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Do physical habitat complexity and predator cues influence the baseline and stress-induced glucocorticoid levels of a mangrove-associated fish?

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ABSTRACT

As human populations continue to expand, increases in coastal development have led to the alteration of much of the world's mangrove habitat, creating problems for the multitude of species that inhabit these unique ecosystems. Habitat alteration often leads to changes in habitat complexity and predation risk, which may serve as additional stressors for those species that rely on mangroves for protection from predators. However, few studies have been conducted to date to assess the effects of these specific stressors on glucocorticoid (GC) stress hormone levels in wild fish populations. Using the checkered puffer as a model, our study sought to examine the effects of physical habitat complexity and predator environment on baseline and acute stress-induced GC levels. This was accomplished by examining changes in glucose and cortisol concentrations of fish placed in artificial environments for short periods (several hours) where substrate type and the presence of mangrove roots and predator cues were manipulated. Our results suggest that baseline and stress-induced GC levels are not significantly influenced by changes in physical habitat complexity or the predator environment using the experimental protocol that we applied. Although more research is required, the current study suggests that checkered puffers may be capable of withstanding changes in habitat complexity and increases in predation risk without experiencing adverse GC-mediated physiological effects, possibly as a result of the puffers' unique morphological and chemical defenses that help them to avoid predation in the wild.

1. Introduction

Human urbanisation has led to dramatic alterations in biodiversity and ecosystem structure and function (Hooper et al., 2012; Vitousek et al., 1997). Although all ecosystems have been altered, coastal marine systems have been particularly impacted (Crain et al., 2009; Gray, 1997). Coastal ecosystems have been subjected to shoreline modification (shoreline hardening, removal of vegetation), dredging, and input of pollutants (including chemicals, silt and nutrients; Alongi, 2002; Buchan, 2000), causing negative impacts on biota that span multiple levels of biological organization (i.e. from the ecosystem to the cell; Helmuth, 2009).

Mangrove habitats in particular are being threatened by coastal development, having undergone global declines of about one third since the early 1950s (Alongi, 2002). The destruction of mangrove habitats is devastating for natural systems, as these environments support thousands of species of flora and fauna (Katherisan and Bingham, 2001; Nagelkerken et al., 2008). This is particularly salient for those species of marine fishes and invertebrates whose early life-stages use the complex mangrove prop-root systems as habitat during early ontogeny. Mangroves therefore contribute directly to biodiversity and can also generate massive economic benefits (Rönnbäck, 1999). For example, it is estimated that 80% of global commercial fish catches rely directly or indirectly on mangrove systems (Sandilyan and Katherisan, 2012). Given that many aquatic prey species rely on the physical complexity of the mangrove ecosystem for protection from predators (Buchan, 2000; MacDonald et al., 2009), destruction of mangrove communities may influence the stress physiology of these prey species.

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When fish are exposed to a stressor, activation of the hypothalamic-pituitary-interrenal (HPI) axis results in the release of glucocorticoid (GC) stress hormones such as cortisol into the blood, leading to a mobilization of energy stores such as glucose that help fish restore homeostasis and thereby cope with the stressor (Barton, 2002; Wendelaar Bonga, 1997). Although this process, hereafter referred to as the stress response, plays a fundamental role in helping fish respond to changes in their environment (Wingfield, 2008; Wingfield et al., 2011), chronically elevated levels of stress hormones can have negative fitness implications (Breuner et al., 2008; Romero et al., 2009). While there is a general understanding that baseline and post-stress GC levels increase in response to environmental change (Bonier et al., 2009; Breuner et al., 2008), few studies have been conducted on wild animals to determine the effects of specific changes on GC secretion (Boonstra, 2013).

Given the importance of mangroves to fish populations around the globe, the negative fitness implications of elevated stress, and the ecosystem services generated by healthy fish populations (Holmlund and Hammer, 1999), there is a growing desire to understand how stressors associated with coastal development affect GC secretion in wild fish populations. Development in tropical regions often involves the removal of mangrove trees (Alongi, 2002), resulting in a loss of physical habitat complexity and, in theory, an associated increase in the risk of predation for prey fish species. While a number of studies have been conducted on terrestrial animals examining the effects of predation risk on prey physiology and demography (e.g. Clinchy et al., 2013; Creel et al., 2009; Sheriff et al., 2011; Zanette et al., 2011), comparatively few studies have been conducted on aquatic species examining how factors such as habitat alteration and the predator environment influence baseline GC levels and the ability of fish to respond to other stressors.

The objective of our study was therefore to investigate whether exposure to varying levels of physical habitat complexity and predation risk influences the stress responsiveness of fish from a mangrove community. This was accomplished by examining the baseline and stress-induced changes in glucose and cortisol concentrations of fish placed in artificial environments in which substrate type and the presence of mangrove roots and predator cues were manipulated. The checkered puffer (*Sphoeroides testudineus*) was chosen as a model species because of its wide distribution, ease of capture, and abundance in mangrove communities (MacDonald et al., 2009; Shipp, 1974). Checkered puffers are thought to rely heavily on mangrove habitats for protection from predators (MacDonald et al., 2009), sheltering in the roots and blending in with the heterogeneous substrate using their cryptic dorsal colouration (Austin and Austin, 1971; Targett, 1978). Therefore, we hypothesized that homogeneous substrate, the absence of mangrove roots, and the presence of predator cues would result in higher baseline and post-stress glucose and cortisol concentrations in the puffers as well as a magnified physiological stress response.

2. Materials and methods

2.1. Fish capture and acclimation

This study was conducted in July 2014 at the Cape Eleuthera Institute (CEI) located on Eleuthera, The Bahamas. All research was conducted in accordance with an approved Canadian Council for Animal Care protocol (B12-08) and with a Scientific Collection permit furnished by the Bahamas Department of Marine Resources. A total of 110 checkered puffers (TL = 178 ± 20 mm, mass = 130 ± 37 g; mean ± standard error of the mean [SEM]) were captured from Page Creek (N 24°49′ 04.7″, W 076°18′51.6″), an undisturbed, mangrove-lined tidal creek at the southern end of Eleuthera. Capture was achieved by directing fish down the creek into a seine net during low tide. All fish were then transported to CEI in aerated coolers. At CEI, fish were held in a 1831-L tank that was constantly aerated and supplied with filtered, UV-sterilized flow-through seawater (28.8 ± 0.4 °C). This tank was devoid of any substrate or other habitat characteristics, and fish were held in the tank for a minimum of 72 h prior to experimentation to allow sufficient time for acclimation to laboratory conditions (see Fig. 1 for schematic of the experimental protocol). The puffers were maintained on a diet of chopped sardines (*Sardinella aurita*) throughout the study period, with feeding and cleaning of the holding tank occurring every other day at least 12 h prior to the beginning of the subsequent day's trials. All fish were returned to Page Creek at the conclusion of the study.

2.2. Experimental design

2.2.1. Habitat complexity experiment

In this experiment, we examined the effect of the presence of mangrove roots and substrate type on the baseline and stress-induced GC levels of the puffers. The substrate in mangrove communities is often heterogeneous, with components such as leaf litter, grasses, and small stones that help the checkered puffer to camouflage itself against the bottom (Austin and Austin, 1971). However, the dredging that often accompanies the removal of mangrove trees may result in a homogeneous substrate, making it more difficult for checkered puffers to use the substrate to avoid predators. To examine the effects of mangrove removal and substrate homogenization on the puffers' baseline and stress-induced GC levels, fish ($n = 44$) were assigned to one of four treatment groups: roots present/natural substrate (control group), roots present/homogeneous substrate, roots absent/natural substrate, and roots absent/homogeneous substrate.

Trials were conducted using four opaque experimental aquaria, each filled with approximately 40 L of seawater. Aquaria were constantly aerated using airstones, and the bottom of each aquarium was covered with a 2.0–2.5 cm-deep layer of beach sand. Sand in the 'natural substrate' treatments was scattered with small leaves, rocks, and twigs gathered from a nearby mangrove habitat, while the 'homogeneous substrate' treatments contained plain sand. Mangrove cover was provided by one of two sets of live red mangrove (*Rhizophora mangle*) roots that had 5 branches extending under the water, providing shelter for approximately 25–30% of the aquarium.

2.2.2. Predator environment experiment

In this experiment, we examined the effect of the presence of mangrove roots and chemical predator cues on the baseline and stress-induced GC levels of the puffers. Fish are capable of detecting or inferring the presence of predators using a variety of chemical olfactory cues, including predator odours, disturbance pheromones from conspecifics, and injury-released alarm cues from conspecifics (Wisenden, 2000). This experiment used a conspecific alarm cue in the form of a whole-body extract to simulate the presence of a predator. To examine the effects of mangrove removal and increased predation risk on the puffers' baseline and stress-induced GC levels, fish ($n = 45$; different from fish used in the habitat complexity experiment) were assigned to one of four treatment groups: roots present/predator cue absent (control group), roots present/predator cue present, roots absent/predator cue absent, and roots absent/predator cue present.

The conspecific alarm cue used in this experiment was taken from one of the puffers captured at Page Creek. Preparation of the cue was carried out similar to the methods of Brown and Smith (1997). However, because the presence of a damage-released alarm cue has not previously been tested in checkered puffers, the entire fish was used to account for the possibility of alarm cues located outside of the skin and visceral tissue (Meuthen et al., 2014). The puffer (mass = 102 g) was euthanized via cerebral percussion and blended with 350 mL of tap water. The skin of the puffer had to be removed because it was too tough to blend. Once removed, fifty 1-inch cuts were made in the skin with a scalpel; the skin was then rinsed with 50 mL of water, which was added to the blender, and the mixture was homogenized for an additional 1 min. After homogenization, the solution was poured through a cotton filter and more water was added to bring the filtrate to a final

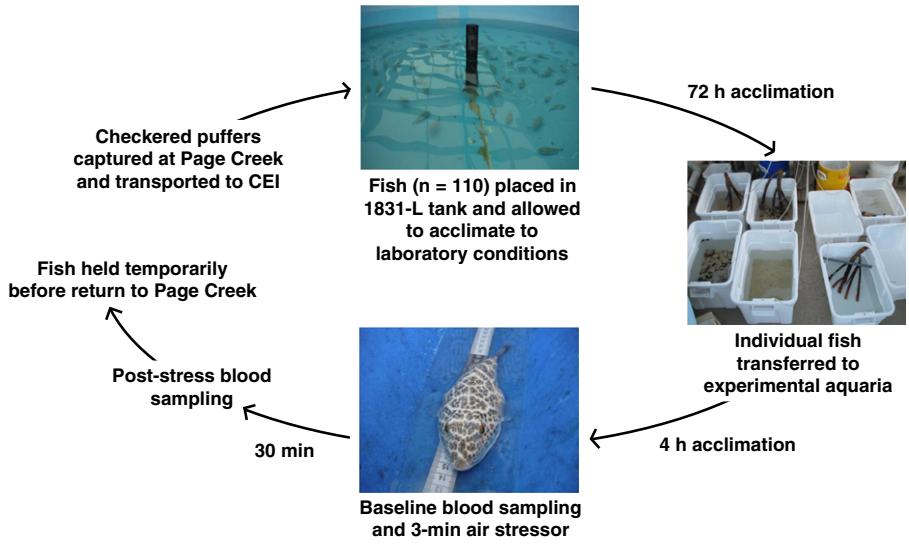

Fig. 1. Outline of experimental procedure used for the habitat complexity experiment and the predator environment experiment.

volume of 1 L. The filtrate was divided and frozen in 12-mL aliquots until needed in the trials. Regular tap water was also frozen in 12-mL aliquots as a control cue.

Before being used in the trials, the effectiveness of the predator cue was tested by observing the behaviour of a subset of the puffers before and after addition of the cue to the experimental aquaria (as per Mirza et al., 2003). We observed individual puffers every 60 s for 10 min before and 10 min after addition of either a control or alarm cue to the aquarium, noting the position of the fish in the aquarium and classifying its movement as either 'directed' (fast, often linear movement), 'undirected' (slow, often random movement), or 'still.' Addition of the control cue did not result in any obvious changes in the behaviour of the puffers, while addition of the alarm cue was followed by a noticeable decrease in activity characteristic of an antipredator response. Similar to Mirza et al. (2003), this response was taken to indicate that the alarm cue was detectable by the puffers and was perceived as an indicator of increased predation risk. Behavioural responses were strongest when 24 mL of the alarm cue was added, so this was the concentration used for the trials.

Trials were conducted using four opaque experimental aquaria, each filled with approximately 30 L of seawater. Aquaria were constantly aerated using airstones, and did not contain substrate to avoid adsorption of the alarm cue to the substrate. Mangrove cover was provided by one of two sets of live red mangrove roots (different from the roots used in the habitat complexity experiment) that had 5 branches extending under the water, providing shelter for approximately 50% of the aquarium. The 'predator cue present' treatments were supplied with 24 mL of the conspecific alarm cue while the 'predator cue absent' treatments were supplied with 24 mL of the control cue. Cues were introduced into the aquaria by pouring the thawed mixture into the water close to the airstone 5 min prior to the addition of the fish.

2.3. Data collection

Trials were conducted from July 7 to 23. For both experiments, fish were removed from the common tank using hand nets; each individual was then randomly assigned to a treatment group and transferred in water to the corresponding experimental aquarium, with one fish per aquarium. Fish were acclimated in the aquaria for approximately 4 h. Although this may seem like a relatively short acclimation time, tidal creek environments are extremely dynamic and fish in these environments are often forced to leave the shelter of the mangroves and move in and out of the creek according to the tides (Meynecke et al., 2008; Reis-Filho et al., 2016; Sheaves, 2005). Therefore, a shorter acclimation period may realistically represent the amount of time that wild checkered puffers spend in a particular habitat before being forced to move to one of differing complexity as the tide changes. We also considered 4 h sufficient to avoid any significant effects of handling stress on baseline results, since cortisol concentrations in checkered puffers return to baseline levels 2 h after exposure to a major stressor (Cull et al., 2015).

Following the 4 h acclimation, fish were subjected to a 3-min air stressor that consisted of holding the fish in a foam-lined trough supplied with a small amount of water that left the gills partially exposed. During the stressor, a non-lethal 0.1–0.5 mL baseline blood sample was collected from each fish by caudal venipuncture using a heparinized 1-mL syringe and a 22-gauge, 22 mm needle. To avoid biases associated with sampling-induced stress, efforts were made to collect all blood samples within 3 min of the onset of the stressor (Romero and Reed, 2005). All samples were collected without the use of anaesthesia to avoid any interference with the stress response of the fish (Cooke et al., 2005). Following the stressor, fish were returned to their respective experimental aquaria. A post-stress blood sample was obtained from each fish 30 min after the initial stressor, at the time of peak cortisol concentration (Cull et al., 2015), after which the fish's length and mass were measured. Used fish were given a tail fin clip before being returned to the common tank, and all experimental aquaria were emptied and rinsed before being used for subsequent trials. Water in the common tank was cycled constantly, limiting exposure of untested fish to any alarm cues or disturbance pheromones that might be released by used fish.

Immediately following collection, whole-blood glucose measurements were taken for each sample using an Accu-Chek® Compact Plus glucose meter (Roche Diagnostics, Basel, Switzerland), a method previously validated for use in fish (Cooke et al., 2008; Stoot et al., 2014). Blood samples were centrifuged for 5 min with a TSZ-C-01 Mini Centrifuge 3K (TSZ Scientific LLC, Framingham, Massachusetts, USA), and plasma samples were then isolated and frozen until time of analysis. Plasma cortisol concentrations were quantified using a commercial radioimmunoassay kit (ImmuChem™ Cortisol RIA Kit 07221106; MP Biomedicals, Santa Ana, California, USA), with samples run in duplicate. The intra- and inter-assay coefficients of variation were 8% and 10%, respectively, and samples were read using a 2470 WIZARD^2 automatic gamma counter (PerkinElmer, Waltham, Massachusetts, USA).

2.4. Statistical analyses

Statistical analyses were conducted using *R* 3.1.0. (R Core Team, 2014). Two-way analysis of variance (ANOVA) tests with an interaction were conducted for each experiment to determine whether baseline and stress-induced glucose and plasma cortisol concentrations differed between treatments. Presence of mangrove roots and substrate type were used as independent variables for the habitat complexity ANOVAs, while presence of mangrove roots and presence of the predator cue were independent variables for the predator environment ANOVAs. Tests for both experiments used glucose level and cortisol concentration as the dependent variable. Values identified as outliers were removed if they were deemed to be due to abnormalities in the experimental procedure (e.g. if more than 3 min were required to collect a blood sample).

Normality and homogeneity of variance of residuals were assessed visually, and where necessary data were log-transformed to meet the assumptions of the statistical test. All values are reported as mean ± standard error of the mean (SEM). The level of significance for all statistical tests was evaluated at a level of $\alpha = 0.05$.

3. Results

There was no significant difference in mean total lengths or masses of fish between treatments for either experiment (Table 1). All fish mounted a stress response following exposure to the standardized stressor, as demonstrated by the dramatic difference in baseline and post-stress glucose and cortisol levels observed for both the habitat complexity experiment (Table 2) and the predator environment experiment (Table 3). On average, baseline glucose levels were 2.0 ± 0.0 mmol L^{-1} and increased 70% to a post-stress value of 3.4 ± 0.1 mmol L^{-1}. Mean baseline cortisol concentrations were 22.31 ± 0.46 ng mL^{-1}, and increased 845% to a post-stress concentration of 210.74 ± 10.18 ng mL^{-1}.

3.1. Habitat complexity experiment

Baseline levels of whole-blood glucose were not significantly affected by mangrove root cover ($F_{1,40} = 0.0001$; $p = 0.993$) or substrate type ($F_{1,40} = 0.225$; $p = 0.638$), and there was no significant interaction between these two factors ($F_{1,40} = 1.058$; $p = 0.310$). Similarly, post-stress glucose levels did not show a significant root cover effect ($F_{1,39} = 0.546$; $p = 0.464$) or substrate type effect ($F_{1,39} = 0.049$; $p = 0.826$), and there was no significant interaction effect ($F_{1,39} = 0.079$; $p = 0.780$). The magnitude of the glucose stress response also demonstrated no significant root cover effect ($F_{1,39} = 0.455$; $p = 0.504$), substrate type effect ($F_{1,39} = 0.012$; $p = 0.913$), or interaction effect ($F_{1,39} = 0.636$; $p = 0.430$).

Data for baseline plasma cortisol concentrations were log-transformed for analysis in order to meet the assumptions of the ANOVA test. The baseline cortisol concentrations were approximately 2.2 times higher when roots were present than when roots were absent, but the root cover effect was non-significant ($F_{1,37} = 3.374$; $p = 0.074$). There was also no significant substrate type effect ($F_{1,37} = 0.184$; $p = 0.671$) or interaction effect ($F_{1,37} = 0.151$; $p = 0.700$). Post-stress cortisol concentrations were not significantly affected by mangrove root cover ($F_{1,39} = 2.073$; $p = 0.158$). Concentrations were 1.3 times higher for the natural substrate treatments than for the homogeneous substrate treatments, but the substrate type effect was non-significant ($F_{1,39} = 3.840$; $p = 0.057$). Similarly, no significant interaction effect was found for post-stress cortisol concentrations ($F_{1,39} = 1.490$; $p = 0.230$). The magnitude of the cortisol stress response was unaffected by the presence of mangrove roots ($F_{1,38} = 0.226$; p = 0.638). Cortisol increase was 1.34 times higher for natural substrate than for homogeneous substrate, but the substrate type effect was non-significant ($F_{1,38} = 3.189$; $p = 0.082$) and there was no evidence of a significant interaction effect for the cortisol stress response ($F_{1,38} = 0.593$; $p = 0.446$).

3.2. Predator environment experiment

Baseline whole-blood glucose levels were not significantly affected by mangrove root cover ($F_{1,41} = 0.208$; $p = 0.651$) or the predator cue ($F_{1,41} = 2.990$; $p = 0.091$), and there was no significant interaction effect ($F_{1,41} = 0.159$; $p = 0.692$). Post-stress glucose levels did not show a significant root cover effect ($F_{1,37} = 0.036$; $p = 0.851$), predator cue effect ($F_{1,37} = 0.630$; $p = 0.432$), or interaction effect ($F_{1,37} = 0.232$; $p = 0.632$). Similarly, the magnitude of the glucose stress response was not significantly affected by mangrove root cover ($F_{1,38} = 0.002$; $p = 0.969$) or the predator cue ($F_{1,38} = 0.188$; $p = 0.668$), and there was no significant interaction effect ($F_{1,38} = 0.572$; $p = 0.454$).

Baseline plasma cortisol concentrations were more than twice as high when roots were absent compared to when roots were present, but the root cover effect was non-significant ($F_{1,39} = 3.359$; $p = 0.074$). Similarly, there was no significant predator cue effect ($F_{1,39} = 1.262$; $p = 0.268$) or interaction effect for baseline cortisol concentrations ($F_{1,39} = 0.403$; $p = 0.529$). Post-stress cortisol concentrations were not significantly affected by the presence of mangrove roots ($F_{1,35} = 0.653$; $p = 0.424$) or the predator cue ($F_{1,35} = 0.110$; $p = 0.742$) and there was no evidence of a significant interaction effect ($F_{1,35} = 0.005$; $p = 0.944$). Finally, the magnitude of the cortisol stress response did not show a significant root cover effect ($F_{1,35} = 1.642$; $p = 0.209$), predator cue effect ($F_{1,35} = 0.006$; $p = 0.939$), or interaction effect ($F_{1,35} = 0.003$; $p = 0.961$).

4. Discussion

Checkered puffers are commonly thought to rely both on mangrove roots and heterogeneous substrate for protection from predators (Austin and Austin, 1971; MacDonald et al., 2009; Targett, 1978). Therefore, we hypothesized that the puffers' GC concentrations would be highest when fish were exposed to homogeneous substrate, the absence of mangrove roots and the presence of conspecific alarm cues.

Table 1
Mean length and mass data according to treatment for checkered puffers used in the habitat complexity experiment and the predator environment experiment, and results of one-way ANOVAs testing for differences in size between treatments for both experiments.

Treatment	*n*	Length (mm)			Mass (g)		
		Mean ± SEM	*F*	*p*	Mean ± SEM	*F*	*p*
Habitat complexity experiment							
Roots present/natural substrate	12	179 ± 4	0.218	0.884	128 ± 10	0.115	0.951
Roots present/homogeneous substrate	11	183 ± 7			137 ± 17		
Roots absent/natural substrate	11	183 ± 4			135 ± 11		
Roots absent/homogeneous substrate	10	185 ± 4			137 ± 8		
Predator environment experiment							
Roots present/predator cue absent	12	179 ± 5	0.374	0.772	130 ± 12	0.317	0.813
Roots present/predator cue present	11	174 ± 4			117 ± 9		
Roots absent/predator cue absent	10	175 ± 5			127 ± 12		
Roots absent/predator cue present	12	179 ± 4			126 ± 7		

Table 2
Mean baseline, post-stress and stress response (post-stress – baseline) values of whole-blood glucose and plasma cortisol for checkered puffers exposed to different combinations of mangrove roots and substrate type in the habitat complexity experiment.

Treatment	Sampling period	Glucose (mmol L^{-1})		Cortisol (ng mL^{-1})	
		n	Mean ± SEM	*n*	Mean ± SEM
Roots present/natural substrate	Baseline	12	1.9 ± 0.1	11	34.56 ± 12.86
	Post-stress	12	3.7 ± 0.3	12	271.43 ± 33.60
	Response	12	1.7 ± 0.2	12	217.78 ± 34.80
Roots present/homogeneous substrate	Baseline	11	2.0 ± 0.1	10	40.36 ± 21.22
	Post-stress	10	3.5 ± 0.2	10	182.43 ± 24.30
	Response	10	1.5 ± 0.2	9	143.92 ± 22.02
Roots absent/natural substrate	Baseline	11	2.0 ± 0.1	10	10.58 ± 3.49
	Post-stress	11	3.4 ± 0.3	11	200.05 ± 25.31
	Response	11	1.3 ± 0.3	11	186.55 ± 26.21
Roots absent/homogeneous substrate	Baseline	10	1.9 ± 0.1	10	22.84 ± 14.67
	Post-stress	10	3.4 ± 0.3	10	179.92 ± 18.04
	Response	10	1.5 ± 0.3	10	157.08 ± 17.54

However, our results show that the puffers' baseline and post-stress GC levels and the magnitude of the stress response were not significantly affected by any of the above parameters, suggesting that the stress physiology of checkered puffers may not be strongly influenced by changes in habitat complexity and predation risk within the context of our experimental protocol.

These results run counter to those obtained by the small number of existing studies that have examined the relationship between perceived predation risk and GC levels in fish, most of which point to an increase in glucose and cortisol levels with increased predation risk. For example, Rehnberg and Schreck (1986) found that exposure to chemical stimuli from predatory fish resulted in a significant elevation in glucose and plasma cortisol concentrations in juvenile coho salmon (*Oncorhynchus kisutch*). Woodley and Peterson (2003) observed that longnose killifish (*Fundulus majalis*) that were fully exposed to the sight of a predator had significantly higher plasma cortisol levels than killifish that were partially hidden behind aquatic vegetation. More recently, Barcellos et al. (2007) found that zebrafish (*Danio rerio*) exposed to visual contact with a predator had significantly higher whole-body cortisol than fish that were completely isolated from predators. However, while the results obtained by this study clearly conflict with those mentioned above, there is reason to believe that the response of checkered puffers to increased predation risk may not be characteristic of that of the majority of mangrove-associated fish.

When compared to most other mangrove-associated fishes, checkered puffers are unusual in that they possess a variety of morphological and chemical defense mechanisms that help them to avoid predation. Morphologically and chemically defended species tend to respond less intensely to increased predation risk than species that do not possess special defenses (e.g. Abrahams, 1995; Cotton et al., 2004; Mowles et al., 2011; Rundle and Brönmark, 2001), a phenomenon referred to as 'trait compensation' (DeWitt et al., 1999). Checkered puffers possess a variety of such defenses, including an exceptionally tough epidermal layer, the ability to rapidly inflate their bodies upon capture (Brainerd, 1994), and a potent neurotoxin called tetrodotoxin (TTX) found in the skin (Katz and Miledi, 1967; Narahashi et al., 1964), all of which may help these fish to successfully escape or evade predation attempts in the wild (Caley and Schluter, 2003; Gladstone, 1987; Recher and Recher, 1968; Wainwright and Turingan, 1997). Given their low risk of predation-induced mortality, it is possible that environments with a high risk of predator encounter are not perceived as stressful by checkered puffers, allowing these fish to avoid any unnecessary negative effects associated with increased GC secretion (Bonier et al., 2009; Wingfield and Sapolsky, 2003).

The degree to which checkered puffers rely on mangroves for protection, and therefore the degree to which they are expected to show a physiological response to decreased shelter and increased predation risk, may be size-dependent. Predation risk in mangrove-associated fish decreases with increasing body size, meaning that larger fish do not rely as heavily on the shelter of the mangrove roots (MacDonald et al., 2009) and often choose not to seek shelter in the presence of predators (Laegdsgaard and Johnson, 2001; Werner et al., 1983). It is likely that the majority of individuals used in this study were in the upper half of the size spectrum for checkered puffers, as the smallest puffer that was captured from Page Creek was 140 mm TL despite the fact that these fish are known to range in size from 20 to 300 mm TL (MacDonald et al., 2009; Shipp, 1974). The piscivore community in shallow tropical estuarine systems such as tidal creeks is also composed primarily of smaller predators (Baker and Sheaves, 2005) that may be incapable of consuming adult checkered puffers. Although checkered puffers are often cited to rely on mangrove roots for shelter from predators, it is possible that the puffers' unique collection of morphological

Table 3
Mean baseline, post-stress and stress response (post-stress – baseline) values of whole-blood glucose and plasma cortisol for checkered puffers exposed to different combinations of mangrove roots and conspecific alarm cues in the predator environment experiment.

Treatment	Sampling period	Glucose (mmol L^{-1})		Cortisol (ng mL^{-1})	
		n	Mean ± SEM	*n*	Mean ± SEM
Roots present/predator cue absent	Baseline	12	2.2 ± 0.1	12	8.69 ± 3.29
	Post-stress	10	3.3 ± 0.2	9	217.58 ± 27.55
	Response	10	1.2 ± 0.2	9	207.47 ± 30.57
Roots present/predator cue present	Baseline	11	1.9 ± 0.1	10	12.66 ± 5.68
	Post-stress	11	3.2 ± 0.2	11	225.36 ± 24.29
	Response	11	1.2 ± 0.1	11	211.04 ± 25.75
Roots absent/predator cue absent	Baseline	10	2.1 ± 0.1	10	17.64 ± 6.87
	Post-stress	9	3.5 ± 0.5	9	191.75 ± 30.98
	Response	9	1.4 ± 0.4	9	172.63 ± 29.81
Roots absent/predator cue present	Baseline	12	1.9 ± 0.1	11	31.68 ± 11.75
	Post-stress	11	3.1 ± 0.2	10	203.69 ± 29.25
	Response	12	1.1 ± 0.2	10	173.36 ± 21.82

and chemical defenses combined with the lack of relatively large predators in tidal creek ecosystems significantly diminishes the protective value of the roots for larger individuals of this species.

To further test the role that body size plays in mediating the puffers' GC secretion, future studies should examine the stress response of juvenile checkered puffers exposed to similar experimental conditions. The importance of mangrove habitats as nurseries for a wide variety of fish species, partially due to their provision of shelter, has been well established in the literature (e.g. Adams and Tobias, 1999; Laegdsgaard and Johnson, 1995; Nagelkerken et al., 2000; Robertson and Duke, 1987). Although both juvenile and adult checkered puffers are found in mangrove habitats, it is possible that the protective function of the mangrove roots applies primarily to juveniles, as the predators found in Page Creek may still be capable of consuming smaller checkered puffers. Given the lack of juveniles in the sample taken from Page Creek, it may be necessary for future studies to sample at a different time of year when juveniles are known to be more abundant in the creek, or attempt capture from a different tidal creek that drains more completely at low tide.

Future studies may also benefit from the use of visual contact with a predator as an indicator of predation risk, or a combination of visual and chemical cues. Although the conspecific alarm cue used in this study appeared to induce antipredator behaviours in the puffers during a preliminary trial, it is possible that chemical cues alone are not enough to elicit a significant physiological fright response in these fish due to their unique antipredator defenses. This idea is similar to that proposed by Brown et al. (2005), who observed that fish from high-predation areas had a lower opercular beat frequency than fish from low-predation areas following exposure to a mild stressor, and concluded that the fish from high-predation regions have likely evolved to conserve energy except in the case of the most extreme stressors. Similarly, it is possible that checkered puffers may only exhibit a noticeable elevation in GC secretion when physically exposed to a predator. Juvenile lemon sharks (*Negaprion brevirostris*), great barracuda (*Sphyraena barracuda*) and houndfish (*Tylosurus crocodilus*) are the main predators known to reside in Page Creek (Harborne et al., 2015; Murchie et al., 2010) and could be captured for this purpose in future studies.

Another limitation of the current study is the lack of habitat structure in the common tank where the puffers were held for a majority of the experimental period. Initially we wished to supply this tank with substrate and mangrove roots to create an environment similar to the habitat in Page Creek, but this was deemed to be infeasible due to the difficulty that such structure would present in capturing fish for the experimental trials. As a consequence, it is possible that acclimating the fish to an environment devoid of cover may have altered their responses to the various levels of habitat complexity presented in the experimental trials. However, given that checkered puffers occasionally occupy open habitats outside of the mangrove roots (Murchie et al., 2015), and that previous studies on predation-induced stress and habitat complexity have achieved significant results despite holding fish in a structureless tank (Woodley and Peterson, 2003), it is unlikely that this lack of structure had a substantial effect on the results. Nevertheless, improvements may be made in future studies to more accurately assess the response of the fish to the various habitat treatments.

Although limited access to transportation to and from Page Creek necessitated that all fish be collected at the beginning of the study period for the current study, future studies may wish to collect fish at multiple intervals in order to avoid acclimating them to a predator- and structure-free environment for an extended period of time (Woodley and Peterson, 2003). It may also be beneficial for future studies to allow for longer acclimation times in a more natural setting (e.g. a mesocosm) during the experimental trials, as the use of laboratory versus field settings can sometimes yield different results in biological studies (Calisi and Bentley, 2009). Finally, future studies may also wish to sample fish at different time points. While our study collected all of its blood samples following the 4 h acclimation period, taking a sample both before and after acclimation to the experimental aquaria may give a better idea of the effects of different habitat components on baseline cortisol concentrations. This may be particularly useful if implemented in conjunction with longer acclimation times.

Although the results of our study suggest that stress levels in checkered puffers are not significantly affected by the removal of habitat complexity, this does not mean that these fish will be unaffected by habitat degradation. The destruction of mangrove habitats such as tidal creeks may impact the puffers by limiting access to food sources or by forcing them into areas where they are constantly exposed to larger predators that are normally unable to navigate in the narrow tidal creeks. Indeed, limited research currently suggests that checkered puffer abundance is negatively affected by environmental change and habitat degradation (Layman et al., 2010; Villéger et al., 2010), although the precise reason for these declines is unclear. While a number of studies have shown a connection between predation-induced stress and population declines in other taxa (e.g. Relyea, 2003; Sheriff et al., 2009; Zanette et al., 2011), further research is needed to determine the role that stress plays in mediating the population dynamics of checkered puffers and other coastal fish species.

In conclusion, the baseline and stress-induced glucose and cortisol levels of checkered puffers do not appear to be strongly influenced by changes in physical habitat complexity or the predator environment on the scale used in this experiment. This study is one of the first to attempt to connect changes in habitat complexity and predation risk with measures of glucocorticoid levels in fish, challenging the idea that mangrove roots play a significant protective function for adult checkered puffers. If this trend is reflective of the response that puffers would have to these stressors in the wild, the results suggest that checkered puffers may be a robust species capable of withstanding changes in habitat complexity and associated increases in predation risk without experiencing adverse stress-induced physiological effects. However, we also suggest that the effects of these stressors must be examined over a larger temporal scale (we only acclimated fish for 4 h to a given habitat type) and ideally in a field setting or large mesocosm. Given that the destruction of mangrove habitats and other coastal ecosystems is expected to continue well into the future as a result of increasing anthropogenic disturbances, more research is needed to better understand the effects of these changes on the stress physiology of juvenile puffers and other, more vulnerable aquatic species found in these systems.

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