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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This manuscript has been submitted for review to the Journal of Functional Ecology. The project was conceived by Cooke, O'Connor and Cull with the support of Suski and Danylchuk. Field work was completed by Cooke, Shultz and Cull. All analysis conducted by Cull.

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 adrenocorticotrophic hormone and

93 are controlled by negative feedback of corticosteroids on the hypothalamic-pituitary axis
94 (Fryer and Peter 1977, Donaldson 1981, Wendelaar Bonga 1997). Cortisol is the
95 glucocorticoid in Actinopterygii that increases metabolic rate via several biochemical
96 processes (Idler and Truscott 1972, Hanson and Fleming 1979, Barton et al. 1998). The
97 synthesis and release of cortisol is delayed by several minutes and generally peaks within
98 0.5 to 1 hr after an acute stressor (Wedemeyer et al. 1990, Barton and Iwama 1991,
99 Gamperl et al. 1994). The secondary response consists of changes in metabolism,
100 hydromineral balance, as well as cardiovascular, respiratory and immune functions; for
101 instance, measurable changes in concentrations of blood glucose, lactate and major ions
102 (chloride, sodium, and potassium), and tissue levels of glycogen and heat shock proteins
103 (Pickering 1981, Iwama et al. 1997 and 1998, Mommsen et al. 1999). The tertiary
104 response consists of changes in performance, including growth, disease resistance, overall
105 health and behaviour (Wedemeyer and McLeay 1981, Wedemeyer et al. 1990). Primary
106 responses are sometimes directly responsible for secondary responses, and tertiary
107 responses result from the primary and secondary responses and may even influence an
108 individual's state of survival (Sumpter 1997, Hontela 1997, Wendelaar Bonga 1997).
109 Stress itself cannot be quantified. However, the response to stress can be measured
110 on the primary, secondary and tertiary levels to determine the amount of stress
111 experienced by a fish (Barton and Iwama 1991). Blood cortisol concentration is
112 commonly used as a physiological stress indicator as it is highly responsive to acute
113 stressors, easy to quantify, and its delayed release allows for proper sampling of resting
114 levels in fish (Wedemeyer et al. 1990, Barton and Iwama 1991, Gamperl et al. 1994,
115 Wendelaar Bonga 1997).

116 The stress response is separated into three categories – recognition of a threat to
117 homeostasis, the stress response, and the consequences of stress (Moberg 1985). These
118 responses are often considered adaptive, however, if the stressor is severe in intensity and
119 duration, responses may cause a state of distress and become detrimental or maladaptive
120 (Selye 1973, Deitinger and McCauley 1990, Barton and Iwama 1991). Stress response
121 can be influenced by genetic, developmental and environmental factors. The stress
122 response varies between and within species, develops early in life and may sharpen
123 during periods of metamorphosis. It is also influenced by almost all environmental
124 factors, both external and internal. Internal factors include features of the animal's overall
125 health, and external factors include a variety of abiotic and biotic variations imposed by
126 the inhabited ecosystem (see Barton 2002 for overview). This study will focus on biotic
127 and abiotic factors as these are most likely to be influenced by the pending global
128 warming phenomenon.
129 Early research on stress (cortisol in particular) in fish focused on identifying
130 factors that elicited a stress response and characterizing the elevation and eventual
131 recovery of cortisol to pre-stress levels. However, a recent framework for examining
132 stress in fish, though it has been used for some time with birds and reptiles, has been to
133 examine individual variation in baseline cortisol and maximal stress responsiveness in
134 order to understand the extent to which they are associated with various fitness-oriented
135 endpoints (e.g., Bonier et al. 2009). Such research is focused on elucidating the functional
136 significance of variation in responsiveness given the growing recognition that not all
137 individuals respond to stress in the same manner. For fish, baseline cortisol as well as
138 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 branchiostegal ray – and four cranial musculature features – muscles positioned near the articulation, the well-developed hyohyoideus abductor, protractor 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

207 comprising of 3 to 20 cycles and taking between 10 and 34 sec to reach full inflation.
208 Once the fish is fully inflated and its skin is tight to the touch, 3 to 4 more inflation
209 attempts are made before the fish accepts the resistance (Wainwright 1992). Upon
210 deflation, skin exerts little force to help the fish return to its original length as it is
211 inelastic. Water is expelled from the mouth by several repeated buccal cavity
212 compressions and lordotic movements (Brainerd 1994). Inflation has interested biologists
213 for some time (Thilo 1899, Parr 1927, Brainerd, 1994), however it has not yet been
214 studied as a stress response correlate.

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

224 The checkered puffer is an omnivorous benthic predator using its beak-like teeth
225 to feed mainly on crustaceans and molluscs likely during dawn and dusk, tidal extremes,
226 and in non-mangrove habitats (Targett 1978, MacDonald et al. 2009). Other prey items
227 include sipunculids, tunicates, seagrass, and detritus. Diet varies with size as larger
228 puffers (>150 mm) have a more powerful bite and are able to feed on a larger variety of
229 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 al. 1986, Pauly 1991, Figueiredo and Menezes 2000, Vendel et al. 2002, Spach et al. 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, 2004 and 2006, Prodocimo et al. 2008, Contente et al. 2011, Lopes 2000, Vega-Candejas and de Santillana 2004). The checkered puffer efficiently regulates its plasma osmolality

252 and chloride concentration in decreasing salinities of 29.5, 14, and 4.5ppt, along a 5 to 6
253 hr period (Prodocimo and Freire 2001, 2004 and 2006, Prodocimo et al. 2008).
254 Independent of all other water conditions, salinity has been shown to be the main
255 factor structuring fish assemblages in several estuarine systems (Blaber 1997, Jaureguizar
256 et al. 2004, Paperno and Brodie 2004, Barletta et al. 2005, Sosa-Lopez et al. 2007,
257 Mendoza et al. 2009, Contente et al. 2011). Other factors influencing estuarine fish
258 abundance and richness include tide regime (Barletta et al. 2003), dissolved oxygen
259 (Barletta et al. 2008), turbidity (Marchand 1993), aquatic vegetation (Castellanos and
260 Rozas 2001), food availability (Scharf et al. 2004, Martinetto et al. 2005), and sediment
261 type (Scharf et al. 2004). Nevertheless, global warming will create yet another
262 environmental challenge influencing coastal ecosystems – temperature. Temperature is
263 one of the most prevalent conditions influencing biological processes thereby impacting
264 the performance of organisms and overall fitness (Haynie 2001, Brown et al. 2004,
265 Angilletta et al. 2006).
266 The checkered puffer is an interesting model for studying stress to environmental
267 change as they reside in habitats prone to high fluctuations in temperature independent of
268 global climate change over their entire lifespan. Unlike other coastal species, all life
269 history stages of the checkered puffer are subjected to the extremes imposed by their
270 coastal environment. On top of their high tolerance to external environmental changes,
271 the checkered puffer also displays remarkable defensive behaviours, including biting
272 performance and the unique predator avoidance strategy of “puffing”.
273
274

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.

366 1990, Barton and Iwama 1991, Gamperl et al. 1994), making it relatively easy to quantify
367 elevated post-stress levels.

368 Increased glucocorticoid (GC) release in response to a stressor is thought to
369 promote survival through heightened performance during a challenge (e.g., facilitating
370 escape from acute stressors), as well as to influence recovery once the challenge has been
371 overcome (Pagnotta et al. 1994, Wingfield et al.1998, Sapolsky et al. 2000, Breuner et al.
372 2008). While these responses are considered adaptive on a short time scale, if the stressor
373 is severe in intensity and duration, increased GC release may also come at a cost to other
374 functions, such as performance, immunocompetency, disease resistance, growth, overall
375 health and reproduction (Wedemeyer and McLeay 1981, Wedemeyer et al. 1990, Barton
376 and Iwama 1991, Sapolsky et al. 2000, Romero et al. 2009).

377 The implications of individual variation in GC secretion on performance and
378 overall fitness are complex (see reviews by Ricklefs and Wikelski 2002, Bruener et al.
379 2008, Bonier et al. 2009). Increased baseline GCs through diet manipulation are highly
380 correlated with heightened performance and increased survival in the mountain chickadee
381 (*Parus gambelii*; Saldanha et al. 2000), captive white-crowned sparrow (*Zonotrichia*
382 *leucophrys gambelii*; Lynn et al. 2003), and rainbow trout (*Oncorhynchus mykiss*; Overli
383 et al. 2002). However, elevated post-stress GCs have also been found to have no effect on
384 fitness (e.g., Moore et al. 2000), and even negative effects on measures of fitness (e.g.,
385 Blas et al. 2007, Roberts et al. 2007). Furthermore, measures of fitness have been found
386 to be influenced by elevated baseline GCs (e.g., Brown et al. 2005) and manipulated GCs
387 (e.g., Saino et al. 2005, Wada and Breuner, 2008). Much of the GC research performed to
388 date has examined individual variation in stress response and the associated fitness-

389 oriented endpoints using birds (Angelier et al. 2007, Groscolas et al. 2008, Williams et al.
390 2008), reptiles (Romero and Wikelski, 2001, Meylan and Clobert 2005, Lancaster et al.
391 2008) and mammals (Pride 2005, Cabezas et al. 2007, Rogovin et al. 2008) as model
392 species, with considerably less GC work done in fish (see Breuner et al. 2008 and Bonier
393 et al. 2009 for overviews, McConnachie et al. 2012). Such research is focused on
394 elucidating the functional significance of variation in GC secretion given the growing
395 recognition that not all individuals respond to stress in the same manner.
396 The checkered puffer (*Sphoeroides testudineus*) is an interesting model for
397 studying stress in an ecological context because of its unique predator avoidance
398 strategies, which are readily measurable and have direct implications for individual
399 survival and fitness. The 'puffing' of pufferfish serves as effective predator avoidance
400 behaviour by increasing body size and making them difficult to subdue and consume
401 (Randall 1967, Recher and Recher 1968, Myer 1989). In this study, puffing will be
402 measured in terms of intensity over time, and the time at which it takes the fish to deflate
403 once released. Checkered puffers are also durophagous, feeding on hard-shelled prey. In
404 all durophagus vertebrates, bite force is an important component of feeding performance
405 (Wainwright 1988, Hernandez and Motta 1997, Grubich 2005, Berumen and Pratchett
406 2008) and expanding dietary range (see Mara et al. 2010 for overview). Increased bite
407 force allows exploitation of prey unavailable to conspecifics and other species
408 (Hernandez and Motta 1997, Berumen and Pratchett 2008), thereby potentially reducing
409 inter- and intraspecific competition and increasing fitness (Wainwright 1988, Grubich
410 2005). Bite force has also been studied extensively in the reptile field as an indicator of
411 fitness, specifically dewlap size and combat success in several Anolis lizard species

412 (Vanhooydonck et al. 2005, Lailvaux and Irschick 2007), and as a strong correlate to
413 increased dietary range, body condition and reproductive output in the northern map turtle
414 (*Graptemys geographica*; Bulte et al. 2008). Furthermore, bite-force has been studied as
415 temperature-dependent anti-predator behavioural correlate in reptiles (Greene 1988, Hertz
416 et al. 1982). As bite force has been linked to measures of fitness in vertebrates, I predict
417 that bite force will be an accurate fitness proxy for durophagous fish. While the
418 relationship between the GC stress response and fitness has not been well-documented in
419 fish (see reviews by Breuner et al. 2008, Bonier et al. 2009), the GC stress response has
420 been shown to be repeatable through time, and to be correlated with individual size,
421 behavioural traits, reproductive success and overall fitness (Cook et al. 2011a, 2011b,
422 O'Connor et al. 2012). To date for fish, relationships between GC responsiveness and
423 fitness endpoints have been observed in Pacific salmon (*Oncorhynchus nerka* and *O.*
424 *gorbuscha*; McConnachie et al. 2012, Cook et al. in review). Therefore, to build on the
425 foundational syntheses published by Breuner et al. (2008) and Bonier et al. (2009), I
426 attempted to define the relationship between GC stress response and two established
427 fitness proxies in the checkered puffer: inflation performance (i.e., puffing intensity and
428 time to deflate) and bite force.
429

430 **2.3 Methods**

431

432 **2.3.1 Study site and sampling**

433 Between February 22-25, and June 1-12, 2012, checkered puffers (n=110) were collected
434 from Plum and Page Creeks, on Eleuthera Island, Bahamas (Plum: N24°45'45.79"

W076°15'6.65", Page: N24°49'04.7" W076°18'51.6"). 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±2.7ºC), 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

458 portions of the study. To generate this profile, pufferfish were placed in individual opaque
459 experimental chambers (12.5 L) with ample aeration and a constant flow of saltwater 24 h
460 before experimentation. Fish were then randomly assigned to one of six treatment groups:
461 (1) control (n=8), (2) 15 min post-stressor (n=7), (3) 30 min post-stressor (n=8), (4) 1 h
462 post-stressor (n=7), (5) 2 h post-stressor (n=7), and (6) 4 h post-stressor (n=5). Pufferfish
463 in each of the treatment groups were subjected to an acute standardized stressor by
464 holding them at the air-water interface for 5 min in a rubber-mesh dip net, and then
465 returning them to their individual chambers for the designated duration. Fish in each
466 treatment were then non-lethally sampled for 0.5 mL of blood by caudal venipuncture
467 using a heparinized 1-mL syringe and 21-gauge, 2.5-cm needle. To avoid sampling-
468 induced stress, each blood sample was withdrawn in under 3 min (Romero and Reed
469 2005). Control fish remained in their chambers for 24 h, but received no net holding.
470 Collected blood samples were held in syringes in water-ice slurries for no more than 1 h
471 before analysis. Based on data from this series of preliminary samples (n=48), I
472 determined that maximum values of stress-induced GC concentrations occurred 30 min
473 post-stressor (Fig. 1); all sampling for maximal cortisol concentrations during successive
474 trials therefore occurred 30 min after the onset of a stressor.
475

476 **2.3.3 Glucocorticoid responsiveness relative to two fitness proxies**

477 To identify the relationship between GC stress response and fitness proxies, pufferfish
478 (n=48) were collected, held, and placed in individual opaque experimental chambers
479 (12.5 L) as described above. After 12 hours of acclimation to these chambers, all fish
480 were air-exposed for 3 min, during which time their baseline bite force (N) was measured

481 with a custom built force transducer system composed of a load cell and a custom built
482 DC amplifier. The load cell was constructed from a (75 × 12 × 12 mm) aluminum block
483 with material removed from the center portion to create a thin-walled (1mm) c. 15 mm
484 long, channel. Loads applied to one end of the aluminum block therefore caused
485 deformation of the thinned regions in the centre that were detected by thin-foil type
486 resistive strain gauges bonded to adjacent surfaces of the block at the thinned regions. The
487 paired strain gauges were connected in a Wheatstone bridge configuration. The amplifier
488 unit supplied an excitation voltage to the bridge and changes in resistance of the strain
489 gauges produced a change in voltage proportional to the load applied to the cell. A
490 multimeter (Agilent True RMS Multimeter, Model U1233A) was used to display voltage
491 changes from the load cell. The bite force meter was calibrated using a series of loads of
492 known weight. The calibrated output of the unit was linear and the drift due to thermal
493 instability was small (less than 0.05 % of full scale). All pufferfish were also sampled for
494 0.5 mL of blood within this 3 min period, which served as a baseline sample.
495 In addition, the intensity of the fish's 'puff' over the course of the 3 min sampling
496 period was monitored to generate a baseline puff score. Puffs were assigned a score from
497 0 to 3, with 0 being no puff, 1 being equal to or less than half a full puff, 2 being greater
498 than half a full puff, and 3 being a full puff; a full puff was assigned once the fish was
499 maximally inflated (i.e., the fish's skin was tight to the touch and subsequent inflation
500 attempts resulted in no further expansion). Each puff score (0-3) was assigned a
501 percentage of time used over the 3 min, and then weighted according to its score. As a
502 result, each puff score is presented as a value between 0 and 3 (i.e., 0 being no puff at all
503 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 -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, 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:

$$K = 100 \times (W/L3);$$

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 ± 31.0ng ml-1 30 min post-stressor, and a subsequent return to baseline levels by the 1 h time point (Fig. 2.1 A). 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. 2.1 B). 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

573 increased 2-fold relative to pre-stress concentrations (Table 2.1). In contrast, fitness
574 proxies (bite force and puff score) decreased following a standardized stressor relative to
575 pre-stress performance (Table 2.1, Figs 2.2 A and B). While there was a similar trend for
576 puff time to deflate once released, this trend was not statistically significant (Table 2.1).
577 Baseline and post-stress puffing performances, including puff scores and puff times to
578 deflate once released, were significantly correlated with one another (two-tailed Pearson
579 correlations: $R = 0.389$, $P < 0.05$, and $R = 0.379$, $P < 0.05$, respectively; Table 2.1),
580 indicating that these fitness proxies are repeatable through time. The correlation between
581 baseline and post-stress bite force exhibited similar trends (two-tailed Pearson
582 correlations: $R = 0.315$, $P = 0.054$; Table 2.1).
583 Prior to the standardized air exposure challenge, checkered puffers did not
584 demonstrate any significant relationships among baseline values of different fitness
585 proxies (bite force, puff score, and puff time to deflate once released; Table 2.2).
586 Following a 5 min air exposure challenge, however, post-stress fitness proxies of
587 checkered puffers showed significant correlations. Specifically, checkered puffers with
588 elevated post-stress puff scores also showed stronger post-stress bite forces and longer
589 times to deflate (Table 2.2, Figs 2.3 A and B).
590 The total length, body mass and condition of the checkered puffers did not
591 significantly influence baseline physiological indices and baseline fitness proxies, with
592 the exception of bite force (Table 2.3). Following a standardized 5 min stressor, however,
593 longer and heavier fish did produce higher levels of cortisol and glucose, and exhibited
594 significantly stronger bite forces and higher puff scores (Table 2.3). In addition, pufferfish

595 that had higher condition scores also demonstrated lower post-stress glucose levels (Table
596 2.3).

597 I found that larger checkered puffers with lower post-stress concentrations of
598 cortisol and glucose exhibited significantly stronger post-stress bite forces ($R^2 = 0.165$, F
599 = 4.667, $P < 0.05$; Figs 2.3 C and D). Also, I found that larger pufferfish with higher
600 baseline cortisol concentrations and higher post-stress glucose concentrations exhibited
601 greater puff score responsiveness (i.e. Significantly lower post-stress puff scores
602 compared to that exhibited in the baseline treatment; $t = -2.057$, $P < 0.05$ and $t = -2.396$, P
603 < 0.05, respectively). Physiological metrics were not related to other aspects of fitness
604 proxies (see Supplemental Materials). Consequences of the physiological responsiveness
605 (i.e., the difference between stress-induced and resting cortisol and glucose levels) on
606 checkered puffer performances before and after the standardized stressor were also
607 examined. When accounting for the total length of the fish, physiological responsiveness
608 had no significant influence on changes in fitness proxies (regressions: $P > 0.05$), and
609 appeared to influence the bite force of stress-induced checkered puffers, although the
610 relationship was not statistically significant ($R^2 = 0.338$, $F = 5.774$, $P = 0.089$).
611 Nevertheless, total length of checkered puffers was found to predict over 50 % of the
612 change in baseline bite forces ($R^2 = 0.509$, $F = 37.278$, $P < 0.001$; Fig. 2.3 A), over 20 %
613 of the change in post-stress bite forces ($R^2 = 0.236$, $F = 11.131$, $P < 0.01$; Fig. 2.3 A), and
614 nearly 30 % of the change in post-stress puff scores ($R^2 = 0.281$, $F = 14.047$, $P < 0.001$;
615 Fig. 2.3 B).
616
617

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

641 that larger checkered puffers with higher post-stress cortisol concentrations and lower
642 post-stress glucose concentrations exhibited significantly stronger post-stress bite forces.
643 Physiological metrics were not related to other aspects of fitness proxies. When
644 accounting for the total length of the fish, physiological responsiveness had no significant
645 influence on changes in fitness proxies.

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

653 Following a 5 min air exposure challenge, the physiological stress response of the
654 checkered puffer was characterized by increased plasma cortisol and blood glucose
655 concentrations; results comparable to studies with other teleost fish (Barton 2002).
656 Checkered puffers with elevated plasma cortisol and glucose levels post-stress displayed
657 significantly weaker bite forces before and after the stressor, as well as lower puff scores
658 following the stressor. These findings establish an unexpected negative relationship
659 between the GC response and fitness related endpoints, where fitness proxies significantly
660 decrease following an acute stressor. Similar negative relationships between the GC
661 response and fitness related endpoints have been documented in the European white stork
662 nestlings (*Ciconia ciconia*; Blas et al. 2007) and the zebra finch (*Taeniopygia guttata*;
663 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

687 over the following winter (Mackereth et al. 1999). Similarly, somatic energy reserves
688 greatly decrease in Atlantic salmon (*Salmo salar*) during upstream migration and
689 spawning (Johnsson et al. 1997). Although significant differences were not established
690 between baseline and post-stress puff time to deflate once released, there is a considerable
691 increase worth noting. Puff time data likely yielded insignificant results due to the
692 immense variability displayed in the post-stress treatment. Over the course of the
693 experiment, trends of shorter baseline puff times and longer post-stress puff times were
694 apparent. Puff time to deflate once released may be associated with reduced fitness as this
695 behaviour will inhibit the pufferfish from escaping the predator, in this case, the
696 experimenter.

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

707 Total length and mass of the checkered puffer were significantly predictive of
708 both baseline and stress-induced bite force, as well as stress-induced puff scores. Many
709 performance measures were exclusively influenced by the size of the checkered puffer.

710 Following the 5 minute air exposure challenge, checkered puffers with increasing
711 physiological responsiveness (i.e., a large difference between pre- and post-stress cortisol
712 and glucose levels) displayed significantly weaker baseline bite forces, as well as weaker
713 bite forces, lower puff scores, and shorter puff times once released following the
714 standardized stressor. However, when accounting for the total length of the fish, the
715 physiological responsiveness of the checkered puffer has no significant influence on
716 changes in performance before nor following the standardized challenge. The size of the
717 checkered puffer predicted up to 50% of the change in performance observed. Bite force
718 is often strongly associated with the size of an animal as larger individuals generally have
719 larger jaw structures and thus stronger bite forces (e.g., Wainwright et al 2004, Grubich et
720 al. 2008). Similarly, larger pufferfish seemed to hold stronger ‘puffs', although it is
721 currently unclear why that was the case. Fulton’s condition factor (K) is considered as a
722 long-term indicator of an individual’s general well-being (Suthers, 2000), and therefore
723 was only measured once for each checkered puffer. As a result, the change in condition of
724 the individual pufferfish between baseline and stress-induced treatment groups could not
725 be monitored. However, the predictive value of the pufferfish’s general condition (i.e.,
726 Fulton’s condition factor) to successfully respond to an acute stressor could be examined.
727 Fulton’s condition factor was only found to be significantly predictive of stress-induced
728 glucose and glucose responsiveness levels. In response to an acute stressor, fish with
729 greater energy reserves (inferred from Fulton’s condition factor) released less glucose
730 when compared to fish in lower condition.
731 To effectively quantify the relationship between individual variation in GC stress
732 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.

	Mean±SE			Paired t-test		Two-tailed Pearson correlation	
	Baseline	Post-stress	df	*t-statistic*	*P*	*R*	*P*
Cortisol (ng ml^{-1})	16.3±4.6	198.4±23.7	37	12.507	**<0.001**	-0.210	0.109
Glucose (mmol L^{-1})	1.1±0.0	3.2±0.1	37	17.372	**<0.001**	0.258	0.062
Bite force (N)	78.5±3.5	53.4±4.5	37	5.258	**<0.001**	0.315	0.054
Puff score	2.1±0.1	1.7±0.1	37	3.132	**0.003**	0.389	**0.016**
Puff time to deflate once released (min)	79±15	321±184	37	1.324	0.194	0.379	**0.019**

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

	R		
	Bite force (N)	Puff score	Puff time to deflate once released (min)
Baseline treatment			
Bite force (N)		0.053	-0.094
Puff score			0.201
Puff time to deflate once released (min)			
Post-stress treatment			
Bite force (N)		0.573***	0.062
Puff score			0.383*
Puff time to deflate once released (min)			

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

778

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.

	Total length (mm)		**Mass (g)**		**Fulton's condition factor (mg mm^{-3})**	
	F	R^2	*F*	R^2	*F*	R^2
Baseline treatment						
Cortisol (ng ml^{-1})	0.876	0.024	0.940	0.025	0.029	0.001
Glucose (mmol L^{-1})	0.658	0.018	0.440	0.012	0.123	0.003
Bite force (N)	37.278	0.509***	26.558	0.425***	0.223	0.006
Puff score	2.426	0.063	1.684	0.045	0.302	0.008
Puff time to deflate once released (min)	0.181	0.005	0.261	0.007	0.403	0.011
Post-stress treatment						
Cortisol (ng ml^{-1})	4.439	0.110*	6.789	0.159*	1.757	0.047
Glucose (mmol L^{-1})	6.214	0.147*	13.096	0.267**	10.047	0.218**
Bite force (N)	11.131	0.236**	9.294	0.205**	0.001	0.000
Puff score	14.047	0.281**	15.265	0.298***	0.495	0.014
Puff time to deflate once released (min)	0.263	0.007	0.237	0.007	0.035	0.001

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

2.7 Figures

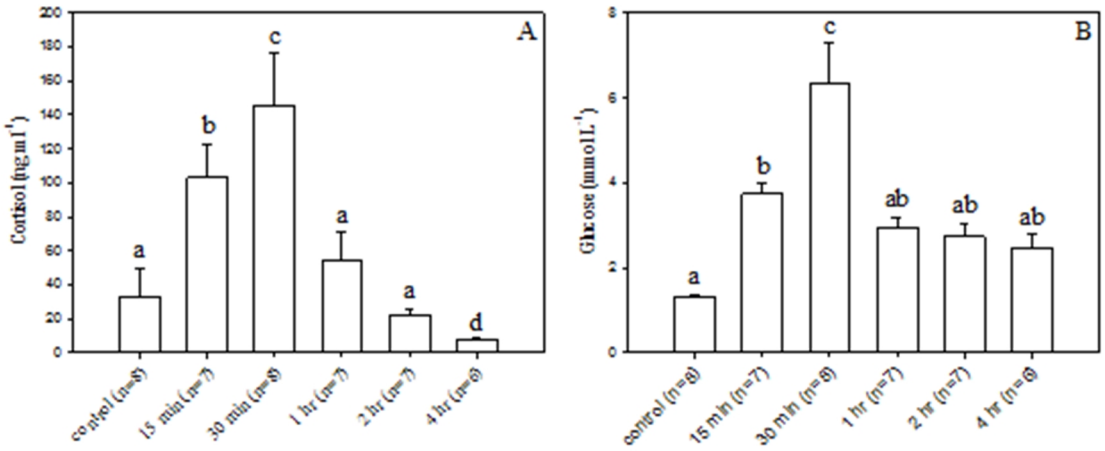
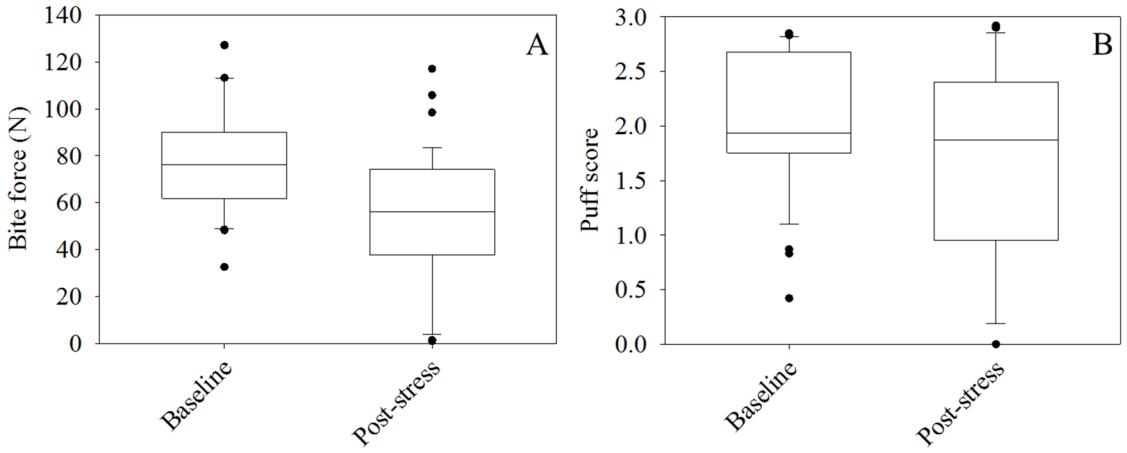


Figure 2.1 Plasma cortisol (A) and blood glucose (B) secretion and recovery in the checkered puffer (*Sphoeroides testudineus*) following a 5 min standardized stressor. Error bars represent standard error from the mean, and different letters indicate statistically significant differences among sampling time points (Tukey's HSD post-hoc test following a significant ANOVA; α=0.05)



799

800 **Figure 2.2** Baseline and post-stress bite force (A) and puff score (B) of the the checkered
801 puffer (*Sphoeroides testudineus*). Baseline values were collected from acclimated
802 pufferfish within 3 min. Fish were then immediately 'stressed' by holding them at the air-
803 water interface for 5 min in a rubber-mesh dip net. Pufferfish were then resampled 30 min
804 post-stressor to collect post-stress values. A total of 38 fish were sampled. Boxes
805 represent 25th and 75th percentiles with median enclosed within, and whiskers represent
806 10th and 90th percentiles.

807

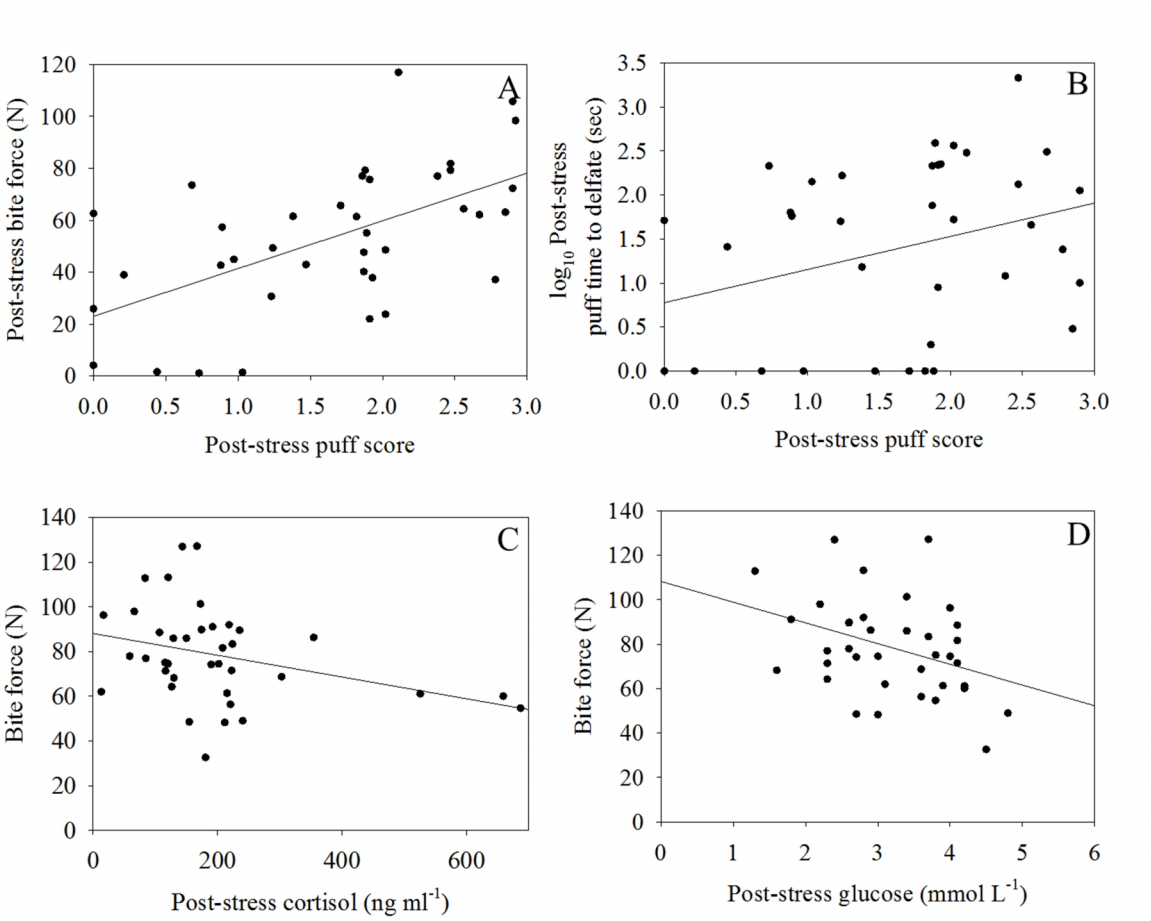


Figure 0.1 Correlations among baseline and post-stress fitness proxies of the checkered puffer (*Sphoeroides testudineus*). Significant two-tailed correlations were found between post-stress puff score and bite force ($R = 0.573$; $P < 0.001$; A), as well as between post-stress puff score and puff time 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 ºC 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";

Plum: N24°45'45.79" W076°15'6.65"; Kemps: N24 48'54.29" W76 18'03.09"; Fig. 1). 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 ± 2.0ºC), 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

955 field-based studies using cortisol manipulations in fish (e.g., Dey et al. 2010,
956 McConnachie et al. 2012a and 2012b, O'Connor et al. 2012). Preliminary tests in
957 pufferfish, however, indicated that intra-peritoneal injection would not work for this
958 species due to its ability to inflate and deflate its intra-peritoneal cavity. The pufferfish
959 generally inflate upon injection, and eject the cortisol through the mouth and gills upon
960 deflation. Based on these observations, an intra-muscular injection of the dorsal muscle
961 anterior to the dorsal fin was used to experimentally administer exogenous cortisol to the
962 checkered puffer. All treatment fish were weighed using a portable electronic balance,
963 and then placed in water-filled foam-lined trough to obtain a total length (TL)
964 measurement, and for injection with 5 mL of cocoa butter per kg of fish body weight.
965 Sham treatment fish were injected with heated pure cocoa butter only. Low-dose cortisol
966 treatment fish were injected with 5 mg mL^{-1} cortisol in heated cocoa butter (i.e., 25 mg
967 kg^{-1} fish body weight), while high-dose cortisol treatment fish were injected with 10 mg
968 mL^{-1} cortisol in heated cocoa butter (i.e., 50 mg kg^{-1} fish body weight). Low and high
969 cortisol dose concentrations were chosen based on the work done on smallmouth bass
970 (*Micropterus dolomieu*; Dey et al. 2010), largemouth bass (*M. salmonides*; O'Connor et
971 al. 2012) and bluegill sunfish (*Lepomis macrochirus*; McConnachie et al. 2010). Control
972 fish received no injection, but were handled in an identical manner to the treatment fish.
973 Using a rubber-mesh dip net, all fish were placed in individual opaque experimental
974 chambers (12.5 L) supplied with aeration and a constant flow of saltwater within 10 s of
975 treatment. After 48 hrs, to determine circulating cortisol levels, fish were non-lethally
976 sampled for 0.5 mL of blood by caudal venipuncture using a heparinized 1-mL syringe
977 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±1.5ºC), 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 (*Lepomis macrochirus*; McConnachie et al. 2012b), bonefish (*Albula spp.*; Shultz et al. 2011) and two species of cardinalfish (*Ostorhinchus doederleini* and *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±1.5ºC), checkered puffers were randomly assigned to the following treatment groups: control at 1) ambient temperature (n=8); 2) -5ºC from ambient temperature (n=6); and 3) +5ºC from ambient temperature (n=8); 4) cortisol treatment at ambient temperature (n=8); 5) cortisol treatment at -5ºC from ambient temperature (n=6); and 6) cortisol treatment at +5ºC 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 ± 1ºC 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 ºC 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 ºC, while the others (model no. DS1921Z) had a range of -5 to 26 ºC. Factory-stated resolution of all thermal loggers is 0.125 ± 1 °C; previous calibration by our team

1070 reveals actual mean accuracy of 0.4 ± 0.3 ºC and mean precision of 0.2±0.0 ºC
1071 (Donaldson et al. 2009).
1072 On December 31, 2012, pufferfish were tagged with either unmodified iButtons
1073 (model nos. DS1921H and DS1921Z; n=18) and iButtons that were miniaturized
1074 according to Lovegrove (2009; model no. DS1921H; n=19). All iButtons were covered in
1075 Plasti Dip, and fastened to a backing plate. All iButtons were set to log temperature every
1076 30 min over a 20-day period. Fish were randomly assigned to one of two treatment
1077 groups: 1) control (n=19); and 2) cortisol treatment (n=18). Thermal loggers were
1078 randomly distributed between groups and were externally attached to the dorsal surface of
1079 the fish, immediately posterior to the dorsal fin on the caudal peduncle (Thiem et al.
1080 2013). Following iodine disinfection, two hypodermic stainless steel needles (16 gauge)
1081 were pushed through the dermis and 9 kg monofilament line (previously inserted through
1082 the tag via pre-made holes) was passed through the lumen of the needles and secured
1083 using multiple knots (see Thiem et al. 2013 for tagging validation of checkered puffer).
1084 Cortisol-treated fish were then weighed, measured, and given a 5 mL kg^{-1} intramuscular
1085 injection of 10 mg mL^{-1} cortisol in cocoa butter as described above. Control fish were
1086 handled identically, but were not given injections. All fish were released in Page Creek
1087 (N24°49'1.90" W76°18'48.80") upon recovery. After fish were at liberty for a 20-day
1088 period, control (n=10) and cortisol-treated pufferfish (n=13) were recovered from Page
1089 Creek on January 19, 2013. The recapture rate for control and cortisol-treated fish was
1090 58% and 72%, respectively. Despite promising trials on the benchtop, modified iButtons
1091 failed to log temperature 70% of the time when deployed in the field. Therefore, of the
1092 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 (MO_2) 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 ºC below, and 5 ºC 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 26ºC, all temperature recorded values equal to or above 26ºC were identified and marked as 26ºC. The proportion of temperature values equal to or above 26ºC 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 26ºC 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 26ºC were considered 26ºC. 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-days following deployment) as an indicator of general well being using the following equation:

$$K = 100 \times (W/L^3);$$

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 5ºC 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: $Ps > 0.05$; 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 then 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 ± 5ºC) were 6ºC cooler when compared to control fish (1089 ± 6ºC), and 9ºC cooler than that logged by the habitat (1092 ± 4ºC; Table 5).

3.4.8 Field experiment: Change in body condition

The initial condition of control and cortisol-treated puffers (2.05 ± 0.09 and 2.05 ± 0.07 mg mm^{-3}, respectively) was similar to their final condition (1.97 ± 0.06 and 1.91 ± 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 ºC 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.

1976, Donaldson et al. 2008 for overview). 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 then 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

1392 possible trade-offs made by the pufferfish – chronically stress-induced fish may have
1393 selected refuge in the mangrove habitat, away from intra- and inter-specific competition,
1394 regardless of sub-optimal fluctuations in temperature. In addition, elevated levels of
1395 cortisol are known to suppress levels of hsps in several species of fish (Ackerman et al.
1396 2001, Basu et al. 2001, Basu et al. 2001), suggesting that cortisol may mediate hsp levels
1397 following times of physiological stress (see Basu et al. 2001 and 2002 for details). This
1398 may explain why cortisol implanted pufferfish selected cooler temperatures in the wild.
1399 Although a severe metabolic cost was not associated with the cortisol implant,
1400 cortisol dosed pufferfish may have selected cooler temperatures to reduce metabolic
1401 energy expenditure when subjected to the additional stressors of their natural
1402 environment. Few fish studies have documented thermal preferences in the field,
1403 however, Roscoe et al. (2010) found that reproductively advanced female sockeye salmon
1404 (*Oncorhynchus nerka*) with lower levels of energy similarly selected cooler temperatures
1405 compared to less mature females with high levels of energy, possibly to reduce metabolic
1406 energy expenditure and delay final maturation. Similar to fish, stressed reptiles respond
1407 with increases in plasma glucocorticoid levels (i.e., corticosterone), affecting the animal's
1408 physiology and behaviour (Greenberg and Wingfield, 1987). Increased levels of
1409 glucocorticoids have been associated with a variety of consequences in lizards, including
1410 exposure to predation (Montgomerie and Wheatherhead 1988), a reduction in fat stores
1411 (Guillette et al. 1995), and immune system depression (Zuk 1996, Oppliger et al. 1998).
1412 Furthermore, increased levels of glucocorticoids due to experimental corticosterone
1413 manipulation have been found to increase activity and thermoregulation in the common
1414 lizard (*Lacerta vivipara*; Belliure et al. 2004), enhance locomotor activity and reduce

1415 thermoregulatory behaviour in juvenile wall lizards (*Podarcis muralis*; Belliure and
1416 Colbert, 2004), and increase metabolic rate and increase thermoregulatory behaviour in
1417 females of the New Zealand common gecko (*Hoplodactylus maculatus*; Preest and Cree
1418 2008). Although we did not measure any of those specific endpoints here, our data
1419 support the idea that cortisol-treated pufferfish selected marginally cooler temperatures to
1420 potentially reduce further metabolic costs and seek refuge from predation or other forms
1421 of competition. Curiously however, we were unable to document any significant burden
1422 linked to the cortisol implant in the laboratory, suggesting that metabolic costs were not
1423 measurable in terms of aerobic activity 48 hrs post-treatment.

1424 Although a slight decrease in fish condition was observed over the 20 day period,
1425 the decline in condition was not found to be significant, nor to be significantly dissimilar
1426 between control and cortisol-treated pufferfish. The minor decline in condition across
1427 control and cortisol-treated fish is likely due to the handling stress of the experiment and a
1428 small tagging burden (Thiem et al. 2013).

1429 As the checkered puffer has been observed to rarely venture out of the study
1430 creek, we can likely assume the recapture rate is a measure of survival. The recapture rate
1431 for control and cortisol dosed fish was 58% and 72%, respectively; indicating that cortisol
1432 implanted fish may have a better survival rate than controls. Cortisol implanted fish may
1433 have sought refuge due to the unidentified burden of the implant, taking fewer risks and
1434 thus suffering less predation than controls. Other plausible speculations include the notion
1435 that control pufferfish were more mobile and thus less likely to be in the same area at the
1436 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.

3.6 Tables

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.

Fish treatment group	TL (mm)	*F*	*P*	Mass (g)	*F*	*P*
Validation of cortisol implant dose						
Control	186 ± 5	1.136	0.358	149 ± 9	0.966	0.427
Sham	182 ± 8			155 ± 12		
Low-dose	180 ± 6			140 ± 14		
High-dose	176 ± 4			130 ± 7		
Validation of cortisol implant depletion timeline						
Control	184 ± 5^{a}	4.826	**0.005**	145 ± 9^{ab}	5.444	**0.002**
2 days post-implant	176 ± 4^{a}			130 ± 7^{a}		
5 days post-implant	178 ± 5^{a}			105 ± 6^{a}		
10 days post-implant	191 ± 10^{ab}			136 ± 12^{ab}		
20 days post-implant	215 ± 11^{b}			192 ± 27^{b}		
Metabolic cost and swimming performance						
Control	152 ± 4	0.004	0.948	74 ± 3	10.605	**0.006**
Cortisol-dosed	152 ± 11			93 ± 5		
Lab experiment: Thermal tolerance						
Control at *amb. T*	182 ± 4	1.333	0.271	142 ± 8^{a}	2.744	**0.033**
Control at -5ºC	187 ± 6			124 ± 13^{ab}		
Control at +5ºC	191 ± 7			137 ± 14^{ab}		
Cortisol-dosed at *amb. T*	175 ± 4			130 ± 6^{ab}		
Cortisol-dosed at -5ºC	181 ± 2			106 ± 5^{ab}		
Cortisol-dosed at +5ºC	176 ± 6			102 ± 10^{b}		
Field experiment: Thermal preference						
Deployed control	183 ± 3	0.084	0.969	125 ± 4	0.131	0.941
Deployed cortisol-dosed	185 ± 4			130 ± 9		
Recaptured control	184 ± 3			124 ± 7		
Recaptured cortisol dosed	186 ± 5			125 ± 12		

1462 **Table 3.2** Checkered puffer (*Sphoeroides testudineus*) hematocrit levels at 5-, 10- and 20-

1463 days post-implantation.

Fish treatment group	Hematocrit (%)
5 days post-implant	19.16 ± 1.64
10-days post-implant	19.25 ± 4.36
20 days post-implant	23.49 ± 2.61

1464

1465

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 5ºC 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).

	R^2	Adjusted R^2	DF	F	
Cortisol					
Corrected model	0.453	0.381	5	6.288	***
Fish treatment			1	15.962	***
Thermal treatment			2	1.412	
Fish treatment * Thermal treatment			2	4.540	*
Glucose					
Corrected model	0.876	0.860	5	53.772	***
Fish treatment			1	3.609	
Thermal treatment			2	130.217	***
Fish treatment * Thermal treatment			2	2.955	
Hematocrit					
Corrected model	0.011	-0.113	3	0.085	
Fish treatment			1	0.043	
Thermal treatment			1	0.149	
Fish treatment * Thermal treatment			1	0.077	
Puff score					
Corrected model	0.521	0.461	3	8.695	***
Fish treatment			1	0.160	
Thermal treatment			1	25.713	***
Fish treatment * Thermal treatment			1	0.160	
Puff time to deflate once released					
Corrected model	0.211	0.112	3	2.134	
Fish treatment			1	0.303	
Thermal treatment			1	5.696	*
Fish treatment * Thermal treatment			1	0.303	

*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$

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.

	+5 °C		-5 °C	
	Control	**Cortisol implanted**	**Control**	**Cortisol implanted**
Puff score	1.29 ± 0.29 [a]	1.15 ± 0.22 [a]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]
Puff time to deflate once released (sec)	97 ± 49 [a]	31 ± 11 [ab]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]
Hematocrit (%)	24.00 ± 2.36	24.10 ± 2.21	22.69 ± 1.71	25.77 ± 2.95

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.

		Temperature metrics		
Treatment	**ATUs**	**Minimum**	**Maximum**	**Range**
Control fish	1089.25 ± 6.01	19.85 ± 0.13	25.65 ± 0.40	4.81 ± 0.55
Dosed fish	1082.80 ± 4.56	19.88 ± 0.13	25.82 ± 0.40	4.82 ± 0.51
Habitat	1092.07 ± 4.27	20.00 ± 0.12	25.67 ± 0.28	4.61 ± 0.38

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.

	DF	F	
ATUs			
Day	19	71.175	***
Day * Treatment	38	2.663	***
Minimums			
Day	19	2447.665	***
Day * Treatment	38	1.216	
Maximum			
Day	19	100.510	***
Day * Treatment	38	.863	
Ranges			
Day	19	61.471	***
Day * Treatment	38	.862	

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

3.7 Figures

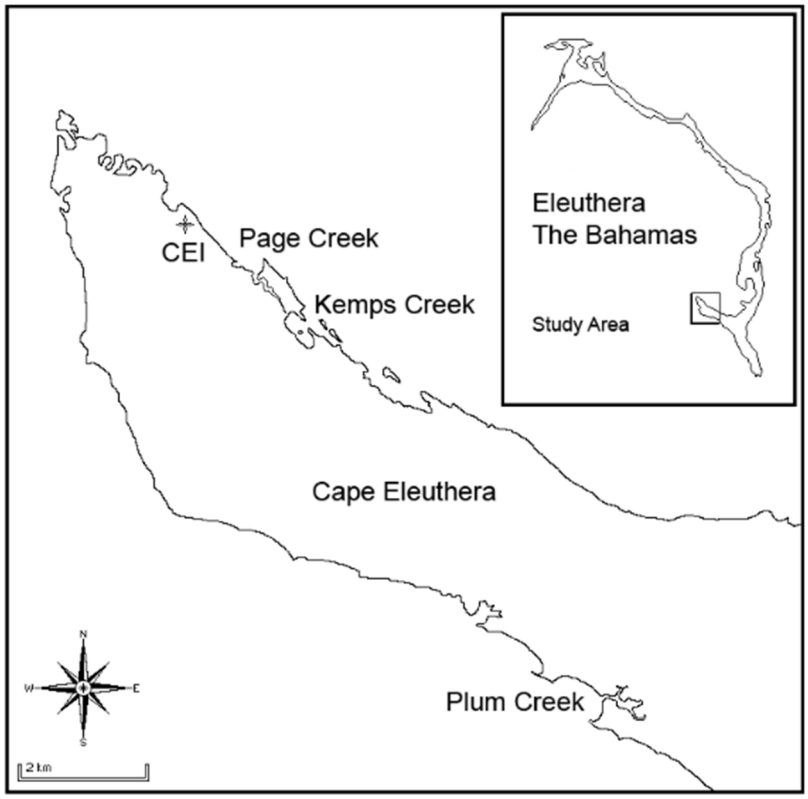


Figure 3.1 Study area along the coast of Cape Eleuthera, Eleuthera, The Bahamas, showing the locations of the sampled Page, Kemps and Plum Creeks, and the location of the Cape Eleuthera Institute (CEI) research facility (black star). The inset map displays the entire island of Eleuthera with the study area highlighted.

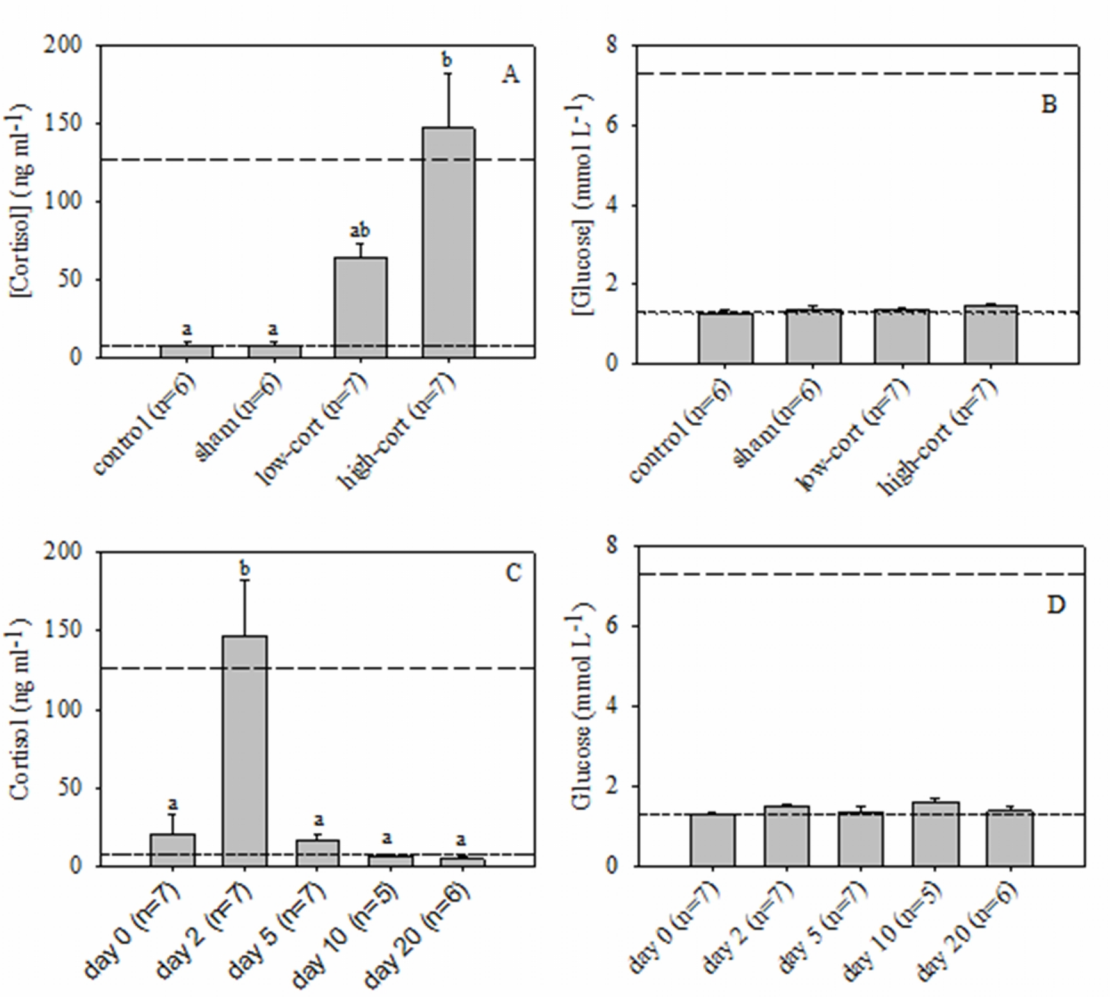
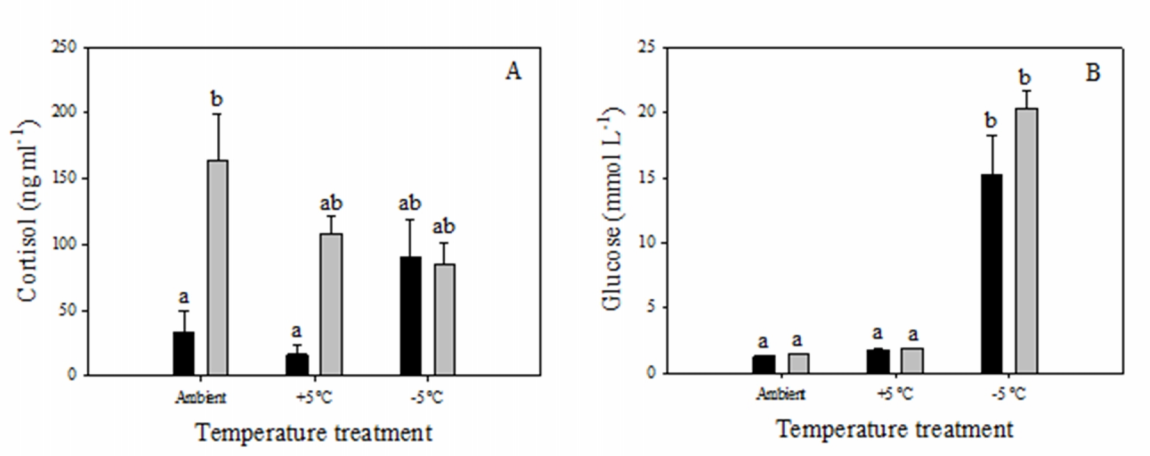


Figure 3.2 Checkered puffer (*Sphoeroides testudineus*) plasma cortisol (A) and blood glucose (B) concentrations of control and cocoa butter sham treated fish, as well as low-cort, low dose cortisol-cocoa butter (25 mg kg^{-1} fish, 5 ml melted cocoa butter kg^{-1} fish) injected fish and high-cort, 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.



1508

1509 **Figure 3.3** Plasma cortisol (A) and blood glucose (B) concentrations of control (black)

1510 and cortisol implanted (gray) checkered puffers (*Sphoeroides testudineus*) in ambient

1511 conditions, as well as +5 and -5 °C changes from ambient temperature.

1512

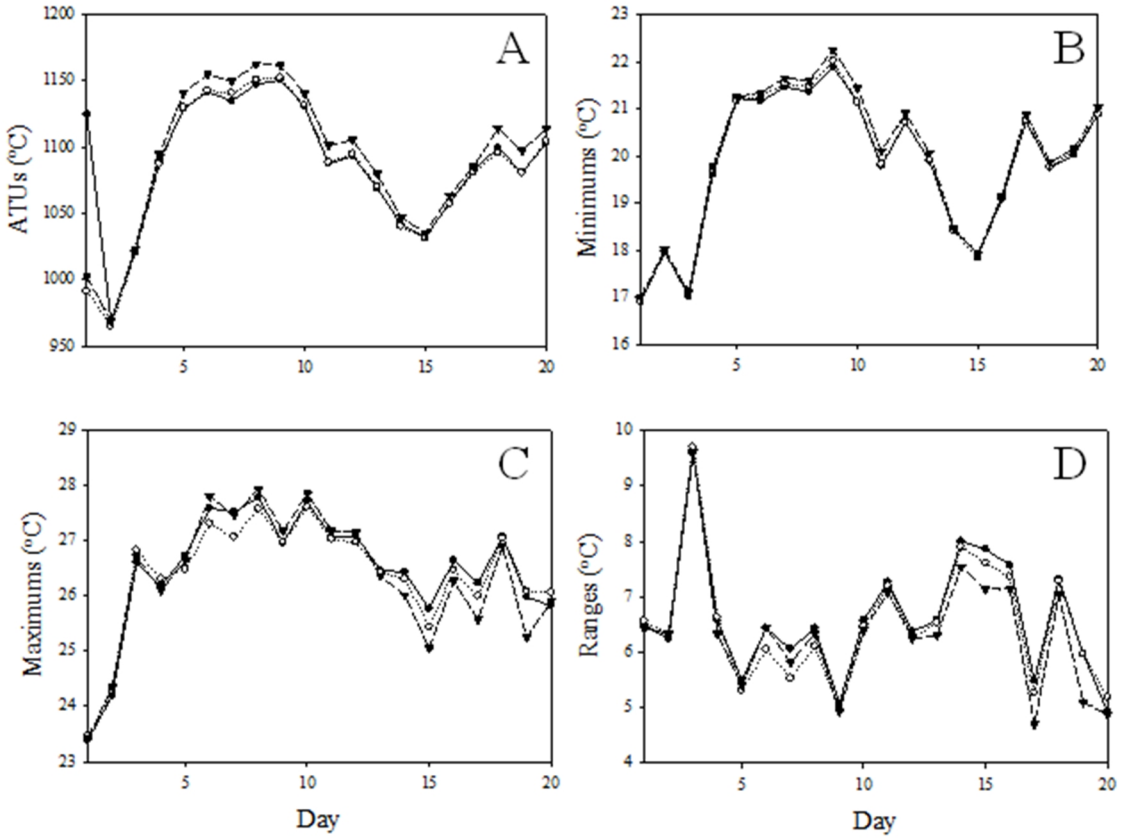


Figure 3.4 Thermal variables, including the accumulated thermal units (ATUs; A), thermal minimums (B), maximums (C) and ranges (D), for control (dark circles) and cortisol dosed (light circles) checkered puffers (*Sphoeroides testudineus*), as well as for the habitat loggers (dark triangle), over the 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

1542 measurable and have direct implications for individual survival and fitness. In chapter 2,
1543 wild checkered puffers were subjected to a standardized stress protocol to quantify baseline
1544 and post-stress physiological stress indices (circulating GCs and glucose), and to evaluate
1545 whether these indices were correlated with fitness proxies (i.e., bite force and puffing
1546 performance). I determined the variability and repeatability of post-stress cortisol in
1547 pufferfish brought into a controlled laboratory setting and attempted to define the
1548 relationship between the variation in the GC response and fitness proxies.
1549 Moreover, checkered puffers are adapted to extreme environmental gradients
1550 imposed by their coastal and tropical environment, and their distribution is often governed
1551 by their tolerances to these conditions, particularly rapid changes in temperature. Given
1552 that anthropogenic environmental change will not occur in isolation of other stressors,
1553 eurythermal and heat-tolerant species, like the pufferfish, may be more vulnerable to
1554 increasing temperatures because these species typically live closer to their thermal limits
1555 (Tomanek and Somero 1999, Stillman 2002, Harley et al. 2006). To clarify the thermal-
1556 related characteristics of stress in a wild fish population, I focused on controlled
1557 laboratory experiments and a complementary field study to evaluate the physiological,
1558 behavioural and ecological consequences of thermal stress (chapter 3). Stress influences
1559 physiology, behaviour and overall fitness of wild fish populations (Boonstra 2013), and
1560 this manuscript helps contribute to our understanding of the ecology of stress in wild
1561 animals inhabiting extreme environments. In chapter 3, plasma cortisol was
1562 experimentally manipulated to physiological post-stress levels to investigate the
1563 physiological, behavioural and ecological consequences of a thermal challenge on the
1564 checkered puffer as a secondary stressor. First, the sub-lethal consequences of climate-
1565 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.

1632 2008) and mammals (Pride 2005, Cabezas et al. 2007, Rogovin et al. 2008) as model
1633 species, with considerably less GC work done in fish (see Breuner et al. 2008 and Bonier
1634 et al. 2009 for overviews, McConnachie et al. 2012). Only recently, scientists have
1635 seriously identified the weak connections between stress and other overlapping areas of
1636 biology, including genetics, physiology, behaviour and evolution (Boonstra 2013). The
1637 ecology of stress is an important underpinning of ecology that overlaps with these four
1638 major areas of biology (Krebs 2009, Boonstra 2013), highlighting the significance of
1639 multidisciplinary stress studies to further our understanding of ecosystem dynamics. Of
1640 special importance is the finding that the checkered puffer has a highly variable GC response
1641 and is negatively correlated with fitness proxies (chapter 2). Variable GCs are likely
1642 beneficial from an evolutionary standpoint, due to the nature of their highly fluctuating
1643 coastal habitat.

1644 Most thermal stress work in fish has been restricted to laboratory studies
1645 (Ackerman et al. 2000, Vijayan et al. 2000, Basu et al. 2001), and cortisol-implanted fish
1646 have been found to be more susceptible to thermal stress (Basu et al. 2001, McConnachie
1647 et al. 2012). However, no studies have been conducted on a free-swimming fish
1648 population. Chapter 3 outlines a series of experiments revealing the costs of experimental
1649 GC manipulation and using these manipulations to determine the thermal-related
1650 characteristics of pufferfish in the laboratory, as well as in a complementary field study.
1651 Pufferfish responded to thermal stress differently when in the laboratory and in their
1652 natural habitat, suggesting that laboratory studies, although easy to interpret, may not be
1653 fully applicable to dynamic fish populations in the wild.

1654 As with most studies, the findings from my experiments raise additional questions
1655 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 adaptions, 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.

	Cortisol (ng ml^{-1})							Glucose (mmol L^{-1})						
	Baseline		Post-stress		Responsiveness			Baseline		Post-stress			Responsiveness	
	B	t	B	t	B	t		B	t	B	t		B	t
Baseline treatment														
Bite force (N)	0.024	0.196	-0.109	-0.880	-0.123	-0.997		0.018	0.148	-0.120	-0.945		-0.131	-1.018
Puff score	0.235	1.459	0.097	0.559	0.051	0.292		0.060	0.367	0.256	1.488		0.240	1.364
Puff time to deflate once released (min)	0.014	0.080	-0.010	-0.057	-0.014	-0.078		0.111	0.656	-0.710	-0.388		-0.119	-0.640
Post-stress treatment														
Bite force (N)	0.216	1.488	**0.262**	**1.746**	* 0.233	1.543		-0.193	-1.325	-0.250	-1.624		-0.181	-1.127
Puff score	-0.133	-0.926	-0.156	-1.040	-0.137	-0.917		-0.152	-1.065	-0.186	-1.221		-0.130	-0.831
Puff time to deflate once released (min)	0.136	0.803	-0.173	-0.983	-0.217	-1.247		0.032	0.187	0.000	-0.002		-0.013	-0.072
Responsiveness														
Bite force (N)	0.189	1.124	**0.329**	**1.937**	* **0.312**	**1.829**	*	-0.197	-1.177	-0.152	-0.839		-0.077	-0.413
Puff score	**-0.312**	**-2.057**	** -0.231	-1.410	-0.177	-1.074		-0.199	-1.273	**-0.381**	**-2.396**	**	**0.314**	**-1.878** *
Puff time to deflate once released (min)	0.127	0.746	-0.167	-0.994	-0.209	-1.191		-0.050	-0.296	0.052	0.286		0.075	0.401

*P < 0.10; **P < 0.05

	Cortisol (ng ml-1) and Glucose (mmol L^{-1})								
	Baseline			Post-stress			Responsiveness		
	R^2	F		R^2	F		R^2	F	
Baseline treatment									
Bite force (N)	0.001	0.031		0.018	0.630		0.022	0.794	
Puff score	0.058	1.131		0.056	1.084		0.047	0.905	
Puff time to deflate once released (min)	0.012	0.215		0.004	0.075		0.012	0.203	
Post-stress treatment									
Bite force (N)	0.079	1.957		**0.165**	**4.667**	**	**0.101**	**2.601**	*
Puff score	0.041	1.038		0.039	0.982		0.024	0.598	
Puff time to deflate once released (min)	0.019	0.337		0.029	0.517		0.044	0.791	
Responsiveness									
Bite force (N)	0.070	1.283		**0.157**	**3.179**	*	0.110	2.112	
Puff score	**0.139**	**3.184**	*	**0.137**	**3.147**	*	0.090	1.933	
Puff time to deflate once released (min)	0.018	0.306		0.035	0.616		0.054	0.976	

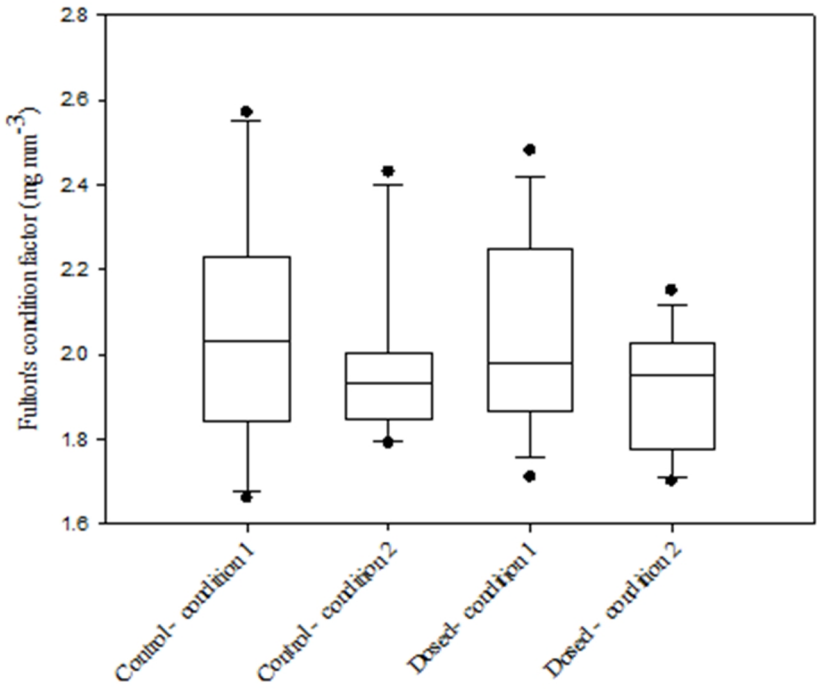
*P < 0.10; **P < 0.05

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.

ID No.	iButton Model	Coordinates	Depth During Low Tide (cm)		Location Description	Proximity To Mangrove Shelter
			Water Depth	Tag Depth		
1	DS1921H-F5	N24 49.047 W76 18.840	15.0	6.5	Mouth of creek; North side	Near
2	DS1921H-F5	N24 49.035 W76 18.830	40.5	25.5	Mouth of creek; South side	Near
3	DS1921H-F5	N24 49.028 W76 18.814	10.3	4.5	Within large mangrove patch where pufferfish are normally spotted	Within shelter
4	DS1921H-F5	N24 49.019 W76 18.813	35.4	28.5	Deep area in first bend where pufferfish often seek refuge	Far
5	DS1921Z-F5	N24 49.026 W76 18.795	12.7	10.8	Past first bend; North side	Near
6	DS1921H-F5	N24 49.020 W76 18.779	11.0	4.2	Past first bend; South side	Near
7	DS1921Z-F5	N24 49.000 W76 18.762	14.4	9.1	Upper part of creek; North side	Near
8	DS1921Z-F5	N24 48.976 W76 18.751	10.5	10.5	Upper part of creek; Middle of creek	Far
9	DS1921Z-F5	N24 48.970 W76 18.730	22.6	19.0	Upper part of creek; Within large mangrove bush	Within shelter
10	DS1921Z-F5	N24 48.947 W76 18.715	18.0	15.0	Most upper reach of creek; no adult pufferfish were spotted beyond this point	Near

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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