Ecology of stress in the tropical coastal marine fish, the checkered puffer (*Sphoeroides testudineus*)

by

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Abstract

It is imperative that we understand the physiological, behavioural and ecological consequences of stress in wild animals. This thesis presents an integrative and multidisciplinary study on the ecology of stress in a tropical coastal marine fish, the checkered pufferfish (*Sphoeroides testudineus*). By incorporating physiological and behavioural tools, I quantified individual variation in the glucocorticoid (GC) stress response and established a negative relationship between the GC stress response and two established fitness proxies of the pufferfish (chapter 2). GCs were then experimentally elevated for the purpose of investigating the thermal-related consequences on the pufferfish in the laboratory and in their natural coastal habitat (chapter 3). Various consequences were documented including fluctuating GCs and weakened fitness proxies to thermal shock, and minor variations in ecosystem dynamics. As a whole, this thesis improves our understanding of the ecology of stress in a wild tropical fish population.
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Co-Authorship Statement

The presented work is a manuscript-based thesis, and chapters 2 and 3 have been prepared for submission to peer-reviewed journals; therefore, a degree of repeatability between chapters is to be expected. All presented material is a product of my own work but chapters 2 and 3 were conducted as a collaborative effort. As specified below, each co-author played an important role and provided valuable comments and feedback on the manuscript. Co-author permission to include these manuscripts in my thesis can be found in Appendix B.

Chapter 2: The relationship between the glucocorticoid stress response and fitness proxies in checkered puffer (*Sphoeroides testudineus*)

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Chapter 3: Consequences of experimental cortisol manipulations on the thermal biology of the checkered puffer (*Sphoeroides testudineus*) in field and laboratory environments

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Chapter 1. General Introduction

1.1 Changing coastal ecosystems

Coastal marine ecosystems represent the transition zone between land and water, from the intertidal zone to the continental shelf, and their biodiversity is shaped by the dynamic processes that create and sustain them (Harley et al. 2006, Burkett et al. 2008). These ecosystems are among the most ecologically and socio-economically rich on the planet, providing approximately US$14 trillion worth of ecosystem goods and services per year (Costanza et al. 1997). Coastal biodiversity is specifically adapted to the extreme environmental conditions imposed along the gradients of these coastal boundaries, and the distribution is often governed by the tolerances to these environmental conditions, including water parameters (such as temperature, salinity and pH), light availability, storm disturbance, tides, water depth and nutrient availability (Burkett et al. 2008). These coastal ecosystems are threatened by anthropogenic environmental change (IPCC 2001). Climate change, due to natural variability and human activity, will likely increase over the subsequent decades due to cumulating greenhouse gas emissions and land use alterations (IPCC 2007a and 2007b). Such loss of equilibrium is threatening the Earth’s genetic, species and ecosystem biodiversity (IPCC 2001, 2007a), risking the loss of marine ecosystems around the world. Elevated greenhouse gas emissions will likely result in increased global mean temperature, causing a variety of physical and chemical changes in marine systems (Harley et al. 2006). Atmospheric carbon dioxide concentrations are expected to increase from a pre-industrial level of 280 to 540–970 ppm by the year 2100 (IPCC 2001). Approximately half of all atmospheric carbon dioxide will eventually be
taken up by the Earth’s oceans (Sabine et al. 2004, Feely et al. 2004) and given that
marine plants, except seagrasses, are carbon-saturated (Gattuso and Buddemeier 2000),
increasing oceanic carbon dioxide will consequently lower oceanic pH (Andersson et al.
2003, Caldeira and Wickett 2005, Burkett et al. 2008). Reduced pH will disturb many
physiological processes in marine organisms including decreased protein synthesis and
ion exchange (see Portner and Langenbuch 2005 for review), as well as change the
saturation limits of aragonite, calcite and other minerals necessary to calcifying organisms
(Kleypas et al. 1999, Feely et al. 2004).

Atmospheric and ocean temperature are increasing and expected to accelerate in
the current century (IPCC 2001), altering biological diversity at every level in the food
web. Temperature can affect basic physiological processes (Hochachka and Somero
2002), thereby influencing the growth, survival, reproduction and distribution of biota
(Brander et al. 2003, Reid 2003). Eurythermal (specifically heat-tolerant) and low-latitude
species may be more vulnerable to increasing temperatures as compared to more
temperate species because they live closer to their thermal limits (Tomanek and Somero
1999, Stillman 2002, Harley et al. 2006). There is a lack of information on how tropical
fish will respond to increases in temperature (Cambers et al. 2007). However, species
distribution is expected to expand toward cooler environments to evade such
consequences (Parmesan and Yohe 2003, IPCC 2007a). Increasing temperatures may also
cause a cascade of subsequent disturbances, including increased severity of diseases as
pathogens are often favoured by warmer temperatures relative to their hosts (Harvell et al.
2002), rising sea level and increasing storm systems (Burkett et al. 2008).
Oceans are estimated to rise approximately 2 mm per year due to ocean expansion through freshwater input of melting polar ice caps (IPCC 2001). Rises in sea level are expected to negatively impact mangrove habitats through flooding and erosion, likely threatening species that require mangroves for food and protection (Field 1994, Bacon 1994, Lugo 2002, Diop 2003, Yáñez-Arancibia et al. 1998, Piedra and Piedra 2007). In addition, storms, particularly tropical storms and hurricanes, are predicted to intensify in terms of wind speed and rainfall as sea surface temperature increases in the main hurricane origins of the North Atlantic and the Gulf of Mexico (Smith and Reynolds 2004, Webster et al. 2005, Bell et al. 2007, IPCC 2007b, Knutson et al. 2008). Changes in storm frequency along ocean coasts have already been documented (Bromirski et al. 2003) and this trend is expected to continue (IPCC 2001). These storms often severely damage coastal systems through hydrodynamic disturbances and thus, threaten the range of coastal biota (Burkett et al. 2008). Intensifying storms may also influence shifts in nutrient upwelling (Roemmich and McGowan 1995, Lotze and Worm 2002, Nielsen 2003), precipitation patterns (Harley et al. 2006), wave regimes (Komar and Allan, 2007) and coastal run-off patterns (Burkett et al. 2008). Coastal run-off is predicted to change (Milly et al. 2005), thereby altering coastal salinity (Contente et al. 2011), turbidity, water residence time, vertical stratification, overall productivity (risk of eutrophication and algal blooms), and inflow of terrestrial nutrients and pollutants (Harley et al. 2006, Nicholls et al. 2007, Burkett et al. 2008).

Evidently, climate change will affect the Earth’s biota in several ways on the individual, population and community levels, through changes in physiology and performance, processes of reproduction and dispersal, as well as species interactions.
(Harley et al. 2006). The combination of stressors caused by gradual anthropogenic climate change may provoke complex non-linear responses in coastal systems (Lee et al. 2001, Harley et al. 2006, Burkett et al. 2008). Wild populations will need to rely on micro-evolution, phenotypic plasticity and phenotypic flexibility to cope with the various challenges imposed by global climate change (i.e., new types, increased frequency and a wider range of stressors; Angelier and Wingfield 2013). The consequences to such stressors have been documented to be highly variable and context-dependent, likely attributable to the role of the glucocorticoid (GC) response. The GC response is known to mediate rapid physiological and behavioural changes to regain homeostasis and benefit survival following a challenge (refer to Angelier and Wingfield 2013).

1.2 The stress response in fish

Stress in vertebrates is a state of threatened homeostasis that is restored via a set of elaborate and complex responses (Chrousos 1998). Depending on the magnitude and duration of the stress experienced by an individual, all levels of biological organization may be affected, from direct physiological to indirect ecological effects. The effects of stress manifested at the ecological level may be the most difficult to determine, however these effects are at the pinnacle of conservation concern (Adams 1990).

The stress response is a complex mechanism that allows an individual to manage real or perceived stressors in order to maintain homeostasis (Barton 2002). In fish, the primary response consists of endocrine changes, resulting in quantifiable levels of circulating catecholamines and corticosteroids (Donaldson 1981, Randall and Perry 1992, Wendelaar Bonga 1997). Corticosteroids are released primarily from interrenal cells of head kidney tissue in fish following stimulation by the adrenocorticotropic hormone and
are controlled by negative feedback of corticosteroids on the hypothalamic-pituitary axis (Fryer and Peter 1977, Donaldson 1981, Wendelaar Bonga 1997). Cortisol is the glucocorticoid in Actinopterygii that increases metabolic rate via several biochemical processes (Idler and Truscott 1972, Hanson and Fleming 1979, Barton et al. 1998). The synthesis and release of cortisol is delayed by several minutes and generally peaks within 0.5 to 1 hr after an acute stressor (Wedemeyer et al. 1990, Barton and Iwama 1991, Gamperl et al. 1994). The secondary response consists of changes in metabolism, hydromineral balance, as well as cardiovascular, respiratory and immune functions; for instance, measurable changes in concentrations of blood glucose, lactate and major ions (chloride, sodium, and potassium), and tissue levels of glycogen and heat shock proteins (Pickering 1981, Iwama et al. 1997 and 1998, Mommsen et al. 1999). The tertiary response consists of changes in performance, including growth, disease resistance, overall health and behaviour (Wedemeyer and McLeay 1981, Wedemeyer et al. 1990). Primary responses are sometimes directly responsible for secondary responses, and tertiary responses result from the primary and secondary responses and may even influence an individual’s state of survival (Sumpter 1997, Hontela 1997, Wendelaar Bonga 1997).

Stress itself cannot be quantified. However, the response to stress can be measured on the primary, secondary and tertiary levels to determine the amount of stress experienced by a fish (Barton and Iwama 1991). Blood cortisol concentration is commonly used as a physiological stress indicator as it is highly responsive to acute stressors, easy to quantify, and its delayed release allows for proper sampling of resting levels in fish (Wedemeyer et al. 1990, Barton and Iwama 1991, Gamperl et al. 1994, Wendelaar Bonga 1997).
The stress response is separated into three categories—recognition of a threat to homeostasis, the stress response, and the consequences of stress (Moberg 1985). These responses are often considered adaptive, however, if the stressor is severe in intensity and duration, responses may cause a state of distress and become detrimental or maladaptive (Selye 1973, Deitinger and McCauley 1990, Barton and Iwama 1991). Stress response can be influenced by genetic, developmental and environmental factors. The stress response varies between and within species, develops early in life and may sharpen during periods of metamorphosis. It is also influenced by almost all environmental factors, both external and internal. Internal factors include features of the animal’s overall health, and external factors include a variety of abiotic and biotic variations imposed by the inhabited ecosystem (see Barton 2002 for overview). This study will focus on biotic and abiotic factors as these are most likely to be influenced by the pending global warming phenomenon.

Early research on stress (cortisol in particular) in fish focused on identifying factors that elicited a stress response and characterizing the elevation and eventual recovery of cortisol to pre-stress levels. However, a recent framework for examining stress in fish, though it has been used for some time with birds and reptiles, has been to examine individual variation in baseline cortisol and maximal stress responsiveness in order to understand the extent to which they are associated with various fitness-oriented endpoints (e.g., Bonier et al. 2009). Such research is focused on elucidating the functional significance of variation in responsiveness given the growing recognition that not all individuals respond to stress in the same manner. For fish, baseline cortisol as well as responsiveness have been shown to be repeatable through time and to be correlated with
individual size, behavioural traits, reproductive success and overall fitness (Cook 2007, O’Connor et al. 2009). Given that glucocorticoids influence the expression of nearly 10% of the genome, targeting metabolism, growth, repair, reproduction and resource allocation (Le et al. 2005), the consequences of elevated glucocorticoids have also been studied, particularly in the context of aquaculture. For example, much research has examined the potential for mortality associated with inhibited disease resistance, growth, reproduction, and general health (Barton and Iwama 1991). By studying the consequences of stress, aquaculture practices have aimed to increase survival rates and optimize overall production (Barton and Iwama 1991, Iwama et al. 1997, Pickering 1998).

Much work has studied the stress responsiveness to a single stressor, however, an area of concern is the effect of chronic or repeated stressors on coastal marine animals that are already living in challenging environmental conditions. Fish reveal an uncertain cumulative response to multiple and repeated stressors (Carmichael et al. 1983, Flos et al. 1988, Maule et al. 1988). Fish may experience a desensitized or weakened stress response to multiple or repeated exposures to stressors (Reid et al. 1998) and chronic stress may even intensify or attenuate the response to a secondary stressor (Barton et al. 1985, Pickering and Pottinger 1987, Wilson et al. 1998, Angelier and Wingfield 2013). Human activities (i.e. aquaculture and fisheries), urban development and the increasing effects of climate change may be external environmental factors triggering chronic stress in fish (Pickering and Pottinger 1989). To study the stress response in fish as well as its consequences, it is typical to expose fish to stressors (physical, environmental, chemical) in a controlled manner. For example, if studying stress responses to hypoxia one would manipulate the dissolved oxygen concentration in the water and then measure various
endpoints. Such stressors can be acute (seconds to hours – e.g., a simulated predation event, transient hypoxia) or chronic (e.g., days to weeks – e.g., starvation, long-term hypoxia). Another approach for studying the consequences of the stress response is to experimentally elevate cortisol, typically using an injection of exogenous cortisol or incorporating cortisol into food (Gamperl et al. 1994). This thesis will involve two approaches: First, an examination of individual baseline and maximal stress responsiveness as they relate to individual fitness and secondly, the experimental elevation of cortisol to examine physiological, behavioural and ecological consequences of stress.

1.3 Checkered puffer biology

The checkered puffer (*Sphoeroides testudineus*) belongs to the family Tetraodontidae meaning four tooth plates (Shipp 1974). Tetraodontidae are Actinopterygii comprising 19 genera and approximately 160 species, inhabiting tropical and subtropical areas of the Atlantic, Indian and Pacific oceans (Nelson 1984). The two fused teeth on both upper and lower jaws of the pufferfish form a solid beak. The checkered puffer has dorsal (8 rays) and anal (7 rays) fins set anteriorly near the caudal fin, reduced scales and prickles covering the anterior half of the body. This pufferfish is cryptic in colouration with a brown to black dorsal side covered in a light regular geometric pattern, and a white to yellow ventral side (Shipp 1974, Robins et al. 1986). The checkered puffer can reach a total length of up to 300 mm (Shipp 1974, Robins et al. 1986), but most specimens studied are much smaller (180 ± 20 mm).
Pufferfish are named for their ability to inflate by taking water or air into their stomachs through numerous rhythmic buccal cavity expansions and compressions (Jackson 1848, Gabriel 1940, Brainerd 1994, Wainwright 1992). Body inflation is restricted to the taxa Tetraodontidae and Diodontidae, and serves as a mechanical defense against piscine predators (Winterbottom 1974, Brainerd 1994). Wainwright (1992) studied the inflation pump of *Chilomycterus* (a member of the Diodontidae family) and found that there were three principal skeletal characters – the buccal cavity, two pectoral girdle features and the first large, jointed branchiostegals ray – and four cranial musculature features – muscles positioned near the articulation, the well-developed hyohyoideus abductor, protracter hyoideus and valvulus muscle, as well as the oral valve – contributing to the inflation mechanism. The valvulus muscle is found only in the Tetraodontidae and Diodontidae families (Winterbottom 1974) and reinforces the oral valve by preventing water or air from exiting the mouth upon inflation (Wainwright 1992). Pufferfish belonging to the family Tetraodontidae have large expandable stomachs with strong esophageal, pyloric and horizontal sphincters (Rosen 1912, Breder and Clark 1947). The ventral and dorsal portions of the stomach are called the inflatable sac and the stomach proper, respectively, thereby implying division of stomach function (Breder and Clark 1947). Many other biological structures within the puffer allow the fish to inflate, including specialized unconstrained skin forming an orthogonal arrangement of dermal collagen sheets, loose connective tissue (aureolar tissue), large folds in the peritoneum, expandable stomach and peritoneal cavity, axial skeleton and musculature, as well as the absence of pleural ribs and a pelvis (Brainerd 1994). The inflation sequence is characterized by a cyclic pattern of decreasing and unusually increasing pressure pulses.
comprising of 3 to 20 cycles and taking between 10 and 34 sec to reach full inflation. Once the fish is fully inflated and its skin is tight to the touch, 3 to 4 more inflation attempts are made before the fish accepts the resistance (Wainwright 1992). Upon deflation, skin exerts little force to help the fish return to its original length as it is inelastic. Water is expelled from the mouth by several repeated buccal cavity compressions and lordotic movements (Brainerd 1994). Inflation has interested biologists for some time (Thilo 1899, Parr 1927, Brainerd, 1994), however it has not yet been studied as a stress response correlate.

Large fish and birds likely prey on pufferfish, however, ‘puffing’ serves as an effective predator avoidance behaviour thereby increasing body size and making them difficult to subdue and consume (Randall 1967, Myer 1989). Piscine predators rarely feed on puffers (Hutchinson 1972), with the exception of the occasional large shark and grouper (Randall 1967). Similarly, avian predators were found to rarely feed on pufferfish (Recher and Recher 1968). Recher and Recher (1968) reported eleven pufferfish (Spheoroides) captures by herons; 5 of which were able to escape by means of ‘puffing’, and 6 successful predations as the puffer was small relative to the size of the predator or the predator was able to spear the inflated pufferfish with its beak.

The checkered puffer is an omnivorous benthic predator using its beak-like teeth to feed mainly on crustaceans and molluscs likely during dawn and dusk, tidal extremes, and in non-mangrove habitats (Targett 1978, MacDonald et al. 2009). Other prey items include sipunculids, tunicates, seagrass, and detritus. Diet varies with size as larger puffers (>150 mm) have a more powerful bite and are able to feed on a larger variety of prey items (Targett 1978). Qualitative observations found a notable biting performance in
the checkered puffer, most likely due to structural reinforcement, diet preferences and
possible defensive or intraspecific competition strategy. Bite force has been studied
extensively in the reptile field as a fitness proxy (Herrel et al. 2001, Erickson et al. 2003,
Vanhooydonck et al. 2005, Lailvaux and Irschick 2007, Bulte et al. 2008), however this
metric not yet been thoroughly investigated in durophagus fish.

The checkered puffer is a highly successful species inhabiting bays, seagrass beds,
tidal creeks, mangrove lagoons and freshwater areas, ranging along the Atlantic coastline
as far North as Rhode Island and as far South as the southeastern coasts of Brazil, and
throughout the Gulf of Mexico and Caribbean sea (Shipp 1974, Targett, 1978, Robins et
2003, Felix et al. 2006, MacDonald et al. 2009). This species of pufferfish does not
typically migrate to coral reefs at any point in its lifecycle (Froese and Pauly, 2008).
Instead, it spends its life resting on shallow turbid substrate to assist with its cryptic
defense (Austin and Austin 1971, Pauly 1991) and uses mangroves for protection
(MacDonald et al. 2009). As pufferfish grow in size, vulnerability to predation decreases
and more time is thus spent higher in the water column and outside the mangrove root
system (Laegdsgaard and Johnson 2001, MacDonald et al. 2009).

The expansive distribution of the checkered puffer is credited to its broad
physiological tolerance. This euryhaline pufferfish has been found to dwell in less saline,
lower estuary habitats ranging from 0 to 67 ppt (Blaber 1997, Prodocimo and Freire 2001,
and de Santillana 2004). The checkered puffer efficiently regulates its plasma osmolality
and chloride concentration in decreasing salinities of 29.5, 14, and 4.5ppt, along a 5 to 6 hr period (Prodocimo and Freire 2001, 2004 and 2006, Prodocimo et al. 2008).

Independent of all other water conditions, salinity has been shown to be the main factor structuring fish assemblages in several estuarine systems (Blaber 1997, Jaureguizar et al. 2004, Paperno and Brodie 2004, Barletta et al. 2005, Sosa-Lopez et al. 2007, Mendoza et al. 2009, Contente et al. 2011). Other factors influencing estuarine fish abundance and richness include tide regime (Barletta et al. 2003), dissolved oxygen (Barletta et al. 2008), turbidity (Marchand 1993), aquatic vegetation (Castellanos and Rozas 2001), food availability (Scharf et al. 2004, Martinetto et al. 2005), and sediment type (Scharf et al. 2004). Nevertheless, global warming will create yet another environmental challenge influencing coastal ecosystems – temperature. Temperature is one of the most prevalent conditions influencing biological processes thereby impacting the performance of organisms and overall fitness (Haynie 2001, Brown et al. 2004, Angilletta et al. 2006).

The checkered puffer is an interesting model for studying stress to environmental change as they reside in habitats prone to high fluctuations in temperature independent of global climate change over their entire lifespan. Unlike other coastal species, all life history stages of the checkered puffer are subjected to the extremes imposed by their coastal environment. On top of their high tolerance to external environmental changes, the checkered puffer also displays remarkable defensive behaviours, including biting performance and the unique predator avoidance strategy of “puffing”.
1.4  Research objective and predictions

The purpose of this thesis is to examine the ecology of stress in a population of wild checkered puffer with a focus on fitness-related endpoints. Specifically, I will determine the individual variation in the relationship between the physiological stress response and two fitness proxies. In addition, I will use experimental cortisol manipulations to evaluate the physiological, behavioural and ecological consequences on the thermal biology of the checkered puffer in a controlled laboratory setting and in a tidal creek in Eleuthera, The Bahamas.

1.4.1  Rationale and hypotheses for chapter 2

The purpose of chapter 2 was first to quantify individual variation in the GC stress response in checkered puffer, and then to determine whether there is a relationship between the GC stress response and two established fitness proxies, puffing metrics and bite force. Given that checkered puffer have not been well-studied in the context of stress, a number of important methodological issues had to be resolved before commencing experimentation. For example, the GC response and recovery of the checkered puffer had to be determined by subjecting the animal to a standardized acute stressor that would simulate a typical stress response exhibited in the wild. It was necessary to determine if fish can be held in laboratory conditions in a manner that results in low GCs, and the time course for recovery needed to be documented to identify the time period at which sampling should occur to obtain maximal values. Barton and his colleagues (1987) found that exposing a fish to air for a standard length of time causes a fish to display a standard and repeatable stress response to characteristic and challenging environmental conditions.
By standardizing the stressor, individual variation in baseline and maximum stress responses in this species was also be determined. Responses of interest are concentrations of cortisol and glucose, as well as two fitness proxies – inflation or ‘puff’ metrics and bite force.

1.4.2 Rationale and hypotheses for chapter 3

The purpose of chapter 3 was to quantify the effects of multiple and chronic stressors on thermal-related characteristics in the checkered puffer using experimentally manipulated cortisol techniques. First, I identified the appropriate method and concentration of cortisol delivery needed to raise blood plasma cortisol levels to physiologically relevant limits in the pufferfish. The depletion timeline of the cortisol implant was examined over a 20 day period, and the energetic cost associated with this cortisol 2 days post-administration was determined. In the laboratory, I compared indicators of energy use (i.e., blood glucose concentrations and intermittent-flow respirometry experiments) between control and cortisol-implanted fish. Once the details of the cortisol implant were uncovered, the effect of multiple and chronic stressors on thermal-related characteristics in the pufferfish were elucidated by means of controlled laboratory experiments and a complimentary field study. In the laboratory, I compared stress indicators (i.e., cortisol titres, and behavioural consequences) between control and cortisol-implanted fish, in response to thermal challenges (i.e., heat- or cold-shock). In a tidal creek in Eleuthera, I used small thermal loggers affixed to fish over a 20-day period to compare thermal habitat use between control and cortisol-implanted fish. To that end, I tested the null hypothesis that
checkered puffer’s thermal sensitivity and thermal habitat use was independent of whether cortisol levels had been experimentally elevated.

Chapter 2. The relationship between the glucocorticoid stress response and fitness proxies in checkered puffer (*Sphoeroides testudineus*)

2.1 Abstract

Individual variation in the endocrine stress response (i.e., the change in circulating glucocorticoids [GCs] following a challenge) has been linked to survival and fitness in a variety of species. However, the strength and the direction of this relationship have proven to be highly context dependent. The checkered puffer (*Sphoeroides testudineus*) is an interesting model for studying stress in an ecological context because it has a unique predator avoidance strategy. Pufferfish will not hesitate to bite and inflate or ‘puff’ to deter potential predators. These behaviours are readily measurable and have direct implications for individual survival and fitness. The purpose of this study was first to quantify individual variation in the GC stress response in checkered puffer, and then to determine whether there was a relationship between the GC stress response and two established fitness proxies, puffing metrics and bite force. Wild checkered puffer from Eleuthera Island, The Bahamas, were subjected to a standardized stress protocol, and baseline and post-stress physiological stress indices (circulating GCs and glucose) were subsequently quantified. To evaluate whether these indices were correlated with fitness proxies, bite force and puffing metrics were assessed prior to and following the standardized stressor. As expected, physiological stress indices were significantly elevated following the
standardized stressor. Interestingly, both bite force and the extent of puffing were reduced following the standardized stress protocol. Furthermore, the magnitude of individual physiological stress response was negatively correlated with post-stress fitness proxies. I also documented that puff metrics for individuals are repeatable through time. This study highlights the negative consequences of an acute stressor on fitness proxies, demonstrates a negative relationship between GC response and fitness proxies, and establishes the checkered puffer as a valuable model for future research on the ecology of stress in wild vertebrates.

2.2 Introduction

The physiological stress response is a complex mechanism that allows an individual to maintain homeostasis in the face of real or perceived challenges (Selye 1937, Sapolsky et al. 2000). In brief, the physiological stress response of vertebrates involves the release of glucocorticoids (GCs). In fish, cortisol is the primary GC that increases metabolic rate via several biochemical processes (Barton 2002), thereby causing changes in metabolism, hydromineral balance, as well as cardiovascular, respiratory and immune function; for instance, measurable changes in concentrations of blood glucose, lactate and major ions (Pickering 1981, Iwama et al. 1997 and 1998, Mommsen et al. 1999, Barton 2002). GC concentrations are commonly used as a physiological stress indicator as it is highly responsive to acute stressors, easy to quantify, and its delayed release allows for proper sampling of resting, baseline levels (Wedemeyer et al. 1990, Barton and Iwama 1991, Gamperl et al. 1994, Wendelaar Bonga 1997). Furthermore, maximum post-stress GC concentrations generally peak within 0.5 to 1 h after an acute stressor (Wedemeyer et al.

Increased glucocorticoid (GC) release in response to a stressor is thought to promote survival through heightened performance during a challenge (e.g., facilitating escape from acute stressors), as well as to influence recovery once the challenge has been overcome (Pagnotta et al. 1994, Wingfield et al. 1998, Sapolsky et al. 2000, Breuner et al. 2008). While these responses are considered adaptive on a short time scale, if the stressor is severe in intensity and duration, increased GC release may also come at a cost to other functions, such as performance, immunocompetency, disease resistance, growth, overall health and reproduction (Wedemeyer and McLeay 1981, Wedemeyer et al. 1990, Barton and Iwama 1991, Sapolsky et al. 2000, Romero et al. 2009).

The implications of individual variation in GC secretion on performance and overall fitness are complex (see reviews by Ricklefs and Wikelski 2002, Bruener et al. 2008, Bonier et al. 2009). Increased baseline GCs through diet manipulation are highly correlated with heightened performance and increased survival in the mountain chickadee (Parus gambelii; Saldanha et al. 2000), captive white-crowned sparrow (Zonotrichia leucophrys gambelii; Lynn et al. 2003), and rainbow trout (Oncorhynchus mykiss; Overli et al. 2002). However, elevated post-stress GCs have also been found to have no effect on fitness (e.g., Moore et al. 2000), and even negative effects on measures of fitness (e.g., Blas et al. 2007, Roberts et al. 2007). Furthermore, measures of fitness have been found to be influenced by elevated baseline GCs (e.g., Brown et al. 2005) and manipulated GCs (e.g., Saino et al. 2005, Wada and Breuner, 2008). Much of the GC research performed to date has examined individual variation in stress response and the associated fitness-

The checkered puffer (Sphoeroides testudineus) is an interesting model for studying stress in an ecological context because of its unique predator avoidance strategies, which are readily measurable and have direct implications for individual survival and fitness. The ‘puffing’ of pufferfish serves as effective predator avoidance behaviour by increasing body size and making them difficult to subdue and consume (Randall 1967, Recher and Recher 1968, Myer 1989). In this study, puffing will be measured in terms of intensity over time, and the time at which it takes the fish to deflate once released. Checkered puffers are also durophagous, feeding on hard-shelled prey. In all durophagous vertebrates, bite force is an important component of feeding performance (Wainwright 1988, Hernandez and Motta 1997, Grubich 2005, Berumen and Pratchett 2008) and expanding dietary range (see Mara et al. 2010 for overview). Increased bite force allows exploitation of prey unavailable to conspecifics and other species (Hernandez and Motta 1997, Berumen and Pratchett 2008), thereby potentially reducing inter- and intraspecific competition and increasing fitness (Wainwright 1988, Grubich 2005). Bite force has also been studied extensively in the reptile field as an indicator of fitness, specifically dewlap size and combat success in several Anolis lizard species.
(Vanhooydonck et al. 2005, Lailvaux and Irschick 2007), and as a strong correlate to increased dietary range, body condition and reproductive output in the northern map turtle (Graptemys geographica; Bulte et al. 2008). Furthermore, bite-force has been studied as temperature-dependent anti-predator behavioural correlate in reptiles (Greene 1988, Hertz et al. 1982). As bite force has been linked to measures of fitness in vertebrates, I predict that bite force will be an accurate fitness proxy for durophagous fish. While the relationship between the GC stress response and fitness has not been well-documented in fish (see reviews by Breuner et al. 2008, Bonier et al. 2009), the GC stress response has been shown to be repeatable through time, and to be correlated with individual size, behavioural traits, reproductive success and overall fitness (Cook et al. 2011a, 2011b, O’Connor et al. 2012). To date for fish, relationships between GC responsiveness and fitness endpoints have been observed in Pacific salmon (Oncorhynchus nerka and O. gorbuscha; McConnachie et al. 2012, Cook et al. in review). Therefore, to build on the foundational syntheses published by Breuner et al. (2008) and Bonier et al. (2009), I attempted to define the relationship between GC stress response and two established fitness proxies in the checkered puffer: inflation performance (i.e., puffing intensity and time to deflate) and bite force.

2.3 Methods

2.3.1 Study site and sampling

Between February 22-25, and June 1-12, 2012, checkered puffers (n=110) were collected from Plum and Page Creeks, on Eleuthera Island, Bahamas (Plum: N24°45'45.79"
Pufferfish were corralled into a seine net set at the mouth of the creeks on an outgoing tide and transported to the Cape Eleuthera Institute (CEI: N24°50'05" W076°20'32") in aerated coolers. At CEI, pufferfish were held in 1250 L flow-through tanks with ample aeration (29.2±2.7°C), and were allowed to acclimate to laboratory conditions between 2 and 7 days before experimentation. During acclimation, pufferfish were fed an assortment of sardines (*Sardinella aurita*), juvenile bonefish (*Albula vulpes*) and mottled mojarra (*Eucinostomus lefroyi*) every 2 days. The holding tank was cleaned every 4 days until 3 days prior to sampling, and fish were starved 48 hours before experimentation to avoid disturbing the fish before experimentation. Following experiments, all pufferfish were weighed (g) using a portable electronic balance and then placed in a foam-lined trough to obtain a total length measurement (mm). All techniques were performed without anesthesia (see Cooke et al. 2005 for rationale), all samples were collected in accordance with the guidelines of the Canadian Council on Animal Care as administered by Carleton University (B12-01), and all fish were released back into the ocean upon recovery at the conclusion of the experiment.

### 2.3.2 Maximal glucocorticoid response

At the outset, I performed a preliminary study to define the cortisol secretion profile and subsequent recovery timelines of pufferfish by subjecting them to a standardized stress challenge and then sampling them during the recovery period. This preliminary stress challenge served to identify the maximum cortisol concentration for pufferfish, the time at which this maximum occurs, and aided in defining the sampling interval for subsequent
portions of the study. To generate this profile, pufferfish were placed in individual opaque experimental chambers (12.5 L) with ample aeration and a constant flow of saltwater 24 h before experimentation. Fish were then randomly assigned to one of six treatment groups: (1) control (n=8), (2) 15 min post-stressor (n=7), (3) 30 min post-stressor (n=8), (4) 1 h post-stressor (n=7), (5) 2 h post-stressor (n=7), and (6) 4 h post-stressor (n=5). Pufferfish in each of the treatment groups were subjected to an acute standardized stressor by holding them at the air-water interface for 5 min in a rubber-mesh dip net, and then returning them to their individual chambers for the designated duration. Fish in each treatment were then non-lethally sampled for 0.5 mL of blood by caudal venipuncture using a heparinized 1-mL syringe and 21-gauge, 2.5-cm needle. To avoid sampling-induced stress, each blood sample was withdrawn in under 3 min (Romero and Reed 2005). Control fish remained in their chambers for 24 h, but received no net holding. Collected blood samples were held in syringes in water-ice slurries for no more than 1 h before analysis. Based on data from this series of preliminary samples (n=48), I determined that maximum values of stress-induced GC concentrations occurred 30 min post-stressor (Fig. 1); all sampling for maximal cortisol concentrations during successive trials therefore occurred 30 min after the onset of a stressor.

2.3.3 Glucocorticoid responsiveness relative to two fitness proxies

To identify the relationship between GC stress response and fitness proxies, pufferfish (n=48) were collected, held, and placed in individual opaque experimental chambers (12.5 L) as described above. After 12 hours of acclimation to these chambers, all fish were air-exposed for 3 min, during which time their baseline bite force (N) was measured
with a custom built force transducer system composed of a load cell and a custom built DC amplifier. The load cell was constructed from a (75 × 12 × 12 mm) aluminum block with material removed from the center portion to create a thin-walled (1 mm) c. 15 mm long, channel. Loads applied to one end of the aluminum block therefore caused deformation of the thinned regions in the centre that were detected by thin-foil type resistive strain gauges bonded to adjacent surfaces of the block at the thinned regions. The paired strain gauges were connected in a Wheatstone bridge configuration. The amplifier unit supplied an excitation voltage to the bridge and changes in resistance of the strain gauges produced a change in voltage proportional to the load applied to the cell. A multimeter (Agilent True RMS Multimeter, Model U1233A) was used to display voltage changes from the load cell. The bite force meter was calibrated using a series of loads of known weight. The calibrated output of the unit was linear and the drift due to thermal instability was small (less than 0.05 % of full scale). All pufferfish were also sampled for 0.5 mL of blood within this 3 min period, which served as a baseline sample.

In addition, the intensity of the fish’s ‘puff’ over the course of the 3 min sampling period was monitored to generate a baseline puff score. Puffs were assigned a score from 0 to 3, with 0 being no puff, 1 being equal to or less than half a full puff, 2 being greater than half a full puff, and 3 being a full puff; a full puff was assigned once the fish was maximally inflated (i.e., the fish’s skin was tight to the touch and subsequent inflation attempts resulted in no further expansion). Each puff score (0-3) was assigned a percentage of time used over the 3 min, and then weighted according to its score. As a result, each puff score is presented as a value between 0 and 3 (i.e., 0 being no puff at all and 3 being a consistent full puff over the course of the 3 min sampling period). Given
that pufferfish rarely maintained the same level of puff over the entire sampling period, puff scores were weighted to account for the varying puff intensity over the 3 min sampling period. Following this, all fish were immediately given a stress challenge by holding them at the air-water interface for 5 min in a rubber-mesh dip net, and subsequently returned to their individual chambers. Once released into the chamber, the time the fish required to deflate (sec) was recorded. Thirty minutes after the standardized stressor, all pufferfish were again collected to record their post-stress bite force (N), and sampled for 0.5 mL of blood while monitoring their post-stress puff score; this blood sample was considered the post-stress sample. Pufferfish were then returned to their individual chambers where the time to deflate (sec) was again recorded. Out of the 48 fish, 10 failed to yield one of the samples, resulting in a final sample size of 38 fish.

2.3.4 Sample analysis

Whole blood glucose concentrations were quantified on site using an Accu-Chek® Compact Plus glucose meter (Roche Diagnostics, Basel, Switzerland; see Cooke et al. 2008 for validation), and remaining whole blood was centrifuged at 2000 g for 5 min to separate erythrocytes from plasma (Capsule HF-120, Tomy Seiko Co., LTD, Tokyo, Japan). Plasma samples were stored at -20°C until cortisol immunoassay analysis. Plasma cortisol was quantified using colorimetric competitive enzyme-linked immunoassay (ELISA; Enzo Life Sciences Cortisol ELISA Kit ADI-900-071; Farmingdale, New York, USA), a technique previously validated for measuring cortisol concentrations in a variety of fish species (Sink et al. 2008). Samples were read by a SpectraMax Plus384 absorbance microplate reader as per manufacturer recommendations.
2.3.5 Statistical analyses

One-way analysis of variance (ANOVA) tests were performed to identify differences in cortisol concentration in control and post-stress fish 15 min, 30 min, 1 h, 2 h and 4 h post-stressor. Following a significant omnibus test, Tukey's HSD post-hoc tests were used to quantify differences across treatment groups. Paired t-tests were performed to quantify differences between baseline and post-stress values for cortisol, glucose, bite force, puff score and puff time to deflate once released. Pearson's correlations were used to determine fitness proxies repeatability between baseline and post-stress treatments, as well as to quantify the relationships among the fitness proxies (i.e., bite force, puff score, and time to deflate once released) within the baseline and post-stress treatments.

Fulton’s condition factor (K) was calculated for each pufferfish as an indicator of general well-being using the following equation:

\[ \text{K} = 100 \times (W/L^3); \]

where (W) is body mass (mg) and (L) is total length (mm) (Ricker 1975). Simple regression was then used to determine the predictive value of total length, mass and Fulton’s condition factor on baseline and post-stress physiological indices and fitness proxies. The total length and mass of the pufferfish both proved to be highly predictive of all physiological and behavioural measures, with the exception of puff time; therefore, only total length was used as a covariate in subsequent regression analyses. While controlling for the total length of the pufferfish, several multiple regression analyses were conducted to determine physiological predictors (i.e., cortisol and glucose concentrations) of fitness proxies (i.e., bite force, puff score and puff time) in both baseline and post-stress treatments. The effect of cortisol and glucose responsiveness (i.e., the difference
between baseline and post-stress samples) on these baseline and post-stress fitness proxies was also determined using similar regression analyses. Statistical analyses were conducted using IBM SPSS Statistics 20.0 (2011). Residuals were examined for normal distributions using the Shapiro-Wilk test. The Levene’s and Brown-Forsythe tests were used to assess homogeneity of variance within variables of normally and non-normally distributed data, respectively. Variables were log or square root transformed to meet assumptions of normality and homogeneity of variance. The level of significance for all statistical analyses (α) was assessed at 0.05. Means ± standard error of the mean (SEM) are reported.

2.4 Results

Following the standardized stress challenge checkered puffers displayed significant differences in cortisol concentration across the 4 h timeline (ANOVA: F = 7.580, P < 0.001), with a maximum glucocorticoid (GC) response of 145.9 ± 31.0 ng ml⁻¹ 30 min post-stressor, and a subsequent return to baseline levels by the 1 h time point (Fig. 2.1A). Furthermore, pufferfish exhibited significant differences in blood glucose concentration across the 4 h timeline (ANOVA: F = 13.078, P < 0.001), peaking at 6.3 ± 1.0 mmol L⁻¹ 30 min post-stressor, and a subsequent return to baseline levels by the 1 h time point (Fig. 2.1B). Therefore, I used a conservative 12 h acclimation period, and a 30 min time point to assess maximum post-stress physiological and behavioural measures for all subsequent aspects of the study. Following the standardized stressor (i.e., 5 min air-water interface challenge), plasma cortisol concentration increased 12-fold, and blood glucose concentrations...
increased 2-fold relative to pre-stress concentrations (Table 2.1). In contrast, fitness proxies (bite force and puff score) decreased following a standardized stressor relative to pre-stress performance (Table 2.1, Figs 2.2 A and B). While there was a similar trend for puff time to deflate once released, this trend was not statistically significant (Table 2.1). Baseline and post-stress puffing performances, including puff scores and puff times to deflate once released, were significantly correlated with one another (two-tailed Pearson correlations: $R = 0.389$, $P < 0.05$, and $R = 0.379$, $P < 0.05$, respectively; Table 2.1), indicating that these fitness proxies are repeatable through time. The correlation between baseline and post-stress bite force exhibited similar trends (two-tailed Pearson correlations: $R = 0.315$, $P = 0.054$; Table 2.1).

Prior to the standardized air exposure challenge, checkered puffers did not demonstrate any significant relationships among baseline values of different fitness proxies (bite force, puff score, and puff time to deflate once released; Table 2.2). Following a 5 min air exposure challenge, however, post-stress fitness proxies of checkered puffers showed significant correlations. Specifically, checkered puffers with elevated post-stress puff scores also showed stronger post-stress bite forces and longer times to deflate (Table 2.2, Figs 2.3 A and B).

The total length, body mass and condition of the checkered puffers did not significantly influence baseline physiological indices and baseline fitness proxies, with the exception of bite force (Table 2.3). Following a standardized 5 min stressor, however, longer and heavier fish did produce higher levels of cortisol and glucose, and exhibited significantly stronger bite forces and higher puff scores (Table 2.3). In addition, pufferfish
that had higher condition scores also demonstrated lower post-stress glucose levels (Table 2.3).

I found that larger checkered puffers with lower post-stress concentrations of cortisol and glucose exhibited significantly stronger post-stress bite forces ($R^2 = 0.165$, $F = 4.667$, $P < 0.05$; Figs 2.3 C and D). Also, I found that larger pufferfish with higher baseline cortisol concentrations and higher post-stress glucose concentrations exhibited greater puff score responsiveness (i.e. significantly lower post-stress puff scores compared to that exhibited in the baseline treatment; $t = -2.057$, $P < 0.05$ and $t = -2.396$, $P < 0.05$, respectively). Physiological metrics were not related to other aspects of fitness proxies (see Supplemental Materials). Consequences of the physiological responsiveness (i.e., the difference between stress-induced and resting cortisol and glucose levels) on checkered puffer performances before and after the standardized stressor were also examined. When accounting for the total length of the fish, physiological responsiveness had no significant influence on changes in fitness proxies (regressions: $P > 0.05$), and appeared to influence the bite force of stress-induced checkered puffers, although the relationship was not statistically significant ($R^2 = 0.338$, $F = 5.774$, $P = 0.089$).

Nevertheless, total length of checkered puffers was found to predict over 50% of the change in baseline bite forces ($R^2 = 0.509$, $F = 37.278$, $P < 0.001$; Fig. 2.3 A), over 20% of the change in post-stress bite forces ($R^2 = 0.236$, $F = 11.131$, $P < 0.01$; Fig. 2.3 A), and nearly 30% of the change in post-stress puff scores ($R^2 = 0.281$, $F = 14.047$, $P < 0.001$; Fig. 2.3 B).
2.5 Discussion

Variability in GC levels is well established and thought to mediate ecological and evolutionary trade-offs in individuals (see Ricklefs and Wikelski 2002, McConnachie 2010). In the current study, I noted considerable variation in GC concentrations (both baseline and post-stress), providing the foundation to ask questions related to the correlates and consequences of the diversity of GC responses. The relationship between acute GC stress response and fitness is highly context dependent (e.g., Overli et al. 2002, Blas et al. 2007, Wada and Breuner, 2008). Research on teleost fish lags behind that of other taxa, but may prove to be a valuable comparative group for the study of stress and fitness. Individual variation in the acute GC response in fish has been shown to be repeatable through time (Cook et al. 2011a), and to be correlated with individual size, behavioural traits, and reproductive success (Cook et al. 2011a, 2011b, O’Connor et al. 2012). In an attempt to build on this area of research, individual variation in the GC response of the checkered puffer (*Sphoeroides testudineus*), as well as its relationship with two fitness proxies (i.e., inflation and bite force) were investigated. I found that checkered puffers displayed a maximum GC response 30 min following a standardized stressor and a subsequent return to baseline levels by the 4 h time point. Following a standardized stressor, physiological stress indices, including plasma cortisol and blood glucose concentrations, increased, and fitness proxies (bite force and puff score) decreased. Fitness proxies were found to be repeatable through time, and highly correlated with one another following the standardized stressor. Furthermore, the total length and mass of the pufferfish significantly predicted bite force, and all physiological indices as well as fitness proxies post-stressor. While controlling for fish length, I found
that larger checkered puffers with higher post-stress cortisol concentrations and lower post-stress glucose concentrations exhibited significantly stronger post-stress bite forces. Physiological metrics were not related to other aspects of fitness proxies. When accounting for the total length of the fish, physiological responsiveness had no significant influence on changes in fitness proxies.

Baseline GC levels are examined as physiological indices of the relative condition of an individual and/or population, where low GC levels indicate relatively good condition and fitness, and high GC levels suggest poor condition and decreased fitness (Bonier et al. 2009). In the current study, baseline plasma cortisol and blood glucose levels of the checkered puffer were not predictive of individual fitness proxies before or following the standardized stressor, and it is possible that this reflects that all of the animals included in the current study were in relatively good condition.

Following a 5 min air exposure challenge, the physiological stress response of the checkered puffer was characterized by increased plasma cortisol and blood glucose concentrations; results comparable to studies with other teleost fish (Barton 2002). Checkered puffers with elevated plasma cortisol and glucose levels post-stress displayed significantly weaker bite forces before and after the stressor, as well as lower puff scores following the stressor. These findings establish an unexpected negative relationship between the GC response and fitness related endpoints, where fitness proxies significantly decrease following an acute stressor. Similar negative relationships between the GC response and fitness related endpoints have been documented in the European white stork nestlings (*Ciconia ciconia*; Blas et al. 2007) and the zebra finch (*Taeniopygia guttata*; Roberts et al. 2007). Post-stress fitness proxies were characterized by decreasing bite
forces and puff scores, as well as increasing puff times once released. Inflation (i.e., puffing) has interested biologists for some time (Thilo 1899, Parr 1927, Brainerd 1994). Puffing is a mechanical defense against piscine predators (Winterbottom 1974, Brainerd 1994), and is known to engage several muscles to achieve a full and effective puff (Wainwright et al. 1995). Similarly, bite force has been examined as a performance measure that mediates diet, growth and fitness (Vanhooydonck et al. 2005, Bulte et al. 2008, Mara et al. 2010), and requires a significant amount of energy (Huber et al. 2005). Therefore, it is possible that puff score and bite force decreased following the acute stressor due to reduced somatic energy reserves. Cortisol increased following the stressor, however the underlying mechanism was presumably physiological exhaustion. Although not measured here, tissue energy stores (e.g., adenosine triphosphate (ATP), phosphocreatine (PCr), glycogen) would have been depleted and metabolites such as lactate would have been generated. In the presence of an intense challenge such as the standardized stressor, it is known that the stress response required to regain homeostasis is energetically costly (see Schreck 2010 for overview). Somatic energy reserves are known to be consistently correlated with fish size (Brett 1995, Mackereth et al. 1999, Crossin et al. 2004), and to decline following acute and chronic stressors, resulting in fitness-related consequences. For example, glycogen stores were found to quickly mobilized in food-deprived rainbow trout (Oncorhynchus mykiss) to meet the initial energy demand imposed by an acute handling stressor (Vijayan and Moon 1992), leaving significantly reduced energy budgets for subsequent challenges. Furthermore, parental defense in smallmouth bass (Micropterus dolomieu) over the parental care period suffers due to declining somatic energy reserves, thereby decreasing their probability of survival.
over the following winter (Mackereth et al. 1999). Similarly, somatic energy reserves
greatly decrease in Atlantic salmon (Salmo salar) during upstream migration and
spawning (Johnsson et al. 1997). Although significant differences were not established
between baseline and post-stress puff time to deflate once released, there is a considerable
increase worth noting. Puff time data likely yielded insignificant results due to the
immense variability displayed in the post-stress treatment. Over the course of the
experiment, trends of shorter baseline puff times and longer post-stress puff times were
apparent. Puff time to deflate once released may be associated with reduced fitness as this
behaviour will inhibit the pufferfish from escaping the predator, in this case, the
experimenter.

Prior to the onset of a stressor, baseline fitness proxies were found to have no
correlation with one another. However, these fitness proxies were significantly correlated
with one another following exposure to an acute stressor. Baseline fitness proxies likely
had no relation to each other as a result of their basal GC levels and nondepleted energy
stores. To this end, no tradeoffs were necessary to increase the pufferfish’s chance of
survival in the baseline treatment (Breuner et al. 2008). Stress-induced puff scores were
both highly correlated with stress-induced bite force and puff time; however stress-
induced bite force and puff time were not correlated with one another. The significant link
among stress-induced fitness proxies highlights the common decrease in performance
once exposed to an acute stressor, and therefore an overall reduction in fitness.

Total length and mass of the checkered puffer were significantly predictive of
both baseline and stress-induced bite force, as well as stress-induced puff scores. Many
performance measures were exclusively influenced by the size of the checkered puffer.
Following the 5 minute air exposure challenge, checkered puffers with increasing physiological responsiveness (i.e., a large difference between pre- and post-stress cortisol and glucose levels) displayed significantly weaker baseline bite forces, as well as weaker bite forces, lower puff scores, and shorter puff times once released following the standardized stressor. However, when accounting for the total length of the fish, the physiological responsiveness of the checkered puffer has no significant influence on changes in performance before nor following the standardized challenge. The size of the checkered puffer predicted up to 50% of the change in performance observed. Bite force is often strongly associated with the size of an animal as larger individuals generally have larger jaw structures and thus stronger bite forces (e.g., Wainwright et al 2004, Grubich et al. 2008). Similarly, larger pufferfish seemed to hold stronger ‘puffs’, although it is currently unclear why that was the case. Fulton’s condition factor (K) is considered as a long-term indicator of an individual’s general well-being (Suthers, 2000), and therefore was only measured once for each checkered puffer. As a result, the change in condition of the individual pufferfish between baseline and stress-induced treatment groups could not be monitored. However, the predictive value of the pufferfish’s general condition (i.e., Fulton’s condition factor) to successfully respond to an acute stressor could be examined. Fulton’s condition factor was only found to be significantly predictive of stress-induced glucose and glucose responsiveness levels. In response to an acute stressor, fish with greater energy reserves (inferred from Fulton’s condition factor) released less glucose when compared to fish in lower condition.

To effectively quantify the relationship between individual variation in GC stress responsiveness and fitness proxies, it is essential to establish appropriate controls through
several stress response parameters (see Adams 1990), as well as determine suitable fitness proxies that accurately predict successful fitness proxies. I found the GC response to an acute, standardized stressor in the checkered puffer to be well within the range found in other studies for other teleost fish species (Pickering et al. 1982, Pickering and Pottinger 1989, Barton 2002, Shultz et al. 2011). Similar studies have also shown that plasma cortisol concentrations return to resting levels within 4 hours post-stressor (Pickering et al. 1982, Barton 2002).

Inflation and bite force are logical fitness metrics for the checkered puffer as inflation serves as an effective predator avoidance behaviour by increasing body size and making them difficult to consume (Randall 1967, Recher and Recher 1968, Myer 1989), and bite force is an important component of feeding performance (Wainwright 1988, Hernandez and Motta 1997, Grubich 2005, Berumen and Pratchett 2008) and expanding dietary range (see Mara et al. 2010 for overview). As the link between the GC response and such fitness proxies is tenuous, studying quantifiable metrics of fitness relative to baseline and post-stress GC levels is necessary.

2.5.1 Conclusion

GCs are often measured in individuals to monitor the relative condition of species and populations of conservation concern (Walker et al. 2005, Breuner et al. 2008). However, few studies examine individual fitness proxies or intermediate performance consequences of individual variation in GC concentrations. In the current study, I found that exposure to an acute stressor reduced subsequent fitness proxies in the checkered puffer, likely due to reduced somatic energy reserves. Furthermore, post-stress circulating
cortisol values were negatively correlated with post-stress fitness proxies. This study highlights the importance of discovering the link between GCs and fitness in fish, and establishes the checkered puffer as a valuable model for future research on the ecology of stress in wild vertebrates.

2.6 Tables

Table 2.1 Means (± SE), paired sample t-test and Pearson correlation results for baseline and post-stress physiological parameters (cortisol and glucose) and fitness proxies (bite force, puff score and puff time) in the checkered puffer (*Sphoeroides testudineus*). Baseline values were collected from acclimated pufferfish within 3 min. Fish were then immediately ‘stressed’ by holding them at the air-water interface for 5 min in a rubber-mesh dip net. Pufferfish were then resampled 30 min post-stressor within 3 min to collect post-stress values. A total of 38 fish were sampled.

<table>
<thead>
<tr>
<th></th>
<th>Mean±SE Baseline</th>
<th>Post-stress</th>
<th>df</th>
<th>Paired t-test</th>
<th>Two-tailed Pearson correlation</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>t-statistic</td>
<td>R</td>
</tr>
<tr>
<td><strong>Cortisol (ng ml⁻¹)</strong></td>
<td>16.3±4.6</td>
<td>198.4±23.7</td>
<td>37</td>
<td>12.507</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-0.210</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>0.109</td>
</tr>
<tr>
<td><strong>Glucose (mmol L⁻¹)</strong></td>
<td>1.1±0.0</td>
<td>3.2±0.1</td>
<td>37</td>
<td>17.372</td>
<td>&lt;0.001</td>
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<td></td>
<td></td>
<td>0.258</td>
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<td></td>
<td></td>
<td>0.062</td>
</tr>
<tr>
<td><strong>Bite force (N)</strong></td>
<td>78.5±3.5</td>
<td>53.4±4.5</td>
<td>37</td>
<td>5.258</td>
<td>&lt;0.001</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td>0.315</td>
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<td></td>
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<td></td>
<td>0.054</td>
</tr>
<tr>
<td><strong>Puff score</strong></td>
<td>2.1±0.1</td>
<td>1.7±0.1</td>
<td>37</td>
<td>3.132</td>
<td>0.003</td>
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<td>0.389</td>
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<td></td>
<td></td>
<td>0.016</td>
</tr>
<tr>
<td><strong>Puff time to deflate once released (min)</strong></td>
<td>79±15</td>
<td>321±184</td>
<td>37</td>
<td>1.324</td>
<td>0.194</td>
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</tbody>
</table>

34
Table 2.2 Pearson correlation results for fitness proxies (bite force, puff score and puff time) of the checkered puffer (*Sphoeroides testudineus*) in the baseline and post-stress treatments. Baseline values were collected from acclimated pufferfish within 3 min. Fish were then immediately ‘stressed’ by holding them at the air-water interface for 5 min in a rubber-mesh dip net. Pufferfish were then resampled 30 min post-stressor within 3 min to collect post-stress values. A total of 38 fish were sampled.

<table>
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<tbody>
<tr>
<td></td>
<td>Bite force (N)</td>
<td>Puff score</td>
<td>Puff time to deflate once released (min)</td>
</tr>
<tr>
<td><strong>Baseline treatment</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Bite force (N)</td>
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<td>0.053</td>
<td>-0.094</td>
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<td>Puff score</td>
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<td>0.201</td>
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<td>Puff time to deflate once released (min)</td>
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<tr>
<td><strong>Post-stress treatment</strong></td>
<td></td>
<td>0.573***</td>
<td>0.062</td>
</tr>
<tr>
<td>Bite force (N)</td>
<td></td>
<td>0.573***</td>
<td>0.062</td>
</tr>
<tr>
<td>Puff score</td>
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<td></td>
<td>0.383*</td>
</tr>
<tr>
<td>Puff time to deflate once released (min)</td>
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<td>0.383*</td>
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</tr>
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</table>

* *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001
Table 2.3 The effect of body measures, including total length, mass and condition (Fulton’s condition factor calculated from total length and mass measurements), on baseline and post-stress physiological indices and fitness proxies. Simple regression results are presented. Baseline values were collected from acclimated pufferfish within 3 min. Fish were then immediately ‘stressed’ by holding them at the air-water interface for 5 min in a rubber-mesh dip net. Pufferfish were then resampled 30 min post-stressor within 3 min to collect post-stress values. A total of 38 fish were sampled.

<table>
<thead>
<tr>
<th>Total length (mm)</th>
<th>Mass (g)</th>
<th>Fulton's condition factor (mg mm⁻³)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>R²</td>
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<tr>
<td><strong>Baseline treatment</strong></td>
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</tr>
<tr>
<td>Cortisol (ng ml⁻¹)</td>
<td>0.876</td>
<td>0.024</td>
</tr>
<tr>
<td>Glucose (mmol L⁻¹)</td>
<td>0.658</td>
<td>0.018</td>
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<tr>
<td>Bite force (N)</td>
<td>37.278</td>
<td>0.509***</td>
</tr>
<tr>
<td>Puff score</td>
<td>2.426</td>
<td>0.063</td>
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<tr>
<td>Puff time to deflate once released (min)</td>
<td>0.181</td>
<td>0.005</td>
</tr>
<tr>
<td><strong>Post-stress treatment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortisol (ng ml⁻¹)</td>
<td>4.439</td>
<td>0.110*</td>
</tr>
<tr>
<td>Glucose (mmol L⁻¹)</td>
<td>6.214</td>
<td>0.147*</td>
</tr>
<tr>
<td>Bite force (N)</td>
<td>11.131</td>
<td>0.236**</td>
</tr>
<tr>
<td>Puff score</td>
<td>14.047</td>
<td>0.281**</td>
</tr>
<tr>
<td>Puff time to deflate once released (min)</td>
<td>0.263</td>
<td>0.007</td>
</tr>
</tbody>
</table>

* P < 0.05; ** P < 0.01; *** P < 0.001
Figures

Figure 2.1 Plasma cortisol (A) and blood glucose (B) secretion and recovery in the checkered puffer (*Sphoeroides testudineus*) following a 5 min standardized stressor. Error bars represent standard error from the mean, and different letters indicate statistically significant differences among sampling time points (Tukey's HSD post-hoc test following a significant ANOVA; \( \alpha = 0.05 \))
Figure 2.2 Baseline and post-stress bite force (A) and puff score (B) of the checkered puffer (*Sphoeroides testudineus*). Baseline values were collected from acclimated pufferfish within 3 min. Fish were then immediately ‘stressed’ by holding them at the air-water interface for 5 min in a rubber-mesh dip net. Pufferfish were then resampled 30 min post-stressor to collect post-stress values. A total of 38 fish were sampled. Boxes represent 25th and 75th percentiles with median enclosed within, and whiskers represent 10th and 90th percentiles.
Figure 0.1 Correlations among baseline and post-stress fitness proxies of the checkered puffer (*Sphoeroides testudineus*). Significant two-tailed correlations were found between post-stress puff score and bite force ($R = 0.573; P < 0.001; A$), as well as between post-stress puff score and puff time to deflate once release ($R = 0.325; P < 0.05; B$). Also, the effect of post-stress physiological indices, including cortisol (C) and glucose (D) concentrations, on bite force following the standardized stressor.
Chapter 3. Consequences of experimental cortisol manipulations on the thermal biology of the checkered puffer (*Sphoeroides testudineus*) in field and laboratory environments

3.1 Abstract

Given that anthropogenic environmental change will not occur in isolation of other stressors, it is necessary to explore the potential consequences of stress on the thermal-related characteristics (i.e., physiological and behavioural stress indices) of tropical marine fish. In this study, we used exogenous cortisol manipulations to investigate the effects of a thermal challenge on checkered puffers (*Sphoeroides testudineus*) as a secondary stressor. Two days post-treatment, the implanted pufferfish exhibited reduced swimming performance, however did not show any energetic costs in terms of changes in blood glucose concentrations and standard metabolic rate. In the lab, we tested the thermal tolerance of wild pufferfish by subjecting control and cortisol dosed individuals to abrupt 5 °C changes in temperature (i.e., cold and heat shock treatments). Following cold shock, control fish exhibited post-stress cortisol levels and weak ‘puff’ performances. Whereas, fish dosed with cortisol exhibited post-stress cortisol levels at ambient temperature and contrary to our predictions, attenuated cortisol levels when subjected to the secondary thermal challenge. The 20-day complementary field study conducted in their natural habitat – a tidal creek in Eleuthera, The Bahamas – revealed that cortisol implanted fish generally selected cooler temperatures in their natural habitat when compared to controls. Although the physiological and behavioural consequences documented in the laboratory were not comparable to the ecological trends observed in
the field, these results highlight the need to establish the link between laboratory and field
data to successfully develop management policies and conservation initiatives with
regards to anthropogenic climate change. This study is the first to use experimental
cortisol manipulation to investigate the effects of stress on the thermal biology in a wild
fish population in a controlled laboratory setting, as well as on free-swimming fish in
their natural habitat.

3.2 Introduction

Coastal marine ecosystems represent the transition zone between land and water, with
their biodiversity shaped by dynamic physical and chemical processes (Harley et al. 2006,
Burkett et al. 2008). Coastal biodiversity is specifically adapted to the extreme
environmental conditions imposed along the gradients of these coastal boundaries, and
the distribution of organisms within coastal ecosystems is governed by tolerances to
varying environmental conditions, including changes in water parameters (such as
temperature, salinity and pH), light availability, storm disturbance, tides, water depth, and
nutrient availability (Burkett 2008). However, coastal ecosystems are currently threatened
by a suite of anthropogenic environmental changes including coastal development,
contamination, and changes in environmental parameters (IPCC 2001). Despite the
tolerance of coastal biota to naturally variable environments, coastal biodiversity is
vulnerable to these anthropogenic impacts. In particular, rising global temperatures are
provoking complex, non-linear responses among many biota in coastal ecosystems (Lee
et al. 2001, Harley et al. 2006, Burkett et al. 2008). Temperature influences the growth,
survival, reproduction and distribution of organisms (Brander et al. 2003, Reid, 2003).
Eurythermal and heat-tolerant species may be more vulnerable to increasing temperatures as compared to more temperate species, because these species typically live closer to their thermal limits (Tomanek and Somero 1999, Stillman 2002, Harley et al. 2006). Ectothermic animals, whose basic physiological processes are influenced by external temperatures, are of particular interest (Hochachka and Somero 2002). While it is unclear how tropical marine fishes will respond to increases in temperature (Cambers et al. 2007), species distributions are expected to expand toward cooler environments (Parmesan and Yohe 2003, IPCC 2007a).

Given that climate change will not occur in isolation of other stressors (e.g., habitat alteration, contamination), it is necessary to determine the effects of multiple environmental challenges on the temperature tolerances of coastal fishes. This information is necessary to predict thresholds for survival of individuals under changing climate regimes, and predict the associated ecological consequences on coastal ecosystems. To date, most research on thermal stress among fish has been restricted to laboratory studies, and most have been conducted in vitro (e.g., Ackerman et al. 2000, Vijayan et al. 2000, Basu et al. 2001). However, it is valuable to study the effects of multiple stressors on animals in their natural habitat. Field-based studies have the potential to provide a more comprehensive understanding of the impacts of multiple stressors within the natural ecosystem, and thus may provide better predictions of the consequences of climate change. Tools now exist for monitoring temperature selection in field settings. It is possible to tag individual fish with thermal logging devices to quantify thermal preferences. It is also possible to experimentally manipulate baseline physiological stress levels through the use of exogenous glucocorticoid hormone
implants. Glucocorticoids are the primary stress hormones in vertebrates. During a challenge, these hormones are released, and orchestrate a suite of physiological processes that promote survival and recovery of the individual through the challenge (Sapolsky et al. 2000). Glucocorticoid implants mimic a natural stress response in that they elevate circulating glucocorticoid concentrations to levels seen during a typical endogenous stress response, and initiate the same suite of downstream physiological processes. In fish, the primary glucocorticoid is cortisol (Mommsen et al. 1999, Barton 2002), and the use of cortisol implants has been established as a method of elevating circulating cortisol levels in fish for approximately 3-7 days in both laboratory (Gamperl et al. 1994) and field (e.g., O’Connor et al. 2009 and 2013, Dey et al. 2010) settings. In a laboratory environment, cortisol-implanted fish are more susceptible to thermal stress than un-manipulated controls (Basu et al. 2001, McConnachie et al. 2012). However, no studies have investigated the effects of thermal stress in conjunction with other stressors in wild free-swimming fish.

In the current study, circulating cortisol was experimentally manipulated within physiologically relevant limits to quantify the effects of multiple and chronic stressors on thermal-related characteristics in the checkered puffer (*Sphoeroides testudineus*). The checkered puffer has an expansive distribution throughout the Gulf of Mexico and Caribbean sea, and ranges along the Atlantic coastline as far north as Rhode Island, and as far south as the south eastern coasts of Brazil (Shipp 1974, Targett, 1978, Pauly 1991). This distribution is credited to the pufferfish’s broad physiological tolerance. Here, we focussed on characterising the thermal tolerances and preferences in control and cortisol-implanted checkered puffers in both the laboratory and in the field. In the laboratory, we
compared indicators of energy use (blood glucose concentrations, oxygen use and swimming performance) and stress indicators (blood plasma cortisol concentrations, behavioural changes) in response to thermal challenges between control and cortisol-implanted fish. We predicted that experimental cortisol implants would reduce the thermal tolerances of checkered puffers in a laboratory setting. In the field, we used small thermal loggers affixed to fish over a 20-day period to compare thermal habitat use between treatment groups. To that end, we tested the null hypothesis that the checkered puffer’s thermal sensitivity and habitat use was independent of whether cortisol levels had been artificially elevated. To test our hypothesis, we completed a series of complementary experiments validating the cortisol implant, including the required dosage, the depletion timeline, and the energetic cost in terms of blood glucose concentrations, swimming performance and standard metabolic rate measurements using intermittent-flow respirometry. Once the caveats of the cortisol implant were established, experimental cortisol manipulations were used to focus on the thermal-related consequences in the laboratory as well as in the natural habitat of the pufferfish. Collectively, these experiments assist in our understanding of the physiological, behavioural and ecological consequences of climate-induced stress in a wild tropical fish.

3.3 Methods

3.3.1 Study site and study animals

For all experiments, checkered puffers (n=143) were collected from Page, Plum and Kemps Creeks on the island of Eleuthera, Bahamas (Page: N24°49'04.7" W076°18'51.6";
Pufferfish were corralled into seine nets set at the mouths of the creeks on an outgoing tide, and transported to the Cape Eleuthera Institute (CEI: N24°50'06.70" W76°19'31.69) in aerated coolers. At CEI, pufferfish were held in 1250 L aerated flow-through tanks, and were allowed to acclimate to laboratory conditions. Temperatures in tanks reflected ambient coastal conditions. During acclimation, pufferfish were fed an assortment of dead sardines (*Sardinella aurita*), juvenile bonefish (*Albula vulpes*) and mottled mojarra (*Eucinostomus lefroyi*) every 2 days. Fish were starved 48 hrs before experimentation. Given that fish could be easily handled and most sampling occurred with the fish submerged in a water-filled trough, all techniques were performed without anesthesia (see Cooke et al. 2005). All samples were collected in accordance with the guidelines of the Canadian Council on Animal Care as administered by Carleton University (B12-01), and all fish were released back into the ocean alive upon recovery at the conclusion of the experiment.

### 3.3.2 Validation study of cortisol implant dose

In February 2012 (water temperature: 23.2 ± 2.0°C), checkered puffers were randomly assigned to one of four treatment groups to validate the dose of the cortisol implant: 1) control (n=6); 2) sham (n=6); 3) low-dose cortisol treatment (n=7); and 4) high-dose cortisol treatment (n=7). Treatment fish were air-exposed for administration of an intramuscular injection of heated cocoa butter containing cortisol (hydrocortisone 21-hemisuccinate; Sigma H2882, Sigma-Aldrich, St. Louis, MO). Intra-peritoneal injection of cortisol dissolved in cocoa butter has been the vehicle method of choice for several
field-based studies using cortisol manipulations in fish (e.g., Dey et al. 2010, McConnachie et al. 2012a and 2012b, O’Connor et al. 2012). Preliminary tests in pufferfish, however, indicated that intra-peritoneal injection would not work for this species due to its ability to inflate and deflate its intra-peritoneal cavity. The pufferfish generally inflate upon injection, and eject the cortisol through the mouth and gills upon deflation. Based on these observations, an intra-muscular injection of the dorsal muscle anterior to the dorsal fin was used to experimentally administer exogenous cortisol to the checkered puffer. All treatment fish were weighed using a portable electronic balance, and then placed in water-filled foam-lined trough to obtain a total length (TL) measurement, and for injection with 5 mL of cocoa butter per kg of fish body weight. Sham treatment fish were injected with heated pure cocoa butter only. Low-dose cortisol treatment fish were injected with 5 mg mL\(^{-1}\) cortisol in heated cocoa butter (i.e., 25 mg kg\(^{-1}\) fish body weight), while high-dose cortisol treatment fish were injected with 10 mg mL\(^{-1}\) cortisol in heated cocoa butter (i.e., 50 mg kg\(^{-1}\) fish body weight). Low and high cortisol dose concentrations were chosen based on the work done on smallmouth bass (*Micropterus dolomieu*; Dey et al. 2010), largemouth bass (*M. salmoides*; O’Connor et al. 2012) and bluegill sunfish (*Lepomis macrochirus*; McConnachie et al. 2010). Control fish received no injection, but were handled in an identical manner to the treatment fish. Using a rubber-mesh dip net, all fish were placed in individual opaque experimental chambers (12.5 L) supplied with aeration and a constant flow of saltwater within 10 s of treatment. After 48 hrs, to determine circulating cortisol levels, fish were non-lethally sampled for 0.5 mL of blood by caudal venipuncture using a heparinized 1-mL syringe and 21-gauge, 2.5-cm needle. To avoid sampling-induced stress, each blood sample was
withdrawn in under 3 min (Romero and Reed 2005). Data were compared to previous
studies of checkered puffers where cortisol was measured in the blood following a 5 min
air exposure challenge (Cull et al. submitted).

3.3.3 Validation of cortisol implant depletion timeline
From April to June 2012 (water temperature: 29.1±1.5°C), checkered puffers were
randomly assigned one of the following treatment groups to verify the time course of
cortisol elevation over a 20 day holding period: 1) control (i.e., sampling of resting fish
on day 0; n=7); 2) sampling at 2 days (n=7); 3) sampling at 5 days (n=7); 4) sampling at
10 days (n=5); and 5) sampling at 20 days (n=6) post-implantation. Fish from all
treatment groups were measured as described above, and briefly air-exposed (30 s) while
a 5 mL kg\(^{-1}\) intramuscular injection of 10 mg mL\(^{-1}\) cortisol in cocoa butter was
administered (dosage selected based on above validation). Control fish were handled in an
identical manner, but received no injections. Treatment fish were then placed in a
common holding tank where they were cared for as described above. To minimize
disturbances prior to blood sampling, fish were placed in 12.5 L individual opaque
experimental chambers 12 hrs prior to sampling. Previous work on checkered puffer
revealed that fish recover from handling stressors within 3 hrs (Cull et al., submitted). The
method of holding fish in communal tanks before introducing them temporarily to
individual holding chambers has been successfully used with bluegill (\textit{Lepomis
macrochirus}; McConnachie et al. 2012b), bonefish (\textit{Albula spp.}; Shultz et al. 2011) and
two species of cardinalfish (\textit{Ostorhinchus doederleini} and \textit{O. cyanosoma}; Munday et al.
2009). All pufferfish were sampled for blood as described above.
3.3.4 Metabolic cost of cortisol implant

The metabolic burden imposed by the cortisol-cocoa butter implant (10 mg mL\(^{-1}\)) on standard metabolic rate (SMR) was determined using intermittent-flow respirometry on checkered puffers in June 2012 (water temperature: 28.6±1.8ºC). The SMR of cortisol-dosed individuals (n=8) was compared with that of control individuals (n=8). Cortisol treated fish were dosed (10 mg mL\(^{-1}\); methods described above) 48 hrs prior to SMR measurement. The respirometry system, operating procedures and calculations were identical to those previously described by Shultz et al. (2011), with the exception of the duration of individual cycles that consisted of an 18 min flush, 1 min wait and 20 min measurement cycle. Oxygen consumption rate (MO\(_2\), mg O\(_2\) kg\(^{-1}\) h\(^{-1}\)) for each fish was calculated using the average of the six lowest values recorded overnight (i.e., between 20:00 and 06:00; Schurmann and Steffensen 1997), and when the coefficient of determination (R\(^2\)) for slope measurements was >0.95 during each measurement cycle. All calculated dissolved oxygen values were corrected for background oxygen consumptions generated for each specific fish and chamber prior to commencing experiments.

3.3.5 Swimming performance

Following 24 hrs of intermittent-flow respirometry, cortisol burden was further assessed by quantifying swimming ability using a chase to exhaustion protocol on the same group of pufferfish. Individually, fish were dip netted from their respirometry chamber and quickly placed into a shallow circular tank (1.22 m diameter filled with 15 cm of water). A chase test was performed, and the time to exhaustion (i.e., the time at which three
consecutive tail grabs could be performed without a reflex response; Kieffer 2000) was recorded for each pufferfish. This protocol provided a comparative swimming performance measure between control and cortisol-treated pufferfish and has previously been validated in a variety of fish species (Heath et al. 1993, Portz 2007, Thiem et al. 2013).

3.3.6 Lab experiment: Thermal tolerance

From April to June 2012 (water temperature: 29.1±1.5°C), checkered puffers were randomly assigned to the following treatment groups: control at 1) ambient temperature (n=8); 2) -5°C from ambient temperature (n=6); and 3) +5°C from ambient temperature (n=8); 4) cortisol treatment at ambient temperature (n=8); 5) cortisol treatment at -5°C from ambient temperature (n=6); and 6) cortisol treatment at +5°C from ambient temperature (n=8). Cortisol-treated fish were measured and briefly air-exposed while a 5 mL kg⁻¹ intramuscular injection of 10 mg mL⁻¹ cortisol in cocoa butter was administered. Fish were then returned to communal tanks for 36-60 hrs. Control fish were handled identically, but received no treatment. All fish were captured from communal tanks and placed in individual opaque experimental chambers (12.5 L) supplied with ample aeration and a constant flow of saltwater. The experimental chambers were cooled by pumping water through a copper coil submerged in ice water, and warmed by heaters. These methods provided the appropriate temperature accurate to ± 1°C of the target thermal treatment. After 4 hrs (chosen because previous work on checkered puffers revealed that fish recover from handling stressors within 3 hrs; Cull et al. submitted), fish were sampled for blood as described above. For temperatures 5 °C below and above ambient, a
puff score was recorded during the 3 min sampling period by noting the time and intensity of the ‘puff’ (i.e., body inflation). More specifically, puffs were assigned a score from 0 to 3, with 0 being no puff, 1 being equal to or less than half a full puff, 2 being greater than half a full puff, and 3 being a full puff. A full puff was assigned once the fish was maximally inflated (i.e., its skin was tight to the touch and subsequent inflation attempts resulted in no further expansion). Each puff score (0-3) was assigned a percentage of time used over the 3 min, and then weighted according to its score. As a result, each puff score is presented as a value between 0 and 3 (i.e., 0 being no puff at all and 3 being a consistent full puff over the course of the 3 min sampling period). The fish were then released back into their respective chambers, and the time to fully deflate was recorded.

3.3.7 Field experiment: Thermal preference

From December 31, 2012, to January 19, 2013, thermal preferences of checkered puffers in their natural habitat were monitored in Page Creek. Page Creek is a shallow tidal water channel with a single opening to the ocean. This creek system consists of an expansive mangrove habitat undergoing two tidal cycles per day. The creek almost drains entirely at low tide, causing large variability in water parameters. To assess the thermal characteristics of the tidal creek, thermal loggers (iButton, Maxim Integrated Products, Inc., Sunnyvale, CA; n=10) were covered in a synthetic rubber coating (Plasti Dip International, Performix Brand products, Blaine, MN) and placed throughout Page creek, covering a range of habitat types. Five of the iButtons (model no. DS1921H) had a range of 15 to 46 °C, while the others (model no. DS1921Z) had a range of -5 to 26 °C. Factory-stated resolution of all thermal loggers is 0.125 ± 1 °C; previous calibration by our team
reveals actual mean accuracy of 0.4 ± 0.3 °C and mean precision of 0.2±0.0 °C (Donaldson et al. 2009).

On December 31, 2012, pufferfish were tagged with either unmodified iButtons (model nos. DS1921H and DS1921Z; n=18) and iButtons that were miniaturized according to Lovegrove (2009; model no. DS1921H; n=19). All iButtons were covered in Plasti Dip, and fastened to a backing plate. All iButtons were set to log temperature every 30 min over a 20-day period. Fish were randomly assigned to one of two treatment groups: 1) control (n=19); and 2) cortisol treatment (n=18). Thermal loggers were randomly distributed between groups and were externally attached to the dorsal surface of the fish, immediately posterior to the dorsal fin on the caudal peduncle (Thiem et al. 2013). Following iodine disinfection, two hypodermic stainless steel needles (16 gauge) were pushed through the dermis and 9 kg monofilament line (previously inserted through the tag via pre-made holes) was passed through the lumen of the needles and secured using multiple knots (see Thiem et al. 2013 for tagging validation of checkered puffer). Cortisol-treated fish were then weighed, measured, and given a 5 mL kg⁻¹ intramuscular injection of 10 mg mL⁻¹ cortisol in cocoa butter as described above. Control fish were handled identically, but were not given injections. All fish were released in Page Creek (N24°49'1.90" W76°18'48.80") upon recovery. After fish were at liberty for a 20-day period, control (n=10) and cortisol-treated pufferfish (n=13) were recovered from Page Creek on January 19, 2013. The recapture rate for control and cortisol-treated fish was 58% and 72%, respectively. Despite promising trials on the benchtop, modified iButtons failed to log temperature 70% of the time when deployed in the field. Therefore, of the fish that were recaptured, useable data covering the entire 20-day period was only
obtained for 7 control (n=3 DS1921H iButtons; n=4 DS1921Z iButtons; TL=186±3mm; mass=128±8g) and 8 cortisol-treated (n=3 DS1921H iButtons; n=5 DS1921Z iButtons; TL=188±8mm; mass=131±19g) fish. Upon capture, fish were re-measured and re-weighed using the methods described above, so that changes in condition over the course of the study could be calculated.

3.3.8 Sample analyses

Whole blood glucose concentrations were quantified on site using an Accu-Chek® Compact Plus glucose meter (Roche Diagnostics, Basel, Switzerland; see Cooke et al. 2008 for validation). Whole blood hematocrit (% packed cell volume, PCV) was also determined on-site (LW Scientific Zipocrit, model # ZO-1, 10,000 r min⁻¹; Lawrenceville, GA). The remaining blood was centrifuged at 2000 g for 5 min to separate erythrocytes from plasma (Capsule HF-120, Tomy Seiko Co., LTD, Tokyo, Japan). Plasma samples were stored at -20ºC until cortisol immunoassay analysis. Plasma cortisol was quantified using colorimetric competitive enzyme-linked immunoassay (ELISA; Enzo Life Sciences Cortisol ELISA Kit ADI-900-071; Farmingdale, NY) using a technique previously validated for measuring cortisol concentrations in largemouth bass (Sink et al. 2008). Samples were read by a SpectraMax Plus384 absorbance microplate reader (Molecular Devices, LLC; Sunnyvale, CA) following ELISA manufacturer recommendations.

3.3.9 Data handling and statistical analysis

All statistical analyses were conducted using IBM SPSS Statistics 20.0 (2011). For all tests, residuals were examined for normal distributions using the Shapiro-Wilk test, and
Levene’s and Brown-Forsythe tests were used to assess homogeneity of variance for variables with normally and non-normally distributed data, respectively. Variables were transformed (log or square root transformed) to meet assumptions of normality and homogeneity of variance. The level of significance for all statistical analyses was assessed at $\alpha=0.05$. All values are reported as mean ± standard error of the mean (SEM).

For all experiments, difference in the size of pufferfish used in control and cortisol treatment groups was assessed using one-way analysis of variance (ANOVA) tests. In cases where differences were found among treatment groups, the test was followed by a Tukey’s post-hoc test of honestly significant differences (Tukey’s HSD test) to determine which treatments differed.

**Validation of cortisol implant dose.** To validate the cortisol implant dose in the checkered puffer, a one-way ANOVA followed by a Tukey’s HSD test was performed to quantify differences in plasma cortisol and blood glucose concentrations in fish treated with high (10 mg mL$^{-1}$) and low (5 mg mL$^{-1}$) implant doses, sham treated fish and controls. Data were compared to previous studies of the checkered puffer where endogenous circulating cortisol was measured in the blood following a 5 min air exposure challenge (Cull et al. submitted). The high dose (10 mg mL$^{-1}$) resulted in circulating plasma cortisol concentrations similar to those seen during a natural stressor, and was therefore used for all subsequent experiments.

**Validation of cortisol implant depletion timeline.** To assess the depletion timeline of a cortisol implant over a 20-day time course, an ANOVA followed by a Tukey’s HSD test
was used to define for differences in plasma cortisol and blood glucose concentrations in control fish as well as fish at 2-, 5-, 10- and 20-days post-implantation. Differences in hematocrit at 5-, 10- and 20-days post-implantation were also identified using an ANOVA followed by a Tukey’s HSD post-hoc test where appropriate.

Metabolic cost of cortisol implant. To determine the metabolic cost of the high cortisol implant dose, independent sample t-tests were used to compare SMR (MO₂) between control and cortisol implanted pufferfish.

Swimming performance. An independent sample t-test was used to compare the time until exhaustion of control and cortisol implanted fish in the chase experiments.

Lab experiment: Thermal tolerance. To quantify the interactive effect of cortisol manipulation and thermal stress on the physiological and behavioural responses of the checkered puffer in the laboratory, two-way ANOVAs were used to identify the effect of multiple stressors (i.e., cortisol implant, and thermal stress) on physiological stress indices and ‘puffing’ performance. Independent variables included in the model were endocrine stress treatment (i.e., control vs. cortisol implant), and thermal treatment (i.e., ambient temperature, 5 °C below, and 5 °C above ambient temperature). The interaction between these two variables (stress treatment × thermal treatment) was included in the model. Dependent variables were circulating cortisol concentration, circulating glucose concentration, hematocrit, puff score, and puff time to deflate once released.
Field experiment: Thermal preference. To quantify the impact of cortisol manipulations on the thermal preferences of checkered puffers in a field setting, thermal data from both fish and habitat iButtons were recovered using the Java application, One Wire Viewer (Maxim Integrated, San Jose, CA). A variety of thermal parameters were then compared among the iButtons collected from the habitat, control, and cortisol-treated fish.

First, as the iButton model no. DS1921Z has a maximum temperature reading of 26°C, all temperature recorded values equal to or above 26°C were identified and marked as 26°C. The proportion of temperature values equal to or above 26°C was then calculated for each fish and habitat iButton over the 20-day sampling period. Proportions were logit-transformed (Warton and Hui 2011), and a one-way ANOVA was used to compare the ratio of time spent at or above 26°C among habitat, control and cortisol-dosed fish.

The daily accumulated thermal units (ATUs) were calculated for each fish and habitat iButton for each day by summing every temperature value. The number of recordings per iButton was consistent across all fish and habitat iButtons. All temperature values equal to or above 26°C were considered 26°C. For only the iButton model no. DS1921H, the average daily maximum was calculated for all groups. For all iButtons, the average daily minimum was calculated. For only the iButton model no. DS1921H, the average daily range was calculated for all groups by determining the difference between average daily maximum and minimum values. One-way ANOVAs were used to compare the ATUs, maximum, minimum and range temperatures among habitat, control and cortisol-treated fish. To control for daily fluctuations in temperature, repeated measure ANOVAs were also used to compare the ATUs, maximum, minimum and range temperatures among habitat, control and cortisol-treated fish.
Field experiment: Change in body condition. Fulton’s condition factor (K) was calculated twice for each pufferfish (once before tagging and deploying fish; and once retrieved 20-1187 days following deployment) as an indicator of general well being using the following 1188 equation:

\[ K = 100 \times \left( \frac{W}{L^3} \right); \]

where (W) is body mass (in mg) and (L) is total length (in mm; Ricker 1975). A two-way repeated measures ANOVA test was then used to identify possible differences between the initial and final (i.e., following the 20 day period) conditions of control and cortisol-implanted checkered puffers.

3.4 Results

3.4.1 Size differences among treatment groups

Although distinct individuals of checkered puffers were randomly assigned to each treatment group (i.e. No pufferfish were repeatedly sampled), significant differences in size among groups were identified (one-way ANOVAs: Ps < 0.05; Table 1). In the cortisol implant depletion timeline validation experiment, pufferfish in the 20 days post-implantation treatment averaged 38 mm longer and 75 g heavier than fish included in the 2 and 5 days post-implantation treatments, as well as 30 mm longer than fish in the control treatment (Tukey test: Ps < 0.05). In the metabolic cost and swimming performance experiments, cortisol-treated pufferfish were averaged 19 g heavier than controls (one-way ANOVA: F = 10.605; P < 0.01). In the thermal tolerance laboratory experiment, control pufferfish treatments were averaged 20 g heavier than cortisol-dosed
pufferfish treatments. Notably, cortisol-treated fish subjected to 5°C changes in temperature were averaged 30 g lighter than other treatments (Tukey test: Ps < 0.05).

3.4.2 Validation study of cortisol implant dose

Intra-muscular cortisol manipulations successfully raised plasma cortisol titers in pufferfish 2 days post-implantation (F = 13.997, P < 0.001; Fig. 2 A). Low and high cortisol doses (25 and 50 mg kg\(^{-1}\) fish, respectively) caused circulating cortisol concentrations to increase by 8 and 18 times, respectively, when compared to control and sham-treated fish (Fig. 2 A). However, only the high cortisol dosed fish exhibited plasma cortisol levels that were statistically higher than other treatment groups (Tukey’s HSD tests, P < 0.01). The checkered puffer has been reported to naturally release 126 ± 34 ng ml\(^{-1}\) of plasma cortisol in response to an acute standardized stressor (Cull et al., submitted). Circulating cortisol concentrations following the high cortisol dose were 147 ± 35 ng ml\(^{-1}\) (Fig. 2 A). Thus, this dose resulted in physiologically relevant post-stress level of plasma cortisol, and the 10 mg mL\(^{-1}\) cortisol dose was used for the remainder of the study. Control, sham treated, as well as low and high cortisol dosed pufferfish displayed no differences in blood glucose levels (P > 0.05; Fig. 2 B).

3.4.3 Validation of cortisol implant depletion timeline

The high cortisol implant dose (50 mg kg\(^{-1}\) fish) resulted in significant changes to the stress response of pufferfish over the 20-day time course (one-way ANOVA: F = 15.100, P < 0.001; Fig. 2 C). More specifically, following cortisol implantation, pufferfish exhibited over 20 times higher circulating plasma cortisol on day 2 when compared to
baseline levels (i.e., day 0; Tukey’s HSD test: \( P < 0.001 \)), and then dropped to baseline levels over days 5, 10 and 20 (Tukey’s HSD tests: \( P > 0.05 \); Fig. 2 C), Pufferfish implanted with a high cortisol implant dose displayed no significant difference in blood glucose concentrations at any of the sampling periods over the 20-day period (one-way ANOVA: \( P > 0.05 \); Fig. 2 D). Similarly, no differences were found among groups in hematocrit (one-way ANOVA: \( P > 0.05 \); Table 2).

### 3.4.4 Metabolic cost of cortisol implant

The standard metabolic rate (SMR) of control (183.6 ± 22.9 mg \( O_2 \) kg\(^{-1}\) h\(^{-1}\)) and high cortisol (151.9 ± 22.3 mg \( O_2 \) kg\(^{-1}\) h\(^{-1}\)) implanted puffers was similar (independent sample t-test: \( P > 0.05 \)).

### 3.4.5 Swimming performance

During the chase experiments, control and cortisol implanted fish showed no significant difference in swimming performance in terms of time until exhaustion (control: 189.50 ± 37.08 s; cortisol-dosed: 171.38 ± 13.62 s; independent samples t-test: \( P > 0.05 \)).

### 3.4.6 Lab experiment: Thermal tolerance

Plasma cortisol levels in the checkered puffer were primarily influenced by the cortisol implant, as well as the interaction between the cortisol implant and thermal treatment (Table 3; Fig. 3 A). Control pufferfish exhibited similar plasma cortisol levels at all temperatures (Fig. 3 A). Pufferfish dosed with cortisol exhibited increased plasma cortisol levels (164.20 ± 21.10 ng ml\(^{-1}\)) at ambient temperature and lower levels of cortisol when
subjected to changes in temperature. Blood glucose levels in the checkered puffer were primarily influenced by thermal treatment (Table 3; Fig. 3 B). Pufferfish exposed to a 5 ºC decrease in temperature had significantly higher levels of blood glucose than pufferfish exposed to ambient temperature and a 5 ºC increase in ambient temperature. There was no significant difference in blood glucose concentrations of pufferfish exposed to ambient temperature and a 5 ºC increase in ambient temperature. The cortisol implant and thermal treatments had no significant effect on hematocrit in the checkered puffer (Table 3).

The puffing performances of the checkered puffer, including puff score and time required to deflate once released, were primarily influenced by thermal treatment (Tables 3 and 4). Pufferfish were unable to perform any anti-predator puffing behaviour in response to decreasing temperatures. The cortisol implant, as well as the interaction between the cortisol implant and thermal treatment, did not significantly contribute to changes in puff performance (Table 4).

3.4.7 Field experiment: Thermal preference

The proportion of temperature values equal to or above 26ºC was similar for control (12.77 ± 1.63 %) and cortisol dosed (13.28 ± 1.67 %) fish, as well as to habitat iButtons (11.98 ± 1.02 %; one-way ANOVA: P > 0.05). Furthermore, the daily ATUs, as well as minimum, maximum and range of temperatures experienced by control and cortisol dosed fish did not differ from one another, or to the habitat temperature recordings (one-way ANOVA: P > 0.05; Table 5). The ATUs, minimum, maximum and ranges values were found to significantly differ across each day over the 20 day study (repeated measures ANOVA: Ps < 0.0001; Table 6 and Fig. 4). However, significant differences between
groups were only apparent for ATUs (repeated measures ANOVA: $P < 0.0001$; Table 5 and Fig. 4). On average, daily ATUs for cortisol-treated fish ($1083 \pm 5^\circ C$) were $6^\circ C$ cooler when compared to control fish ($1089 \pm 6^\circ C$), and $9^\circ C$ cooler than that logged by the habitat ($1092 \pm 4^\circ C$; Table 5).

### 3.4.8 Field experiment: Change in body condition

The initial condition of control and cortisol-treated puffers ($2.05 \pm 0.09$ and $2.05 \pm 0.07$ mg mm$^{-3}$, respectively) was similar to their final condition ($1.97 \pm 0.06$ and $1.91 \pm 0.05$ mg mm$^{-3}$, respectively). Although a slight decrease in fish condition was observed over the 20-day period, the decline in condition was not found to be significant, nor to be significantly dissimilar among control and cortisol-treated pufferfish (two-way repeated measures ANOVA: $P > 0.05$).

### 3.5 Discussion

In the current study, cortisol was experimentally manipulated to physiological post-stress levels to elucidate the effects of a thermal challenge as a secondary stressor on the checkered puffer. We compared indicators of energy use among control and cortisol-implanted fish to evaluate the effects of the cortisol implant on pufferfish, and found that pufferfish did not show any energetic costs in terms of changes in blood glucose concentrations, standard metabolic rate, nor swimming performance. We then tested the thermal tolerance of pufferfish in a controlled laboratory setting, and the thermal preferences of wild pufferfish in a complementary field study. In the lab, we found, contrary to our predictions, that fish dosed with cortisol exhibited lower levels of cortisol
when subjected to the secondary thermal challenge. In the field, we found that cortisol implanted fish generally selected cooler temperatures when compared to controls. Collectively, these results tell an interesting story about how a wild tropical fish species can deal with multiple climate-induced stressors.

3.5.1 Validation study of cortisol implant

Due to the unique anatomy and physiology of the checkered puffer, we used intramuscular injections of cortisol in the current study. Other cortisol manipulation studies have employed intra-peritoneal cortisol implants, and cortisol-spiked food to raise plasma cortisol levels in fish (reviewed in Gamperl et al. 1994). Although intramuscular injection is rather uncommon for cortisol implants in fish, it worked quite well for pufferfish. Indeed, cortisol levels were elevated for between 2 to 4 days over control levels, a period similar to intra-peritoneal implants (e.g., O’Connor et al. 2009, 2013; McConnachie et al. 2012).

3.5.2 Metabolic cost of cortisol implant

The cortisol implant caused a peak in plasma cortisol levels of the checkered puffer 2 days post-injection. Therefore, we predicted a significant metabolic cost associated with the cortisol implant at this time point. It is well established that exposure of fish to experimentally manipulated cortisol titres initiates an endocrine response which in turn induces metabolic and osmotic disturbances (i.e., the secondary stress response; Mazeaud et al. 1977, Barton and Iwama 1991). However, there was no difference in O2 consumption between cortisol-treated and control fish 2 days post-injection. Furthermore,
there was no difference in swimming performance between cortisol-treated and control fish. Generally, cortisol implanted fish tired more rapidly than control fish, and therefore may have reduced swimming performance; previous studies have linked chronically increased cortisol titres to impaired performance in juvenile rainbow trout (*Oncorhynchus mykiss*; Basu et al. 2002) and adult female pink salmon (*O. gorbuscha*; McConnachie et al. 2012).

### 3.5.3 Thermal biology

Few studies have established clear links between the documented consequences of thermal stressors in the laboratory, and the parallel consequences found at the ecosystem level by means of field studies (Pörtner 2009). By both quantifying the behavioural and physiological consequences to multiple stressors in the laboratory, and establishing thermal preferences under these different stressed states in a natural habitat, we are able to better predict alterations in performance and overall fitness that may be expected of climate change in the checkered puffer.

**Laboratory experiment.** We predicted that the cortisol implant would alter the short-term thermal tolerance of the pufferfish, and increase the physiological stress response to the thermal challenge (i.e., as a secondary stressor). We found that decreases in temperature has the most significant physiological consequences on the checkered puffer, whereas similar increases in temperature has little impact. Fish dosed with cortisol exhibited high cortisol levels at ambient temperature, and lower levels of cortisol when subjected to changes in temperature. Implanted pufferfish exhibited lower levels of circulating cortisol
following heat and cold challenges likely due to the additive response and rapid clearance
of cortisol similar to that documented in bluegill sunfish (*Lepomis macrochirus*; McConnachie et al. 2012).

Elevated levels of cortisol (manipulated through intra-peritoneal injection) have been previously found to significantly suppress levels of heat shock proteins (hsp) in cutthroat trout (*O. clarki clarki*; Ackerman et al. 2001), mossambique tilapia (*Oreochromis mossambicus*; Basu et al. 2001) and rainbow trout (Basu et al. 2001), suggesting that cortisol may mediate hsp levels in fish tissues following times of physiological stress through a series of cellular processes (see Basu et al. 2001 and 2002 for details). Furthermore, Basu et al. (2001) found that mossambique tilapia exhibited a milder physiological stress response relative to rainbow trout, likely due to the fact that tilapia are known to be a stress-tolerant fish (Bruton and Boltt, 1975, Basu et al. 2001).

Like tilapia, the checkered puffer also prefers a high thermal range, which may account for their ability to better deal with a heat shock challenge in comparison to the equivalent but opposite cold shock challenge.

In response to the acute decrease in temperature, pufferfish were notably more active than fish placed in the other treatments (i.e., constantly swimming or struggling to exit the experimental chamber). This anecdotal increase in activity is likely an attempt to cope with the acute change in temperature, and responsible for the inability to puff following the 5 °C decrease in temperature. In response to decreasing temperature, fish have been previously reported to show hyperresponsiveness, uncoordinated swimming (e.g., bumping into tank walls and spontaneous circling), difficulty maintaining equilibrium, complete loss of equilibrium, and induction of coma (see Friedlander et al.
An acute decrease in temperature (i.e., cold shock) may influence the reliability of neuronal activity and the reliability of cellular responses, leading to compromised anti-predator behaviour (Preuss and Faber 2003).

Field experiment. Generally, pufferfish selected cooler temperatures than the average thermal profile of the creek, and cortisol-treated fish favoured cooler temperatures than controls. The interpretation of the thermal preferences of checkered puffers in an ecological context is complicated by the fact that this is the first study of its kind, and that the laboratory and field studies reveal dissimilar findings. Based on the laboratory study, control and cortisol dosed pufferfish seemed to easily cope with heat shock, but less so to cold shock; therefore, we might expect cortisol implanted pufferfish and control fish to select warmer temperatures within their natural habitat. We would also predict that cortisol implanted fish would avoid secondary stressors, thereby moving within the habitat to seek smaller thermal fluctuations.

In variable environments, evolutionary theory predicts that ectotherms prefer a body temperature slightly below the physiological optimum (e.g., intertidal snails (Chlorostoma funebralissnails; Tepler et al. 2011) and Australian skinks (Lygosominae; Huey and Bennett, 1987)). These less than optimal thermal choices are often strongly associated with habitat-seeking behaviour that bears a competitive or anti-predation advantage (Tepler et al. 2011). While these animals have shown to possess a high thermal optimum and preference in the laboratory, like the checkered puffer, their habitat selection in the field limits them to lower temperatures, sometimes leading to physiological impairment (Angilletta et al., 2006). These findings may correlate to
possible trade-offs made by the pufferfish – chronically stress-induced fish may have
selected refuge in the mangrove habitat, away from intra- and inter-specific competition,
regardless of sub-optimal fluctuations in temperature. In addition, elevated levels of
cortisol are known to suppress levels of hsp's in several species of fish (Ackerman et al.
2001, Basu et al. 2001, Basu et al. 2001), suggesting that cortisol may mediate hsp levels
following times of physiological stress (see Basu et al. 2001 and 2002 for details). This
may explain why cortisol implanted pufferfish selected cooler temperatures in the wild.

Although a severe metabolic cost was not associated with the cortisol implant,
cortisol dosed pufferfish may have selected cooler temperatures to reduce metabolic
energy expenditure when subjected to the additional stressors of their natural
environment. Few fish studies have documented thermal preferences in the field,
however, Roscoe et al. (2010) found that reproductively advanced female sockeye salmon
(Oncorhynchus nerka) with lower levels of energy similarly selected cooler temperatures
compared to less mature females with high levels of energy, possibly to reduce metabolic
energy expenditure and delay final maturation. Similar to fish, stressed reptiles respond
with increases in plasma glucocorticoid levels (i.e., corticosterone), affecting the animal's
physiology and behaviour (Greenberg and Wingfield, 1987). Increased levels of
glucocorticoids have been associated with a variety of consequences in lizards, including
exposure to predation (Montgomerie and Wheatherhead 1988), a reduction in fat stores
(Guillete et al. 1995), and immune system depression (Zuk 1996, Oppliger et al. 1998).
Furthermore, increased levels of glucocorticoids due to experimental corticosterone
manipulation have been found to increase activity and thermoregulation in the common
lizard (Lacerta vivipara; Belliure et al. 2004), enhance locomotor activity and reduce
thermoregulatory behaviour in juvenile wall lizards (*Podarcis muralis*; Belliure and Colbert, 2004), and increase metabolic rate and increase thermoregulatory behaviour in females of the New Zealand common gecko (*Hoplodactylus maculatus*; Preest and Cree 2008). Although we did not measure any of those specific endpoints here, our data support the idea that cortisol-treated pufferfish selected marginally cooler temperatures to potentially reduce further metabolic costs and seek refuge from predation or other forms of competition. Curiously however, we were unable to document any significant burden linked to the cortisol implant in the laboratory, suggesting that metabolic costs were not measurable in terms of aerobic activity 48 hrs post-treatment.

Although a slight decrease in fish condition was observed over the 20 day period, the decline in condition was not found to be significant, nor to be significantly dissimilar between control and cortisol-treated pufferfish. The minor decline in condition across control and cortisol-treated fish is likely due to the handling stress of the experiment and a small tagging burden (Thiem et al. 2013).

As the checkered puffer has been observed to rarely venture out of the study creek, we can likely assume the recapture rate is a measure of survival. The recapture rate for control and cortisol dosed fish was 58% and 72%, respectively; indicating that cortisol implanted fish may have a better survival rate than controls. Cortisol implanted fish may have sought refuge due to the unidentified burden of the implant, taking fewer risks and thus suffering less predation than controls. Other plausible speculations include the notion that control pufferfish were more mobile and thus less likely to be in the same area at the time of recapture, or cortisol implanted fish may have simply been easier to capture.
3.5.4 Conclusion

The combination of stressors caused by gradual anthropogenic climate change may provoke complex non-linear responses in coastal systems on the individual, population and community levels (Lee et al. 2001, Harley et al. 2006, Burkett et al. 2008). Through experimental cortisol manipulations, we were able to highlight the detrimental physiological and behavioural consequences of multiple and repeated thermal stressors (i.e., heat and cold shock challenges) in the checkered pufferfish in a controlled laboratory, and for the first time, relate it to a comparable and ecologically relevant field study monitoring the thermal preferences and condition of fish. The disparity in findings between the lab and the field suggests that in field environments, animals have greater opportunity to select their environments, and that any physiological consequences associated with experimentation have the potential to be modulated by behaviour more so than in a confined laboratory settings. These findings highlight the need to establish the link between laboratory findings and ecologically relevant information in order to develop appropriate management policies and conservation initiatives with regards to anthropogenic climate change.
### Table 3.1
The mass and total length (TL) of checkered puffers (*Sphoeroides testudineus*) treatment groups included in all experiments. One-way ANOVAs followed by a Tukey’s HSD tests were conducted to quantify differences among groups. Letters identify statistical differences across treatment groups.

<table>
<thead>
<tr>
<th>Fish treatment group</th>
<th>TL (mm)</th>
<th>F</th>
<th>P</th>
<th>Mass (g)</th>
<th>F</th>
<th>P</th>
</tr>
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<tr>
<td><strong>Validation of cortisol implant dose</strong></td>
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<tr>
<td>Control</td>
<td>186 ± 5</td>
<td>1.136</td>
<td>0.358</td>
<td>149 ± 9</td>
<td>0.966</td>
<td>0.427</td>
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<td>Sham</td>
<td>182 ± 8</td>
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<td>155 ± 12</td>
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<tr>
<td>Low-dose</td>
<td>180 ± 6</td>
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<td>140 ± 14</td>
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<td>High-dose</td>
<td>176 ± 4</td>
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<td></td>
<td>130 ± 7</td>
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<td><strong>Validation of cortisol implant depletion timeline</strong></td>
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<tr>
<td>Control</td>
<td>184 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.826</td>
<td>0.005</td>
<td>145 ± 9&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>5.444</td>
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<td>2 days post-implant</td>
<td>176 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>130 ± 7&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>5 days post-implant</td>
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<td>105 ± 6&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>10 days post-implant</td>
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<td>136 ± 12&lt;sup&gt;ab&lt;/sup&gt;</td>
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<tr>
<td>20 days post-implant</td>
<td>215 ± 11&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>192 ± 27&lt;sup&gt;b&lt;/sup&gt;</td>
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<td><strong>Metabolic cost and swimming performance</strong></td>
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<tr>
<td>Control</td>
<td>152 ± 4</td>
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<td>0.948</td>
<td>74 ± 3</td>
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</tr>
<tr>
<td>Cortisol-dosed</td>
<td>152 ± 11</td>
<td></td>
<td></td>
<td>93 ± 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lab experiment: Thermal tolerance</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control at <em>amb. T</em></td>
<td>182 ± 4</td>
<td>1.333</td>
<td>0.271</td>
<td>142 ± 8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.744</td>
<td>0.033</td>
</tr>
<tr>
<td>Control at -5ºC</td>
<td>187 ± 6</td>
<td></td>
<td></td>
<td>124 ± 13&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control at +5ºC</td>
<td>191 ± 7</td>
<td></td>
<td></td>
<td>137 ± 14&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortisol-dosed at <em>amb. T</em></td>
<td>175 ± 4</td>
<td></td>
<td></td>
<td>130 ± 6&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortisol-dosed at -5ºC</td>
<td>181 ± 2</td>
<td></td>
<td></td>
<td>106 ± 5&lt;sup&gt;ab&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortisol-dosed at +5ºC</td>
<td>176 ± 6</td>
<td></td>
<td></td>
<td>102 ± 10&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Field experiment: Thermal preference</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deployed control</td>
<td>183 ± 3</td>
<td>0.084</td>
<td>0.969</td>
<td>125 ± 4</td>
<td>0.131</td>
<td>0.941</td>
</tr>
<tr>
<td>Deployed cortisol-dosed</td>
<td>185 ± 4</td>
<td></td>
<td></td>
<td>130 ± 9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recaptured control</td>
<td>184 ± 3</td>
<td></td>
<td></td>
<td>124 ± 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recaptured cortisol-dosed</td>
<td>186 ± 5</td>
<td></td>
<td></td>
<td>125 ± 12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.2 Checkered puffer (*Sphoeroides testudineus*) hematocrit levels at 5-, 10- and 20-days post-implantation.

<table>
<thead>
<tr>
<th>Fish treatment group</th>
<th>Hematocrit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 days post-implant</td>
<td>19.16 ± 1.64</td>
</tr>
<tr>
<td>10-days post-implant</td>
<td>19.25 ± 4.36</td>
</tr>
<tr>
<td>20 days post-implant</td>
<td>23.49 ± 2.61</td>
</tr>
</tbody>
</table>


Table 3.3 Two-way ANOVA outputs identifying the effect of multiple stressors (i.e., fish treatment (control and cortisol implanted pufferfish) and thermal treatment (ambient temperature, as well as 5°C below and above ambient temperature)) on physiological and behavioural stress indices, including cortisol, glucose, hematocrit and ‘puff’ performances (i.e., puff score and puff time to deflate once released).

<table>
<thead>
<tr>
<th></th>
<th>( R^2 )</th>
<th>( \text{Adjusted } R^2 )</th>
<th>DF</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cortisol</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected model</td>
<td>0.453</td>
<td>0.381</td>
<td>5</td>
<td>6.288***</td>
</tr>
<tr>
<td>Fish treatment</td>
<td></td>
<td></td>
<td>1</td>
<td>15.962***</td>
</tr>
<tr>
<td>Thermal treatment</td>
<td></td>
<td></td>
<td>2</td>
<td>1.412</td>
</tr>
<tr>
<td>Fish treatment * Thermal treatment</td>
<td></td>
<td></td>
<td>2</td>
<td>4.540*</td>
</tr>
<tr>
<td><strong>Glucose</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected model</td>
<td>0.876</td>
<td>0.860</td>
<td>5</td>
<td>53.772***</td>
</tr>
<tr>
<td>Fish treatment</td>
<td></td>
<td></td>
<td>1</td>
<td>3.609</td>
</tr>
<tr>
<td>Thermal treatment</td>
<td></td>
<td></td>
<td>2</td>
<td>130.217***</td>
</tr>
<tr>
<td>Fish treatment * Thermal treatment</td>
<td></td>
<td></td>
<td>2</td>
<td>2.955</td>
</tr>
<tr>
<td><strong>Hematocrit</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected model</td>
<td>0.011</td>
<td>-0.113</td>
<td>3</td>
<td>0.085</td>
</tr>
<tr>
<td>Fish treatment</td>
<td></td>
<td></td>
<td>1</td>
<td>0.043</td>
</tr>
<tr>
<td>Thermal treatment</td>
<td></td>
<td></td>
<td>1</td>
<td>0.149</td>
</tr>
<tr>
<td>Fish treatment * Thermal treatment</td>
<td></td>
<td></td>
<td>1</td>
<td>0.077</td>
</tr>
<tr>
<td><strong>Puff score</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected model</td>
<td>0.521</td>
<td>0.461</td>
<td>3</td>
<td>8.695***</td>
</tr>
<tr>
<td>Fish treatment</td>
<td></td>
<td></td>
<td>1</td>
<td>0.160</td>
</tr>
<tr>
<td>Thermal treatment</td>
<td></td>
<td></td>
<td>1</td>
<td>25.713***</td>
</tr>
<tr>
<td>Fish treatment * Thermal treatment</td>
<td></td>
<td></td>
<td>1</td>
<td>0.160</td>
</tr>
<tr>
<td><strong>Puff time to deflate once released</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected model</td>
<td>0.211</td>
<td>0.112</td>
<td>3</td>
<td>2.134</td>
</tr>
<tr>
<td>Fish treatment</td>
<td></td>
<td></td>
<td>1</td>
<td>0.303</td>
</tr>
<tr>
<td>Thermal treatment</td>
<td></td>
<td></td>
<td>1</td>
<td>5.696*</td>
</tr>
<tr>
<td>Fish treatment * Thermal treatment</td>
<td></td>
<td></td>
<td>1</td>
<td>0.303</td>
</tr>
</tbody>
</table>

*** \( P < 0.001; ** P < 0.01; * P < 0.05 \)

1471

1472
Table 3.4 Puffing performance (i.e., puff score and puff time to deflate once released) and hematocrit levels of control and cortisol implanted checkered puffers (*Sphoeroides testudineus*) when subject to +5 and -5 °C changes from ambient temperature. Letters identify statistical differences across treatment groups.

<table>
<thead>
<tr>
<th></th>
<th>+5 °C</th>
<th>-5 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Cortisol implanted</td>
</tr>
<tr>
<td>Puff score</td>
<td>1.29 ± 0.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.15 ± 0.22&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Puff time to deflate once released (sec)</td>
<td>97 ± 49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31 ± 11&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>24.00 ± 2.36</td>
<td>24.10 ± 2.21</td>
</tr>
</tbody>
</table>

Table 3.5 Temperature metrics, including the daily accumulated thermal units (ATUs), thermal minimums, maximums and ranges, recorded by the iButtons of control and cortisol implanted checkered puffers (*Sphoeroides testudineus*), as well as that of the habitat.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ATUs</th>
<th>Temperature metrics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MINIMUM</td>
<td>MAXIMUM</td>
</tr>
<tr>
<td>Control fish</td>
<td>1089.25 ± 6.01</td>
<td>19.85 ± 0.13</td>
</tr>
<tr>
<td>Dosed fish</td>
<td>1082.80 ± 4.56</td>
<td>19.88 ± 0.13</td>
</tr>
<tr>
<td>Habitat</td>
<td>1092.07 ± 4.27</td>
<td>20.00 ± 0.12</td>
</tr>
</tbody>
</table>
Table 3.6 Repeated measures general linear model statistical output, where the effect of
day (i.e., each day over the 20 day period) on the calculated thermal variables (i.e., ATUs, minimums, maximums and ranges) of the different treatments (i.e., control and cortisol implanted fish, as well as the habitat thermal loggers) were established.

<table>
<thead>
<tr>
<th></th>
<th>DF</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ATUs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day</td>
<td>19</td>
<td>71.175 ***</td>
</tr>
<tr>
<td>Day * Treatment</td>
<td>38</td>
<td>2.663 ***</td>
</tr>
<tr>
<td><strong>Minimums</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day</td>
<td>19</td>
<td>2447.665 ***</td>
</tr>
<tr>
<td>Day * Treatment</td>
<td>38</td>
<td>1.216</td>
</tr>
<tr>
<td><strong>Maximum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day</td>
<td>19</td>
<td>100.510 ***</td>
</tr>
<tr>
<td>Day * Treatment</td>
<td>38</td>
<td>.863</td>
</tr>
<tr>
<td><strong>Ranges</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day</td>
<td>19</td>
<td>61.471 ***</td>
</tr>
<tr>
<td>Day * Treatment</td>
<td>38</td>
<td>.862</td>
</tr>
</tbody>
</table>

*** P < 0.001; ** P < 0.01; * P < 0.05
Figure 3.1 Study area along the coast of Cape Eleuthera, Eleuthera, The Bahamas, showing the locations of the sampled Page, Kemps and Plum Creeks, and the location of the Cape Eleuthera Institute (CEI) research facility (black star). The inset map displays the entire island of Eleuthera with the study area highlighted.
Figure 3.2 Checkered puffer (*Sphoeroides testudineus*) plasma cortisol (A) and blood glucose (B) concentrations of control and cocoa butter sham treated fish, as well as low-cort, low dose cortisol-cocoa butter (25 mg kg\(^{-1}\) fish, 5 ml melted cocoa butter kg\(^{-1}\) fish) injected fish and high-cort, high dose cortisol-cocoa butter (50 mg kg\(^{-1}\) fish, 5 ml melted cocoa butter kg\(^{-1}\) fish) injected fish. Plasma cortisol (C) and blood glucose (D) concentrations of high dose cortisol-cocoa butter (50 mg kg\(^{-1}\) fish, 5 ml melted cocoa butter kg\(^{-1}\) fish) injected checkered puffers over a 20 day period. Dashed lines indicate physiological baseline and post-stress concentrations.
Figure 3.3 Plasma cortisol (A) and blood glucose (B) concentrations of control (black) and cortisol implanted (gray) checkered puffers (Sphoeroides testudineus) in ambient conditions, as well as +5 and -5 °C changes from ambient temperature.
Figure 3.4 Thermal variables, including the accumulated thermal units (ATUs; A), thermal minimums (B), maximums (C) and ranges (D), for control (dark circles) and cortisol dosed (light circles) checkered puffers (*Sphoeroides testudineus*), as well as for the habitat loggers (dark triangle), over the 20 day sampling period.
Chapter 4. General discussion

4.1 Overview

This thesis is a compilation of two distinct yet related studies that help characterize the ecological consequences of stress in a wild tropical fish. In chapter 2, I quantified individual variation in the glucocorticoid (GC) stress response in checkered puffers (Sphoeroides testudineus) and determined whether there is a relationship between the GC stress response and two established fitness proxies; two puffing metrics and bite force. In chapter 3, I attempted to elucidate the consequences of the stress response on the thermal biology of the pufferfish in a controlled laboratory setting and in the field. Through the use of experimental cortisol manipulations, I revealed some of the possible physiological, behavioural and ecological consequences of climate-induced stress.

Individual variation in the endocrine stress response (i.e., the change in circulating GCs following a challenge) has been linked to survival and fitness in a variety of species. However, the strength and the direction of this relationship has proven to be highly context dependent (e.g., Cockrem 2007, Øverli et al. 2007, Blas et al. 2007, Wada and Breuner, 2008). To date, the majority of research assessing the link between the GC stress response and fitness uses birds, reptiles and mammals, while only few examine fish (see Breuner et al. 2008 and Bonier et al. 2009 for overviews, McConnachie et al. 2012). In chapter 2, I focused on elucidating the functional significance of variation in GC secretion using wild checkered puffers. The checkered puffer is an interesting model for studying stress because it has unique predator avoidance strategies. Pufferfish will not hesitate to bite and 'puff' (i.e., inflate) to deter potential predators. These behaviours are readily
measurable and have direct implications for individual survival and fitness. In chapter 2, wild checkered puffers were subjected to a standardized stress protocol to quantify baseline and post-stress physiological stress indices (circulating GCs and glucose), and to evaluate whether these indices were correlated with fitness proxies (i.e., bite force and puffing performance). I determined the variability and repeatability of post-stress cortisol in pufferfish brought into a controlled laboratory setting and attempted to define the relationship between the variation in the GC response and fitness proxies.

Moreover, checkered puffers are adapted to extreme environmental gradients imposed by their coastal and tropical environment, and their distribution is often governed by their tolerances to these conditions, particularly rapid changes in temperature. Given that anthropogenic environmental change will not occur in isolation of other stressors, eurythermal and heat-tolerant species, like the pufferfish, may be more vulnerable to increasing temperatures because these species typically live closer to their thermal limits (Tomanek and Somero 1999, Stillman 2002, Harley et al. 2006). To clarify the thermal-related characteristics of stress in a wild fish population, I focused on controlled laboratory experiments and a complementary field study to evaluate the physiological, behavioural and ecological consequences of thermal stress (chapter 3). Stress influences physiology, behaviour and overall fitness of wild fish populations (Boonstra 2013), and this manuscript helps contribute to our understanding of the ecology of stress in wild animals inhabiting extreme environments. In chapter 3, plasma cortisol was experimentally manipulated to physiological post-stress levels to investigate the physiological, behavioural and ecological consequences of a thermal challenge on the checkered puffer as a secondary stressor. First, the sub-lethal consequences of climate-induced stress (i.e., heat and cold shock) on the physiology and behaviour of the checkered
puffers were identified by experimentally manipulating cortisol levels in controlled laboratory experiments. Next, a complementary 20-day field study was conducted in a tidal creek to evaluate the effect of increased cortisol levels, through experimental manipulation, on the preferred thermal profiles of pufferfish in their natural habitat.

4.2 Findings and implications

In summary, the results of chapter 2 indicated that following an acute standardized stressor, pufferfish exhibited increased physiological stress indices and interestingly, reduced bite force and the extent of puffing. Furthermore, the magnitude of individual physiological stress response was negatively correlated with post-stress fitness proxies. I also documented that puff metrics for individuals are repeatable through time. Although the acute stress response is thought to be adaptive (Wingfield et al. 1998), I documented negative consequences in response to an acute standardized stressor, similar to other studies (Blas et al. 2007, MacDougall-Shackleton et al. 2009, Cook et al. 2011). In chapter 3, I verified the cortisol implant required to raise plasma cortisol titres to physiological post-stress levels 2-day post treatment, the depletion timeline of the implant over a 20-day period, and the energetic cost of the implant 2 days post-treatment. The physiological consequences of the implant appeared to dissipate between 2 and 5 days post-treatment, and the implant had no measurable energetic cost. The research I conducted revealed the consequences of experimental cortisol manipulations on the thermal biology of the pufferfish in laboratory and field environments. Control fish exhibited resting physiological and behavioural stress indices following the heat shock treatment, and post-stress cortisol levels and weak “puff” performances after the cold shock treatment. Whereas, fish dosed with cortisol exhibited post-stress cortisol levels at ambient
temperature, and contrary to the collective prediction that additional stressors increase the GC response, lower levels of cortisol when subjected to the secondary thermal challenge. Furthermore, cortisol implanted fish generally selected cooler temperatures in their natural habitat when compared to controls.

Together, the results of chapters 2 and 3 suggest that the primary endocrine stress response to acute and chronic stressors is associated with negative secondary and tertiary consequences (i.e., variable glucose release, weakened fitness proxies and decreased condition) and may translate to ecological consequences, in terms of wild population dynamics. Although variation is a vital issue when using wild fish populations in their natural habitat, an experimental field approach allows us to understand how stress influences behaviour and survival of fish in the wild. These findings increase the application of the results to real conservation issues (see Cooke and O’Connor, 2010) and highlight the need to establish the link between laboratory findings and ecologically relevant information needed for the development of management policies and conservation initiatives with regards to anthropogenic climate change.

To date, most research on tropical and intertidal species examines thermal limits. To my knowledge, no studies have attempted to elucidate the effect of additional stressors on the thermal biology of animals that live in extreme environments. These studies are novel in that I:
1. Used the checkered puffer as a valuable species to elucidate the consequences of stress on a wild tropical population in an extremely fluctuating coastal environment;

2. Determined simple protocols to experimentally manipulate GCs and quantify unique performances associated with fitness (i.e., bite force and puffing) in a pufferfish, and;

3. Elucidated the link between stress and the thermal biology of a tropical marine species nearing its thermal limits in a controlled laboratory setting and in the wild.

4.3 **Future directions and further questions**

The concepts investigated in this manuscript elucidate the intra- and inter-individual variation in the physiological and behavioural stress response to an acute standardized stressor, and the consequences this response may have on the physiology, performance, and ecology of a wild population. By clarifying the role of GCs in a wild tropical fish population, I have broadened our understanding of the ecology of stress in wild animals that may be increasingly exposed to multiple and chronic stressors due to anthropogenic climate change.

To date, the majority of stress research has focused on elucidating the functional significance of variation in GC secretion given the growing recognition that not all individuals respond to stress in the same manner. The effect of individual variation in GC secretion on performance and overall fitness is complex (see reviews by Ricklefs and Wikelski 2002, Bruener et al. 2008, Bonier et al. 2009). Much of the GC research performed has examined individual variation in stress response and the associated fitness-oriented endpoints using birds (Angelier et al. 2007, Groscolas et al. 2008, Williams et al. 2008), reptiles (Romero and Wikelski, 2001, Meylan and Clobert 2005, Lancaster et al.
2008) and mammals (Pride 2005, Cabezas et al. 2007, Rogovin et al. 2008) as model species, with considerably less GC work done in fish (see Breuner et al. 2008 and Bonier et al. 2009 for overviews, McConnachie et al. 2012). Only recently, scientists have seriously identified the weak connections between stress and other overlapping areas of biology, including genetics, physiology, behaviour and evolution (Boonstra 2013). The ecology of stress is an important underpinning of ecology that overlaps with these four major areas of biology (Krebs 2009, Boonstra 2013), highlighting the significance of multidisciplinary stress studies to further our understanding of ecosystem dynamics. Of special importance is the finding that the checkered puffer has a highly variable GC response and is negatively correlated with fitness proxies (chapter 2). Variable GCs are likely beneficial from an evolutionary standpoint, due to the nature of their highly fluctuating coastal habitat.

Most thermal stress work in fish has been restricted to laboratory studies (Ackerman et al. 2000, Vijayan et al. 2000, Basu et al. 2001), and cortisol-implanted fish have been found to be more susceptible to thermal stress (Basu et al. 2001, McConnachie et al. 2012). However, no studies have been conducted on a free-swimming fish population. Chapter 3 outlines a series of experiments revealing the costs of experimental GC manipulation and using these manipulations to determine the thermal-related characteristics of pufferfish in the laboratory, as well as in a complementary field study. Pufferfish responded to thermal stress differently when in the laboratory and in their natural habitat, suggesting that laboratory studies, although easy to interpret, may not be fully applicable to dynamic fish populations in the wild.

As with most studies, the findings from my experiments raise additional questions that could further clarify the variability and consequences of the stress response.
1. Why do studies continuously find different links between the physiological stress response and behavioural consequences? What link are we missing? This challenge may be due to the ambiguity in our definitions of an acute versus a chronic stressor, and our limited understanding on how wild populations manage repeated and multiple stressors. At what point does the stress response becomes maladaptive?

2. The physiological and behavioural variability of the stress response established among individuals of a wild checkered puffer population was noteworthy. Are there covariates influencing this variability other than size? Maybe other covariates should be considered, such as other hormones, sex, measures of condition and sexual reproductive state?

3. The physiological consequence of the cortisol implant had returned to resting titres by day 5 post-implantation. Do pufferfish have an increased ability to excrete cortisol relative to other fish species? Although fish appear to physiologically recover from the implant 5 days post-treatment, is there evidence that fish may be burdened by the stressor on other levels beyond day 5, in terms of energetic cost, performance and habitat selection? It would be interesting to expose these fish to different treatments beyond the 5 day mark, once cortisol has returned to resting baseline levels, and compare them to controls. Treatments could include exposure to a pathogen or an epidermal laceration, to elucidate immune function. It would be simple to include this within controlled laboratory and field environments. Other treatments possible in the laboratory are thermal shock (or other water parameter variations such as dissolved oxygen, pH, silt, etc.) and predator encounters.

4. Given that the checkered puffer is known to endure frequent temperature fluctuations in their natural habitat and that thermal shock had a significant impact on the stress response
of the pufferfish, would slower and larger changes in temperature have similar
consequences? What are the thermal thresholds if you take fluctuation time into
consideration?

5. The thermal biology laboratory and field studies were conducted in the summer and
winter months, respectively. Given the change in temperature between summer and
winter, I would suggest that future studies compare within the same season to maintain
consistency between studies. In the summer, fish were found to easily cope with heat
shock over cold shock in the laboratory. Is this because heat shock likely occurs more
often during the summer? Would I have found opposite results (i.e., fish easily coping
with cold shock over heat shock) if the laboratory study was conducted in the winter?

6. The checkered puffer is the first group of fish species to have their genome sequenced
(citation?). The pufferfish have little genetic variation with small intron spacing, and
therefore are unique candidates for extinction. The inconsistency between the reduced
genetic variation and the large ecological distribution suggests that season and
population specific isoforms may exist. Further laboratory and field studies on the
ecology of stress in pufferfish must be conducted on different ecological systems,
spanning a range of habitat types (i.e., varying thermal fluctuations, local adaptations,
water parameters, predator burdens, etc.), and implementing common garden
experiments. Experiments would include individuals from different marine habitats in
Eleuthera (i.e., Page, Plum, and Kemps creeks, as well as Poison flats), and other
habitats nearing the northern and southern limits of the species’ range. In addition,
Page creek provides a promising study site for a long-term pufferfish monitoring
program, and may give us the means to answer questions with regards to the
consequences of chronic stress in the wild.
7. Checkered puffers were not sexed in this study as no apparent sexual size dimorphism was observed in this species. However, as fitness proxies were found to be highly correlated with size in the checkered puffer, future work should consider sexing individuals to monitor the possible confounding role of sex and its relationship with size.
Appendices

Appendix A

A.1 Supplementary materials for chapter 2.

The relationships between physiological and fitness proxies for baseline, post-stress and responsiveness treatments. Multiple regression results are presented. Baseline values were collected from acclimated pufferfish within 3 min. Fish were then immediately ‘stressed’ by holding them at the air-water interface for 5 min in a rubber-mesh dip net. Pufferfish were then resampled 30 min post-stressor within 3 min to collect post-stress values. The difference between baseline and post-stress treatment was labeled as the responsiveness treatment. A total of 38 fish were sampled.
<table>
<thead>
<tr>
<th></th>
<th>Baseline treatment</th>
<th>Post-stress treatment</th>
<th>Responsiveness</th>
<th>Baseline treatment</th>
<th>Post-stress treatment</th>
<th>Responsiveness</th>
<th>Baseline treatment</th>
<th>Post-stress treatment</th>
<th>Responsiveness</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline</strong></td>
<td>B  t</td>
<td>B  t</td>
<td>B  t</td>
<td>B  t</td>
<td>B  t</td>
<td>B  t</td>
<td>B  t</td>
<td>B  t</td>
<td>B  t</td>
</tr>
<tr>
<td>Bite force (N)</td>
<td>0.024 0.196</td>
<td>-0.109 -0.880</td>
<td>-0.123 -0.997</td>
<td>0.018 0.148</td>
<td>-0.120 -0.945</td>
<td>-0.131 -1.018</td>
<td>0.023 0.129</td>
<td>-0.109 -0.880</td>
<td>-0.123 -0.997</td>
</tr>
<tr>
<td>Puff score</td>
<td>0.235 1.459</td>
<td>0.097 0.559</td>
<td>0.051 0.292</td>
<td>0.060 0.367</td>
<td>0.256 1.488</td>
<td>0.240 1.364</td>
<td>0.235 1.459</td>
<td>0.097 0.559</td>
<td>0.051 0.292</td>
</tr>
<tr>
<td>Puff time to deflate</td>
<td>0.014 0.080</td>
<td>-0.010 -0.057</td>
<td>-0.014 -0.078</td>
<td>0.111 0.656</td>
<td>-0.710 -0.388</td>
<td>-0.119 -0.640</td>
<td>0.014 0.080</td>
<td>-0.010 -0.057</td>
<td>-0.014 -0.078</td>
</tr>
<tr>
<td><strong>Post-stress treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bite force (N)</td>
<td>0.216 1.488</td>
<td><strong>0.262 1.746</strong></td>
<td>*0.233 1.543</td>
<td>-0.193 -1.325</td>
<td>-0.250 -1.624</td>
<td>-0.181 -1.127</td>
<td>0.136 0.803</td>
<td>-0.173 -0.983</td>
<td>-0.217 -1.247</td>
</tr>
<tr>
<td>Puff score</td>
<td>-0.133 -0.926</td>
<td>-0.156 -1.040</td>
<td>-0.137 -0.917</td>
<td>-0.152 -1.065</td>
<td>-0.186 -1.221</td>
<td>-0.130 -0.831</td>
<td>-0.133 -0.926</td>
<td>-0.156 -1.040</td>
<td>-0.137 -0.917</td>
</tr>
<tr>
<td>Puff time to deflate</td>
<td>0.136 0.803</td>
<td>-0.173 -0.983</td>
<td>-0.217 -1.247</td>
<td>0.032 0.187</td>
<td>0.000 -0.002</td>
<td>-0.013 -0.072</td>
<td>0.136 0.803</td>
<td>-0.173 -0.983</td>
<td>-0.217 -1.247</td>
</tr>
<tr>
<td><strong>Responsiveness</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bite force (N)</td>
<td>0.189 1.124</td>
<td><strong>0.329 1.937</strong></td>
<td>*0.312 1.829</td>
<td>-0.197 -1.177</td>
<td>-0.152 -0.839</td>
<td>-0.077 -0.413</td>
<td>0.127 0.746</td>
<td>-0.167 -0.994</td>
<td>-0.209 -1.191</td>
</tr>
<tr>
<td>Puff score</td>
<td><strong>-0.312 -2.057</strong></td>
<td><strong>-0.231 -1.410</strong></td>
<td><strong>-0.177 -1.074</strong></td>
<td>-0.199 -1.273</td>
<td><strong>-0.381 -2.396</strong></td>
<td><strong>0.314 -1.878</strong></td>
<td>-0.127 0.746</td>
<td>-0.167 -0.994</td>
<td>-0.209 -1.191</td>
</tr>
<tr>
<td>Puff time to deflate</td>
<td><strong>0.127 0.746</strong></td>
<td><strong>-0.167 -0.994</strong></td>
<td><strong>-0.209 -1.191</strong></td>
<td><strong>-0.050 -0.296</strong></td>
<td>0.052 0.286</td>
<td>0.075 0.401</td>
<td><strong>0.127 0.746</strong></td>
<td><strong>-0.167 -0.994</strong></td>
<td><strong>-0.209 -1.191</strong></td>
</tr>
</tbody>
</table>

*P < 0.10; **P < 0.05
<table>
<thead>
<tr>
<th>Cortisol (ng ml-1) and Glucose (mmol L⁻¹)</th>
<th>Baseline</th>
<th>Post-stress</th>
<th>Responsiveness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R²</td>
<td>F</td>
<td>R²</td>
</tr>
<tr>
<td><strong>Baseline treatment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bite force (N)</td>
<td>0.001</td>
<td>0.031</td>
<td>0.018</td>
</tr>
<tr>
<td>Puff score</td>
<td>0.058</td>
<td>1.131</td>
<td>0.056</td>
</tr>
<tr>
<td>Puff time to deflate once released (min)</td>
<td>0.012</td>
<td>0.215</td>
<td>0.004</td>
</tr>
<tr>
<td><strong>Post-stress treatment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bite force (N)</td>
<td>0.079</td>
<td>1.957</td>
<td><strong>0.165</strong></td>
</tr>
<tr>
<td>Puff score</td>
<td>0.041</td>
<td>1.038</td>
<td>0.039</td>
</tr>
<tr>
<td>Puff time to deflate once released (min)</td>
<td>0.019</td>
<td>0.337</td>
<td>0.029</td>
</tr>
<tr>
<td><strong>Responsiveness</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bite force (N)</td>
<td>0.070</td>
<td>1.283</td>
<td><strong>0.157</strong></td>
</tr>
<tr>
<td>Puff score</td>
<td><strong>0.139</strong></td>
<td><strong>3.184</strong></td>
<td><strong>0.137</strong></td>
</tr>
<tr>
<td>Puff time to deflate once released (min)</td>
<td>0.018</td>
<td>0.306</td>
<td>0.035</td>
</tr>
</tbody>
</table>

*P < 0.10; **P < 0.05
A.2 Supplementary materials for chapter 3.

Information on the habitat iButton loggers in a tropical and shallow tidal creek, including model, coordinates, depth (cm), location description, and proximity to shelter from mangroves.

<table>
<thead>
<tr>
<th>ID No.</th>
<th>iButton Model</th>
<th>Coordinates</th>
<th>Depth During Low Tide (cm)</th>
<th>Location Description</th>
<th>Proximity To Mangrove Shelter</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DS1921H-F5</td>
<td>N24 49.047 W76 18.840</td>
<td>15.0 6.5</td>
<td>Mouth of creek; North side</td>
<td>Near</td>
</tr>
<tr>
<td>2</td>
<td>DS1921H-F5</td>
<td>N24 49.035 W76 18.830</td>
<td>40.5 25.5</td>
<td>Mouth of creek; South side</td>
<td>Near</td>
</tr>
<tr>
<td>3</td>
<td>DS1921H-F5</td>
<td>N24 49.028 W76 18.814</td>
<td>10.3 4.5</td>
<td>Within large mangrove patch where pufferfish are normally spotted</td>
<td>Within shelter</td>
</tr>
<tr>
<td>4</td>
<td>DS1921H-F5</td>
<td>N24 49.019 W76 18.813</td>
<td>35.4 28.5</td>
<td>Deep area in first bend where pufferfish often seek refuge</td>
<td>Far</td>
</tr>
<tr>
<td>5</td>
<td>DS1921Z-F5</td>
<td>N24 49.026 W76 18.795</td>
<td>12.7 10.8</td>
<td>Past first bend; North side</td>
<td>Near</td>
</tr>
<tr>
<td>6</td>
<td>DS1921H-F5</td>
<td>N24 49.020 W76 18.779</td>
<td>11.0 4.2</td>
<td>Past first bend; South side</td>
<td>Near</td>
</tr>
<tr>
<td>7</td>
<td>DS1921Z-F5</td>
<td>N24 49.000 W76 18.762</td>
<td>14.4 9.1</td>
<td>Upper part of creek; North side</td>
<td>Near</td>
</tr>
<tr>
<td>8</td>
<td>DS1921Z-F5</td>
<td>N24 48.976 W76 18.751</td>
<td>10.5 10.5</td>
<td>Upper part of creek; Middle of creek</td>
<td>Far</td>
</tr>
<tr>
<td>9</td>
<td>DS1921Z-F5</td>
<td>N24 48.970 W76 18.730</td>
<td>22.6 19.0</td>
<td>Upper part of creek; Within large mangrove bush</td>
<td>Within shelter</td>
</tr>
<tr>
<td>10</td>
<td>DS1921Z-F5</td>
<td>N24 48.947 W76 18.715</td>
<td>18.0 15.0</td>
<td>Most upper reach of creek; no adult pufferfish were spotted beyond this point</td>
<td>Near</td>
</tr>
</tbody>
</table>
Fulton’s condition factor for control and cortisol dosed checkered puffers (*Sphoeroides testudineus*) before treatment (i.e. condition 1) and post-treatment, following the 20 day period inhabiting Page creek (i.e. condition 2). Boxes represent 25th and 75th percentiles with median enclosed within, and whiskers represent 10th and 90th percentiles.
Appendix B

B.1 Permission from co-authors

9:17 PM November 16, 2013
From: Felicia St-Louis <felicia.stlouis@gmail.com>
To: Cory Suski, Andy Danylchuk, Constance O’Connor, Aaron Shultz
Hello,
I would like to include the following two papers you co-authored as chapters 2 and 3 in my MSc thesis:
1) The relationship between the glucocorticoid stress response and anti-predator behaviours in checkered pufferfish (*Sphoeroides testudineus*)
2) Consequences of experimental cortisol manipulations on the thermal biology of the checkered pufferfish (*Sphoeroides testudineus*) in field and laboratory environments
I would be grateful if you could contact me with a short email to give your permission to include these papers in my thesis by Monday morning. I apologize for the late notice.
Thank you and I look forward to hearing from you.

10:39 PM November 16, 2013
From: Cory Suski
To: me
Hi. Permission granted – thanks for keeping this all moving forward.
Let me know if I can help & I’m looking forward to hearing back from the referees.

11:32 AM November 17, 2013
From: Andy Danylchuk
To: me
No prob.

7:23 PM November 17, 2013
From: Constance O’Connor
To: me
Permission granted.

10:00 AM November 18, 2013
From: Aaron Shultz
To: me
You have my permission to include these papers in your thesis. Good luck with your defense!
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