Puffed and bothered: Personality, performance, and the effects of stress on checkered pufferfish

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HIGHLIGHTS

- Wild checkered pufferfish have consistent, individual-level differences in behaviour.
- Personality, puff response, and swimming performance did not form coping styles.
- Behaviours and performance in the lab were not related to movements in the field.
- A physiological dose of cortisol did not modify personality or performance.

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ABSTRACT

Although consistent individual-level differences in behaviour are widespread and potentially important in evolutionary and ecological processes, relatively few studies focus on the physiological mechanisms that might underlie and regulate these individual-level differences in wild populations. We conducted experiments to determine whether checkered pufferfish (*Sphoeroides testudineus*), which were collected from a dynamic (in terms of depth and water temperature) tidal mangrove creek environment in The Bahamas, have consistent individual-level differences in locomotor activity and the response to a simulated predator threat, as well as swimming performance and puffing in response to stressors. The relationships between personality and performance traits were evaluated to determine whether they represented stress-coping styles or syndromes. Subsequently, a displacement study was conducted to determine how personality and performance in the laboratory compared to movements in the field. In addition, we tested whether a physiological dose of the stress hormone cortisol would alter individual consistency in behavioural and performance traits. We found that pufferfish exhibited consistent individual differences in personality traits over time (e.g., activity and the duration of a response to a threat) and that performance was consistent between the lab and the natural enclosure. Locomotor activity and the duration of startled behaviour were not associated with swimming and puffing performance. Locomotor activity, puffing performance, and swimming performance were not related to whether fish returned to the tidal creek of capture after displacement. Similarly, a cortisol treatment did not modify behaviour or performance in the laboratory. The results reveal that consistent individual-level differences in behaviour and performance were present in a population from a fluctuating and physiologically challenging environment but that such traits are not necessarily correlated. We also determined that certain individual performance traits were repeatable between the lab and a natural enclosure. However, we found no evidence of a relationship between exogenous cortisol levels and behavioural traits or performance in these fish, which suggests that other internal and external mechanisms may underlie the behaviours and performance tested.

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1. Introduction

There is growing recognition that consistent individual-level differences in behaviour are widespread in natural populations and that they are important for ecological and evolutionary processes. Consistent individual-level differences in behaviour have been described in the contexts of animal personality [1], temperament [2], behavioural syndromes [3], and stress-coping styles [4], and have been documented in a wide variety of invertebrates [5] and vertebrates [6], including fishes [7]. We define animal personality as individual-level differences in behaviour that are consistent over time and between contexts [1–3],

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whereas we define behavioural syndromes as correlations between different personality traits [3]. Personality traits and physiological stress responses that form coherent sets within groups of individuals are defined as stress-coping styles [4]. A key evolutionary consequence of personalities and coping styles is that they can at times represent limited plasticity, so that an animal might not be able to express the most suitable behaviour in all contexts [8], which could incur significant fitness consequences for individuals [9,10,11]. The fitness consequences of such individual-level variation in behaviour also have the potential to influence higher-order processes such as population growth and persistence, interspecific interactions, community dynamics, and rates of evolution [11,12].

Yet, despite the potential costs of behavioural consistency, accumulating evidence suggests that diverse personalities emerge and persist within natural populations [6], possibly as a result of factors such as fluctuating environmental conditions [13] and frequency-dependent selection [14]. However, compared to captive-bred mice and rats, relatively few studies focus on the physiological mechanisms that might underpin personality [15] and coping styles in wild animals, especially ectotherms [16,17]. Furthermore, there is a paucity of studies that validate personality in the field despite concerns that personality traits observed in stressful lab conditions may not reflect personality traits expressed wild animals in natural conditions [18].

Careau and Garland [19] suggest that individual differences in performance can help elucidate the relationships between selection, physiology, and consistent behaviour. These authors describe how efforts have been made to include behaviour in the pace-of-life syndrome hypothesis [20], which posits that combinations of physiological characteristics have evolved with particular life-history traits within species [21,22]. In general, this integration appears to be supported by studies that indicate that there is a positive relationship between activity, aggressiveness, boldness, and metabolic rates [23] as well as life-history traits [10] but not in all cases, as these relationships tend to be highly context specific [19]. Given the difficulty in establishing a direct, causal relationship between metabolism and personality, and given that selection acts more directly on performance than the mechanisms that limit performance, Careau and Garland [19] advocate that researchers examine the relationships between physiology and performance as well as performance and personality to elucidate the links between physiology, personality, and selection. Despite the promise of this integrative approach and evidence that personality and individual differences in performance exist in natural environments [24–28], few studies address the ecological relevance of consistent individual differences in both stress-coping behaviours and performance.

In addition to performance capabilities, individual differences in endocrine stress responses can also be proximate causes of divergent personalities. Relationships between endocrine reactivity and the consistent stress coping behaviours of individuals suggest that, for some behaviours, consistent individual-level differences may be related to hormone levels [4,29]. When an animal's homeostasis is at risk, endocrine responses orchestrate a range of changes that help the organism cope with stress [30], including alterations in behaviour. In fish, these endocrine response systems include the brain-sympathetic-chromaffin cell axis and the hypothalamic-pituitary-interrenal axis (HPI; [31]). Catecholamines and corticosteroids are important end products of these axes which mediate changes in metabolism and ion balance, cardiovascular, respiratory, and immune functions [32] and ultimately induce changes in behaviour [33]. The activity of the HPI axis in fishes appears to have a particularly strong relationship with stress-coping behaviours, which is consistent with its analogues in other animals [4,30].

Despite evidence of a relationship between the activity of the HPI axis and behaviour, only a few studies have tested the causal link between cortisol, the primary glucocorticoid in fish, and styles of stress-coping behaviour in isolation from other parts of the stress response, such as the perception of a stressor and hormones that stimulate the production of cortisol [7]. These include studies that have explored the effects of cortisol on behaviour in fish using exogenous cortisol treatments. This approach initiates the cortisol-mediated responses to stress without activating the onset of the HPI axis and without a sensory perception of a stressor and can thus be used to isolate the cortisol-mediated effects on behaviour. Several of these studies provide evidence that cortisol is related to altered behaviour in fish. For example, an intraperitoneal cortisol implant increased the probability of social subordination in juvenile captive bred rainbow trout (*Oncorhynchus mykiss*) in size-matched pairs but this effect was abolished with the administration of the glucocorticoid receptor antagonist, RU486 [34]. In another study, a chronic cortisol treatment from an implant resulted in reduced feeding in rainbow trout but did not alter their swimming performance [35]. Other studies that explore the relationships between specific stressors, physiological characteristics, and behaviour reveal that there can also be an interaction between stressors and the effects of an individual's metabolism on behaviour (see review in [36]). Most of the studies on the effects of cortisol and stressors on behaviour have been conducted using captive-bred animals (but see examples for animals from wild populations [37–40], reviewed in [41]). Factors such as past experiences with predation threats and fluctuations in environmental conditions could affect the behavioural response of wild animals to stress [42,14].

Checkered pufferfish (*Sphoeroides testudineus*) provide a useful model to study whether animals from a fluctuating and potentially stressful environment will demonstrate consistency in behaviour and performance and whether a cortisol treatment will alter this consistency. This species is common in tropical and sub-tropical mangrove habitats throughout the Americas, with populations being found from Florida to Brazil [43]. Checkered pufferfish are well adapted to withstand frequent and drastic changes in temperature [44], water depth (tides), and salinity [45,46], and must also withstand frequent natural and anthropogenic alterations to their habitat [47]. In addition to these environmental stressors, pufferfish are subject to predation by herons (Ardeidae) [48] and fish [49], despite their defensive toxin, tetrodotoxin [50], and their ability to increase their size by pumping water or air into their stomachs [51].

In this study, we tested whether or not pufferfish exhibit consistent individual-level differences in activity and anti-predator responses (simulated aerial and aquatic predations events) as well as performance under stress (i.e., puff magnitude and duration, duration of a chase to exhaustion) over time. We also tested whether or not individual activity and performance were repeatable between a laboratory environment and a natural, enclosed environment. We conducted a displacement study to determine how activity and performance traits corresponded to individual differences in movement and recapture in the wild. Lastly, we tested whether a cortisol treatment would alter individual consistency in behaviour and performance. To do this, we injected the fish with a physiological dose of cortisol for an intermediate duration (3 days) to activate the cortisol-mediated components of the stress response (as per [52]) and compared the behaviour and performance of individuals before the cortisol treatment, during peak cortisol, and after the cortisol treatment was exhausted.

2. Methods

2.1. Study site and study animals

Checkered puffers (n = 60; January 2014) were collected in Page Creek (24°49′04.7″N, 76°18′51.6″W) (Fig. 1), a mangrove-lined creek at the south end of Eleuthera, The Bahamas, using a large (20 m) beach seine during the outgoing tide. Only fish weighing 50 g and over were retained, to prevent mortality from tagging and cortisol injection procedures. Captured fish were transported to the wetlab research facilities at the Cape Eleuthera Institute (24°50′06.70″N, 76°19′31.69″W) in coolers with frequent water changes (30% water volume every 5 min)
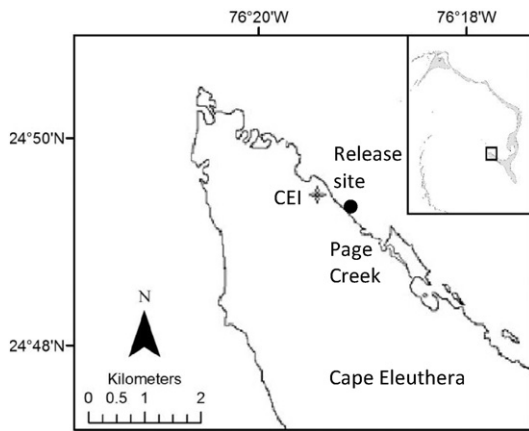
Fig. 1. Map of the research sites on Cape Eleuthera, Eleuthera, The Bahamas. Page Creek is the fish collection location. A star indicates the Cape Eleuthera Institute (CEI) and a black circle marks the release site for the displacement experiment. The inset shows Eleuthera with the study region highlighted.
This figure is adapted from Cull et al., 2015a.

to maintain temperature and aeration. On arrival at the laboratory, fish were all held together in a large circular 1831 L tank until they were marked with individual tags, after which they were alternated between a ~440 L rectangular raceway and a ~910 L circular tank. All tanks were supplied with UV-treated flow-through seawater whose temperature was that of ambient coastal conditions. Fish were fed to satiation with chopped sardines (*Sardinella aurita*) every 2 days. Each fish was tagged for identification using surgical tools, numbered vinyl laminated tags (Floy Tag & Mfg. Inc., Seattle, WA), and tagging line that were disinfected with diluted povidone-iodine and rinsed in fresh water before use. Each fish had a numbered tag sutured to the caudal peduncle using a reverse cutting needle and 17 lb. fused polyethylene braid (Berkley® Nanofil; Pure Fishing, Inc., Columbia, SC). Fish were not anaesthetized during any procedure because anaesthesia was not needed to immobilize the fish to facilitate handling and to prevent harm, and because there is no clear indication that anaesthetization prevents physiological stress responses to handling [53,54].

2.2. Repeatability of behaviour and performance in the laboratory

The fish were allowed to recover for approximately 24 h after being tagged before being tested for behaviour and performance, which was considered to be sufficient because the cortisol response to handling stress in checkered pufferfish and the aerobic recovery from puffing in the black-saddled pufferfish (*Canthigaster valentini*) last less than six hours [55,56]. Each fish was individually tested for puff performance, activity, startle duration, and the time it took to chase to exhaustion (swimming performance). Behaviours were recorded for analyses using a camera mounted directly overhead (GoPro HD Hero; GoPro Inc., San Mateo, CA).

Before being placed in an arena, each fish was air exposed and gently squeezed three times to induce puffing. Puff duration was recorded based on the video footage and the extent of the puff was scored (puff score) by one researcher based on a visual and tactile assessment during the experiment. To score the extent of the inflation, 0 described no puff, 1 described less than half a full puff during which the skin surrounding the stomach was loose, 2 described an intermediate level of inflation during which the skin surrounding the stomach was loose, and 3 described a full puff during which the skin was taut and further attempts to inflate by the fish did not increase its volume [55]. Puffing is an important performance metric for pufferfish, as it can help these fish avoid consumption by predators after capture [48].

After scoring the puff, each fish was placed into an individual 30 × 50 cm plastic experimental arena with a 10 cm grid marked on

the bottom and filled to a depth of 10 cm with seawater. The fish were allowed to acclimate to the experimental arena for two minutes before activity was measured. Activity was quantified by counting the number of 10 cm grid line units the fish crossed with the tip of its nose within five minutes. Various metrics for quantifying activity have been demonstrated as ecologically relevant measures which are related to risk-taking and exploration in natural conditions (e.g., [57–59]).

To simulate a predation threat from above, after determining the position of the fish in the arena taking care not to startle the fish, a lead weight tied to a fishing line at the end of a pole held at waist height was dropped into each arena within the view of the fish and then immediately removed. The weight made both a splash on the surface of the water and a sound as it hit the bottom of the arena. Startle duration was measured as the time required for the fish to resume the same level of swimming activity as that exhibited several seconds prior to the test stimulus. Startle behaviour was distinctive, generally consisting of a brief sprint followed by one or more periods of immobility before returning to prior behaviour, which generally consisted of slow swimming around the periphery of the arena. Observations ceased after one minute. The response to a threat from above is a relevant measure of predator evasion behaviour because herons are suspected to be one of the more frequent predators of checkered pufferfish [48].

After the startle-response test we measured the time required to chase each fish to exhaustion. The duration of a chase to exhaustion was measured in order to quantify swimming performance. The fish were chased individually by hand within the 30 × 50 cm arena until they either puffed or there was no body-flex response after three consecutive tail grabs and exposure to air [60]. In this way we were able to quantify the swimming endurance of each fish and compare the effect of a cortisol treatment (see below) on the swimming performance of the treatment groups. This test was adapted from other studies that effectively compared the swimming performance of checkered pufferfish [60] as well as other fishes [61,62]. Each fish was returned to the holding tank at the end of each trial. After the initial trial, these behaviour and performance trials were repeated with the same fish two days later, after an exogenous cortisol treatment, and again once the cortisol treatment was exhausted (see below).

2.3. Cortisol treatment

Immediately after the initial set of behaviour trials, all fish were weighed and assigned to two treatment groups, to one of which was administered a physiological dose of cortisol (n = 32), whereas the other group was kept as a control (n = 28). No sham treatment was included in the study because it has been demonstrated that giving checkered pufferfish a sham cocoa butter injection does not cause cortisol levels to differ from minimally handled control fish 48 h after the treatment [63]. Furthermore, the stress associated with handling and sham injections in fish has been demonstrated to cause short-term increases in cortisol levels that are intermediate between controls treated individuals and cortisol treated individuals (e.g., [64–67]). Thus, a sham-treated group would essentially be an acute, intermediate cortisol treatment, and for this reason we chose not to include this treatment in the study. Care was given in assigning fish to treatment groups so that the weight distribution of fish between groups was approximately equal (Mann–Whitney $U = 310$; control treatment: mean = 121 g, SD = ±34, n = 28; cortisol treatment: mean = 129 g, SD = ±35, n = 27, p = 0.26 two-tailed; fish removed from the study are excluded from this test, see below).

Cortisol treated fish received a heated 50 mg per ml cortisol (hydrocortisone 21-hemisuccinate; Sigma H2882, Sigma-Aldrich, St. Louis, MO) in cocoa butter intramuscular implant from a 1 ml syringe with an 18-gauge, 2.5-cm needle. Each treated fish received a 5 ml per kg body weigh dose to simulate the increase in cortisol experienced after an acute stressor. Cull et al. [63] determined that this dose increases the plasma cortisol of wild-caught checkered pufferfish to 147 ±

35 ng ml^{-1}, which is similar to the rise in plasma cortisol to 126 ± 34 ng ml^{-1} that they observed in response to an acute stressor. Behaviour trials were repeated for both groups approximately 48 h after the cortisol injection, when circulating cortisol from the implant had reached its peak [63]. Behaviour trials were also repeated four days after the cortisol treatment, when the cortisol treatment was exhausted [63].

2.4. Validation in a natural enclosure

In July 2014, fish were collected (n = 99), housed, and tagged as described above, with the exception that fish were distributed between two ~440 L rectangular raceways after tagging. Each fish was tested once for puff duration, activity, and swimming performance in the laboratory over the course of two days as described in the first portion of the study at a temperature of approximately 29 °C. In this series of trials the fish were gently squeezed five times before measuring puff duration, to increase the likelihood that individuals would inflate. Two days after the trials in the laboratory, each fish was tested again for puff duration, activity, and swimming performance in a natural habitat over the duration of three days. To do this, fish were transported in a pail of seawater to a nearby wetland mesocosm. The wetland area receives constant seawater input from the wetlab and empties into the ocean. The wetland contains a variety of fish and invertebrate species as well as both red (*Rhizophora mangle*) and black mangrove (*Avicennia germinans* and *Halimeda* spp.). In this semi-natural habitat the puff duration test was repeated immediately after transport and then each fish was placed in a 35×50 cm mesh pen with the bottom filled with wetland substrate and a 10 cm grid marked on the bottom with string. The pens were filled to a depth of 20 cm with seawater. Activity and swimming performance were measured as described above, with the exception that the number of squares crossed was converted to distance and rounded to the nearest 10 cm to account for grid squares in the mesh pens that were smaller than 10 cm. Throughout the trials in the mesocosm, temperatures in the pens ranged from 28.2 °C to 35.2 °C, fluctuations which pufferfish are regularly subject to in tidal creek systems [68].

2.5. Recapture study

We used a displacement and recapture procedure to test whether activity, puff duration, and swimming performance during a trial in the laboratory and a trial in the natural enclosure were related to whether fish would return to the tidal creek where they were originally captured. Of the fish used to validate performance tests in the natural enclosures, 70 fish were released near the shoreline approximately 625 m up-current from the mouth of Page Creek (24°49′16.6″N, −76°19′8.5″W) in an area with rocky, sandy substrate and no mangroves (Fig. 1). A control group of 20 fish were released in Page Creek. We attempted to recapture fish in Page Creek 2, 3, 19, 20, and 23 days after the initial release using seine nets. Checkered pufferfish are relatively weak swimmers and thus their performance abilities have the potential to predict their ability to swim distances and seek out the desirable Page Creek habitat. Furthermore, more active individuals might be more likely either to explore their environment and locate the desirable habitat in their original capture creek or to continue to explore the coastline once the creek was found. In the first scenario, more active fish would be more likely to be recaptured in Page Creek, whereas in the second scenario, more active fish would be less likely to be recaptured in Page Creek.

2.6. Analysis

Data were analysed using R (Version 3.0.3; [69]) and Sigmaplot (Version 11.0; [70]). To assess the repeatability of behaviours and performance, we calculated point estimates for repeatability using mixed-models based on recommendations by Nakagawa and Schielzeth [71]. Because the activity data collected in January and July were both approximately normally distributed, we estimated repeatability for each on the original scale data using a linear mixed-model. For activity (January) we estimated adjusted repeatability for the null model, as well as models that controlled for trial period (trial periods being before cortisol treatment, peak circulating cortisol at 48 h, and after the cortisol treatment had been extinguished at 4 days), for trial period and body condition (Fulton's K), and for trial period and total length. We also estimated adjusted repeatability for activity (July) using models that controlled for trial period, trial period and temperature, trial period and body condition, and trial period and total length. To estimate the repeatability of non-Gaussian count data (all puff duration, duration of a startle response, and time to exhaustion data), we used a generalized linear mixed-effects model for multiplicative overdispersion, using a log-link or a square root-link, depending on whether the mean of the data was greater than 5 (see [71]). For each model, the 95% confidence intervals were estimated using parametric bootstrapping (1000 permutations). Also, Pearson's correlation or Spearman's rank correlation, depending on the distribution of the data, were used to examine the correlations of behaviours and performance of individual fish in the laboratory between each of the three trials (January), and between the trials in the laboratory and the natural enclosure (July). To determine whether any of the behaviours measured formed syndromes or coping styles, we used Spearman's rank correlation to quantify the relationships between the mean behaviours and performance over the three trials (January), and the mean behaviours and performance over the trials in the laboratory and the natural enclosure (July). The confidence intervals for the Spearman's correlation coefficients (ρ) were estimated by bootstrapping (1000 permutations).

The proportion of fish recaptured from the control and displaced groups were compared in Sigmaplot using a chi-square test. Recapture or no recapture of the displaced group in the original capture tidal creek was modelled using a logistic regression with a logit link. A backwards single-term deletion procedure was used to select factors using likelihood-ratio tests at $\alpha = 0.05$ (drop1 command in R), [72] with puff duration, activity, swimming performance, total length, and Fulton's condition factor as fixed-effects. We tested the linearity of the logit of each factor using the method described in Field et al. [73]. Laboratory and natural enclosure fixed-effects were modelled separately to avoid collinearity. Because total length and Fulton's condition factor were not collinear ($r = -0.196$, n = 69, p = 0.11) they were both added to the full models. The data was examined for outliers and influential data using the studentized residual, hat values (leverage), and the DFBeta of each case for each predictor.

In the puff duration data, the number of individuals inflated decayed with time (seconds after the beginning of the puff), and there were individuals that did not puff. For these reasons we used the Peto and Peto modification of the Gehan–Wilcoxon test to compare the left-censored puff duration survival curves (cendiff command in R), [74] between control and cortisol treatments within trials to test the null hypothesis that the survival curves of the two treatments were not different within trials. We used survival analyses rather than multiple regressions to compare survival curves of behaviour and performance data because survival analyses are more effective at dealing with censored data. Using the Cochran–Mantel–Haenszel test for stratified categorical data (mantelhaen.test in R), [75,76], we tested the null hypothesis that the relative proportions of puff scores are the same between treatment groups within trials. As the activity data met the assumptions of normality and heterogeneity (Levene's test), we ran a type III ANOVA on the activity data with subject, treatment, trial, and the interaction between trial and treatment to determine whether there was a significant interaction effect. Because there was no interaction effect, we ran a type II ANOVA with subject, treatment, and trial as factors to determine the effects of treatment and trial on activity, using the Greenhouse–Geisser method to correct for sphericity. The number

of individuals who continued to express altered behaviour in response to a simulated threat decayed with time (seconds after the threatening event) and observations ended 60 s after the threat. For these reasons we used a log-rank test to compare all the right-censored survival curves of startle behaviour duration of the two treatments within trials. If the curves of cortisol and control treatments differed significantly from each other, the curves were compared using a log-rank test within treatments between trials and within trials between treatments to determine which factor was the source of the difference. A log-rank test was also used to compare the survival curves of the time to chase a fish to exhaustion with treatment and trial as factors. If the observed data from the two treatments differed significantly from each other, the curves were compared within trial by treatment and among trials. In the event of fish mortality (January, n = 5), such individuals were removed from the full dataset and not included in the analyses.

3. Results

Based on the point repeatability estimates, activity, puff duration, and the duration of a chase to exhaustion were repeatable within individuals over time for the data collected in the lab during the cortisol experiment (January) (Table 2). Puff duration and the duration of a chase to exhaustion were repeatable the data collected in the trials for the lab and natural enclosure experiment (July) (Table 2), although we note that models for activity in July failed to converge. All behaviours and performance metrics had at least one highly significant correlation between two trials (Tables 3 and 4), with the exception of the correlation between activity in the laboratory and the field in July (r = 0.096, n = 90, p = 0.37). A portion of the repeatability in the activity of individuals (January data) was accounted for by body condition (Table 1). Rank puff duration ($\rho = 0.225$, n = 90, p = 0.03) and activity (r = 0.52, n = 90, p = <0.001) in the natural enclosure were correlated with water temperature, but swimming performance was not (r = 0.163, n = 90, p = 0.125). The individuals differed from each other in their behaviour and performance, in some cases quite distinctly, as indicated by the ranges of the individual behaviour and performance assessed in January (Fig. 2). There were no significant correlations between behaviours or performance metrics when cortisol and control test groups were pooled and these measures were averaged for individuals over the three trials. Of puff duration, locomotor activity, and swimming performance measured in July, only mean puff duration and mean swimming performance of individuals tested in the lab and a natural enclosure were related ($\rho = -0.219$, n = 88, p = 0.04).

We recaptured 58% of the 70 displaced fish and 80% of the 20 control fish released in the tidal creek of original capture. However, power was low at $\alpha = 0.05$ (0.27) and we did not detect a significant difference between the proportion of displaced and control fish that were recaptured (χ^2 (1) = 1.973, p = 0.16). Neither behaviour nor performance metrics in the laboratory and in a natural enclosure could be included in a model for predicting the recapture of displaced fish in the original creek of capture (Fig. 3, Tables 5 and 6). Only total length was a significant predictor of recapture of the displaced fish in the original creek of capture in both models, with longer fish being more likely to be recaptured.
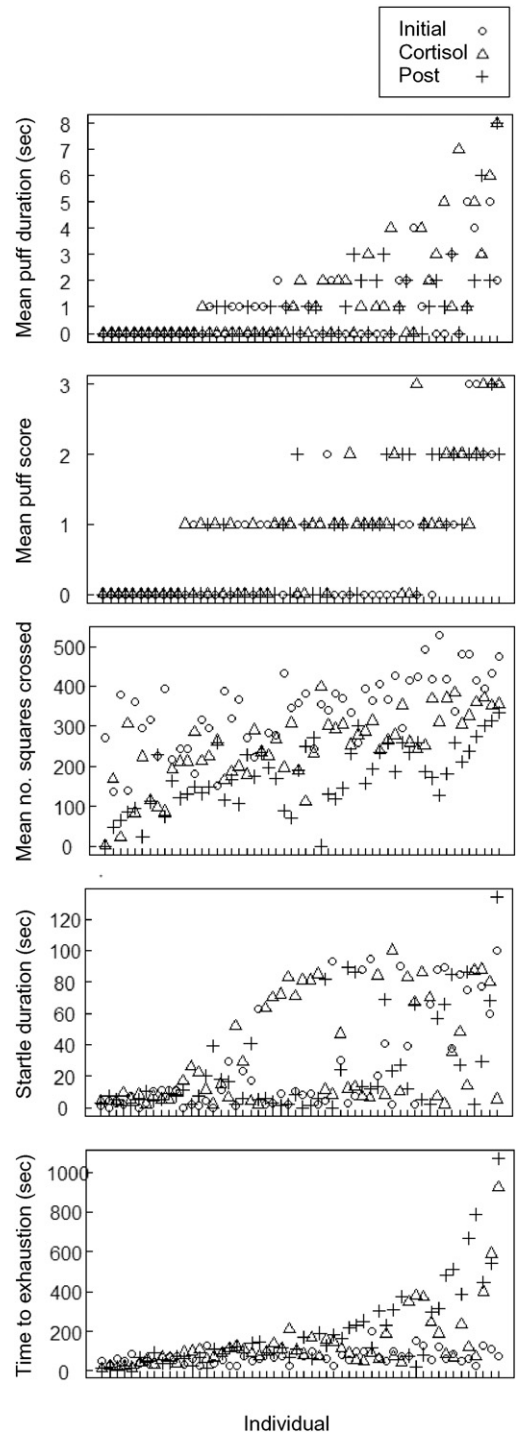

Fig. 2. Behaviours and performance measured at three time points for individual checkered pufferfish (January).

Table 1

The repeatability estimates of measures of behaviour and performance of checkered pufferfish in the laboratory (January) between an initial trial, a trial at peak cortisol levels, and a trial after the cortisol treatment had been exhausted.

Behaviour	Controlled factors	Model link function	Repeatability (SE)	95% CI	n
Activity	None	None	0.046 (0.065)	0, 0.215	55
	Trial	None	0.331 (0.081)	0.17, 0.483	55
	Trial, total length	None	0.337(0.085)	0.162, 0.490	55
	Trial, Fulton's K	None	0.287 (0.084)	0.117, 0.445	55
Puff duration	None	Log	0.481 (0.138)	0.263, 0.789	54
Duration of startle response	None	Square root	0.033 (0.068)	0, 0.215	54
Time to exhaustion	None	Square root	0.221 (0.105)	0.053, 0.460	53

Table 2
The repeatability estimates of measures of behaviour and performance of checkered pufferfish in the laboratory (July) between a trial in the lab and a trial in a natural enclosure.

Behaviour	Controlled factors	Model link function	Repeatability (SE)	95% CI	n
Activity	Trial	None	0.082 (0.086)	0, 0.268	90
	Trial, total length	None	0.064 (0.080)	0, 0.272	90
	Trial, Fulton's K	None	0.082 (0.084)	0, 0.282	90
	Trial, temperature	None	0.051 (0.078)	0, 0.256	90
Time to exhaustion	None	Square root	0.351 (0.102)	0.156, 0.555	88
Puff duration	None	Log	0.463 (0.060)	0.487, 0.639	93

There was no significant difference between the puff duration of the cortisol treated fish and the control fish within trials ($\chi^2 = 5.8$, df = 5, p = 0.322; Fig. 4). In addition, there was no significant difference between the puff scores of the cortisol treated group and the control group within trials ($\chi^2 = 2.76$, df = 3, p = 0.430). The cortisol treatment did not have any significant effect on activity relative to the control group (F = 2.11, df = 53, p = 0.151, ges = 0.021; Fig. 5), whereas the trial repetition did have a significant effect on activity (F = 65.1, df = 106, GG p = <0.001, ges = 0.357). The duration of startle behaviour of the cortisol treated fish did not differ significantly from that of the control group within each trial ($\chi^2 = 8$, df = 5, p = 0.156; Fig. 4). In the control group, the proportion of fish that remained startled for at least 60 s increased with each successive trial. In the cortisol treated group, the proportion of fish that remained startled at 60 s decreased between the initial trial and the trial at peak cortisol and subsequently increased to an intermediate proportion during the trial at which the cortisol treatment was exhausted. There was no significant difference in the time to chase a fish to exhaustion between the cortisol and control treatments within the initial ($\chi^2 = 0.03$, df = 1, p = 0.913), peak cortisol ($\chi^2 = 0.1$, df = 1, p = 0.761), and post cortisol trials ($\chi^2 = 1.5$, df = 1, p = 0.215) (Fig. 4), but there was a significant difference between trials ($\chi^2 = 25.2$, df = 2, p = <0.001).

4. Discussion

Our results reveal that individual checkered pufferfish have consistent ranks in behaviour and performance over time with respect to puff score, puff duration, locomotor activity, startle duration, and swimming performance. However, we did not observe any significant correlations between different personality traits, which indicate that the behaviours tested may not form behavioural syndromes in these fish. Neither were there correlations between personality and performance traits, which suggest that these individual characteristics do not combine to form coping styles. However, there was evidence of a negative relationship between puffing performance and swimming performance. The puffing and swimming performance of individual fish were consistent between trials in the laboratory and in a natural enclosure. Activity was not repeatable between the lab and a natural enclosure, but it should be taken into consideration that the models of these data failed to converge. Individual differences in these behaviours and performances were not related to whether a displaced fish was recaptured in the original capture stream. In addition, a physiological dose of cortisol did not have a significant effect on any of the consistent behaviours or performance measured either at peak cortisol or after the cortisol treatment was exhausted, although there may have been a biologically relevant effect on the duration of a startle response.

Our study demonstrates that pufferfish exhibit consistent between-individual differences in behaviour (i.e., personality). We recognize that the range of behaviours observed may not be representative of the entire population because individuals with certain personality traits are likely to be more catchable by seining than others (e.g., [77]). Nevertheless, the results indicate that the personality traits measured exist in a wild population from a variable and physiologically challenging environment. Diverse personalities may persist because they increase the likelihood that adaptive traits will be present in at least some of the members of the population when conditions change [78], or as a result of frequency-dependent selection (see [14]).

Although individuals had consistent differences in behaviour, certain behaviours changed over successive trials for both treatment groups. For example, activity in January trials decreased over time (Fig. 5). For this reason, a fixed effect was added to the models of activity to account for temporal changes. The inclusion of a time effect increased the repeatability estimates of activity relative to the null model (Table 1). Because we were not able to account for temporal changes in the models used to estimate the repeatability of the non-Gaussian data, our measures of repeatability for these data likely underestimate the consistency of individual behaviour when the mean behaviour of the group changed with time [79].

Variability in motivation may have contributed to differences in performance among trials and between individuals. For example, it

Table 3
Correlations between repeated measures of behaviour of checkered pufferfish in the laboratory (January) between an initial trial (initial), a trial at peak cortisol levels (peak cortisol), and a trial after the cortisol treatment had been exhausted (post cortisol).

Pearson correlation	Behaviour	Trial comparison	Correlation coefficient r	95% CI	p-value	n
	Activity	Initial/peak cortisol	0.326	0.066, 0.544	0.02	55
		Peak/post cortisol	0.444	0.200, 0.633	<0.001	55
		Initial/post cortisol	0.238	−0.029, 0.474	0.08	55

Spearman rank correlation	Behaviour	Trial comparison	ρ	95% CI	p-value	n
	Puff duration	Initial/peak cortisol	0.166	−0.120, 0.433	0.23	55
		Peak/post cortisol	0.374	0.111, 0.602	0.005	55
		Initial/post cortisol	0.339	0.072, 0.573	0.01	55
	Duration of startle response	Initial/peak cortisol	−0.0561	−0.341, 0.237	0.69	54
		Peak/post cortisol	−0.146	−0.382, 0.108	0.29	54
		Initial/post cortisol	0.292	0.009, 0.543	0.03	53
	Time to exhaustion	Initial/peak cortisol	0.158	−0.141, 0.435	0.25	54
		Peak/post cortisol	0.363	0.057, 0.613	0.007	55
		Initial/post cortisol	0.126	−0.145, 0.399	0.36	54
	Puff score	Initial/peak cortisol	0.271	−0.027, 0.527	0.05	55
		Peak/post cortisol	0.411	0.159, 0.633	0.002	55
		Initial/post cortisol	0.333	0.049, 0.568	0.01	55

Table 4

Spearman rank correlations between repeated measures of behaviour of checkered pufferfish in the laboratory and in a natural enclosure (July).

Behaviour	Correlation coefficient	95% CI	p-value	n
Activity	0.0964	−0.130, 0.302	0.37	90
Time to exhaustion	0.349	0.152, 0.534	0.001	88
Puff duration	0.305	0.102, 0.488	0.003	94

Table 6

Summary of the terms included in the full model used to predict recapture of checkered pufferfish based on total length, body condition (Fulton's K), puff duration, locomotor activity, and swimming performance (time to exhaustion) in a natural enclosure.

Model term	L-ratio	Df	p-value (χ^2)
Total length	6.236	1	0.01
Fulton's K	0.004	1	0.95
Puff duration	0.106	1	0.60
Activity	0.246	1	0.92
Time to exhaustion	0.0948	1	0.62

may be that, although efforts were made to chase the fish as vigorously as possible, changes in the motivation to swim contributed to longer chases over successive trials. If so, this would reflect the findings of other studies that have found that some lizards do not exhibit maximum performance even in challenges such as predator evasion [80–85]. Changes in motivation within individuals over time would cause us to underestimate the repeatability of performance. Conversely, consistent differences between individuals in their motivation to perform would lead us to overestimate repeatability. For example, fish puffed during the chase to exhaustion, at which time they ceased to swim or their swimming was impaired. We assume that the fish puffed when they were exhausted, because puffing impairs their ability to swim and evade predators. Yet individuals likely differ in their motivation to swim and to puff during an encounter with a predator. Although differences in motivation between trials may lead us to underestimate performance abilities, measures of performance that incorporate individual differences in motivation are useful in exploring the relationships between personality and performance because they can reveal important individual differences in behaviour as they occur in the field [19,86].

We did not observe correlations between different consistent behaviours and thus these did not form behavioural syndromes. Whereas behavioural syndromes have been observed in certain wild populations, they do not appear in all populations; for example, the activity-aggression-boldness syndrome is present in populations of sticklebacks from large ponds with predators, but not small ponds without predators [87]. The absence of behavioural syndromes suggests that the individual-level differences in behaviour in the present study have different proximate mechanisms, which might be adaptive in terms of avoiding potential constraints on an individual's behavioural response to environmental fluctuation [3,88]. It is also possible that environmental factors, such as captivity and aspects of the experimental arena, obscured relationships between behaviours. However, our design does not include controls that would enable us to test for these environmental effects.

Similarly, we did not find relationships between performance or a cortisol treatment and consistent behaviour (i.e., coping styles). Measures of puffing and swimming performance were not related to the personality traits measured, which suggests that these individual differences in performance and behaviour were not shaped by the same physiological mechanisms, such as circulating cortisol levels. Our finding that cortisol levels did not modulate individual differences in the behaviour of checkered pufferfish reflects the findings of several studies on fish (e.g., [89–91]), but not others (e.g., [29,34,35]). As the dose of cortisol for the treatment was validated using checkered pufferfish from the same population, it is unlikely that the dose was

Table 5

Summary of the terms included in the full model used to predict recapture of checkered pufferfish based on total length, body condition (Fulton's K), puff duration, locomotor activity, and swimming performance (time to exhaustion) in the laboratory.

Model term	L-ratio	Df	p-value (χ^2)
Total length	7.360	1	0.007
Fulton's K	0.003	1	0.96
Puff duration	0.139	1	0.41
Activity	0.0365	1	0.95
Time to exhaustion	1.533	1	0.12

incorrect. Other possible explanations as to why cortisol treatment may not have had an effect on behaviour are discussed below, and include interactions with the environment, other aspects of the stress response which were not activated by the treatment, or other physiological factors that may be more tightly linked to behaviour than cortisol (see [41] for discussion of diverse responses associated with cortisol manipulation). These results also indicate that puffing and swimming performance abilities were not paired with the behaviour traits measured through correlational selection for adaptive behavioural strategies. It may be that fish with such effective defences against predators do not receive added benefits from matching individual behavioural strategies to avoid predators with differences in performance capacities. Thus, despite speculation that correlations between the HPA/HPI axis characteristics, behaviour, and life-history strategies form a pace-of-life syndrome in some species [20], we did not find evidence of this in checkered pufferfish.

Whereas swimming and puffing performance were not related to other behaviours measured, we did observe relationships between body condition and behaviour and performance. It might be that fish in better condition have more available energy and are more motivated to actively explore in a novel environment, but that this motivation decreases as the fish becomes familiar with the environment. The negative relationship between body condition and swimming performance in the trial after cortisol had been exhausted is counter-intuitive, as one would expect fish in better condition to have greater swimming endurance. It may be that fish with greater body condition had more robust sprints in response to a threat but tired quickly during the chase, whereas fish with lower condition swim more slowly to conserve energy and are thus able to swim for a longer period, although we were unable to test this in the current study.

Fish size was also related to performance and behaviour in our study. Total length was related to puff duration in the natural enclosure. This is similar to the results of another study that revealed that the weight of checkered pufferfish was related to puffing performance [55]. In the current study, longer fish were also more likely to be recaptured in the original capture creek after displacement. It may be that, although we did not observe a relationship between body length and swimming performance during a chase to exhaustion in captivity, there was a relationship between length and swimming performance, capacity, or motivation in other contexts. In addition, despite efforts to capture all the pufferfish in Page Creek during seining, some fish were able to avoid capture by hiding among the mangrove roots. It could be that certain fish, such as smaller individuals, were less catchable. Other intrinsic factors such as sex and ontogeny may contribute to individual differences in personality and performance (e.g., [92–94]) but these cannot be reliably identified in checkered pufferfish without sacrificing them.

An important consideration for comparing the results of the current study to others is the effects of the environmental context, such as captivity, on fish behaviour and performance in general and on the behavioural response to elevated plasma cortisol in particular. For example, rainbow trout bred for a high cortisol response showed higher levels of activity than low cortisol response fish in the presence of an intruder, but not in isolation [95]. Similarly, captivity can be a chronic stressor for wild fish [96] and might have led to desensitization towards cortisol in our study fish [97]. However, baseline cortisol levels
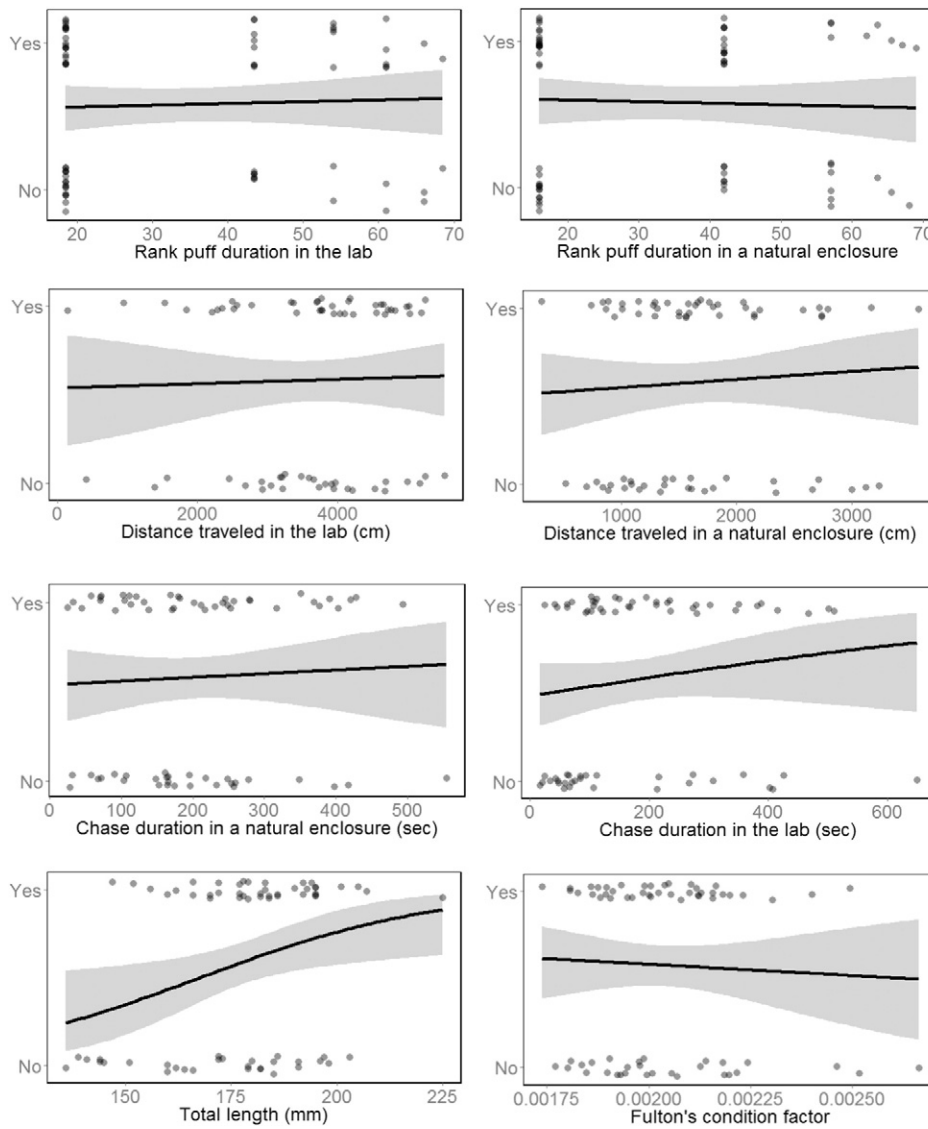
Fig. 3. The behaviour and performance of individual checkered pufferfish in laboratory arenas and natural enclosures categorized by capture success in their original tidal creek. Fish were recaptured 2, 3, 19, 20, and 23 days after being displaced ~625 m up-current along the coast. The black line represents the logistic regression and the 95% confidence interval is shaded in grey. Data points have been spaced vertically to reduce overlap.

measured in wild checkered pufferfish in captivity resemble baseline levels sampled from wild checkered pufferfish in the field ([55]; Jennifer Magel, Carleton University, unpublished data), which suggests that captivity does not induce chronic increases of cortisol levels in these fish.

A stress response also incorporates a suite of endocrine and other physiological changes and it is possible that cortisol must act in combination with these processes in order to affect changes in behaviour. For example, Øverli et al. [29] posit that the high levels of corticotropin releasing hormone in fish with a high cortisol response to stress contributes to their high levels of activity. There are also studies which suggest that baseline or chronic cortisol treatments have a greater effect on behaviour than an acute treatment [89–91], including another study on checkered pufferfish, which indicates that puff score and duration are not related to stress-induced cortisol levels but that puff score might be related to baseline cortisol [55]. It is possible that long term exposure to cortisol has implications for behaviour that short-term exposure does not, such as the effects of energy depletion on energetically demanding behaviours [98].

A general lack of effect by cortisol implants could indicate that this hormone is not involved in the regulation of the traits measured in our study. For example, studies on other fishes have found that

individual cortisol levels were not related to behavioural responses to a predator cue [37], swimming performance [35,63,99], and activity [95, 100,101]. As such, certain behaviours might be more tightly associated with other physiological factors such as circulating levels of catecholamines [37].

Startle duration is a possible exception in that there may have been an effect of the cortisol treatment. Although there was no significant difference between the duration of startled behaviour of the treatment groups, there were distinct changes between trials in the proportions of fish that remained startled for 60 s within each treatment which may be biologically significant. It is possible that energy stores were depleted in the cortisol treated fish during the trial at peak cortisol levels, reducing their ability to alter their behaviour in response to a threat. However, results from a study by Cull et al. [63] reveal that an exogenous cortisol treatment did not raise blood glucose levels in checkered pufferfish, which casts doubt on this explanation. Alternately, rises in circulating glucocorticoids that exceed acute timescales may suppress behavioural responses to a threat through other pathways. The increasing duration of startled behaviour in the control treated fish over time may be the result of captive conditions; for example, greater densities of fish in captivity relative to the field may increase
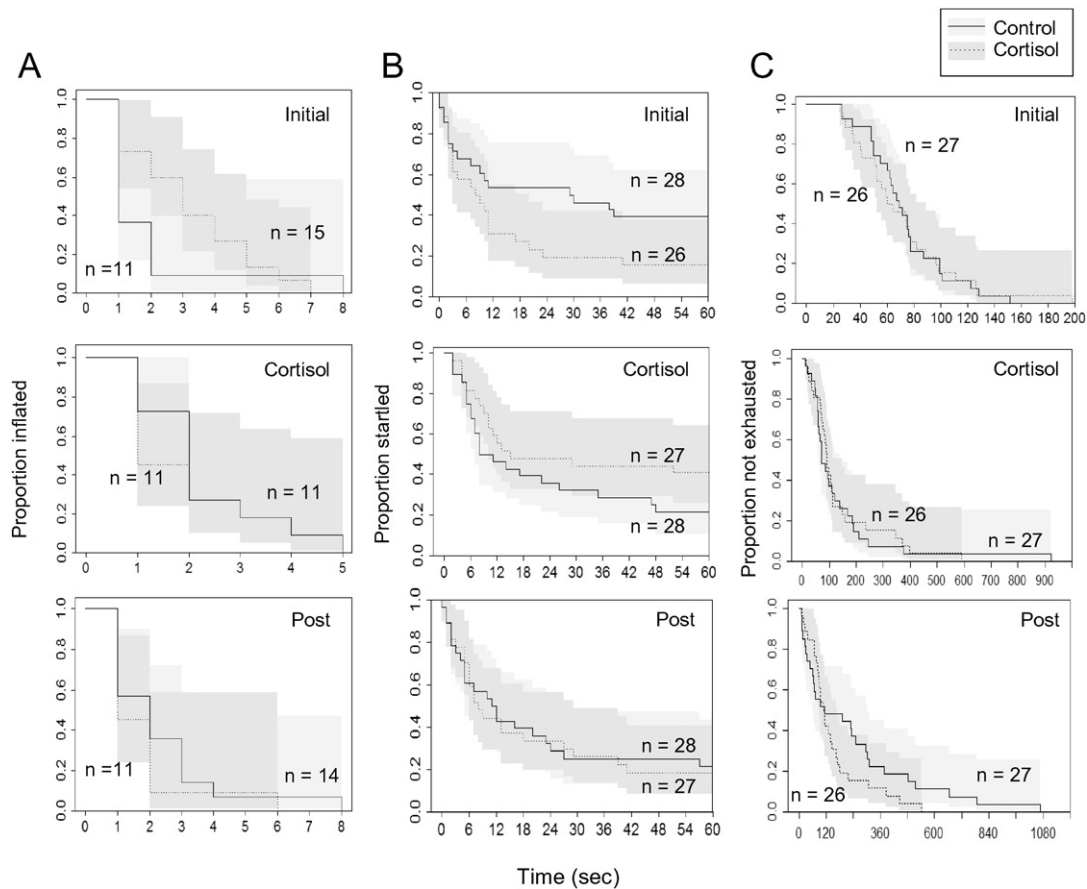
Fig. 4. Survival curves of the (A) puff duration, (B) proportion displaying startled behaviour, and (C) duration of a chase to exhaustion of checkered pufferfish. These tests were repeated before treatment (Initial), when the cortisol treated group was experiencing peak circulating cortisol (Cortisol) at 48 h, and after the cortisol does had been extinguished (Post) at 4 days. Shaded areas represent the 95% confidence interval. Fish that did not inflate were not included in survival curves of puff duration. The observations of startled behaviour were censored after 60 s.

aggression between fish over time and cause individuals to be more sensitive to threatening stimuli. Another possible explanation is that frequent feeding in captivity gave fish the opportunity to store energy and thus they had more energy available for longer responses to a threat in successive trials.

Our results reveal that consistent individual-level differences in behaviour and performance were present in a wild population of pufferfish from a variable and physiologically challenging environment. Although individuals' differences in activity, anti-predator behaviour, and performance were consistent over time, behaviours and performance

of pufferfish changed over successive trials for some measures, possibly in response to holding conditions or familiarity with the trials. Personality traits were generally not correlated with each other or related to performance and thus did not form behavioural syndromes. Individual performance was consistent between the lab and an in-situ natural enclosure, however we found that activity was influenced by temperature in the natural enclosure. Whether a fish returned to the tidal creek of capture after displacement was related to fish length but not observed behaviour or performance. A cortisol treatment did not alter most personality traits or performance in these fish, with the possible exception of duration of a startle response, which suggests that other mechanistic factors might underlie observed individual differences in behaviour and performance in this species. We recommend that the effects of environmental context, such as captivity, on the behaviour, performance, and stress physiology of wild-caught subjects should be taken into careful consideration when designing behaviour studies. This study is one of the few studies currently available that examines the effect of a cortisol treatment on personality and performance in wild animals, and we suggest that this is an important area of research need. Future studies must examine the impact of both anthropogenic and natural environmental heterogeneity and disturbance as the effects of these factors on animal habitats will increasingly become key factors in understanding animal behavioural plasticity and stress responsiveness in a changing world.
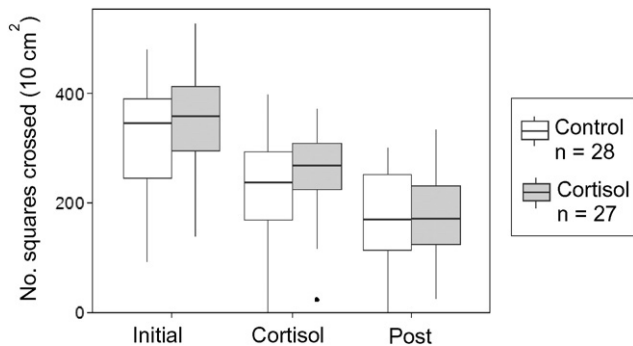

Fig. 5. The number of 10 cm squares crossed by checkered pufferfish from control and cortisol treated groups during the initial trial (Initial), when cortisol was elevated in the treatment group (Cortisol) at 48 h, and when cortisol levels had returned to baseline (Post) at 4 days.

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