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

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ABSTRACT

Anthropogenic climate change is altering temperature regimes for coastal marine fishes. However, given that temperature changes will not occur in isolation of other stressors, it is necessary to explore the potential consequences of stress on the thermal tolerances and preferences of tropical marine fish in order to understand the thresholds for survival, and predict the associated coastal ecological consequences. In this study, we used exogenous cortisol injections to investigate the effects of a thermal challenge on checkered puffers (*Sphoeroides testudineus*) as a secondary stressor. There were no significant differences between control and cortisol-treated fish 48 h following cortisol treatment for swimming ability (using a chase to exhaustion protocol), blood glucose concentrations or standard metabolic rate. In the lab, control and cortisol-treated puffers were exposed to ambient (29.1 ± 1.5 °C), ambient +5 °C (heat shock) and ambient −5 °C (cold shock) for 4 h and to evaluate the consequences of abrupt temperature change on puff performance and blood physiology. Following cold shock, control fish exhibited increases in cortisol levels and weak 'puff' performance. Conversely, fish dosed with cortisol exhibited consistently high cortisol levels independent of thermal treatment, although there was a trend for an attenuated cortisol response in the cortisol-treated fish to the cold shock treatment. A 20-day complementary field study conducted in the puffer's natural habitat, a tidal creek in Eleuthera, The Bahamas, revealed that cortisol-injected fish selected significantly cooler temperatures, measured using accumulated thermal units, when compared to controls. These results, and particularly the discrepancies between consequences documented in the laboratory and the ecological trends observed in the field, highlight the need to establish the link between laboratory and field data to successfully develop management policies and conservation initiatives with regards to anthropogenic climate change.

1. Introduction

Coastal marine ecosystems represent the transition zone between land and water, with their biodiversity shaped by dynamic physical and chemical processes (Harley et al., 2006; Burkett et al., 2008). The distribution of organisms within coastal ecosystems is governed by the tolerances to changes in abiotic factors such as water temperature, salinity, pH, light availability, tides, water depth and nutrient availability (Burkett et al., 2008). Although inhabitants of coastal marine areas are well suited to life in these dynamic ecosystems, anthropogenic disturbances such as coastal development, contamination and changes in environmental parameters threaten species diversity, abundance and distribution in these areas (IPCC, 2001). In particular, rising global temperatures are provoking complex, non-linear responses among many biota in coastal ecosystems (Lee et al., 2001; Harley et al., 2006; Burkett et al., 2008). Temperature influences the growth, survival, reproduction and distribution of organisms (Brander et al., 2003;

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Reid, 2003). Heat-tolerant species from the tropics with narrow thermal ranges may be more vulnerable to increasing temperatures, as well as more vulnerable to temperature variability (e.g., cold shock events) as compared to more temperate species, because these tropical species typically live closer to their thermal limits, and with relative annual thermal stability (Tomanek and Somero, 1999; Stillman, 2002; Harley et al., 2006). Ectothermic animals, whose basic physiological processes are influenced by external temperatures, are of particular interest (Hochachka and Somero, 2002). While it is unclear how tropical marine fishes will respond to increases in temperature (Chambers et al., 2007), species distributions are expected to expand toward cooler environments (Parmesan and Yohe, 2003; IPCC, 2007).

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

In the current study, circulating cortisol was experimentally manipulated within physiologically relevant limits to quantify the effects of elevated cortisol titers (the primary stress hormone in fish) on the thermal physiology and habitat use in the checkered puffer (*Sphoeroides testudineus*). The checkered puffer has an expansive distribution throughout the Gulf of Mexico and Caribbean sea, and ranges along the Atlantic coastline as far north as Rhode Island, and as far south as the south eastern coasts of Brazil (Shipp, 1974; Targett, 1978; Pauly, 1991). This distribution is credited to the broad physiological tolerance of checkered puffers. Here, we focused on characterizing the responses of control and cortisol-injected puffers to cold and heat shock in the laboratory and thermal habitat use of puffers in the field. First, we completed a series of preliminary experiments validating the cortisol injection, including the required dosage, the depletion timeline, and the energetic cost in terms of blood glucose concentrations, swimming ability (measured using a chase protocol) and standard metabolic rate measurements (using intermittent-flow respirometry). Once the caveats of the cortisol injection were established, experimental cortisol manipulations were used to focus on the thermal-related consequences in the laboratory and in the natural habitat of the checkered puffer. In the laboratory, we compared indicators of energy use (blood glucose concentrations, standard metabolic rate and anaerobic swimming ability) and stress indicators (blood plasma cortisol concentrations, behavioral changes) in response to thermal challenges (i.e., cold shock and heat shock) between control and cortisol-injected fish. We predicted that experimental cortisol injections would increase the sensitivity of checkered puffers to temperature change in a laboratory setting. In the field, we used small thermal loggers affixed to fish over a 20-day period to compare thermal habitat use between treatment groups to test the prediction that experimental cortisol injections would increase the thermal sensitivity of checkered puffers and alter their habitat use relative to control fish. Collectively, these experiments assist in our understanding of the physiological, behavioral and ecological consequences of climate-induced stress in a wild tropical fish.

2. Methods

2.1. Study site and study animals

For all experiments, checkered puffers (total $n=143$) were collected from Page, Plum and Kemps Creeks (details on experiment-specific capture dates provided below) on the island of Eleuthera, Bahamas (Page: 24°49′04.7″N, 76°18′51.6″W; Plum: 24°45′45.79″N, 76°15′6.65″W; Kemps: 24°48′54.29″N, 76°18′03.09″W; Fig. 1). All field experiments were conducted in Page Creek. Checkered puffers were corralled into seine nets set at the mouths of the creeks on an outgoing tide, and transported to the Cape Eleuthera Institute (CEI: 24°50′06.70″N, 76°19′31.69″W) in aerated coolers. At CEI, checkered puffer fish were held in 1250 L aerated flow-through tanks, and were allowed to acclimate to laboratory conditions. Temperatures in tanks reflected ambient coastal conditions, which are more stable than the temperatures observed in the tidal creeks, and typically fluctuated by 2.5 °C daily. During acclimation, checkered puffers were fed an assortment of dead sardines (*Sardinella aurita*), juvenile bonefish (*Albula vulpes*) and mottled mojarra (*Eucinostomus lefroyi*) every 2 days. Fish were starved 48 h before experimentation. Given that fish could be easily handled and most sampling occurred with the fish submerged in a water-filled trough, all techniques were performed without anesthesia (see Cooke et al., 2005). All samples were collected in accordance with the guidelines of the Canadian Council on Animal Care as administered by Carleton University (B12-01), and all fish were released back into the ocean alive upon recovery at the conclusion of the experiment.

2.2. Validation of cortisol injection: dose

In February 2012 (ambient water temperature: 23.2 ± 2.0 °C [mean ± SD, presented here and throughout paper when reporting temperatures]), checkered puffer fish were randomly assigned to one of four treatment groups to validate the dose of the cortisol injection: (1) control ($n=6$); (2) sham ($n=6$); (3) low-dose cortisol treatment ($n=7$); and (4) high-dose cortisol treatment ($n=7$). Treatment fish were air-exposed for administration of an intramuscular injection of heated cocoa butter containing cortisol (hydrocortisone 21-hemisuccinate; Sigma H2882, Sigma-Aldrich,

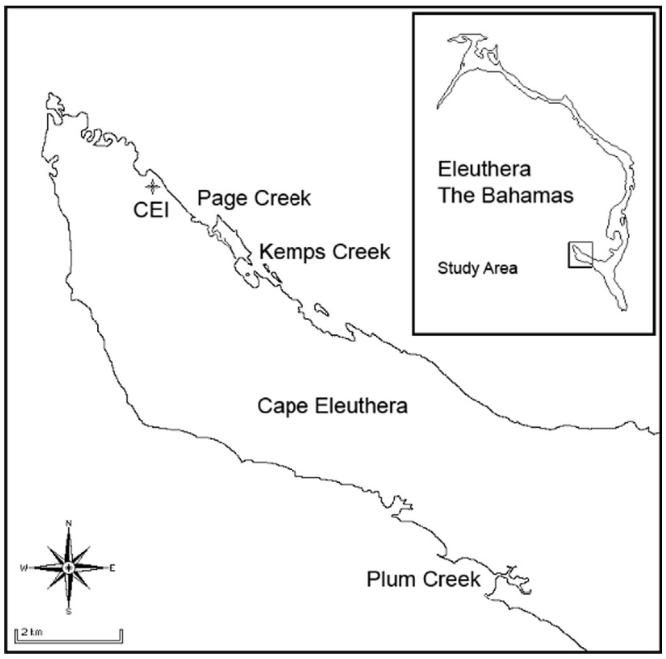

Fig. 1. Study area along the coast of Cape Eleuthera, Eleuthera, The Bahamas, showing the locations of fish collection from Page, Kemps and Plum Creeks, and the location of the Cape Eleuthera Institute (CEI) research facility where fish were taken for experimentation (black star). The inset map displays the entire island of Eleuthera with the study area highlighted.

St. Louis, MO). Intra-peritoneal injection of cortisol dissolved in cocoa butter has been the vehicle method of choice for several field-based studies using cortisol manipulations in fish (e.g., Dey et al., 2010; McConnachie et al., 2012a, 2012b; O'Connor et al., 2013). Preliminary tests in checkered puffers, however, indicated that intra-peritoneal injection would not work for this species due to its ability to inflate and deflate its intra-peritoneal cavity. The checkered puffer fish generally inflate upon injection, and eject the cortisol through the mouth and gills upon deflation. Based on these observations, an intramuscular injection of the dorsal muscle anterior to the dorsal fin was used to experimentally administer exogenous cortisol to checkered puffers. All treatment fish were weighed using a portable electronic balance, and then placed in water-filled foam-lined trough to obtain a total length (TL) measurement, and for injection with 5 mL of cocoa butter per kg of fish body mass. Sham treatment fish were injected with heated pure cocoa butter only. Low-dose cortisol treatment fish were injected with 5 mg mL^{-1} cortisol in heated cocoa butter (i.e., 25 mg kg^{-1} fish body mass), while high-dose cortisol treatment fish were injected with 10 mg mL^{-1} cortisol in heated cocoa butter (i.e., 50 mg kg^{-1} fish body mass). Low and high cortisol dose concentrations were chosen based on work done on smallmouth bass (*Micropterus dolomieu*; Dey et al., 2010), largemouth bass (*M. salmonides*; O'Connor et al., 2013) and bluegill sunfish (*Lepomis macrochirus*; McConnachie, 2010). Control fish received no injection, but were handled in an identical manner to the treatment fish. Using a rubber-mesh dip net, all fish were placed in individual opaque experimental chambers (12.5 L) supplied with aeration and a constant flow of saltwater within 10 s of treatment. After 48 h, to determine circulating cortisol levels, fish were non-lethally sampled for 0.5 mL of blood by caudal venipuncture using a heparinized 1 mL syringe and 21 gauge, 2.5 cm needle. To avoid sampling-induced stress, each blood sample was withdrawn in under 3 min (Romero and Reed, 2005). Data were compared to previous studies of checkered puffer fish where cortisol was measured in the blood following a 5 min air exposure challenge (Cull, unpublished data).

2.3. Validation of cortisol injection: depletion timeline

From April to June 2012 (ambient water temperature: 29.1 ± 1.5 °C), checkered puffers were randomly assigned to one of the following treatment groups to verify the time course of cortisol elevation over a 20 day holding period: (1) control (i.e., sampling of resting fish on day 0; $n=7$); (2) sampling at 2 days ($n=7$); (3) sampling at 5 days ($n=7$); (4) sampling at 10 days ($n=5$); and (5) sampling at 20 days ($n=6$) post-injection. Fish from all treatment groups were measured as described above, and briefly air-exposed (30 s) while a 5 mL kg^{-1} intramuscular injection of 10 mg mL^{-1} cortisol in cocoa butter was administered (dosage selected based on above validation; yielded a dosage of 50 mg cortisol kg^{-1} fish body mass). Control fish were handled in an identical manner, but received no injections. Treatment fish were then placed in a common holding tank where they were cared for as described above. To minimize disturbances prior to blood sampling, fish were placed in 12.5 L individual opaque experimental chambers 12 h prior to sampling. Previous work on checkered puffers revealed that fish recover from handling stressors within 3 h (Cull et al., unpublished data). The method of holding fish in communal tanks before introducing them temporarily to individual holding chambers has been successfully used with bluegill (McConnachie et al., 2012b), bonefish (*Albula* spp.; Shultz et al., 2011) and two species of cardinalfish (*Ostorhinchus doederleini* and *O. cyanosoma*; Munday et al., 2009). All checkered puffers were sampled for blood as described above.

2.4. Lab experiment: metabolic cost of cortisol injection

The metabolic burden imposed by the cortisol-cocoa butter injection on standard metabolic rate (SMR) was determined using intermittent-flow respirometry on checkered puffers in June 2012 (water temperature: 28.6 ± 1.8 °C). The SMR of cortisol-dosed individuals ($n=8$) was compared with that of control individuals ($n=8$). Cortisol treated fish were dosed (5 mL kg^{-1} intramuscular injection of 10 mg mL^{-1} cortisol in cocoa butter, for a total dose of 50 mg cortisol kg^{-1} fish body mass; Section 2.3) 48 h prior to SMR measurement. The respirometry system, operating procedures and calculations were identical to those previously described by Shultz et al. (2011), with the exception of the duration of individual cycles that consisted of an 18 min flush, 1 min wait and 20 min measurement cycle. Oxygen consumption rate (MO_2, mg O_2 kg^{-1} h^{-1}) for each fish was calculated using the average of the six lowest values recorded overnight (i.e., between 20:00 and 06:00; Schurmann and Steffensen, 1997), and when the coefficient of determination (R^2) for slope measurements was >0.95 during each measurement cycle. All calculated dissolved oxygen values were corrected for background oxygen consumptions generated for each specific fish and chamber prior to commencing experiments.

2.5. Lab experiment: effects of cortisol injection on swimming ability

Following 24 h of intermittent-flow respirometry, cortisol burden was further assessed by quantifying anaerobic swimming ability, using a chase to exhaustion protocol on the same group of checkered puffers that were used for respirometry measurements. Individually, fish were dip netted from their respirometry chamber and quickly placed into a shallow circular tank (1.22 m diameter filled to a height of 15 cm with water). A chase test was performed, and the time to exhaustion (i.e., the time at which three consecutive tail grabs could be performed without a reflex response; Kieffer, 2000) was recorded for each fish. This protocol provided a

comparative swimming ability measure between control and cortisol-treated fish and has previously been validated in a variety of fish species (Heath et al., 1993; Portz, 2007; Thiem et al., 2013).

2.6. Lab experiment: cold and heat shock

From April to June 2012 (ambient water temperature: 29.1 ± 1.5 °C), checkered puffers were randomly assigned to the following treatment groups: control at (1) ambient temperature ($n=8$); (2) −5 °C from ambient temperature ($n=6$); and (3) +5 °C from ambient temperature ($n=8$); (4) cortisol treatment at ambient temperature ($n=8$); (5) cortisol treatment at −5 °C from ambient temperature ($n=6$); and (6) cortisol treatment at +5 °C from ambient temperature ($n=8$). Cortisol-treated fish were measured and briefly air-exposed while cortisol in cocoa butter (i.e., high dose as above; 50 mg cortisol kg^{-1} fish body mass) was administered. Fish were then returned to communal tanks for 36–60 h and held at ambient temperature (i.e., 29.1 ± 1.5 °C). Control fish were handled identically, but received no treatment. All fish were captured from communal tanks and placed in individual opaque experimental chambers (12.5 L) supplied with ample aeration and a constant flow of saltwater. The experimental chambers were cooled by pumping water through a copper coil submerged in ice water, and warmed by heaters. These methods provided the appropriate temperature accurate to ±1 °C of the target thermal treatment. After 4 h (chosen because previous work on checkered puffer fish revealed that fish recover from handling stressors within 3 h; Cull et al., unpublished data), fish were sampled for blood as described above. For temperatures 5 °C below and above ambient, a 'puff score' was recorded during the 3 min sampling period by noting the time and intensity of the 'puff' (i.e., body inflation). More specifically, puffs were assigned a score from 0 to 3, with 0 being no puff, 1 being equal to or less than half a full puff, 2 being greater than half a full puff, and 3 being a full puff. A full puff was assigned once the fish was maximally inflated (i.e., its skin was tight to the touch and subsequent inflation attempts resulted in no further expansion). Each puff score (0–3) was assigned a percentage of time used over the 3 min, and then weighted according to its score. As a result, each puff score is presented as a value between 0 and 3 (i.e., 0 being no puff at all and 3 being a consistent full puff over the course of the 3 min sampling period). The fish were then released back into their respective chambers, and the time to fully deflate was recorded.

2.7. Field experiment: thermal habitat use and change in body condition

From December to January 2013, thermal habitat use of checkered puffers in their natural habitat was monitored in Page Creek. Page Creek is a shallow tidal water channel with a single opening to the ocean. This creek system consists of an expansive mangrove habitat undergoing two tidal cycles per day. The creek almost drains entirely at low tide, causing relatively large diel fluctuations in temperature, driven by a combination of solar radiation and tidal cycles. Mean winter water temperatures in Page Creek tend to be around 22 °C during the winter, while in the summer are closer to 32 °C and will occasionally exceed 40 °C (Murchie and Shultz, unpublished data). Water temperatures are cooler towards the mouth of the creek, but predator burden also appears to be higher in this area. To assess the thermal characteristics of the tidal creek, thermal loggers (iButton, Maxim Integrated Products, Inc., Sunnyvale, CA; $n=10$) were covered in a synthetic rubber coating (Plasti Dip International, Performix Brand products, Blaine, MN) and placed throughout Page creek, covering a range of habitat types. Five of the iButtons (model no. DS1921H) had a range of 15–46 °C, while the others (model no. DS1921Z) had a range of −5 to 26 °C. Factory-stated resolution of all thermal loggers is 0.125 ± 1 °C; previous calibration by our team reveals actual mean accuracy of 0.4 ± 0.3 °C and mean precision of 0.2 ± 0.0 °C (Donaldson et al., 2009).

On December 31, 2012, checkered puffer fish were tagged with either unmodified iButtons (model no. DS1921H and no. DS1921Z; $n=18$) and iButtons that were miniaturized according to Lovegrove (2009; model no. DS1921H; $n=19$). All iButtons were covered in Plasti Dip, and fastened to a backing plate. All iButtons were set to log temperature every 30 min over a 20-day period. Fish were randomly assigned to one of two treatment groups: (1) control ($n=19$) and (2) cortisol treatment ($n=18$). Thermal loggers were randomly distributed between groups and were externally attached to the dorsal surface of the fish, immediately posterior to the dorsal fin on the caudal peduncle (Thiem et al., 2013). Following iodine disinfection, two hypodermic stainless steel needles (16 gauge) were pushed through the dermis and 9 kg monofilament line (previously inserted through the tag via premade holes) was passed through the lumen of the needles and secured using multiple knots (see Thiem et al. (2013) for tagging validation of checkered puffers). Cortisol-treated fish were then weighed, measured, and given a 5 mL kg^{-1} intramuscular injection of cortisol in cocoa butter (i.e., 50 mg cortisol kg^{-1} fish body mass) as described above. Control fish were handled identically, but were not given injections. All fish were released within Page Creek upon recovery. After fish were at liberty for a 20-day period, control ($n=10$) and cortisol-treated checkered puffers ($n=13$) were recovered from Page Creek on January 19, 2013. The recapture rate for control and cortisol-treated fish was 58% and 72%, respectively. Despite promising trials on the benchtop, modified iButtons failed to log temperature 70% of the time when deployed in the field. Therefore, of the fish that were recaptured, useable data covering the entire 20-day period was only obtained for 7 control ($n=3$ DS1921H iButtons; $n=4$ DS1921Z iButtons; TL = 186 ± 3 mm; mass = 128 ± 8 g) and 8 cortisol-treated ($n=3$ DS1921H iButtons; $n=5$ DS1921Z iButtons; TL = 188 ± 8 mm; mass = 131 ± 19 g) fish. Upon capture (fish were either "chased" down and dipnetted, or captured by seine as above), fish were re-measured and re-weighed using the methods described above, so that changes in condition over the course of the study could be calculated. Given the variable efforts needed to recapture individual fish, it would have been difficult to control for capture stress, and so blood samples were not collected.

2.8. Sample analyses

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

2.9. Data handling and statistical analysis

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

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

2.9.1. Validation of cortisol injection: dose

To validate the cortisol injection dose in the checkered puffer, a one-way ANOVA followed by a Tukey's HSD test was performed to quantify differences in plasma cortisol and blood glucose concentrations among fish treated with high (10 mg mL^{-1}) and low (5 mg mL^{-1}) injection doses, sham treated fish, and controls. Data were compared to previous studies of the checkered puffer where endogenous circulating cortisol was measured in the blood following a 5 min air exposure challenge (Cull et al., unpublished data).

2.9.2. Validation of cortisol injection: depletion timeline

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

2.9.3. Lab experiment: metabolic cost of cortisol injection

To determine the metabolic cost of the high cortisol injection dose, independent sample *t*-tests were used to compare SMR (MO_2) between control and cortisol-injected checkered puffers.

2.9.4. Lab experiment: effects of cortisol injection on swimming ability

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

2.9.5. Lab experiment: cold and heat shock

To quantify the interactive effect of cortisol manipulation and thermal stress on the physiological and behavioral responses of the checkered puffer in the laboratory, two-way ANOVAs were used to identify the effect of the cortisol injection and thermal shock on physiological stress indices and 'puffing' performance. Independent variables included in the model were endocrine stress treatment (i.e., control vs. cortisol injection), and thermal treatment (i.e., ambient temperature, 5 °C below, and 5 °C above ambient temperature). The interaction between these two variables (stress treatment × thermal treatment) was included in the model. Dependent variables were circulating cortisol concentration, circulating glucose concentration, hematocrit, puff score, and puff time to deflate once released.

2.9.6. Field experiment: thermal habitat use

To quantify the potential consequences of cortisol manipulations on the thermal habitat use of checkered puffers in a field setting, thermal data from both fish and habitat iButtons were recovered using the Java application, One Wire Viewer (Maxim Integrated, San Jose, CA). A variety of thermal parameters were then compared among the iButtons collected from the habitat, control, and cortisol-treated fish.

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

The daily accumulated thermal units (ATUs) were calculated for each fish and habitat iButton for each day by summing every temperature value (i.e., 48 per day given that the iButtons were set to record at 30 min intervals). The number of recordings per iButton was consistent across all fish and habitat iButtons. All temperature values equal to or above 26 °C were considered 26 °C. For only the iButton model no. DS1921H, the average daily maximum was calculated for all groups. For all iButtons, the average daily minimum was calculated. For only the iButton model no. DS1921H, the average daily range was calculated for all groups by determining the difference between average daily maximum and minimum values. To control for daily fluctuations in temperature, repeated measure ANOVAs were used to compare the ATUs, maximum, minimum and range temperatures among habitat, control and cortisol-treated fish.

2.9.7. Field experiment: change in body condition

Fulton's condition factor (*K*) was calculated twice for each checkered puffer (once before tagging and deploying fish; and once retrieved 20-days following deployment) as an indicator of general well being using the following equation:

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

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

3. Results

3.1. Size differences among treatment groups

Details on fish size in the various treatments are presented in Table 1. Although individuals were randomly assigned to each treatment group (i.e., no checkered puffers were repeatedly sampled), some small but significant differences in size among groups were identified (one-way ANOVAs: Table 1).

3.2. Validation study of cortisol injection: dose

Intramuscular cortisol manipulations successfully raised plasma cortisol titers in pufferfish two days post-injection ($F = 13.997$, $p < 0.001$; Fig. 2A). Low and high cortisol doses (25 and 50 mg kg^{-1} fish, respectively) caused circulating cortisol concentrations to increase by 8 and 18 times, respectively, when compared to control and sham-treated fish (Fig. 2A). However,

Table 1
The mass and total length (TL) of checkered puffer (*Sphoeroides testudineus*) treatment groups included in experiments related to validation of the cortisol injection dose, validation of the cortisol depletion timeline, lab experiments related to metabolic costs and thermal shock (heat and cold) and field studies of thermal habitat preference. One-way ANOVAs followed by a Tukey's HSD tests were conducted to quantify differences among groups. Letters identify statistical differences across treatment groups.

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

only the high cortisol dosed fish exhibited plasma cortisol levels that were statistically higher than other treatment groups (Tukey's HSD tests, all $p < 0.01$). The checkered puffer has been reported to naturally release 126 ± 34 ng mL^{-1} of plasma cortisol in response to an acute standardized stressor (Cull et al., unpublished data). Circulating cortisol concentrations following the high cortisol dose were 147 ± 35 ng mL^{-1} (Fig. 2A). Thus, this dose resulted in physiologically relevant post-stress level of plasma cortisol, and the 10 mg mL^{-1} cortisol dose (i.e., 50 mg kg^{-1} fish) was used for the remainder of the study. Control, sham treated, as well as low and high cortisol dosed fish displayed no differences in blood glucose levels ($p > 0.05$; Fig. 2B).

3.3. Validation of cortisol injection: depletion timeline

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

3.4. Validation of cortisol injection: metabolic cost

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

3.5. Validation of cortisol injection: swimming ability

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

3.6. Lab experiment: cold and heat shock

There was a significant interaction between the cortisol injection and thermal treatment for cortisol titers (Table 3; Fig. 3A). Control fish exhibited rather similar plasma cortisol levels except when exposed to cold shock, where cortisol levels were elevated (Fig. 3A). Checkered puffers dosed with a high concentration of cortisol generally exhibited the highest plasma cortisol levels (164 ± 21 ng mL^{-1}) when held at ambient temperature and lower levels of cortisol when subjected to heat or cold shock, but those differences were not statistically significant. Blood glucose levels in the fish were primarily influenced by thermal treatment independent of cortisol treatment (Table 3 and Fig. 3B). Pufferfish exposed to a 5 °C cold shock had significantly higher levels of blood glucose then fish held at ambient temperature or a 5 °C heat shock. The cortisol injection and thermal treatments had no significant effect on hematocrit in the checkered puffer (Table 3).

The puffing performance of the checkered puffer, including puff score and time required to deflate once released, were primarily influenced by thermal treatment (Tables 3 and 4). Fish were unable to perform any anti-predator puffing behavior when exposed to the cold shock. The cortisol injection, as well as the interaction between the cortisol injection and thermal treatment, did not significantly contribute to changes in puff performance (Table 4).

3.7. Field experiment: thermal habitat use

The proportion of temperature values equal to or above 26 °C was similar for control ($12.77 \pm 1.63\%$) and cortisol dosed ($13.28 \pm 1.67\%$) fish, as well as to habitat iButtons ($11.98 \pm 1.02\%$; one-way ANOVA: $p > 0.05$). When we examined how ATUs, minimum, maximum and ranges varied across the 20-day study, it was apparent that "day" was consistently a significant factor in the repeated measures ANOVAs (all $p < 0.0001$; Table 5 and Fig. 4). However, significant differences in thermal experience among control and cortisol treatment groups as well as the habitat temperature loggers were only apparent for ATUs (repeated measures ANOVA: $p < 0.0001$; Table 5 and Fig. 4). On average, daily ATUs for cortisol-treated fish (1083 ± 5 °C) were 6 °C cooler when compared to control fish (1089 ± 6 °C), and 9 °C cooler than that logged by sensors placed within their habitats (1092 ± 4 °C; Table 6).

3.8. Field experiment: change in body condition

The initial condition of control and cortisol-treated checkered puffers (2.05 ± 0.09 and 2.05 ± 0.07 mg mm^{-3}, respectively) was generally similar to their final condition (1.97 ± 0.06 and 1.91 ± 0.05 mg mm^{-3}, respectively). Although a slight decrease in fish condition was observed over the 20-day period, the decline in condition was not found to be significant, nor to be significantly

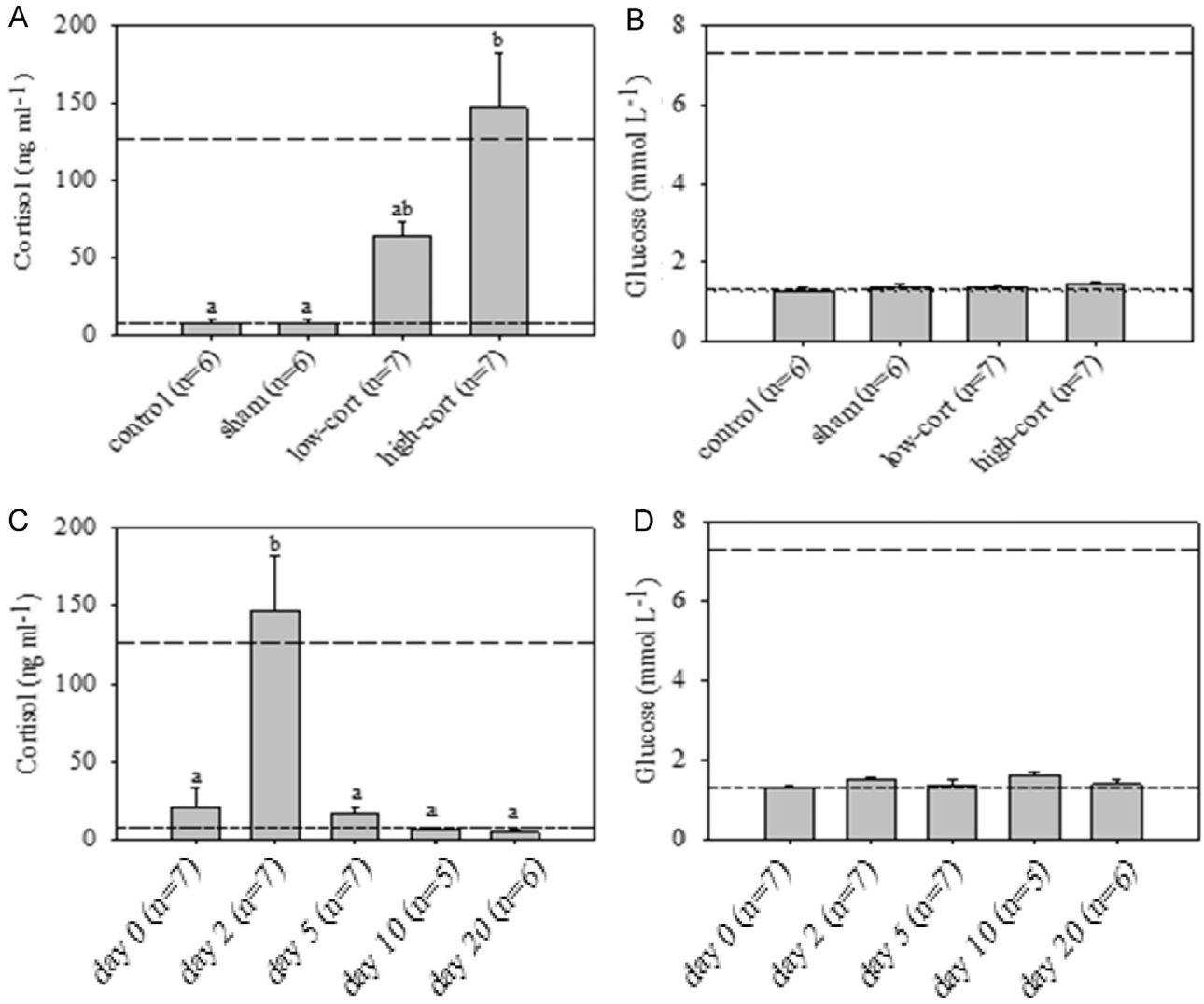

Fig. 2. Validation of different cortisol doses (A, B) and time course recovery (C, D) for checkered puffer (*Sphoeroides testudineus*) at Cape Eleuthera Institute, The Bahamas. Plasma cortisol (A) and blood glucose (B) concentrations of control and cocoa butter sham treated fish, as well as low-cort. (i.e., 25 mg cortisol kg^{-1} fish body mass) and high-cort. (i.e., 50 mg cortisol kg^{-1} fish body mass) treatments sampled 48 h after treatment (e.g., injection). Plasma cortisol (C) and blood glucose (D) concentrations of high-cort. (i.e., 50 mg cortisol kg^{-1} fish body mass) injected checkered puffers are visualized over a 20 day period. Dashed lines indicate physiological baseline and mean post-stress. concentrations arising from an acute stress challenge (based on Cull, unpublished data). Within a panel, dissimilar letters indicate statistically significant differences (all $p < 0.05$). Bars represent means with whiskers representing 1 standard deviation (SD).

Table 2
Checkered puffer (*Sphoeroides testudineus*) hematocrit levels at 5-, 10- and 20-days post-injection of cortisol at a dose of 50 mg cortisol kg^{-1} fish body mass.

Fish treatment group	Hematocrit (%)
5 days post-injection	19.2 ± 1.6
10 days post-injection	19.3 ± 4.4
20 days post-injection	23.5 ± 2.6

dissimilar among control and cortisol-treated fish (two-way repeated measures ANOVA: $p > 0.05$).

4. Discussion

In the current study, cortisol was experimentally manipulated to physiological post-stress levels to elucidate the effects of a thermal challenge as a secondary stressor on the checkered puffer. We compared indicators of energy use among control and cortisol-injected fish to evaluate the effects of the cortisol injection on checkered puffers, and found that fish did not show any energetic costs in terms of changes in blood glucose concentrations, standard metabolic rate, nor anaerobic swimming ability. We then tested the response of fish (with and without cortisol treatment) to a cold and heat shock in a controlled laboratory setting, and the thermal habitat use of wild checkered puffer fish in a complementary field study. In the lab, we found no statistical evidence of differences in cortisol response to thermal challenge (e.g., heat or cold shock) but from a biological perspective, we did note that that fish dosed with cortisol exhibited lower levels of cortisol when subjected to the thermal challenge ($\sim$50 ng mL^{-1} less when compared to cortisol-treated fish held at ambient temperatures). In the field, we found that cortisol-injected fish generally selected cooler temperatures when compared to controls in terms of ATUs, but the difference was minor. Collectively, these results suggest that despite raising cortisol to physiologically-relevant levels, we observed little evidence that it had even modest biologically relevant consequences on the thermal biology or physiological ecology of checkered puffer.

Table 3
Two-way ANOVA outputs identifying the effect of multiple stressors (i.e., fish treatment [control and cortisol-injected checkered puffers] and thermal treatment [ambient temperature, as well as 5 °C below and above ambient temperature]) on physiological and behavioral stress indices, including cortisol, glucose, hematocrit and 'puff' performances (i.e., puff score and puff time to deflate once released). Cortisol injection was intramuscularly at a dose of 50 mg cortisol kg^{-1} fish body mass.

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

**$p < 0.01$.
*** $p < 0.001$.
* $p < 0.05$.

Table 4
Puffing performance (i.e., puff score and puff time to deflate once released) and hematocrit levels of control and cortisol-injected (dose of 50 mg cortisol kg^{-1} fish body mass) checkered puffers (*Sphoeroides testudineus*) when subject to +5 and −5 °C changes from ambient (i.e., 29.1 ± 1.5 °C) temperature. Dissimilar letters indicate statistically significant differences among treatment groups.

	+5 °C		−5 °C	
	Control	Cortisol injected	Control	Cortisol injected
Puff score	1.29 ± 0.29 [a]	1.15 ± 0.22 [a]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]
Puff time to deflate once released (s)	97 ± 49 [a]	31 ± 11 [ab]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]
Hematocrit (%)	24.0 ± 2.4	24.1 ± 2.2	22.7 ± 1.7	25.8 ± 3.0

Table 5
Statistical output for field study of comparative thermal habitat selection of checkered puffer (*Sphoeroides testudineus*) in The Bahamas. 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., accumulated thermal units [ATUs], minimums, maximums and ranges) of the different treatments (i.e., control and cortisol-injected fish [dose of 50 mg cortisol kg^{-1} fish body mass], as well as the habitat thermal loggers) were established.

	DF	F
ATUs		
Day	19	71.175***
Day × treatment	38	2.663***
Minimum		
Day	19	2447.665***
Day × treatment	38	1.216
Maximum		
Day	19	100.510***
Day × treatment	38	.863
Ranges		
Day	19	61.471***
Day × treatment	38	.862

** $p < 0.01$.
* $p < 0.05$.
*** $p < 0.001$.

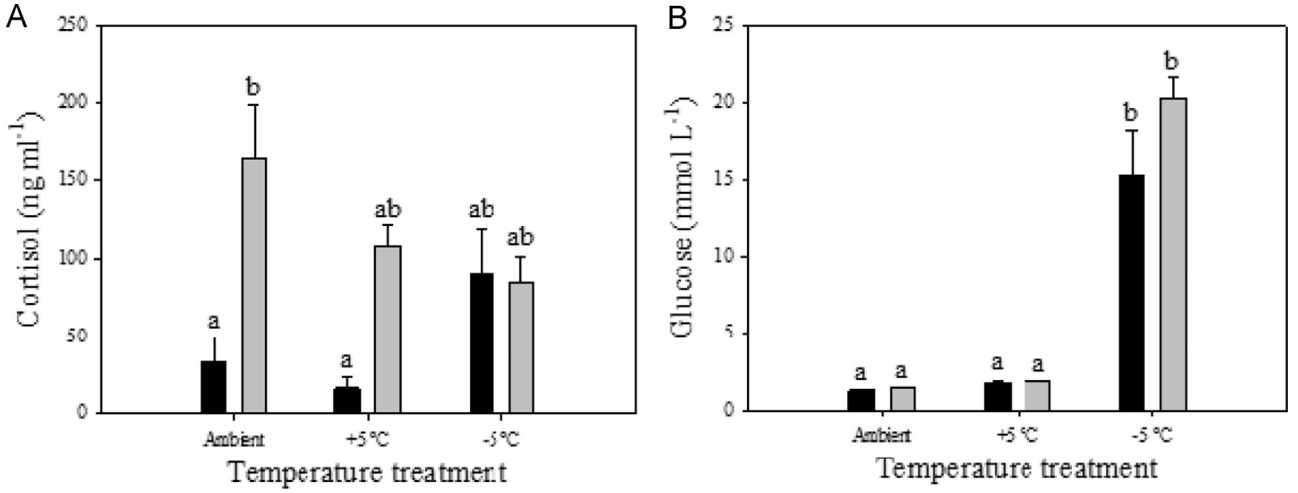

Fig. 3. Blood physiology of checkered puffers (*Sphoeroides testudineus*) held at the Cape Eleuthera Institute in The Bahamas. Plasma cortisol (A) and blood glucose (B) concentrations of control (black) and cortisol-injected. (gray; dose of 50 mg cortisol kg^{-1} fish body mass) fish in ambient conditions (29.1 ± 1.5 °C), as well as +5 and −5 °C changes from ambient temperature. Blood was collected 4 h post-thermal challenge. Within a panel dissimilar letters indicate statistically significant differences (all $p < 0.05$). Bars represent means with whiskers representing 1 SD.

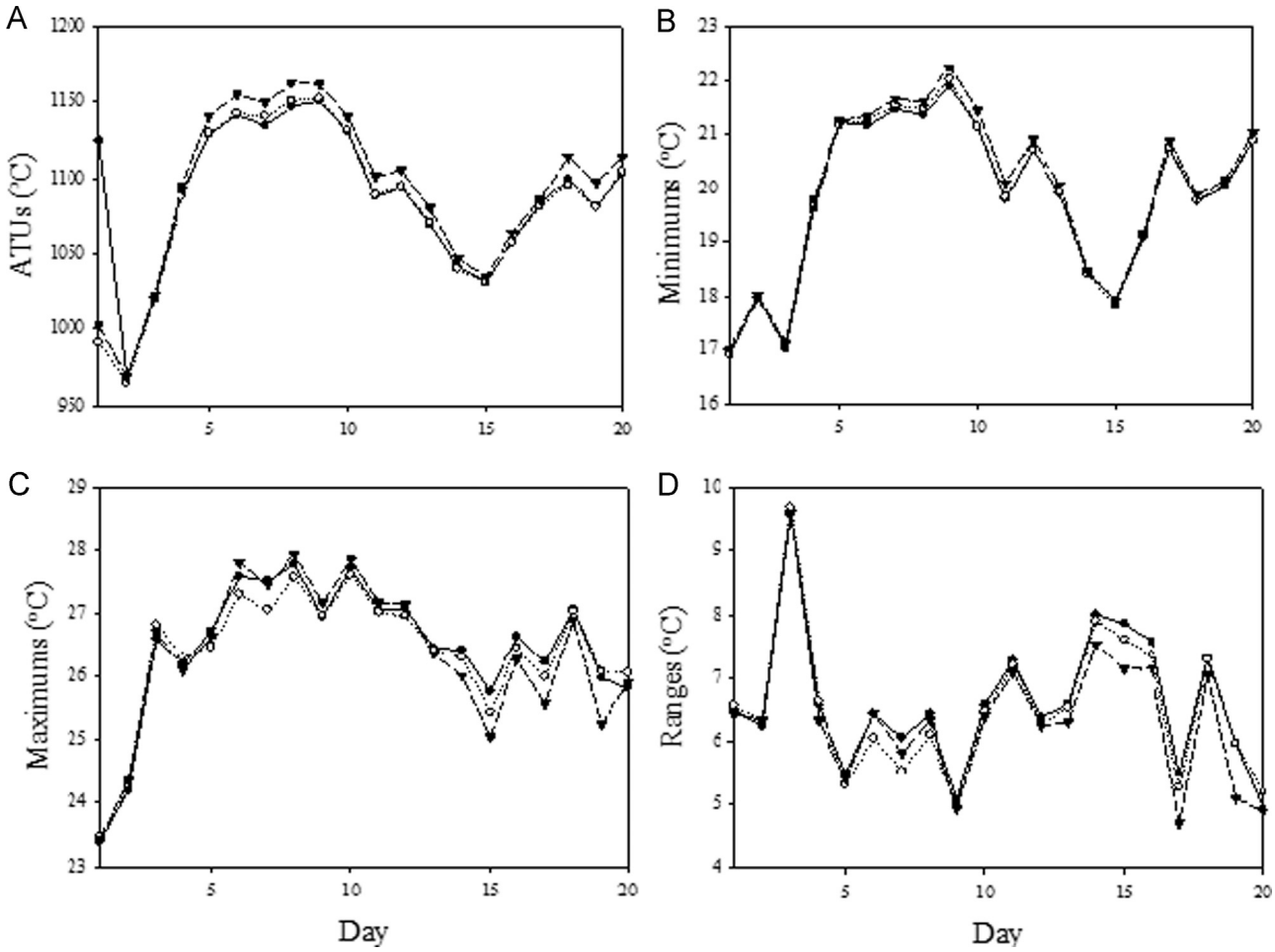

Fig. 4. Thermal biology of checkered puffers (*Sphoeroides testudineus*) collected over a 20 day sampling period in Kemps Creek, Eleuthera, The Bahamas. Thermal variables, including the accumulated thermal units (ATUs; A), thermal minimums (B), maximums (C) and ranges (D), for control (dark circles) and cortisol-dosed. (dose of 50 mg cortisol kg^{-1} fish body mass; light circles) were obtained for fish as well as for the habitat loggers (dark triangle). Data points represent means for all fish in a given treatment on a given day.

Table 6
Temperature metrics, including the daily accumulated thermal units (ATUs), thermal minimums, maximums and ranges, recorded by the iButtons of control and cortisol-injected [dose of 50 mg cortisol kg^{-1} fish body mass] checkered puffers (*Sphoeroides testudineus*), as well as that of their habitat. For ATUs dissimilar letters indicate statistically significant differences ($p < 0.05$).

Treatment	Temperature metrics			
	ATUs	Minimum	Maximum	Range
Control fish	1089.3 ± 6.0a	19.85 ± 0.13	25.65 ± 0.40	4.81 ± 0.55
Dosed fish	1082.8 ± 4.6b	19.88 ± 0.13	25.82 ± 0.40	4.82 ± 0.51
Habitat	1092.1 ± 4.2c	20.00 ± 0.12	25.67 ± 0.28	4.61 ± 0.38

4.1. Validation study of cortisol injection

Due to the unique anatomy and physiology of the checkered puffer, we used intramuscular injections of cortisol in the current study. Other cortisol manipulation studies have employed intraperitoneal cortisol injections, and cortisol-spiked food to raise plasma cortisol levels in fish (reviewed in Gamperl et al., 1994). Although intramuscular injection is rather uncommon for cortisol manipulations in fish, it worked quite well for checkered puffers. Indeed, cortisol levels were elevated for at least 2 days over control levels and were back to pre-manipulation levels by 5 days, a period similar to intra-peritoneal injections (e.g., O'Connor et al., 2009, 2013; McConnachie et al., 2012a, 2012b).

Given that elevated cortisol, particularly in a semi-chronic state as would presumably have been elicited by the cortisol injection (Gamperl et al., 1994), is well known to result in glucose elevations (e.g., Barton and Iwama, 1991; Barton, 2002), it was peculiar that we observed no such elevation in glucose after 2 days (or indeed as any part of the initial validation study; see Fig. 2D). Interestingly, when checkered puffer are exposed to an acute stressor (e.g., struggling at air–water interface for 3 min) cortisol and glucose both become elevated (glucose typically reaches 7 mmol L^{-1} within 30 min). The levels of glucose we observed with cortisol injection were similar to baseline values observed when animals are sampled rapidly in the field or held in sensory deprivation chambers for 24 h (i.e., 1.5 mmol L^{-1}). It is conceivable that the glucose is no longer being mobilized (e.g., due to exhaustion of resources or failure of cortisol to stimulate glucose mobilization beyond an acute event) or that the injection protocol for some reason did not generate the stress response we were attempting to achieve. With so little such work on checkered puffers to date, we can only identify this peculiarity and speculate on its basis.

The cortisol injection caused a peak in plasma cortisol levels of the checkered puffer 2 days post-injection. Therefore, we predicted a significant metabolic cost associated with the cortisol injection at this time point. It is well established that exposure of fish to experimentally manipulated cortisol titers initiates an endocrine response which in turn induces metabolic and osmotic disturbances (i.e., the secondary stress response; Mazeaud et al.,

1977; Barton and Iwama, 1991). However, there was no difference in blood glucose concentration and standard metabolic rate between cortisol-treated and control fish 2 days post-injection. Furthermore, there was no difference in anaerobic swimming ability (measured using a chase protocol; Portz, 2007) between cortisol-treated and control fish despite there being a general trend towards cortisol-treated fish tiring more rapidly than control fish. Previous studies have linked chronically increased cortisol titers to impaired locomotor performance in juvenile rainbow trout (*Oncorhynchus mykiss*; Basu et al., 2002).

4.2. Thermal biology

Few studies have established clear links between the documented consequences of thermal stressors in the laboratory, and the parallel consequences found at the ecosystem level by means of field studies (Pörtner et al., 2005). By both quantifying the behavioral and physiological consequences to multiple stressors in the laboratory, and establishing thermal habitat use under these different stressed states in a natural habitat, we are able to better predict alterations in performance and overall fitness that may be expected of climate change in the checkered puffer.

4.3. Laboratory experiment

We predicted that the cortisol injection would alter the sensitivity of puffers to cold and heat shock, and increase the physiological stress response to the thermal challenge (i.e., as a secondary stressor). We found that decreases in temperature had the most significant physiological consequences on the checkered puffer, whereas similar increases in temperature had little impact. Fish dosed with cortisol exhibited high cortisol levels when held at ambient temperature, and comparatively lower (but not statistically different) levels of cortisol when subjected to thermal manipulation. Although this observation of a ~50 ng mL mean difference in cortisol titers between ambient and thermal shock for cortisol-treated fish is counter-intuitive, it may reveal an attenuation of the cortisol response or rapid clearance of circulating cortisol levels when fish are exposed to thermal change. Unfortunately there is little relevant literature to draw upon to inform the interpretation. Moreover, the variance and sample size limited our ability to discern a statistical difference, so it is possible that the trend we observed was simply an artefact. Future research should explore this interesting observation.

In response to the acute decrease in temperature, checkered puffers were notably more active than fish placed in the other treatments (i.e., constantly swimming or struggling to exit the experimental chamber). This anecdotal increase in activity is likely an attempt to cope with the acute change in temperature, and responsible for the inability to puff following the 5 °C decrease in temperature. In response to decreasing temperature, fish have been previously reported to show hyper-responsiveness, uncoordinated swimming (e.g., bumping into tank walls and spontaneous circling), difficulty maintaining equilibrium, complete loss of equilibrium, and induction of coma (see Friedlander et al., 1976; Donaldson et al., 2008, for overview). An acute decrease in temperature (i.e., cold shock) may influence the reliability of neuronal activity and the reliability of cellular responses, leading to compromised anti-predator behavior (Preuss and Faber, 2003).

4.4. Field experiment

Checkered puffer fish selected cooler temperatures then the average thermal profile of the creek as measured by ATUs, and cortisol-treated fish favoured cooler temperatures than controls. The interpretation of the thermal habitat use of checkered puffers in an ecological context is complicated by the fact that this is the first study of its kind, and that the laboratory and field studies reveal dissimilar findings. Elevated levels of cortisol are known to suppress levels of heat shock proteins (HSPs) in several species of fish (Ackerman et al., 2000; Basu et al., 2001), suggesting that cortisol may mediate HSP levels following times of physiological stress (see Basu et al., 2001, 2002 for details). This may explain why cortisol-injected checkered puffers selected cooler temperatures in the wild in terms of ATUs. Future work should incorporate other aspects of habitat use including physical cover, food availability and predator risk to understand the patterns in thermal biology observed here.

We would also predict that cortisol-injected checkered puffers would avoid secondary stressors, thereby moving within the habitat to seek smaller thermal fluctuations. The differences in ATUs noted here between the three groups on a daily basis are relatively minor, and across longer time periods (e.g., seasons or years) this could be meaningful. Indeed, if the mean ATUs reported in Table 6 were to persist at the same level over an entire year for the control and cortisol-treated fish, the cortisol treated fish would experience 2354 fewer ATUs which equates to just over two days of growth potential (assuming that the control fish are operating around a thermal optima for growth). It is unclear how this difference may have been magnified had we conducted the research in the summer when average water temperatures in Page Creek are 4–5 °C warmer (Shultz, unpublished data). Importantly, the cortisol validation study revealed that cortisol was only elevated for 2 days and by 5 days was back to baseline levels. Certainly that reflects more than an acute stressor, but the cortisol levels were not elevated for the full 20 day field study period. Nonetheless, differences in ATUs were identified including during some of the days after the cortisol titers would have been at baseline levels. The exact mechanism is unclear but the subsequent alteration in thermal habitat use even when cortisol values were back to baseline levels may represent a carryover effect, whereby past experience (i.e., the cortisol injection) influences future "performance", in this case thermal preference (O'Connor et al., 2014).

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

metabolic costs and seek refuge from predation or other forms of competition. Curiously, however, we were unable to document any significant burden linked to the cortisol injection in the laboratory, suggesting that metabolic costs were not measurable in terms of aerobic activity 48 h post-treatment, nor was blood glucose elevated.

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

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

4.5. Conclusion

The combination of stressors caused by gradual anthropogenic climate change may provoke complex non-linear responses in coastal systems on the individual, population and community levels (Lee et al., 2001; Harley et al., 2006; Burkett et al., 2008). Through experimental cortisol manipulations we were able to highlight the detrimental physiological and behavioral consequences of multiple and repeated thermal stressors (i.e., heat and cold shock challenges) in the checkered puffer in a controlled laboratory experiment, and for the first time, relate it to a comparable and ecologically relevant field study monitoring the thermal habitat use and condition of fish. The disparity in findings between the lab and the field suggests that in field environments, animals have greater opportunity to select their environments, and that any physiological consequences associated with experimentation have the potential to be modulated by behavior more so than in a confined laboratory settings. Indeed, it is well known that physiology and behavior are interconnected (Gilmour et al., 2005; Cooke et al., 2014b). These findings highlight the need to establish the link between laboratory findings and ecologically relevant processes (including behavior) in the field to help scale physiological knowledge to higher levels of biological organization such as populations and ecosystems (Cooke and O'Connor, 2010; Cooke et al., 2014a). Unfortunately there are still comparatively few studies of thermal biology that combine laboratory and field components (but see Farrell et al. (2008) for example) despite their ability to generate cause-and-effect relationships and test or contextualize them in field settings (Pörtner and Peck, 2010). Comprehensive approaches that generate ecologically-relevant evidence are needed to inform the development of appropriate management policies and conservation initiatives in the face of anthropogenic climate change (Pörtner and Farrell, 2008; Somero, 2012).

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