular and organ functions cannot be met anymore and organisms hit lethal limits (Figure I1; Pörtner 2010).

Energetic costs associated with environmental challenges are best quantified by investigating changes in metabolic scope (difference between active metabolic rates and resting metabolic rates, Figure I2; Angilletta & others 2002; Pörtner 2002; Pörtner & Farrell 2008; Chown *et al.* 2010), as it represents the amount of energy an organism expends to undertake fitness-related activities (such as foraging and reproduction).

Figure I1. Performance curve. Performance reaction norm shape depends on thermal limits (CTmin and CTmax), resting metabolic rates (RMR) and temperature optimum (Topt) within the preferred temperatures range (Tpref) at different temperatures. Temperature pejus (Tpejus) indicates loss of performance.



Moreover, performance curves are useful to analyze spatial variation of responses to environmental challenges between populations and sister species (Somero 2010). For example, it is known that many species with wide geographic ranges that span across latitudinal (and thermal) gradients and exhibit high site-fidelity may show local adaptation in performance curves (Pörtner 2002; Angilletta *et al.* 2004; Fangue, Hofmeister & Schulte 2006; Fangue, Richards & Schulte 2009; Baumann & Conover 2011; Todgham & Stillman 2013; Rummer *et al.* 2014). Then, provided enough genetic

variation, reaction norms in latitudinally separated populations should differ along geographic (and thermal) clines.

If local adaptation in performance curves is detected within a species, it usually takes of two forms, countergradient variation or thermal adaptation (Angilletta 2009). The countergradient variation concept was developed in relation to responses of organisms to the length of the favorable growth season (Conover *et al.* 2005; Arnott, Chiba & Conover 2006; Baumann & Conover 2011). In this type of adaptation to local environment, different populations maintain the same thermal optimum but the northern population outperforms the most southern populations. In this scenario, northern population individuals exhibit larger body size and increased performance (for example growth) when compared to individuals of southern populations (Gardiner, Munday & Nilsson 2010; Baumann & Conover 2011). On the other hand, populations could exhibit thermal adaptation to temperature gradients where performance optima shift and match average local temperatures (Angilletta 2009). These two different adaptations to local thermal environment will have profound consequences in the response to current climate change (Somero 2010; Chown *et al.* 2010).

E. Knowledge gaps

Quite a few studies have examined the effect of ocean warming and acidification over the past five years, however our understanding of the impacts of projected climate change on fish species is still limited (Todgham & Stillman 2013). Most of the studies on

fishes have been limited to understanding of thermal tolerance and sensitivity of adult fishes, especially from lower latitudes with little consideration for the most vulnerable life stages (embryonic, larval and juvenile), thus impairing our ability to predict long term impacts on natural populations (Pörtner & Farrell 2008; Dalziel, Rogers & Schulte 2009; Somero 2010; Todgham & Stillman 2013). To improve predictions on the effect of climate change on fish communities there is an urgent need for implementation of multi-stressor studies on different life stages and latitudinally-separated populations to identify life stages that represent the bottleneck for survival, as well as the potential for acclimation and adaptation (through pre-adaptation) to environmental change (Melzner *et al.* 2009; Checkley *et al.* 2009; Baumann & Conover 2011; Baumann *et al.* 2011; Nilsson *et al.* 2012). To date, research shows that ocean warming and acidification have a deleterious effect on fishes, but most often this is sub-lethal and variable across species (Pörtner & Knust 2007; Somero 2010; Kroeker, Micheli & Gambi 2012; Todgham & Stillman 2013). Analysis of the effect of multistressors on fish physiology at varying life stages and at different locations, may allow identification of underlying mechanisms that determine resilience or vulnerability to warming and acidification, and ultimately can aid in determining 'winner and loser' physiotypes under a changing climate.

F. Cleaner Gobies as a model system to investigate the advantage of body size in warming climate

The gobies of the genus *Elacatinus* are known to exhibit a mutualistic relationship with two types of invertebrates. Some reside within large tube sponges. Others are associated with hard coral and remove ectoparasites, scales, and mucus from the body surface, gills and mouth of larger cooperating fishes (commonly defined as 'hosts' or 'clients') at specific sites on the reef, known as cleaning stations (Arnal and Côté, 2000; Grutter and Hendrikz, 1999; Grutter, 1999). Cleaner fishes reduce parasite load (Grutter, 1999) and decrease stress in hosts (Soares et al., 2011), and therefore play an important role in shaping fish communities. The first cleaning behavior was observed by Longley (1918) in the neon goby *Elacatinus oceanops*, although the significance of this behavior was only understood later (Colin 1975). The neon goby *E. oceanops* was observed to inhabit shallow reef areas (1-40 m) in south Florida and in Belize, until recent genetic data (Taylor 2003; Taylor & Hellberg 2005) and morphological description (Randall & Colin 2009) recognized the gobies in these two areas as separate species (*E. oceanops* and *E. lobeli*). Moreover, the two species are considered to be more closely related to each other than to any other *Elacatinus* in the area (Taylor & Hellberg 2005).

Past geographic conditions may be key when considering the distribution of these sister species. In fact, during the Pleistocene a vast area of land that is currently submerged was exposed, thus reducing the available shallow areas for gobies and coral reefs to about 10% of the area of today (Colin 1975; Taylor & Hellberg 2005; Marsili

2008; Gaither *et al.* 2014). At that time, water temperatures in south Florida were more representative of current temperate latitudes (Lynts, Judd & Stehman 1973). It is possible that the separation of the two areas (Florida Keys and Belize) could have favored differentiation and speciation in these two fishes. In fact, in ectotherms, high average temperatures (typical of Belizean microclimate) select for smaller body size, while thermal fluctuations favor larger body mass (this type of environment is more representative of waters adjacent to south Florida) (Daufresne, Lengfellner & Sommer 2009). The main difference in *E. oceanops* and *E. lobeli* is in fact size, where *E. lobeli* is considered a dwarf-size neon goby. These sister species pair therefore provides an opportunity to test intra- and inter-specifically whether smaller body mass in adults provides physiological advantages that may benefit marine fishes in warming environments, as may occur in global warming.

G. Little skate as a model system to investigate the impacts of ocean warming and acidification on locally adapted populations

1. Biology

The little skate, *Leucoraja erinacea* Mitchill 1825, belongs to the family Rajidae, the most speciose family of batoids with more than 230 species. *Leucoraja erinacea* is demersal, inhabiting sandy, muddy, or gravelly bottoms of coastal and shallow waters between the Gulf of Maine and Cape Hatteras (Frisk & Miller 2009). It is found at depth of 0 to 90 meters (Bigelow, Schroeder & Hole 1953). They prey upon a variety of benthic

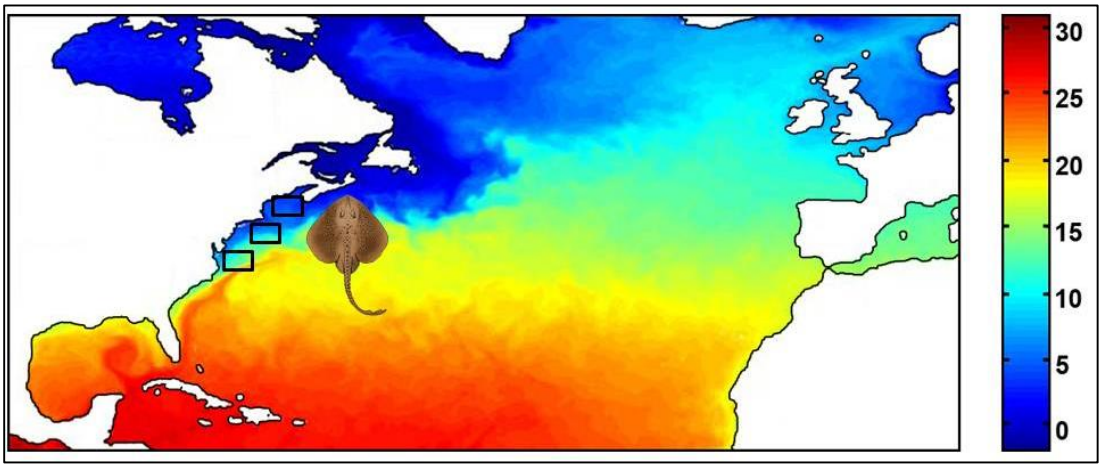
organisms, including polychaetes, crustaceans, and fishes (Ebert & Bizzarro 2009). Compared to other Rajidae, this species is small (max length 57 cm), short lived (average 12.5 years), and has low fecundity (between 21 to 57 eggs/year). *Leucoraja erinacea* reaches maturity at 6 years of age, with a reproductive peak of two years when they are 7-8 years old (Frisk 2002; Frisk & Miller 2006, 2009). Eggs are laid throughout the year, usually in pairs at one week intervals (Bigelow *et al.* 1953; Palm *et al.* 2011). After a relatively long development (five to twelve months depending on temperature), hatchlings exit the egg through the posterior opening of the case (Luer & Gilbert 1985; Leonard, Summers & Koob 1999). The hatchlings have a stomach full of yolk and their tails still possess a whip-like appendage that disappears within about two of weeks (Bigelow *et al.* 1953; Luer & Gilbert 1985; Leonard *et al.* 1999).

2. Spatial Ecology

Compared to viviparous batoids, oviparous elasmobranchs such as skates have a reduced geographic distribution (Goodwin, Dulvy & Reynolds 2005). This is thought to occur because oviparous elasmobranchs are typically smaller than livebearers, and size correlates with the ability to maintain wider geographic ranges (Musick, Harbin & Compagno 2004). Additionally, skates have to restrict their ranges to suitable spawning habitats, because embryos are spatially and temporally constrained in the egg case for a prolonged period of time that spans two or more seasons. For *L. erinacea*, remaining in an optimal spawning site throughout the year is particularly important because they are serial spawners (Frisk & Miller 2009; Palm *et al.* 2011). As a consequence, although

skates that exhibit a well-defined spawning season undertake seasonal latitudinal migrations, *L. erinacea* only show weak seasonal distribution patterns that consist of short distance movements from coastal and shallow waters to offshore and deeper waters during colder months (McEachran 2002). Perhaps because of these reproduction-linked spatial constraints, latitudinal gradients in growth rates and maturation times in this species have been observed. Indeed, although laboratory-controlled studies have not yet confirmed observations made in wild specimens, a few studies have documented regional variations in life history patterns in this species (Bigelow *et al.* 1953; McEachran 2002; Frisk & Miller 2006, 2009). The latitudinally-separated skates may represent populations that have adapted to the local environments (Figure I2).

Figure I2. Spatial distribution of potential populations of *Leucoraja erinacea*. Three potential populations have been suggested by Frisk and Miller (2009) for *Leucoraja erinacea*. Colors represent different temperatures.



Locally-adapted populations within a species have been described in several teleost species, such as the minnow *Fundulus heteroclitus*, the silverside *Menidia menidia*, the salmon *Oncorhynchus nerka* (Fangue *et al.* 2006, 2009; Baumann & Conover 2011; Eliason *et al.* 2011), as well as a few shark species (Tanaka & Mizue 1979; Carlson, Cortés & Johnson 1999; Yamaguchi, Taniuchi & Shimizu 2000; Lombardi-Carlson *et al.* 2003). Differences in growth rates along latitudinal gradients are thought to be the result of temperature-induced changes in metabolic functions (Arnott *et al.* 2006; Baumann & Conover 2011). A few studies have now documented that populations of little skates at colder (higher) latitudes exhibit larger sizes and longer life spans than populations at warmer latitudes (Frisk & Miller 2006, 2009), but the nature of these differences, whether genetic or the result of phenotypic plasticity still needs to be investigated. Frisk and Miller (2006) identified three potential populations of *L. erinacea* along its geographic range: the northern population in the Gulf of Maine (GoM), the middle population in Georges Bank to southern New England (GB) and the southern population in the Mid-Atlantic to Cape Hatteras (MA-CH). Interestingly, differences in growth and other vital patterns overlap with sharp thermal discontinuities in the environment (Figure I2), thus supporting the hypothesis that these differences in the environment might be temperature-induced through either adaptation or plasticity.

3. Vulnerability to exploitation

The conservation of marine fishes presents a challenge because it is relatively difficult to determine population declines and how many individuals are remaining in a population. Because the vast majority of fishes are not managed, local extinctions of marine fishes tend to be ignored (Dulvy & Reynolds 2002; Dulvy, Sadovy & Reynolds 2003). Many species are thought to be resilient to local extirpation because of large geographic ranges and dispersal mechanisms (especially for teleosts). Skates generally reach a large size and have slow maturation. Although *L. erinacea* has a wide geographic range, this species may be vulnerable to local extirpation given the large scale mortality due to fishing and bycatch, and because they have limited capacity for shifting its range. In fact, skates tend to be philopatric, with only short distance (less than 50-100 miles) movements (Dulvy & Reynolds 2002; Frisk & Miller 2006), and there is little evidence for recolonization after local extirpation despite the presence of nearby populations (Dulvy & Reynolds 2002).

H. Specific aims

The overall aim of this dissertation is to identify physiological responses of fish to ocean warming and acidification. Retrospective analyses of organismal adaptation are difficult to conduct because of a lack of replication; however, it is possible to evaluate variation in physiological traits inter-individually by testing extant sister species (chapters 1 and 2)

and intra-specifically by looking at different populations across spatial gradients (chapters 3 and 4).

Chapter 1 I investigate the acute and intrinsic thermal tolerance of two sister species of cleaner gobies acclimated to common temperatures and address the following question: do dwarf fishes have an advantage in warming environment?

Chapter 2 I investigate a potential mechanism that makes dwarf species more tolerant to thermal variability by comparing thermal reaction norms (in this case digestive performance) of two sister species of cleaner gobies.

Chapter 3 Do different populations respond differently to climate change stressors (local adaptation)? I expose little skate eggs from different populations to present and predicted temperatures and carbon dioxide levels in a crossed-factors design, and compare mortality, growth and reaction norms.

Chapter 4 Juvenile little skates' active and standard metabolic rates are tested to determine the effect of local adaptation (different populations) and increased carbon dioxide (synergistic climate change stressors) on thermal reaction norms during intense exercise.

Chapter 1: Body size affects thermal tolerance in tropical cleaner gobies

Abstract

In this study, we investigated the effect of body size on thermal tolerance of same age but different size adult neon gobies, *Elacatinus oceanops* and *E. lobeli*, acclimated at the same temperatures. We used Critical Thermal Methodology to test the hypothesis that intra-specifically as well as inter-specifically, smaller fish acclimated over the same temperature range are better able to tolerate wide thermal fluctuations than larger individuals, thus investigating possible physiological advantages associated with decreased body size in ectotherms. Climatic models project an increase in the frequency and intensity of heat and cold events over the next century as a consequence of anthropogenic climate change. These events have the potential to disrupt community dynamics, by affecting survival of species. It is important therefore to determine whether there is an advantage to having a smaller body size for to rapid changes in temperature. Results from this study show that both species exhibited a limited capacity for enhanced thermal tolerance following acclimation. Additionally, the smaller *E. lobeli* was able to tolerate higher temperatures than *E. oceanops*. However, only *E. oceanops* showed intraspecific difference in thermal tolerance, with smaller individuals being more tolerant to higher temperatures. Although the two fish species used in this study have diverged and have lived in geographically distinct locations in the Caribbean basin for about 800,000 years ago,

they provide further evidence supporting the prediction of increasing thermal windows with a reduced body size.

Introduction

Climate change is considered by many researchers to be the greatest current threat to biodiversity and ecosystem stability across biota. Therefore, predicting its impacts at the organismal level has become a major research goal for evolutionary and conservation biologists (Kappelle, Van Vuuren & Baas 1999; Thomas *et al.* 2004; Somero 2010; Wernberg *et al.* 2011). Current physical models predict an increase in frequency, duration and magnitude of sudden and rapid thermal fluctuations as a consequence of anthropogenic global warming (Harley *et al.* 2006; Karl *et al.* 2011; IPCC 2013). Most studies on the effect of environmental stress on organisms focus on the direct and indirect consequences of increased average temperatures on organismal physiology (Beitinger & Bennett 2000; Sears & Angilletta 2004), but it is the occurrence of rapid temperature changes (e.g. cold fronts, heat waves) that has the potential to disrupt ecosystem stability by altering species composition and dynamics through elimination of keystone species (Sazima *et al.* 2010; Rossi *et al.* 2013).

As nearly every physiological process is temperature-sensitive in aquatic ectotherms (Fry 1971; Magnuson, Crowder & Medvick 1979; Ohlberger *et al.* 2008a; Somero 2010; Di Santo & Bennett 2011a), rapid thermal changes are thought to profoundly impact fishes, with consequences for the stability of the whole community (Perry *et al.*

2005; Gilman *et al.* 2010). Climatic models project a 2-3°C increase in average ocean temperatures at low latitudes by the end of the century (Allen *et al.* 2013). It is apparent from phylogenetic and geographic patterns that current rapid warming is already responsible for shifts in species distribution, phenology, and a reduced body size across marine taxa (Daufresne, Lengfellner & Sommer 2009; Genner *et al.* 2010; Ohlberger *et al.* 2012; Cheung *et al.* 2012). As tropical fishes are known to live close to their thermal limits (Rummer *et al.* 2014), it is plausible to expect that they will be at high risk in warming scenarios.

The relationship between temperature, metabolic rate and body size is one of the most overlooked phenomena among climate change driven adaptations (Gardner *et al.* 2011). During past major warming shifts, both marine and terrestrial organisms became smaller, suggesting a universal response to climate change (Daufresne, Lengfellner & Sommer 2009; Gardner *et al.* 2011; Clark *et al.* 2012). In particular, fossil evidence suggests that during the warming phase of the Palaeocene-Eocene Thermal Maximum (PETM), ectotherms' mass shrank by 50–75% (Sheridan & Bickford 2011). Although current warming is occurring at a much faster rate than in previous periods, reduction in body size as a response to the same magnitude of temperature increase could be very useful to predict changes in mass expected for the next century (Sheridan & Bickford 2011). A few mechanisms have been proposed to explain shrinking body size with environmental temperature. For instance, growth is closely linked to ambient temperature. If food resources are limited, increased temperature may put a constraint on maximum body mass as the energy allocated for growth is reduced at higher temperature (Billerbeck, Lankford

& Conover 2001; Angilletta, Steury & Sears 2004). Consequently, even though it is not surprising to observe shrinking in organisms' body size in response to increasing temperature, the question is whether or not smaller organisms may acquire a physiological advantage over larger-bodied organisms in global warming.

Demonstrating an increase in thermal tolerance in smaller fish has been controversial when tested intra-specifically, as studies disagree on the effect of body size on tolerance (Taylor *et al.* 2005; Pörtner & Knust 2007; Calosi, Bilton & Spicer 2008; Genner *et al.* 2010). Past studies were unable to discern the effect of differing metabolic requirements at each life stage from the simple effect of body mass on tolerance (for instance when comparing juveniles vs. adults). To overcome the complications caused by differing life stages metabolic requirements, it is possible to evaluate variations in physiological traits in individuals of the same species or of sister species that occupy similar thermal niches but possess different adult sizes (Shields & Underhill 1993; Chouinard, Pigeon & Bernatchez 1996; Landry, Vincent & Bernatchez 2007). By testing the effect of body size in a laboratory-controlled setting, it is possible to exclude differences derived by dissimilar acclimation history and life stage from those resulting from body mass (Baumann & Conover 2011). In fact, it has been argued that size patterns observed in the wild might be the product of phenotypic plasticity (Shields & Underhill 1993) rather than a representation of genetic diversity produced by natural selection (Conover, Duffy & Hice 2009). Consequently, it is of utmost importance to disentangle the relationship between temperature sensitivity and body size by measuring thermal tolerance of different sized but same-age fishes reared in "common garden" conditions (i.e., similar laboratory-

controlled conditions). In these conditions, the adaptive advantage of a small body size may be directly tested in pair sister species as well as within a species.

On a global scale, the marine realm offers clearly defined thermal niches and therefore an ideal basis for comparisons. Studies on closely related species help elucidate the underlying physiological principles of thermal tolerance windows and mechanisms of thermal limitation. Overall capacity for oxygen supply and delivery (respiration and circulation) is set to be optimal only within a narrow window of temperatures. Beyond these limits, organisms can survive acute short-term thermal stress by resorting to unsustainable anaerobic pathways (Pörtner and Knust 2008). Since speciation history cannot be directly studied, it is useful to investigate traits that may be exposed to divergent natural selection and the ecological conditions that promote diversification of traits (Ohlberger *et al.* 2008a). This type of diversification is often driven by competition for resources and habitat. For instance, size dimorphism in congeneric fishes living in post glacial temperate freshwater lakes is most likely associated with resource partitioning, including competition for thermal microhabitats (whitefish and dwarf ciscoes; (Landry *et al.* 2007; Ohlberger *et al.* 2008a; b). Indeed, pair species are commonly used to unveil mechanisms behind morphological and/or physiological divergence that promote differential resource utilization (Bernatchez *et al.* 2010). Temperature is a fundamental component of the ecological niche of fishes and should be treated as an ecological resource that organisms exploit to enhance different physiological processes (Magnuson *et al.* 1979; Ohlberger *et al.* 2008a; Di Santo & Bennett 2011a; b). In fact, temperature is the preferred choice over food for most fishes (Krause *et al* 1998, Crowder and Magnuson et al. 1979).

Within the next 50 years, about 75% of marine ectotherms are expected to decrease body weight up to 39% (with an average of 10% decrease) (Perry et al. 2010) and it is therefore important to identify specific advantages that small fish might possess to overcome environmental change.

Until recently, the Caribbean goby, *Elacatinus lobeli* Randall & Colin 2009, was considered a dwarf variant of the well-known neon goby, *Elacatinus oceanops* Jordan 1904, until genetic data revealed that the two clades diverged 800,000 years ago (Taylor & Hellberg 2006), which along with morphological characters, supported a species level designation (Randall & Colin 2009). The two species are ecologically equivalent; they reside in colonies on shallow live coral heads where they wait for potential hosts to be cleaned of external parasites (Whiteman & Côté 2002, 2004; Olivotto *et al.* 2005). They are also morphologically similar except for differences in body length (Figure 1.1), with *E. oceanops* being larger than *E. lobeli* (Randall & Colin 2009). In this study we tested thermal tolerance of adult *E. lobeli* and *E. oceanops* acclimated at the same temperatures. We hypothesized that a smaller body size in fish will correlate with wider thermal tolerance windows.

Figure 1.1. Photograph of *Elacatinus lobeli* and *Elacatinus oceanops.*

(A) *Elacatinus lobeli*. Photo by P.S. Lobel

(B) *Elacatinus oceanops*. Photo by V. Di Santo

Materials and Methods

Holding conditions of experimental animals

Juvenile *Elacatinus lobeli* (n=48) were collected at Wee Wee Caye, Belize (16.76N, 88.14W) and transported using Kordon® Breathing Bags™, while juvenile *Elacatinus oceanops* (n=48) were collected in Key Largo, Florida, USA (25.16N, 80.29W) and shipped using live fish shipping bags with oxygen. Fish of both species were reared at three different temperature treatments for one year (i.e. until they became adults). Fish were divided by species and randomly assigned to three temperature acclimation groups. All groups were maintained in well aerated and filtered 130-L aquaria. Aquaria were kept at diel photoperiod of 12 h light: 12 h dark and constant temperature. Water quality in each tank was monitored weekly to test for ammonia, nitrites, and nitrates (Table 1.1). Temperature was initially set at 24 ± 0.5°C with a submersible Ebo Jager 50-W aquarium heater. After a two-week period at 24°C, water temperatures were unchanged, or increased or decreased 0.5 °C per day until reaching acclimation temperatures of 20, 24, and 28°C. These temperatures were chosen because they are experienced by both species in the wild. Thermal profiles (over a period of about 10 years) for both sites were analyzed to determine mean maximum and minimum temperature as well as the most commonly experienced temperature in these two species. Water thermal data from Wee Wee Caye were recorded every hour using HOBO temperature loggers while temperature from the reef at Key Largo were obtained from NOAA (http://www.nodc.noaa.gov). Fish were fed a mixed diet of fresh frozen mysis shrimp and marine flakes twice daily *ad libitum* throughout the

acclimation period but were fasted for 24h prior to experimentation to ensure measurements were taken while the animals were in post-absorptive state.

Table 1.1. Water parameters in experimental tanks.

Water parameter	Treatment 1	Treatment 2	Treatment 3
Temperature (°C)	20	24	28
pH	8.1	8.1	8.1
Salinity	34	34	34
Ammonia	0	0	0
Nitrites	0	0	0
Nitrates	<30	<30	<30
Photoperiod	12L:12D	12L:12D	12L:12D

Critical Thermal Methodology

To quantify high and low temperature tolerance we identified the critical thermal maximum (CTmax) and minimum (CTmin) of *E. oceanops* and *E. lobeli*, as the mean temperature at which fish exhibit loss of equilibrium (LOE) or muscle spasm after steady temperature increase or decrease (Becker & Genoway 1979; Beitinger & Bennett 2000). For each trial, fish from each respective treatment were placed one each into 1 L glass beakers filled with water held at the same start acclimation temperature and suspended in

a re-circulating water bath. Oxygen saturation was maintained and thermal stratification prevented by providing moderate aeration to each beaker. Water temperature was increased or decreased 0.3 °C per minute by heating or cooling in a re-circulating bath equipped with a TE-10D Techne Heater or a DS-4 Aqua Logic Delta Star Chiller. Traceable® NIST calibrated thermometer continuously monitored temperatures inside the beaker during trials. Water temperature was increased or decreased until fish exhibited LOE or muscle spasm (Beitinger & Bennett 2000), at which time water temperature was recorded and the fish immediately transferred to the original acclimation temperature. Gobies were then weighed (± 0.01 g), measured (standard length: snout-last vertebra; ± 0.1 mm) and returned to their acclimation tank. Critical thermal maxima and minima are the arithmetic mean of the collective temperatures at which LOE or muscle spasm was observed. Some authors suggest muscle spasms should be used for CTM determinations (Paladino *et al.* 1980; Bonin, Lee & Rinne 1981), and in this study both species experienced LOE and muscle spasm nearly simultaneously with no temperature difference between the two endpoints.

Construction of thermal polygons

The thermal tolerance niche of gobies was quantified by constructing an ecological thermal tolerance polygon (Beitinger & Bennett 2000). The thermal tolerance polygon was assembled with both thermal tolerance scope (difference between the CTMax and CTMin) and acclimation range, and was expressed quantitatively using area units (C^2). The regression model of CTMax or CTMin on acclimation temperature of fish used in constant-temperature trials was used to define the upper and lower boundaries of the polygon.

Division of the polygon was that of thermal tolerance independent of previous thermal acclimation history (i.e., intrinsic tolerance zone) and thermal tolerance gained through acclimation (i.e., acquired tolerance zone) by drawing two horizontal boundary lines across at the lowest CTMax and highest CTMin constant-temperature values (Fangue & Bennett 2003).

Statistical analyses

One-way analysis of variance (ANOVA) was used to test for significant differences between CTmax or CTmin, mean wet mass and mean standard lengths of treatment groups. A Tukey-Kramer multiple comparisons test (Tukey-Kramer MCT) was used to discriminate between means. Analysis of covariance (ANCOVA) using length or weight as covariates followed to assess the effects these variables on temperature tolerance. The potential relationship between temperature tolerance and acclimation temperature was quantified using regression analysis. Temperature data collected at sites of fish collection (WWC and Key Largo) were analyzed to determine mean monthly and yearly temperatures, maximum and minimum temperatures, 10th and 90th quantiles, temperature trends. Parameters were analyzed using least squares regression and mean values were compared between sites using Student's t-test. All statistical decisions were based on an alpha of 0.05. All statistical analyses were performed in JMP Pro version 11.

Results

Standard length and mass of *E. oceanops* were greater than *E. lobeli* (one-way ANOVA, $p<0.0001$) regardless of temperature treatment (Table 1.2). Critical thermal maxima (±SD) of *E. oceanops* and *E. lobeli* acclimated at temperatures between 20 and 28 °C ranged from 31.8 ± 0.47°C to 35.5 ± 1.47°C and 34.9 ± 0.83 °C to 39.1 ± 0.72°C, respectively (Table 1); CTmax significantly increased at higher acclimation temperatures (one-way ANOVA, $p < 0.0001$) and were distinct (Tukey-Kramer MCT, $p<0.05$). Critical thermal minima (±SD) of *E. oceanops* and *E. lobeli* ranged from 14.7 ± 0.25 °C to 17.0 ± 0.54°C and 13.8 ± 0.24°C to 17.0 ± 0.24°C, respectively (Table 1.2); CTmin were significantly lower at cooler acclimation temperatures (one-way ANOVA, $p < 0.0001$) and distinct at each acclimation temperature (Tukey-Kramer MCT, $p<0.05$) within species but means were not statistically different across species (Tukey-Kramer MCT, $p>0.05$). Low temperature tolerances (CTmin) are represented by the regression models: CTmin = 8.85 + 0.28 x acclimation temperature ($R^2=0.88$, $p<0.0001$) in *E. oceanops* and CTmin = 5.9 + 0.39 x acclimation temperature ($R^2=0.95$, $p<0.0001$) (Figure 1.2). High temperature tolerances (CTmax) are represented by the regression models: CTmax = 22.62 + 0.46 x acclimation temperature ($R^2=0.9$, $p<0.0001$) in *E. oceanops* and CTmax = 24.31 + 0.526 x acclimation temperature ($R^2=0.88$, $p<0.0001$) (Figure 1.2).

Standard length had no significant effect on CTmin or Ctmax in either species ($p>0.05$); however both CTmin and CTmax were significantly affected by mass in *E. oceanops* ($p<0.01$) but not in *E. lobeli* ($p=0.09$) (Figures 1.3 and 1.4). Larger *E. oceanops*

were more tolerant to lower temperatures while smaller E. *oceanops* were more tolerant to higher temperatures ($p<0.01$). Acclimation temperature did not affect weight of fish in both species ($p>0.05$) however inter-individual variations in weight were more pronounced across treatments in *E. oceanops* ($p<0.01$) (Table 1.2).

Data from CTM were combined to construct thermal polygons and overlapped to show differences in thermal tolerance (Figure 1.2). Total polygon area for *E. oceanops* was 137.6 $°C^2$ and only 18.6% of the tolerance was acquired through acclimation (25.6$°C^2$). Total polygon area for *E. lobeli* was 172.8$°C^2$ and only 17.13% was acquired through acclimation (29.6$°C^2$). Both species are stenotherms with low acclimation capacity to change in temperature. However, *E. lobeli* exhibits higher intrinsic tolerance area (143.2$°C^2$) when compared to *E. oceanops* (112$°C^2$).

Overall, mean annual temperature, 10th and 90th quantile and minimum and maximum temperatures at WWC and Key Largo differ (Least Squares Regression, $p<0.01$). Temperature decreased to 19.7 ± 0.4°C in Key Largo and 24.6 ± 0.3°C at WWC (T q10: $p < 0.0001$; $R^2=0.79$) during the coldest month (January) with an mean T_{min} of 21.6 ± 0.4°C (Key Largo) and 25.3 ± 0.3 °C (WWC) (mean T_{min}: $p < 0.0001$, $R^2=0.70$; Table 1.2, Figures 1.5 and 1.6). Temperature increased to 31.9 ± 0.1°C in Key Largo and 30.2 ± 0.1°C at WWC (T q90: $p < 0.0001$; $R^2=0.77$) during the warmest month (August) with an mean T_{max} of 31.4 ± 0.2°C (Key Largo) and 30.0 ± 0.1 °C (WWC) (mean T_{min}: $p < 0.0001$; $R^2=0.59$; Table 1.2, Figures 1.5 and 1.6). Temperature trend over about ten

years shows a significant increase in temperature at WWC (least squares regression, $p<0.0001$) but not in Key Largo (Figures 1.5 and 1.6).

Figure 1.2. Ecological thermal polygon of two gobies. Ecological thermal polygon with critical thermal minima (white) and maxima (black) values for *Elacatinus oceanops* (triangles) and *E. lobeli* (circles) acclimated to temperatures between 20 and 28 °C. Vertical bars represent 95% confidence intervals. Regression models on acclimation temperature were based on 8 fish each species per acclimation groups (n=48 fish per species).

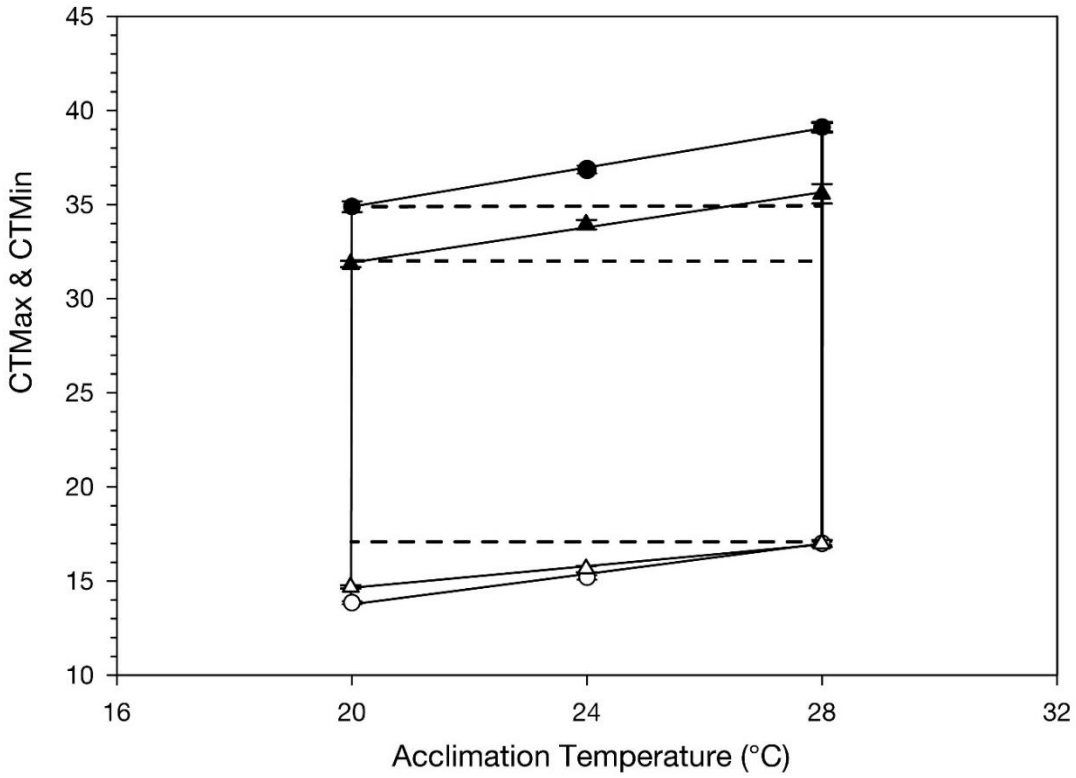
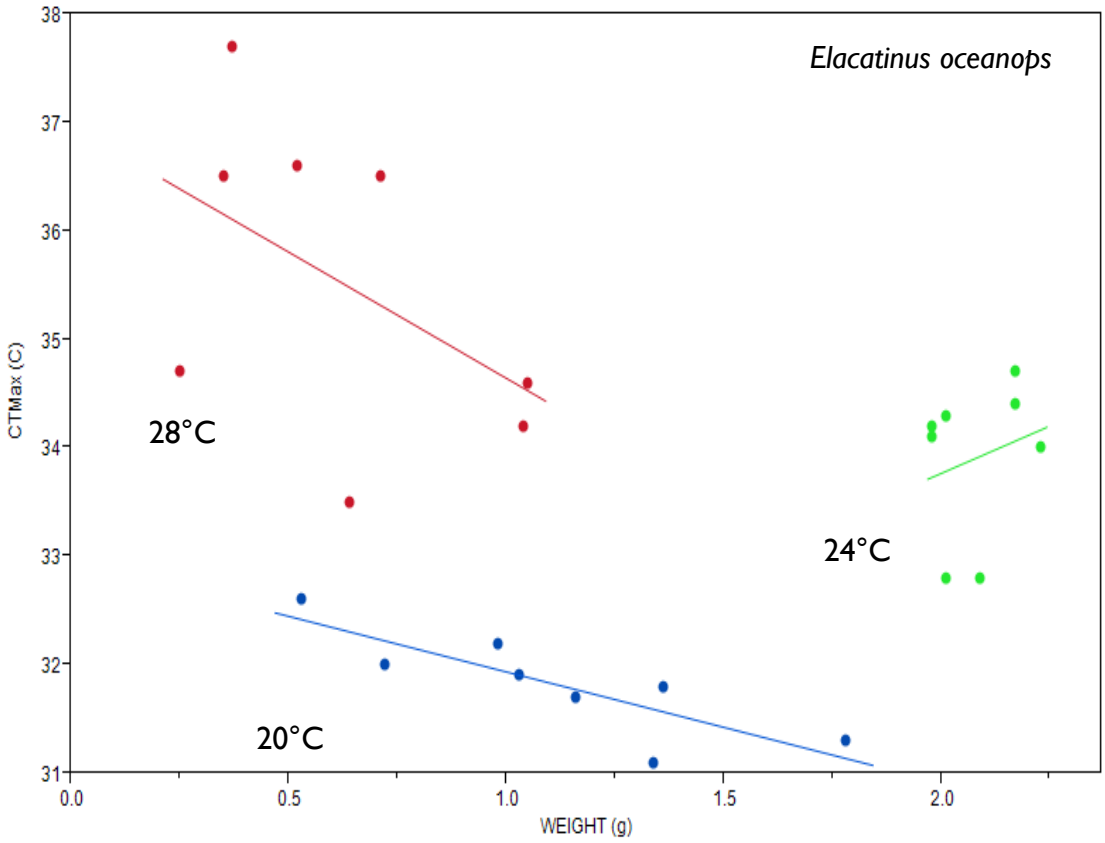
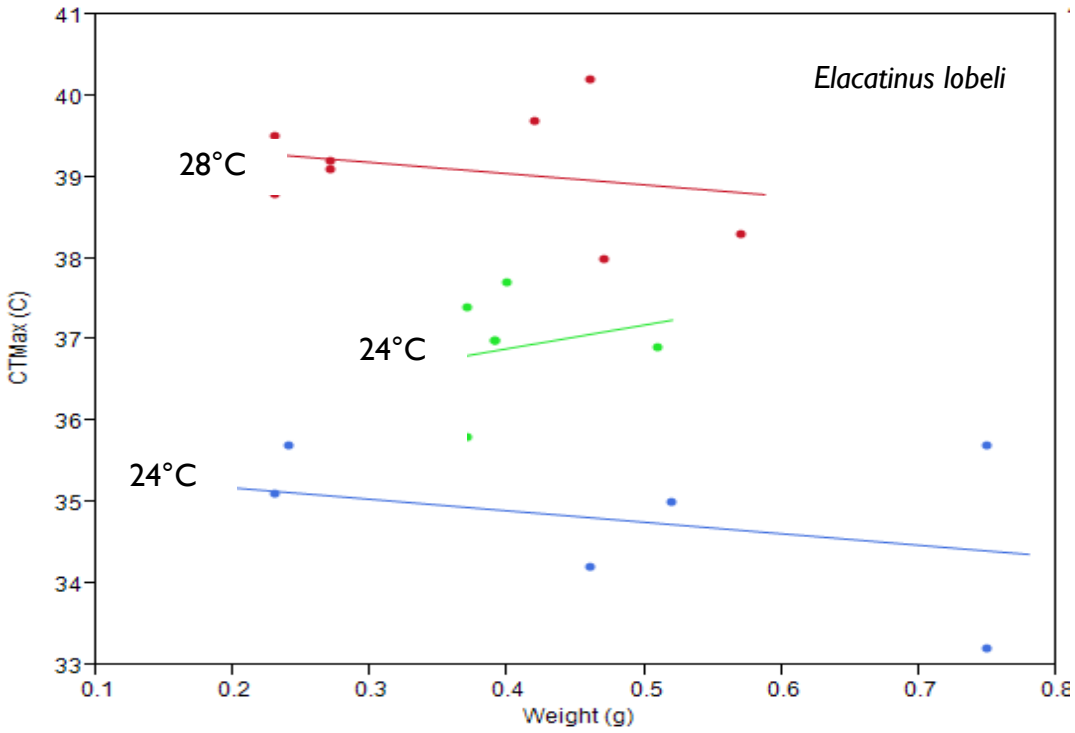


Figure 1.3. Effect of weight on CTMax in *Elacatinus oceanops* (A) and *E. lobeli* (B). At each acclimation temperature (red: 28°C, green: 24°C, blue: 20°C) weight (g) has a significantly effect on CTMax in *Elacatinus oceanops* (A, $p<0.01$, one way ANOVA, n=24) but not in *E. lobeli* (B, $p=0.09$, one way ANOVA, n=24)

(A)

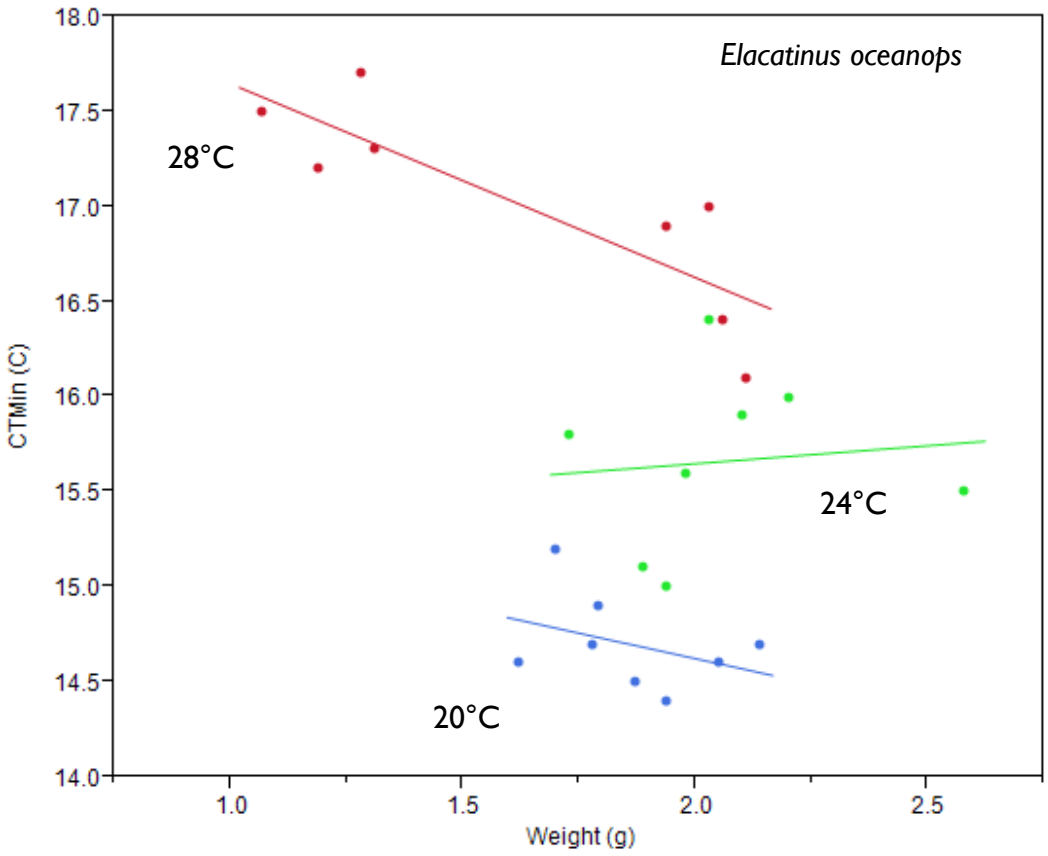



Elacatinus lobeli
28°C
24°C
24°C
CTMax (C)
Weight (g)
41
40
39
38
37
36
35
34
33
0.1
0.2
0.3
0.4
0.5
0.6
0.7

(B)

Figure 1.4. Effect of weight on CTMin in *Elacatinus oceanops* (A) and *E. lobeli* (B). At each acclimation temperature (red: 28°C, green: 24°C, blue: 20°C) weight (g) has a significantly effect on CTMin in *Elacatinus oceanops* acclimated at 20 and 28 °C ($p<0.01$, one way ANOVA, n=24) but not in *E. lobeli* (B, $p=0.09$, one way ANOVA, n=24).

(A)



(B)

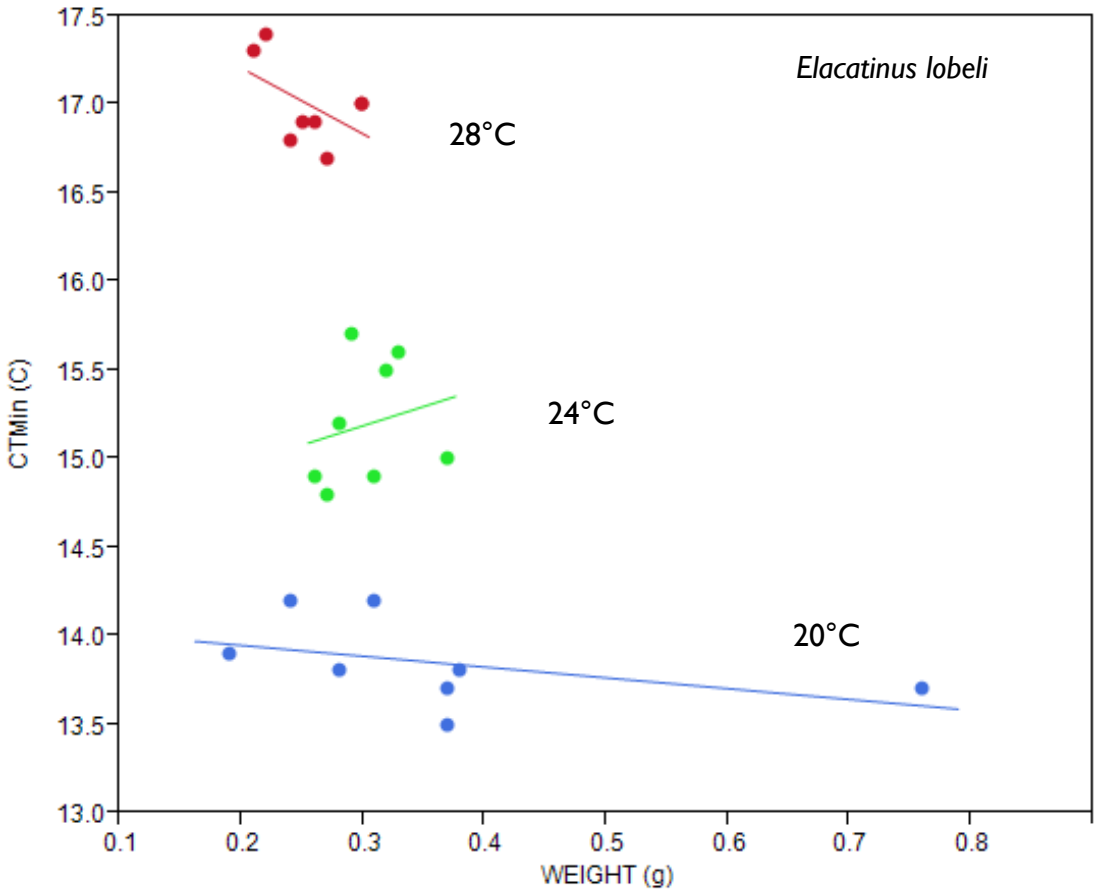
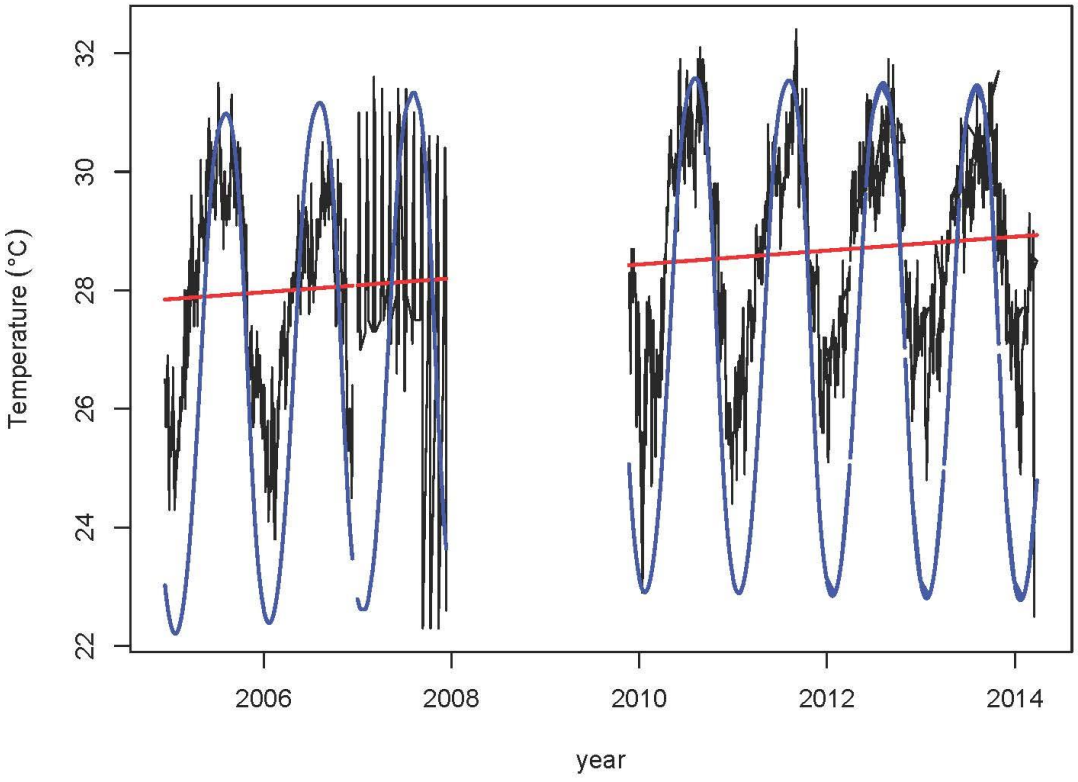

Elacatinus lobeli
28°C
24°C
20°C
CTMin (C)
WEIGHT (g)
17.5
17.0
16.5
16.0
15.5
15.0
14.5
14.0
13.5
13.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8

Figure 1.5. Daily water temperatures at Wee Wee Caye and Key Largo. Water temperatures (black line: mean daily temperatures; blue line: model that describes mean daily temperatures; red line: mean annual temperature) at Wee Wee Caye, Belize (A) and Key Largo, USA (B). Mean annual temperature increase over time is statistically significant only for Wee Wee Caye (Least Squares Regression, $p<0.0001$). Data loggers were lost at Wee Wee Caye between 2009 and 2010.

(A)



(B)

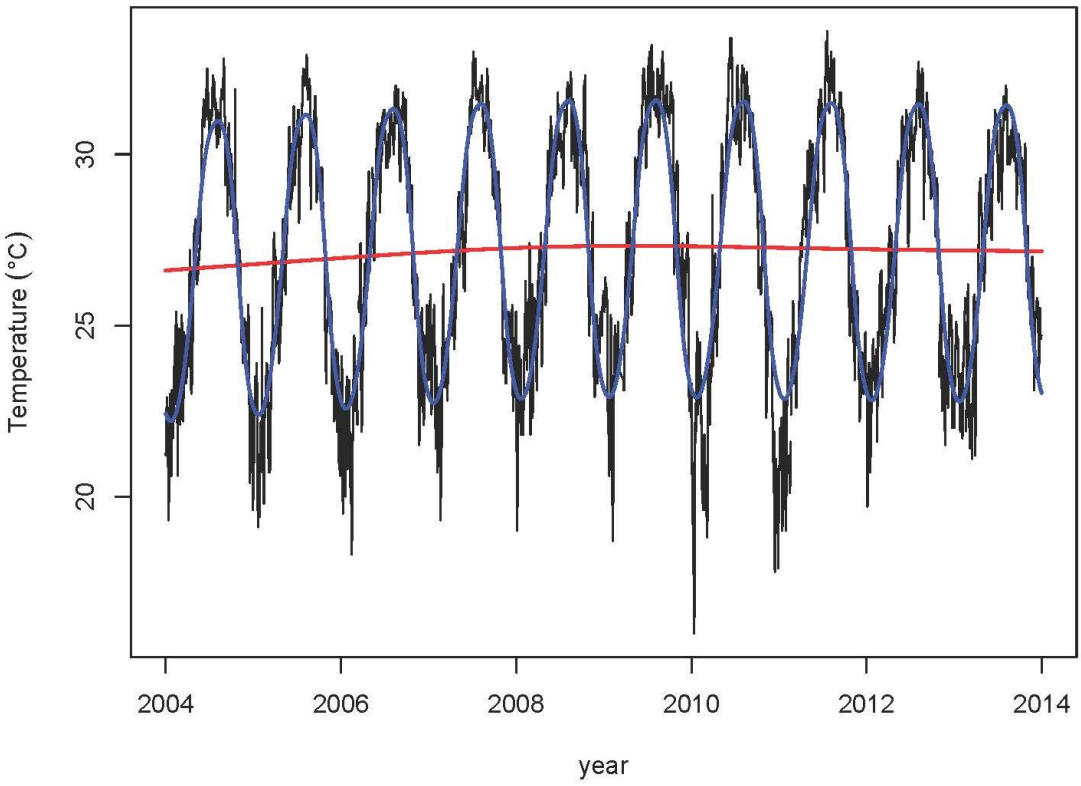
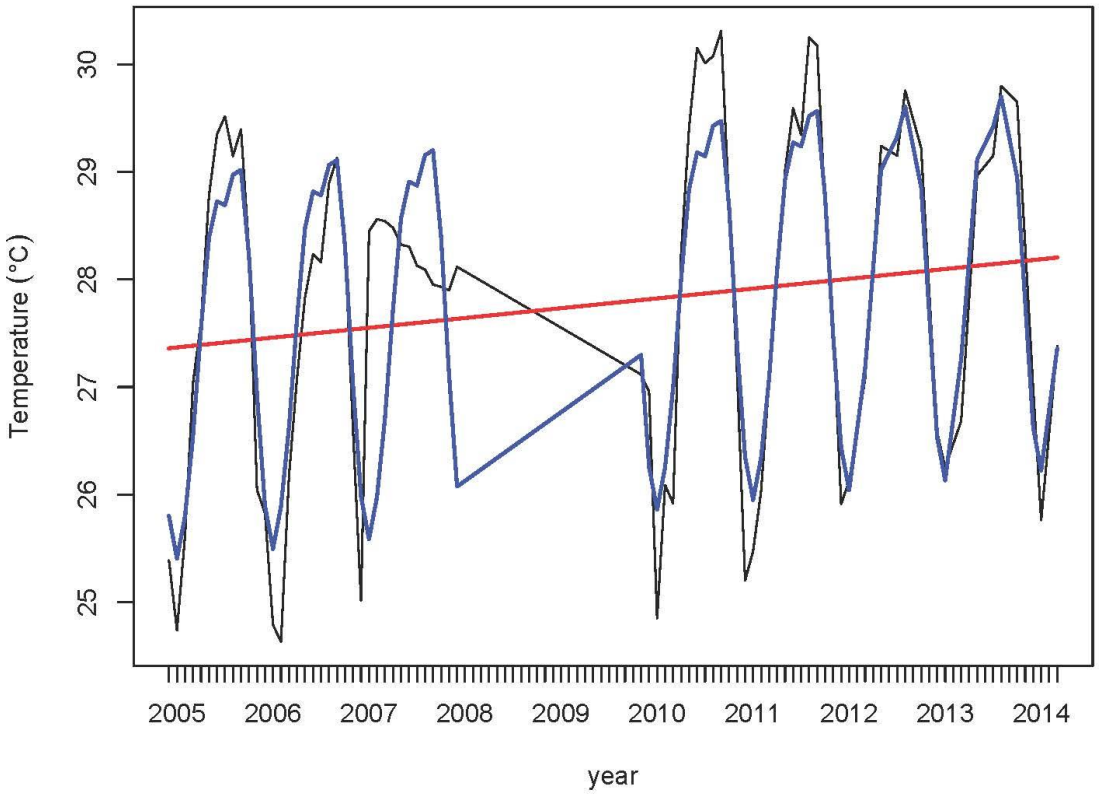


Figure 1.6. Monthly water temperatures at Wee Wee Caye and Key Largo. Water temperature (black line: mean monthly temperatures; blue line: model that describes mean monthly temperatures; red line: mean annual temperature) at Wee Wee Caye, Belize (A) and Key Largo, USA (B). Mean annual temperature increase over time is statistically significant only for Wee Wee Caye (Least Squares Regression, $p<0.0001$). Data loggers were lost at Wee Wee Caye between 2009 and 2010.

(A)



(B)

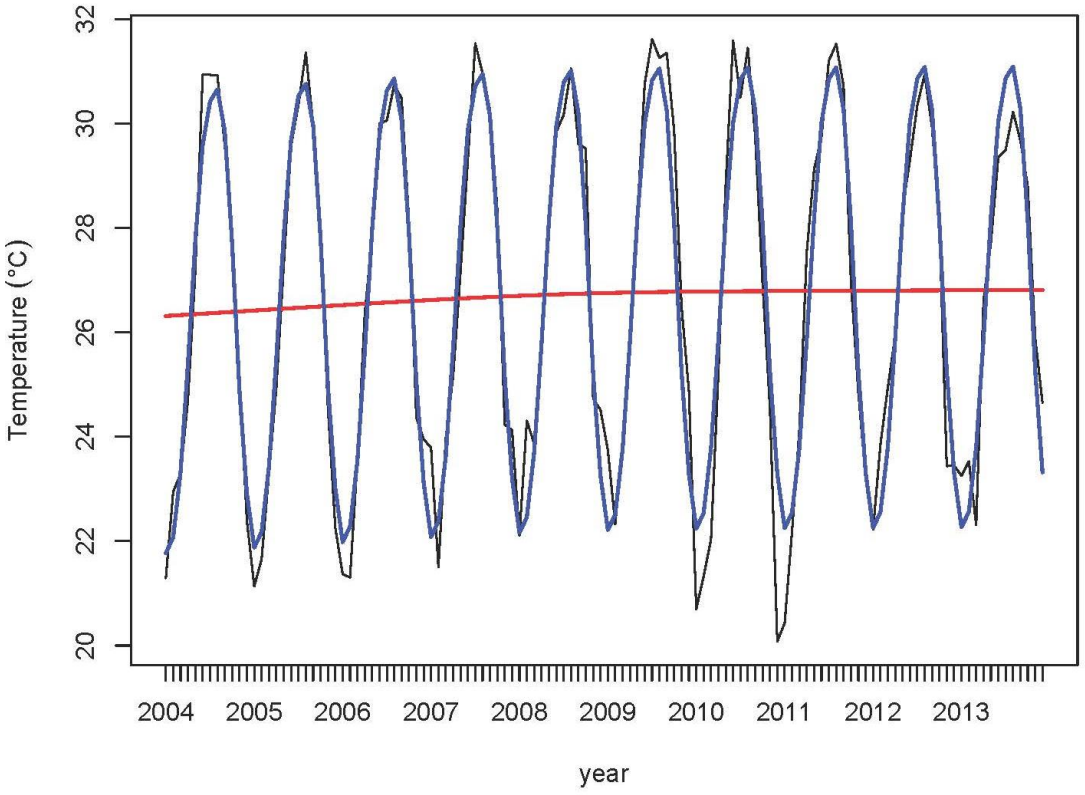

Temperature (°C)
20
22
24
26
28
30
32
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
year

Table 1.2. Critical Thermal Methodology results and body size. Critical thermal maxima (CTMax), critical thermal minima (CTMin), standard length measurements (SLM), and wet weight mass (WWM) for *Elacatinus oceanops* and *E. lobeli* are grouped by acclimation temperature. The number of fish used in each trial (per acclimation temperature) is indicated below the species name. All experimental values are reported as mean ± standard deviation ($\bar{x} \pm SD$). The effect of acclimation temperature, SLM and WWM on CTMax and CTMin were explored using analysis of covariance (ANCOVA) within and between species. Different superscripts denote groups that differ significantly within species (lowercase) and between species (uppercase) (Tukey–Kramer multiple comparison test; α=0.05).

Acclimation temperature (°C)	CTMax $\bar{x} \pm SD$		CTMin $\bar{x} \pm SD$		SLM (cm) $\bar{x} \pm SD$		WWM (g) $\bar{x} \pm SD$	
	E. oceanops (n=8)	*E. lobeli* (n=8)	*E. oceanops* (n=8)	*E. lobeli* (n=8)	*E. oceanops* (n=8)	*E. lobeli* (n=8)	*E. oceanops* (n=8)	*E. lobeli* (n=8)
20	31.8 ± 0.47	34.9 ± 0.83	14.7 ± 0.25 ^{A}	13.8 ± 0.24 ^{A}	4.40 ± 0.415^{b}	2.79 ± 0.502^{c}	1.48 ± 0.485^{d}	0.38 ± 0.206^{e}
24	33.9 ± 0.71	36.9 ± 0.59	15.7 ± 0.46 ^{A}	15.2 ± 0.35 ^{A}	4.26 ± 0.406 ^{b}	2.60 ± 0.245 ^{c}	2.06 ± 0.186 ^{d}	0.34 ± 0.062^{e}
28	35.5 ± 1.47	39.1 ± 0.72	17.0 ± 0.54^{A}	17.0 ± 0.24^{A}	3.75 ± 0.580 ^{b}	2.74 ± 0.334 ^{c}	1.12 ± 0.638 ^{d}	0.31 ± 0.108 ^{e}
			Overall $\bar{x} \pm SD$:		4.13 ± 0.295	2.71 ± 0.142	1.56 ± 0.610	0.35 ± 0.140

Table 1.3. Mean temperature (T) (±s.e.m) over ten years at Wee Wee Caye (WWC) and Key Largo. Underlined mean pairs are *not statistically different* (t-test; α=0.05).

Mo	Mean T (°C)		T q10 (°C)		T q90 (°C)		Mean T_{min} (°C)		Mean T_{max} (°C)	
	WWC Belize	*Key Largo USA*	*WWC Belize*	*Key Largo USA*	*WWC Belize*	*Key Largo USA*	*WWC Belize*	*Key Largo USA*	*WWC Belize*	*Key Largo USA*
Jan	25.8 ± 0.3	22.0 ± 0.4	24.6 ± 0.3	19.7 ± 0.4	26.8 ± 0.3	23.8 ± 0.4	25.3 ± 0.3	21.6± 0.4	26.3 ± 0.3	22.5 ± 0.4
Feb	26.4 ± 0.3	22.5 ± 0.3	25.3 ± 0.3	20.4 ± 0.4	27.4 ± 0.3	24.4 ± 0.4	25.9 ± 0.3	21.8 ± 0.4	27.0 ± 0.3	22.7 ± 0.4
Mar	27.0 ± 0.2	23.5 ± 0.2	26.1 ± 0.2	21.7 ± 0.3	28.0 ± 0.2	25.3 ± 0.3	26.4 ± 0.2	23.1 ± 0.3	27.6 ± 0.2	24.0 ± 0.3
Apr	28.0 ± 0.2	25.8 ± 0.2	27.1 ± 0.2	24.3 ± 0.2	28.9 ± 0.2	27.2 ± 0.3	27.5 ± 0.1	25.3 ± 0.2	28.6 ± 0.1	26.1 ± 0.2
May	28.8 ± 0.1	28.0 ± 0.2	28.1 ± 0.2	26.4 ± 0.3	29.5 ± 0.2	29.6 ± 0.2	28.3 ± 0.1	27.8 ± 0.2	29.4 ± 0.2	28.6 ± 0.2
Jun	29.2 ± 0.2	30.0 ± 0.2	28.4 ± 0.3	28.6 ± 0.4	30.0 ± 0.2	31.2 ± 0.2	28.7 ± 0.2	29.7 ± 0.2	29.8 ± 0.2	30.5 ± 0.2
Jul	28.8 ± 0.1	30.6 ± 0.6	28.2 ± 0.3	29.6 ± 0.3	29.9 ± 0.2	31.6 ± 0.2	28.6 ± 0.2	30.3 ± 0.2	29.6 ± 0.2	31.0 ± 0.2
Aug	29.4 ± 0.2	31.0 ± 0.2	28.7 ± 0.2	29.8 ± 0.3	30.2 ± 0.1	31.9 ± 0.1	28.9 ± 0.2	30.7 ± 0.2	30.0 ± 0.1	31.4 ± 0.2
Sep	29.6 ± 0.2	30.1 ± 0.2	28.7 ± 0.2	29.0 ± 0.3	30.5 ± 0.2	31.1 ± 0.2	29.0 ± 0.2	29.9 ± 0.2	30.2 ± 0.2	30.5 ± 0.2
Oct	28.7 ± 0.2	28.2 ± 0.2	27.6 ± 0.3	26.1 ± 0.3	29.7 ± 0.2	29.7 ± 0.2	28.2 ± 0.2	28.0 ± 0.2	29.3 ± 0.2	28.6 ± 0.3
Nov	27.2 ± 0.2	24.8 ± 0.2	26.1 ± 0.2	23.2 ± 0.3	28.2 ±0.3	26.5 ± 0.3	26.7 ± 0.2	24.5 ± 0.2	27.7 ± 0.2	25.2 ± 0.2
Dec	26.2 ± 0.3	23.3 ± 0.4	25.3 ± 0.3	21.6 ± 0.4	27.1 ±0.3	24.9 ± 0.4	25.7 ± 0.3	23.0 ± 0.4	26.8 ± 0.3	23.7 ± 0.3
Avg	27.8 ± 0.2	26.7 ± 0.2	26.0 ± 0.2	25.0 ± 0.2	28.8 ± 0.2	28.1 ± 0.2	27.3 ± 0.2	26.4 ± 0.2	28.4 ± 0.2	27.2 ± 0.2

Discussion

This study shows a significant difference in thermal tolerance between two sister species of neon gobies confirming genetic and morphological data that suggest separation of these two fishes in warmer habitat (*E. lobeli* on the Mesoamerican Barrier Reef) and cooler habitat (*E. oceanops* on the Florida Key reef) (Fig. 2). The smaller *E. lobeli* had higher critical thermal maxima when compared to *E. oceanops* acclimated at the same temperatures. Overall, critical thermal minima was affected by acclimation temperature. The goby *E. lobeli* exhibited a wider thermal tolerance window than *E. oceanops* but in both species most of the polygon area was accounted for by intrinsic rather than acquired tolerance following acclimation. *Elacatinus lobeli* and *oceanops* are both stenotherms, having a narrow capacity to adjust their tolerance even after a prolonged period of acclimation, thus rendering them susceptible to extreme thermal events. The ecological thermal polygons of these two gobies are comparable to extreme stenotherms such as Antarctic icefish of the family Nototheniidae (Fangue and Bennett 2003). Although average and mean maximum temperatures across the past decade are significantly higher at WWC when compared to Key Largo (~1°C), *E. oceanops* at Key Largo experience significantly higher temperatures during the hottest month of the year (~1.5°C) and live closest to their thermal limits making them more vulnerable to extirpation. Therefore, in this system, mass rather than T_{max} or mean temperature seems to justify the differences in thermal tolerance observed. In fact trends in intraspecific thermal tolerances might suggest that same-age but smaller individuals of *E. oceanops* are better able to cope with high temperatures.

This difference in scale-tolerance relationship might be explained by the correlation between body mass and oxygen-limited active metabolic rates (Oxygen-Limited-Thermal-Tolerance Theory; Pörtner 2004). As temperature increases, fishes accelerate ventilation to deliver oxygen to metabolically demanding tissues (Pörtner and Knust 2008). As the environmental temperature reaches a thermal maximum, the conformational structure of fish hemoglobin changes and consequently becomes incapable of binding and delivering sufficient oxygen to tissues, therefore impairing basic locomotion (loss of equilibrium and muscle spasms) (Pörtner and Knust 2008, Rummer et al. 2014). Although the two species of neon gobies investigated in this study diverged 800,000 years (Taylor & Hellberg 2006) and natural selection might have acted to shape different thermal phenotypes, the negative trend correlation between mass and critical thermal maxima observed within species suggest that a reduced body size is at least implicated in conferring an advantage during extreme heat waves. It is also possible that small changes in dissolved oxygen that were not detected but that could result with increasing temperature could have affected tolerance limits in the two species. Moreover, the two gobies were transported to the laboratory in different way and transport stress may have triggered different stress responses in the two species.

Previous studies on the effect of size on thermal tolerance seem to support this hypothesis. For example, larger eelpouts are more likely to exhibit a reduced tolerance to changes in temperature (Pörtner and Knust 2008). Thermal biology theory predicts that thermal windows increase with reduced body size thereby explaining why smaller animals survived the evolutionary crisis better than larger ones (Pörtner 2004).

Additionally, previous studies have documented that juvenile fishes spend a prolonged period of time (sometimes years) in shallow nursery areas and are likely to experience wide thermal fluctuations (Fangue and Bennett 2004, Wallman and Bennett 2006, DiGirolamo et al. 2011). In freshwater post-glacial lakes, normal-size and dwarf salmonid fishes of the genus *Coregonus* are known to partition resources according to temperature by segregating by depth and have modified digestive and growth processes to exploit their thermal niche (Ohlberger et al. 2008).

As this study shows, smaller individuals are likely to survive higher temperatures when compared to larger conspecifics and congenerics. A shift towards smaller body size will have a significant effect on marine communities interactions by reducing fish biomass as larger individuals produce more eggs (Perry *et al.* 2005). Furthermore, smaller fishes are capable of eating only a fraction of the parasite biomass that larger fishes can ingest (Jobling 1981). Although it is difficult to predict future interactions between parasite-host-cleaner solely on temperature, given the complexity of the systems, results from this and previous studies can help identify sub-lethal effects of reduced cleaning behavior in tropical environments. Fishes with parasites are known to be more susceptible to secondary bacterial infections (Bandilla *et al.* 2006) and predation, have lower body condition and fecundity than fishes without parasites (Vaughan & Coble 1975; Brassard, Rau & Curtis 1982). Moreover, ectoparasites increase drag during swimming, thereby elevating metabolic costs and energetic requirements in tropical reef fishes (Binning, Roche & Layton 2013). Finally, parasitized fishes exhibit reduced thermal tolerance (Vaughan & Coble 1975), with implications for survival in warming

oceans. Although gobies of the genus *Elacatinus* are not the only cleaners in the Caribbean, recent data on the main cleaner, the shrimp *Lysmata amboinensis*, suggest high vulnerability to warming temperature as well (Rosa *et al.* 2014). In conclusion, a reduction in cleaning symbiosis as a consequence of ocean warming has the potential to decrease the health of reef fishes.

Chapter 2: Increasing temperature favors digestion in smaller fishes

Abstract

Digestive metabolism is considered key to resilience of fishes as it determines energy and nutrient availability for growth and survival. Therefore, understanding the influence of temperature on digestion metabolic scope (energy allocated to digestive processes) is crucial to predict responses of fish communities to increasing ocean temperatures. As body size can affect many physiological processes and is thought to decrease with increasing temperature, we examined two sister species of the genus *Elacatinus* and tested the hypothesis that smaller but same age fishes will have an advantage at higher temperature by increasing digestive scope. The dwarf-size *E. lobeli* increased digestive metabolic rates and scope while the larger *E. oceanops* decreased metabolic scope with warming. Intra-specifically, larger *E. lobeli* also showed a decreased metabolic scope when compared to smaller individuals. Results from this study suggest that smaller fishes may have a digestive and metabolic advantage at higher temperatures and may be more resilient under warming temperatures.

Introduction

Cleaning symbiosis is an important mutualistic cooperation and plays an important role in the health of coral reef fish communities (Grutter 1999; Cheney & Côté 2005). Cleaner fishes are known to remove ectoparasites, scales, and mucus from the body surface, gills and mouth of larger cooperating fishes (commonly referred to as 'hosts') at specific sites on the reef, called cleaning stations (Grutter 1999; Grutter & Hendrikz 1999; Arnal & Côté 2000). In order to work, this symbiotic relationship relies on cleaners' immunity against predation and therefore these fishes have evolved a stereotypical bright colored stripe along their body so that hosts can easily recognize them (Arnal & Côté 2000; Whiteman & Côté 2004). Cleaner fishes reduce parasite load (Grutter 1999) and decrease stress in clients (Soares *et al.* 2011), and therefore play an important role in shaping fish communities. Members of the genus *Elacatinus* are the most abundant cleaner fishes in the Caribbean (Sazima *et al.* 2000; Taylor & Hellberg 2005). Some live in socially monogamous pairs while others live in large groups and are highly social (Whiteman & Côté 2004). They provide cleaning services to many species on the reef from herbivores to predatory groupers fishes (Sazima *et al.* 2000). Although their role in reef community resilience is fundamental, to date it is unclear if cleaner fishes are affected by climate change.

It has been suggested that tropical organisms are more sensitive to warming than their temperate counterparts because they live in thermally stable environments (Graham *et al.* 2007; Somero 2010; Donelson *et al.* 2011; Rummer *et al.* 2014). However, the role of thermal acclimatization on resilience of cleaner fishes in ocean warming still needs to

be defined (Beitinger & Bennett 2000; Somero 2010; Donelson *et al.* 2011). It has been shown that the metabolic scope of some tropical reef fish decreases when temperature increases, thus limiting development and reproductive capacity (Farrell *et al.* 2008; Rummer *et al.* 2014). While previous studies analyzed locomotion as an index of metabolic performance, an alternative way to assess the degree of biological impairments caused by warming temperatures in benthic, site-attached fishes, is to measure metabolic scope during the specific dynamic action (SDA) of digestion. It is known that metabolic rates peak during SDA, and termination of SDA is implicated with return of appetite in fish (Jobling 1981; Ferry-Graham & Gibb 2001; Papastamatiou & Lowe 2004; SIMS *et al.* 2006; Di Santo & Bennett 2011a). As return of appetite is fundamental to trigger cleaning behavior (Sazima *et al.* 2000; Arnal & Côté 2000), and the amount of energy available for digestion and the duration of SDA peak is critical to feeding rates (Sims & Davies 1994; Pang, Cao & Fu 2011), identifying the effect of temperature on digestive metabolism will enhance predictions about future dynamics of cleaning symbiosis.

Recent studies have documented a decrease in body size with increasing temperature in many ectotherms (Daufresne, Lengfellner & Sommer 2009; Gardner *et al.* 2011). As body size affects metabolism, food requirements, and competition (Daufresne, Lengfellner & Sommer 2009; Ohlberger *et al.* 2012) it is plausible that smaller fishes may gain an advantage under projected warming. Two sister species of cleaner gobies in the genus *Elacatinus* have different adult size, the larger *E. oceanops* inhabiting coral reefs in the Florida Keys, and the dwarf-size *E. lobeli* living in the Belizean Meso-American Barrier Reef. The two fishes were considered different morphs of the same

species until genetic data revealed that the two species diverged about 800,000 years ago (Taylor & Hellberg 2005, 2006). Here we tested the hypothesis that smaller size gobies increase their digestion scope at higher temperatures, which may provide evidence for an advantage for smaller ectotherms in warmer climates.

Methods

Study system and experimental design

Juvenile *Elacatinus lobeli* (n=12) were collected at Wee Wee Caye, Belize (16.76N, 88.14W),) and transported using Kordon® Breathing Bags™, while juvenile *Elacatinus oceanops* (n=12) were collected in the Florida Keys, USA (25.16N, 80.29W) and shipped using live fish shipping bags with oxygen. Fish were divided by species and randomly assigned to three acclimation groups containing four individuals each. All groups were maintained in well aerated and filtered 130-L aquaria. Water quality in each tank was monitored weekly to test for ammonia, nitrites, and nitrates (Table 2.1). Aquaria were kept at diel photoperiod of 12 h light: 12 h dark and temperature was initially set at 24 ± 0.5°C with a submersible Ebo Jager 50-W aquarium heater. After a two-week period at 24°C, water temperatures were unchanged, or increased or decreased 0.5 °C per day until reaching acclimation temperatures of 20, 24, and 28°C. These temperatures were chosen because they are experienced by both fishes in the wild (Chapter 1). Fish were held at final acclimation conditions a minimum of 21 days before trials to ensure full acclimation to experimental conditions (Beitinger & Bennett 2000;

Fangue & Bennett 2003). Fish were fed a mixed diet of fresh frozen mysis shrimp and marine flakes twice daily throughout the acclimation period.

Table 2.1. Water parameters in experimental tanks.

Water parameter	Treatment 1	Treatment 2	Treatment 3
Temperature (°C)	20	24	28
pH	8.1	8.1	8.1
Salinity	34	34	34
Ammonia	0	0	0
Nitrites	0	0	0
Nitrates	<30	<30	<30
Photoperiod	12L:12D	12L:12D	12L:12D

Measurement of resting and digestive metabolic rates

Oxygen consumption rates (MO_2) at rest (RMR) were determined at each temperature (i.e. 20, 24, 28°C) using a static respirometer. Gobies (n=4 per acclimation temperature per species) were individually placed in an open static respirometer for 24 hours to allow them to get accustomed to the experimental set up. Water was continuously filtered and aerated to maintain normoxic conditions. The respirometer chamber was submersed in a temperature controlled water bath and fitted with a Pro-ODO YSI oxygen probe that does not consume oxygen while measuring concentration in water. Fish RMR was first measured for 30 min prior to feeding. After RMR measurements were completed, the

respirometer chamber was open, and fish were fed 0.5% of body weight (mysis shrimp). Food ingestion was confirmed by direct observation, and the respirometer was immediately closed and oxygen measurements resumed. Oxygen consumption was measured at 30 min intervals for 4 hours.

Statistical analysis

We quantified the following parameters: MO_2 at rest, MO_2 peak, digestive metabolic scope ($MO_{2\ peak}$ - $MO_{2\ rest}$), and duration — calculated as the time from feeding to when the MO_2 of fed fish was not significantly different from pre-fed levels. Digestive metabolic scope was calculated as the difference between $MO_{2\ peak}$ and $MO_{2\ rest}$. The effect of taxon, size, and temperature on metabolic parameters were determined using a 3-WAY ANOVA. Return to resting state after $MO_{2\ peak}$ was determined by repeated measure ANOVA followed by the Dunnett's test. All statistical decisions were based on $\alpha=0.05$. All statistical analyses were performed in JMP Pro version 11.

Results

Mass-adjusted $MO_{2\ rest}$ were not statistically different between species at each temperature treatment (1-WAY ANOVA, $p=0.08$) or among temperatures when analyzed intraspecifically (1-WAY ANOVA, $p>0.05$; Figure 2.1). Following food ingestion, MO_2 increased to a peak after about 60 minutes in both species regardless of temperature before returning to pre-fed levels (Figure 2.2). Digestive $MO_{2\ peak}$ was significantly affected by temperature, species, and mass (3-WAY ANOVA, $p<0.0001$; Figure 2.3). In

particular, mass had a stronger effect across species (p=0.0005) but we also observed interactions between species and temperature (p=0.03). Intraspecifically, *E. lobeli* increased digestive $MO_{2\ peak}$ with temperature (p=0.002) and decreased with mass (p=0.01) with no interactions between temperature and mass (p=0.1). On the other hand, $MO_{2\ peak}$ in *E. oceanops* did not significantly increase with temperature (p=0.07) but decreased with mass (p=0.001).Within temperature treatments, higher body mass reduced $MO_{2\ peak}$ of *E. lobeli* at 28°C (p=0.02) and $MO_{2\ peak}$ of *E. oceanops* at 20 (p=0.006) and 24°C (p=0.02). Finally, digestive metabolic scope was significantly affected by temperature, species and mass (3-WAY ANOVA, p=0.0001) with the strongest effect being species (p=0.005; Figure 2.4 and 2.5). In *E. lobeli*, temperature significantly increased metabolic scope (p=0.02) but mass had no significant effect (p=0.2). In *E. oceanops*, only temperature had a significant effect on metabolic scope by reducing energy available for digestion (p=0.004).

Figure 2.1. Mass-adjusted resting metabolic rates of *Elacatinus oceanops* and *E. lobeli* at three different temperatures. Mass-adjusted resting metabolic rates of *Elacatinus oceanops* (red circles) and *E. lobeli* (blue circles) are not significantly affected by temperatures experienced in their habitat ($p>0.05$, one way ANOVA). Circles represent mean and bars are standard error.

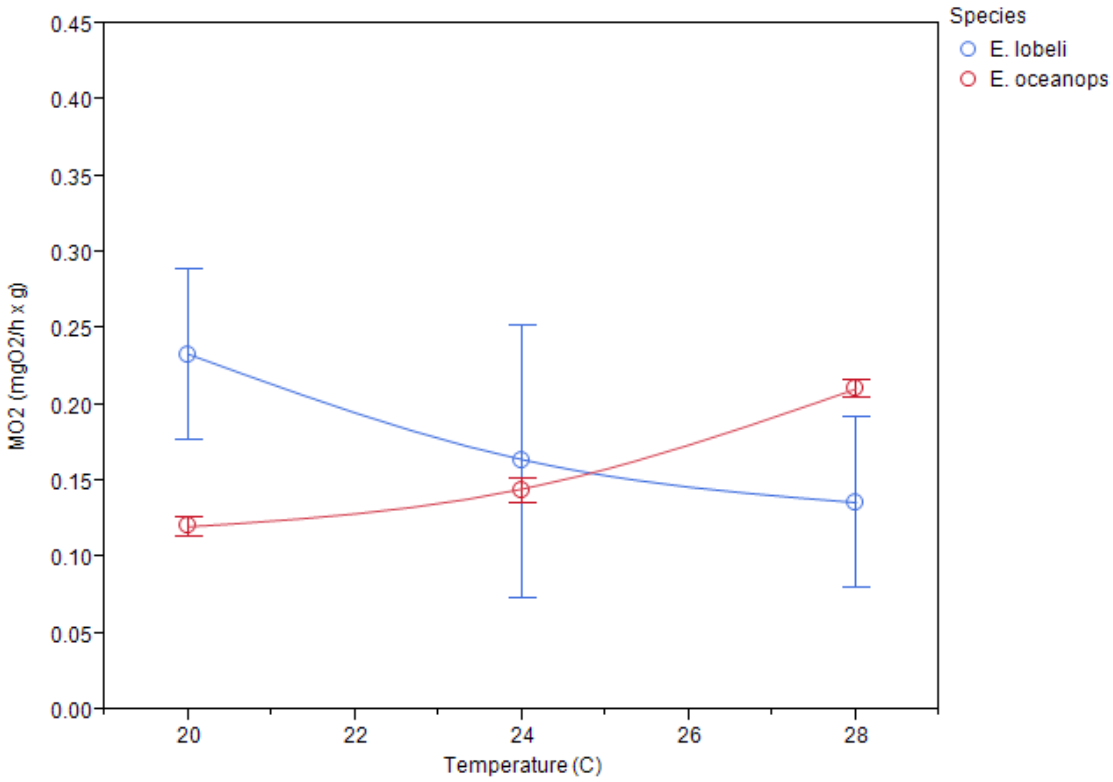


Figure 2.2. Digestive oxygen consumption responses (SDA: mean ± s.e.m.) in adult *E. lobeli* (A) and *E. oceanops* (B) acclimated at three temperatures over time. Temperature significantly affects mean SDA only at 60 minutes post-feeding (p=0.01, repeated measures ANOVA, n=12). Food ingestion occurred at time 0 after 30 minutes of resting oxygen consumption measurements. Scaling of y-axis is different in the two panels.

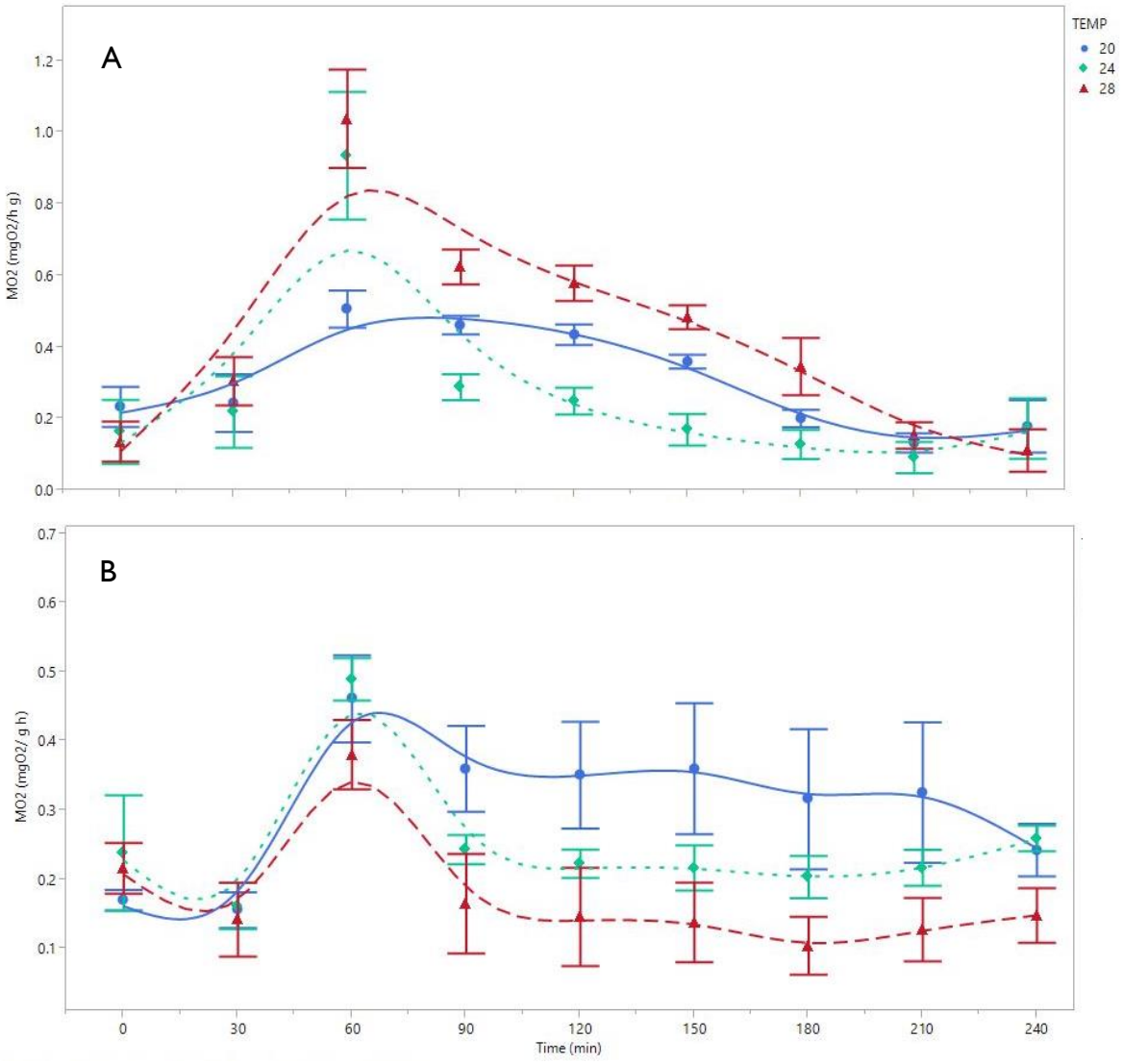


Figure 2.3. Effect of temperature and mass on SDA. Maximum digestive metabolic peak (Max SDA) is affected by temperature and mass in *Elacatinus oceanops* ($p<0.0001$, two way ANOVA, n=12) but only by temperature in *E. lobeli* (temperature: $p=0.04$, mass: $p=0.4$, two way ANOVA, n=12).

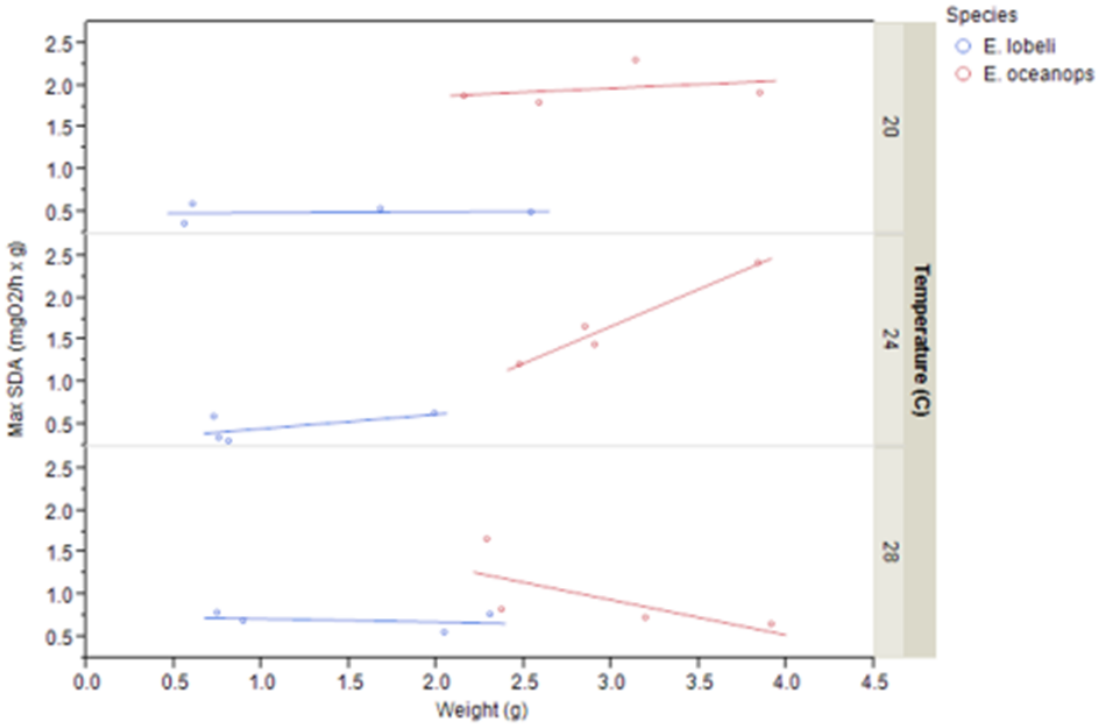


Figure 2.4. Digestive aerobic scope in two gobies at three temperatures. Digestive metabolic scope (mean ± s.e.m.) in adult *Elacatinus lobeli* and *E. oceanops* acclimated at three temperatures. Temperature significantly affects aerobic scope in both species ($p<0.01$, one way ANOVA, n=12 per species).

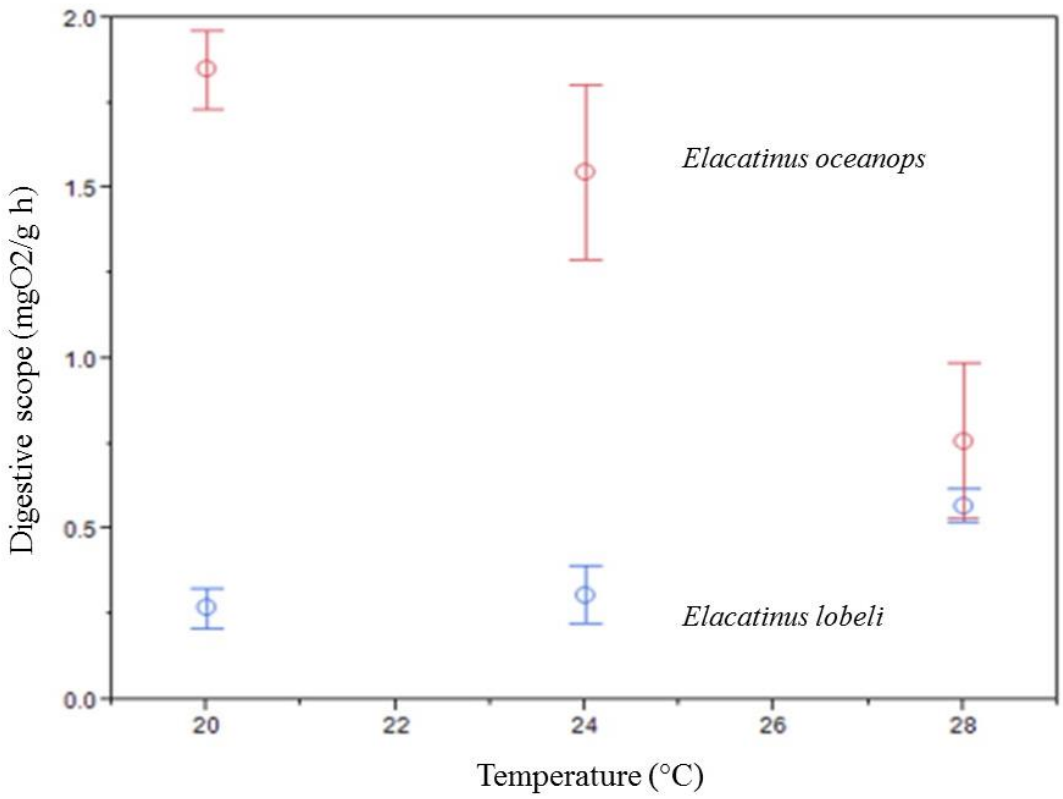
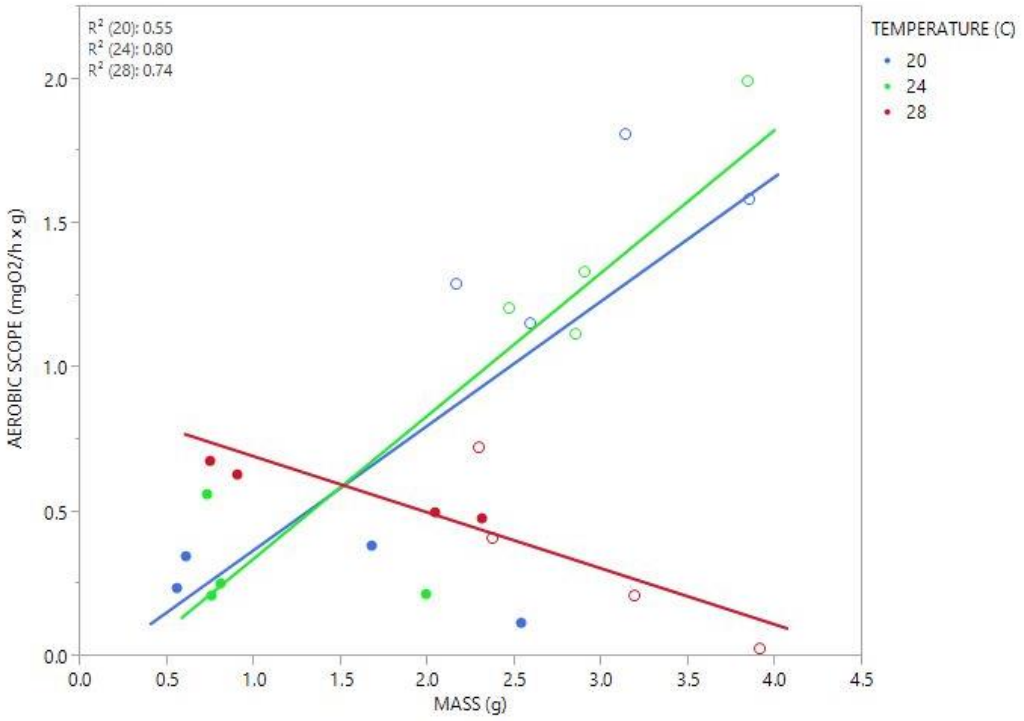


Figure 2.5. Effect of mass on digestive aerobic scope in two gobies. Digestive aerobic scope (Max SDA-RMR) in *Elacatiuns lobeli* (closed circles) and *E. oceanops* (open circles) is affected by mass at each acclimation temperatures (20°C: p=0.03, 24°C: p=0.002, 28°C: p=0.006, one way ANOVA, n=24).



Discussion

This study aimed to evaluate the effect of temperature and size on resting and digestion metabolism in two sister species of cleaner gobies of the genus *Elacatinus*. We found that temperature had a significant effect on digestive metabolic rate and scope, but the effects were different between species. *Elacatinus oceanops* decreased digestive metabolic scope with temperature while *E. lobeli* increased it at the highest temperature

(28°C). Fish mass had a significant effect on digestive metabolic rates by increasing metabolic scope at 20, and 24°C with mass. However digestive scope had an inverse relationship with mass at 28°C. When analyzed intraspecifically, only E. *lobeli* showed a significant effect of body mass on digestive scope.

Body temperature in aquatic ideal poikilotherms (such as fishes) closely matches the thermal environment and most physiological processes are profoundly influenced by temperature (Fry & Hart 1948; Fry 1971; Magnuson, Crowder & Medvick 1979; Di Santo & Bennett 2011b; Pang *et al.* 2011). In particular, digestive processes are known to be sensitive to temperature in fishes that experience thermally fluctuating environments (Wurtsbaugh & Neverman 1988; Neverman & Wurtsbaugh 1994; SIMS *et al.* 2006; Di Santo & Bennett 2011a). Nonetheless, it is not unusual to observe low sensitivity of metabolic rates to a range of temperatures in some fishes (Windell, Foltz & Sarokon 1978; Di Santo & Bennett 2011a) especially if these have evolved in relatively stable thermal environments (Di Santo & Bennett 2011a; b).

Results from this study further advance our understanding of the role of thermal adaptation in physiological processes as they clearly show differences in digestive metabolic responses in closely-related tropical fishes acclimated at the same temperatures. In fact, warming reduced the digestive scope of *E. oceanops* but enhanced it in *E. lobeli*. For both species, post-prandial metabolism peaked after about 60 minutes of ingestion, regardless of temperature suggesting a temperature-insensitive but time-dependent increase in digestive activity. Digestive peak metabolism ($MO_{2\ peak}$) was significantly affected by temperature in both species but only by body mass in *E.*

oceanops. Additionally, *E. oceanops* only showed a clear peak in digestive metabolism at 24°C, and exhibited high variability in $MO_{2\ peak}$ over time at 20 and 28°C. These results are not unusual, as coral reef fishes living close to the limit of their geographic ranges often show high variability in responses to threshold temperatures (Rummer et al. 2014).

As observed during thermal tolerance tests, *E. oceanops* show a reduced thermal window when compared to *E. lobeli* (Chapter 1). According to the oxygen limited thermal tolerance hypothesis (OLTT hp) advanced by Pörtner and Farrell (2008), reduced thermal tolerance windows are associated with a mismatch between oxygen demand and supply at highest temperatures. In fact, the post-prandial metabolic rates of fish acclimated at 28°C are determined by the capacity of the cardio-respiratory system to deliver oxygen to tissues (Pörtner & Farrell 2008). At the highest temperature, oxygen becomes a limiting factor which sets the upper limit of digestive metabolisms in aquatic ectotherms (Pörtner *et al.* 2006; Kassahn *et al.* 2009). At these *pejus* temperatures (where metabolic performance transitions from optimum to an increasingly deleterious range; Frederich & Pörtner 2000), fishes increase energy demand to reestablish ion balance, thereby limiting the capacity to increase digestive capacities beyond their thermal *optimum* (Jobling 1981; Pang *et al.* 2011). In the present study, *E. oceanops* only showed a clear peak at the mid temperature of 24°C, suggesting this may represent the thermal optimum for digestion in this fish. Conversely, *E. lobeli* exhibit an increase in metabolic scope at the highest temperature as well (28°C), thereby demonstrating an enhanced metabolic capacity at high temperatures when compared to its congeneric. These results suggest that *E. lobeli* may be a hyperthermal specialist that thrives at warmer temperature

when compared to *E. oceanops*. However, we cannot exclude the possibility that small changes in dissolved oxygen that were not detected but that could result with increasing temperature could have affected size and digestion in the two species. Moreover, the two gobies were transported to the laboratory in different ways and transport stress may have triggered different responses in the two species.

In both species, low temperatures reduced digestive scope while increasing digestion time. Even though a prolonged contact of food with digestive surfaces is known to enhance nutrient absorption (Di Santo and Bennett 2011), this strategy is thought to be more beneficial to intermittent feeders such as large predators (Di Santo and Bennett 2011, Sims et al., 2007). For continuous feeders such as cleaner gobies, faster digestion rates and therefore quicker return of appetite are to be considered more advantageous than a more efficient absorption as these fishes remove parasites from their clients throughout the day. Cessation of the SDA response (end of the $MO_{2\ peak}$) is key to return of appetite and initiation of feeding in fishes (Sims & Davies 1994). It is therefore plausible to predict that cleaning rates (as the rate of parasite removal from clients) would peak at 24°C in *E. oceanops* but would increase in *E. lobeli* with warming. Given that *E. oceanops* live close to their thermal maximum (Lobel and Di Santo, in prep) and are the main parasite removers on the Florida Keys coral reef fishes (Rüber, Tassell & Zardoya 2007), further warming might slow cleaning rates with serious repercussions on overall health of fish community. On the other hand, higher temperatures accelerate cleaning rates and digestion in *E. lobeli*, thus favoring parasite removals on coral reefs of Belize.

When both species were analyzed together, mass significantly increased digestive metabolic scope at lowest temperatures while decreasing metabolic scope at 28°C. When analyzed separately only *E. lobeli* showed a decrease in digestive scope with increasing size. It is possible that digestive metabolic scopes decrease beyond a "threshold size" in these cleaner fishes and only the largest individuals of the dwarf-sized *E. lobeli* are penalized by reaching a larger size. This is consistent with the Temperature-Size Rule observed in nature that describes the decrease in body size with increasing temperature (Daufresne, Lengfellner & Sommer 2009). This repeatability of this latitudinal body size pattern coupled with fossil records showing an increase in small bodied organisms during past climatic shifts, have led to the prediction that the current global warming will cause a shift towards a reduced body size (Gardner *et al.* 2011; Clark *et al.* 2012). However the direct causes of a reduced body size have not been resolved yet. It is well known that body size affects metabolism but most studies have analyzed single species or locations and could not control for localized effects on body size (Daufresne, Lengfellner & Sommer 2009; Gardner *et al.* 2011).

Decrease in body mass in fish communities as the result of warming can be the product of different processes. For instance, small individuals could gain some reproductive or physiological advantage and increase in number in the population. This outcome could be achieved through a shift in size-at-age (observable in adult individuals) and/or through an increase in juveniles in a population (thereby reducing the life span of individuals). It is possible that in reality both processes are occurring. Older and larger eelpot showed a reduced tolerance to warming when compared to younger and smaller

individuals (Pörtner 2001; Pörtner 2002; Pörtner & Farrell 2008). It is also plausible that smaller adult individuals may be better able to cope with warming because of the reduced mismatch between oxygen demanding tissues and delivery by the cardio-respiratory system (Pörtner 2001). The latter hypothesis would explain the reduction in digestive metabolic scope observed in cleaner gobies in the present study. In fact, according to the OLTT hp, ectotherms reduce metabolic performance beyond a thermal pejus and larger individuals experience lower performance with warming faster because their cardio-respiratory system cannot keep pace with the oxygen demand of tissues (Pörtner & Farrell 2008). Therefore, results from this study point out that in warmer environments smaller fish may gain a physiological advantage. It is plausible to predict a shift towards smaller cleaner fishes in warming ocean scenarios. It is still unknown if cleaner gobies communities will be able to increase in density to compensate for slower rates of cleaning and smaller biomass of ectoparasites removed from other coral reef fishes.

Chapter 3: Ocean acidification exacerbates the impacts of global warming on embryonic skates

Abstract

Ocean acidification and warming have the potential to profoundly impact marine fishes by reducing embryo fitness and survival. However, local adaptation to thermal gradients may reduce the impact of global warming, but whether fish from different populations may respond differently to climatic stressors remains unknown. Here I test the hypothesis that acidification and warming may have an effect on development, aerobic scope, and survival of little skate (*Leucoraja erinacea*) embryos from two latitudinally separated populations. Temperature had the strongest effect on development, survival and metabolic rates, but acidification further exacerbated stress on embryos from the Gulf of Maine population by increasing the costs of activity and reducing body condition newly hatched skates. Aerobic metabolic rates of both populations exhibited countergradient variation with peak of performance at 18°C but were affected differently by acidification. These findings demonstrate that even adjacent fish populations may respond differently to increasing temperatures and acidification and emphasize the need for multi-stressor studies on different populations of fishes with wide geographic range to understand complex responses to climate change and other environmental challenges.

Introduction

Since the industrial revolution, atmospheric concentrations of carbon dioxide (pCO_2) have risen by 41% to ~400ppm, thus exceeding levels experienced over the past 650,000 years (Smith *et al.* 2009; Baumann, Talmage & Gobler 2011). In addition to accelerating atmospheric ad thus oceanic warming, about 30% of CO_2 introduced in the atmosphere enters the oceans causing a decrease in pH, a phenomenon known as ocean acidification (Rosa *et al.* 2014). As warming and acidification may act synergistically to decrease fitness of fishes, researchers are investigating their combined effect on physiological processes (Todgham & Stillman 2013; Rosa *et al.* 2014). Fishes are particularly vulnerable to warming as nearly every metabolic function depends on temperature (Fry 1971; Di Santo & Bennett 2011a; b), and shifts in migration and reproductive timing as well as in geographic ranges have already been widely observed (Perry *et al.* 2005; Greenstein & Pandolfi 2008; Gregory, Christophe & Martin 2009). Furthermore, acidification has the potential to exacerbate the effect of warming by increasing osmoregulatory costs to buffer body fluid acidosis (Claiborne, Edwards & Morrison-Shetlar 2002). However, to date, there is little direct evidence that the CO_2 levels projected by the end of the century will significantly affect adult fishes because of their efficient acid-base capacities (Ishimatsu *et al.* 2004; Rummer *et al.* 2013). On the other hand, recent data suggest that when reared at low pH, teleosts exhibit a reduction in survival and tissue function (Baumann *et al.* 2011; Chambers *et al.* 2013), impaired olfactory abilities (Munday *et al.* 2008) and abnormal otolith growth (Checkley *et al.*

2009; Bignami *et al.* 2013) suggesting that early life stages (i.e., embryos and juveniles) may be particularly vulnerable to this consequence of increased pCO_2.

Whereas, an understanding of the separated effects of increasing temperature and ocean acidification on fish performance are generally understood (Fry 1971; Ishimatsu *et al.* 2004; Di Santo & Bennett 2011a), there is still an urgent need to investigate the combined effect of these two major climatic stressors on morphology, survival, and metabolic rates (Todgham & Stillman 2013). In fact, synergistic stressors may trigger complex responses in fishes, by reducing, enhancing or even causing no changes in different physiological processes (Rummer *et al.* 2013; Allan *et al.* 2014). Furthermore, local adaptation to thermal gradients could have important consequences in climate change scenarios as warm-adapted individuals may survive rapid increase in temperature and replace cold-adapted conspecifics (Angilletta, Oufiero, C. E & Sears, M. W 2004). Yet, empirical data are still needed to resolve the effect of local adaptation on chronic stress and survival in fishes challenged by changes in the environment. Such data may provide evidence that stress responses and physiological performance following climate change may depend on local adaptations in fish species that have a relatively wide geographic range, a phenomenon already documented in marine invertebrates (Dong & Somero 2009; Sorte, Jones & Miller 2011). To answer this crucial question it is necessary to conduct "common garden" experiments where physiological responses to environmental changes are measured in individuals from different populations that are reared at the same conditions (Angilletta Jr 2001; Baumann & Conover 2011).

Here, I exposed little skate *Leucoraja erinacea* (Mitchill) embryos from two latitudinally separated populations to current and projected pH (8.1, 7.7) and temperature (15, 18, 20°C) according to the model RCP 8.5 (Meehl & Collins; Meinshausen, Raper & Wigley 2008) in a fully-crossed experimental design to quantify the combined and individual effects of acidification and warming on key morphological and physiological traits. The little skate is an oviparous elasmobranch that is found along transitional regions of the northwestern Atlantic such as the Gulf of Maine (GM) and Georges Bank (GB), where impacts of sharp thermal discontinuities are evident (Frisk 2002). Furthermore, the little skate exhibits increasing size with latitude (Frisk & Miller 2006, 2009), which suggest that this species is split into populations with locally-adapted metabolic functions. A critical stage in the life of oviparous elasmobranchs is during the relatively long development (between about five months and one year for this species; Luer & Gilbert 1985), because embryos are unable to utilize their thermal environment by undertaking thermotaxic behavior (Di Santo & Bennett 2011a). Although increasing temperature is likely to reduce survival and aerobic performance in embryos (Luer & Gilbert 1985; Palm *et al.* 2011), there is as yet no evidence that low pH conditions expected by the end of the century will affect embryonic skates. Additionally, by investigating separated populations from two geographic locations (North and South), it is possible to test the hypothesis that local adaptation can affect responses to climate change. Comparisons between treatments will allow us to determine individual and combined effects of acidification and warming on different physiological processes that are linked to fitness and survival. More specifically, I examined how ocean warming and

acidification affect: i) embryonic development and survival, ii) embryonic aerobic scope, and iii) newborn body condition and initiation of feeding.

Materials and Methods

Egg incubation and experimental system

Newly laid (~1 week old) little skate eggs were obtained from wild caught females at two distinct locations, Gulf of Maine (43°N, 68°W) and Georges Bank (41.21°N, 67.38°W), USA (northern and southern populations, respectively), and reared in the laboratory under controlled conditions (Table 3.1). Viable eggs were randomly assigned to a treatment group in a fully crossed experimental design, to match the current and projected temperatures (15, 18, 20 °C) and pH (8.1, 7.7) as suggested by the *Guide to best practices for ocean acidification research and data reporting* (Riebesell *et al.* 2010) according to high emission scenarios by year 2100 model RCP 8.5 (IPCC 2013). Each tank (150L) had independent temperature and CO_2 control. Embryos were held in a temperature-controlled environmental chamber (Harris Environmental Systems, Inc., Andover, MA, USA) set at 12°C and each experimental tank was maintained at constant temperature (either 15, 18 or 20°C) by a submersible titanium heater unit (Finnex 300W) controlled by a digital thermostat (Aqua Logic Inc., San Diego, CA, USA). In addition, each tank was provided with a mix of air:CO_2 (water pH = 7.7 ±0.05 which resulted in pCO_2 ~1100ppm) or ambient air (water pH = 8.1 ±0.05 which resulted in pCO_2 ~400ppm) and controlled by an Aqua Medic pH computer (Aqua Medic of North

America, Loveland, CO). Skates were reared at constant salinity (33ppt) and photoperiod (14L:10D) and fed frozen mysis shrimp twice daily after hatching.

Table 3.1. Water parameters in experimental tanks.

Water parameter	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6
Temperature (°C)	15	15	18	18	20	20
pH	8.1	7.7	8.1	7.7	8.1	7.7
Salinity (ppt)	33	33	33	33	33	33
Ammonia (ppm)	0	0	0	0	0	0
Nitrites (ppm)	0	0	0	0	0	0
Nitrates (ppm)	<30	<30	<30	<30	<30	<30
Photoperiod	14L:10D	14L:10D	14L:10D	14L:10D	14L:10D	14L:10D

Development, survival and body condition

Yolk area was initially measured in a subsample of embryos from both populations (n=10 each) under a light source. Each egg was monitored daily to detect mortality. Survival was then measured again 30 days after hatching. Within 24 hours of hatching, skates were weighed and measured to determine body condition as mass (g) x disc area^{-1} (cm^2).

Metabolic performance curves

Skate embryos possess a long whip-like appendage on the tail which is inserted into a horn of the egg case where it is rapidly oscillated (Leonard, Summers & Koob 1999). This activity can increase oxygen consumption by 81% at 15°C from resting state (Leonard *et al.* 1999). Therefore, as classic swimming performance tests to determine aerobic costs are not feasible in embryos, the approach in this study was to quantify oxygen consumption of embryos moving in the egg case, or active metabolic rate (AMR) and compare it to standard metabolic rate (SMR). To achieve this goal, individual embryos were placed in a custom-made 1cm-thick acrylic intermittent-closed respirometer (0.465L) fitted with a YSI ProODO oxygen meter. In both experiment series, embryonic metabolic rates were measured every 30 minutes for 2 hours after a 1 hour adjustment to experimental conditions (Leonard *et al.* 1999); oxygen saturation never fell under 80% (Steffensen 1989; Di Santo & Bennett 2011b). To measure SMR, embryos were anesthetized using tricaine methansulfonate (MS-222) buffered with $NaHCO_3$ and NaOH to stop voluntary tail beating while retaining gill movement (Benetti, Brill & Kraul 1995; Leonard *et al.* 1999). Benetti et al. (1995) showed that MS-222 had no significant effect on fish RMR. Only near-hatch embryos (with yolk diameter ~1mm) were used to determine metabolic rates (Leonard *et al.* 1999). Metabolic rates (MO_2) were calculated following the formula:

$$MO_2 = (O_{2\,start} - O_{2\,end}) \times volume \times time^{-1} \times mass^{-0.67};$$

where O_{2start} and O_{2end} are oxygen concentration at the start and the end (mg L^{-1}), volume represent the total volume of the respirometer (L), time is expressed in hours and mass is

expressed in g. The mass exponent of 0.67 was used to correct for the allometric relationships between metabolic rates and mass in elasmobranchs (Di Santo & Bennett 2011a; b). Performance curves were constructed by fitting a binomial curve to metabolic data (Baumann & Conover 2011).

Statistical analyses

The effect of temperature and pH on morphological and physiological responses were explored by analysis of variance (ANOVA) using fish population, temperature, and pH as factors. Percentage data (% survival) were subjected to arc sine square root transformation prior to analysis. Statistical significance was determined based on $\alpha = 0.05$. Data are shown as mean ± standard error. Statistical analyses were run in JMP Pro (version 11).

Results

The 3-WAY ANOVA revealed that acidification, temperature and origin of population had a significant but complex effect on embryonic development ($F_{7,39}=10.09$, $p<0.0001$), aerobic performance ($F_{7,51}=3.01$, $p=0.01$) and newborn body condition ($F_{7,106}=7.87$, $p<0.0001$). Water parameters and potential effects on fish are included (Table 3.2).

Table 3.2. Changes in water parameters associated with warming and acidification, and potential effect on fishes.

Water parameter	Effect on fishes	Levels in the tank	References
Reduction in oxygen	Lower oxygen depresses metabolic rates (if below 40% saturation) and reduces growth	>80%	Cheung et al. (2009) Perry et al. (2005) Di Santo et al. (in prep)
Reduction in bicarbonate	Reduction in water absorption in teleosts	---	Kurita et al. (2008)
Increase in carbonic acid	Anesthetic (150 to 600 mg/L)	---	Post (2011)
Ammonia	lethal	0 ppm	Fry (1971) Smith et al. (2004)
Nitrites	lethal	0 ppm	Fry (1971) Smith et al. (2004)
Nitrates	No effect below 40 ppm in elasmobranchs	20-25 ppm	Smith et al. (2004)

(a) Development, survival and body condition

At current oceanic pH (8.1), GM embryos developed faster than GB embryos at all temperatures, showing countergradient variation between northern and southern populations ($F_{7,39}$=10.09, p=0.001; Figure 3.1). Additionally, low pH had a significant effect only in the GM embryos by increasing development time, and thus reducing performance, across temperatures (p=0.03; Figure 3.1). Low pH did not significantly decrease hatching success in either population (2-WAY ANOVA, p=0.6).

Even accounting for the ~20% mortality that occurred in the control treatment (15°C, pH 8.1), embryonic survival declined at highest temperature in both populations (3-WAY ANOVA, $F_{7,49}$=1.12, temperature: p=0.01), suggesting this temperature may represent the thermal pejus for performance and survival (Figure 3.2A). Likewise, post-hatch survival measured decreased at the highest temperature regardless of pH in GB population (2-WAY ANOVA, $F_{3,26}$=5.76, p=0.0004) while survival was not significantly affected by either stressors in GM population (2-WAY ANOVA, $F_{3,21}$=0.59, p=0.6; Figure 3.2B). Although initial yolk area of newly-laid embryos did not differ significantly between populations (GM: 26.08 ±0.41cm^2, GB: 25.96 ±0.43cm^2; 1-WAY ANOVA, $F_{1,18}$=0.04, p=0.8), newborns from GM population had higher weight and larger disc size ($F_{7,106}$=45.05, p<0.0001), than GB population, regardless of treatment (p>0.05; Table 3.3). However, warming and acidification reduced newborn body condition at low pH ($F_{1,106}$=14.6, p=0.0002) and high temperature ($F_{1,106}$=17.18, p<0.0001) in both populations. Additionally there was a linear relationship between body condition and the

latency of newborns to begin feeding in both populations ($F_{1,106}$=8.46, p<0.0001, Figure 3.3).

Table 3.3. Effect of temperature and pH on key morphological traits in two skate populations.

treatment T(°C); pH	population	weight (g)	disc width (cm)	body condition (g/cm^2)
15; 8.1	**GM**	**6.442 ±0.089** ^{a,‡}	**6.19 ±0.112** ^{a}	**0.168 ±0.005** ^{a}
control	**GB**	**4.203 ±0.112** ‡,*	**4.24 ±0.069** ^{a,‡,*}	**0.234 ±0.006** ^{a,‡,*}
18; 8.1	**GM**	**6.218 ±0.201** ^{b}	**6.01 ±0.106** ^{b,‡}	**0.172 ±0.005** ^{b,‡}
	GB	**3.859 ±0.109** ‡,*	**6.16 ±0.230** ^{a,‡}	**0.106 ±0.006** ^{b,‡,*}
20; 8.1	**GM**	**5.564 ±0.131** ^{c,‡}	**7.00 ±0.25** ^{c}	**0.115 ±0.011** ^{c}
	GB	**4.716 ±0.170** ‡,*	**5.06 ±0.156** ^{c,‡,*}	**0.187 ±0.011** ^{c,‡,*}
15; 7.7	**GM**	**6.077 ±0.089** ^{A}	**6.54 ±0.26** ^{A}	**0.114 ±0.012** ^{A}
	GB	**3.855 ±0.047** ^{A,*}	**5.26 ±0.041** ^{A,*}	**0.138 ±0.001**
18; 7.7	**GM**	**6.16 ±0.080** ^{B}	**6.35 ±0.078** ^{A}	**0.152 ±0.004** ^{B}
	GB	**3.538 ±0.097** ^{B,*}	**5.33 ±0.046** ^{B,*}	**0.124 ±0.002** *
20; 7.7	**GM**	**5.038 ±0.044** ^{C}	**7.12 ±0.083** ^{B}	**0.099 ± 0.003** ^{C}
	GB	**5.234 ±0.141** ^{C}	**6.15 ±0.181** ^{C,*}	**0.141 ±0.007** *

Mean (±s.e.m.) for little skate (Leucoraja erinacea) exposed to different temperature levels (T) and pH. GM= Gulf of Maine population (n=37); GB= Georges Bank population (n=77). Different lower and upper case letters represent significant differences within high and low pH condition, respectively; double daggers represent significant differences between pH treatment at each temperature; asterisks represent significant differences between populations (p<0.05).

Figure 3.1. Effect of acidification and warming on embryonic development time. Developmental time (mean ± s.e.m) of *Leucoraja erinacea* embryos from two populations (Georges Bank n=24, Gulf of Maine n=23), at three temperatures and two pH conditions. Different lower and upper case letters represent significant differences within high and low pH condition, respectively; double daggers represent significant differences between pH treatment at each temperature; asterisks represent significant differences between populations ($p<0.05$).

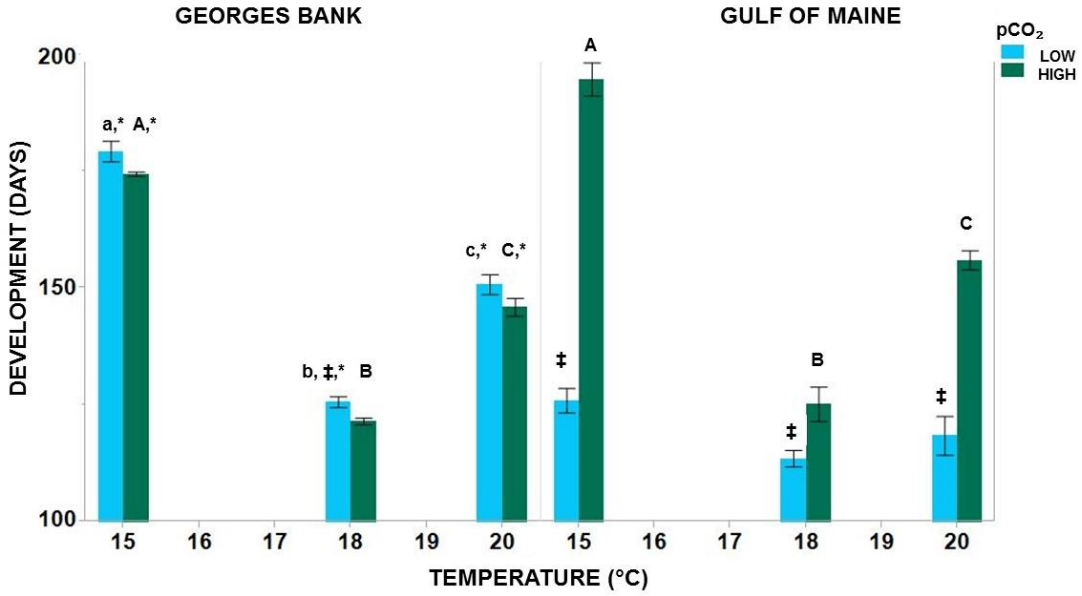


Figure 3.2. Pre- and post-hatching survival in the little skate. (a) Hatching success and (b) 30 days post-hatching survival of *Leucoraja erinacea* from two populations (Georges Bank n=77, Gulf of Maine n=37), at three temperatures and two pH conditions. Different lower and upper case letters represent significant differences within high and low pH condition, respectively; double daggers represent significant differences between pH treatment at each temperature; asterisks represent significant differences between populations ($p<0.05$).

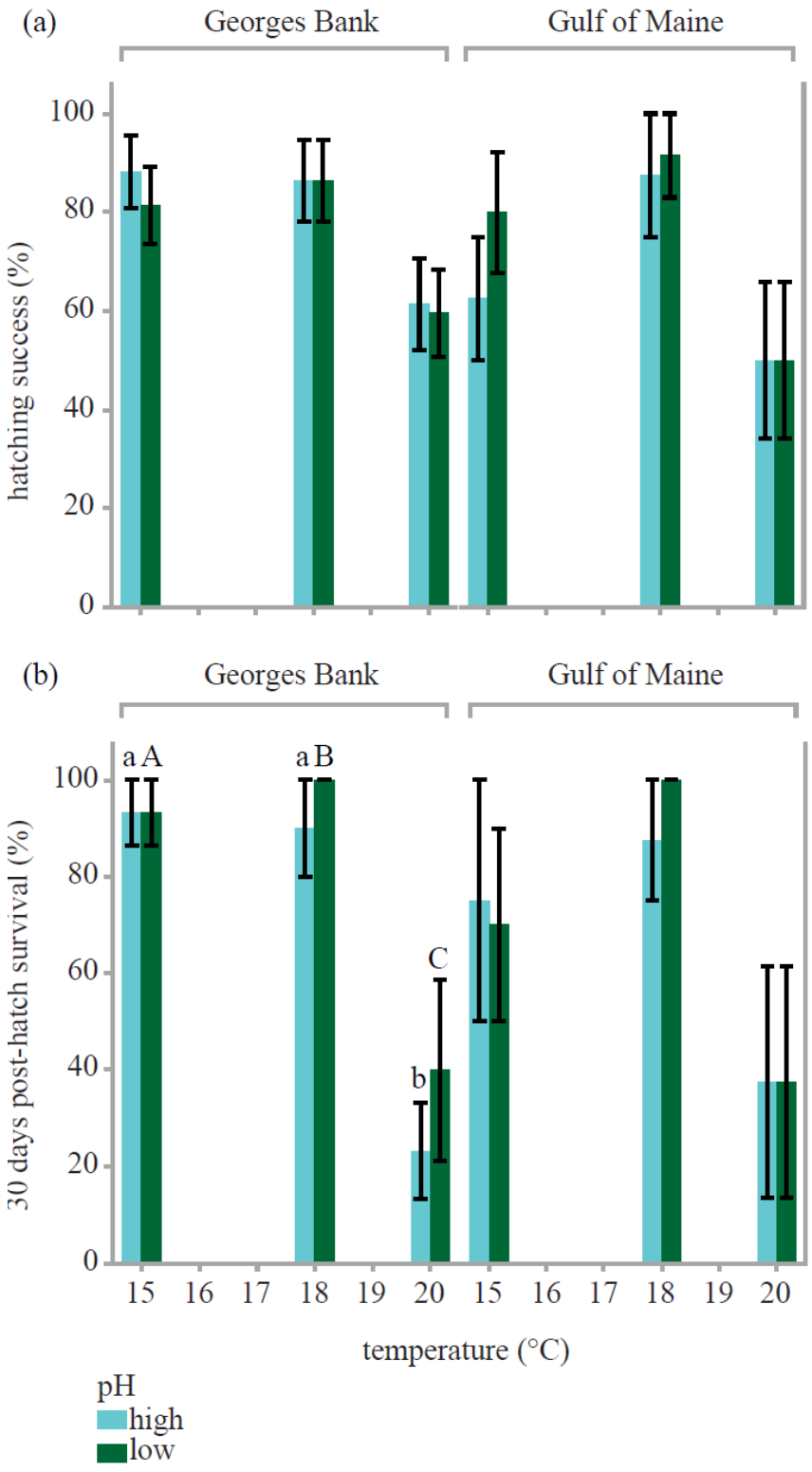
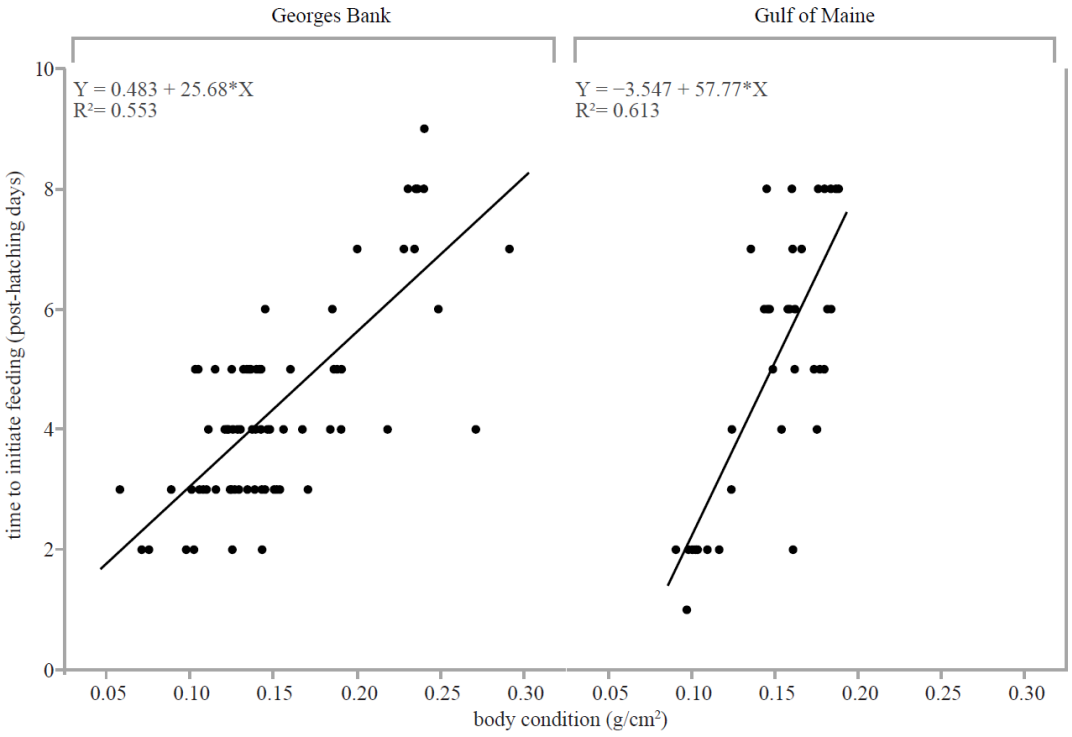

(a)
Georges Bank
Gulf of Maine
hatching success (%)
100
80
60
40
20
0
(b)
Georges Bank
Gulf of Maine
30 days post-hatch survival (%)
a A
a B
C
b
100
80
60
40
20
0
15
16
17
18
19
20
15
16
17
18
19
20
temperature (°C)
pH
high
low

Figure 3.3. Body condition determines when newly-hatched skates start to eat. The time elapsed from hatching to first feeding in newborn *Leucoraja erinacea* from the Gulf of Maine (n=37) and the Georges Bank (n=77).

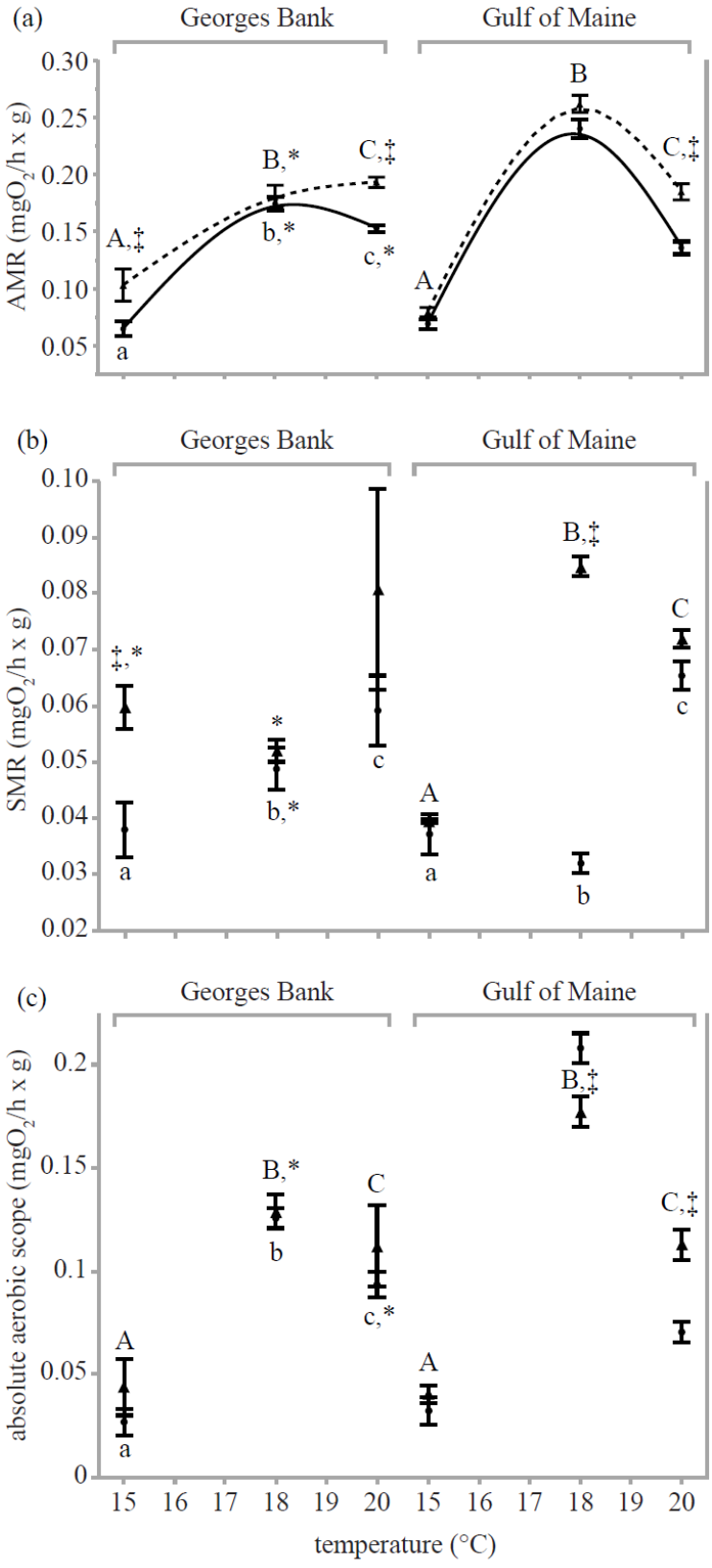


(b) Metabolic performance curves

Active metabolic rates peaked at 18°C, again showing countergradient variation between GM and GB populations (Figure 3.4a). Overall, there was a significant effect of treatments on AMR (3-WAY ANOVA, $F_{7,58}$=7.63, p<0.0001), with temperature and population having the highest impact (p<0.0001, p=0.005, respectively). Active metabolic rates were significantly affected by temperature (p<0.0001) and pH (p=0.01) in

GB embryos (2-WAY ANOVA, $F_{3,26}$=21.62, p<0.0001), but only significantly affected by temperature (p=0.0008) in GM embryos (2-WAY ANOVA, $F_{3,25}$=5.30, p<0.0001; Figure 3.4a). Low pH significantly increased AMR at 20°C in the GM population when compared to high pH (2-WAY ANOVA, $F_{1,8}$=31.93, p=0.0005). Low pH significantly increased SMR at 15°C in GB embryos ($F_{1,8}$=12.23, p=0.008, Figure 3.4b) but had no significant effect on SMR at the optimal temperature for performance, 18°C (2-WAY ANOVA, $F_{1,8}$=0.57, p=0.4, Figure 3.4b). Conversely, in GM embryos, low pH only significantly increased SMR at the peak of their performance (2-WAY ANOVA, $F_{1,8}$=469.33, p<0.0001, 18°C). Overall, the aerobic scope (AMR-SMR) increased up to the optimal temperature (18°C) and declined at highest temperature (20°C) in both populations (3-WAY ANOVA, $F_{7,51}$=3.01, p=0.01), while low pH increased the costs of activity of GM embryos at higher temperatures (2-WAY ANOVA, $F_{1,8}$=8.87, p=0.01, 18°C; $F_{1,8}$=23.25, p=0.001, 20°C; Figure 3.4c).

Figure 3.4. Metabolic rates of little skate at here temperatures and two acidification levels. Mass-adjusted (a) active metabolic rates, (b) standard metabolic rates, (c) aerobic scopes (mean ±s.e.m) of *Leucoraja erinacea* from the Gulf of Maine (n=29) and the Georges Bank (n=30) at three temperatures and two pH (high: circle and continuous line, low: triangle and dashed line).


(a)
Georges Bank
Gulf of Maine
AMR (mgO2/h x g)
0.30
0.25
0.20
0.15
0.10
0.05
A,‡
B,*
C,‡
a
b,*
c,*
A
B
C,‡
(b)
Georges Bank
Gulf of Maine
SMR (mgO2/h x g)
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
‡,*
*
a
b,*
c
A
a
B,‡
b
C
c
(c)
Georges Bank
Gulf of Maine
absolute aerobic scope (mgO2/h x g)
0.2
0.15
0.1
0.05
0
A
a
B,*
b
C
c,*
A
B,‡
C,‡
15
16
17
18
19
20
temperature (°C)

DISCUSSION

This study demonstrates a significant effect of stressors associated with climate change on elasmobranch embryos by providing empirical evidence that, when exposed to increased warming and acidification, little skate embryos exhibit: 1) increased developmental time outside optimal conditions, 2) higher metabolic costs with decreasing pH, 3) decline in body condition, and 4) decreased survival. Furthermore, although initial yolk area did not differ between populations when raised in common garden conditions, newborns from the southern population (GB) showed smaller body size than the ones from the northern population (GM). This suggests local adaptation in metabolic processes by countergradient variation (Baumann & Conover 2011), a pattern also observed in the wild by Frisk and Miller (Frisk 2002; Frisk & Miller 2006, 2009). As embryos were collected from sets of mothers held in different laboratories, feeding and size could not be determined for the parental generation. Maternal effect of different populations is generally measured by looking at the yolk size of embryos (Bengtson, Barkman & Berry 1987; Angilletta *et al.* 2004) . In this study, yolk size of embryos from the two populations were not statistically different, which implies that mothers' condition did not significantly affect energy reserves in embryos, which are key for growth and metabolic activities (Storm & Angilletta 2007). In addition, both labs maintained skates at 15-16°C thus reducing the potential effect of different thermal acclimation across generations (Donelson *et al.* 2011). Small differences in water quality caused by changes in temperature and CO_2/pH such as bicarbonate, carbonic acid, dissolved oxygen could have also affected performance in embryos and therefore cannot be discounted (Table 3.2).

However, larger body size and increased performance (aerobic and developmental) in the GM skates have significant tradeoffs. Body condition was overall lower in the GM population, and skates suffered long-term high mortality outside optimal conditions. Additionally, acidification had a stronger effect on the GM population suggesting that local adaptation may also have a role in the response to decreased pH. Given that embryos were reared at the same conditions, the responses should be attributed to genetic differences rather than physiological plasticity (Baumann & Conover 2011). It is possible that, given the higher metabolic costs associated with activity in the GM embryos when compared to GB ones, the additional stress induced by acidification may have exacerbated chronic stress in the northern population. Therefore, even though weight and disc size were greater at high pCO_2 and temperature, the relative change was different. In fact, at projected levels of pCO_2, skates grew in disc area much more than in weight, which resulted in reduced body condition. These results corroborate previous findings on *L. erinacea* that showed smaller but healthier newborns at lower temperatures when compared to higher temperatures (Palm *et al.* 2011). Poor body conditions may have far-reaching consequences for skate populations. In this study, newborn body condition had a direct relationship with the time elapsed from hatching to first feeding event, likely as a way to compensate for low stored energy. In nature, the necessity to quickly initiate exploration of the environment in order to procure prey, may dramatically increase predation risks and mortality in newborns (Munch & Conover 2003). Furthermore, it is likely that low body condition may be a consequence of faster depleted yolk reserves as higher temperature and pCO_2 elevate embryonic metabolic rates.

In both populations, aerobic scope increased up to 18°C (*thermal optimum*) but decreased at 20°C (*thermal pejus*) (Pörtner & Knust 2007). However, the southern population was less sensitive to the higher temperature suggesting a narrower thermal window for the northern population. These results support the 'oxygen and capacity limitation of thermal tolerance' (OCLTT) hypothesis advanced by Pörtner and Knust (2007). According to the OCLTT hypothesis, the metabolic rates of aquatic ectotherms might be constrained by a reduced capacity of the cardio-respiratory system to extract oxygen at high temperatures thereby causing a mismatch between oxygen demand and supply to tissues (Pörtner & Knust 2007; Rosa *et al.* 2014). Increasing hypercapnia is also known to further increase metabolic costs and therefore reduce the amount of energy for growth (Baumann *et al.* 2011; Rosa *et al.* 2014). In this study, high CO2 only had a significant effect on aerobic scopes of the northern population at higher temperatures indicating that GM skates may be less resilient in future acidification and warming conditions.

Finally, mortality in embryos resulted in the first five weeks of development when the egg case plugs were not absorbed yet, suggesting that perhaps temperature rather than pCO_2 determined survival. These findings are different from previously observed results in which acidification decreased embryonic teleost survival (Baumann *et al.* 2011; Chambers *et al.* 2013). A possible interpretation is that embryonic skates are initially protected from chemical changes in the environment due to their closed hard shells (Luer & Gilbert 1985). Each horn of the egg case has a plug with albumen that protects the embryos from biochemical changes in the environment until they develop competent gills

to maintain homeostasis (about one third of the embryonic period; Luer & Gilbert 1985). On the contrary, teleost embryos are directly exposed to the surrounding environment, making them more vulnerable to changes in pCO_2 (Baumann *et al.* 2011; Bignami *et al.* 2013; Chambers *et al.* 2013).

In summary, results from this study suggest that embryonic development and aerobic scope are affected differently by increasing warming and acidification in two little skate populations, and low pH exacerbates the effect of increasing temperature in at least the northern population. Decreased body condition, as a result of the synergistic effect of acidification and warming, triggers newborn skates to start feeding sooner therefore making them more vulnerable to predation. Furthermore, performance curves in the two populations suggest local adaptation by countergradient variation. Lastly, in light of this study it is apparent that an increase in temperature beyond 18°C will likely reduce fitness and survival of little skates and that the Gulf of Maine population may be more vulnerable at acidification levels expected by the end of the century.

Chapter 4: Geographic variation in performance curves determines vulnerability to climate change in a benthic elasmobranch

Abstract

Understanding the combined effects of increasing temperature and ocean acidification on performance of fishes is central to our understanding of how fish species will respond to global climate change. Metabolic costs associated with intense and short exercise, a measure of energy required to escape predators is a key performance parameter linked to many aspects of fish life history. I compared performance of juvenile little skate *Leucoraja erinacea* raised at current and predicted temperatures and pH conditions by using a chasing protocol. Results from this study suggest countergradient variation in growth between two populations. Aerobic scope and performance declines in a northern population at expected temperatures and acidification while a southern population was less sensitive to these stressors. This study demonstrates that even neighbouring populations will show substantial differences in energetic costs of exercise and that the northern population may more vulnerable to directional increase in temperature and acidification.

Intoduction

Climate change is regarded as the biggest threat to ecosystem stability and biodiversity (Somero 2010; Chown *et al.* 2010). As carbon dioxide levels and temperature increase in the oceans, the likelihood of single populations of marine organisms to survive extirpation may be linked to intraspecific variation in physiological responses and the capacity for acclimatization and rapid adaptation to these major climate-related stressors (Baumann & Conover 2011; Donelson *et al.* 2011; Rummer *et al.* 2014). Temperature is an important ecological factor, known to affect many metabolic processes in aquatic ectotherms, such as fishes (Magnuson, Crowder & Medvick 1979; Di Santo & Bennett 2011a). Concurrently, several studies have presented data suggesting that increasing ocean acidification caused by accelerating rates of CO_2 introduction in marine systems, potentially exert an adverse effect on fish skeletogenesis (Chambers *et al.* 2013), survival (Baumann, Talmage & Gobler 2011) and several other important behavioral and physiological traits, such as swimming and predator avoidance (Ferrari *et al.* 2012; Hamilton, Holcombe & Tresguerres 2014).

In particular, predator evasion is considered the key factor in fish survival, especially juveniles (Svendsen *et al.* 2012; Binning, Roche & Layton 2013). In elasmobranch fishes the effect of warming and acidification on predator escape performance in juveniles poses additional challenges because of their typical slow growth and development which increase and prolong the chance of being predated upon before reaching sexual maturity (DIBATTISTA *et al.* 2007). Although the consequences of rapid ocean acidification and warming on escape performance could be high, no studies

to date have evaluated their combined effect on an elasmobranch fish. To address this issue and improve predictions of species response to environmental challenges, it is necessary to implement multistressor studies that quantify responses of individuals to simultaneous warming and acidification (Todgham & Stillman 2013). Moreover, as individuals are likely to respond to abiotic stressors depending on their environmental history, it has become increasingly important to test individuals from different localities raised in common garden conditions (i.e., similar laboratory-controlled conditions) to account for local adaptation of metabolic functions (Baumann & Conover 2011).

The little skate *Leucoraja erinacea* (Mitchill 1825) is a small benthic oviparous elasmobranch inhabiting near-shore waters along the northwestern Atlantic from Cape Hatteras (CH) to the Gulf of Maine (GoM). Frisk and Miller (Frisk & Miller 2006, 2009) found a latitudinal gradient in growth of L. erinacea with size-at-age increasing with latitude. In particular, *L.erinacea* from the GoM show significantly larger body size than conspecifics in the Georges Bank (GB) and Mid Atlantic (MA) suggesting low population exchange and migration in the fish (Frisk & Miller 2009). Interestingly, although geographically contiguous, the GoM and GB show different thermal environments that, combined with low migration rates, might have favored local thermal adaptation of metabolism and growth (Baumann & Conover 2011). As metabolic rates are key in resilience and success of organisms in their environment, differences in fine scale geographic variations in whole-organismal performance may determine individual vulnerability to ocean warming and acidification (Somero 2010; Rummer *et al.* 2014).

The maximum and resting metabolic rates (MMR and RMR, respectively) are key measures of performance that relate to fitness. Although various methods are used to induce MMR, experiments that use chasing protocols have been found to elicit exhaustion and higher metabolic rates than classic critical speed swimming protocols in benthic fishes (Cutts, Metcalfe & Taylor 2002; Svendsen *et al.* 2012; Roche *et al.* 2013). Moreover, manually chasing fish to exhaustion is the most appropriate method to test predator escape performance (Cutts *et al.* 2002). Here I quantified the effects of ocean warming, acidification and population on performance of *L. erinacea* by employing a fully-crossed experimental design that compared i) maximum and resting metabolic rates, ii) aerobic scope, iii) exercise intensity, and iv) recovery time after exhaustion in juvenile skates from the GoM and GB. I hypothesized that *L. erinacea* would exhibit local adaptation in metabolic traits and would respond differently to exhaustive exercize when challenged with increased temperatures and acidification.

MATERIALS and METHODS

(a) Animals and experimental set up

Leucoraja erinacea (n=24 per population) were obtained from two localities, the GoM (43°N, 68°W) and GB (41.21°N, 67.38°W) as newly laid embryos (<1 week old), transpoted in a constant-temperature tank, and were held in common garden conditions (Table 4.1) in a temperature-controlled environmental chamber (Harris Environmental Systems) at Boston University. Temperatures of 15 and 18°C were chosen because previous data showed high fecundity and lowest mortality in *L. erinacea* at these

temperatures (Palm *et al.* 2011) and are overlapping temperatures at the two localities (Baumann & Conover 2011). Fish were maintained at constant temperature (either 15, 18 or 20°C) by a submersible titanium heater unit (Finnex 300W) controlled by a digital thermostat (Aqua Logic Inc.), and pH conditions (either 8.1 or 7.7) to simulate current and projected year 2100 conditions according to the climatic model RCP 8.5 (Riebesell *et al.* 2010; IPCC 2013). To maintain the appropriate pH, each tank was independently supplied with a mix of air:CO_2 (water pH=7.7 ±0.05 which resulted in pCO2 ~1100ppm) or ambient air (water pH=8.1 ±0.05 which resulted in pCO2 ~400ppm) and controlled by an Aqua Medic pH computer. Temperature and pH data were collected every 12 hours. Water quality (ammonia, nitrites, nitrates, salinity, dissolved oxygen) was monitored and 30% water changes were made weekly (Table 4.1). Water parameters and potential effects on fish are included (Table 4.2). Skates were reared at constant salinity (33ppt) and photoperiod (14L:10D) and, once hatched, were fed daily a diet of frozen mysis shrimp *ad libitum* but were fasted for 24 hours prior to experiments.

Table 4.1. Water parameters in experimental tanks.

Water parameter	Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5	Treatment 6
Temperature (°C)	15	15	18	18	20	20
pH	8.1	7.7	8.1	7.7	8.1	7.7
Salinity (ppt)	33	33	33	33	33	33
Ammonia (ppm)	0	0	0	0	0	0
Nitrites (ppm)	0	0	0	0	0	0
Nitrates (ppm)	<30	<30	<30	<30	<30	<30
Photoperiod	14L:10D	14L:10D	14L:10D	14L:10D	14L:10D	14L:10D

Table 4.2. Changes in water parameters associated with warming and acidification, and potential effects on fishes.

Water parameter	Effect on fishes	Levels in the tank	References
Reduction in oxygen	Lower oxygen depresses metabolic rates (if below 40% saturation) and reduces growth	>80%	Cheung et al. (2009) Perry et al. (2005) Di Santo et al. (in prep)
Reduction in bicarbonate	Reduction in water absorption in teleosts	---	Kurita et al. (2008)
Increase in carbonic acid	Anesthetic (150 to 600 mg/L)	---	Post (2011)
Ammonia	lethal	0 ppm	Fry (1971) Smith et al. (2004)
Nitrites	lethal	0 ppm	Fry (1971) Smith et al. (2004)
Nitrates	No effect below 40 ppm in elasmobranchs	>30 ppm	Smith et al. (2004)

(b) Respirometry and chasing protocol

Mass-adjusted metabolic rates (MO_2 in $mg \times g^{-1} \times h^{-1}$) were determined using intermittent-closed respirometry (Steffensen 1989) and calculated using the formula: $MO_2 = (O2start – O2end) \times volume\ of\ respirometer \times mass^{-0.67} \times time^{-1}$;
the mass exponent of 0.67 was used to correct for the allometric relationship between metabolic rates and mass in elasmobranchs (Meloni *et al.* 2002; Di Santo & Bennett

2011b). Resting metabolic rate (RMR) and maximum metabolic rate (MMR) were measured at the three temperatures and two pH conditions. Individual skates were placed in a custom-made 1-cm thick acrylic respirometer chamber (0.465L) fitted with an optical oxygen probe (model ProODO YSI) submersed in a temperature- and pH-controlled water bath.

Resting metabolic rate was measured for 30 minutes after a 12 hours acclimation period to the experimental chamber (Di Santo & Bennett 2011b). Maximum metabolic rate of juvenile skates was measured using the chasing protocol described by Cutts and co-authours (2002) and modified to accommodate skate behaviour. Individual fish were quickly placed in a round tank and rotated on their back forcing them to continually right themselves, while a second researcher was measuring time with a stop-watch. *Leucoraja erinacea* juveniles produced short intense swimming bursts as they right themselves until they reached fatigue (righting behaviour was no longer observed). The modified chasing protocol was necessary to elicit escape behavior as juvenile skates do not respond to simple hand or net chase (Cutts *et al.* 2002; Svendsen *et al.* 2012; Roche *et al.* 2013). Upon exhaustion, *L. erinacea* were transferred to the respirometer chamber where O_2 measurements started immediately. Sampling continued for one hour at 5 min intervals. MMR was calculated using the highest MO_2 measurement for each fish. The absolute aerobic scope (AAS) was calculated by subtracting the RMR from the MMR, and gives an estimation of the amount of energy that an individual allocates to perform an activity at different environmental conditions.

(c) Statistical analysis

All experimental values are reported as means ± s.e.m. The effects of temperature, pH, and population were explored using a 3-way analysis of variance (ANOVA) followed by a Tukey-Kramer MCT to identify statistical difference between treatment group means. Metabolic rates following fatigue were compared to resting metabolic rates (controls) using a repeated measures ANOVA followed by a Dunnett's test. All statistical comparisons were based on α=0.05. All analyses were performed in JMP Pro version 11 (SAS Institute Inc.).

Results

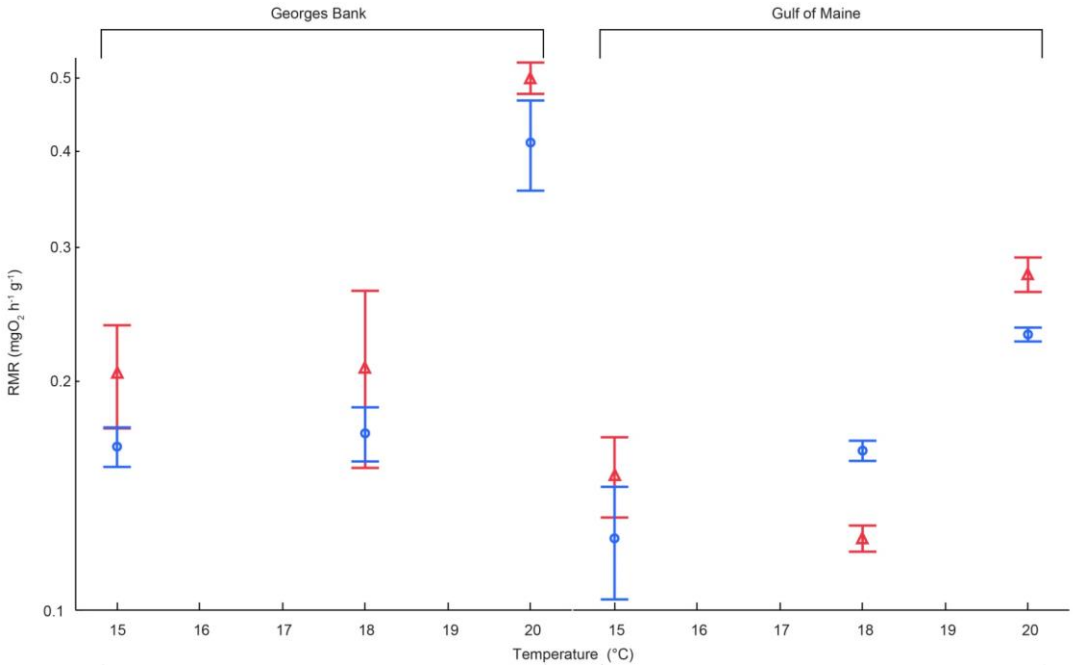
Wet mass of juvenile (3 month old) *L. erinacea* showed a typical pattern of countergradient variation (i.e. the population at higher latitudes outperforms the population at lower latitudes at the same conditions), as the northern population (GoM) skates exhibiting significantly larger body mass when compared to the lower latitude population (GB) regardless of temperature and pH (24.68 ±0.27g, 12.61 ±0.51g respectively; 3-way ANOVA: $F_{3,40}$= 60.90, p<0.0001). Overall, temperature and pH had no significant effect on mass of juvenile skates (2-way ANOVA, p>0.05). Low pH exacerbated the effect of high temperature (20 ºC) on growth in juvenile *L. erinacea* from the GoM resulting in lower body weight (low pH: 22.70 ±1.43g, high pH 26.50 ±0.45g; Dunnett's test, p=0.04).

Maximum metabolic rate following exhaustion, differ between two populations as well (3-way ANOVA, $F_{7,40}$= 9.54, p<0.0001; Figure 4.1a). However, temperature had no

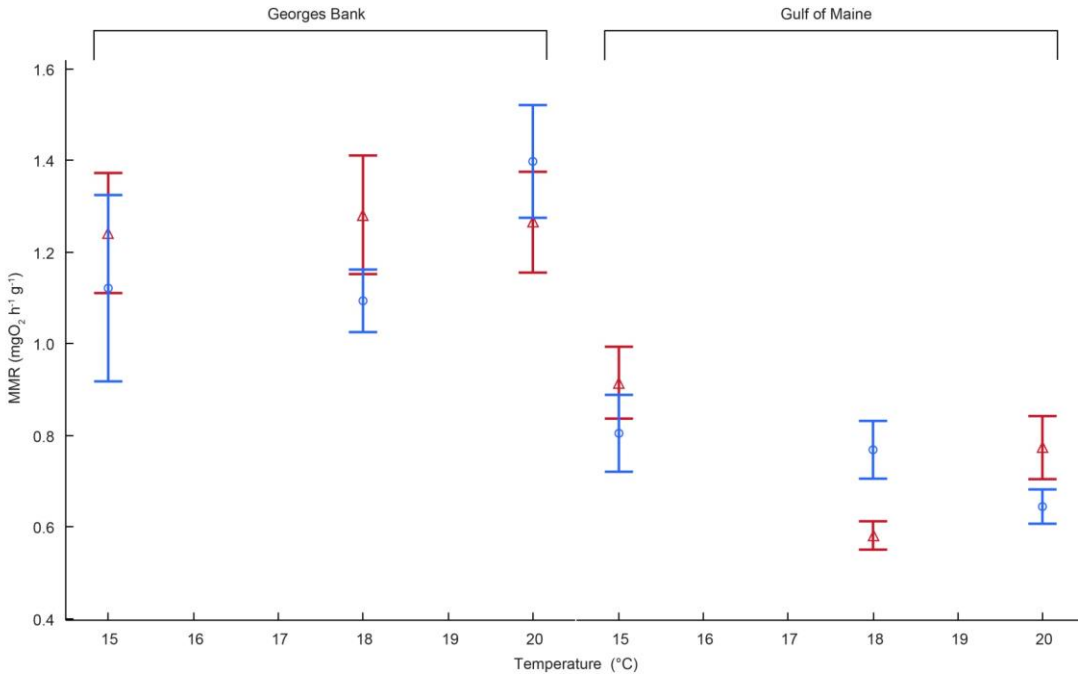
significant effect on maximum oxygen consumption at either pH level in both populations (1-way ANOVA, $p>0.05$). Oxygen consumption at rest (RMR) was significantly affected by temperature, acidification and population (3-way ANOVA, $F_{7,40}= 9.33$, $p<0.0001$; Figure 4.1b). Resting metabolic rates significantly increased with temperature ($p<0.001$) and were higher in the GB population ($p<0.0001$). Furthermore, there was a significant interaction between population and temperature ($p=0.01$). Low pH significantly increased RMR at highest temperatures in the GoM population (Figure 4.1b). Absolute aerobic scope differed between populations at different conditions (3-way ANOVA; $F_{7,40}= 6.73$, $p<0.0001$; Figure 1c). Only the AAS of the GoM population was significantly affected by acidification and warming (2-way ANOVA, $F_{3,20}= 5.92$, $p=0.004$), however pH conditions did not have a statistically significant effect on AAS ($p>0.05$). The AAS of the GoM population was also significantly affected by length of exercise (time to fatigue) by increasing MMR (1-way ANOVA, $p=0.02$). Overall, all exercise metrics (time to fatigue, number of turns to reach fatigue and intensity of exercise as turns per minute) were affected by treatments across populations (3-way ANOVA, $p<0.0001$; Figure 4.2a-c). Overall, fish from GB recovered faster from exercise (Figure 3). Acidification increased recovery time in both population; however skates from GM exhibited elevated metabolic rates for as much as double the time individuals from GB showed at higher temperatures and acidification conditions (Dunnett's test, $p>0.05$; Figure 4.3).

Figure 4.1. Metabolic rates of juvenile little skates. Mass-adjusted (a) maximum metabolic rates (MMR), (b) resting metabolic rates (RMR), (c) absolute aerobic scopes (mean ±s.e.m) of *Leucoraja erinacea* from the Gulf of Maine (n=24) and the Georges Bank (n=24) at three temperatures and two pH conditions (high: circle, low: triangle).

(a)



(b)


Georges Bank
Gulf of Maine
MMR (mgO2 h-1 g-1)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
15
16
17
18
19
20
Temperature (°C)

(c)

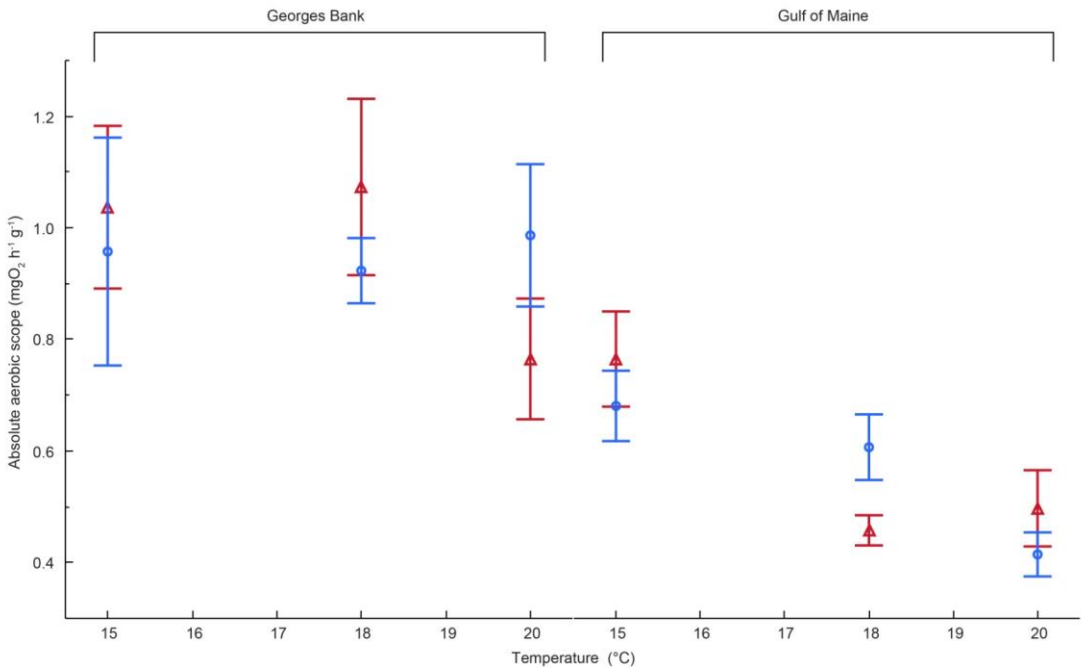
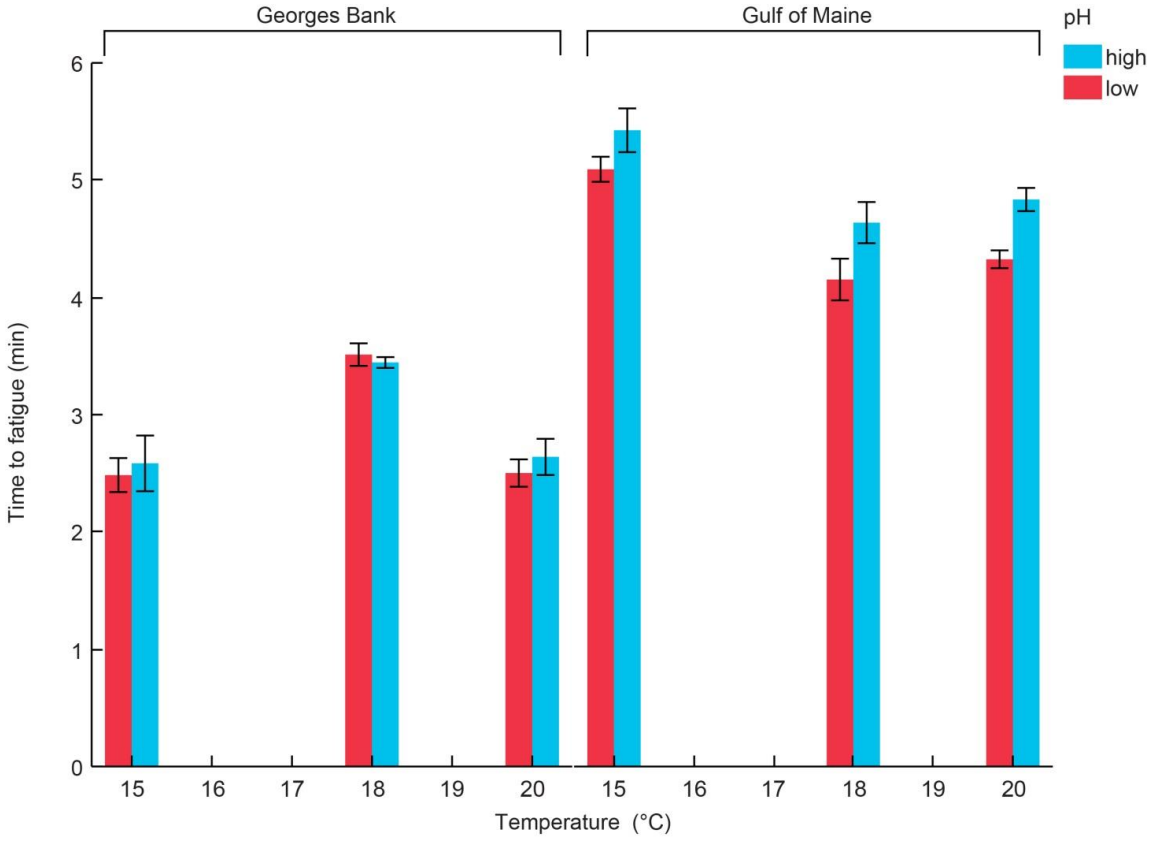
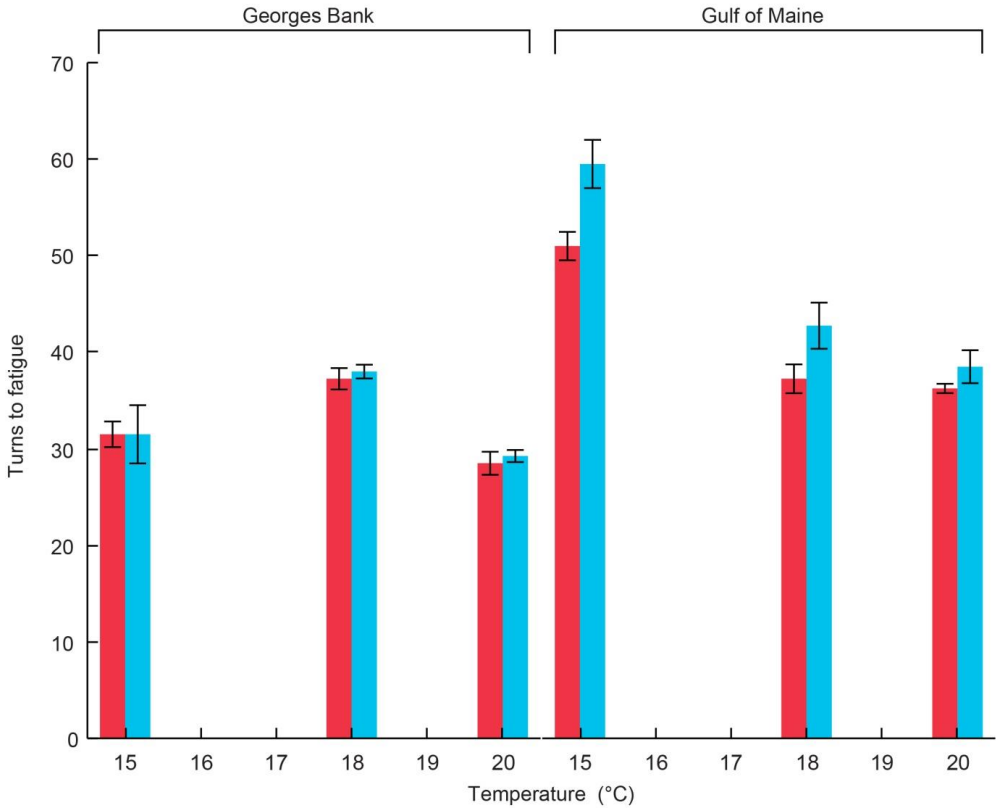

Georges Bank
Gulf of Maine
Absolute aerobic scope (mgO2 h-1 g-1)
1.2
1.0
0.8
0.6
0.4
15
16
17
18
19
20
15
16
17
18
19
20
Temperature (°C)

Figure 4.2. Exercize parameters of juvenile little skate. (a) Time to fatigue, (b) turns to fatigue, (c) intensity of exercise (mean ±s.e.m) of *Leucoraja erinacea* from the Gulf of Maine (n=24) and the Georges Bank (n=24) at three temperatures and two pH conditions (high: blue, low: red).

(a)



(b)


Georges Bank
Gulf of Maine
Turns to fatigue
70
60
50
40
30
20
10
0
15
16
17
18
19
20
15
16
17
18
19
20
Temperature (°C)

(c)

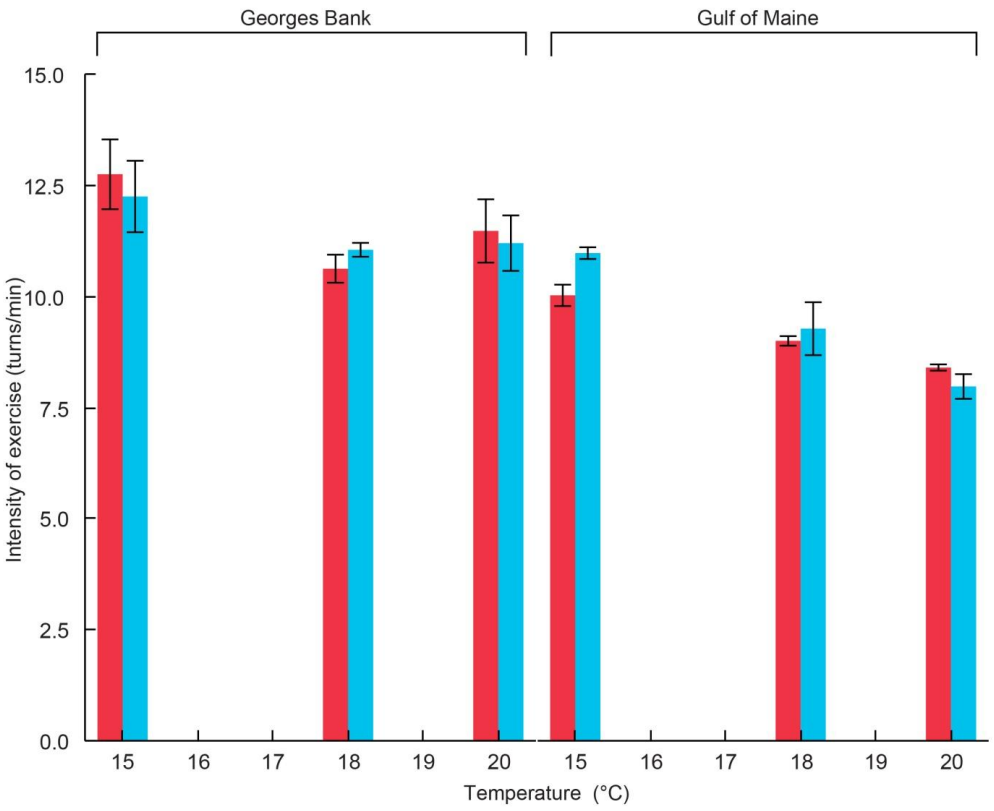
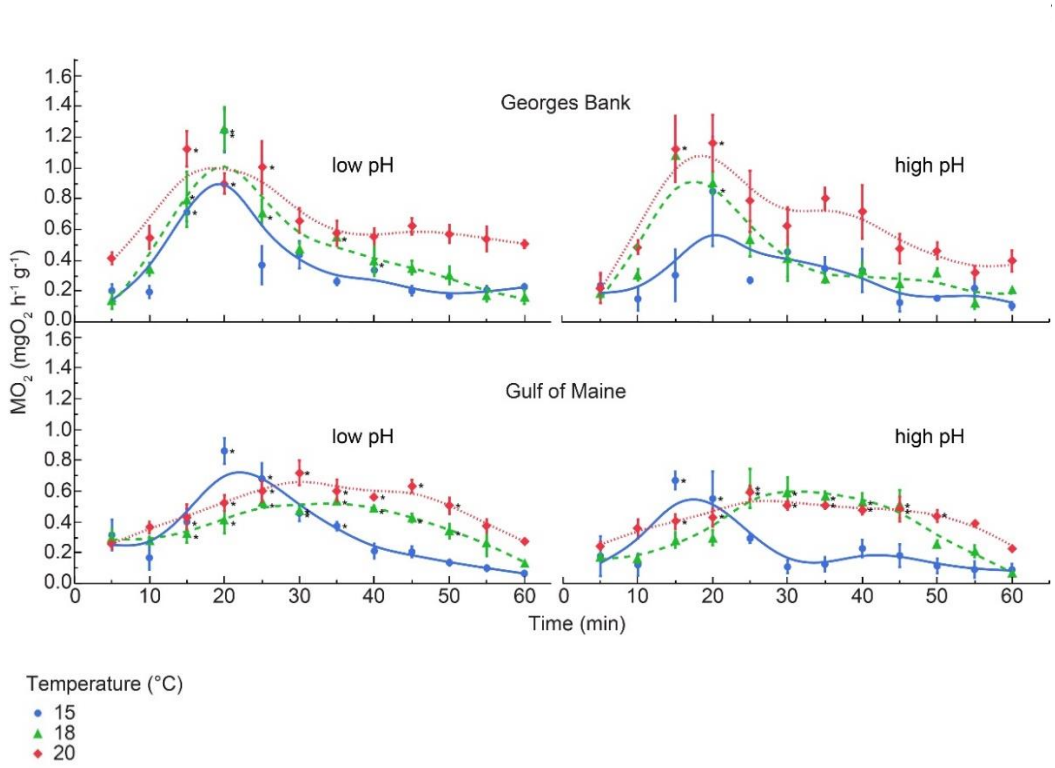

Georges Bank
Gulf of Maine
15.0
12.5
10.0
7.5
5.0
2.5
0.0
Intensity of exercise (turns/min)
15
16
17
18
19
20
15
16
17
18
19
20
Temperature (°C)

Figure 4.3. Post-exhaustion oxygen consumption responses in two populations of juvenile little skate *Leucoraja erinacea*. Post-exhaustion oxygen consumption responses (mean ±s.e.m) in two populations (Georges Bank and Gulf of Maine) of juvenile little skate *Leucoraja erinacea* (n=24 per population) raised in common garden conditions to mimic current and future levels of warming and acidification. The symbol * indicates significant diffence in mean oxygen consumption between resting metabolic rates (controls) and post-exhaustion metabolic rates (repeated measures ANOVA followed by Dunnett's test, $p<0.05$).



Discussion

The present multistressor study demonstrated the presence of two locally-adapted populations of *L. erinacea* even within a small geographic distance. Growth in this elasmobranch showed a countergradient variation pattern with the northern population (GoM) exhibiting larger body mass at the same age. This empirical evidence supports observations in the field that described the same pattern but could not discern the effect of plasticity from local adaptation (Frisk & Miller 2006, 2009). In the present study, fish from two populations were raised from newly laid embryos in the same conditions, therefore eliminating the effect of different acclimatization levels on fish.

Interestingly, similar patterns of variation in life history traits have developed in several fishes along latitudinal gradients as the result of thermal adaptation in growth and metabolic rates. For instance countergradient variation is observed in growth of the Atlantic silverside *Menidia menidia* along the coast in the northwestern Atlantic (Baumann & Conover 2011) and respiratory performance of several coral reef fishes in the Great Barrier Reef (Gardiner, Munday & Nilsson 2010). On the other hand, there is the potential for important trades-off in other physiological traits (Fangue, Richards & Schulte 2009). For instance, aerobic scopes were greater in skates from the GB population and were significantly depressed by increasing temperature in the GoM population. GoM skates were able to respond to chasing longer and right themselves more times before fatiguing. Individuals from GB showed highest endurance (time and turns to fatigue) at 18°C while GoM skates showed a decline in all performance parameters with temperature increase. Even though 3-5 °C increase in average

temperature was not lethal for the GoM population, it reduced endurance during escape response, prolonged recovery time after exahustion and lowered aerobic scope with the potential to substantially decrease organism fitness and population persistence in near-future climate change. Moreover, in both populations low pH increased the recovery time after exahustive exercise. In fact, increased acidification may have reduced pH of body fluids, thereby increasing the costs associated with homeostasis. It is therefore possible that skates increased the time to 'pay off' their oxygen debt after exhaustive anaerobic exercize.

High energy output is required during anaerobic bursts such as predator escapes (Binning *et al.* 2013). At higher acidification and temperature conditions, *L. erinacea* from the GoM will be strongly disadvantaged as their energy scope is low. Moreover, GoM skates will also have a reduced capacity for intense activity which will likely impact their ability to escape predators. Although righting and burst exercise involve anaerobic pathways, these will be negatively impacted by a reduced aerobic scope (Svendsen *et al.* 2012). In fact, intensive anaerobic exercise results in an oxygen debt that must be accounted for at the expenses of other important activities such as foraging (Svendsen *et al.* 2012; Binning *et al.* 2013). It is possible, however, that some fishes at higher latitudes would benefit from allocating most of their energy towards growth at the expense of other metabolic activities. For example, this might allow the fish to develop faster as compensation for a shorter growth season.

Lastly, although increasing ocean acidification and warming are likely to have a substantial effect across species at different latitudes, empirical evidence on

elasmobranchs has been lacking. Here I showed that combined climate-related stressors can have important and quantifiable consequences on performance of a benthic elasmobranch and that the individual response depends on local adaptation. By reducing aerobic scope and escape endurance, ocean warming and acidification are likely to compromise vital activities such as predator evasion. This study shows that in light of projected climate change, the northern population may be at higher risk of extirption because it is more sensitive to acidification than the southern population and performance declines at temperatures above 15 °C. It is possible that little skates from the Georges Bank are already pre-adapted to high fluctuations in pH in their environment as a consequence of upwelling in the area. Alternatively, skates from Gulf of Maine have reduced aerobic capacity given the faster growth rates (and therefore higher energy allocated for growth) and the additional stress caused by high acidification in their body fluids is not as readily compensated as in Georges Bank skates. However it is possible Small differences in water quality caused by changes in temperature and CO_2/pH such as bicarbonate, carbonic acid, and dissolved oxygen could have also affected performance in juvenile skates and therefore cannot be discounted (Table 1).

The higher capacity for resilience at higher acidification and warming in the southern population seems to suggest that ther is a low possibility of complete extinction of little skate as a species.

Conclusions

Summary

Climatic models project an increase in frequency and duration of extreme thermal events as a consequence of global warming (Greenstein & Pandolfi 2008). It has been suggested that one of the predicted responses of ectotherms to global warming may be a decrease in body size (Gardner *et al.* 2011). However more studies are needed to answer the question of whether a reduced body size is a consequence of or an advantage to counteract rapid temperature increases.

In the first study (chapter 1), I investigated the effect of body size on thermal tolerance of two tropical neon gobies *Elacatinus oceanops* and *E. lobeli* reared under the same controlled environmental conditions in the laboratory, known as 'common garden' conditions , . I hypothesized that smaller adults would be better able to cope with extreme temperature. Results from this study showed that both species are stenothermic, with little capacity for acclimation. Additionally, the smaller *E. lobeli* was able to tolerate higher temperatures better than *E. oceanops*. Yet, only *E. oceanops* showed an intraspecific difference in thermal tolerance, with smaller adults being more tolerant to rapid increases in temperature. Although the two fish species used in this study diverged about 800,000 years ago and therefore a difference in thermal tolerance might be expected as a consequence of adaptation to different thermal environments, the intra-specifically differences in temperature tolerance between same-age but different size adults provide further evidence supporting thermal biology theories that predict an increase of thermal windows with a reduced body size.

In the second chapter, I compared performance curves of same age but different size adult neon gobies acclimated to three temperatures in a common garden condition experiment. As swimming performance experiments are not feasible with these benthic gobies, I measured active metabolic rates as the oxygen consumed during digestion peaks, known as specific dynamic

action. Acclimation temperature did not significantly affect adult size of gobies. Although temperature is known to affect body size, food ration were not constrained in this study and therefore might have masked the effect of temperature on growth observed in natural populations of fishes (Cheung *et al.* 2012). Smaller individuals increase digestive metabolic scopes at the highest temperature tested. . This may suggest that warming can favor digestion in smaller individuals.

In the third chapter, I evaluated the effect of multiple climate-related stressors on different populations of the little skate *Leucoraja erinacea.* Although the individual effects of acidification and temperature on fish have been evaluated in a number of species (Domenici *et al.* 2011; Baumann, Talmage & Gobler 2011; Ferrari *et al.* 2012; Munday, McCormick & Nilsson 2012), there is an urgent need for studies that more closely approximate nature by elucidating the consequences of simultaneous stressors on the most vulnerable stages of fish life history (i.e., embryos). In addition, experiments that can identify the role of local adaptation in providing specific advantages to populations are needed. Here I show the combined effects of acidification and warming on embryonic little skates from two native populations. Temperature had the strongest effect on development, survival and metabolic rates, but acidification further exacerbated stress on embryos. Thermal performance curves of populations exhibited countergradient variation (Baumann & Conover 2011) and were affected differently by acidification. These findings emphasize the need for multi-stressor studies to understand complex responses of fish to climate change.

Increasing anthropogenic CO_2 in the ocean has the potential to decrease the respiratory capacity of fish by increasing metabolic costs related to acid-base balance (Claiborne, Edwards & Morrison-Shetlar 2002; Rummer *et al.* 2013). Therefore, in the fourth chapter, I investigated the effect of synergistic warming and acidification on the aerobic scope of two latitudinally separated

populations of the little skate *Leucoraja erinacea* by employing a chasing protocol to induce exhaustion (Svendsen *et al.* 2011). Surprisingly, performance curves of northern and southern population juveniles shifted from the countergradient variation exhibited during embryonic development (Chapter 3) to thermal adaptation, suggesting that fish may change their preferred temperature as they become able to thermoregulate and exploit thermal variation in their environment (Di Santo & Bennett 2011). Similarly to aerobic performance in embryonic skates, juvenile aerobic scopes decreased at high CO_2 suggesting that acidification may exacerbate the effect of warming on metabolic functions.

These studies provide empirical evidence that body size affects thermal tolerance and digestive metabolic rates of same age but different size adult fishes. In both studies, smaller fishes had an advantage at higher temperatures suggesting that long-term warming may favor smaller fishes. Furthermore, my study extended our knowledge about the effect of local adaptation on the response of fishes to simultaneous ocean warming and acidification, and represent the first physiological climate-change study on an elasmobranch species. Based on the results presented here, I can conclude that although subtle, differences in body size can affect long term resilience to directional ocean warming as previously hypothesized, and shrinking body mass may indeed represent an evolutionary response to warming (Daufresne, Lengfellner & Sommer 2009; Gardner *et al.* 2011). This could have significant consequences for trophic relationships in marine ecosystems thereby potentially reducing long-term stability of marine communities as shifts toward smaller body mass may reduce fish fecundity, recruitment and populations' biomass.

Finally, embryos seem to represent the most vulnerable life stage in elasmobranchs. Skates that survive the developmental stage and hatch, shift performance curves of escape response toward cooler temperatures suggesting that this population may be more susceptible to predicted warming. Losing the northern population could severely decrease little skate biomass

and potentially resilience because they reach larger size and fecundity faster than the southern population. The latter may have an advantage in acidifying oceans as Georges Bank already experiences fluctuations in pH and may have selected for low-pH resistant skates.

Appendices

Figure A1. Critical thermal maxima in *Elacatinus oceanops* depends on acclimation temperature. Critical thermal maxima values (CTMax) for *Elacatinus oceanops* acclimated to temperatures between 20 and 28 °C for three weeks. Vertical bars represent 95% confidence intervals. Regression models of CTMax on acclimation temperature were based on eight fish at three constant temperature acclimation groups. CTMax were significantly different (one-way ANOVA, $p < 0.0001$, n=24) and distinct at each acclimation temperature (Tukey-Kramer MRT, $\alpha=0.05$) and explained by the model: CTMax = 22.62 + 0.46 x acclimation temperature ($R^2=0.9$, $p<0.0001$).

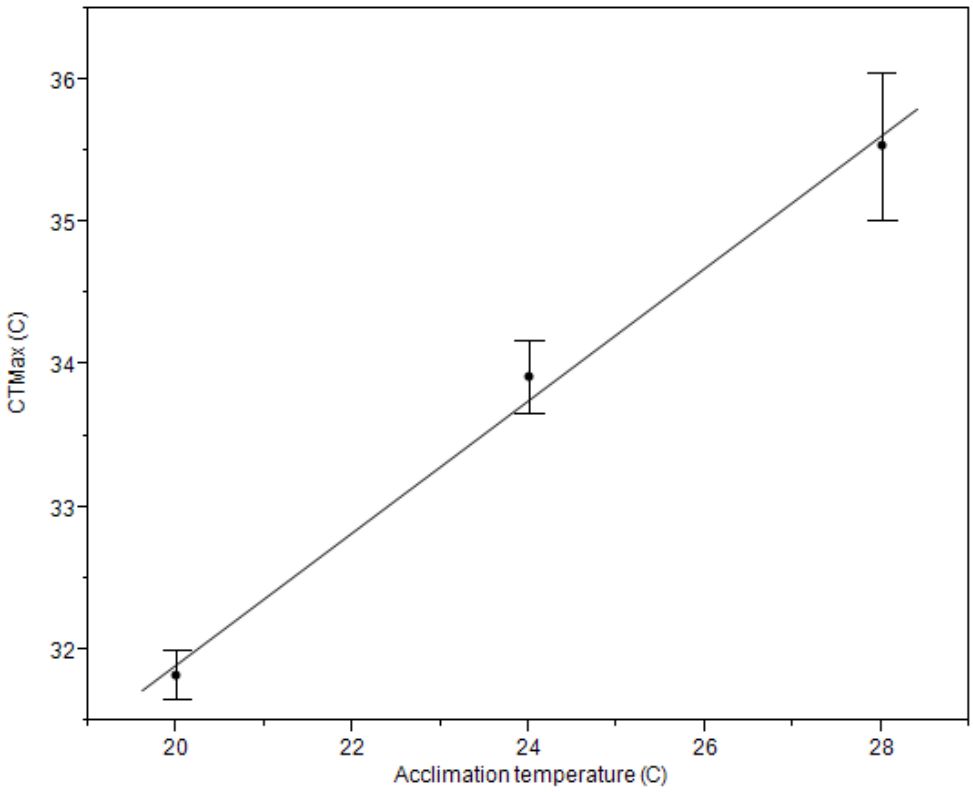


Figure A2. Critical thermal minima in *Elacatinus oceanops* depends on acclimation. Critical thermal minima values (CTMin) for *Elacatinus oceanops* acclimated to temperatures between 20 and 28 °C for three weeks. Vertical bars represent 95% confidence intervals. Regression models of CTMin on acclimation temperature were based on eight fish at three constant temperature acclimation groups. CTMin were significantly different (one-way ANOVA, $p < 0.0001$, n=24) and distinct at each acclimation temperature (Tukey-Kramer MRT, $\alpha=0.05$) and explained by the model CTMin = 8.85 + 0.28 x acclimation temperature ($R^2=0.88$, $p<0.0001$).

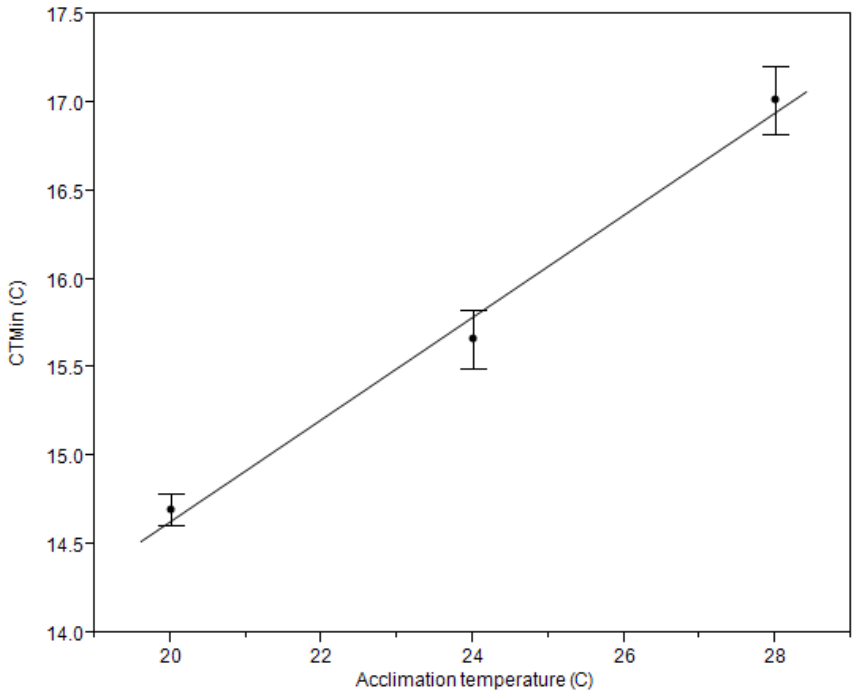


Figure A3. Critical thermal maxima in *Elacatinus lobeli* depends on acclimation temperature. Critical thermal maxima values (CTMax) for *Elacatinus lobeli* acclimated to temperatures between 20 and 28 °C for three weeks. Vertical bars represent 95% confidence intervals. Regression models of CTMax on acclimation temperature were based on eight fish at three constant temperature acclimation groups. CTMax were significantly different (one-way ANOVA, $p < 0.0001$, n=24) and distinct at each acclimation temperature (Tukey-Kramer MRT, $\alpha=0.05$) and explained by the model: CTmax = 24.31 + 0.526 x acclimation temperature ($R^2=0.88$, $p<0.0001$).

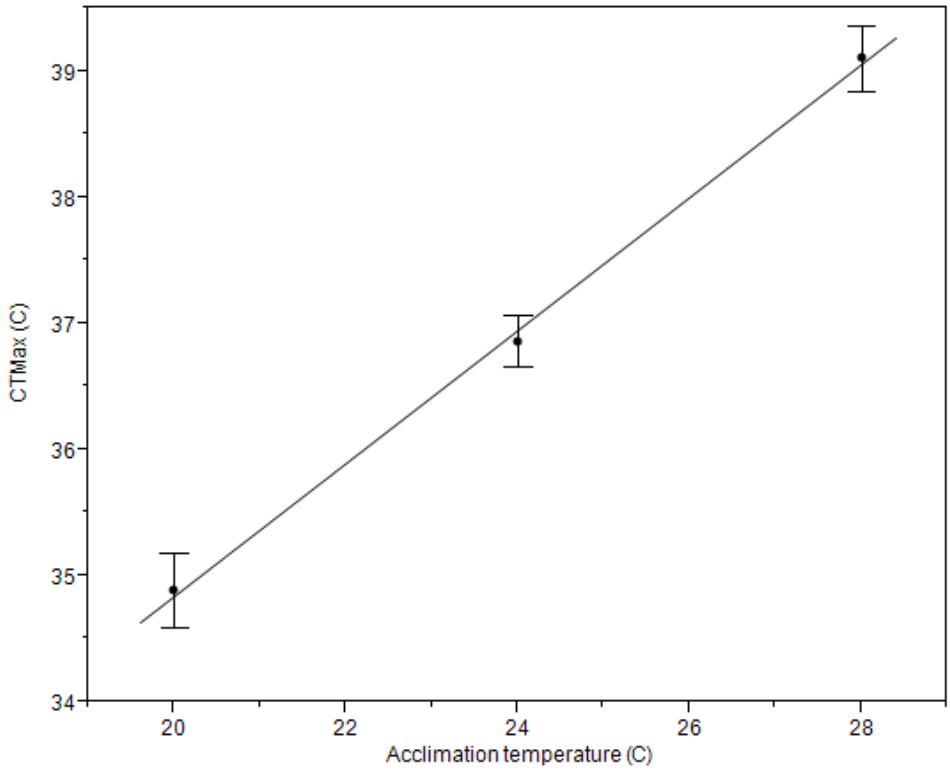


Figure A4. Critical thermal minima in *Elacatinus lobeli* depends on acclimation temperature. Critical thermal minima values (CTMin) for *Elacatinus lobeli* acclimated to temperatures between 20 and 28 °C for three weeks. Vertical bars represent 95% confidence intervals. Regression models of CTMin on acclimation temperature were based on eight fish at three constant temperature acclimation groups. CTMin were significantly different (one-way ANOVA, $p < 0.0001$, n=24) and distinct at each acclimation temperature (Tukey-Kramer MRT, $\alpha=0.05$) and explained by the model: CTmin = 5.9 + 0.39 x acclimation temperature ($R^2=0.95$, $p<0.0001$).

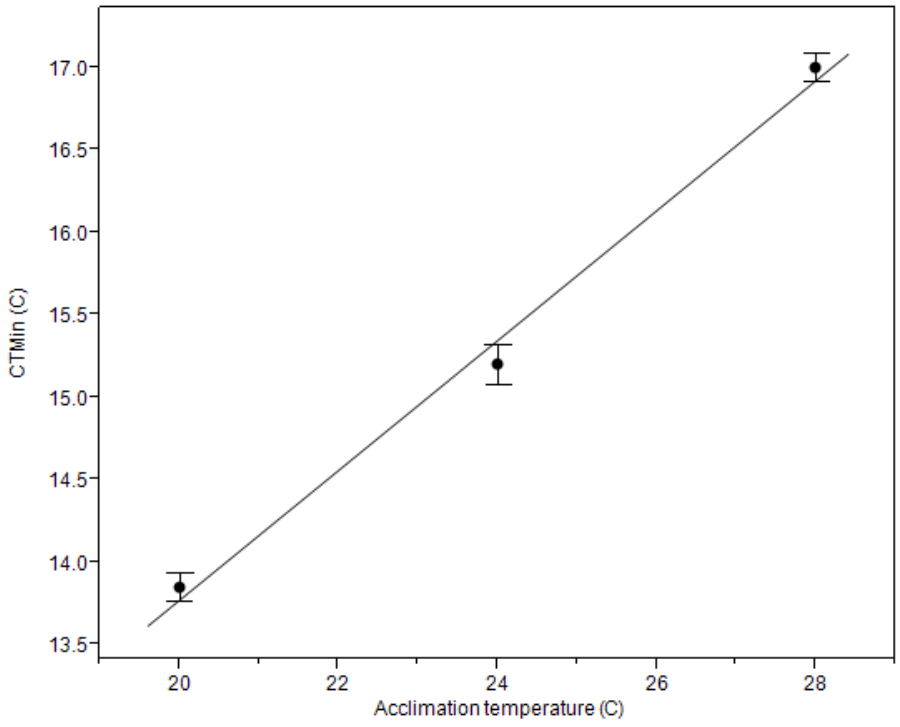


Figure A5. Standard length does not affect tolerance to high temperatures in *Elacatinus oceanops*. Standard length had no significant effect on Ctmax in *E. oceanops* ($p>0.05$, one way ANOVA, n=24).

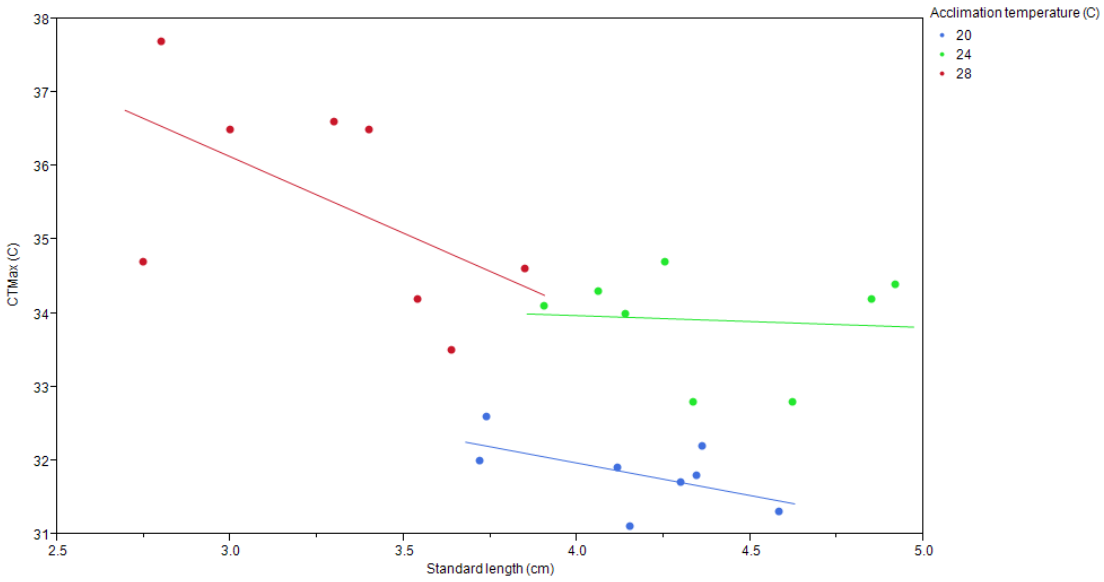


Figure A6. Standard length does not affect tolerance to low temperatures in *Elacatinus oceanops*. Standard length has no significant effect on CTmin in *E. oceanops* ($p>0.05$, one way ANOVA, n=24).

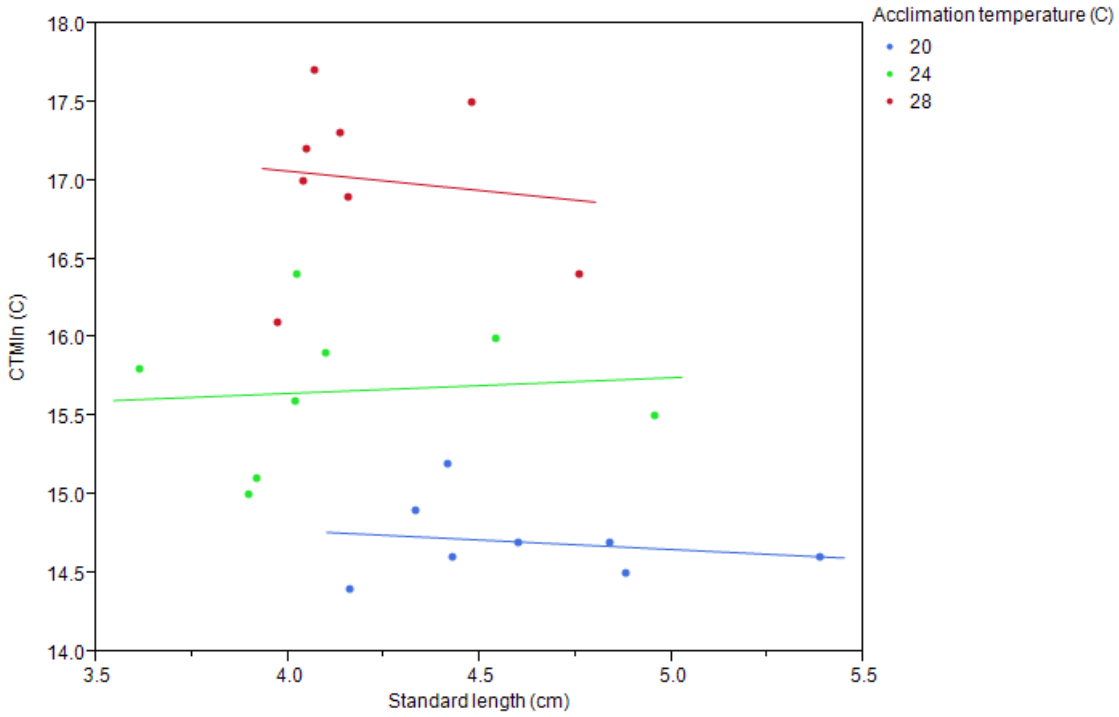


Figure A7. Standard length does not affect tolerance to low temperatures in *Elacatinus lobeli*. Standard length had no significant effect on CTmin in *E. lobeli* ($p>0.05$, one way ANOVA, n=24).

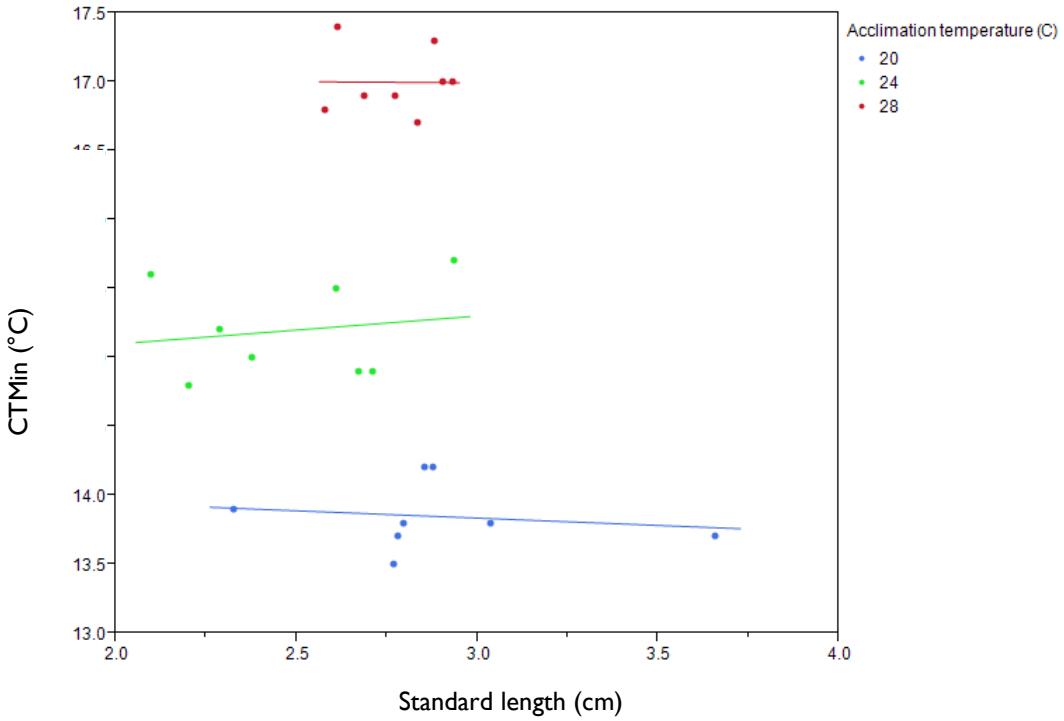


Figure A8. Standard length does not affect high temperature tolerance in *Elacatinus lobeli*. Standard length does not significant affect CTMax in *E. lobeli* ($p>0.05$, one way ANOVA, n=24).

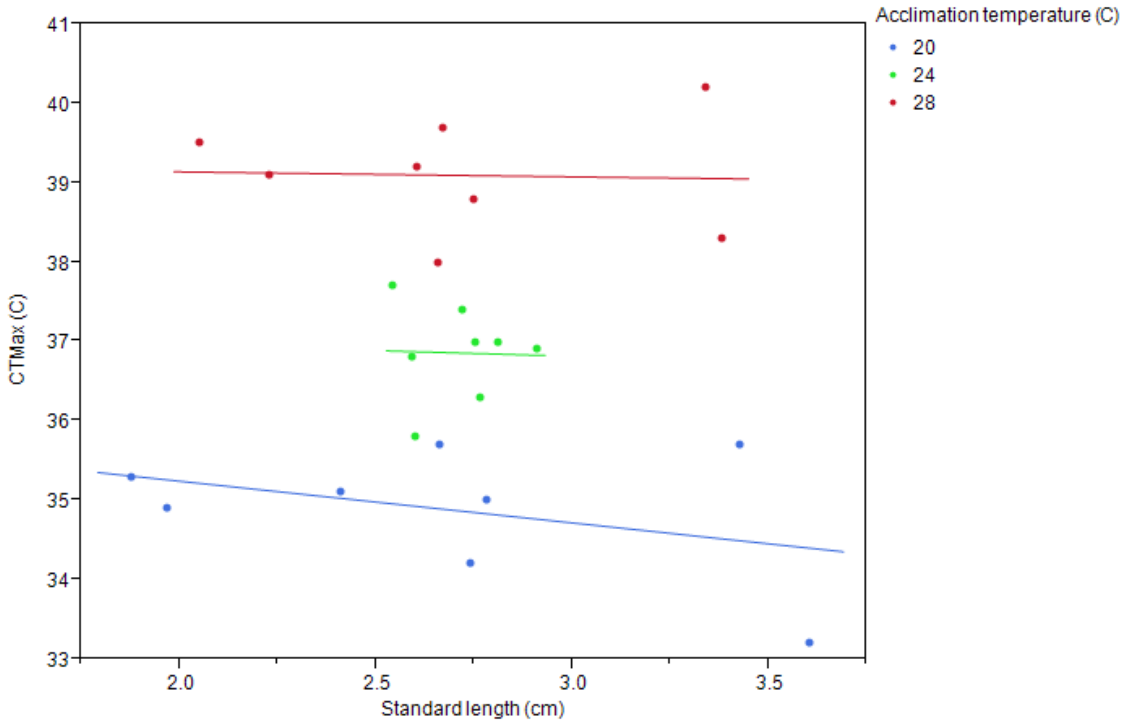


Figure A9. Adult standard length of *Elacatinus lobeli* and *E. oceanops* reared at common garden condition differs. Standard length (±s.e.m.) of 1-year old *E. oceanops* and *E. lobeli* is significantly different (one-way ANOVA, $p<0.0001$, n=48 fish per species).

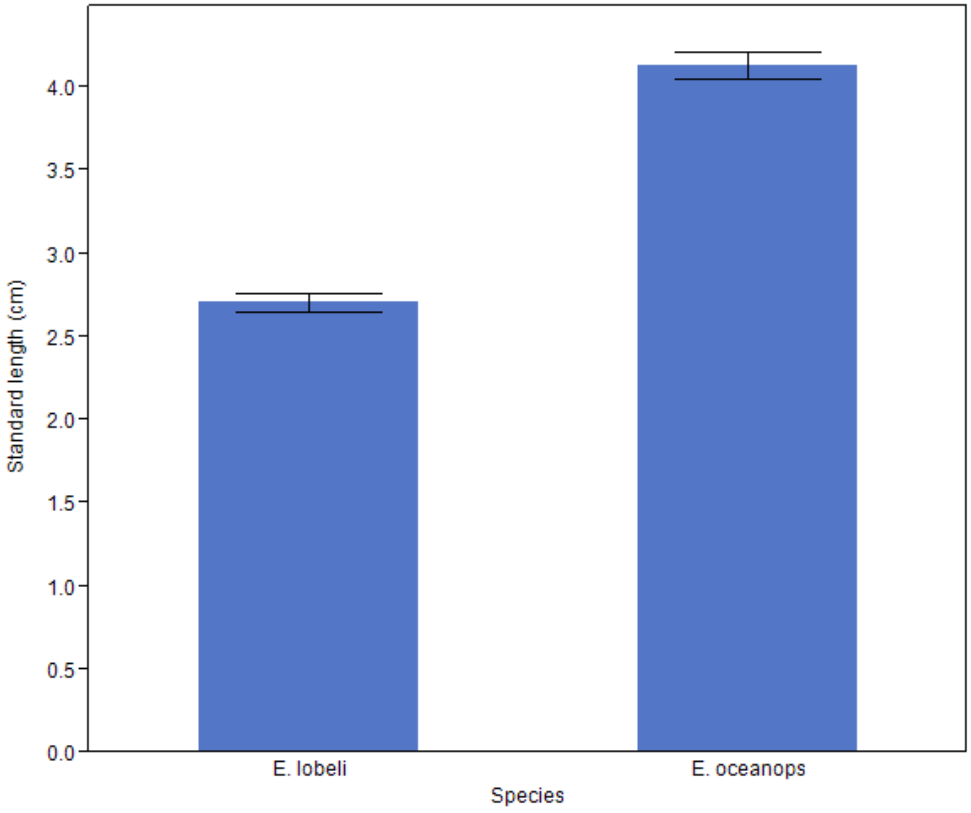


Figure A10. Adult mass of *Elacatinus lobeli* and *E. oceanops* reared at common garden condition differs. Average mass (±s.e.m.) of 1 year old *E. oceanops* and *E. lobeli* is significantly different (one-way ANOVA, $p<0.0001$, n=48 fish per species).

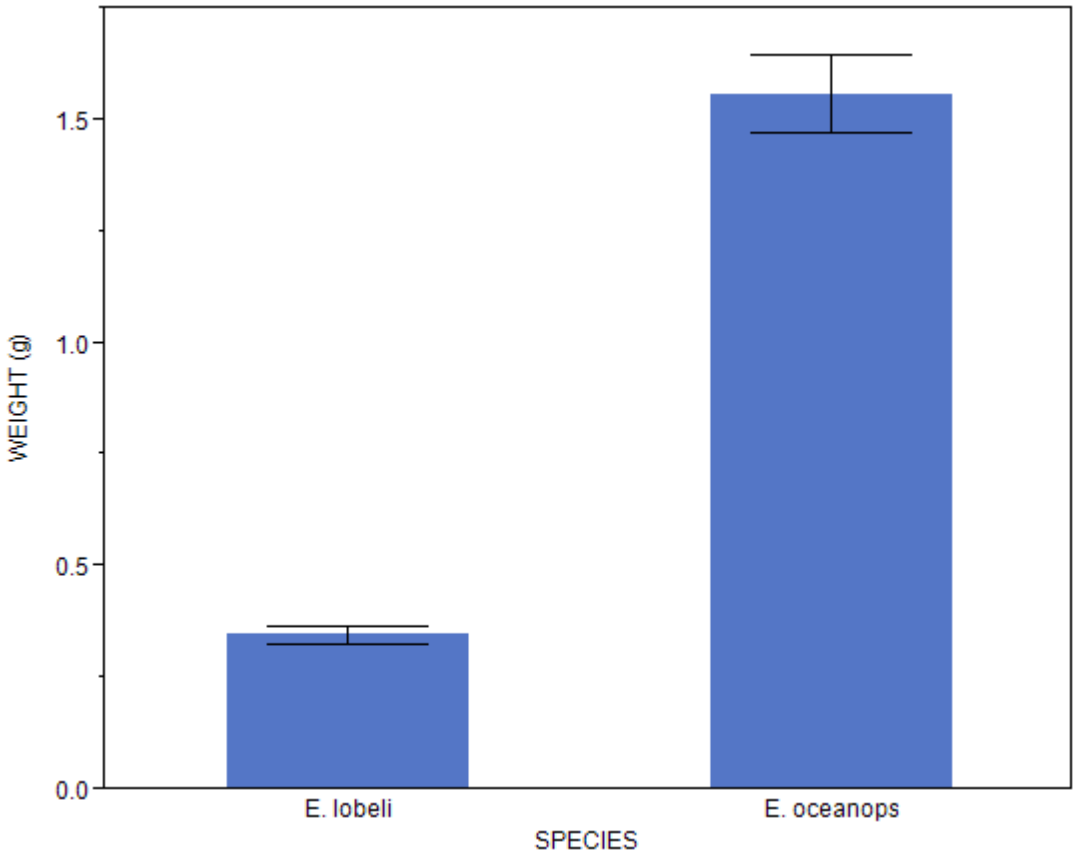


Figure A11. Yolk area of newly laid skate eggs does not differ between sites.

Comparison of yolk area (mean ±s.e.m.) in newly laid skate eggs from two sites (Georges Bank and Gulf of Maine) does not differ (t-test, $p>0.05$, $n=10$).

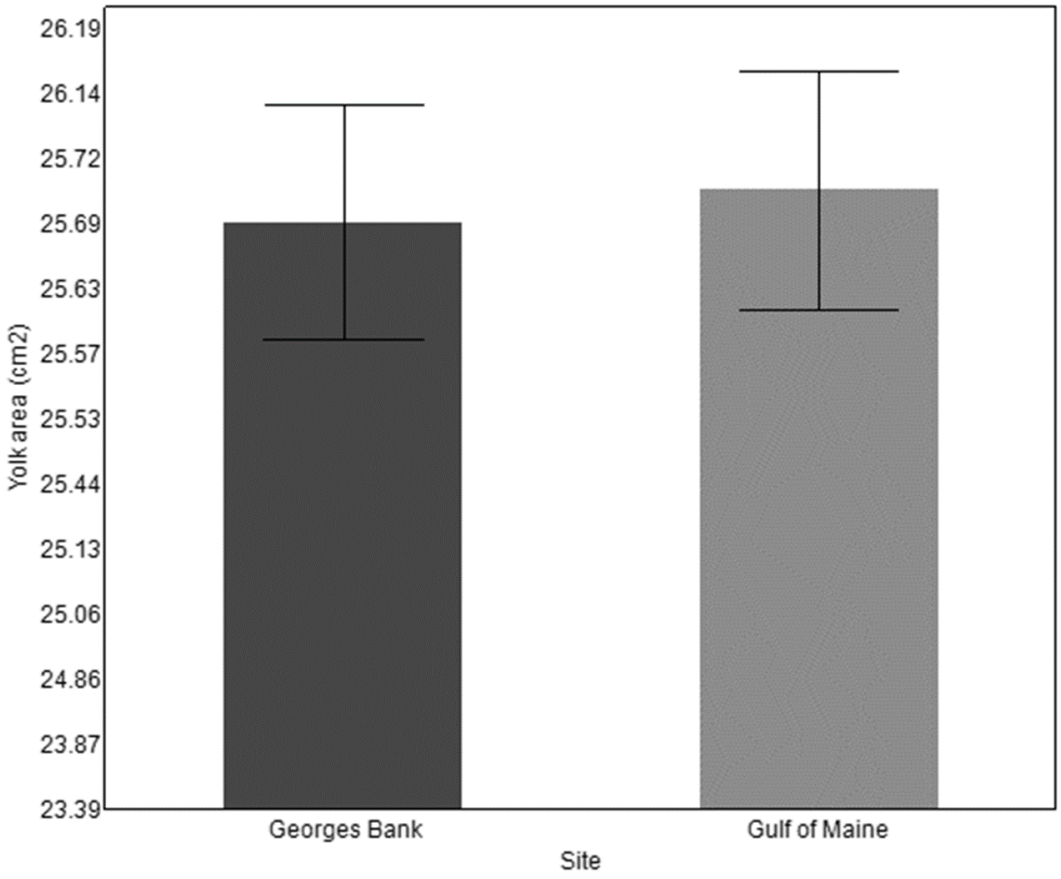


Figure A12. Effect of ocean warming and acidification on newborn weight in two skate populations. Weights (mean ±s.e.m.) of newborn *Leucoraja erinacea* significantly differ between sites (GB: Georges Bank, GM: Gulf of Maine) and treatments ($p<0.0001$ three-way ANOVA, n=114). Within sites, temperature has the strongest effect on newborn weight ($p<0.0001$, two-way ANOVA, GB: n=77, GM: n=37). Carbon dioxide significantly affect newborn weight at each temperature ($p<0.05$, one-way ANOVA) with the exception of the 18°C treatment in the Gulf of Maine population ($p=0.8$, one way ANOVA).

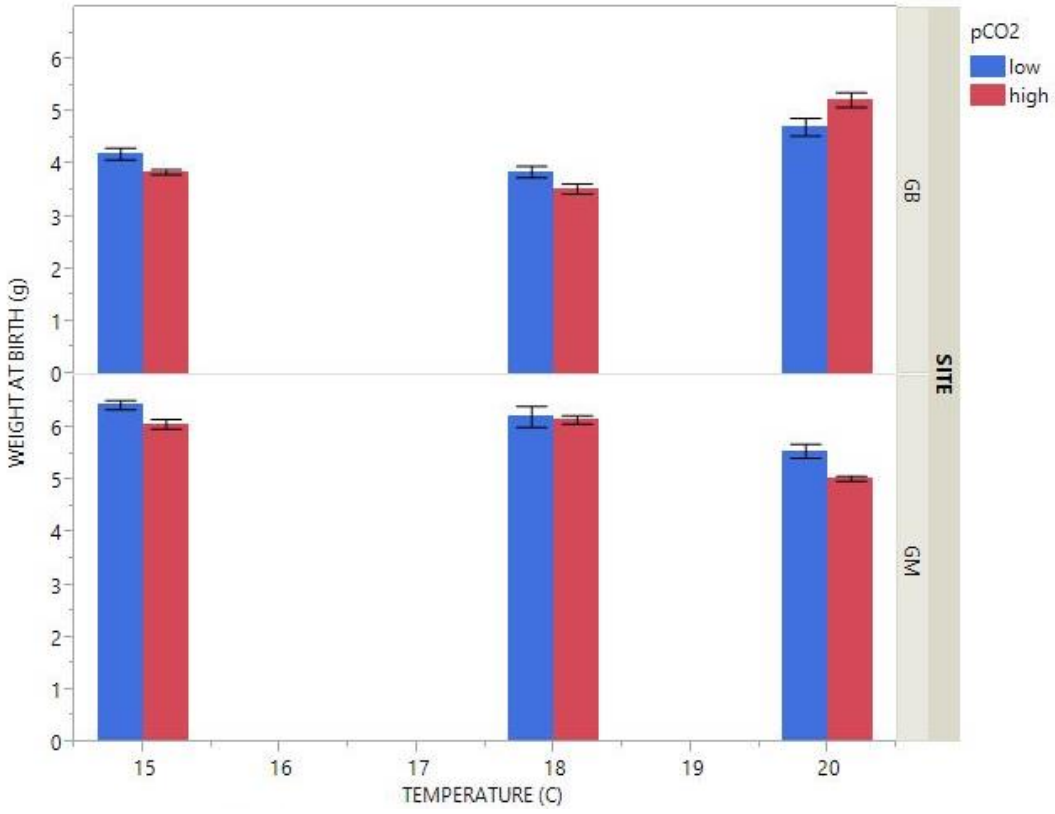


Figure A13. Effect of ocean warming and acidification on newborn body condition in two skate populations. Body condition indices (g/cm^2; mean ±s.e.m.) of newborn *Leucoraja erinacea* significantly differ between treatments ($p<0.001$ three-way ANOVA, n=114). For the Gulf of Maine population, pCO_2 only significantly affects body condition at 18°C ($p=0.01$, one-way ANOVA), while pCO_2 affects body condition across temperatures in the Georges Bank population ($p<0.05$, one-way ANOVA). Temperature significantly affects body condition across populations ($p<0.01$, one-way ANOVA) with the exception of the high pCO_2 treatment in the Georges Bank population ($p=0.9$, one-way ANOVA).

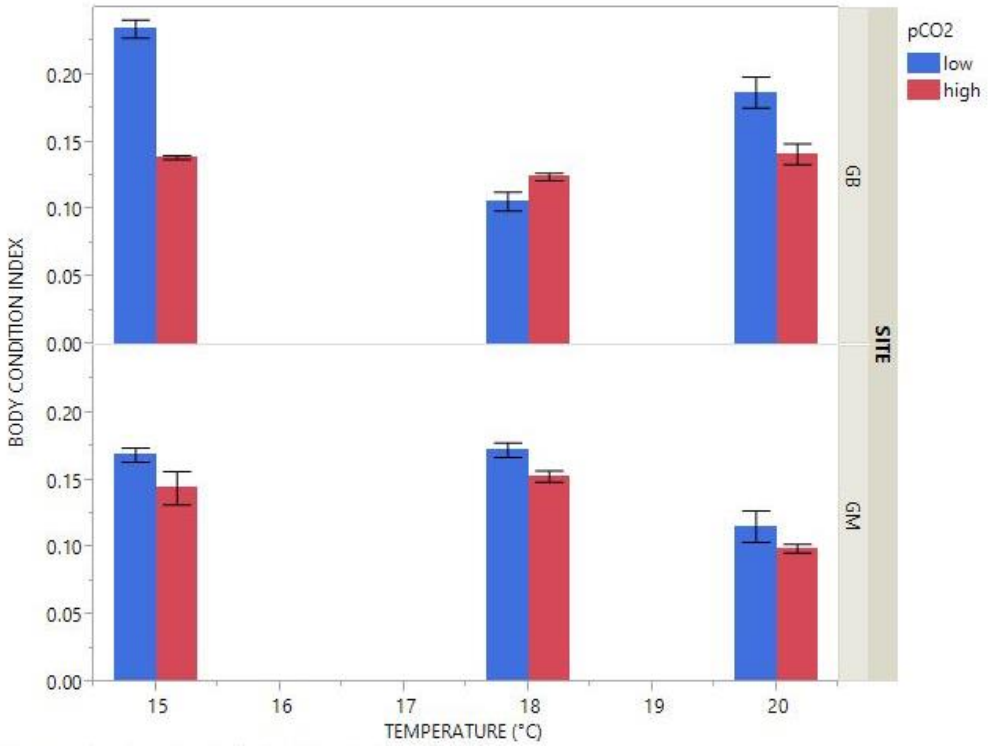


Figure A14. Factorial aerobic scopes of *Leucoraja erinacea* juveniles exposed to current and future level of warming and acidification. Factorial aerobic scopes (mean ± s.e.m.) of juvenile *Leucoraja erinacea* reared at three temperatures and two pH conditions. Factorial aerobic scopes did not differ between populations (p=0.1) and pH (p=0.9) but are significantly affected by temperature (p<0.0001) (three-way ANOVA, n=60).

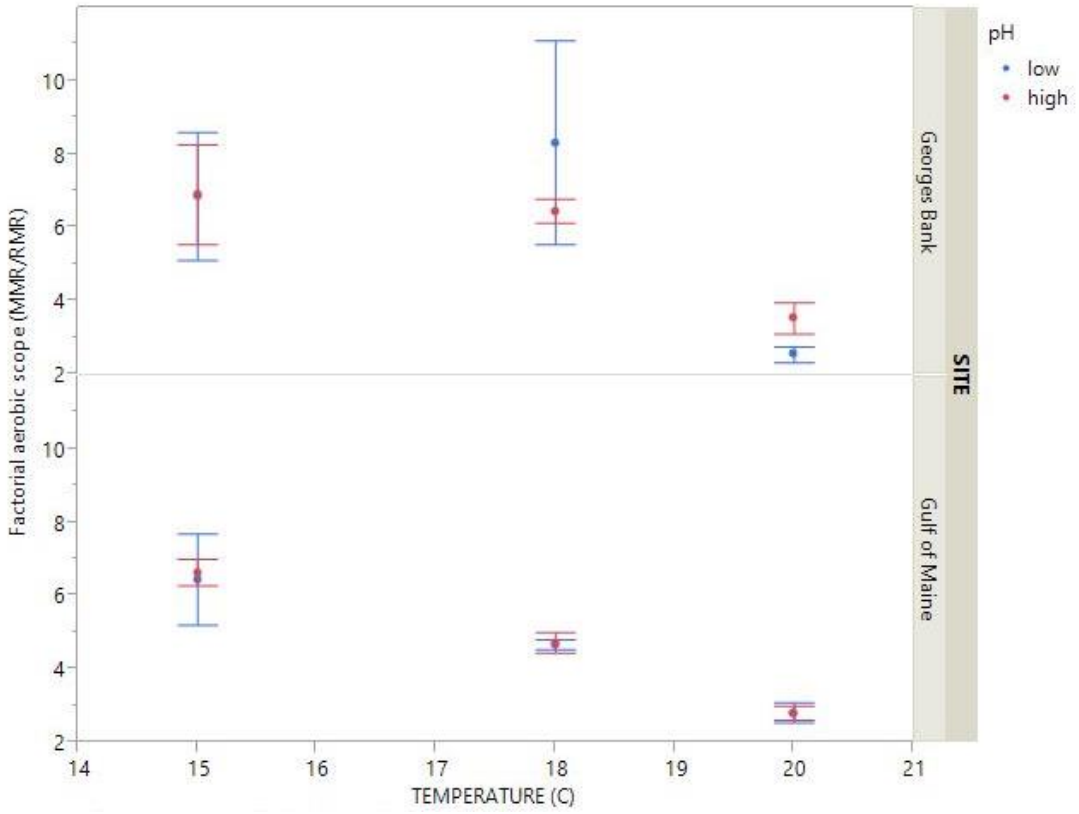


Figure A15. Effect of exercise time on maximum metabolic rates in two populations of little skates. Time to fatigue during exercise significantly elevates maximum metabolic rates in little skates from the Gulf of Maine population (p=0.01, n=24) but not from the Georges Bank population (p=0.1, n=24, one-way ANOVA).

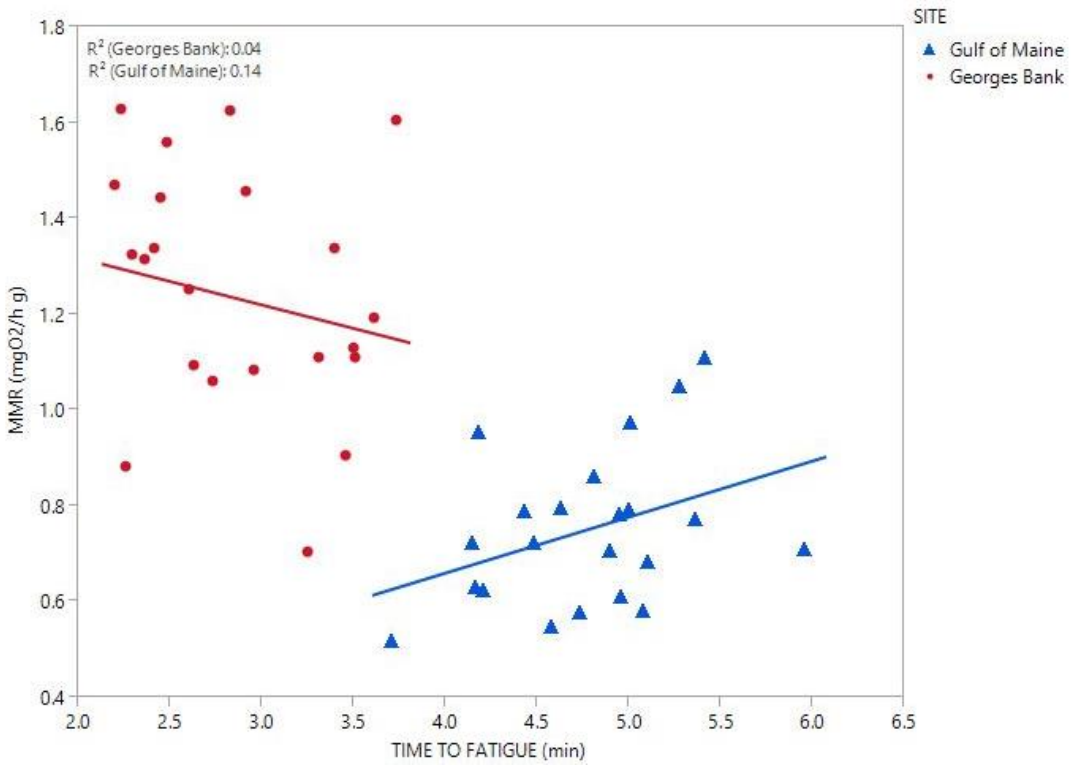


Figure A16. Effect of number of turns on maximum metabolic rates in two populations of little skates. Number of turns to fatigue during exercise significantly elevates maximum metabolic rates in little skates from the Gulf of Maine population (p=0.01, n=24) but not from the Georges Bank population (p=0.1, n=24, one-way ANOVA).

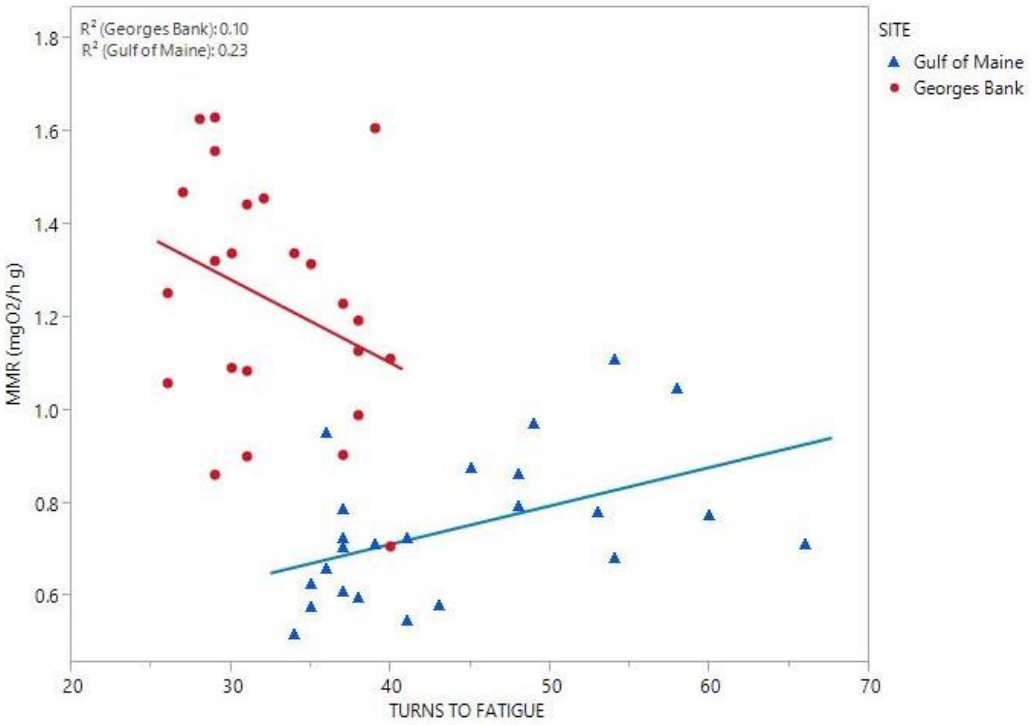


Figure A17. Effect of exercise intensity on maximum metabolic rates in two populations of little skates. Intensity of exercise (turns/minute) significantly elevates maximum metabolic rates in little skates from the Gulf of Maine population (p=0.02, n=24) but not from the Georges Bank population (p=0.7, n=24, one-way ANOVA).

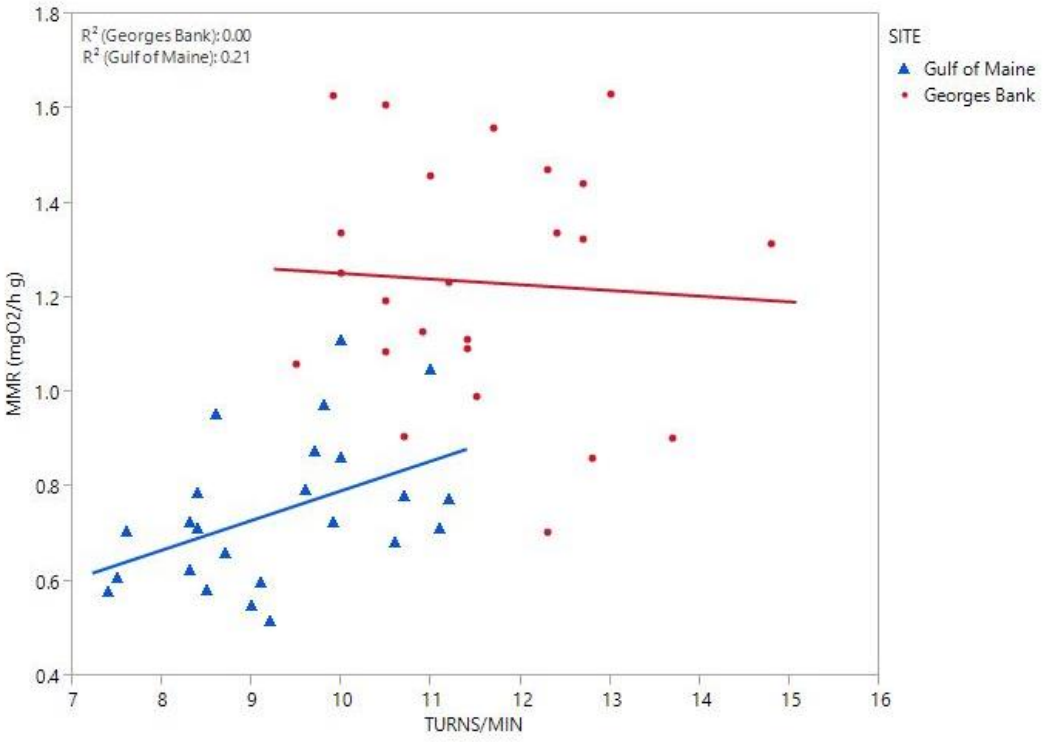
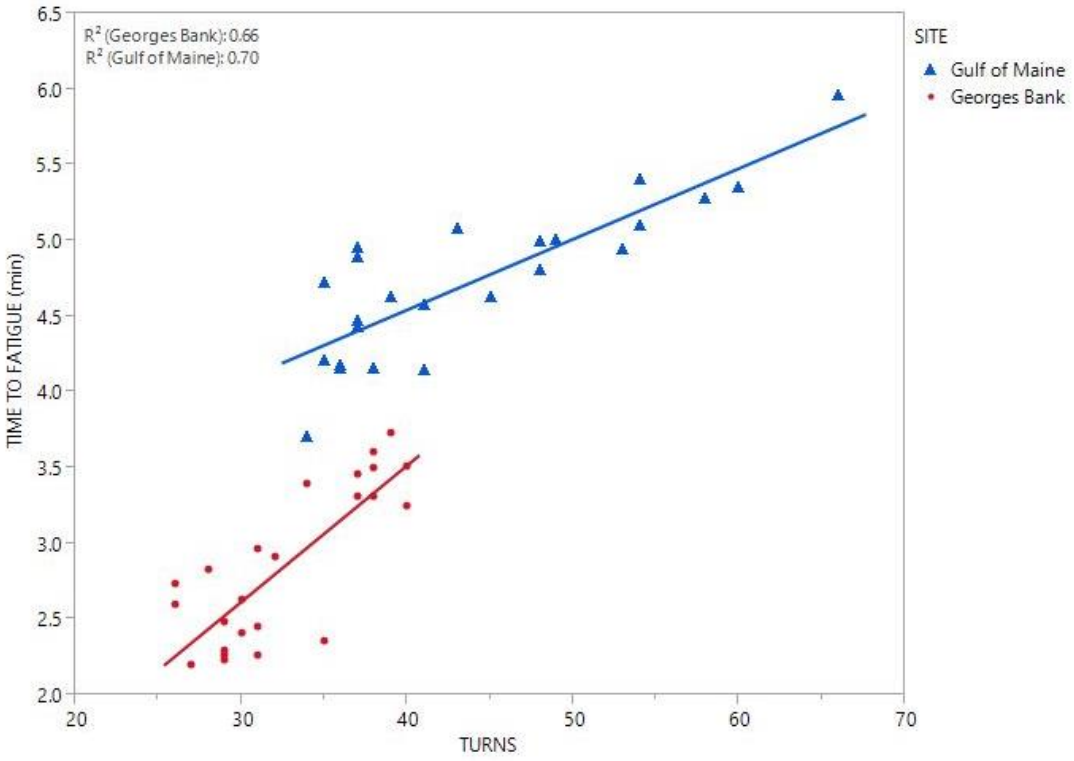


Figure A18. Time to fatigue differs between populations of *Leucoraja erinacea*. Juvenile skates from the Georges Bank fatigue faster and after fewer turns than the Georges Bank population (p=0.005, two-way ANOVA, n=48). In both skate populations time to fatigue and number of turns are positively correlated (p<0.0001, one way ANOVA, n=24).



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Di Santo, V. & Bennett, W. (2011b) Effect of rapid temperature change on resting routine metabolic rates of two benthic elasmobranchs. *Fish Physiology and Biochemistry*, 1–6.

Simpson, S.D., Munday, P.L., Wittenrich, M.L., Manassa, R., Dixson, D.L., Gagliano, M. & Yan, H.Y. (2011) Ocean acidification erodes crucial auditory behaviour in a marine fish. *Biology Letters*, **7**, 917–920.

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SIMS, D.W., WEARMOUTH, V.J., SOUTHALL, E.J., HILL, J.M., MOORE, P., RAWLINSON, K., HUTCHINSON, N., BUDD, G.C., RIGHTON, D., METCALFE, J.D., NASH, J.P. & MORRITT, D. (2006) Hunt warm, rest cool: bioenergetic strategy underlying diel vertical migration of a benthic shark. *Journal of Animal Ecology*, **75**, 176–190.

Somero, G.N. (2010) The physiology of climate change: how potentials for acclimatization and genetic adaptation will determine "winners" and "losers." *Journal of Experimental Biology*, **213**, 912–920.

Svendsen, J.C., Steffensen, J.F., Aarestrup, K., Frisk, M., Etzerodt, A. & Jyde, M. (2012) Excess posthypoxic oxygen consumption in rainbow trout (Oncorhynchus mykiss): recovery in normoxia and hypoxia. *Canadian Journal of Zoology*, **90**, 1–11.

Tanaka, S. & Mizue, K. (1979) Studies on sharks, 15: Age and growth of Japanese dogfish Mustelus manazo Bleeker in the East China Sea. *Bulletin of the Japanese Society of Scientific Fisheries (Japan).*

Taylor, M.S. (2003) Genetic Evidence for Local Retention of Pelagic Larvae in a Caribbean Reef Fish. *Science*, **299**, 107–109.

Taylor, M.S. & Hellberg, M.E. (2005) MARINE RAIATIONS AT SMALL GEOGRAPHIC SCALES: SPECIATION IN NEOTROPICAL REEF GOBUIES (ELACATINUS). *Evolution*, **59**, 374–385.

Todgham, A.E. & Stillman, J.H. (2013) Physiological Responses to Shifts in Multiple Environmental Stressors: Relevance in a Changing World. *Integrative and Comparative Biology*, **53**, 539–544.

Wallman, H. & Bennett, W. (2006) Effects of Parturition and Feeding on Thermal Preference of Atlantic Stingray, *Dasyatis sabina* (Lesueur). *Environmental Biology of Fishes*, **75**, 259–267.

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Yamaguchi, A., Taniuchi, T. & Shimizu, M. (2000) Geographic variations in reproductive parameters of the starspotted dogfish, Mustelus manazo, from five localities in Japan and in Taiwan. *Environmental Biology of Fishes*, **57**, 221–233.

Yu, P.C., Matson, P.G., Martz, T.R. & Hofmann, G.E. (2011) The ocean acidification seascape and its relationship to the performance of calcifying marine invertebrates: Laboratory experiments on the development of urchin larvae framed by environmentally-relevant pCO2/pH. *Journal of Experimental Marine Biology and Ecology*, **400**, 288–295.

Curriculum Vitae

Valentina Di Santo

PROFESSIONAL ADDRESS

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PROFESSIONAL PREPARATION

Università degli Study di Firenze	Natural Sciences	B.S. 2005
University of West Florida	Biology	M.S. 2009
Boston University	Biology	Ph.D. 2014

APPOINTMENTS

Teaching Assistant, Harvard University	2013 – 2014
Research and Teaching Fellow, Boston University	2009 – 2014
Marine Research Facility Supervisor, University of West Florida	2008 – 2009
Instructor of Biology, University of West Florida	2007 – 2009
Laboratory Manager, University of West Florida	2007 – 2009
Visiting Researcher, Netherlands Institute of Ecology	2006
Research Assistant, Bimini Biological Field Station, University of Miami	2005

PUBLICATIONS

Di Santo, V. in review. Ocean acidification exacerbates the impacts of global warming on skate embryos.

Di Santo, V. in prep. Synergistic effect of ocean acidification and warming on aerobic scopes of little skate.

Di Santo, V. in prep. Abiotic stressors and the conservation of elasmobranch fishes.

Rossi, F., Gribsholt, B, Gazeau, F., **Di Santo, V.**, Middelburg, JJ. 2013. Complex effects of ecosystem engineer loss on benthic ecosystem response to detrital macroalgae. PLoS ONE 8(6): e66650.

Di Santo, V. and Bennett, W. A. 2011. Effect of rapid temperature change on resting routine metabolic rates of two benthic elasmobranchs. Fish Physiology and Biochemistry, 37: 929–934.

Di Santo, V. and Bennett, W. A. 2011. Is post-feeding thermotaxis advantageous in elasmobranch fishes? Journal of Fish Biology, 78: 195–207.

Wells, D. L., El-Sheikh, E. M., Sutton, M. A., **Di Santo, V.** and Bennett, W. A. 2009. Automated image processing of X-radiographics of digestion in stingrays. Proceedings of International Conference on Artificial Intelligence, 2: 715-719.

Di Santo, V., Pomory, C. M. and Bennett, W. A. 2009. Algal garden cultivation and guarding behavior of dusky damselfish on coral rubble and intact reef in Dry Tortugas National Park. Proceedings of the American Academy of Underwater Sciences, 2009: 222-228.

RESEARCH AWARDS AND HONORS

2014 Flying Sharks Research Fund
2014 Director's Outstanding Teaching Award in the Marine Program
2014 George R. Bernard, Jr. Travel Award (BU)
2013 Ryan Kelley Memorial Scholarship
2013 Dana Wright Fellowship (BU)
2012 American Elasmobranch Society Research Award
2012 Warren-McLeod Summer Research Fellowship (BU)
2012 Ryan Kelley Memorial Scholarship
2011 Steven Berkeley Marine Conservation Fellowship – American Fisheries Society
2011 George R. Bernard Jr. Travel Award (BU)
2011 Ryan Kelley Memorial Scholarship
2011 Raney Award – American Society of Ichthyologists and Herpetologists
2010 Flying Sharks Research Fund
2010 George R. Bernard, Jr. Travel Award (BU)
2009 Marine Ecology Research Society "Technical Skill Advancement" grant
2009 Student Government Association Academic Travel Fund (UWF)
2009 Graduate Students Scholarly and Creative Activity Award – Travel (UWF)
2009 Who's Who Among Students in American Universities & Colleges
2009 Student Government Association Academic Travel Fund (UWF)
2009 Department of Biology's Honors Board (UWF)
2008 PADI Project AWARE Foundation – declined
2008 Student Government Association Academic Travel Fund (UWF)
2008 University of West Florida Travel Grant
2008 Florida Institute of Oceanography Research Grant
2008 Graduate Merit Award (UWF)
2008 Department of Biology's Honors Board (UWF)
2007 Graduate Students Scholarly and Creative Activity Award – Research (UWF)
2007 Marine Ecology Research Society travel grant
2007 Graduate Merit Award (UWF)
2004 Erasmus/Socrates fellowship (host institution: UEvora)
2001-04 ARDSU award (UniFi)

TEACHING APPOINTMENTS

Teaching Assistant, Harvard University 2013 – 2014
-Patterns and Processes in Fish Diversity (OEB 130)
-Darwin and Contemporary Evolutionary Biology (BIOS S-113) in Oxford, UK

Teaching Fellow, Boston University 2009–2014
- Tropical Marine Invertebrates (taught in Boston and Belize) (BI 569)
- Coral Reef Dynamics (taught in Boston and Belize) (BI 539)
- Ichthyology: Behavior, Ecology, and Evolution of Fish (BI 531)
- Field Biology of Belize Coral Reefs: Expeditionary Ichthyology (BI 532)

- Biology I (BI 107)
- Biology II (BI 108)

Laboratory Instructors Coordinator, University of West Florida 2009
- Comparative Animal Physiology (PCB4723, PCB5990)

Instructor, University of West Florida 2007–2009
- General Biology (BSC1005)
- Comparative Animal Physiology (PCB4723, PCB5990)
- Marine Ecological Physiology (PCB4364)

Teaching Assistant, University of West Florida 2007–2009
- Contemporary Lab Skills (BSC6002)
- Biochemistry: Metabolism (BCH3034)
- Introduction to Marine Biology and Oceanography (BSC2311)
- Anatomy and Physiology I (BSC1085)

SYNERGISTIC ACTIVITIES

(i) Educational Activities:

-*Boston University:* I have mentored six undergraduate thesis and I have supervised over 40 undergraduate projects during courses in Belize

-*University of West Florida*: I was the undergraduate independent study supervisor for the Biology Department

(ii) Web-based Education and Citizen Science

"*Shark Tails*" scientific advisor and collaborator, project that aims to creating children educational videos on the effect of global warming on coral reefs and sharks.

(iii) Manuscripts Reviewer:

Biological Conservation, Environmental Biology of Fishes

(iv) Community Outreach, Conservation, Service:

-Committee member for the Oscar Elton Sette Award – American Fisheries Society
-BIOBUGS volunteer for high school science education program, Boston University
-Graham and Parks Science Fair, Cambridge, MA – Judge
-Volunteer at the American Elasmobranch Society Meeting to fund student awards
-Shark Night. Organized a fundraising and shark finning awareness night
-Festival on the Green. Marine fauna touch tank for children. Pensacola, Florida
-Santa Rosa Pre-Kindergarten Thanksgiving Day. Touch tank to teach children how to handle and appreciate marine fauna
-Oceans Day. Representative for the Marine Biology Program at the University of West Florida
-Annual West Florida Panhandle Regional Science & Engineering Fair – Judge

-World Wildlife Fund Natural Reserve Torre Salsa, Italy. Rescue and care of injured sea turtles. Raise public awareness about the conservation project and collaboration with Regional Center for Wildlife Rescue "FICUZZA", Sicily

PRESENTATIONS AT PROFESSIONAL MEETINGS

Di Santo, V. 2014. Ocean acidification exacerbates the effect of warming on little skate performance. Joint Meeting of Ichthyologists and Herpetologists, Chattanooga, TN

Di Santo, V., Cooper, B. and Bennett, W. A. 2011. Thermal tolerance of the red-bellied pacu in relation to its survival in the United States. Joint Meeting of Ichthyologists and Herpetologists, Minneapolis, MN

Di Santo, V. and Bennett, W. A. 2010. Comparison of farming and guarding behavior of dusky damselfish on coral rubble and intact reef in Dry Tortugas National Park. Joint Meeting of Ichthyologists and Herpetologists, Providence, RI.

Di Santo, V., Cooper, B. and Bennett, W. A. 2010. Thermal tolerance of the red-bellied pacu in relation to its survival in the United States. International Congress on the Biology of Fish, Barcelona, Spain.

Di Santo, V. and Bennett, W. A. 2009. Effects of thermotaxis on digestion efficiency in two elasmobranchs. Joint Meeting of Ichthyologists and Herpetologists, Portland, OR.

Di Santo, V. and Bennett, W. A. 2009. Temperature effect on resting routine metabolic rates of two benthic elasmobranchs. Joint Meeting of Ichthyologists and Herpetologists, Portland, OR.

Di Santo, V. and Bennett, W. A. 2009. Effects of post-feeding shuttling behavior on elasmobranchs' digestion. Scholars of Engineering, Applied Sciences & Technology Annual Research Symposium, University of West Florida, Pensacola, Florida.

Wells, D. L., El-Sheikh, E. M., Sutton, M. A., **Di Santo, V.** and Bennett, W. A. 2009. An application for automated image processing of stingray digestion X-rays. Scholars of Engineering, Applied Sciences & Technology Annual Research Symposium, University of West Florida, Pensacola, Florida.

Wells, D. L., El-Sheikh, E. M., Sutton, M. A., **Di Santo, V.** and Bennett, W. A. 2009. Automated image processing of X-radiographics of digestion in stingrays. The 2009 International Conference on Artificial Intelligence, Las Vegas, NV.

Di Santo, V., Pomory, C. M. and Bennett, W. A. 2009. Algal garden cultivation and guarding behavior of dusky damselfish on coral rubble and intact reef in Dry Tortugas National Park. American Academy of Underwater Sciences Symposium, Atlanta, GA.

Di Santo, V. and Bennett, W. A. 2008. Is post-feeding thermotaxis advantageous in elasmobranchs? European Elasmobranch Association Meeting, Lisbon, Portugal.

Di Santo, V. and Bennett, W. A. 2007. Effects of temperature on elasmobranch fishes: overview and future prospects. Joint Meeting of Ichthyologists and Herpetologists, St. Louis, MO.