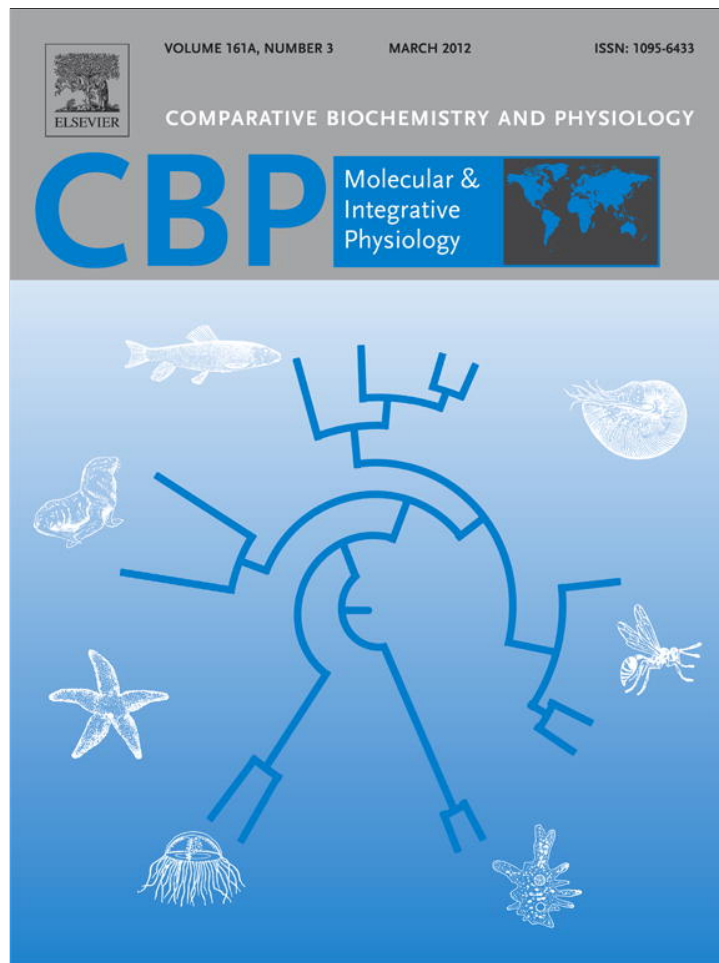


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## Condition dependent intra-individual repeatability of stress-induced cortisol in a freshwater fish

K.V. Cook<sup>a,\*</sup>, C.M. O'Connor<sup>a</sup>, S.H. McConnachie<sup>a</sup>, K.M. Gilmour<sup>b</sup>, S.J. Cooke<sup>a,c</sup><sup>a</sup> Fish Ecology and Conservation Physiology Laboratory, Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, ON, Canada K1S 5B6<sup>b</sup> Department of Biology, University of Ottawa, 30 Marie Curie, Ottawa, ON, Canada K1N 6N5<sup>c</sup> Institute of Environmental Science, Carleton University, 1125 Colonel By Drive, Ottawa, ON, Canada K1S 5B6

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## ABSTRACT

The glucocorticoid (GC) stress response is thought to be an individual trait associated with behaviour and life history strategies. Studies exploring such relationships typically assume measured hormone values to be repeatable within an individual. However, repeatability of GCs has proven variable in wild animals and underlying reasons remain unknown. We assessed individual repeatability of circulating stress-induced cortisol, the primary GC in teleost fish, and glucose concentrations in a wild teleost fish held under consistent laboratory conditions. We also tested the hypothesis that the magnitude of intra-individual variability in stress-induced cortisol concentrations ("cortisol variability") is influenced by body condition. Wild-caught bluegill sunfish (*Lepomis macrochirus*) were subjected to repeated standardized stressors and blood sampled (3 times over 6 days) once cortisol concentrations peaked. Various indicators of fish condition, both whole body and physiological, were also measured. Overall, stress-induced circulating cortisol concentrations were repeatable but stress-induced glucose was not. Cortisol variability was related to Fulton's condition factor and size (eviscerated mass) where smaller fish in poor condition exhibited increased cortisol variability. The findings have implications for the interpretation of studies that examine correlates of GC concentrations as they suggest consistency in stress responsiveness is influenced by factors such as size and condition.

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## 1. Introduction

Physiological diversity at the individual level is often ignored and largely attributed to inconvenient variance or sampling error (Bennett, 1987; Williams, 2008). Glucocorticoid (GC) hormones, best known for their role during the physiological stress response (Sapolsky et al., 2000), have received attention for the magnitude of variation present between individuals. Variation in GC hormone concentrations in response to a stressor is thought to be a defining individual trait with consistent consequences and correlates. In fish, exposure to an acute stressor stimulates the hypothalamic–pituitary–interrenal axis (HPI axis; the teleost homolog of the mammalian hypothalamic–pituitary–adrenal axis) and results in the synthesis and release of GC hormones into circulation (Wendelaar Bonga, 1997; Mommsen et al., 1999; Barton, 2002). Cortisol is the primary GC in teleost fish and along with glucose, a metabolite associated with the secondary stress response, is the most commonly measured hormone used to quantify the magnitude of perceived threat from a stressor (Mommsen et al., 1999). The physiological stress response acts to provide the energy required to meet challenges associated with the stressor (Barton, 2002).

However, with energy resources being finite, overcoming a stressor occurs at the cost of other energy-demanding processes and results in life history trade-offs (Ketterson and Nolan, 1999; Ricklefs and Wikelski, 2002) and behavioural correlates (Koolhaas et al., 1999; Øverli et al., 2007). Ultimately, the GC response is thought to influence individual fitness (Breuner et al., 2008).

To assess physiological diversity in relation to evolutionary theory (e.g. Ketterson and Nolan, 1999; Zera and Harshman, 2001), the measured physiological trait must be consistent and repeatable within an individual. Alternatively, mechanisms underlying its variability must be known. However, studies exploring individual traits influenced by GC variability usually rely upon measurements of GCs in a single sample from each specimen, especially for wild populations. Underlying this methodology is the assumption that the stress response is repeatable within an individual. Repeatability of the stress response has been confirmed in captive populations of fish (Pottinger et al., 1992; Schjolden et al., 2005) but it is rare to examine this in wild populations (but see Kralj-Fiser et al., 2007; Cockrem et al., 2009 for examples in colonial birds) and has just recently been confirmed in wild fish (Cook et al., 2011). However, results are sometimes variable within a population. Repeatability of the stress response in zebra finches (*Taeniopygia guttata*) from nestlings to adults was sex-dependent (Wada et al., 2008) and in another experiment differed with experimental conditions (e.g. short day vs. long day) for baseline GC concentrations in house sparrows

\* Corresponding author.

E-mail address: [katrina.vcook@gmail.com](mailto:katrina.vcook@gmail.com) (K.V. Cook).

(*Passer domesticus*; Romero and Reed, 2008). Differences in the magnitude of intra-individual variability also exist in gilted sea bream (*Sparus aurata*) where some fish exhibit almost identical concentrations of stress indicators across trials and others a high degree of variation (Tort et al., 2001).

The reasons underlying inconsistency in an individual's response to an identical stressor remain unclear. The hypothesis that irregularity in hormone titres within an individual is related to measures of behaviour (While et al., 2010) and condition (Collier et al., 2010) has recently emerged in the literature, primarily in the field of biomedical research. Assessing intra-individual variability in GC concentrations with respect to measures of condition is a novel approach that may yield explanations for inconsistencies in repeatability. Using wild bluegill sunfish (*Lepomis macrochirus*) as a model species, individual condition was quantified and individuals were repeatedly sampled for both stress-induced cortisol and glucose concentrations. We aimed to determine the degree of repeatability of both stress-induced cortisol and glucose concentrations and to explore condition correlates of differential intra-individual variability in stress-induced cortisol concentrations. Bluegill sunfish were chosen as a model species as they are abundant, adapt easily to holding conditions, and tolerate repeated sampling (Fobert et al., 2009). Each individual fish was sampled three times for peak cortisol concentrations following exposure to a standardized stressor. As peak GC concentrations in response to an acute stressor vary according to species (Romero, 2004), a time course analysis of stress-induced cortisol concentrations was also conducted.

## 2. Materials and methods

Research was conducted at Queen's University Biological Station in south-eastern Ontario, Canada (44°34'N, 76°19'W) and fish were captured from Lake Opinicon. In this lake, bluegill (*L. macrochirus*) begin forming breeding colonies in mid-May and breed through to July (Cargnelli and Neff, 2006). While fish were showing signs of sexual maturity and colonies had begun to form at the time of collection, fish used in the current study had not yet spawned. All fish were collected in mid-May 2010 by rod and reel angling using barbless circle hooks baited with a small piece of earthworm (*Lumbricus* sp.). Fish were landed within 10 s, immediately placed in a 50 Litre coolers filled with lake water, and transported by boat to the laboratory. Such rapid collection is common for sunfish (Cooke et al., 2003) and generally, the greater the duration of an angling event, the greater the magnitude of physiological disturbance and recovery time required (Cooke and Suski, 2005). If total handling time from hooking exceeded 10 s or if a fish sustained any injury as a result of hooking or handling, they were not used in the experiment and released. No fish was held in the cooler for more than 2 h from capture to relocation to the laboratory. Once in the laboratory, fish were held in a 100 Litre fibreglass tank for 24 h following capture to allow for recovery. Holding tanks were exposed to ambient sunlight and a flow-through system provided continuous fresh water from the lake. This short time period was chosen as we wished to ensure appropriate recovery while minimizing total holding duration. Previous physiological assessments of bluegill angled from Lake Opinicon validate that 24 h is a sufficient recovery time. Wilson et al. (2011) quantified baseline cortisol concentrations of  $92 \pm 108 \text{ ng} \cdot \text{mL}^{-1}$  (mean  $\pm$  SD) following a 24-h holding period in tanks identical to those used in the presented study. These values are similar to those obtained from another study obtaining baseline concentrations 2 min following capture ( $47.7 \pm 34 \text{ ng} \cdot \text{mL}^{-1}$ ; range of 0.3 to  $552.9 \text{ ng} \cdot \text{mL}^{-1}$ ; McConnachie, 2010). Generally, bluegill exhibit large inter-individual variability.

To elicit a stress response, all fish were exposed to a standardized stressor. This consisted of 3 min of air exposure while held in a small, covered container lined with moistened padding to prevent

desiccation and injury. Immediately following air exposure, fish were held individually in 20 Litre buckets regularly refreshed with lake water. This artificial stressor was employed to induce maximal elevations of cortisol concentrations. Two experiments were conducted; an initial study evaluated the time course of cortisol elevation and repeatability was assessed on a separate group of fish.

### 2.1. Time course of cortisol response (Study 1)

Fish greater than 130 mm (total length; TL) were captured and subjected to the standardized stressor as detailed above. A blood sample was collected on groups of fish after 10 (n=7), 20 (n=8), 30 (n=7), 40 (n=8), or 50 (n=8) min of the standardized stressor. Approximately 0.5 mL of blood was collected via caudal puncture using a 1 mL luer-lock sodium-heparinized (10,000 USP Units/mL, Sandoz, QC, Canada) syringe with a 25 gauge 1½ inch needle (BD, Franklin Lakes, NJ, USA). Blood samples were collected within 2 min of approaching the fish and placed in a water-ice slurry for no more than 1 h. Samples were then centrifuged for 5 min at 2000 g (Fisher Scientific Micro-fuge), and the resultant plasma was stored at  $-80^\circ\text{C}$  until analysis of plasma cortisol concentrations (see below).

### 2.2. Repeatability of stress-induced circulating cortisol concentrations (Study 2)

Only larger individuals over 160 mm (TL) were used to reduce the effects of repeated sampling. Fish were captured, held, and exposed to the standardized stressor as detailed above. Based on the results of the time course experiment, a blood sample was collected at 45 min following acute stress exposure. Due to repeated sampling and small size, a baseline sample was not taken and the total blood drawn ( $\sim 0.2 \text{ mL}$ ) was minimal. Fish were then placed into individual sensory deprivation chambers (115 cm  $\times$  22 cm  $\times$  22 cm) made of opaque black Perspex and supplied with a constant flow of lake water. The experimental chambers were located in an entry-controlled room that ensured fish were not exposed to external stimuli. Previous research has validated that wild bluegill held in sensory deprivation chambers exhibit relatively low baseline cortisol concentrations (i.e.  $44.0 \pm 117.9 \text{ ng} \cdot \text{mL}^{-1}$  after 24 h and  $76.9 \pm 89.1 \text{ ng} \cdot \text{mL}^{-1}$  after 4 days; McConnachie, 2010). After 48 h in the black boxes, fish were sequentially removed, again exposed to the standardized stressor and a blood sample was collected. This process was repeated a third time after another 48 h. The result was 3 post-stress blood samples, all 48 h apart and in response to identical stressors. In all cases, blood samples were collected within 2 min of approaching the fish and placed in a water-ice slurry for no more than 1 h prior to analysis.

Blood glucose concentration was measured using a drop ( $\sim 0.05 \text{ mL}$ ) of whole blood with a hand-held glucose metre (ACCU-CHEK glucose metre; Roche Diagnostics, Basel, Switzerland), a device previously validated for use in fish (Cooke et al., 2008). Haematocrit (HCT), a measure of the oxygen carrying-capacity of blood (Evans, 1993), was determined using microhaematocrit capillary tubes centrifuged for 5 min (CritSpin-Micro-Haematocrit Centrifuge). Blood was then centrifuged at 2000 g for 5 min. Plasma protein concentration, a measure of general nutritional health (Adams et al., 1993) was assessed using a hand-held protein refractometer. The remaining plasma was frozen immediately in liquid nitrogen and stored at  $-80^\circ\text{C}$  for further analysis.

Following collection of the third blood sample, fish were euthanized by cerebral percussion and dissected to obtain measures of body condition including hepato-somatic index (HSI), splenic-somatic index (SSI), Fulton's condition factor (K), and several other physiological and body condition indices combined to form a health assessment index (HAI) modified from Adams et al. (1993). HSI (the ratio of liver mass to body mass, %) is associated with the nutritional state of the fish and provides an estimate of its energy status

(Chellappa et al., 1995). SSI (the ratio of spleen mass to body mass, %) is used to evaluate splenic enlargement, an indicator of disease (Adams et al., 1993). Fulton's condition factor ( $K = \text{mass} \cdot \text{TL}^{-3} \cdot 10^6$ , where mass is expressed in g and TL in mm) was calculated as an additional metric that has been reported to be a good indicator of individual energetic state and overall quality for bluegill in Lake Opinicon (Neff and Cargnelli, 2004). The HAI is a quantitative index in which necropsy observations are scored numerically enabling statistical comparisons (Adams et al., 1993). The index was modified to focus on six main variables expected to reveal condition differences among individuals: average haematocrit and plasma protein concentration across the three trials (see Adams et al., 1993 for the rating system used), skin condition (an average of external parasite load and % coverage of the fish's skin by fungus [10 = low, 20 = moderate, 30 = high]), gill condition (an average of level of gill fray [10 = low, 20 = moderate, 30 = high]), presence of necrotic gill tissue [10 = low, 20 = moderate, 30 = high], and parasite load [10 = low, 20 = moderate, 30 = high]), liver colour (0 = normal, 10 = spotted cream colouration, 30 = completely discoloured throughout) and internal parasite load (an average of parasite load from the liver, kidney, heart and intestine [0 = none, 10 = low, 20 = moderate, 30 = high]). All six variables were added together to yield the total HAI where higher numbers represent individuals in poorer condition.

Plasma cortisol concentration was measured using a commercial radioimmunoassay kit (immunoChem Cortisol  $^{125}\text{I}$ -RIA Kit, MP Bio-medicals, Orangeburg, NY, USA) and a Cobra Auto-Gamma counter (Hewlett-Packard, Palo Alto, CA, USA) following the methods outlined by Gamperl et al. (1994). All samples were measured in a single assay, and intra-assay variability (% CV) was 9.7%.

### 2.3. Statistical analyses

Statistical analyses were conducted using SPSS Statistics 19.0 (2010). Residuals were tested for deviations from a normal distribution using Shapiro–Wilk goodness-of-fit tests, and variables were log-transformed where necessary. If log-transformation did not achieve a normal distribution, non-parametric statistics were used. A Levene's test was used to check for homogeneity of variances and sphericity was tested in repeated-measures analyses using a Mauchly's test. The level of significance for all tests ( $\alpha$ ) was assessed at 0.05. Data are reported as mean values  $\pm 1$  standard error of the mean (SEM).

For the time course experiment, Kruskal–Wallis tests were used as sample sizes were small. Differences in size and cortisol concentrations among sampling times were tested. Post-hoc Mann–Whitney tests compared the subsequent time period to the previous following a significant result. In the main experiment, a mixed design analysis of variance (MD ANOVA) tested for effects of sampling period, sex, and the interaction of these two factors in variables measured after each sampling period with post hoc pairwise comparisons. Student's *t*-tests explored sex differences in condition parameters. Repeatability of stress-induced cortisol and glucose was assessed using the repeatability statistic (*r*) according to Lessells and Boag (1987). To determine *r*, individuals were ranked according to the magnitude of stress-induced cortisol concentration for each trial. A one-way ANOVA was used to assess rank consistency within an individual across sampling periods with individual identity as an independent variable and rank as a dependant. A non-significant ANOVA would support the null hypothesis that individual rank is not consistent and therefore, that the measured value is not repeatable (Romero and Reed, 2008). The value of *r* is calculated according to the formula  $r = s^2_A / (s^2 + s^2_A)$  where  $s^2_A$  is the among-group variance and  $s^2$  is the within-group variance (Lessells and Boag, 1987).

Intra-individual variability of stress-induced cortisol concentrations was determined by calculating the standard deviation of all three measures of cortisol for each individual, and was termed

'cortisol variability'. As cortisol is the focus of this paper, condition correlates of 'glucose variability' were not explored. We used a backward stepwise multiple regression model (*p* to enter <0.05 and *p* to remove >0.1) to determine associations between condition and cortisol variability. From initial saturated models comprising all dependent variables (condition measures), the least significant terms were sequentially eliminated until obtaining a model where all retained variables had a significant effect on the independent variable. Predictors included K, HAI, HSI, SSI, plasma protein, haematocrit, sex and size (eviscerated mass). Eviscerated mass was used as size as opposed to length or total weight as partial correlations were comparably stronger as well as the additional benefit of eliminating sex-bias of total weight due to differential gonad size. For variables measured from all 3 blood samples for each individual (haematocrit, plasma protein and glucose), only those from initial capture were included. All variables except sex (coded as 0 = males, 1 = females) were log-transformed.

## 3. Results

### 3.1. Time course of stress-induced cortisol concentrations

Fish ranged in size from 129 mm to 220 mm (TL; Mean =  $155.4 \pm 3.7$  mm). Size did not significantly differ across sampling times (Kruskal–Wallis:  $H_4 = 1.1$ ,  $p > 0.05$ ). A significant effect of sampling time on stress-induced cortisol concentration was detected (Kruskal–Wallis:  $H_4 = 21.8$ ,  $p < 0.001$ ; Fig. 1). Post-hoc tests revealed a significant increase from 30 to 40 min (Mann–Whitney:  $U = 5.0$ ,  $p < 0.05$ ), but not between any other time period. Therefore, measured maximal cortisol levels were not reached until ~40 min after the standardized stressor (Fig. 1) and 45 min was selected as the time to sample blood for detection of maximal cortisol concentrations post-stress.

### 3.2. Repeatability of stress-induced cortisol concentrations

Fish ranged in size from 161 mm to 214 mm (TL =  $187 \pm 1.4$  mm). Of all physiological and condition parameters measured, sex differences were only apparent in stress-induced cortisol and glucose, both being greater in females (Table 1). In both sexes, experimental protocols influenced individual physiology. There was an effect of sampling period on both stress-induced cortisol concentrations and haematocrit (MD ANOVA:  $F_{(2, 66)} = 19.2$ ,  $p < 0.0001$  and  $F_{(2, 66)} =$

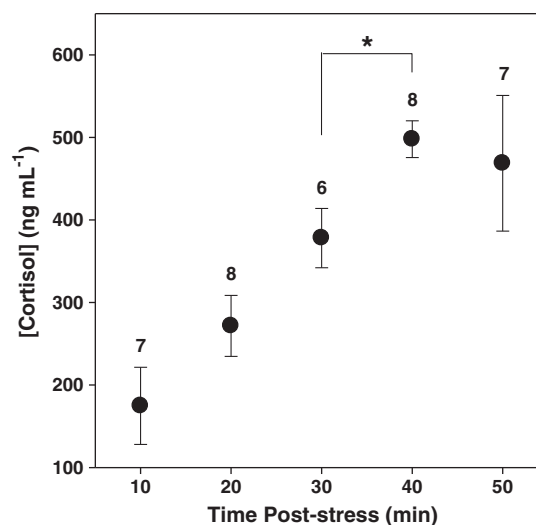


Fig. 1. Plasma cortisol concentrations measured 10–50 min following exposure to a standardized acute stressor (3 min of air exposure) in bluegill sunfish (*Lepomis macrochirus*). Values are means  $\pm 1$  SEM. An asterisk (\*) indicates a significant difference between the indicated points (Mann–Whitney U;  $p = 0.014$ ). Sample sizes indicated above each point.

**Table 1**

Differences in physiological and condition metrics between male and female bluegill sunfish (*Lepomis macrochirus*) were sampled following three exposures to a standardized stressor. Indicated variables were quantified following each stress exposure and all other only upon termination of the experimental period. P-values represent differences between sexes (Student's *t* test). Bold text is used to indicate statistically significant differences and grand mean is presented for those variables not significantly different between sexes.

	Males (Mean ± SEM)	Females (Mean ± SEM)	P-value
Stress-induced cortisol (ng mL <sup>-1</sup> ) <sup>a</sup>	260.5 ± 34.7	545.6 ± 58.4	< <b>0.0001</b>
Stress-induced glucose (mmol L <sup>-1</sup> ) <sup>a</sup>	4.6 ± 0.4	6.0 ± 0.4	<b>0.014</b>
Cortisol variability <sup>b</sup>	163.9 ± 29.3	304.9 ± 80.9	0.06
Hepato-somatic index	1.3 ± 0.06	1.6 ± 0.07	0.08
Eviscerated mass (g)	115.39 ± 23.8	87.27 ± 19.0	< <b>0.0001</b>
Total body mass (g)	134.1 ± 29.7	104.9 ± 29.7	< <b>0.0001</b>
	Grand mean ± SEM		P-value
Condition factor	1.7 ± .02		0.60
Splenic-somatic index	0.1 ± .02		0.94
Plasma protein (mg dL <sup>-1</sup> ) <sup>a</sup>	54.9 ± 1.1		0.16
Haematocrit <sup>a</sup>	31.9 ± 1.1		0.46
Health assessment index	42.1 ± 2.3		0.51

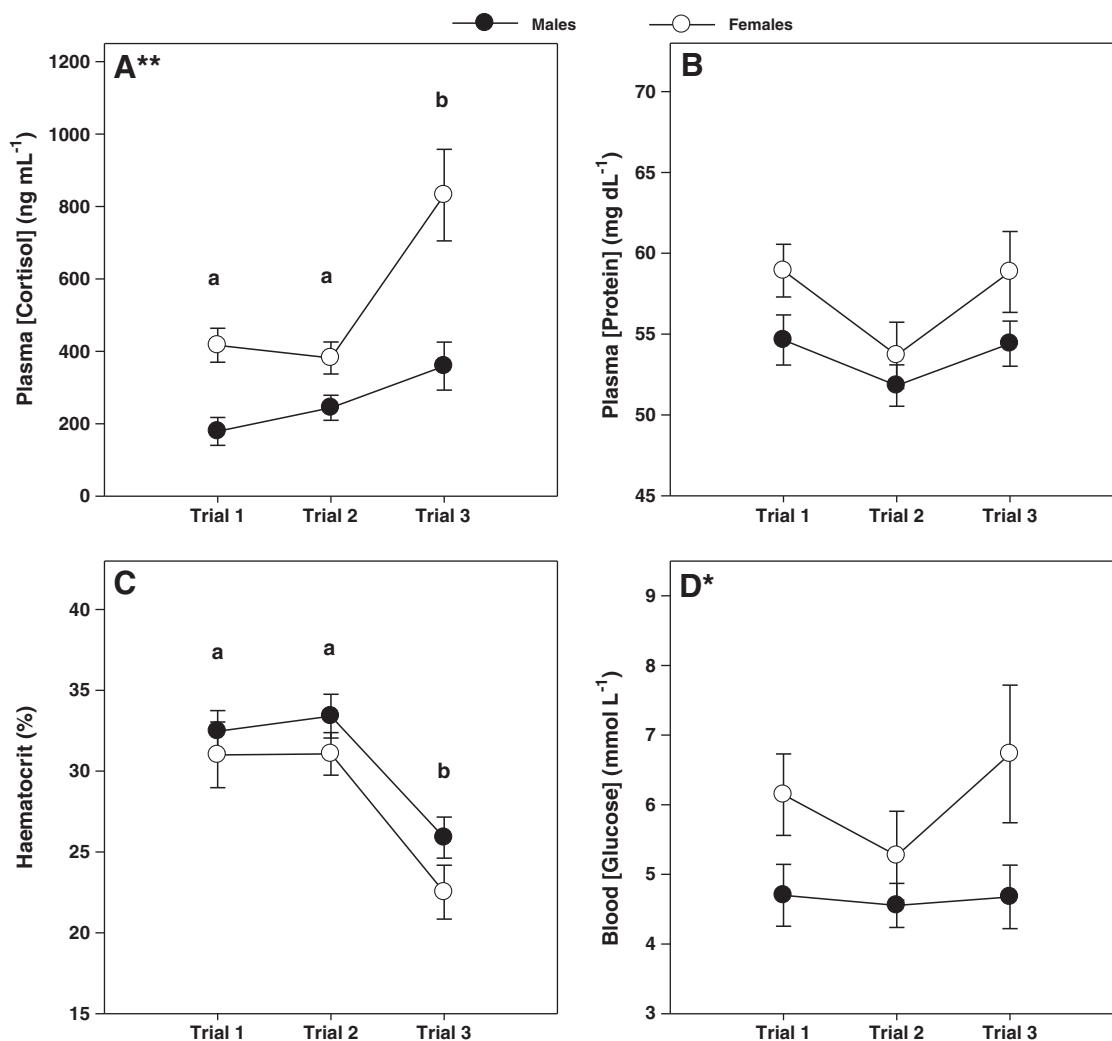
<sup>a</sup> Mean from all three sampling periods for each fish.

<sup>b</sup> Standard deviation of stress-induced cortisol for each individual fish across three sampling periods.

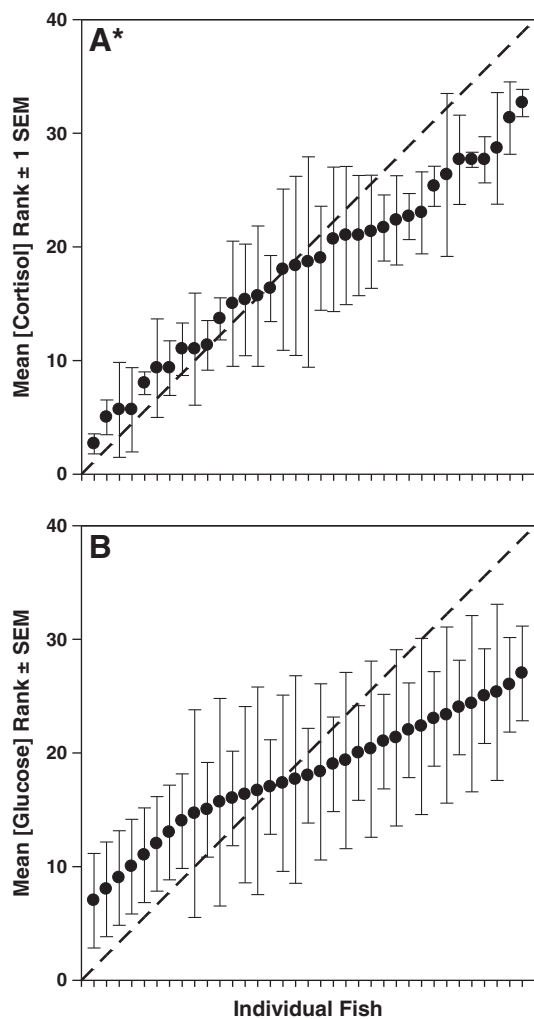
25.1,  $p < 0.0001$ , respectively; Fig. 2). Planned contrasts revealed that in the third trial stress-induced cortisol increased but haematocrit decreased. Glucose and plasma protein did not change across sampling periods ( $p$ 's > 0.3 in both cases; Fig. 2). For stress-induced cortisol, there was a significant interaction effect between sampling period and sex (MD ANOVA:  $F_{(2, 66)} = 5.2$ ,  $p = 0.008$ ). Sexes responded to repeated stress differently; males showed a steady increase where females remained consistent until the third trial (Fig. 2). Regardless of this sampling effect, repeatability analyses according to Lessells and Boag (1987) determined that ranked stress-induced cortisol concentrations were consistent among individuals across all trials (ANOVA:  $F_{(34, 70)} = 3.2$ ,  $p < 0.001$ ; Fig. 3) with a calculated repeatability statistic of  $r = 0.432$ . This was not true of stress-induced glucose rankings (ANOVA:  $F_{(34, 70)} = 0.7$ ,  $p = 0.8$ ; Fig. 3). Although stress-induced cortisol was repeatable, there was considerable intra-individual variation which was explained by both *K* and eviscerated weight (multiple regression:  $F_{3, 31} = 11.6$ ,  $R^2 = 0.5$ ,  $p < 0.0001$ ; Table 2). Smaller fish with a reduced condition factor had greater intra-individual variability in stress-induced cortisol concentrations.

**4. Discussion**

We tested for the repeatability of stress-induced cortisol concentrations in bluegill sunfish. A significant repeatability statistic



**Fig. 2.** Change in mean A) stress-induced cortisol concentrations (ng mL<sup>-1</sup>), B) plasma protein (mg dL<sup>-1</sup>), C) haematocrit (%) and D) stress-induced glucose concentrations (mmol L<sup>-1</sup>) between sampling periods for both sexes of bluegill sunfish (*Lepomis macrochirus*) exposed to a standard stressor of air emersion on three separate occasions (n = 35). Significant differences across sampling groups are denoted by lower case letters, a double asterisk (\*\*) denotes a significant main effect of sex as well as a significant interaction effect and a single asterisk (\*) denotes a significant effect of sex only (mixed design ANOVA:  $p < 0.05$ ). Values are means + 1 SEM.



**Fig. 3.** Repeatability of ranked A) stress-induced cortisol concentrations ( $\text{ng mL}^{-1}$ ) and B) stress-induced glucose concentrations for bluegill sunfish (*Lepomis macrochirus*) sampled on three occasions following exposure to a standardized stressor of 3 min of air exposure ( $n=35$ ). Each point represents mean rank  $\pm 1$  SEM for an individual fish, ordered on x-axis by mean rank. The dotted line represents perfect repeatability where every fish has identical ranks across all trials (Romero and Reed, 2008). An asterisk (\*) represents significant repeatability overall. However, fish with greater error have a low consistency of measured values and fish with very little error have a highly consistent response to acute stress across the three trials.

confirmed consistency of stress-induced cortisol within an individual (Lessells and Boag, 1987) and was comparable to that of studies in other taxa (Romero and Reed, 2008; Wada et al., 2008; Cockrem et al., 2009). However, there was a considerable degree of intra-individual variation in stress-induced cortisol concentrations or 'cortisol variability' and smaller fish in relatively poor condition (K) exhibited greater cortisol variability.

#### 4.1. Condition and stress-induced cortisol variability

The observed negative relationship between condition and cortisol variability implies the existence of feedback between the ability to respond to stress and current condition. Individual condition is a function of the long-term integration of many intrinsic and extrinsic factors and our results show that HPI reactivity also plays a role. Findings allow for several explanations. Most intuitively, that poor condition alters the normal functioning of the HPI-axis resulting in erratic and irregular reactivity. Indeed, there is a strong association between condition and stress physiology across taxa, including fish (Barton et al., 1987; Barton and Iwama, 1991). Interacting stressors in a short

**Table 2**

Results of backward stepwise multiple regression ( $p$  to enter  $<0.05$   $p$  to remove  $>0.1$ ) testing the effects of physiological and condition parameters on 1) intra-individual variability in responsiveness and 2) stress responsiveness in bluegill sunfish (*Lepomis macrochirus*) repeatedly sampled following exposure to a standardized acute stressor. Variables are listed in the order they were dropped and main effects retained in the final model are indicated in bold. Non-significant results presented are the  $p$ -values just before removal from the model.

Predictor variable <sup>a</sup>	Dependent variable: cortisol variability <sup>b</sup>		
	B	P-value	F
HSI	277.24	0.54	4.26
Stress-induced cortisol <sup>c</sup>	−53.01	0.59	4.81
Sex	−66.10	0.35	5.52
Stress-induced glucose <sup>c</sup>	−167.41	0.36	6.20
HAI	211.85	0.32	7.11
Haematocrit	380.42	0.25	8.33
Plasma protein	−928.89	0.10	9.94
SSI	−192.26	0.07	<b>11.62</b>
Eviscerated weight	<b>−642.43</b>	<b>0.04</b>	
Condition factor	<b>−2675.79</b>	<b>0.01</b>	

Model  $R^2 = 0.53$ ,  $F_{1,30} = 11.65$ ,  $p < 0.0001$ .

<sup>a</sup> All variables except sex (0 = males, 1 = females) are log-transformed.

<sup>b</sup> Standard deviation of stress-induced cortisol for each individual fish across three sampling periods.

<sup>c</sup> Values from the first trial were used to eliminate sampling effects seen in subsequent measurements.

amount of time or repeated stress are known to alter HPI/HPA axis reactivity and do result in unpredictable individual variability in GC responsiveness (Schreck, 2000; Romero, 2004). This unpredictability of responses to acute stress may be exemplified with poor condition. However, an increase in stress-induced cortisol and a decrease in haematocrit in the third trial implies an effect of the experimental protocol (i.e. sampling and holding) on the condition of the study specimens and a lack of physiological recovery between trials. The amount of blood drawn was the minimum amount required to obtain desired measurements and thus did not vary with size. The decrease in haematocrit indicates an effect of repeated blood sampling on body fluid dynamics; one that would be pronounced in smaller fish. It is quite possible that the smaller, poor condition fish were affected more severely by experimental conditions making the effects of repeated stress cumulative, subsequently increasing within-individual variability. On the other hand, larger and healthier individuals were able to maintain stress-induced cortisol levels within a normal range.

Stress-induced glucose did not show overall repeatability and there is considerably less information on the hyperglycaemic stress response. As glucose reflects the metabolic response of the fish and is affected by diet content and blood sugar concentration (Barton et al., 1988), being held without any food resources could differentially affect their abilities to launch a glucose response. The time-course validation was also conducted to determine the peak time for cortisol and thus may not represent the peak time for glucose. Although glucose is a reliable indicator of stress in fish (Silbergeld, 1974), it seems to not be a consistent trait within an individual and thus should not be used as a correlate or predictor of evolutionary and ecological traits in fish, as is cortisol.

#### 4.2. Study limitations

Given the small size of our study specimens, we chose to assess absolute stress-induced cortisol concentrations rather than the overall response (change from baseline to stress-induced concentrations) for animal welfare reasons. However, an understanding the biology of absolute cortisol concentrations is equally important as obtaining two samples is not always possible, especially when working with wild populations. If collection methods don't allow for immediate sampling or capture multiple individuals at once, it is impossible to accurately quantify baseline condition. Additionally, as in this case, some

study specimens are too small to ethically sample both pre- and post-stress physiological state. Although a recent review has actually argued that stress-induced GC concentrations may provide more accurate data interpretation than the overall response (Romero, 2004), the inability to quantify baseline condition introduces limitations in data interpretation.

Study fish were exposed to multiple stressors simultaneously and results do show an effect of holding and/or repeated sampling (i.e. decreased haematocrit and increased stress-induced cortisol in the third trial). Changes in stress-induced cortisol throughout the experimental period could be attributed to the cumulative effects of exposure to repeated acute stressors, the increasing intensity of stressors relating to holding throughout the experimental period, and/or hyperreactivity due to effects of chronic stress. This effect is potentially more pronounced in smaller fish. Furthermore, captive holding coupled with repeated sampling in this experiment could have raised baseline cortisol concentrations. Responses to multiple simultaneous, or repeated stressors are poorly understood (Schreck, 2000). Following exposure to repeated stress, fish have been found to both accumulate (Barton et al., 1986; Maule et al., 1996) and attenuate (Pickering and Stewart, 1984; Jentoft et al., 2005) their response. Without a measure of baseline cortisol, we are unable to clearly identify these patterns or the impact of experimental procedures. Owing to elevated baselines, the stress response could have actually decreased in this study, while stress-induced concentrations rose.

#### 4.3. Implications of results

We proposed several explanations for the presented findings of condition and size as predictors of intra-individual variability in stress-induced cortisol concentrations. Poor condition could alter the regular functioning of the HPI axis or that fish in poor condition respond more severely to the detrimental effects of holding and repeated sampling and thus accumulate their responses over time. Findings are most likely attributed to a combination of the aforementioned explanations. Given a poor understanding of repeatability of cortisol concentrations in wild fish, caution is suggested in assuming a measured value is truly representative of that individual, especially if in a deteriorated condition. Research assessing correlates of individual GC responsiveness, especially in degraded environments or in populations where fish may be in poor condition, should ideally sample each individual more than once. Additionally, the non-repeatability of stress-induced glucose indicates that it should not be used as a sole indicator of stress in fish. Capturing the same individual multiple times is often difficult but results of this study highlight the importance of considering individual condition and size together with GC concentrations measured from a single blood sample

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