



# Seasonal variation in baseline and maximum whole-body glucocorticoid concentrations in a small-bodied stream fish independent of habitat quality



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## ABSTRACT

Alterations to natural habitats are becoming more common due to changes in anthropogenic land use. As such, there is increasing interest in determining how wild animals adapt and respond to environmental stressors. The glucocorticoid (GC) stress response enables animals to react appropriately to environmental challenges but can be affected by many factors, two of which are habitat quality and time of year (i.e., season). This study tested whether baseline and maximum (stress-induced) whole-body cortisol concentrations varied in relation to habitat quality and season using wild central mudminnows (*Umbra limi*) collected from two connected streams differing in habitat quality in each of four seasons. Overall, baseline and maximum cortisol levels did not differ significantly between the two systems but there was evidence of a seasonal effect. Baseline cortisol levels in the fall and summer were significantly ( $P < 0.01$ ) lower than those in winter and spring and maximum cortisol levels in the summer were significantly lower ( $P < 0.01$ ) than those in the spring. Inconsistent with the prevailing paradigm, our results indicate that habitat quality does not always influence baseline GCs or the stress response. In contrast, baseline and maximum GCs in this species do vary seasonally. As such, seasonality should be considered in the interpretation of stress response data especially when using small-bodied stream fish as biological indicators.

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## 1. Introduction

Human activities, past and present, have led to dramatic changes in the biosphere (Vitousek et al., 1997). As the world's population continues to grow and human development accelerates, natural habitats are becoming severely altered. Given the direct relationship between habitat quality and organismal health and condition (Huey, 1991), degraded habitats can result in alterations to organismal physiology (Wingfield, 2005), which has the potential to influence population-level processes (Calow and Forbes, 1998; Ricklefs and Wikelski, 2002; Fefferman and Romero, 2013). Recent studies have begun to unravel some of the specific mechanisms by which changes in habitat quality influence resident biota (Wikelski and Cooke, 2006; Cooke et al., 2013). Evaluating individual stress physiology is one way to quantify the effects of human activities, such as habitat degradation on vertebrates (Homyack, 2010; Baker et al., 2013), thus serving as a useful indicator of ecosystem health (Dale and Beyeler, 2001).

Glucocorticoids (GCs) are found in all vertebrates and changes in GC levels play an important role in responding to and overcoming environmental challenges (Sapolsky et al., 2000). GCs are secreted as a result of activation of the hypothalamic–pituitary–adrenal (HPA) axis in reptiles, birds and mammals, and the hypothalamic–pituitary–interrenal (HPI) axis in fishes and amphibians (Bonga, 1997; Reeder and Kramger, 2005). Corticosterone (or cortisol for fish) is the primary GC stress hormone in vertebrates. This axis is activated when an organism experiences an actual or perceived stressor in its environment (Sapolsky et al., 2000). The acute stress response is widely considered to be beneficial as it is seen across all vertebrates and assists in reacting appropriately to potentially lethal encounters (Wingfield et al., 1998; Breuner et al., 2008). However, it is also accepted that long-term elevation of GCs by chronic stress can be severely detrimental to the health, reproduction and survival of an organism (Breuner et al., 2008). Baseline GC levels regulate basic survival needs (e.g., feeding behaviour, locomotor activity and metabolism; Landys et al., 2006) and maximum GC levels are those that can be measured following the physiological response to a challenge or acute stressor (Sapolsky et al., 2000). Baseline samples are collected immediately following capture (typically within 3 min; Romero and Reed, 2005) and then a stress-induced value is measured some period

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thereafter following exposure to a stressor (e.g., timed handling stressor or air stressor in fish) corresponding to when the GC response is maximal. It is generally considered that lower baseline GC levels indicate an individual or population that is in better condition or exposed to less stress than those with higher baseline GC levels (Bonier et al., 2009a; Busch and Hayward, 2009). As mentioned previously, an acute stress response is beneficial to an organism, though a response that elicits a quick increase of GCs followed by a rapid decrease back to baseline GC levels would intuitively serve the organism better. Such a response would allow animals to respond appropriately to a threat but return to normal functioning quickly, thus avoiding the consequences of chronic stress (Breuner et al., 2008; Angelier and Wingfield, 2013).

Researchers over the past several decades have explored the effects of habitat quality on baseline and stress-induced GCs, particularly among mammals, birds, and herpetofauna. However, the results have been inconclusive and indicate context specificity. In spotted salamanders (*Ambystoma maculatum*), for example, the relationship between stress physiology and habitat quality is sex-specific (Newcomb Homan et al., 2003). Hopkin and DuRant (2011) evaluated baseline and maximum GCs of eastern hellbenders (*Cryptobranchus alleganiensis*) from two stream reaches with differing habitat quality and found no habitat-related differences in GC levels. In the small rodent, the degu (*Octodon degus*), habitats with good cover quality, low ectoparasite loads, and increased food availability were associated with lower baseline and maximum GCs (Bauer et al., 2013). Marra and Holberton (1998) studied baseline and maximal GCs in American redstarts (*Setophaga ruticilla*) from two different habitat types and identified seasonal differences in GCs. Some studies have found no relationship between habitat and GCs, some have found seasonal differences, and in some cases sex differences have been observed. Clearly, interpreting GC concentrations in wild animals is a complex process (Johnstone et al., 2012; Dantzer et al., 2014; Crossin et al., 2015). Interestingly, there are relatively few studies that have examined the influence of habitat quality on wild fish, nor done so across seasons.

The objective of this study was to determine how habitat quality and season affected baseline and maximal (i.e., stress-induced) GC levels in a small-bodied freshwater fish. The model species in this study was the central mudminnow (*Umbra limi*) collected from Watts Creek and Kizell Municipal Drain in Kanata, Ontario, Canada. Central mudminnows (family: Umbridae) are commonly found in freshwater streams, lakes, and ponds of central and eastern North America (Scott and Crossman, 1973). Central mudminnows are typically found in areas with low current and high vegetative cover (Peckham and Dineen, 1957; Martin-Bergmann and Gee, 1985). The two sampling locations in this study join at a confluence and sampling was done 150 m upstream of this confluence in both streams. Previous studies in this area demonstrated that this species travels very little between the two adjoining streams (Bliss et al., 2015). This particular combination of movement and location permitted testing of how habitat quality plays a role in subsequent responses to a standardized stressor while controlling for factors such as weather events, temperature, etc. In this context Kizell Drain was considered to be the more degraded system due to lower levels of sinuosity, habitat complexity, cover, and types of sediment (Goldstein and Meador, 2005; Walsh et al., 2005; Bliss et al., 2015). Although recent advances in techniques allow collection of small sample volumes to estimate plasma GC levels (Sheriff et al., 2011), it is not possible to obtain a sufficiently large blood sample volume for a cortisol assay from small organisms such as the central mudminnow, so a whole-body cortisol measure was conducted (Feist et al., 1990; de Jesus et al., 1991). Given that multiple samples (i.e., baseline and maximum) could not be collected from the same individual, it is not possible to measure individual responsiveness (i.e., the difference between maximum and baseline values) but much information can be obtained from the available whole-body samples.

This work is based on the expectation that organisms in degraded habitats, presumably in poorer condition, will display increased baseline

GC levels (Bonier et al., 2009a) and a depressed response when exposed to a standardized stressor (i.e., Hontela et al., 1992; Norris et al., 1999). This experiment tested the hypothesis that the central mudminnow population collected from Kizell Drain, a more disturbed stream (Bliss et al., 2015), would display higher baseline GC levels and lower maximum GC levels than the population collected from Watts Creek, a relatively less disturbed stream. Moreover, we tested the hypothesis that these GC responses would vary across seasons given the manifold effects of water temperature on fish (Fry, 1947) and the strong influence of the reproductive period on GCs and GC response (Wingfield and Sapolsky, 2003). Specifically, we predicted that winter would be among the most challenging periods (i.e., higher baseline levels). We also predicted that during the reproductive period baseline GCs would be elevated while the stress response (maximum) would be depressed consistent with theory (Wingfield and Sapolsky, 2003).

## 2. Methods

### 2.1. Study area

Watts Creek and Kizell Municipal Drain (45°20'42"N, 75°52'19"W) are located in Kanata, a suburb of Ottawa, Ontario, Canada. Watts Creek is a tributary of the Ottawa River and collects stormwater from surrounding residential areas, including the aforementioned Kizell Drain. All collections took place at minimum 150 m upstream from the confluence of Watts Creek and Kizell Drain to avoid any possible fish movement between sites. Kizell Drain is narrower, shallower, more channelized, and shows lower habitat complexity and cover than Watts Creek (Bliss et al., 2015). For these reasons, as in previous studies (e.g., Bliss et al., 2015), Kizell is considered a more degraded stream when compared to Watts Creek.

Fish for this study were collected on October 24 (fall), December 2 (winter) of 2013, and May 20 (spring) and July 14 (summer) of 2014. Twenty fish were sampled as encountered during electrofishing (backpack shocking, Model 12, Smith-Root, Vancouver, WA, USA) from both Kizell Drain and Watts Creek except for the winter sampling for which only 19 fish were sampled from both Kizell and Watts. The electrofishing crew worked upstream and ceased shocking upon netting of a central mudminnow greater than 35 mm. Shocking commenced again at least 2 m upstream of the last capture location to reduce the likelihood of sampling fish that had already experienced a shock. For both Kizell Drain and Watts Creek, 10 fish were stunned using a cerebral percussion immediately after capture and stored in liquid nitrogen within 3 min (representing baseline cortisol levels; Romero and Reed, 2005). Meanwhile, the other 10 sampled fish were exposed to a 3 min standardized air stressor in a dampened bucket (as per O'Connor et al., 2011) followed by 27 min in ~2 L of water. At 30 min, these fish were stunned as above and stored in liquid nitrogen (representing maximum cortisol). The 30 min time period was selected as it appears to be the most typical period at which maximum values are attained in freshwater temperate fish (reviewed in Barton, 2002). As fish were encountered by electrofishing, they were alternatively assigned to either baseline or maximum groups. All samples were stored in a -80 °C freezer until analysis could be done.

### 2.2. Cortisol analysis

Whole frozen fish were crushed using a mortar and pestle and liquid nitrogen to keep the samples frozen resulting in a powdery extract. Total lipid extraction was performed using the Folch method (Folch et al., 1957) optimized for central mudminnow. First, 30 mL of Folch solution (2 chloroform:1 methanol v/v) was added and the mixture homogenized (Polytron homogenizer; Kinematica, Luzern, Switzerland) for 2 min. After sitting for 20 min, 10 mL KCl with 5 mM EDTA was added and the extract allowed to settle for another 20 min. The lipid fraction was removed using a pipette and transferred to a test tube. The

solvent was then evaporated under a liquid nitrogen stream in a fumehood. The lipids were re-suspended in 30  $\mu\text{L}$  of ethylene glycol monomethyl ether (EGME) and stored until the cortisol assay was performed. When all lipid extractions were completed, whole-body cortisol concentration was further measured using a commercial radioimmunoassay kit (RIA, MP Biomedicals, USA) and subsequently read with a 2480 WIZARD automatic gamma counter (PerkinElmer, Waltham, Massachusetts, USA). The intra- and inter-assay coefficients of variation were 8% and 10%, respectively.

### 2.3. Statistical analysis

A two-way analysis of variance (ANOVA) was used to test if baseline cortisol and maximum cortisol contents differed significantly between fish collected from Watts Creek and Kizell Drain over winter, spring, summer, and fall. Baseline cortisol and maximum cortisol represented the dependent variables and season and collection site (i.e., Watts Creek versus Kizell Drain) were the two independent variables. A Shapiro–Wilk's test was used to ensure normality assumptions were satisfied prior to performing the ANOVA. When not met, square-root and log transformations were utilized to normalize the data. Statistical analyses were performed using R version 3.1.2. When significant, a Tukey HSD test was used to determine which factors differed. Significance levels were set *a priori* as 0.05 for all tests performed and results are presented as mean  $\pm$  standard error.

## 3. Results

Central mudminnow collected from Watts Creek and Kizell Drain did not differ statistically in mass, total length, or condition factor (Tables 1 and 2), although all three measurements did differ significantly ( $P < 0.05$ ) across seasons in a predictable manner (i.e., fish had the longest total length in the summer and smallest in spring) and a significant ( $P < 0.05$ ) interaction effect between site and season was present for fish mass. Baseline cortisol levels were similar between fish collected from Watts Creek and Kizell Drain (Tables 1 and 2; Fig. 1) but differed significantly across seasons (Tables 1 and 2; Fig. 1) with no significant interaction effect. Baseline cortisol concentrations sampled in the winter and spring were significantly ( $P < 0.01$ ) greater than the fall and summer samples (Tables 1 and 2; Fig. 1). Similarly, maximum cortisol levels did not differ between fish collected from Watts Creek and Kizell Drain (Tables 1 and 2; Fig. 2), but differed significantly ( $P < 0.05$ ) across seasons (Tables 1 and 2; Fig. 2). More specifically, maximum cortisol concentrations in the summer samples were significantly ( $P < 0.01$ ) lower than concentrations observed in the spring samples (Fig. 2).

**Table 1**

Results of a two-way ANOVA with capture site and season as the independent variables and weight, baseline cortisol, and maximum cortisol as the measured dependent variables. Comparing pre- and post-stress response of central mudminnows (*Umbra limi*) collected from Watts Creek and Kizell Drain following a standardized air stressor. Significant values ( $P < 0.05$ ) are shown in bold-italics.

Variable	Capture site			Season			Season $\times$ Capture site		
	F	df	P	F	df	P	F	df	P
Mass (g)	0.269	1	0.605	21.489	3	<b>&lt;0.0001</b>	2.810	3	<b>0.042</b>
Total length (mm)	0.441	1	0.508	13.848	3	<b>&lt;0.0001</b>	0.560	3	0.642
Condition factor (g/cm <sup>3</sup> )	0.240	1	0.625	3.330	3	<b>0.0213</b>	2.361	3	0.0737
Baseline cortisol (ng/g)	0.751	1	0.389	8.434	3	<b>&lt;0.0001</b>	0.960	3	0.416
Maximum cortisol (ng/g)	1.568	1	0.215	4.169	3	<b>0.009</b>	1.614	3	0.194

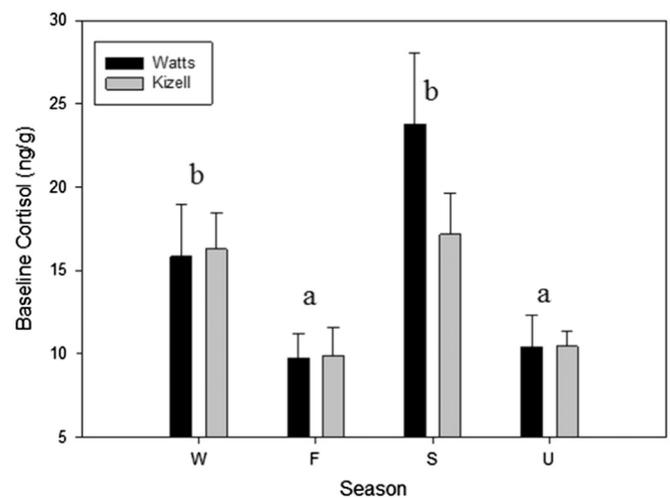
**Table 2**

Summary of biological variables (sample size in parentheses) for central mudminnow collected from two stream reaches (Watts Creek and Kizell Drain) across four seasons (W, winter; F, fall; S, spring; U, summer).

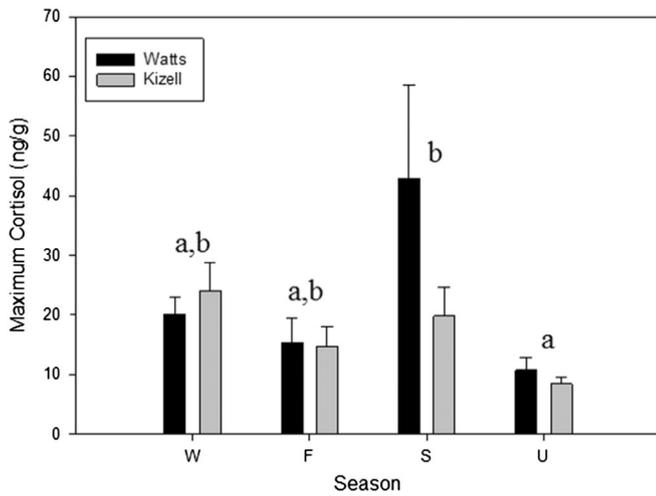
Stream and season	Fish mass (g)	Total length (mm)	Condition factor (g/cm <sup>3</sup> )	Baseline cortisol (ng/g)	Maximum cortisol (ng/g)
<i>Watts</i>					
W (N)	19	19	19	10	9
Mean	7.04	67.5	1.42	15.8	20.2
SE	1.37	3.4	0.22	3.1	2.7
F (N)	20	20	20	10	10
Mean	4.62	63.3	1.68	9.7	15.4
SE	1.56	1.9	0.53	1.4	4.1
S (N)	20	20	20	10	10
Mean	2.43	52.8	1.26	23.7	42.8
SE	0.76	3.3	0.04	4.3	15.6
U (N)	20	20	20	10	10
Mean	5.14	77.4	1.19	10.4	10.7
SE	0.76	7.2	0.07	1.9	2.0
<i>Kizell</i>					
W (N)	19	19	19	10	9
Mean	3.01	63.9	1.13	16.2	24.1
SE	0.21	1.2	0.04	2.2	4.6
F (N)	20	20	20	10	10
Mean	2.95	63.7	1.09	9.8	14.6
SE	0.26	1.5	0.02	1.6	3.3
S (N)	20	20	20	10	10
Mean	2.23	57.1	1.15	17.1	19.7
SE	0.19	1.7	0.03	2.4	4.8
U (N)	20	20	20	10	10
Mean	7.10	83.4	1.36	10.4	8.4
SE	0.66	6.5	0.08	0.9	1.1

## 4. Discussion

This tested the hypothesis that habitat quality influenced baseline and maximum cortisol levels in central mudminnow. Secondly, we tested the hypothesis that baseline and maximum cortisol levels varied over the four seasons. Central mudminnows were sampled four times over the course of a year from Kizell Drain, a more disturbed habitat, and from Watts Creek, a more pristine habitat. As mentioned previously, due to human activity in the area, Kizell Drain displays lower diversity of sediment types and lower levels of sinuosity, habitat complexity, and overhead cover resulting in it being considered more degraded than Watts Creek (Bliss et al., 2015). It was predicted that over the four seasons, organisms in the degraded habitat would presumably



**Fig. 1.** Baseline cortisol (ng/g) for central mudminnows collected from Watts Creek and Kizell Drain over four seasons (winter, W; fall, F; spring, S; summer, U). Dissimilar letters indicate significant differences ( $P < 0.01$ ) among seasons. Data presented are means  $\pm$  1 standard error;  $n = 10$  for Watts and Kizell in each of the seasons.



**Fig. 2.** Maximum cortisol (ng/g) for central mudminnow collected from Watts Creek and Kizell Drain over four seasons (winter, W; fall, F; spring, S; summer, U). Dissimilar letters indicate significant differences ( $P < 0.01$ ) among seasons. Data presented are means  $\pm$  1 standard error;  $n = 10$  for Watts and Kizell in fall spring and summer,  $n = 9$  for Watts and Kizell in winter.

be in poorer condition and therefore show increased baseline cortisol levels (Bonier et al., 2009a) in addition to lower maximum cortisol levels following a standardized air stressor (i.e., Hontela et al., 1992; Norris et al., 1999). Overall, the sampling site did not have a significant effect on either baseline or maximum cortisol levels. However, there were significant differences observed across seasons during which sampling occurred. Winter and spring baseline cortisol concentrations were significantly higher than the summer and fall concentrations. Spring maximum cortisol concentrations were significantly higher than the summer concentrations. For both baseline and maximum cortisol concentrations, the highest values were sampled during the spring while the lowest values were sampled during the fall and summer. The body mass of fish also varied seasonally although it is unclear if body size has any direct functional relationship to GCs.

There were no significant differences for either the baseline or maximum GC levels between Watts Creek and Kizell Drain in any of the four seasons suggesting that under these circumstances, habitat quality is unrelated to whole-body cortisol concentrations. Although few studies have examined the effect of habitat quality on the GC response in fish, freshwater fish are among the species most affected by habitat degradation (Jeffrey et al., 2015). Similar to our findings, Hopkin and DuRant (2011) reported that eastern hellbenders from disturbed and non-disturbed sites displayed no differences in baseline or maximum GC levels or in GC responsiveness in relation to their different habitats. Creek chub (*Semotilus atromaculatus*) demonstrated an attenuated stress response when collected from an agricultural stream (i.e., disturbed) relative to a more pristine system following thermal and hypoxic challenges (Blevins, 2012; Blevins et al., 2014). However, in another study, control and cortisol-injected creek chub exhibited similar responses independent of habitat quality (Nagrodski et al., 2013). Largemouth bass (*Micropterus salmoides*) collected from streams with lower percentage of wetland areas (i.e., degraded habitat) had increased baseline GC levels and decreased GC responsiveness when compared to those from areas with higher wetland percentage (King, 2014). It is clear that the effect of habitat quality on the GC stress response is context dependent and can vary not only between species but also with different measures of habitat quality.

Previous work on Watts Creek and Kizell Drain (i.e., Bliss et al., 2015) indicated that central mudminnow travels between the two reaches but does so rarely. As such, sampling was not conducted in the vicinity of the confluence; however, we cannot exclude the possibility that there was some movement that could influence our results. Watts Creek is

more sinuous and has higher habitat complexity, substrate diversity, and in-stream cover than Kizell Drain resulting in Kizell Drain being designated as a degraded habitat (Goldstein and Meador, 2005; Walsh et al., 2005; Bliss et al., 2015). Though it seems clear that Kizell Drain has been more anthropogenically influenced, it is difficult to determine whether an individual or species perceives the habitat to be unsuitable. In fact, in the Watts Creek watershed, higher numbers of central mudminnow are found in Kizell Drain compared to Watts Creek (Bliss et al., 2015). In light of the results, it is possible that Kizell Drain provides a suitable habitat for the central mudminnow, particularly in the winter when they are found assembled in deeper pools (Martin-Bergmann and Gee, 1985). Along Kizell Drain there are pools that would meet the criteria for central mudminnow overwintering requirements (Bliss et al., 2015) and therefore Kizell Drain may be as desirable a habitat as Watts Creek.

Though no differences were observed between sites, there was a significant difference in both baseline and maximum GC levels among seasons. Baseline and maximum GC levels were highest in the spring, corresponding with the end of the breeding season in the central mudminnow (Peckham and Dineen, 1957). This study did not examine individual gonad development as the whole-body was used for cortisol analysis, but breeding in central mudminnows occurs in the early spring (Peckham and Dineen, 1957). Wingfield et al. (1994) also found the highest observed GC response during the breeding season in common redbolls (*Acanthis flammea*). These findings correspond to the cort-adaptation hypothesis which predicts a positive relationship between reproductive success and baseline cort under the pretext that reproduction itself can act as a stressor, resulting in increased GC concentrations (Bonier et al., 2009a, 2009b). There have been few studies in fish examining seasonal changes in the stress response. Wingfield and Grimm (1977) observed maximum plasma GC levels during pre-spawning in plaice (*Pleuronectes platessa*), similar to what we observed in the central mudminnow. Other studies report maximum baseline GC levels occur in the winter for Australian snapper (*Pagrus auratus*; Pankhurst and Sharples, 1992) or show no seasonal related differences in black bream (*Acanthopagrus butcheri*; Haddy and Pankhurst, 1999). More studies are needed to examine changes in baseline, maximum, and GC responsiveness over the seasons to determine if there are any general patterns in teleost fishes and other vertebrates. Knowing how GC concentrations varied seasonally would potentially enable environmental managers to identify seasons when animals might be particularly sensitive to additional stressors. Moreover, it is necessary to attempt to understand the drivers of seasonal patterns in GC concentrations (e.g., environmental conditions, phenology, reproductive state).

High baseline GC levels are frequently attributed to individuals in poor condition (Bonier et al., 2009a). The highest baseline cortisol levels in this study were seen in the winter and spring. The higher baseline and maximum cortisol levels during the spring observed in this study could be due to reproduction acting as a stressor even though it will also increase individual fitness (Bonier et al., 2009b). Though fish were not sexed in this study, this increase in cortisol around the breeding season has been observed to occur in both sexes (e.g., Wingfield et al., 1994). The higher cortisol concentrations observed in the winter could also be in response to the winter being more challenging in terms of available food sources, low temperatures, and restricted movement. Future studies should include the sex of the individuals in order to determine if this significance between seasons differs between males and females. Examining the breeding season for juvenile and reproductive central mudminnow would enable researchers to observe if it is the stress of reproduction that results in higher GC levels during the spring. Although we did not specifically assess the role of temperature, it certainly changed seasonally (i.e., warmest in summer and coldest in winter). In general, how GCs respond under different thermal scenarios is poorly understood such that it is unclear if using a single time period (i.e., 30 min) post stressor is consistently the time to achieve the maximum cortisol response. There is a need for more basic validation

studies (e.g., on how temperature influences time-course of GC responses) to help with experimental design and data interpretation in future studies of GCs in the wild given the diversity of fishes.

With global changes and extreme weather events becoming more frequent (Easterling et al., 2000), it is important to understand what constitutes baseline GC levels as well as a “normal” stress response. Given that our study as well as others (summarized above) have revealed evidence for seasonal variation in baseline and maximum GCs, it is necessary to consider seasonality when designing and interpreting future research projects. Moreover, with opportunity to incorporate individual-level physiological measures as biological indicators in environmental monitoring programs (e.g., Jeffrey et al., 2015), it is necessary to first understand natural variation in GC concentrations across seasons and other contexts.

### Compliance with ethical standards

The authors confirm that the work conforms to ethical standards.

### Conflict of interest

The authors declare that they have no conflict of interest.

### Ethical approval

All experiments carried out were in accordance with the institutional and national guidelines in Canada and were approved by the Carleton University Animal Care Committee under the auspices of the Canadian Council on Animal Care.

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