

Diel patterns of baseline glucocorticoids and stress responsiveness in a teleost fish (bluegill, *Lepomis macrochirus*)

A. Cousineau, J.D. Midwood, K. Stamplecoskie, G. King, C.D. Suski, and S.J. Cooke

Abstract: Little is known about whether glucocorticoids (GC) and GC responsiveness vary on a diel basis in the wild, especially for fish. Using bluegill (*Lepomis macrochirus* Rafinesque, 1819) as a model freshwater teleost fish, we tested whether baseline concentration and stress responsiveness of GCs (i.e., plasma glucose and cortisol) varied over a 24 h period. Blood samples from lake-dwelling wild bluegill were obtained across six periods representing a complete circadian cycle to determine GC levels in newly captured fish (i.e., within 3 min of capture; baseline), the maximum value (maximum) 45 min following exposure to a standardized aerial exposure stressor, and determining responsiveness (by subtracting minimum from maximum). Our results revealed that baseline glucose concentration did not vary on a diel basis, whereas baseline cortisol concentration did. Maximum and stress-induced glucose responsiveness varied significantly among several time periods with lowest values recorded at midnight and higher values at mid-day. Maximum and stress-induced cortisol responsiveness were consistent across time periods. Collectively, these data suggest that baseline concentrations and stress-induced values of GCs in a freshwater temperate teleost fish tend to be consistent across diel periods such that there is apparently an absence of strong GC diel patterns.

Key words: glucose, cortisol, bluegill, circadian cycle, stress response, *Lepomis macrochirus*.

Résumé : Les connaissances sur d'éventuelles variations journalières des glucocorticoïdes (GC) et de la réactivité de ces derniers chez les espèces sauvages, plus particulièrement les poissons, sont très limitées. En se servant du crapet arlequin (*Lepomis macrochirus* Rafinesque, 1819) comme modèle de poisson téléostéen d'eau douce, nous avons vérifié si la réactivité de fond et au stress des GC (c.-à-d. glucose et cortisol du plasma) variait sur une période de 24 h. Des échantillons sanguins de crapets arlequin sauvages de lac ont été obtenus pour six moments de la journée représentant un cycle circadien complet, afin de déterminer les teneurs en GC dans des poissons tout juste capturés (c.-à-d. dans les 3 min suivant leur capture; valeur de fond), la valeur maximum 45 min après exposition à un stressor aérien standardisé et la réactivité (en soustrayant la valeur minimum de la valeur maximum). Nos résultats révèlent que les teneurs de fond du glucose ne variaient pas sur une base journalière, contrairement aux teneurs de fond du cortisol. Les réactivités maximum et induite par le stress du glucose variaient de manière significative selon le moment de la journée, les teneurs les plus faibles étant enregistrées à minuit et les plus fortes, au milieu de la journée. Les réactivités maximum et induite par le stress du cortisol étaient uniformes pour les différents moments du jour. Collectivement, ces données donnent à penser que les teneurs de fond et induites par le stress des GC chez un poisson téléostéen d'eau douce de milieu tempéré ont tendance à être uniformes d'un moment de la journée à l'autre, de sorte qu'il ne semble pas y avoir de fortes variations journalières des GC. [Traduit par la Rédaction]

Mots-clés : glucose, cortisol, crapet arlequin, cycle circadien, réaction de stress, *Lepomis macrochirus*.

Introduction

Circadian rhythms originate from the transient nature of the light-dark cycle and can have manifold effects on the physiology, behavior, and ecology of vertebrates (Nader et al. 2010). In vertebrates, the hypothalamic-pituitary-adrenal axis (HPA; or hypothalamic-pituitary-interrenal (HPI) axis in fish) modulates the internal circadian clock of organisms (Aschoff 1979), and plays an important role in the maintenance of homeostasis, particularly in the face of stressors (Sapolsky et al. 2000). When a vertebrate perceives a stressor, a cascade of neurotransmitters from the hypothalamus induces a release of glucocorticoids (GC) into the bloodstream (Harbuz and Lightman 1992) that accumulate over time (Cook et al. 2012). Stressors can be subtle changes including,

but not limited to, reduced food intake, temperature extremes, or lighting, and thus fluctuate daily and cyclically (Romero 2004). To survive, an organism must induce an appropriate physiological response, thus perpetuating this endogenous circadian rhythm (Harbuz and Lightman 1992).

Baseline GC concentrations are notably involved in permissive effects (i.e., priming the body for action; Sapolsky et al. 2000), whereas stress-induced GC activation stimulates certain behaviours (e.g., heart rate and breathing) while inhibiting others (e.g., immune response) to help prepare for future stress (Romero 2004). Together, baseline and stress-induced GC inform researchers about the magnitude of the stress response, and the influences of condition and behaviour of fishes in their natural environment

Received 19 March 2014. Accepted 25 March 2014.

A. Cousineau,* J.D. Midwood,* and K. Stamplecoskie. Fish Ecology and Conservation Physiology Laboratory, Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, ON K1S 5B6, Canada.

G. King and C.D. Suski. Department of Natural Resources and Environmental Sciences, University of Illinois at Urbana-Champaign, 1102 South Goodwin Avenue, Urbana, IL 61801, USA.

S.J. Cooke. Fish Ecology and Conservation Physiology Laboratory, Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, ON K1S 5B6, Canada; Institute of Environmental Science, Carleton University, 1125 Colonel By Drive, Ottawa, ON K1S 5B6, Canada.

Corresponding author: J.D. Midwood (e-mail: midwoodj@gmail.com).

*Co-first authors.

on this HPI axis (Romero 2004; Cook et al. 2012). In general, the GC response has been relatively well studied for a variety of taxa because of its proposed role in an animal's entrained circadian rhythm. However, much less is known about how baseline and stress-induced (maximum) GCs, together with stress responsiveness (i.e., the difference between maximum and baseline concentrations; Romero 2004), vary relative to circadian rhythms, particularly for fish.

As noted above, although seasonal patterns in GCs are reasonably well studied, only five studies have analyzed circadian stress responsiveness on a diel basis using baseline and stress-induced glucocorticoid concentrations (brown trout, *Salmo trutta* L., 1758: Pickering and Pottinger 1983; rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792): Laidley and Leatherland 1988; White-crowned Sparrow, *Zonotrichia leucophrys* (J.R. Forster, 1772): Breuner et al. 1999; Great Tit, *Parus major* L., 1758: Carere et al. 2003; Norway rat, *Rattus norvegicus* (Berkenhout, 1769): Weibel et al. 2002). Baseline and stress-induced magnitudes of GCs facilitate complementary physiological responses, thus, when analyzing the health of fish populations, both levels should be assessed (Romero 2004). Nevertheless, most studies have analyzed the circadian patterns of baseline glucocorticoids or maximum glucocorticoids separately. In general, fish species have their highest plasma GC concentrations occurring at night (brown trout: Pickering and Pottinger 1983; rainbow trout: Laidley and Leatherland 1988; green sturgeon, *Acipenser medirostris* Ayres, 1854: Lankford et al. 2003; Mozambique tilapia, *Oreochromis mossambicus* (Peters, 1852): Nikaido et al. 2010) or during both night and day (Atlantic salmon, *Salmo salar* L., 1758: Thorpe et al. 1987; Ebbesson et al. 2008). Together these studies indicate that interspecific differences in peak GCs exist that are likely related to the light–dark cycle.

Field studies of the diel cortisol response are time-constrained and can be difficult to execute amidst other studies; however, the information gathered can be used to gain insight into the evolutionary and ecological behaviours of wild fish (Cook et al. 2012). None of the previously mentioned studies analyzed diel baseline cortisol rhythms in the field; rather, they assessed circadian stress by manipulating light–dark exposure in a laboratory without a component assessing the impacts that natural environmental fluctuations can have on the diel stress response of an animal (Pickering and Pottinger 1983; Thorpe et al. 1987; Laidley and Leatherland 1988). There is no single study that has examined diel patterns of baseline and stress-induced GCs in a wild vertebrate.

In this study, we use bluegill (*Lepomis macrochirus* Rafinesque, 1819) as a model teleost fish to understand how baseline and stress-induced (i.e., stress responsiveness) GCs vary on a diel basis. Bluegill are a commonly used model species in the study of behaviour due to their accessibility, abundance, and geographical range (Cook et al. 2012). Bluegill have been the subject of a previous study on GC responsiveness (Cook et al. 2012) such that methods for measuring responsiveness have been validated. Characterizing the diel rhythms of baseline and stress-induced GC concentrations would help inform experimental design of studies so they could control for such variation, or, at the very least, enable them to understand how the sensitivity of the HPI axis varies relative to time of sampling (Barton et al. 1986; Davis and Parker 1986).

Materials and methods

Fish capture and experimentation

Over a 2-day period in late July 2012, bluegill were collected via pulsed direct current (PDC) boat-electrofishing gear (2.5GPP Electrofisher System, part No. 01868; Smith-Root, Inc., Vancouver, Washington, USA) from Dow's Lake in Ottawa, Ontario, Canada. Sunrise at this time of year was at approximately 05:30 and sunset was at approximately 20:40. To capture daily variations in glucocorticoid concentrations, sampling events were divided into six 90 min time periods at 4 h intervals. Time periods 00:00–04:00

were sampled on 24 July and the remaining periods were sampled on 26 July. Water temperatures were consistent during the sampling period (approximately 26 °C) and there were no precipitation events. We acknowledge that an ideal design would have sampling conducted over a single 24 h period. Unfortunately, that was not possible due to health and safety regulations and the need to provide team members with adequate rest. To ensure that basal glucocorticoid concentrations reflected each individual's true baseline concentrations during the respective time period, several precautions were taken. First, for each 4 h time period, fish were collected within the first 90 min of that time period. Next, no area in Dow's Lake was sampled more than once per day to ensure that individuals were not repeatedly sampled. All sampled fish had the edge of their caudal fin clipped to prevent resampling over the 2-day sampling period. Moreover, bluegill were the most abundant fish in the system (Walker et al. 2010) such that the likelihood of recapture was exceptionally low. Indeed, we did not recapture a clipped fish during the sampling efforts. Finally, blood samples were collected within 3 min of being stunned by electrofishing gear to ensure accuracy and consistency of the baseline reading (Breuner et al. 1999).

The blood sampling occurred during a 3 min aerial emersion. At the end of that period (and assuming successful blood sampling), fish were placed into individual containers (Cook et al. 2012). To quantify stress responsiveness (Breuner et al. 1999), bluegill were held in isolation for 45 min after the air exposure, ensuring a maximum cortisol response before blood was collected for the poststress condition (45 min is maximal response time for bluegill; Cook et al. 2012). After the stressed blood sample was collected, fish total length (mm) and mass (g) were measured and fish were released.

For both the baseline and stress-induced conditions, approximately 0.3 mL of blood was extracted via caudal puncture using a 1 mL Luer-Lock sodium-heparinized (10 000 USP units/mL; Sandoz Canada Inc., Boucherville, Quebec, Canada) syringe (BD (Becton, Dickinson and Company), Franklin Lakes, New Jersey, USA). Blood was immediately expelled into a 1.5 mL microcentrifuge tube and spun for 3 min at 6000 rev/min (Fisher Scientific Microcentrifuge). After spinning, plasma was removed and evenly divided between three microcentrifuge tubes. All three tubes were then placed in a liquid nitrogen dry shipper and kept at –80 °C until analyzed. A Cortisol ELISA (enzyme-linked immunosorbent assay), previously validated and recommended for fishes (Sink et al. 2008), was used to quantify cortisol titres (kit No. 900-071; Enzo Life Sciences, Farmingdale, New York, USA). Baseline and poststress glucose concentrations were quantified on site using a handheld electronic glucose meter (ACCU-CHEK Compact Plus blood glucose meter; Roche Diagnostics, Basel, Switzerland) validated for use on fish (Cooke et al. 2008).

Statistical analysis

To ensure that bluegill sampled were physically similar, the length of fish (mm) caught in each of the six time intervals (i.e., 00:00, 04:00, 08:00, 12:00, 16:00, and 20:00) were compared using a one-way analysis of variance (ANOVA). As a result of baseline and stress-induced GC concentrations being procured from the same fish within each time period, the mean differences between the baseline and maximum GC's were analyzed using a paired *t* test. Stress responsiveness was calculated by subtracting the baseline from the maximum GC response at each time period. To analyze if concentrations of GC were related to the fish size, a Pearson bivariate correlation was conducted. A one-way ANOVA and, where appropriate, a Tukey's honestly significant difference (HSD) multiple comparison post hoc analysis were used to quantify variation in the baseline, maximum, and GC responsiveness of glucose and cortisol concentrations between times periods (i.e., 00:00, 04:00, 08:00, 12:00, 16:00, and 20:00). The assumption of homoscedasticity was not met for three variables: baseline cortisol concentration,

Table 1. Mean and standard error (SE) for each measurement from bluegill (*Lepomis macrochirus*) assessed during the time intervals 00:00, 04:00, 08:00, 12:00, 16:00, and 20:00.

Time of day	Length (mm)	Glucose concentration		Cortisol concentration	
		Basal (mmol/L)	Maximum (mmol/L)	Basal (ng/mL)	Maximum (ng/mL)
00:00	158±2.7	2.0±0.1	5.4±0.6	3.17±0.74	131.25±20.17
04:00	158±3.6	1.9±0.1	7.8±0.4	6.19±0.89	177.46±10.87
08:00	157±5.4	2.1±0.1	7.7±0.6	8.22±1.96*	174.85±33.60
12:00	152±7.8	2.2±0.1	9.6±0.8*	7.77±1.61*	146.36±16.86
16:00	148±3.6	2.1±0.2	8.8±0.6*	8.25±2.02*	168.24±20.71
20:00	162±4.0	2.0±0.1	9.6±0.9*	6.98±0.52*	160.67±22.90
Overall mean ± SE	156±1.9	2.0±0.0	8.1±0.3	6.65±0.58	159.92±8.89

Note: Values with asterisks are significantly higher than those without asterisks in the same column (ANOVA, $p < 0.05$).

stress-induced cortisol concentration, and change in cortisol concentrations. These variables were square-root-transformed and reanalyzed for homogeneity of variance. Baseline cortisol concentration met the assumption of homoscedasticity after being square-root-transformed and was thus analyzed using an ANOVA. Maximum cortisol concentration and cortisol responsiveness did not meet the assumption and thus were analyzed using a Kruskal–Wallis nonparametric test. All data were analyzed using IBM SPSS Statistics version 20 (IBM Corp., Armonk, New York, USA).

Results

A total of 65 bluegills were sampled at 4 h intervals over the course of a 24 h period, with 9–12 unique fish sampled at each period. The length of the specimens sampled during the experiment did not vary across sampling periods (Table 1; $F_{[5,64]} = 1.201$, $p = 0.320$). There was a significant difference between overall baseline glucose and overall maximum glucose concentrations across all sampling periods, with air exposure and confinement resulting in a 4-fold increase in plasma glucose concentrations (paired t test, $t_{[64]} = -18.714$, $p < 0.001$). Similarly, there was a significant difference between overall baseline cortisol and overall maximum cortisol concentrations, with air exposure and confinement inducing a 26-fold increase in plasma cortisol (paired t test, $t_{[64]} = -17.500$, $p < 0.001$). There was no significant correlation between total length and baseline glucose concentration (Pearson correlation, $p = 0.267$), glucose responsiveness (Pearson correlation, $p = 0.486$), baseline cortisol concentration (Pearson correlation, $p = 0.200$), or cortisol responsiveness (Pearson correlation, $p = 0.426$).

Baseline glucose concentration did not vary significantly across sampling periods (Table 1; $F_{[5,64]} = 0.929$, $p = 0.469$); however, post-stress maximum glucose concentrations did vary significantly among sampling periods (Table 1; $F_{[5,64]} = 5.725$, $p < 0.001$). Specifically, maximum glucose concentration was lower at 00:00 than at 12:00 ($p = 0.001$), 16:00 ($p = 0.010$), and 20:00 ($p < 0.001$). Glucose responsiveness followed a similar pattern in that there were differences across periods (Fig. 1a; $F_{[5,64]} = 5.302$, $p < 0.001$) with glucose responsiveness being lower at 00:00 than at 12:00 ($p = 0.002$), 16:00 ($p = 0.017$), and 20:00 ($p = 0.001$). There were significant differences in baseline cortisol values across sampling periods (Table 1; $F_{[5,64]} = 3.556$, $p = 0.007$) such that baseline cortisol concentration was lower at 00:00 than at 08:00 ($p = 0.014$), 12:00 ($p = 0.032$), 16:00 ($p = 0.020$), and 20:00 ($p = 0.032$). Interestingly, maximal poststress cortisol concentrations (Table 1; Kruskal–Wallis test, $p = 0.481$) and cortisol responsiveness did not vary among sampling periods (Fig. 1b; Kruskal–Wallis test, $p = 0.567$).

Discussion

The present study was conducted to analyze the diel patterns of baseline and stress-induced GCs in bluegill. Such research has not previously been conducted in a single integrated study, or on this particular species of fish. Based on experimental evidence

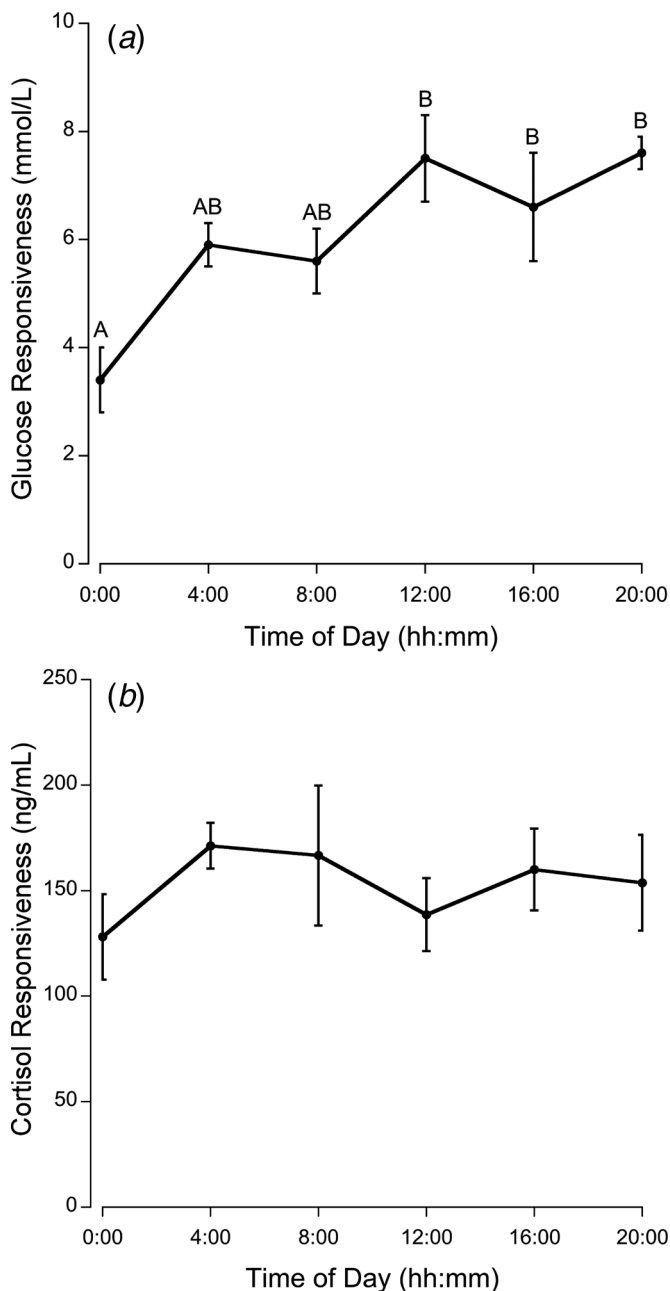
linking teleost fish (e.g., gilthead seabream, *Sparus aurata* L., 1758; López-Olmeda et al. 2009; Mozambique tilapia; Nikaido et al. 2010) with circadian changes in plasma cortisol concentrations, it was hypothesized that there would be variability in the diel GC response of bluegill to a stressor. The results of the present study indicate no presence of a diel pattern in the GC response; however, there is a diel change in the maximum plasma glucose concentration, glucose responsiveness, and baseline cortisol concentration. A diel change in glucose concentration occurs in many species as a result of feeding and activity cycles (Montoya et al. 2010). Bluegill are known to be active feeders who predominantly use their visual sense to locate prey, leading to changes in activity guided by the light–dark cycle (Vinyard and O'Brien 1976); these changes in activity may be responsible for the small changes in GC responses.

The present study found no diel change in baseline glucose concentrations. Baseline GCs are recognized for their contributions to permissive effects of the body (Harbuz and Lightman 1992). Permissive effects may include maintenance of cardiovascular function and normal muscle activation. The counter-regulatory effects of insulin and cortisol help to maintain reserve levels of glucose for permissive effects and spontaneous requisition in case of exposure to an immediate stressor (i.e., prey capture opportunities or predation evasion; Sapolsky et al. 2000; Romero 2004). Thus, no diel pattern in baseline glucose concentrations may reflect the effective homeostatic regulation of circulating glucose by insulin and cortisol.

For the majority of the diel cycle, there was a gradual change in maximum glucose and glucose responsiveness. Interestingly, there was an abrupt decline in maximum glucose concentration between 20:00 to its lowest point at 00:00. The increase in blood glucose responsiveness between 12:00 and 20:00 may result from an increased need for the stress response to maintain activity, compared with the less active time around 00:00 (Harbuz and Lightman 1992). Stress-induced GCs are responsible for stimulating activity (i.e., muscle activation and increased cardiac output), preparing the body for the next stressor (i.e., predictable daily stressors), and suppressing unnecessary behaviour (i.e., feeding; Sapolsky et al. 2000). During times of stress, more energy needs to be allocated to locomotor muscle action, thus glucose storage is inhibited and gluconeogenesis is initiated (Sapolsky et al. 2000). It is likely that higher glucose responsiveness between 12:00 and 20:00 is made possible by the increased glucose concentration associated with food intake and digestion during diurnal feeding. The highest glucose concentrations are seen at 20:00, which may be a result of the delayed digestive activity.

When the stress response of a bluegill collected directly from its natural environment was tested, there was a significant change in baseline cortisol concentrations over the day such that higher concentrations were seen between 08:00 and 20:00. The relationship between cortisol and feeding cycles is widely accepted

Fig. 1. Mean and variability (standard error (SE)) of responsiveness of (a) blood glucose concentration (mmol/L) and (b) cortisol concentration (ng/mL) for bluegill (*Lepomis macrochirus*) at 4 h time intervals between 00:00 and 20:00. Different letters denote significant differences among time periods (ANOVA, $p < 0.05$). There were no significant differences among time periods for cortisol responsiveness, but glucose responsiveness was significantly lower at 00:00 compared with 12:00, 16:00, and 20:00.



(Breuner et al. 1999). Using a light–dark manipulation, Montoya et al. (2010) discovered a synchronization of feeding time and the highest plasma cortisol concentrations in gilthead seabream. As observed, basal glucose concentration is maintained in a narrow range throughout the night, although bluegill foraging radically decreases (Vinyard and O'Brien 1976). Because little to no foraging occurs in bluegill during the night, it follows that there would be a depletion in stored glucose followed by a predictable daily stressor (i.e., hunger). This stressor may lead to the stimulation of the stress response reflected as an increase in circulating cortisol con-

centration in the early morning. In summary, we hypothesize that increased baseline cortisol concentrations between 08:00 and 20:00 induce increased foraging activity during the daylight. It follows that the drop in cortisol concentration at 00:00 is likely a result of decreased need to maintain activity for foraging.

Interestingly, we observed no diel variation in maximum cortisol concentration or cortisol responsiveness. Bluegill sampled in this study had stress-induced cortisol titres within a normal range for teleost fish (normal range is between 40 and 200 ng/mL; Barton and Iwama 1991). However, the mean change in cortisol concentration from baseline concentration to stress-induced cortisol concentration was relatively large. The fact that this study found no significant change in cortisol responsiveness of bluegill on a diel basis, and the evidence for variability among closely related species, supports the need for a case-by-case investigation of stress response in fishes (Barton and Iwama 1991; Barton 2002). It seems bluegill have the same maximum response to stress regardless of the time in the light–dark cycle that stress is induced.

In summary, diel patterns in baseline and stress-induced concentrations of plasma glucocorticoid reflect the maintenance of homeostasis (baseline glucose concentration), the initiation of activity (baseline cortisol concentration), the preparation for stress (stress-induced glucose concentration), and the magnitude of the stress response (stress-induced cortisol concentration) in bluegill. This study also lends some indirect support to previous studies dealing with circadian GC release as a consequence of both feeding and light-cycle entrainment (Montoya et al. 2010). This research is particularly valuable given that field studies of the diel cortisol response are time-constrained and can be difficult to execute (Cook et al. 2012). Indeed, even the current analysis was conducted across multiple days given health and safety constrains for team members. By establishing a circadian pattern of GC release in a temperate teleost fish species, researchers can remove variance attributable to diel patterns in glucose or cortisol concentration that may otherwise mask the result of their studies (Barton et al. 1986; Davis and Parker 1986).

Acknowledgements

S.J.C. is supported by the Canada Research Chairs Program and the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grants Program. Additional support was provided by the U.S. Fish and Wildlife Service Fish Enhancement, Mitigation and Research Fund (FEMRF) program. We thank the Ontario Ministry of Natural Resources for providing a scientific collection permit.

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