

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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# Table of Contents

|   |             |
|---|-------------|
| <b>Abstract .....</b>   | <b>ii</b>   |
| <b>Acknowledgements .....</b>   | <b>iii</b>  |
| <b>Table of Contents.....</b>   | <b>iv</b>   |
| <b>Co-Authorship Statement.....</b>   | <b>vii</b>  |
| <b>List of Tables.....</b>  | <b>viii</b> |
| <b>List of Appendices .....</b>   | <b>xiii</b> |
| <br>  |             |
| <b>Chapter 1. General Introduction.....</b>   | <b>1</b>    |
| 1.1 Changing coastal ecosystems .....   | 1           |
| 1.2 The stress response in fish.....  | 4           |
| 1.3 Checkered puffer biology.....   | 8           |
| 1.4 Research objective and predictions .....  | 13          |
| 1.4.1 Rationale and hypotheses for chapter 2 .....  | 13          |
| 1.4.2 Rationale and hypotheses for chapter 3 .....  | 14          |
| <br>  |             |
| <b>Chapter 2. The relationship between the glucocorticoid stress response and fitness proxies in checkered puffer (<i>Sphoeroides testudineus</i>).....</b> | <b>15</b>   |
| 2.1 Abstract .....  | 15          |
| 2.2 Introduction.....   | 16          |
| 2.3 Methods.....  | 19          |
| 2.3.1 Study site and sampling .....   | 19          |
| 2.3.2 Maximal glucocorticoid response.....  | 20          |
| 2.3.3 Glucocorticoid responsiveness relative to two fitness proxies .....   | 21          |
| 2.3.4 Sample analysis .....   | 23          |

|       |                            |    |
|-------|----------------------------|----|
| 2.3.5 | Statistical analyses ..... | 24 |
| 2.4   | Results .....              | 25 |
| 2.5   | Discussion .....           | 28 |
| 2.5.1 | Conclusion .....           | 33 |
| 2.6   | Tables .....               | 34 |
| 2.7   | Figures .....              | 37 |

**Chapter 3. Consequences of experimental cortisol manipulations on the thermal biology of the checkered puffer (*Sphoeroides testudineus*) in field and laboratory environments 40**

|       |   |    |
|-------|---|----|
| 3.1   | Abstract .....  | 40 |
| 3.2   | Introduction .....                                      | 41 |
| 3.3   | Methods .....   | 44 |
| 3.3.1 | Study site and study animals .....                      | 44 |
| 3.3.2 | Validation study of cortisol implant dose .....         | 45 |
| 3.3.3 | Validation of cortisol implant depletion timeline ..... | 47 |
| 3.3.4 | Metabolic cost of cortisol implant .....                | 48 |
| 3.3.5 | Swimming performance .....                              | 48 |
| 3.3.6 | Lab experiment: Thermal tolerance .....                 | 49 |
| 3.3.7 | Field experiment: Thermal preference .....              | 50 |
| 3.3.8 | Sample analyses .....                                   | 52 |
| 3.3.9 | Data handling and statistical analysis .....            | 52 |
| 3.4   | Results .....   | 56 |
| 3.4.1 | Size differences among treatment groups .....           | 56 |
| 3.4.2 | Validation study of cortisol implant dose .....         | 57 |
| 3.4.3 | Validation of cortisol implant depletion timeline ..... | 57 |
| 3.4.4 | Metabolic cost of cortisol implant .....                | 58 |

|                   |   |           |
|-------------------|---|-----------|
| 3.4.5             | Swimming performance.....                       | 58        |
| 3.4.6             | Lab experiment: Thermal tolerance.....          | 58        |
| 3.4.7             | Field experiment: Thermal preference.....       | 59        |
| 3.4.8             | Field experiment: Change in body condition..... | 60        |
| 3.5               | Discussion.....                                 | 60        |
| 3.5.1             | Validation study of cortisol implant.....       | 61        |
| 3.5.2             | Metabolic cost of cortisol implant.....         | 61        |
| 3.5.3             | Thermal biology.....                            | 62        |
| 3.5.4             | Conclusion.....                                 | 67        |
| 3.6               | Tables.....                                     | 68        |
| 3.7               | Figures.....                                    | 73        |
| <b>Chapter 4.</b> | <b>General discussion.....</b>                  | <b>77</b> |
| 4.1               | Overview.....                                   | 77        |
| 4.2               | Findings and implications.....                  | 79        |
| 4.3               | Future directions and further questions.....    | 81        |
| <b>Appendices</b> | <b>.....</b>                                    | <b>86</b> |
| Appendix A        | .....   | 86        |
| A.1               | Supplementary materials for chapter 2.....      | 86        |
| A.2               | Supplementary materials for chapter 3.....      | 89        |
| Appendix B        | .....   | 91        |
| B.1               | Permission from co-authors.....                 | 91        |
| <b>References</b> | <b>.....</b>                                    | <b>92</b> |

## **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*)**

Cull, F., C.D. Suski, A. Shultz, A.J. Danylchuk, C.M. O'Connor, and S.J. Cooke

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

Cull, F., C.D. Suski, A. Shultz, A.J. Danylchuk, C.M. O'Connor and S.J. Cooke

This manuscript is in preparation for submission to the Journal of Experimental Marine Biology and Ecology. The project was conceived by Cooke, O'Connor and Cull with the support of Suski and Danylchuk. All field work conducted by Cooke, Shultz and Cull. All analyses completed by Cull.

## List of Tables

**Table 2.1** Means ( $\pm$  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..... 34

**Table 2.2** Pearson correlation results for fitness proxies(bite force, puff score and puff time) of the checkered puffer (*Sphoeroides testudineus*) in the baseline and post-stress treatments. Baseline values were collected from acclimated pufferfish within 3 min. Fish were then immediately ‘stressed’ by holding them at the air-water interface for 5 min in a rubber-mesh dip net. Pufferfish were then resampled 30 min post-stressor within 3 min to collect post-stress values. A total of 38 fish were sampled..... 35

**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. .... 36

**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. .... 68

**Table 3.2** Checkered puffer (*Sphoeroides testudineus*) hematocrit levels at 5-, 10- and 20-days post-implantation..... 69

**Table 3.3** Two-way ANOVA outputs identifying the effect of multiple stressors (i.e., fish treatment (control and cortisol implanted pufferfish) and thermal treatment (ambient temperature, as well as 5°C below and above ambient temperature)) on physiological and behavioural stress indices, including cortisol, glucose, hematocrit and ‘puff’ performances (i.e., puff score and puff time to deflate once released). .... 70

**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. .... 71

**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. .... 71

**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. .... 72

## List of Figures

- Figure 2.1** Plasma cortisol (A) and blood glucose (B) secretion and recovery in the checkered puffer (*Sphoeroides testudineus*) following a 5 min standardized stressor. Error bars represent standard error from the mean, and different letters indicate statistically significant differences among sampling time points (Tukey's HSD post-hoc test following a significant ANOVA;  $\alpha=0.05$ ) ..... 37
- Figure 2.2** Baseline and post-stress bite force (A) and puff score (B) of the the checkered puffer (*Sphoeroides testudineus*). Baseline values were collected from acclimated pufferfish within 3 min. Fish were then immediately 'stressed' by holding them at the air-water interface for 5 min in a rubber-mesh dip net. Pufferfish were then resampled 30 min post-stressor to collect post-stress values. A total of 38 fish were sampled. Boxes represent 25<sup>th</sup> and 75<sup>th</sup> percentiles with median enclosed within, and whiskers represent 10<sup>th</sup> and 90<sup>th</sup> percentiles. .... 38
- Figure 2.3** 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..... 39

**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. .... 73

**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<sup>-1</sup> fish, 5 ml melted cocoa butter kg<sup>-1</sup> fish) injected fish and high-cort, high dose cortisol-cocoa butter (50 mg kg<sup>-1</sup> fish, 5 ml melted cocoa butter kg<sup>-1</sup> fish) injected fish. Plasma cortisol (C) and blood glucose (D) concentrations of high dose cortisol-cocoa butter (50 mg kg<sup>-1</sup> fish, 5 ml melted cocoa butter kg<sup>-1</sup> fish) injected checkered puffers over a 20 day period. Dashed lines indicate physiological baseline and post-stress concentrations. .... 74

**Figure 3.3** Plasma cortisol (A) and blood glucose (B) concentrations of control (black) and cortisol implanted (gray) checkered puffers (*Sphoeroides testudineus*) in ambient conditions, as well as +5 and -5 °C changes from ambient temperature..... 75

**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. .... 76

## List of Appendices

|   |    |
|---|----|
| Appendix A .....                                  | 86 |
| A.1    Supplementary materials for chapter 2..... | 86 |
| A.2    Supplementary materials for chapter 3..... | 89 |
| Appendix B.....                                   | 91 |
| B.1    Permission from co-authors.....            | 91 |

1 **Chapter 1. General Introduction**

2

3 **1.1 Changing coastal ecosystems**

4 Coastal marine ecosystems represent the transition zone between land and water, from the  
5 intertidal zone to the continental shelf, and their biodiversity is shaped by the dynamic  
6 processes that create and sustain them (Harley et al. 2006, Burkett et al. 2008). These  
7 ecosystems are among the most ecologically and socio-economically rich on the planet,  
8 providing approximately US\$14 trillion worth of ecosystem goods and services per year  
9 (Costanza et al. 1997). Coastal biodiversity is specifically adapted to the extreme  
10 environmental conditions imposed along the gradients of these coastal boundaries, and  
11 the distribution is often governed by the tolerances to these environmental conditions,  
12 including water parameters (such as temperature, salinity and pH), light availability,  
13 storm disturbance, tides, water depth and nutrient availability (Burkett et al. 2008). These  
14 coastal ecosystems are threatened by anthropogenic environmental change (IPCC 2001).  
15 Climate change, due to natural variability and human activity, will likely increase over the  
16 subsequent decades due to cumulating greenhouse gas emissions and land use alterations  
17 (IPCC 2007a and 2007b). Such loss of equilibrium is threatening the Earth's genetic,  
18 species and ecosystem biodiversity (IPCC 2001, 2007a), risking the loss of marine  
19 ecosystems around the world. Elevated greenhouse gas emissions will likely result in  
20 increased global mean temperature, causing a variety of physical and chemical changes in  
21 marine systems (Harley et al. 2006). Atmospheric carbon dioxide concentrations are  
22 expected to increase from a pre-industrial level of 280 to 540–970 ppm by the year 2100  
23 (IPCC 2001). Approximately half of all atmospheric carbon dioxide will eventually be

24 taken up by the Earth's oceans (Sabine et al. 2004, Feely et al. 2004) and given that  
25 marine plants, except seagrasses, are carbon-saturated (Gattuso and Buddemeier 2000),  
26 increasing oceanic carbon dioxide will consequently lower oceanic pH (Andersson et al.  
27 2003, Caldeira and Wickett 2005, Burkett et al. 2008). Reduced pH will disturb many  
28 physiological processes in marine organisms including decreased protein synthesis and  
29 ion exchange (see Portner and Langenbuch 2005 for review), as well as change the  
30 saturation limits of aragonite, calcite and other minerals necessary to calcifying organisms  
31 (Kleypas et al. 1999, Feely et al. 2004).

32 Atmospheric and ocean temperature are increasing and expected to accelerate in  
33 the current century (IPCC 2001), altering biological diversity at every level in the food  
34 web. Temperature can affect basic physiological processes (Hochachka and Somero  
35 2002), thereby influencing the growth, survival, reproduction and distribution of biota  
36 (Brander et al. 2003, Reid 2003). Eurythermal (specifically heat-tolerant) and low-latitude  
37 species may be more vulnerable to increasing temperatures as compared to more  
38 temperate species because they live closer to their thermal limits (Tomanek and Somero  
39 1999, Stillman 2002, Harley et al. 2006). There is a lack of information on how tropical  
40 fish will respond to increases in temperature (Cambers et al. 2007). However, species  
41 distribution is expected to expand toward cooler environments to evade such  
42 consequences (Parmesan and Yohe 2003, IPCC 2007a). Increasing temperatures may also  
43 cause a cascade of subsequent disturbances, including increased severity of diseases as  
44 pathogens are often favoured by warmer temperatures relative to their hosts (Harvell et al.  
45 2002), rising sea level and increasing storm systems (Burkett et al. 2008).

46           Oceans are estimated to rise approximately 2 mm per year due to ocean expansion  
47 through freshwater input of melting polar ice caps (IPCC 2001). Rises in sea level are  
48 expected to negatively impact mangrove habitats through flooding and erosion, likely  
49 threatening species that require mangroves for food and protection (Field 1994, Bacon  
50 1994, Lugo 2002, Diop 2003, Yáñez-Arancibia et al. 1998, Piedra and Piedra 2007). In  
51 addition, storms, particularly tropical storms and hurricanes, are predicted to intensify in  
52 terms of wind speed and rainfall as sea surface temperature increases in the main  
53 hurricane origins of the North Atlantic and the Gulf of Mexico (Smith and Reynolds  
54 2004, Webster et al. 2005, Bell et al. 2007, IPCC 2007b, Knutson et al. 2008). Changes in  
55 storm frequency along ocean coasts have already been documented (Bromirski et al.  
56 2003) and this trend is expected to continue (IPCC 2001). These storms often severely  
57 damage coastal systems through hydrodynamic disturbances and thus, threaten the range  
58 of coastal biota (Burkett et al. 2008). Intensifying storms may also influence shifts in  
59 nutrient upwelling (Roemmich and McGowan 1995, Lotze and Worm 2002, Nielsen  
60 2003), precipitation patterns (Harley et al. 2006), wave regimes (Komar and Allan, 2007)  
61 and coastal run-off patterns (Burkett et al. 2008). Coastal run-off is predicted to change  
62 (Milly et al. 2005), thereby altering coastal salinity (Contente et al. 2011), turbidity, water  
63 residence time, vertical stratification, overall productivity (risk of eutrophication and algal  
64 blooms), and inflow of terrestrial nutrients and pollutants (Harley et al. 2006, Nicholls et  
65 al. 2007, Burkett et al. 2008).

66           Evidently, climate change will affect the Earth's biota in several ways on the  
67 individual, population and community levels, through changes in physiology and  
68 performance, processes of reproduction and dispersal, as well as species interactions

69 (Harley et al. 2006). The combination of stressors caused by gradual anthropogenic  
70 climate change may provoke complex non-linear responses in coastal systems (Lee et al.  
71 2001, Harley et al. 2006, Burkett et al. 2008). Wild populations will need to rely on  
72 micro-evolution, phenotypic plasticity and phenotypic flexibility to cope with the various  
73 challenges imposed by global climate change (i.e., new types, increased frequency and a  
74 wider range of stressors; Angelier and Wingfield 2013). The consequences to such  
75 stressors have been documented to be highly variable and context-dependent, likely  
76 attributable to the role of the glucocorticoid (GC) response. The GC response is known to  
77 mediate rapid physiological and behavioural changes to regain homeostasis and benefit  
78 survival following a challenge (refer to Angelier and Wingfield 2013).

79

## 80 **1.2 The stress response in fish**

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

87 The stress response is a complex mechanism that allows an individual to manage  
88 real or perceived stressors in order to maintain homeostasis (Barton 2002). In fish, the  
89 primary response consists of endocrine changes, resulting in quantifiable levels of  
90 circulating catecholamines and corticosteroids (Donaldson 1981, Randall and Perry 1992,  
91 Wendelaar Bonga 1997). Corticosteroids are released primarily from interrenal cells of  
92 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

139 individual size, behavioural traits, reproductive success and overall fitness (Cook 2007,  
140 O'Connor et al. 2009). Given that glucocorticoids influence the expression of nearly 10%  
141 of the genome, targeting metabolism, growth, repair, reproduction and resource allocation  
142 (Le et al. 2005), the consequences of elevated glucocorticoids have also been studied,  
143 particularly in the context of aquaculture. For example, much research has examined the  
144 potential for mortality associated with inhibited disease resistance, growth, reproduction,  
145 and general health (Barton and Iwama 1991). By studying the consequences of stress,  
146 aquaculture practices have aimed to increase survival rates and optimize overall  
147 production (Barton and Iwama 1991, Iwama et al. 1997, Pickering 1998).

148        Much work has studied the stress responsiveness to a single stressor, however, an  
149 area of concern is the effect of chronic or repeated stressors on coastal marine animals  
150 that are already living in challenging environmental conditions. Fish reveal an uncertain  
151 cumulative response to multiple and repeated stressors (Carmichael et al. 1983, Flos et al.  
152 1988, Maule et al. 1988). Fish may experience a desensitized or weakened stress response  
153 to multiple or repeated exposures to stressors (Reid et al. 1998) and chronic stress may  
154 even intensify or attenuate the response to a secondary stressor (Barton et al. 1985,  
155 Pickering and Pottinger 1987, Wilson et al. 1998, Angelier and Wingfield 2013). Human  
156 activities (i.e. aquaculture and fisheries), urban development and the increasing effects of  
157 climate change may be external environmental factors triggering chronic stress in fish  
158 (Pickering and Pottinger 1989). To study the stress response in fish as well as its  
159 consequences, it is typical to expose fish to stressors (physical, environmental, chemical)  
160 in a controlled manner. For example, if studying stress responses to hypoxia one would  
161 manipulate the dissolved oxygen concentration in the water and then measure various

162 endpoints. Such stressors can be acute (seconds to hours – e.g., a simulated predation  
163 event, transient hypoxia) or chronic (e.g., days to weeks – e.g., starvation, long-term  
164 hypoxia). Another approach for studying the consequences of the stress response is to  
165 experimentally elevate cortisol, typically using an injection of exogenous cortisol or  
166 incorporating cortisol into food (Gamperl et al. 1994). This thesis will involve two  
167 approaches: First, an examination of individual baseline and maximal stress  
168 responsiveness as they relate to individual fitness and secondly, the experimental  
169 elevation of cortisol to examine physiological, behavioural and ecological consequences  
170 of stress.

171

### 172 **1.3 Checkered puffer biology**

173 The checkered puffer (*Sphoeroides testudineus*) belongs to the family Tetraodontidae  
174 meaning four tooth plates (Shipp 1974). Tetraodontidae are Actinopterygii comprising 19  
175 genera and approximately 160 species, inhabiting tropical and subtropical areas of the  
176 Atlantic, Indian and Pacific oceans (Nelson 1984). The two fused teeth on both upper and  
177 lower jaws of the pufferfish form a solid beak. The checkered puffer has dorsal (8 rays)  
178 and anal (7 rays) fins set anteriorly near the caudal fin, reduced scales and prickles  
179 covering the anterior half of the body. This pufferfish is cryptic in colouration with a  
180 brown to black dorsal side covered in a light regular geometric pattern, and a white to  
181 yellow ventral side (Shipp 1974, Robins et al. 1986). The checkered puffer can reach a  
182 total length of up to 300 mm (Shipp 1974, Robins et al. 1986), but most specimens  
183 studied are much smaller ( $180 \pm 20$  mm).

184 Pufferfish are named for their ability to inflate by taking water or air into their  
185 stomachs through numerous rhythmic buccal cavity expansions and compressions  
186 (Jackson 1848, Gabriel 1940, Brainerd 1994, Wainwright 1992). Body inflation is  
187 restricted to the taxa Tetraodontidae and Diodontidae, and serves as a mechanical defense  
188 against piscine predators (Winterbottom 1974, Brainerd 1994). Wainwright (1992)  
189 studied the inflation pump of *Chilomycterus* (a member of the Diodontidae family) and  
190 found that there were three principal skeletal characters – the buccal cavity, two pectoral  
191 girdle features and the first large, jointed branchiostegal ray – and four cranial  
192 musculature features – muscles positioned near the articulation, the well-developed  
193 hyohyoideus abductor, protractor hyoideus and valvulus muscle, as well as the oral valve  
194 – contributing to the inflation mechanism. The valvulus muscle is found only in the  
195 Tetraodontidae and Diodontidae families (Winterbottom 1974) and reinforces the oral  
196 valve by preventing water or air from exiting the mouth upon inflation (Wainwright  
197 1992). Pufferfish belonging to the family Tetraodontidae have large expandable stomachs  
198 with strong esophageal, pyloric and horizontal sphincters (Rosen 1912, Breder and Clark  
199 1947). The ventral and dorsal portions of the stomach are called the inflatable sac and the  
200 stomach proper, respectively, thereby implying division of stomach function (Breder and  
201 Clark 1947). Many other biological structures within the puffer allow the fish to inflate,  
202 including specialized unconstrained skin forming an orthogonal arrangement of dermal  
203 collagen sheets, loose connective tissue (aureolar tissue), large folds in the peritoneum,  
204 expandable stomach and peritoneal cavity, axial skeleton and musculature, as well as the  
205 absence of pleural ribs and a pelvis (Brainerd 1994). The inflation sequence is  
206 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

230 the checkered puffer, most likely due to structural reinforcement, diet preferences and  
231 possible defensive or intraspecific competition strategy. Bite force has been studied  
232 extensively in the reptile field as a fitness proxy (Herrel et al. 2001, Erickson et al. 2003,  
233 Vanhooydonck et al. 2005, Lailvaux and Irschick 2007, Bulte et al. 2008), however this  
234 metric not yet been thoroughly investigated in durophagus fish.

235         The checkered puffer is a highly successful species inhabiting bays, seagrass beds,  
236 tidal creeks, mangrove lagoons and freshwater areas, ranging along the Atlantic coastline  
237 as far North as Rhode Island and as far South as the southeastern coasts of Brazil, and  
238 throughout the Gulf of Mexico and Caribbean sea (Shipp 1974, Targett, 1978, Robins et  
239 al. 1986, Pauly 1991, Figueiredo and Menezes 2000, Vendel et al. 2002, Spach et al.  
240 2003, Felix et al. 2006, MacDonald et al. 2009). This species of pufferfish does not  
241 typically migrate to coral reefs at any point in its lifecycle (Froese and Pauly, 2008).  
242 Instead, it spends its life resting on shallow turbid substrate to assist with its cryptic  
243 defense (Austin and Austin 1971, Pauly 1991) and uses mangroves for protection  
244 (MacDonald et al. 2009). As pufferfish grow in size, vulnerability to predation decreases  
245 and more time is thus spent higher in the water column and outside the mangrove root  
246 system (Laegdsgaard and Johnson 2001, MacDonald et al. 2009).

247         The expansive distribution of the checkered puffer is credited to its broad  
248 physiological tolerance. This euryhaline pufferfish has been found to dwell in less saline,  
249 lower estuary habitats ranging from 0 to 67 ppt (Blaber 1997, Prodocimo and Freire 2001,  
250 2004 and 2006, Prodocimo et al. 2008, Contente et al. 2011, Lopes 2000, Vega-Candejas  
251 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

275 **1.4 Research objective and predictions**

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

283

284 **1.4.1 Rationale and hypotheses for chapter 2**

285 The purpose of chapter 2 was first to quantify individual variation in the GC stress  
286 response in checkered puffer, and then to determine whether there is a relationship  
287 between the GC stress response and two established fitness proxies, puffing metrics and  
288 bite force. Given that checkered puffer have not been well-studied in the context of stress,  
289 a number of important methodological issues had to be resolved before commencing  
290 experimentation. For example, the GC response and recovery of the checkered puffer had  
291 to be determined by subjecting the animal to a standardized acute stressor that would  
292 simulate a typical stress response exhibited in the wild. It was necessary to determine if  
293 fish can be held in laboratory conditions in a manner that results in low GCs, and the time  
294 course for recovery needed to be documented to identify the time period at which  
295 sampling should occur to obtain maximal values. Barton and his colleagues (1987) found  
296 that exposing a fish to air for a standard length of time causes a fish to display a standard  
297 and repeatable stress response to characteristic and challenging environmental conditions.

298 By standardizing the stressor, individual variation in baseline and maximum stress  
299 responses in this species was also be determined. Responses of interest are concentrations  
300 of cortisol and glucose, as well as two fitness proxies – inflation or ‘puff’ metrics and bite  
301 force.

302

### 303 **1.4.2 Rationale and hypotheses for chapter 3**

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

320 checkered puffer's thermal sensitivity and thermal habitat use was independent of  
321 whether cortisol levels had been experimentally elevated.

322

## 323 **Chapter 2. The relationship between the glucocorticoid stress response** 324 **and fitness proxies in checkered puffer (*Sphoeroides testudineus*)**

325

### 326 **2.1 Abstract**

327 Individual variation in the endocrine stress response (i.e., the change in circulating  
328 glucocorticoids [GCs] following a challenge) has been linked to survival and fitness in a  
329 variety of species. However, the strength and the direction of this relationship have  
330 proven to be highly context dependent. The checkered puffer (*Sphoeroides testudineus*) is  
331 an interesting model for studying stress in an ecological context because it has a unique  
332 predator avoidance strategy. Pufferfish will not hesitate to bite and inflate or 'puff' to deter  
333 potential predators. These behaviours are readily measurable and have direct implications  
334 for individual survival and fitness. The purpose of this study was first to quantify  
335 individual variation in the GC stress response in checkered puffer, and then to determine  
336 whether there was a relationship between the GC stress response and two established  
337 fitness proxies, puffing metrics and bite force. Wild checkered puffer from Eleuthera  
338 Island, The Bahamas, were subjected to a standardized stress protocol, and baseline and  
339 post-stress physiological stress indices (circulating GCs and glucose) were subsequently  
340 quantified. To evaluate whether these indices were correlated with fitness proxies, bite  
341 force and puffing metrics were assessed prior to and following the standardized stressor.  
342 As expected, physiological stress indices were significantly elevated following the

343 standardized stressor. Interestingly, both bite force and the extent of puffing were reduced  
344 following the standardized stress protocol. Furthermore, the magnitude of individual  
345 physiological stress response was negatively correlated with post-stress fitness proxies. I  
346 also documented that puff metrics for individuals are repeatable through time. This study  
347 highlights the negative consequences of an acute stressor on fitness proxies, demonstrates  
348 a negative relationship between GC response and fitness proxies, and establishes the  
349 checkered puffer as a valuable model for future research on the ecology of stress in wild  
350 vertebrates.

351

## 352 **2.2 Introduction**

353 The physiological stress response is a complex mechanism that allows an individual to  
354 maintain homeostasis in the face of real or perceived challenges (Selye 1937, Sapolsky et  
355 al. 2000). In brief, the physiological stress response of vertebrates involves the release of  
356 glucocorticoids (GCs). In fish, cortisol is the primary GC that increases metabolic rate via  
357 several biochemical processes (Barton 2002), thereby causing changes in metabolism,  
358 hydromineral balance, as well as cardiovascular, respiratory and immune function; for  
359 instance, measurable changes in concentrations of blood glucose, lactate and major ions  
360 (Pickering 1981, Iwama et al. 1997 and 1998, Mommsen et al. 1999, Barton 2002). GC  
361 concentrations are commonly used as a physiological stress indicator as it is highly  
362 responsive to acute stressors, easy to quantify, and its delayed release allows for proper  
363 sampling of resting, baseline levels (Wedemeyer et al. 1990, Barton and Iwama 1991,  
364 Gamperl et al. 1994, Wendelaar Bonga 1997). Furthermore, maximum post-stress GC  
365 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"

435 W076°15'6.65", Page: N24°49'04.7" W076°18'51.6"). Pufferfish were corralled into a  
436 seine net set at the mouth of the creeks on an outgoing tide and transported to the Cape  
437 Eleuthera Institute (CEI: N24°50'05" W076°20'32") in aerated coolers. At CEI, pufferfish  
438 were held in 1250 L flow-through tanks with ample aeration (29.2±2.7°C), and were  
439 allowed to acclimate to laboratory conditions between 2 and 7 days before  
440 experimentation. During acclimation, pufferfish were fed an assortment of sardines  
441 (*Sardinella aurita*), juvenile bonefish (*Albula vulpes*) and mottled mojarra (*Eucinostomus*  
442 *lefroyi*) every 2 days. The holding tank was cleaned every 4 days until 3 days prior to  
443 sampling, and fish were starved 48 hours before experimentation to avoid disturbing the  
444 fish before experimentation. Following experiments, all pufferfish were weighed (g)  
445 using a portable electronic balance and then placed in a foam-lined trough to obtain a total  
446 length measurement (mm). All techniques were performed without anesthesia (see Cooke  
447 et al. 2005 for rationale), all samples were collected in accordance with the guidelines of  
448 the Canadian Council on Animal Care as administered by Carleton University (B12-01),  
449 and all fish were released back into the ocean upon recovery at the conclusion of the  
450 experiment.

451

### 452 **2.3.2 Maximal glucocorticoid response**

453 At the outset, I performed a preliminary study to define the cortisol secretion profile and  
454 subsequent recovery timelines of pufferfish by subjecting them to a standardized stress  
455 challenge and then sampling them during the recovery period. This preliminary stress  
456 challenge served to identify the maximum cortisol concentration for pufferfish, the time at  
457 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

504 that pufferfish rarely maintained the same level of puff over the entire sampling period,  
505 puff scores were weighted to account for the varying puff intensity over the 3 min  
506 sampling period. Following this, all fish were immediately given a stress challenge by  
507 holding them at the air-water interface for 5 min in a rubber-mesh dip net, and  
508 subsequently returned to their individual chambers. Once released into the chamber, the  
509 time the fish required to deflate (sec) was recorded. Thirty minutes after the standardized  
510 stressor, all pufferfish were again collected to record their post-stress bite force (N), and  
511 sampled for 0.5 mL of blood while monitoring their post-stress puff score; this blood  
512 sample was considered the post-stress sample. Pufferfish were then returned to their  
513 individual chambers where the time to deflate (sec) was again recorded. Out of the 48  
514 fish, 10 failed to yield one of the samples, resulting in a final sample size of 38 fish.

515

#### 516 **2.3.4 Sample analysis**

517 Whole blood glucose concentrations were quantified on site using an Accu-Chek®  
518 Compact Plus glucose meter (Roche Diagnostics, Basel, Switzerland; see Cooke et al.  
519 2008 for validation), and remaining whole blood was centrifuged at 2000 g for 5 min to  
520 separate erythrocytes from plasma (Capsule HF-120, Tomy Seiko Co., LTD, Tokyo,  
521 Japan). Plasma samples were stored at -20°C until cortisol immunoassay analysis. Plasma  
522 cortisol was quantified using colorimetric competitive enzyme-linked immunoassay  
523 (ELISA; Enzo Life Sciences Cortisol ELISA Kit ADI-900-071; Farmingdale, New York,  
524 USA), a technique previously validated for measuring cortisol concentrations in a variety  
525 of fish species (Sink et al. 2008). Samples were read by a SpectraMax Plus384  
526 absorbance microplate reader as per manufacturer recommendations.

### 527 **2.3.5 Statistical analyses**

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

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

$$539 \quad K = 100 \times (W/L^3);$$

540 where (W) is body mass (mg) and (L) is total length (mm) (Ricker 1975). Simple  
541 regression was then used to determine the predictive value of total length, mass and  
542 Fulton's condition factor on baseline and post-stress physiological indices and fitness  
543 proxies. The total length and mass of the pufferfish both proved to be highly predictive of  
544 all physiological and behavioural measures, with the exception of puff time; therefore,  
545 only total length was used as a covariate in subsequent regression analyses. While  
546 controlling for the total length of the pufferfish, several multiple regression analyses were  
547 conducted to determine physiological predictors (i.e., cortisol and glucose concentrations)  
548 of fitness proxies (i.e., bite force, puff score and puff time) in both baseline and post-  
549 stress treatments. The effect of cortisol and glucose responsiveness (i.e., the difference

550 between baseline and post-stress samples) on these baseline and post-stress fitness proxies  
551 was also determined using similar regression analyses.

552 Statistical analyses were conducted using IBM SPSS Statistics 20.0 (2011).  
553 Residuals were examined for normal distributions using the Shapiro-Wilk test. The  
554 Levene's and Brown-Forsythe tests were used to assess homogeneity of variance within  
555 variables of normally and non-normally distributed data, respectively. Variables were log  
556 or square root transformed to meet assumptions of normality and homogeneity of  
557 variance. The level of significance for all statistical analyses ( $\alpha$ ) was assessed at 0.05.  
558 Means  $\pm$  standard error of the mean (SEM) are reported.

559

## 560 **2.4 Results**

561 Following the standardized stress challenge checkered puffers displayed significant  
562 differences in cortisol concentration across the 4 h timeline (ANOVA:  $F = 7.580$ ,  $P <$   
563  $0.001$ ), with a maximum glucocorticoid (GC) response of  $145.9 \pm 31.0$  ng ml<sup>-1</sup> 30 min  
564 post-stressor, and a subsequent return to baseline levels by the 1 h time point (Fig. 2.1 A).  
565 Furthermore, pufferfish exhibited significant differences in blood glucose concentration  
566 across the 4 h timeline (ANOVA:  $F = 13.078$ ,  $P < 0.001$ ), peaking at  $6.3 \pm 1.0$  mmol L<sup>-1</sup>  
567 30 min post-stressor, and a subsequent return to baseline levels by the 1 h time point (Fig.  
568 2.1 B). Therefore, I used a conservative 12 h acclimation period, and a 30 min time point  
569 to assess maximum post-stress physiological and behavioural measures for all subsequent  
570 aspects of the study.

571 Following the standardized stressor (i.e., 5 min air-water interface challenge),  
572 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

618 **2.5 Discussion**

619 Variability in GC levels is well established and thought to mediate ecological and  
620 evolutionary trade-offs in individuals (see Ricklefs and Wikelski 2002, McConnachie  
621 2010). In the current study, I noted considerable variation in GC concentrations (both  
622 baseline and post-stress), providing the foundation to ask questions related to the  
623 correlates and consequences of the diversity of GC responses. The relationship between  
624 acute GC stress response and fitness is highly context dependent (e.g., Overli et al. 2002,  
625 Blas et al. 2007, Wada and Breuner, 2008). Research on teleost fish lags behind that of  
626 other taxa, but may prove to be a valuable comparative group for the study of stress and  
627 fitness. Individual variation in the acute GC response in fish has been shown to be  
628 repeatable through time (Cook et al. 2011a), and to be correlated with individual size,  
629 behavioural traits, and reproductive success (Cook et al. 2011a, 2011b, O'Connor et al.  
630 2012). In an attempt to build on this area of research, individual variation in the GC  
631 response of the checkered puffer (*Sphoeroides testudineus*), as well as its relationship  
632 with two fitness proxies (i.e., inflation and bite force) were investigated. I found that  
633 checkered puffers displayed a maximum GC response 30 min following a standardized  
634 stressor and a subsequent return to baseline levels by the 4 h time point. Following a  
635 standardized stressor, physiological stress indices, including plasma cortisol and blood  
636 glucose concentrations, increased, and fitness proxies (bite force and puff score)  
637 decreased. Fitness proxies were found to be repeatable through time, and highly  
638 correlated with one another following the standardized stressor. Furthermore, the total  
639 length and mass of the pufferfish significantly predicted bite force, and all physiological  
640 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

664 forces and puff scores, as well as increasing puff times once released. Inflation (i.e.,  
665 puffing) has interested biologists for some time (Thilo 1899, Parr 1927, Brainerd 1994).  
666 Puffing is a mechanical defense against piscine predators (Winterbottom 1974, Brainerd  
667 1994), and is known to engage several muscles to achieve a full and effective puff  
668 (Wainwright et al. 1995). Similarly, bite force has been examined as a performance  
669 measure that mediates diet, growth and fitness (Vanhooydonck et al. 2005, Bulte et al.  
670 2008, Mara et al. 2010), and requires a significant amount of energy (Huber et al. 2005).  
671 Therefore, it is possible that puff score and bite force decreased following the acute  
672 stressor due to reduced somatic energy reserves. Cortisol increased following the stressor,  
673 however the underlying mechanism was presumably physiological exhaustion. Although  
674 not measured here, tissue energy stores (e.g., adenosine triphosphate (ATP),  
675 phosphocreatine (PCr), glycogen) would have been depleted and metabolites such as  
676 lactate would have been generated. In the presence of an intense challenge such as the  
677 standardized stressor, it is known that the stress response required to regain homeostasis  
678 is energetically costly (see Schreck 2010 for overview). Somatic energy reserves are  
679 known to be consistently correlated with fish size (Brett 1995, Mackereth et al. 1999,  
680 Crossin et al. 2004), and to decline following acute and chronic stressors, resulting in  
681 fitness-related consequences. For example, glycogen stores were found to quickly  
682 mobilized in food-deprived rainbow trout (*Oncorhynchus mykiss*) to meet the initial  
683 energy demand imposed by an acute handling stressor (Vijayan and Moon 1992), leaving  
684 significantly reduced energy budgets for subsequent challenges. Furthermore, parental  
685 defense in smallmouth bass (*Micropterus dolomieu*) over the parental care period suffers  
686 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

733 several stress response parameters (see Adams 1990), as well as determine suitable fitness  
734 proxies that accurately predict successful fitness proxies. I found the GC response to an  
735 acute, standardized stressor in the checkered puffer to be well within the range found in  
736 other studies for other teleost fish species (Pickering et al. 1982, Pickering and Pottinger  
737 1989, Barton 2002, Shultz et al. 2011). Similar studies have also shown that plasma  
738 cortisol concentrations return to resting levels within 4 hours post-stressor (Pickering et  
739 al. 1982, Barton 2002).

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

748

#### 749 **2.5.1 Conclusion**

750 GCs are often measured in individuals to monitor the relative condition of species  
751 and populations of conservation concern (Walker et al. 2005, Breuner et al. 2008).

752 However, few studies examine individual fitness proxies or intermediate performance  
753 consequences of individual variation in GC concentrations. In the current study, I found  
754 that exposure to an acute stressor reduced subsequent fitness proxies in the checkered  
755 puffer, likely due to reduced somatic energy reserves. Furthermore, post-stress circulating

756 cortisol values were negatively correlated with post-stress fitness proxies. This study  
 757 highlights the importance of discovering the link between GCs and fitness in fish, and  
 758 establishes the checkered puffer as a valuable model for future research on the ecology of  
 759 stress in wild vertebrates.

760

## 761 2.6 Tables

762

763 **Table 2.1** Means ( $\pm$  SE), paired sample t-test and Pearson correlation results for baseline  
 764 and post-stress physiological parameters (cortisol and glucose) and fitness proxies (bite  
 765 force, puff score and puff time) in the checkered puffer (*Sphoeroides testudineus*).

766 Baseline values were collected from acclimated pufferfish within 3 min. Fish were then  
 767 immediately ‘stressed’ by holding them at the air-water interface for 5 min in a rubber-  
 768 mesh dip net. Pufferfish were then resampled 30 min post-stressor within 3 min to collect  
 769 post-stress values. A total of 38 fish were sampled.

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

770

771

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) |
| <b>Baseline treatment</b>                |                |            |  |
| Bite force (N)                           |                | 0.053      | -0.094                                   |
| Puff score                               |                |            | 0.201                                    |
| Puff time to deflate once released (min) |                |            |  |
| <b>Post-stress treatment</b>             |                |            |  |
| 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

779 **Table 2.3** The effect of body measures, including total length, mass and condition  
 780 (Fulton's condition factor calculated from total length and mass measurements), on  
 781 baseline and post-stress physiological indices and fitness proxies. Simple regression  
 782 results are presented. Baseline values were collected from acclimated pufferfish within 3  
 783 min. Fish were then immediately 'stressed' by holding them at the air-water interface for  
 784 5 min in a rubber-mesh dip net. Pufferfish were then resampled 30 min post-stressor  
 785 within 3 min to collect post-stress values. A total of 38 fish were sampled.

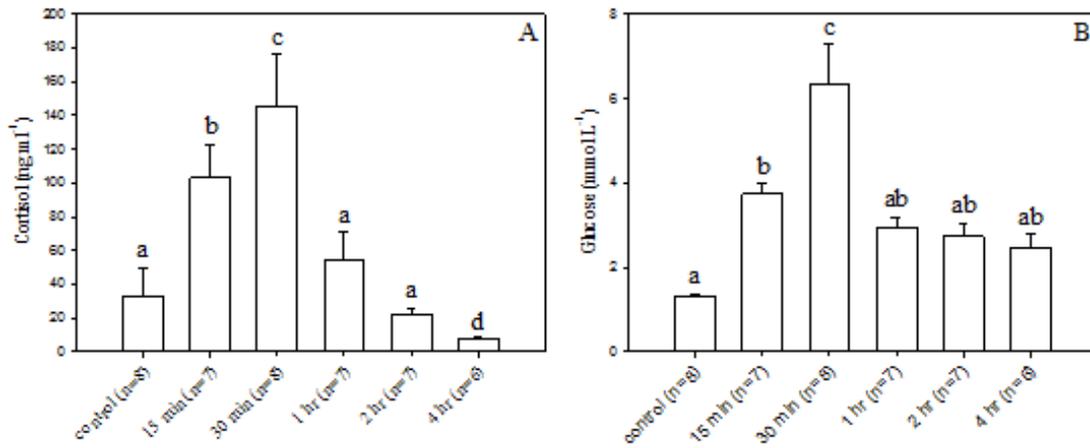
|   | Total length<br>(mm) |                       | Mass<br>(g) |                       | Fulton's condition<br>factor (mg mm <sup>-3</sup> ) |                       |
|---|----------------------|-----------------------|-------------|-----------------------|---|-----------------------|
|   | <i>F</i>             | <i>R</i> <sup>2</sup> | <i>F</i>    | <i>R</i> <sup>2</sup> | <i>F</i>  | <i>R</i> <sup>2</sup> |
| <b>Baseline treatment</b>                   |                      |                       |             |                       |   |                       |
| Cortisol (ng ml <sup>-1</sup> )             | 0.876                | 0.024                 | 0.940       | 0.025                 | 0.029   | 0.001                 |
| Glucose (mmol L <sup>-1</sup> )             | 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<br>once released (min) | 0.181                | 0.005                 | 0.261       | 0.007                 | 0.403   | 0.011                 |
| <b>Post-stress treatment</b>                |                      |                       |             |                       |   |                       |
| Cortisol (ng ml <sup>-1</sup> )             | 4.439                | 0.110*                | 6.789       | 0.159*                | 1.757   | 0.047                 |
| Glucose (mmol L <sup>-1</sup> )             | 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<br>once released (min) | 0.263                | 0.007                 | 0.237       | 0.007                 | 0.035   | 0.001                 |

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

786  
787

788 2.7 Figures

789



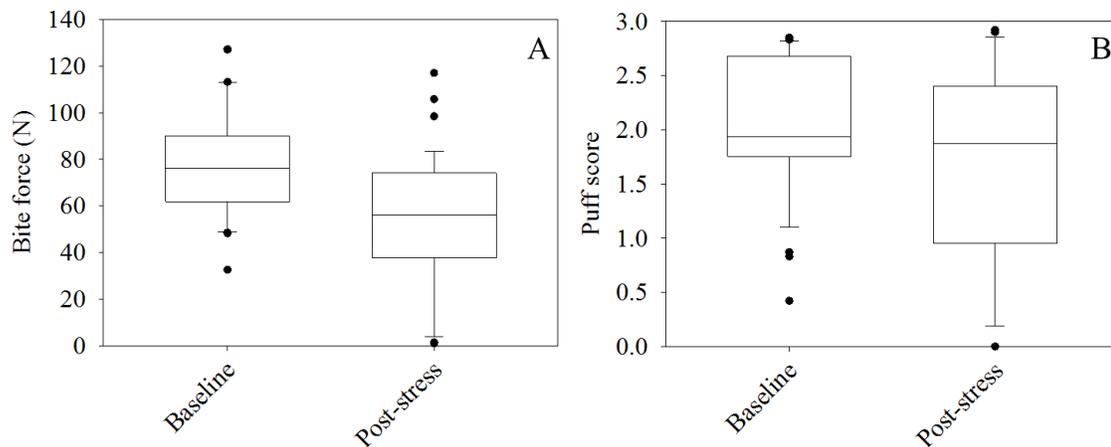
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791 **Figure 2.1** Plasma cortisol (A) and blood glucose (B) secretion and recovery in the  
792 checkered puffer (*Sphaeroides testudineus*) following a 5 min standardized stressor. Error  
793 bars represent standard error from the mean, and different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

796

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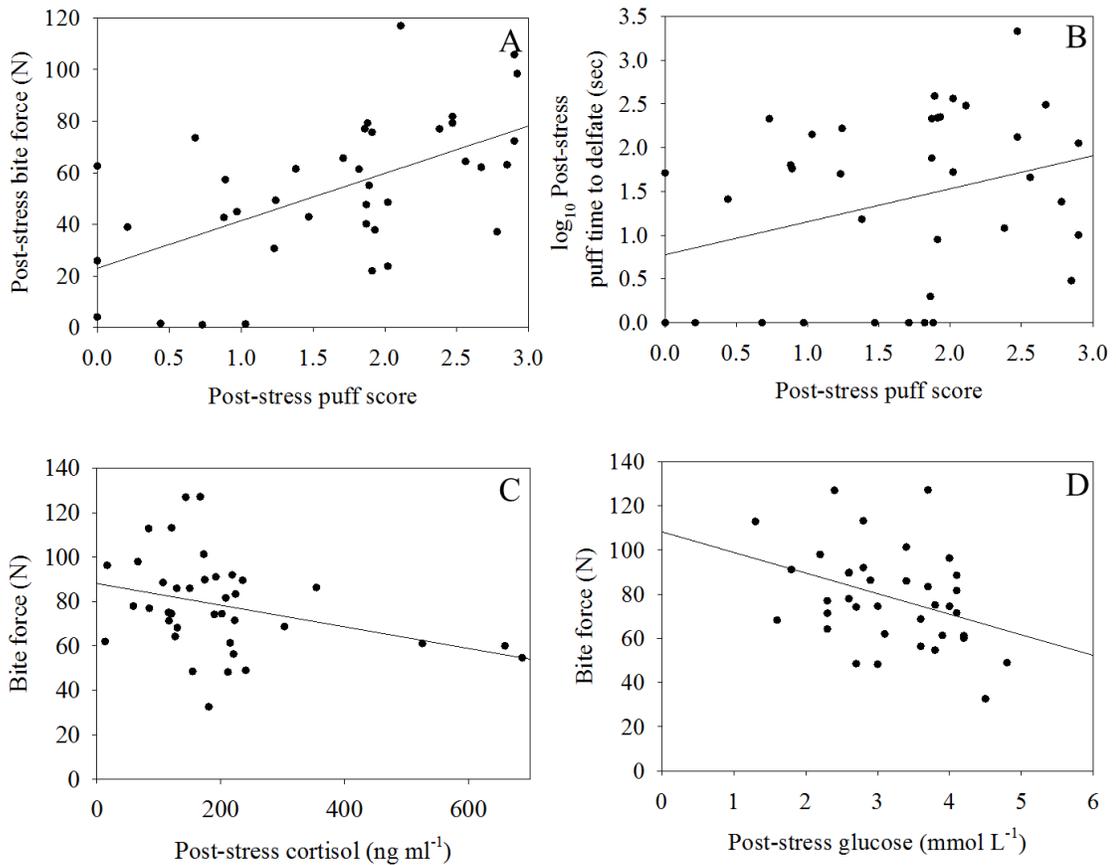
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799

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 25<sup>th</sup> and 75<sup>th</sup> percentiles with median enclosed within, and whiskers represent  
 806 10<sup>th</sup> and 90<sup>th</sup> percentiles.

807



808

809 **Figure 0.1** Correlations among baseline and post-stress fitness proxies of the checked  
 810 puffer (*Sphaeroides testudineus*). Significant two-tailed correlations were found between  
 811 post-stress puff score and bite force ( $R = 0.573$ ;  $P < 0.001$ ; A), as well as between post-  
 812 stress puff score and puff time to deflate once release ( $R = 0.325$ ;  $P < 0.05$ ; B). Also, the  
 813 effect of post-stress physiological indices, including cortisol (C) and glucose (D)  
 814 concentrations, on bite force following the standardized stressor.

815

816

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

820

821 **3.1 Abstract**

822 Given that anthropogenic environmental change will not occur in isolation of other  
823 stressors, it is necessary to explore the potential consequences of stress on the thermal-  
824 related characteristics (i.e., physiological and behavioural stress indices) of tropical  
825 marine fish. In this study, we used exogenous cortisol manipulations to investigate the  
826 effects of a thermal challenge on checkered puffers (*Sphoeroides testudineus*) as a  
827 secondary stressor. Two days post-treatment, the implanted pufferfish exhibited reduced  
828 swimming performance, however did not show any energetic costs in terms of changes in  
829 blood glucose concentrations and standard metabolic rate. In the lab, we tested the  
830 thermal tolerance of wild pufferfish by subjecting control and cortisol dosed individuals  
831 to abrupt 5 °C changes in temperature (i.e., cold and heat shock treatments). Following  
832 cold shock, control fish exhibited post-stress cortisol levels and weak ‘puff’  
833 performances. Whereas, fish dosed with cortisol exhibited post-stress cortisol levels at  
834 ambient temperature and contrary to our predictions, attenuated cortisol levels when  
835 subjected to the secondary thermal challenge. The 20-day complementary field study  
836 conducted in their natural habitat – a tidal creek in Eleuthera, The Bahamas – revealed  
837 that cortisol implanted fish generally selected cooler temperatures in their natural habitat  
838 when compared to controls. Although the physiological and behavioural consequences  
839 documented in the laboratory were not comparable to the ecological trends observed in

840 the field, these results highlight the need to establish the link between laboratory and field  
841 data to successfully develop management policies and conservation initiatives with  
842 regards to anthropogenic climate change. This study is the first to use experimental  
843 cortisol manipulation to investigate the effects of stress on the thermal biology in a wild  
844 fish population in a controlled laboratory setting, as well as on free-swimming fish in  
845 their natural habitat.

846

### 847 **3.2 Introduction**

848 Coastal marine ecosystems represent the transition zone between land and water, with  
849 their biodiversity shaped by dynamic physical and chemical processes (Harley et al. 2006,  
850 Burkett et al. 2008). Coastal biodiversity is specifically adapted to the extreme  
851 environmental conditions imposed along the gradients of these coastal boundaries, and  
852 the distribution of organisms within coastal ecosystems is governed by tolerances to  
853 varying environmental conditions, including changes in water parameters (such as  
854 temperature, salinity and pH), light availability, storm disturbance, tides, water depth, and  
855 nutrient availability (Burkett 2008). However, coastal ecosystems are currently threatened  
856 by a suite of anthropogenic environmental changes including coastal development,  
857 contamination, and changes in environmental parameters (IPCC 2001). Despite the  
858 tolerance of coastal biota to naturally variable environments, coastal biodiversity is  
859 vulnerable to these anthropogenic impacts. In particular, rising global temperatures are  
860 provoking complex, non-linear responses among many biota in coastal ecosystems (Lee  
861 et al. 2001, Harley et al. 2006, Burkett et al. 2008). Temperature influences the growth,  
862 survival, reproduction and distribution of organisms (Brander et al. 2003, Reid, 2003).

863 Eurythermal and heat-tolerant species may be more vulnerable to increasing temperatures  
864 as compared to more temperate species, because these species typically live closer to their  
865 thermal limits (Tomanek and Somero 1999, Stillman 2002, Harley et al. 2006).

866 Ectothermic animals, whose basic physiological processes are influenced by external  
867 temperatures, are of particular interest (Hochachka and Somero 2002). While it is unclear  
868 how tropical marine fishes will respond to increases in temperature (Cambers et al. 2007),  
869 species distributions are expected to expand toward cooler environments (Parmesan and  
870 Yohe 2003, IPCC 2007a).

871         Given that climate change will not occur in isolation of other stressors (e.g.,  
872 habitat alteration, contamination), it is necessary to determine the effects of multiple  
873 environmental challenges on the temperature tolerances of coastal fishes. This  
874 information is necessary to predict thresholds for survival of individuals under changing  
875 climate regimes, and predict the associated ecological consequences on coastal  
876 ecosystems. To date, most research on thermal stress among fish has been restricted to  
877 laboratory studies, and most have been conducted *in vitro* (e.g., Ackerman et al. 2000,  
878 Vijayan et al. 2000, Basu et al. 2001). However, it is valuable to study the effects of  
879 multiple stressors on animals in their natural habitat. Field-based studies have the  
880 potential to provide a more comprehensive understanding of the impacts of multiple  
881 stressors within the natural ecosystem, and thus may provide better predictions of the  
882 consequences of climate change. Tools now exist for monitoring temperature selection in  
883 field settings. It is possible to tag individual fish with thermal logging devices to quantify  
884 thermal preferences. It is also possible to experimentally manipulate baseline  
885 physiological stress levels through the use of exogenous glucocorticoid hormone

886 implants. Glucocorticoids are the primary stress hormones in vertebrates. During a  
887 challenge, these hormones are released, and orchestrate a suite of physiological processes  
888 that promote survival and recovery of the individual through the challenge (Sapolsky et  
889 al. 2000). Glucocorticoid implants mimic a natural stress response in that they elevate  
890 circulating glucocorticoid concentrations to levels seen during a typical endogenous stress  
891 response, and initiate the same suite of downstream physiological processes. In fish, the  
892 primary glucocorticoid is cortisol (Mommsen et al. 1999, Barton 2002), and the use of  
893 cortisol implants has been established as a method of elevating circulating cortisol levels  
894 in fish for approximately 3-7 days in both laboratory (Gamperl et al. 1994) and field (e.g.,  
895 O'Connor et al. 2009 and 2013, Dey et al. 2010) settings. In a laboratory environment,  
896 cortisol-implanted fish are more susceptible to thermal stress than un-manipulated  
897 controls (Basu et al. 2001, McConnachie et al. 2012). However, no studies have  
898 investigated the effects of thermal stress in conjunction with other stressors in wild free-  
899 swimming fish.

900         In the current study, circulating cortisol was experimentally manipulated within  
901 physiologically relevant limits to quantify the effects of multiple and chronic stressors on  
902 thermal-related characteristics in the checkered puffer (*Sphoeroides testudineus*). The  
903 checkered puffer has an expansive distribution throughout the Gulf of Mexico and  
904 Caribbean sea, and ranges along the Atlantic coastline as far north as Rhode Island, and as  
905 far south as the south eastern coasts of Brazil (Shipp 1974, Targett, 1978, Pauly 1991).  
906 This distribution is credited to the pufferfish's broad physiological tolerance. Here, we  
907 focussed on characterising the thermal tolerances and preferences in control and cortisol-  
908 implanted checkered puffers in both the laboratory and in the field. In the laboratory, we

909 compared indicators of energy use (blood glucose concentrations, oxygen use and  
910 swimming performance) and stress indicators (blood plasma cortisol concentrations,  
911 behavioural changes) in response to thermal challenges between control and cortisol-  
912 implanted fish. We predicted that experimental cortisol implants would reduce the  
913 thermal tolerances of checkered puffers in a laboratory setting. In the field, we used small  
914 thermal loggers affixed to fish over a 20-day period to compare thermal habitat use  
915 between treatment groups. To that end, we tested the null hypothesis that the checkered  
916 puffer's thermal sensitivity and habitat use was independent of whether cortisol levels had  
917 been artificially elevated. To test our hypothesis, we completed a series of complementary  
918 experiments validating the cortisol implant, including the required dosage, the depletion  
919 timeline, and the energetic cost in terms of blood glucose concentrations, swimming  
920 performance and standard metabolic rate measurements using intermittent-flow  
921 respirometry. Once the caveats of the cortisol implant were established, experimental  
922 cortisol manipulations were used to focus on the thermal-related consequences in the  
923 laboratory as well as in the natural habitat of the pufferfish. Collectively, these  
924 experiments assist in our understanding of the physiological, behavioural and ecological  
925 consequences of climate-induced stress in a wild tropical fish.

926

### 927 **3.3 Methods**

928

#### 929 **3.3.1 Study site and study animals**

930 For all experiments, checkered puffers (n=143) were collected from Page, Plum and  
931 Kemps Creeks on the island of Eleuthera, Bahamas (Page: N24°49'04.7" W076°18'51.6";

932 Plum: N24°45'45.79" W076°15'6.65"; Kemps: N24 48'54.29" W76 18'03.09"; Fig. 1).  
933 Pufferfish were corralled into seine nets set at the mouths of the creeks on an outgoing  
934 tide, and transported to the Cape Eleuthera Institute (CEI: N24°50'06.70" W76°19'31.69)  
935 in aerated coolers. At CEI, pufferfish were held in 1250 L aerated flow-through tanks, and  
936 were allowed to acclimate to laboratory conditions. Temperatures in tanks reflected  
937 ambient coastal conditions. During acclimation, pufferfish were fed an assortment of dead  
938 sardines (*Sardinella aurita*), juvenile bonefish (*Albula vulpes*) and mottled mojarra  
939 (*Eucinostomus lefroyi*) every 2 days. Fish were starved 48 hrs before experimentation.  
940 Given that fish could be easily handled and most sampling occurred with the fish  
941 submerged in a water-filled trough, all techniques were performed without anesthesia (see  
942 Cooke et al. 2005). All samples were collected in accordance with the guidelines of the  
943 Canadian Council on Animal Care as administered by Carleton University (B12-01), and  
944 all fish were released back into the ocean alive upon recovery at the conclusion of the  
945 experiment.

946

### 947 **3.3.2 Validation study of cortisol implant dose**

948 In February 2012 (water temperature:  $23.2 \pm 2.0^{\circ}\text{C}$ ), checkered puffers were randomly  
949 assigned to one of four treatment groups to validate the dose of the cortisol implant: 1)  
950 control (n=6); 2) sham (n=6); 3) low-dose cortisol treatment (n=7); and 4) high-dose  
951 cortisol treatment (n=7). Treatment fish were air-exposed for administration of an  
952 intramuscular injection of heated cocoa butter containing cortisol (hydrocortisone 21-  
953 hemisuccinate; Sigma H2882, Sigma-Aldrich, St. Louis, MO). Intra-peritoneal injection  
954 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<sup>-1</sup> cortisol in heated cocoa butter (i.e., 25 mg  
967 kg<sup>-1</sup> fish body weight), while high-dose cortisol treatment fish were injected with 10 mg  
968 mL<sup>-1</sup> cortisol in heated cocoa butter (i.e., 50 mg kg<sup>-1</sup> 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

978 withdrawn in under 3 min (Romero and Reed 2005). Data were compared to previous  
979 studies of checkered puffers where cortisol was measured in the blood following a 5 min  
980 air exposure challenge (Cull et al. submitted).

981

### 982 **3.3.3 Validation of cortisol implant depletion timeline**

983 From April to June 2012 (water temperature:  $29.1 \pm 1.5^\circ\text{C}$ ), checkered puffers were  
984 randomly assigned one of the following treatment groups to verify the time course of  
985 cortisol elevation over a 20 day holding period: 1) control (i.e., sampling of resting fish  
986 on day 0;  $n=7$ ); 2) sampling at 2 days ( $n=7$ ); 3) sampling at 5 days ( $n=7$ ); 4) sampling at  
987 10 days ( $n=5$ ); and 5) sampling at 20 days ( $n=6$ ) post-implantation. Fish from all  
988 treatment groups were measured as described above, and briefly air-exposed (30 s) while  
989 a  $5 \text{ mL kg}^{-1}$  intramuscular injection of  $10 \text{ mg mL}^{-1}$  cortisol in cocoa butter was  
990 administered (dosage selected based on above validation). Control fish were handled in an  
991 identical manner, but received no injections. Treatment fish were then placed in a  
992 common holding tank where they were cared for as described above. To minimize  
993 disturbances prior to blood sampling, fish were placed in 12.5 L individual opaque  
994 experimental chambers 12 hrs prior to sampling. Previous work on checkered puffer  
995 revealed that fish recover from handling stressors within 3 hrs (Cull et al., submitted). The  
996 method of holding fish in communal tanks before introducing them temporarily to  
997 individual holding chambers has been successfully used with bluegill (*Lepomis*  
998 *macrochirus*; McConnachie et al. 2012b), bonefish (*Albula spp.*; Shultz et al. 2011) and  
999 two species of cardinalfish (*Ostorhinchus doederleini* and *O. cyanosoma*; Munday et al.  
1000 2009). All pufferfish were sampled for blood as described above.

1001 **3.3.4 Metabolic cost of cortisol implant**

1002 The metabolic burden imposed by the cortisol-cocoa butter implant (10 mg mL<sup>-1</sup>) on  
1003 standard metabolic rate (SMR) was determined using intermittent-flow respirometry on  
1004 checkered puffers in June 2012 (water temperature: 28.6±1.8°C). The SMR of cortisol-  
1005 dosed individuals (n=8) was compared with that of control individuals (n=8). Cortisol  
1006 treated fish were dosed (10 mg mL<sup>-1</sup>; methods described above) 48 hrs prior to SMR  
1007 measurement. The respirometry system, operating procedures and calculations were  
1008 identical to those previously described by Shultz et al. (2011), with the exception of the  
1009 duration of individual cycles that consisted of an 18 min flush, 1 min wait and 20 min  
1010 measurement cycle. Oxygen consumption rate (MO<sub>2</sub>, mg O<sub>2</sub> kg<sup>-1</sup> h<sup>-1</sup>) for each fish was  
1011 calculated using the average of the six lowest values recorded overnight (i.e., between  
1012 20:00 and 06:00; Schurmann and Steffensen 1997), and when the coefficient of  
1013 determination (R<sup>2</sup>) for slope measurements was >0.95 during each measurement cycle.  
1014 All calculated dissolved oxygen values were corrected for background oxygen  
1015 consumptions generated for each specific fish and chamber prior to commencing  
1016 experiments.

1017

1018 **3.3.5 Swimming performance**

1019 Following 24 hrs of intermittent-flow respirometry, cortisol burden was further assessed  
1020 by quantifying swimming ability using a chase to exhaustion protocol on the same group  
1021 of pufferfish. Individually, fish were dip netted from their respirometry chamber and  
1022 quickly placed into a shallow circular tank (1.22 m diameter filled with 15 cm of water).  
1023 A chase test was performed, and the time to exhaustion (i.e., the time at which three

1024 consecutive tail grabs could be performed without a reflex response; Kieffer 2000) was  
1025 recorded for each pufferfish. This protocol provided a comparative swimming  
1026 performance measure between control and cortisol-treated pufferfish and has previously  
1027 been validated in a variety of fish species (Heath et al. 1993, Portz 2007, Thiem et al.  
1028 2013).

1029

### 1030 **3.3.6 Lab experiment: Thermal tolerance**

1031 From April to June 2012 (water temperature:  $29.1 \pm 1.5^\circ\text{C}$ ), checkered puffers were  
1032 randomly assigned to the following treatment groups: control at 1) ambient temperature  
1033 ( $n=8$ ); 2)  $-5^\circ\text{C}$  from ambient temperature ( $n=6$ ); and 3)  $+5^\circ\text{C}$  from ambient temperature  
1034 ( $n=8$ ); 4) cortisol treatment at ambient temperature ( $n=8$ ); 5) cortisol treatment at  $-5^\circ\text{C}$   
1035 from ambient temperature ( $n=6$ ); and 6) cortisol treatment at  $+5^\circ\text{C}$  from ambient  
1036 temperature ( $n=8$ ). Cortisol-treated fish were measured and briefly air-exposed while a 5  
1037  $\text{mL kg}^{-1}$  intramuscular injection of  $10 \text{ mg mL}^{-1}$  cortisol in cocoa butter was administered.  
1038 Fish were then returned to communal tanks for 36-60 hrs. Control fish were handled  
1039 identically, but received no treatment. All fish were captured from communal tanks and  
1040 placed in individual opaque experimental chambers (12.5 L) supplied with ample aeration  
1041 and a constant flow of saltwater. The experimental chambers were cooled by pumping  
1042 water through a copper coil submerged in ice water, and warmed by heaters. These  
1043 methods provided the appropriate temperature accurate to  $\pm 1^\circ\text{C}$  of the target thermal  
1044 treatment. After 4 hrs (chosen because previous work on checkered puffers revealed that  
1045 fish recover from handling stressors within 3 hrs; Cull et al. submitted), fish were  
1046 sampled for blood as described above. For temperatures  $5^\circ\text{C}$  below and above ambient, a

1047 puff score was recorded during the 3 min sampling period by noting the time and intensity  
1048 of the ‘puff’ (i.e., body inflation). More specifically, puffs were assigned a score from 0  
1049 to 3, with 0 being no puff, 1 being equal to or less than half a full puff, 2 being greater  
1050 than half a full puff, and 3 being a full puff. A full puff was assigned once the fish was  
1051 maximally inflated (i.e., its skin was tight to the touch and subsequent inflation attempts  
1052 resulted in no further expansion). Each puff score (0-3) was assigned a percentage of time  
1053 used over the 3 min, and then weighted according to its score. As a result, each puff score  
1054 is presented as a value between 0 and 3 (i.e., 0 being no puff at all and 3 being a  
1055 consistent full puff over the course of the 3 min sampling period). The fish were then  
1056 released back into their respective chambers, and the time to fully deflate was recorded.

1057

### 1058 **3.3.7 Field experiment: Thermal preference**

1059 From December 31, 2012, to January 19, 2013, thermal preferences of checkered puffers  
1060 in their natural habitat were monitored in Page Creek. Page Creek is a shallow tidal water  
1061 channel with a single opening to the ocean. This creek system consists of an expansive  
1062 mangrove habitat undergoing two tidal cycles per day. The creek almost drains entirely at  
1063 low tide, causing large variability in water parameters. To assess the thermal  
1064 characteristics of the tidal creek, thermal loggers (iButton, Maxim Integrated Products,  
1065 Inc., Sunnyvale, CA; n=10) were covered in a synthetic rubber coating (Plasti Dip  
1066 International, Performix Brand products, Blaine, MN) and placed throughout Page creek,  
1067 covering a range of habitat types. Five of the iButtons (model no. DS1921H) had a range  
1068 of 15 to 46 °C, while the others (model no. DS1921Z) had a range of -5 to 26 °C. Factory-  
1069 stated resolution of all thermal loggers is  $0.125 \pm 1$  °C; previous calibration by our team

1070 reveals actual mean accuracy of  $0.4 \pm 0.3$  °C and mean precision of  $0.2 \pm 0.0$  °C  
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  $5 \text{ mL kg}^{-1}$  intramuscular  
1085 injection of  $10 \text{ mg mL}^{-1}$  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

1093 obtained for 7 control (n=3 DS1921H iButtons; n=4 DS1921Z iButtons; TL=186±3mm;  
1094 mass=128±8g) and 8 cortisol-treated (n=3 DS1921H iButtons; n=5 DS1921Z iButtons;  
1095 TL=188±8mm; mass=131±19g) fish. Upon capture, fish were re-measured and re-  
1096 weighed using the methods described above, so that changes in condition over the course  
1097 of the study could be calculated.

1098

### 1099 **3.3.8 Sample analyses**

1100 Whole blood glucose concentrations were quantified on site using an Accu-Chek®  
1101 Compact Plus glucose meter (Roche Diagnostics, Basel, Switzerland; see Cooke et al.  
1102 2008 for validation). Whole blood hematocrit (% packed cell volume, PCV) was also  
1103 determined on-site (LW Scientific Zipocrit, model # ZO-1, 10,000 r min<sup>-1</sup>; Lawrenceville,  
1104 GA). The remaining blood was centrifuged at 2000 g for 5 min to separate erythrocytes  
1105 from plasma (Capsule HF-120, Tomy Seiko Co., LTD, Tokyo, Japan). Plasma samples  
1106 were stored at -20°C until cortisol immunoassay analysis. Plasma cortisol was quantified  
1107 using colorimetric competitive enzyme-linked immunoassay (ELISA; Enzo Life Sciences  
1108 Cortisol ELISA Kit ADI-900-071; Farmingdale, NY) using a technique previously  
1109 validated for measuring cortisol concentrations in largemouth bass (Sink et al. 2008).  
1110 Samples were read by a SpectraMax Plus384 absorbance microplate reader (Molecular  
1111 Devices, LLC; Sunnyvale, CA) following ELISA manufacturer recommendations.

1112

### 1113 **3.3.9 Data handling and statistical analysis**

1114 All statistical analyses were conducted using IBM SPSS Statistics 20.0 (2011). For all  
1115 tests, residuals were examined for normal distributions using the Shapiro-Wilk test, and

1116 Levene's and Brown-Forsythe tests were used to assess homogeneity of variance for  
1117 variables with normally and non-normally distributed data, respectively. Variables were  
1118 transformed (log or square root transformed) to meet assumptions of normality and  
1119 homogeneity of variance. The level of significance for all statistical analyses was assessed  
1120 at  $\alpha=0.05$ . All values are reported as mean  $\pm$  standard error of the mean (SEM).

1121 For all experiments, difference in the size of pufferfish used in control and cortisol  
1122 treatment groups was assessed using one-way analysis of variance (ANOVA) tests. In  
1123 cases where differences were found among treatment groups, the test was followed by a  
1124 Tukey's post-hoc test of honestly significant differences (Tukey's HSD test) to determine  
1125 which treatments differed.

1126

1127 *Validation of cortisol implant dose.* To validate the cortisol implant dose in the checkered  
1128 puffer, a one-way ANOVA followed by a Tukey's HSD test was performed to quantify  
1129 differences in plasma cortisol and blood glucose concentrations in fish treated with high  
1130 ( $10 \text{ mg mL}^{-1}$ ) and low ( $5 \text{ mg mL}^{-1}$ ) implant doses, sham treated fish and controls. Data  
1131 were compared to previous studies of the checkered puffer where endogenous circulating  
1132 cortisol was measured in the blood following a 5 min air exposure challenge (Cull et al.  
1133 submitted). The high dose ( $10 \text{ mg mL}^{-1}$ ) resulted in circulating plasma cortisol  
1134 concentrations similar to those seen during a natural stressor, and was therefore used for  
1135 all subsequent experiments.

1136

1137 *Validation of cortisol implant depletion timeline.* To assess the depletion timeline of a  
1138 cortisol implant over a 20-day time course, an ANOVA followed by a Tukey's HSD test

1139 was used to define for differences in plasma cortisol and blood glucose concentrations in  
1140 control fish as well as fish at 2-, 5-, 10- and 20-days post-implantation. Differences in  
1141 hematocrit at 5-, 10- and 20-days post-implantation were also identified using an  
1142 ANOVA followed by a Tukey's HSD post-hoc test where appropriate.

1143

1144 *Metabolic cost of cortisol implant.* To determine the metabolic cost of the high cortisol  
1145 implant dose, independent sample t-tests were used to compare SMR ( $\text{MO}_2$ ) between  
1146 control and cortisol implanted pufferfish.

1147

1148 *Swimming performance.* An independent sample t-test was used to compare the time until  
1149 exhaustion of control and cortisol implanted fish in the chase experiments.

1150

1151 *Lab experiment: Thermal tolerance.* To quantify the interactive effect of cortisol  
1152 manipulation and thermal stress on the physiological and behavioural responses of the  
1153 checkered puffer in the laboratory, two-way ANOVAs were used to identify the effect of  
1154 multiple stressors (i.e., cortisol implant, and thermal stress) on physiological stress indices  
1155 and 'puffing' performance. Independent variables included in the model were endocrine  
1156 stress treatment (i.e., control vs. cortisol implant), and thermal treatment (i.e., ambient  
1157 temperature, 5 °C below, and 5 °C above ambient temperature). The interaction between  
1158 these two variables (stress treatment  $\times$  thermal treatment) was included in the model.

1159 Dependent variables were circulating cortisol concentration, circulating glucose  
1160 concentration, hematocrit, puff score, and puff time to deflate once released.

1161

1162 *Field experiment: Thermal preference.* To quantify the impact of cortisol manipulations  
1163 on the thermal preferences of checkered puffers in a field setting, thermal data from both  
1164 fish and habitat iButtons were recovered using the Java application, One Wire Viewer  
1165 (Maxim Integrated, San Jose, CA). A variety of thermal parameters were then compared  
1166 among the iButtons collected from the habitat, control, and cortisol-treated fish.

1167         First, as the iButton model no. DS1921Z has a maximum temperature reading of  
1168 26°C, all temperature recorded values equal to or above 26°C were identified and marked  
1169 as 26°C. The proportion of temperature values equal to or above 26°C was then calculated  
1170 for each fish and habitat iButton over the 20-day sampling period. Proportions were logit-  
1171 transformed (Warton and Hui 2011), and a one-way ANOVA was used to compare the  
1172 ratio of time spent at or above 26°C among habitat, control and cortisol-dosed fish.

1173         The daily accumulated thermal units (ATUs) were calculated for each fish and  
1174 habitat iButton for each day by summing every temperature value. The number of  
1175 recordings per iButton was consistent across all fish and habitat iButtons. All temperature  
1176 values equal to or above 26°C were considered 26°C. For only the iButton model no.  
1177 DS1921H, the average daily maximum was calculated for all groups. For all iButtons, the  
1178 average daily minimum was calculated. For only the iButton model no. DS1921H, the  
1179 average daily range was calculated for all groups by determining the difference between  
1180 average daily maximum and minimum values. One-way ANOVAs were used to compare  
1181 the ATUs, maximum, minimum and range temperatures among habitat, control and  
1182 cortisol-treated fish. To control for daily fluctuations in temperature, repeated measure  
1183 ANOVAs were also used to compare the ATUs, maximum, minimum and range  
1184 temperatures among habitat, control and cortisol-treated fish.

1185 *Field experiment: Change in body condition.* Fulton's condition factor (K) was calculated  
1186 twice for each pufferfish (once before tagging and deploying fish; and once retrieved 20-  
1187 days following deployment) as an indicator of general well being using the following  
1188 equation:

$$1189 \quad K = 100 \times (W/L^3);$$

1190 where (W) is body mass (in mg) and (L) is total length (in mm; Ricker 1975). A two-way  
1191 repeated measures ANOVA test was then used to identify possible differences between  
1192 the initial and final (i.e., following the 20 day period) conditions of control and cortisol-  
1193 implanted checkered puffers.

1194

## 1195 **3.4 Results**

1196

### 1197 **3.4.1 Size differences among treatment groups**

1198 Although distinct individuals of checkered puffers were randomly assigned to each  
1199 treatment group (i.e. No pufferfish were repeatedly sampled), significant differences in  
1200 size among groups were identified (one-way ANOVAs:  $P_s < 0.05$ ; Table 1). In the  
1201 cortisol implant depletion timeline validation experiment, pufferfish in the 20 days post-  
1202 implantation treatment averaged 38 mm longer and 75 g heavier than fish included in the  
1203 2 and 5 days post-implantation treatments, as well as 30 mm longer than fish in the  
1204 control treatment (Tukey test:  $P_s < 0.05$ ). In the metabolic cost and swimming  
1205 performance experiments, cortisol-treated pufferfish were averaged 19 g heavier than  
1206 controls (one-way ANOVA:  $F = 10.605$ ;  $P < 0.01$ ). In the thermal tolerance laboratory  
1207 experiment, control pufferfish treatments were averaged 20 g heavier than cortisol-dosed

1208 pufferfish treatments. Notably, cortisol-treated fish subjected to 5°C changes in  
1209 temperature were averaged 30 g lighter than other treatments (Tukey test:  $P_s < 0.05$ ).

1210

### 1211 **3.4.2 Validation study of cortisol implant dose**

1212 Intra-muscular cortisol manipulations successfully raised plasma cortisol titers in  
1213 pufferfish 2 days post-implantation ( $F = 13.997$ ,  $P < 0.001$ ; Fig. 2 A). Low and high  
1214 cortisol doses (25 and 50 mg kg<sup>-1</sup> fish, respectively) caused circulating cortisol  
1215 concentrations to increase by 8 and 18 times, respectively, when compared to control and  
1216 sham-treated fish (Fig. 2 A). However, only the high cortisol dosed fish exhibited plasma  
1217 cortisol levels that were statistically higher than other treatment groups (Tukey's HSD  
1218 tests,  $P < 0.01$ ). The checkered puffer has been reported to naturally release  $126 \pm 34$  ng  
1219 ml<sup>-1</sup> of plasma cortisol in response to an acute standardized stressor (Cull et al.,  
1220 submitted). Circulating cortisol concentrations following the high cortisol dose were  $147$   
1221  $\pm 35$  ng ml<sup>-1</sup> (Fig. 2 A). Thus, this dose resulted in physiologically relevant post-stress  
1222 level of plasma cortisol, and the 10 mg mL<sup>-1</sup> cortisol dose was used for the remainder of  
1223 the study. Control, sham treated, as well as low and high cortisol dosed pufferfish  
1224 displayed no differences in blood glucose levels ( $P > 0.05$ ; Fig. 2 B).

1225

### 1226 **3.4.3 Validation of cortisol implant depletion timeline**

1227 The high cortisol implant dose (50 mg kg<sup>-1</sup> fish) resulted in significant changes to the  
1228 stress response of pufferfish over the 20-day time course (one-way ANOVA:  $F = 15.100$ ,  
1229  $P < 0.001$ ; Fig. 2 C). More specifically, following cortisol implantation, pufferfish  
1230 exhibited over 20 times higher circulating plasma cortisol on day 2 when compared to

1231 baseline levels (i.e., day 0; Tukey's HSD test:  $P < 0.001$ ), and then dropped to baseline  
1232 levels over days 5, 10 and 20 (Tukey's HSD tests:  $P_s > 0.05$ ; Fig. 2 C), Pufferfish  
1233 implanted with a high cortisol implant dose displayed no significant difference in blood  
1234 glucose concentrations at any of the sampling periods over the 20-day period (one-way  
1235 ANOVA:  $P > 0.05$ ; Fig. 2 D). Similarly, no differences were found among groups in  
1236 hematocrit (one-way ANOVA:  $P > 0.05$ ; Table 2).

1237

#### 1238 **3.4.4 Metabolic cost of cortisol implant**

1239 The standard metabolic rate (SMR) of control ( $183.6 \pm 22.9 \text{ mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$ ) and high  
1240 cortisol ( $151.9 \pm 22.3 \text{ mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$ ) implanted puffers was similar (independent sample  
1241 t-test:  $P > 0.05$ ).

1242

#### 1243 **3.4.5 Swimming performance**

1244 During the chase experiments, control and cortisol implanted fish showed no significant  
1245 difference in swimming performance in terms of time until exhaustion (control:  $189.50 \pm$   
1246  $37.08 \text{ s}$ ; cortisol-dosed:  $171.38 \pm 13.62 \text{ s}$ ; independent samples t-test:  $P > 0.05$ ).

1247

#### 1248 **3.4.6 Lab experiment: Thermal tolerance**

1249 Plasma cortisol levels in the checkered puffer were primarily influenced by the cortisol  
1250 implant, as well as the interaction between the cortisol implant and thermal treatment  
1251 (Table 3; Fig. 3 A). Control pufferfish exhibited similar plasma cortisol levels at all  
1252 temperatures (Fig. 3 A). Pufferfish dosed with cortisol exhibited increased plasma cortisol  
1253 levels ( $164.20 \pm 21.10 \text{ ng ml}^{-1}$ ) at ambient temperature and lower levels of cortisol when

1254 subjected to changes in temperature. Blood glucose levels in the checkered puffer were  
1255 primarily influenced by thermal treatment (Table 3; Fig. 3 B). Pufferfish exposed to a 5  
1256 °C decrease in temperature had significantly higher levels of blood glucose than pufferfish  
1257 exposed to ambient temperature and a 5 °C increase in ambient temperature. There was no  
1258 significant difference in blood glucose concentrations of pufferfish exposed to ambient  
1259 temperature and a 5 °C increase in ambient temperature. The cortisol implant and thermal  
1260 treatments had no significant effect on hematocrit in the checkered puffer (Table 3).

1261         The puffing performances of the checkered puffer, including puff score and time  
1262 required to deflate once released, were primarily influenced by thermal treatment (Tables  
1263 3 and 4). Pufferfish were unable to perform any anti-predator puffing behaviour in  
1264 response to decreasing temperatures. The cortisol implant, as well as the interaction  
1265 between the cortisol implant and thermal treatment, did not significantly contribute to  
1266 changes in puff performance (Table 4).

1267

### 1268 **3.4.7 Field experiment: Thermal preference**

1269 The proportion of temperature values equal to or above 26°C was similar for control  
1270 ( $12.77 \pm 1.63$  %) and cortisol dosed ( $13.28 \pm 1.67$  %) fish, as well as to habitat iButtons  
1271 ( $11.98 \pm 1.02$  %; one-way ANOVA:  $P > 0.05$ ). Furthermore, the daily ATUs, as well as  
1272 minimum, maximum and range of temperatures experienced by control and cortisol dosed  
1273 fish did not differ from one another, or to the habitat temperature recordings (one-way  
1274 ANOVA:  $P > 0.05$ ; Table 5). The ATUs, minimum, maximum and ranges values were  
1275 found to significantly differ across each day over the 20 day study (repeated measures  
1276 ANOVA:  $P_s < 0.0001$ ; Table 6 and Fig. 4). However, significant differences between

1277 groups were only apparent for ATUs (repeated measures ANOVA:  $P < 0.0001$ ; Table 5  
1278 and Fig. 4). On average, daily ATUs for cortisol-treated fish ( $1083 \pm 5^\circ\text{C}$ ) were  $6^\circ\text{C}$   
1279 cooler when compared to control fish ( $1089 \pm 6^\circ\text{C}$ ), and  $9^\circ\text{C}$  cooler than that logged by  
1280 the habitat ( $1092 \pm 4^\circ\text{C}$ ; Table 5).

1281

#### 1282 **3.4.8 Field experiment: Change in body condition**

1283 The initial condition of control and cortisol-treated puffers ( $2.05 \pm 0.09$  and  $2.05 \pm 0.07$   
1284  $\text{mg mm}^{-3}$ , respectively) was similar to their final condition ( $1.97 \pm 0.06$  and  $1.91 \pm 0.05$   
1285  $\text{mg mm}^{-3}$ , respectively). Although a slight decrease in fish condition was observed over  
1286 the 20-day period, the decline in condition was not found to be significant, nor to be  
1287 significantly dissimilar among control and cortisol-treated pufferfish (two-way repeated  
1288 measures ANOVA:  $P > 0.05$ ).

1289

### 1290 **3.5 Discussion**

1291 In the current study, cortisol was experimentally manipulated to physiological post-stress  
1292 levels to elucidate the effects of a thermal challenge as a secondary stressor on the  
1293 checkered puffer. We compared indicators of energy use among control and cortisol-  
1294 implanted fish to evaluate the effects of the cortisol implant on pufferfish, and found that  
1295 pufferfish did not show any energetic costs in terms of changes in blood glucose  
1296 concentrations, standard metabolic rate, nor swimming performance. We then tested the  
1297 thermal tolerance of pufferfish in a controlled laboratory setting, and the thermal  
1298 preferences of wild pufferfish in a complementary field study. In the lab, we found,  
1299 contrary to our predictions, that fish dosed with cortisol exhibited lower levels of cortisol

1300 when subjected to the secondary thermal challenge. In the field, we found that cortisol  
1301 implanted fish generally selected cooler temperatures when compared to controls.  
1302 Collectively, these results tell an interesting story about how a wild tropical fish species  
1303 can deal with multiple climate-induced stressors.

1304

### 1305 **3.5.1 Validation study of cortisol implant**

1306 Due to the unique anatomy and physiology of the checkered puffer, we used  
1307 intramuscular injections of cortisol in the current study. Other cortisol manipulation  
1308 studies have employed intra-peritoneal cortisol implants, and cortisol-spiked food to raise  
1309 plasma cortisol levels in fish (reviewed in Gamperl et al. 1994). Although intramuscular  
1310 injection is rather uncommon for cortisol implants in fish, it worked quite well for  
1311 pufferfish. Indeed, cortisol levels were elevated for between 2 to 4 days over control  
1312 levels, a period similar to intra-peritoneal implants (e.g., O'Connor et al. 2009, 2013;  
1313 McConnachie et al. 2012).

1314

### 1315 **3.5.2 Metabolic cost of cortisol implant**

1316 The cortisol implant caused a peak in plasma cortisol levels of the checkered puffer 2  
1317 days post-injection. Therefore, we predicted a significant metabolic cost associated with  
1318 the cortisol implant at this time point. It is well established that exposure of fish to  
1319 experimentally manipulated cortisol titres initiates an endocrine response which in turn  
1320 induces metabolic and osmotic disturbances (i.e., the secondary stress response; Mazeaud  
1321 et al. 1977, Barton and Iwama 1991). However, there was no difference in O<sub>2</sub>  
1322 consumption between cortisol-treated and control fish 2 days post-injection. Furthermore,

1323 there was no difference in swimming performance between cortisol-treated and control  
1324 fish. Generally, cortisol implanted fish tired more rapidly than control fish, and therefore  
1325 may have reduced swimming performance; previous studies have linked chronically  
1326 increased cortisol titres to impaired performance in juvenile rainbow trout (*Oncorhynchus*  
1327 *mykiss*; Basu et al. 2002) and adult female pink salmon (*O. gorbuscha*; McConnachie et  
1328 al. 2012).

1329

### 1330 **3.5.3 Thermal biology**

1331 Few studies have established clear links between the documented consequences of  
1332 thermal stressors in the laboratory, and the parallel consequences found at the ecosystem  
1333 level by means of field studies (Pörtner 2009). By both quantifying the behavioural and  
1334 physiological consequences to multiple stressors in the laboratory, and establishing  
1335 thermal preferences under these different stressed states in a natural habitat, we are able to  
1336 better predict alterations in performance and overall fitness that may be expected of  
1337 climate change in the checkered puffer.

1338

1339 *Laboratory experiment.* We predicted that the cortisol implant would alter the short-term  
1340 thermal tolerance of the pufferfish, and increase the physiological stress response to the  
1341 thermal challenge (i.e., as a secondary stressor). We found that decreases in temperature  
1342 has the most significant physiological consequences on the checkered puffer, whereas  
1343 similar increases in temperature has little impact. Fish dosed with cortisol exhibited high  
1344 cortisol levels at ambient temperature, and lower levels of cortisol when subjected to  
1345 changes in temperature. Implanted pufferfish exhibited lower levels of circulating cortisol

1346 following heat and cold challenges likely due to the additive response and rapid clearance  
1347 of cortisol similar to that documented in bluegill sunfish (*Lepomis macrochirus*;  
1348 McConnachie et al. 2012).

1349       Elevated levels of cortisol (manipulated through intra-peritoneal injection) have  
1350 been previously found to significantly suppress levels of heat shock proteins (hsp) in  
1351 cutthroat trout (*O. clarki clarki*; Ackerman et al. 2001), mossambique tilapia  
1352 (*Oreochromis mossambicus*; Basu et al. 2001) and rainbow trout (Basu et al. 2001),  
1353 suggesting that cortisol may mediate hsp levels in fish tissues following times of  
1354 physiological stress through a series of cellular processes (see Basu et al. 2001 and 2002  
1355 for details). Furthermore, Basu et al. (2001) found that mossambique tilapia exhibited a  
1356 milder physiological stress response relative to rainbow trout, likely due to the fact that  
1357 tilapia are known to be a stress-tolerant fish (Bruton and Bolt, 1975, Basu et al. 2001).  
1358 Like tilapia, the checkered puffer also prefers a high thermal range, which may account  
1359 for their ability to better deal with a heat shock challenge in comparison to the equivalent  
1360 but opposite cold shock challenge.

1361       In response to the acute decrease in temperature, pufferfish were notably more  
1362 active than fish placed in the other treatments (i.e., constantly swimming or struggling to  
1363 exit the experimental chamber). This anecdotal increase in activity is likely an attempt to  
1364 cope with the acute change in temperature, and responsible for the inability to puff  
1365 following the 5 °C decrease in temperature. In response to decreasing temperature, fish  
1366 have been previously reported to show hyperresponsiveness, uncoordinated swimming  
1367 (e.g., bumping into tank walls and spontaneous circling), difficulty maintaining  
1368 equilibrium, complete loss of equilibrium, and induction of coma (see Friedlander et al.

1369 1976, Donaldson et al. 2008 for overview). An acute decrease in temperature (i.e., cold  
1370 shock) may influence the reliability of neuronal activity and the reliability of cellular  
1371 responses, leading to compromised anti-predator behaviour (Preuss and Faber 2003).

1372

1373 *Field experiment.* Generally, pufferfish selected cooler temperatures than the average  
1374 thermal profile of the creek, and cortisol-treated fish favoured cooler temperatures than  
1375 controls. The interpretation of the thermal preferences of checkered puffers in an  
1376 ecological context is complicated by the fact that this is the first study of its kind, and that  
1377 the laboratory and field studies reveal dissimilar findings. Based on the laboratory study,  
1378 control and cortisol dosed pufferfish seemed to easily cope with heat shock, but less so to  
1379 cold shock; therefore, we might expect cortisol implanted pufferfish and control fish to  
1380 select warmer temperatures within their natural habitat. We would also predict that  
1381 cortisol implanted fish would avoid secondary stressors, thereby moving within the  
1382 habitat to seek smaller thermal fluctuations.

1383         In variable environments, evolutionary theory predicts that ectotherms prefer a  
1384 body temperature slightly below the physiological optimum (e.g., intertidal snails  
1385 (*Chlorostoma funebralissnails*; Tepler et al. 2011) and Australian skinks (*Lygosominae*;  
1386 Huey and Bennett, 1987)). These less than optimal thermal choices are often strongly  
1387 associated with habitat-seeking behaviour that bears a competitive or anti-predation  
1388 advantage (Tepler et al. 2011). While these animals have shown to possess a high thermal  
1389 optimum and preference in the laboratory, like the checkered puffer, their habitat  
1390 selection in the field limits them to lower temperatures, sometimes leading to  
1391 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 hsp 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 Weatherhead 1988), a reduction in fat stores  
1411 (Guillete 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.

1437 **3.5.4 Conclusion**

1438 The combination of stressors caused by gradual anthropogenic climate change may  
1439 provoke complex non-linear responses in coastal systems on the individual, population  
1440 and community levels (Lee et al. 2001, Harley et al. 2006, Burkett et al. 2008). Through  
1441 experimental cortisol manipulations, we were able to highlight the detrimental  
1442 physiological and behavioural consequences of multiple and repeated thermal stressors  
1443 (i.e., heat and cold shock challenges) in the checkered pufferfish in a controlled  
1444 laboratory, and for the first time, relate it to a comparable and ecologically relevant field  
1445 study monitoring the thermal preferences and condition of fish. The disparity in findings  
1446 between the lab and the field suggests that in field environments, animals have greater  
1447 opportunity to select their environments, and that any physiological consequences  
1448 associated with experimentation have the potential to be modulated by behaviour more so  
1449 than in a confined laboratory settings. These findings highlight the need to establish the  
1450 link between laboratory findings and ecologically relevant information in order to develop  
1451 appropriate management policies and conservation initiatives with regards to  
1452 anthropogenic climate change.

1453

1454

1455 **3.6 Tables**

1456

1457 **Table 3.1** The mass and total length (TL) of checkered puffers (*Sphoeroides testudineus*)

1458 treatment groups included in all experiments. One-way ANOVAs followed by a Tukey's

1459 HSD tests were conducted to quantify differences among groups. Letters identify

1460 statistical differences across treatment groups.

| <b>Fish treatment group</b>                              | <b>TL<br/>(mm)</b>     | <b>F</b> | <b>P</b>     | <b>Mass<br/>(g)</b>    | <b>F</b> | <b>P</b>     |
|--|------------------------|----------|--------------|------------------------|----------|--------------|
| <b>Validation of cortisol implant dose</b>               |                        |          |              |                        |          |              |
| 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                |          |              |
| <b>Validation of cortisol implant depletion timeline</b> |                        |          |              |                        |          |              |
| Control  | 184 ± 5 <sup>a</sup>   | 4.826    | <b>0.005</b> | 145 ± 9 <sup>ab</sup>  | 5.444    | <b>0.002</b> |
| 2 days post-implant                                      | 176 ± 4 <sup>a</sup>   |          |              | 130 ± 7 <sup>a</sup>   |          |              |
| 5 days post-implant                                      | 178 ± 5 <sup>a</sup>   |          |              | 105 ± 6 <sup>a</sup>   |          |              |
| 10 days post-implant                                     | 191 ± 10 <sup>ab</sup> |          |              | 136 ± 12 <sup>ab</sup> |          |              |
| 20 days post-implant                                     | 215 ± 11 <sup>b</sup>  |          |              | 192 ± 27 <sup>b</sup>  |          |              |
| <b>Metabolic cost and swimming performance</b>           |                        |          |              |                        |          |              |
| Control  | 152 ± 4                | 0.004    | 0.948        | 74 ± 3                 | 10.605   | <b>0.006</b> |
| Cortisol-dosed   | 152 ± 11               |          |              | 93 ± 5                 |          |              |
| <b>Lab experiment: Thermal tolerance</b>                 |                        |          |              |                        |          |              |
| Control at <i>amb. T</i>                                 | 182 ± 4                | 1.333    | 0.271        | 142 ± 8 <sup>a</sup>   | 2.744    | <b>0.033</b> |
| Control at -5°C  | 187 ± 6                |          |              | 124 ± 13 <sup>ab</sup> |          |              |
| Control at +5°C  | 191 ± 7                |          |              | 137 ± 14 <sup>ab</sup> |          |              |
| Cortisol-dosed at <i>amb. T</i>                          | 175 ± 4                |          |              | 130 ± 6 <sup>ab</sup>  |          |              |
| Cortisol-dosed at -5°C                                   | 181 ± 2                |          |              | 106 ± 5 <sup>ab</sup>  |          |              |
| Cortisol-dosed at +5°C                                   | 176 ± 6                |          |              | 102 ± 10 <sup>b</sup>  |          |              |
| <b>Field experiment: Thermal preference</b>              |                        |          |              |                        |          |              |
| 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               |          |              |

1461

1462 **Table 3.2** Checkered puffer (*Sphoeroides testudineus*) hematocrit levels at 5-, 10- and 20-  
1463 days post-implantation.

| <b>Fish treatment group</b> | <b>Hematocrit (%)</b> |
|-----------------------------|-----------------------|
| 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

1466 **Table 3.3** Two-way ANOVA outputs identifying the effect of multiple stressors (i.e., fish  
 1467 treatment (control and cortisol implanted pufferfish) and thermal treatment (ambient  
 1468 temperature, as well as 5°C below and above ambient temperature)) on physiological and  
 1469 behavioural stress indices, including cortisol, glucose, hematocrit and ‘puff’ performances  
 1470 (i.e., puff score and puff time to deflate once released).

|   | <b>R<sup>2</sup></b> | <b>Adjusted<br/>R<sup>2</sup></b> | <b>DF</b> | <b>F</b> |     |
|---|----------------------|-----------------------------------|-----------|----------|-----|
| <b>Cortisol</b>                           |                      |                                   |           |          |     |
| 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    | *   |
| <b>Glucose</b>                            |                      |                                   |           |          |     |
| 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    |     |
| <b>Hematocrit</b>                         |                      |                                   |           |          |     |
| 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    |     |
| <b>Puff score</b>                         |                      |                                   |           |          |     |
| 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    |     |
| <b>Puff time to deflate once released</b> |                      |                                   |           |          |     |
| 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

1471

1472

1473 **Table 3.4** Puffing performance (i.e., puff score and puff time to deflate once released)  
 1474 and hematocrit levels of control and cortisol implanted checkered puffers (*Sphoeroides*  
 1475 *testudineus*) when subject to +5 and -5 °C changes from ambient temperature. Letters  
 1476 identify statistical differences across treatment groups.

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

1477

1478 **Table 3.5** Temperature metrics, including the daily accumulated thermal units (ATUs),  
 1479 thermal minimums, maximums and ranges, recorded by the iButtons of control and  
 1480 cortisol implanted checkered puffers (*Sphoeroides testudineus*), as well as that of the  
 1481 habitat.

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

1482

1483

1484 **Table 3.6** Repeated measures general linear model statistical output, where the effect of  
 1485 day (i.e., each day over the 20 day period) on the calculated thermal variables (i.e., ATUs,  
 1486 minimums, maximums and ranges) of the different treatments (i.e., control and cortisol  
 1487 implanted fish, as well as the habitat thermal loggers) were established.

|                 | <b>DF</b> | <b>F</b> |     |
|-----------------|-----------|----------|-----|
| <b>ATUs</b>     |           |          |     |
| Day             | 19        | 71.175   | *** |
| Day * Treatment | 38        | 2.663    | *** |
| <b>Minimums</b> |           |          |     |
| Day             | 19        | 2447.665 | *** |
| Day * Treatment | 38        | 1.216    |     |
| <b>Maximum</b>  |           |          |     |
| Day             | 19        | 100.510  | *** |
| Day * Treatment | 38        | .863     |     |
| <b>Ranges</b>   |           |          |     |
| Day             | 19        | 61.471   | *** |
| Day * Treatment | 38        | .862     |     |

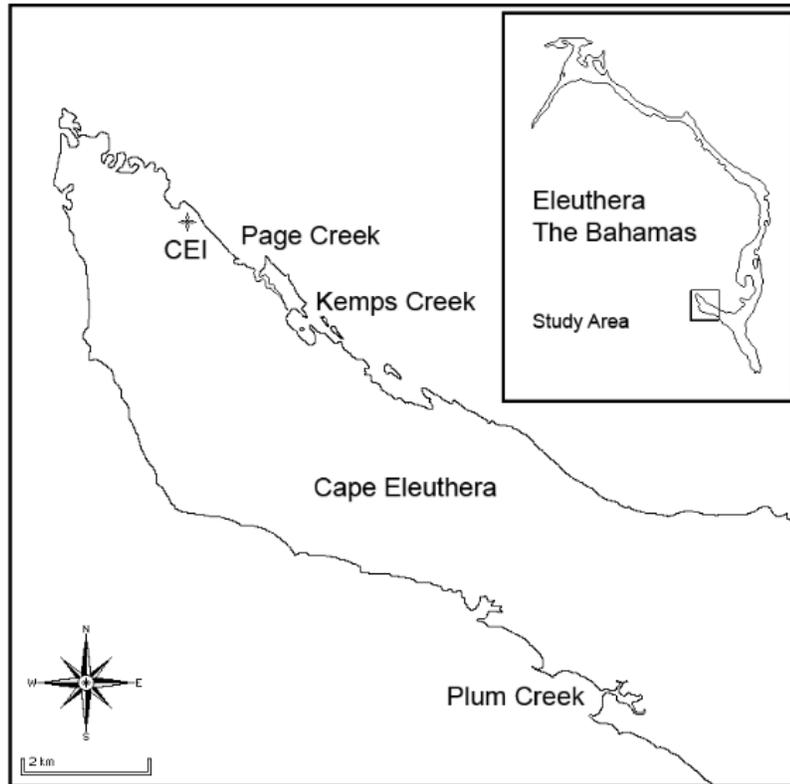
\*\*\* P < 0.001; \*\* P < 0.01; \* P < 0.05

1488

1489

1490 3.7 Figures

1491

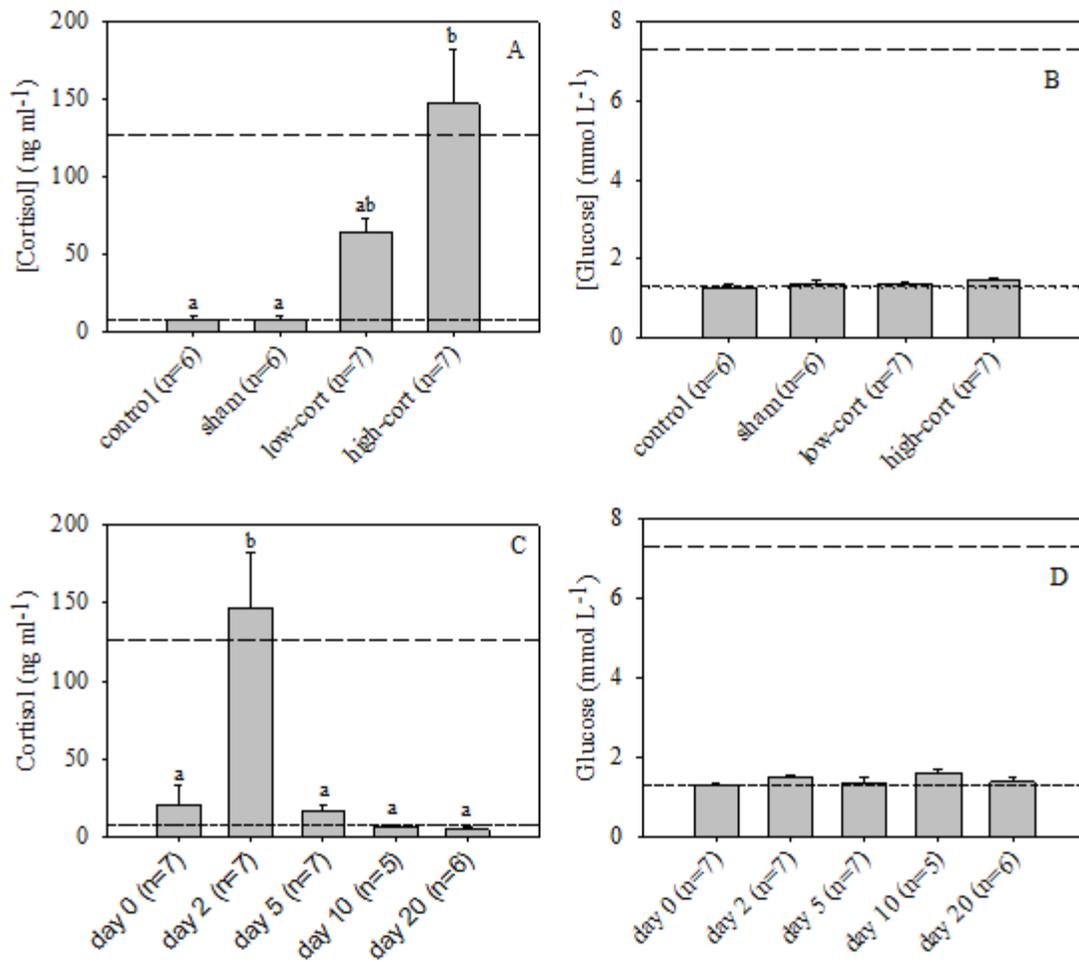


1492

1493 **Figure 3.1** Study area along the coast of Cape Eleuthera, Eleuthera, The Bahamas,  
1494 showing the locations of the sampled Page, Kemps and Plum Creeks, and the location of  
1495 the Cape Eleuthera Institute (CEI) research facility (black star). The inset map displays  
1496 the entire island of Eleuthera with the study area highlighted.

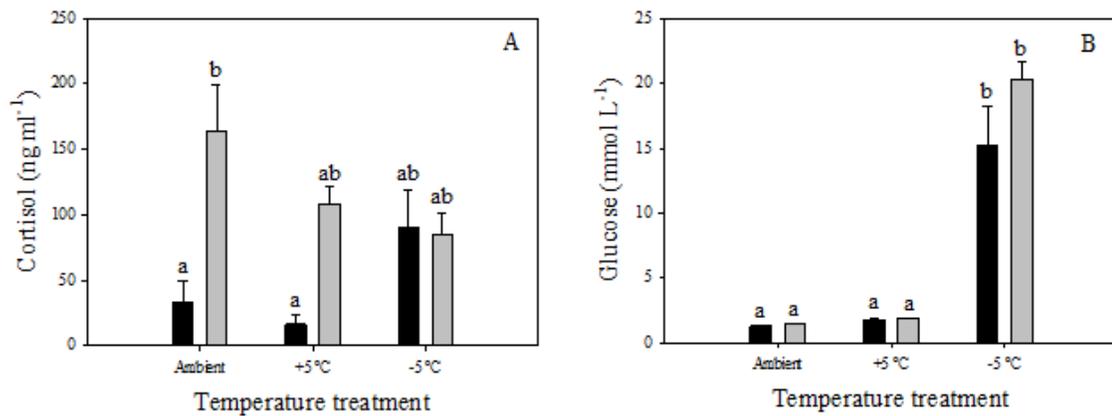
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1499

1500 **Figure 3.2** Checkered puffer (*Sphoeroides testudineus*) plasma cortisol (A) and  
 1501 glucose (B) concentrations of control and cocoa butter sham treated fish, as well as low-  
 1502 cort, low dose cortisol-cocoa butter (25 mg kg<sup>-1</sup> fish, 5 ml melted cocoa butter kg<sup>-1</sup> fish)  
 1503 injected fish and high-cort, high dose cortisol-cocoa butter (50 mg kg<sup>-1</sup> fish, 5 ml melted  
 1504 cocoa butter kg<sup>-1</sup> fish) injected fish. Plasma cortisol (C) and blood glucose (D)  
 1505 concentrations of high dose cortisol-cocoa butter (50 mg kg<sup>-1</sup> fish, 5 ml melted cocoa  
 1506 butter kg<sup>-1</sup> fish) injected checkered puffers over a 20 day period. Dashed lines indicate  
 1507 physiological baseline and post-stress concentrations.



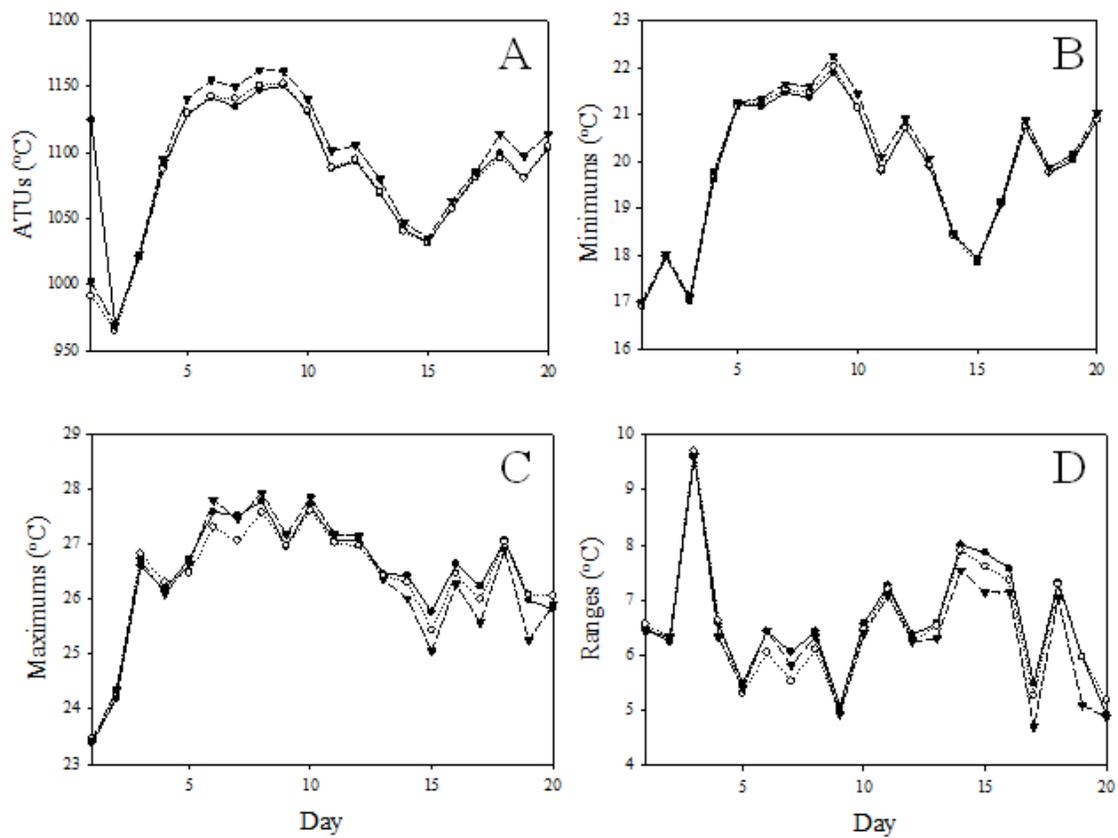
1508

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

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

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

1512



1513

1514 **Figure 3.4** Thermal variables, including the accumulated thermal units (ATUs; A),  
 1515 thermal minimums (B), maximums (C) and ranges (D), for control (dark circles) and  
 1516 cortisol dosed (light circles) checked puffers (*Spherooides testudineus*), as well as for  
 1517 the habitat loggers (dark triangle), over the 20 day sampling period.

1518

## 1519 **Chapter 4. General discussion**

1520

### 1521 **4.1 Overview**

1522 This thesis is a compilation of two distinct yet related studies that help characterize the  
1523 ecological consequences of stress in a wild tropical fish. In chapter 2, I quantified  
1524 individual variation in the glucocorticoid (GC) stress response in checkered puffers  
1525 (*Sphoeroides testudineus*) and determined whether there is a relationship between the GC  
1526 stress response and two established fitness proxies; two puffing metrics and bite force. In  
1527 chapter 3, I attempted to elucidate the consequences of the stress response on the thermal  
1528 biology of the pufferfish in a controlled laboratory setting and in the field. Through the  
1529 use of experimental cortisol manipulations, I revealed some of the possible physiological,  
1530 behavioural and ecological consequences of climate-induced stress.

1531 Individual variation in the endocrine stress response (i.e., the change in circulating  
1532 GCs following a challenge) has been linked to survival and fitness in a variety of species.  
1533 However, the strength and the direction of this relationship has proven to be highly  
1534 context dependent (e.g., Cockrem 2007, Øverli et al. 2007, Blas et al. 2007, Wada and  
1535 Breuner, 2008). To date, the majority of research assessing the link between the GC stress  
1536 response and fitness uses birds, reptiles and mammals, while only few examine fish (see  
1537 Breuner et al. 2008 and Bonier et al. 2009 for overviews, McConnachie et al. 2012). In  
1538 chapter 2, I focused on elucidating the functional significance of variation in GC secretion  
1539 using wild checkered puffers. The checkered puffer is an interesting model for studying  
1540 stress because it has unique predator avoidance strategies. Pufferfish will not hesitate to  
1541 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

1566 puffers were identified by experimentally manipulating cortisol levels in controlled laboratory  
1567 experiments. Next, a complementary 20-day field study was conducted in a tidal creek to  
1568 evaluate the effect of increased cortisol levels, through experimental manipulation, on the  
1569 preferred thermal profiles of pufferfish in their natural habitat.

1570

## 1571 **4.2 Findings and implications**

1572 In summary, the results of chapter 2 indicated that following an acute standardized  
1573 stressor, pufferfish exhibited increased physiological stress indices and interestingly,  
1574 reduced bite force and the extent of puffing. Furthermore, the magnitude of individual  
1575 physiological stress response was negatively correlated with post-stress fitness proxies. I  
1576 also documented that puff metrics for individuals are repeatable through time. Although  
1577 the acute stress response is thought to be adaptive (Wingfield et al. 1998), I documented  
1578 negative consequences in response to an acute standardized stressor, similar to other studies  
1579 (Blas et al. 2007, MacDougall-Shackleton et al. 2009, Cook et al. 2011). In chapter 3, I  
1580 verified the cortisol implant required to raise plasma cortisol titres to physiological post-  
1581 stress levels 2-day post treatment, the depletion timeline of the implant over a 20-day  
1582 period, and the energetic cost of the implant 2 days post-treatment. The physiological  
1583 consequences of the implant appeared to dissipate between 2 and 5 days post-treatment,  
1584 and the implant had no measurable energetic cost. The research I conducted revealed the  
1585 consequences of experimental cortisol manipulations on the thermal biology of the  
1586 pufferfish in laboratory and field environments. Control fish exhibited resting  
1587 physiological and behavioural stress indices following the heat shock treatment, and post-  
1588 stress cortisol levels and weak ‘puff’ performances after the cold shock treatment.  
1589 Whereas, fish dosed with cortisol exhibited post-stress cortisol levels at ambient

1590 temperature, and contrary to the collective prediction that additional stressors increase the  
1591 GC response, lower levels of cortisol when subjected to the secondary thermal challenge.  
1592 Furthermore, cortisol implanted fish generally selected cooler temperatures in their  
1593 natural habitat when compared to controls.

1594           Together, the results of chapters 2 and 3 suggest that the primary endocrine stress  
1595 response to acute and chronic stressors is associated with negative secondary and tertiary  
1596 consequences (i.e., variable glucose release, weakened fitness proxies and decreased  
1597 condition) and may translate to ecological consequences, in terms of wild population  
1598 dynamics. Although variation is a vital issue when using wild fish populations in their natural  
1599 habitat, an experimental field approach allows us to understand how stress influences  
1600 behaviour and survival of fish in the wild. These findings increase the application of the  
1601 results to real conservation issues (see Cooke and O'Connor, 2010) and highlight the need to  
1602 establish the link between laboratory findings and ecologically relevant information  
1603 needed for the development of management policies and conservation initiatives with  
1604 regards to anthropogenic climate change.

1605           To date, most research on tropical and intertidal species examines thermal limits.  
1606 To my knowledge, no studies have attempted to elucidate the effect of additional stressors  
1607 on the thermal biology of animals that live in extreme environments. These studies are  
1608 novel in that I:

- 1609 1. Used the checkered puffer as a valuable species to elucidate the consequences of  
1610 stress on a wild tropical population in an extremely fluctuating coastal environment;  
1611 2. Determined simple protocols to experimentally manipulate GCs and quantify unique  
1612 performances associated with fitness (i.e., bite force and puffing) in a pufferfish, and;  
1613 3. Elucidated the link between stress and the thermal biology of a tropical marine species  
1614 nearing its thermal limits in a controlled laboratory setting and in the wild.

1615

### 1616 **4.3 Future directions and further questions**

1617 The concepts investigated in this manuscript elucidate the intra- and inter-individual  
1618 variation in the physiological and behavioural stress response to an acute standardized  
1619 stressor, and the consequences this response may have on the physiology, performance,  
1620 and ecology of a wild population. By clarifying the role of GCs in a wild tropical fish  
1621 population, I have broadened our understanding of the ecology of stress in wild animals  
1622 that may be increasingly exposed to multiple and chronic stressors due to anthropogenic  
1623 climate change.

1624 To date, the majority of stress research has focused on elucidating the functional  
1625 significance of variation in GC secretion given the growing recognition that not all  
1626 individuals respond to stress in the same manner. The effect of individual variation in GC  
1627 secretion on performance and overall fitness is complex (see reviews by Ricklefs and  
1628 Wikelski 2002, Bruener et al. 2008, Bonier et al. 2009). Much of the GC research  
1629 performed has examined individual variation in stress response and the associated fitness-  
1630 oriented endpoints using birds (Angelier et al. 2007, Groscolas et al. 2008, Williams et al.  
1631 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.

- 1656 1. Why do studies continuously find different links between the physiological stress  
1657 response and behavioural consequences? What link are we missing? This challenge  
1658 may be due to the ambiguity in our definitions of an acute versus a chronic stressor,  
1659 and our limited understanding on how wild populations manage repeated and multiple  
1660 stressors. At what point does the stress response becomes maladaptive?
- 1661 2. The physiological and behavioural variability of the stress response established  
1662 among individuals of a wild checkered puffer population was noteworthy. Are there  
1663 covariates influencing this variability other than size? Maybe other covariates should  
1664 be considered, such as other hormones, sex, measures of condition and sexual  
1665 reproductive state?
- 1666 3. The physiological consequence of the cortisol implant had returned to resting titres by day  
1667 5 post-implantation. Do pufferfish have an increased ability to excrete cortisol relative to  
1668 other fish species? Although fish appear to physiologically recover from the implant 5  
1669 days post-treatment, is there evidence that fish may be burdened by the stressor on other  
1670 levels beyond day 5, in terms of energetic cost, performance and habitat selection? It  
1671 would be interesting to expose these fish to different treatments beyond the 5 day mark,  
1672 once cortisol has returned to resting baseline levels, and compare them to controls.  
1673 Treatments could include exposure to a pathogen or an epidermal laceration, to elucidate  
1674 immune function. It would be simple to include this within controlled laboratory and field  
1675 environments. Other treatments possible in the laboratory are thermal shock (or other  
1676 water parameter variations such as dissolved oxygen, pH, silt, etc.) and predator  
1677 encounters.
- 1678 4. Given that the checkered puffer is known to endure frequent temperature fluctuations in  
1679 their natural habitat and that thermal shock had a significant impact on the stress response

1680 of the pufferfish, would slower and larger changes in temperature have similar  
1681 consequences? What are the thermal thresholds if you take fluctuation time into  
1682 consideration?

1683 **5.** The thermal biology laboratory and field studies were conducted in the summer and  
1684 winter months, respectively. Given the change in temperature between summer and  
1685 winter, I would suggest that future studies compare within the same season to maintain  
1686 consistency between studies. In the summer, fish were found to easily cope with heat  
1687 shock over cold shock in the laboratory. Is this because heat shock likely occurs more  
1688 often during the summer? Would I have found opposite results (i.e., fish easily coping  
1689 with cold shock over heat shock) if the laboratory study was conducted in the winter?

1690 **6.** The checkered puffer is the first group of fish species to have their genome sequenced  
1691 (citation?). The pufferfish have little genetic variation with small intron spacing, and  
1692 therefore are unique candidates for extinction. The inconsistency between the reduced  
1693 genetic variation and the large ecological distribution suggests that season and  
1694 population specific isoforms may exist. Further laboratory and field studies on the  
1695 ecology of stress in pufferfish must be conducted on different ecological systems,  
1696 spanning a range of habitat types (i.e., varying thermal fluctuations, local adaptations,  
1697 water parameters, predator burdens, etc.), and implementing common garden  
1698 experiments. Experiments would include individuals from different marine habitats in  
1699 Eleuthera (i.e., Page, Plum, and Kemps creeks, as well as Poison flats), and other  
1700 habitats nearing the northern and southern limits of the species' range. In addition,  
1701 Page creek provides a promising study site for a long-term pufferfish monitoring  
1702 program, and may give us the means to answer questions with regards to the  
1703 consequences of chronic stress in the wild.

1704 7. Checkered puffers were not sexed in this study as no apparent sexual size dimorphism  
1705 was observed in this species. However, as fitness proxies were found to be highly  
1706 correlated with size in the checkered puffer, future work should consider sexing  
1707 individuals to monitor the possible confounding role of sex and its relationship with  
1708 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 <sup>-1</sup> ) |                  |              |                |                |                | Glucose (mmol L <sup>-1</sup> ) |        |               |                  |                |                 |
|--|---------------------------------|------------------|--------------|----------------|----------------|----------------|---------------------------------|--------|---------------|------------------|----------------|-----------------|
|  | Baseline                        |                  | Post-stress  |                | Responsiveness |                | Baseline                        |        | Post-stress   |                  | Responsiveness |                 |
|  | B                               | t                | B            | t              | B              | t              | B                               | t      | B             | t                | B              | t               |
| <b>Baseline treatment</b>                |                                 |                  |              |                |                |                |                                 |        |               |                  |                |                 |
| 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          |
| <b>Post-stress treatment</b>             |                                 |                  |              |                |                |                |                                 |        |               |                  |                |                 |
| Bite force (N)                           | 0.216                           | 1.488            | <b>0.262</b> | <b>1.746</b> * | 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          |
| <b>Responsiveness</b>                    |                                 |                  |              |                |                |                |                                 |        |               |                  |                |                 |
| Bite force (N)                           | 0.189                           | 1.124            | <b>0.329</b> | <b>1.937</b> * | <b>0.312</b>   | <b>1.829</b> * | -0.197                          | -1.177 | -0.152        | -0.839           | -0.077         | -0.413          |
| Puff score                               | <b>-0.312</b>                   | <b>-2.057</b> ** | -0.231       | -1.410         | -0.177         | -1.074         | -0.199                          | -1.273 | <b>-0.381</b> | <b>-2.396</b> ** | <b>0.314</b>   | <b>-1.878</b> * |
| 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 <sup>-1</sup> ) and Glucose (mmol L <sup>-1</sup> ) |                |                |                 |                |                |
|--|---|----------------|----------------|-----------------|----------------|----------------|
|  | Baseline  |                | Post-stress    |                 | Responsiveness |                |
|  | R <sup>2</sup>  | F              | R <sup>2</sup> | F               | R <sup>2</sup> | F              |
| <b>Baseline treatment</b>                |   |                |                |                 |                |                |
| 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          |
| <b>Post-stress treatment</b>             |   |                |                |                 |                |                |
| Bite force (N)                           | 0.079   | 1.957          | <b>0.165</b>   | <b>4.667</b> ** | <b>0.101</b>   | <b>2.601</b> * |
| 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          |
| <b>Responsiveness</b>                    |   |                |                |                 |                |                |
| Bite force (N)                           | 0.070   | 1.283          | <b>0.157</b>   | <b>3.179</b> *  | 0.110          | 2.112          |
| Puff score                               | <b>0.139</b>  | <b>3.184</b> * | <b>0.137</b>   | <b>3.147</b> *  | 0.090          | 1.933          |
| Puff time to deflate once released (min) | 0.018   | 0.306          | 0.035          | 0.616           | 0.054          | 0.976          |

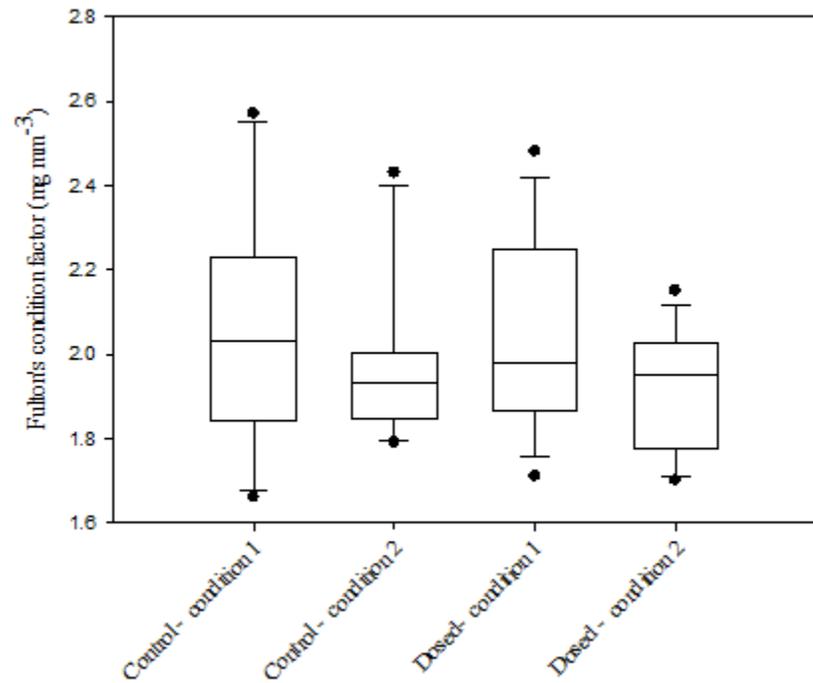
\*P < 0.10; \*\*P < 0.05

## 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 (*Sphaeroides 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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