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# Is winter worse for stressed fish? The consequences of exogenous cortisol manipulation on over-winter survival and condition of juvenile largemouth bass

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## ABSTRACT

Over-winter mortality is an important selective force for warm-water fish (e.g., centrarchids) that live in temperate habitats. Inherent challenges faced by fish during winter may be compounded by additional stressors that activate the hypothalamic–pituitary–interrenal axis, either before or during winter, leading to negative sub-lethal impacts on fish health and condition, and possibly reducing chance of survival. We used experimental cortisol manipulation to test the hypothesis that juvenile largemouth bass (*Micropterus salmoides*) exposed to semi-chronic elevation in cortisol prior to winter would experience higher levels of over-winter mortality, physiological alterations and impaired immune status relative to control and sham-treated bass. Over-winter survival in experimental ponds was high, averaging 83%, and did not differ among treatment groups. Over the study period, bass exhibited an average increase in mass of 19.4%, as well as a slight increase in Fulton's condition factor, but neither measure differed among groups. Hepatosomatic index in cortisol-treated bass was 23% lower than in control fish, suggesting lower energy status, but white muscle lipid content was similar across all groups. Lastly, there was no difference in spleen somatic index or parasite load among treatment groups, indicating no long-term immune impairment related to our cortisol manipulation. The current study adds to a growing body of literature on glucocorticoid manipulations where field-based findings are not consistent with laboratory-based conceptual understanding of multiple stressors. This suggests that field conditions may provide fish with opportunities to mitigate negative effects of some stressors.

## 1. Introduction

In aquatic ecosystems, winter is characterized by a decline in surface water temperature and associated ice conditions in both lotic (Prowse and Beltaos, 2002) and lentic (Duguay et al., 2006) systems, depending on latitude, local geophysical conditions and inter-annual variation in winter severity (Magnuson et al., 2000). In temperate regions, winter temperatures can be sufficiently extreme that they include freezing conditions. From the perspective of ectothermic animals such as fish, winter has manifold effects on their biology. Indeed, water temperature is considered the “master” environmental factor (Brett, 1971) with reductions in water temperature associated with enzyme-mediated decreases in metabolism, locomotor performance, food intake, immune function, and digestion (e.g., Fry, 1971; Brett and Groves, 1979; Le Morvan et al., 1997). Of course, fish in temperate regions anticipate winter as part of their annual phenology and attempt to prepare accordingly (e.g., energy allocation, seasonal habitat shifts; Shuter et al., 2012).

Not all individuals succeed in preparing appropriately for winter, and over-winter mortality is regarded as an important selective force in the life history of fishes (Hurst, 2007). Cold water fishes (e.g., burbot, salmonids) tend to be relatively well adapted to winter conditions, with over-winter mortality being relatively low (McCrimmon and Devitt, 1954; Cunjak et al., 1998; Hurst, 2007), while warm-water fish (e.g., centrarchids) tend to face a greater number of challenges during winter periods that can lead to comparatively higher levels of mortality (summarized in Suski and Ridgway, 2009). Among centrarchids, over-winter mortality is particularly relevant to juvenile fish (reviewed in Sogard, 1997). For example, among juvenile largemouth bass (*Micropterus salmoides*), smaller fish with lower initial energy reserves are particularly prone to over-winter mortality (Miranda and Hubbard, 1994). Moreover, predation can also contribute to increased mortality

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among the smaller juveniles if they need to forage more actively to acquire energetic resources (Garvey et al., 1998; Post et al., 1998). Given that winter is inherently challenging for small centrarchids, any additional stressors, particularly those that activate the hypothalamic–pituitary–interrenal (HPI) axis (reviewed in Barton, 2000), could presumably lead to a variety of negative outcomes. For example, stress experienced before or during winter would require energy to be diverted towards overcoming a given stressor, which may accentuate mortality and result in a variety of sublethal consequences (e.g., compromised immune function, decreases in energy density and condition) in fishes with already-limited energetic resources.

Experimental glucocorticoid manipulations are increasingly being used in wild vertebrates (including fish, birds, reptiles and mammals) to understand ecological processes and conservation threats (Angelier and Wingfield, 2013). Elevation of cortisol (primary stress hormone in fish; Barton, 2000) via intraperitoneal injection of cortisol in a cocoa butter carrier serves as a controlled and consistent means of experimentally challenging fish (reviewed in Gamperl et al., 1994). In the context of winter biology, O'Connor et al. (2011) revealed that adult largemouth bass implanted with cortisol in the fall succumb to a winter-kill event more rapidly than conspecifics without experimental cortisol elevation.

Here, we wanted to know whether winter is worse for physiologically "stressed" fish (i.e., fish that experienced an exogenous elevation of glucocorticoids). Specifically, we tested the hypothesis that juvenile largemouth bass exposed to experimental cortisol elevations prior to winter would experience higher levels of over-winter mortality, physiological alterations and impaired immune status relative to control and sham (carrier but no cortisol). We focused on largemouth bass given the extensive body of research on juvenile over-winter mortality (reviewed above) and the ecological and socio-economic value of the species in North America (Warren, 2009) which could be diminished in the future in the face of new and growing threats.

## 2. Materials and methods

### 2.1. Study site and experimental animals

This study used 140 captive-bred juvenile largemouth bass (*M. salmoides*) from the Sam Parr Biological Station (near Kinmundy, Illinois) to determine the effect of intraperitoneal cortisol implants on over-winter survival and condition. Largemouth bass ranged in mass from 7.0 g to 147.6 g (TL = 83 mm to 213 mm) and were assigned randomly on 2 November 2009 to one of three treatment groups: control, cortisol implant, and sham implant. Control fish were anesthetized in a 50 ppm clove oil solution (1:9 clove oil:ethanol) and then tagged with a 12 mm passive integrated transponder (PIT) tag (Biomark Inc., Idaho, USA) for later identification. Bass in the cortisol implant and sham implant groups underwent the same procedure except that they also received an injection of heat-softened cocoa butter using a 2.3 cm, 16-gauge needle and 5 cc syringe. The volume of cocoa butter injected was relative to the fish's mass, at 5 ml $kg^{-1}$. The sham group received only cocoa butter, while the cortisol group received cocoa butter that had been mixed with 10 mg $ml^{-1}$ of hydrocortisone 21-hemisuccinate (Sigma H4881; Sigma-Aldrich, Inc., St. Louis, MO; O'Connor et al., 2013). Validations with largemouth bass using the same concentrations of cortisol and cocoa butter yielded ecologically-relevant cortisol titers in the blood of 157 ± 47 ng $mL^{-1}$ (mean ± standard error of the mean, SEM; O'Connor et al., 2013), which is consistent with endogenous levels found in largemouth bass following exhaustive exercise (O'Connor et al., 2009). We did not measure the precise time course of the cortisol elevation in our fish; however, based on previous studies in largemouth bass using slightly different protocols (O'Connor et al., 2009), and bluegill sunfish (*Lepomis macrochirus*) using the same protocol (McConnachie et al., 2012), we estimate that the cortisol elevation lasted between one and two weeks. Following the tagging and cocoa butter implantation procedures, fish were transferred to one of two identical on-site 400 $m^2$ experimental ponds (ponds 4 and 6), where they were placed in floating net pens for seven days to monitor acute mortality. At the end of seven days, the fish were released into the ponds and allowed to swim freely for approximately 4.5 months (133 days) without intervention. Air temperature was monitored continuously throughout the study period using a Davis Instruments, Vantage Pro2 weather station.

On the evening of 15 March 2010, drain valves on the two ponds were opened and the fish were flushed into collection tanks for post-experiment processing. During draining, a turtle became lodged in the drain pipe of one of the ponds (pond 6), which caused it to drain at too slow a rate to properly flush the fish. As a result, the majority of fish in that pond became stranded, making them susceptible to bird predation and forcing us to retrieve them manually as the pond finished draining. Following recovery from the ponds, fish were transported to on-site laboratory facilities where they were euthanized, measured for mass (mass of PIT tag and remaining cocoa butter were subtracted from total mass to obtain post-experiment mass) and length, and then dissected. Parasite load on the skin, gill, kidney, liver, and spleen was scored qualitatively on a scale of 0 to 6 (0 = none, 1 = very low, 2 = low, 3 = low to moderate, 4 = moderate, 5 = moderate to high, and 6 = high) for each organ. The spleen and liver were then harvested and measured for mass and a sample of white muscle was excised, wrapped in aluminum foil, and transferred to liquid nitrogen for analysis of muscle lipid content.

### 2.2. Condition calculations and muscle lipid content

General health and condition among fish in the three treatment groups were compared using several indices. Fulton's condition factor ($K$) was calculated for each fish before and after the experiment using the equation $K = 10^5 \cdot W \cdot L^{-3}$, where $W$ is the wet mass of the fish in grams, and $L$ is the total length of the fish in millimeters (Bolger and Connolly, 1989). Somatic indices (ratio of organ mass to whole body mass, expressed as %) for both the liver (HSI) and spleen (SSI) were also calculated for each fish (as per Goede and Barton, 1990). Susceptibility to parasites was compared among treatment groups by comparing parasite load. To simplify the analysis and reduce to risk of type 2 statistical error, individual parasite load scores were collapsed into a single combined parasite load score by summing the parasite scores (qualitative score between 0 and 6) of each of the five individual organs (skin, gill, kidney, liver, and spleen).

Water and lipid content of white muscle samples was determined in duplicate as detailed in Gravel et al. (2010). Briefly, muscle samples (1–2 g wet mass) were dried in an oven at 80 °C until constant mass was achieved (12–16 h) and then were placed in a desiccator for 1–2 h. Percent dry weight was calculated by dividing dry mass by wet mass and multiplying by 100%. Dried muscle samples were then assayed for % lipid content using the methanol–chloroform lipid extraction protocol described in Bligh and Dyer (1959) and Smedes and Askland (1999).

### 2.3. Statistical analysis

All statistical procedures were performed using R statistical software (R project, R version 3.1.2). Initial mass was compared among treatment groups using a 1-factor analysis of variance (ANOVA; $\alpha = 0.05$). Binomial regression was used to compare survival among treatment groups. This analysis was limited to fish from pond 4 because of aforementioned stranding issues in pond 6. The remainder of analyses used all recovered fish. Change in Fulton's condition factor ($F$), HIS, SSI, % dry weight, and % lipid content were compared among treatment groups using Generalized Linear Mixed Models (GLMM) followed by Tukey's tests to compare among treatment groups ($\alpha = 0.05$). GLMMs included post-experiment mass as a covariate to control for allometry and pond as a random factor to account for unexplained variability between the two experimental ponds. Parasite load (combined score) was compared among treatment groups using the non-parametric Kruskal–Wallis rank-sum test ($\alpha = 0.05$).

## 3. Results

Mean daily air temperature at the Sam Par experimental ponds ranged from 17.5 °C to −13.9 °C and averaged 1.3 °C over the duration of the study (Fig. 1). Air temperature was highest in November and March and reached a minimum in early January, being less than 0 °C for 44% of the study period (59 of 133 days).

Mean pre-experiment mass of individuals used in the study did not differ among treatment groups (ANOVA, df = 2, 137, F = 1.48, p = 0.23). Mean mass for control, sham, and cortisol fish was 21.2 ± 2.4 g, 26.2 ± 2.9 g, and 29.1 ± 3.7 g, respectively. Seventy-two fish were released in pond 4 and 68 fish were released in pond 6. There was little acute mortality during the first week post-implantation. Only three individuals did not survive — two control and one cortisol fish.

Based on recovery rate from pond 4, survival of fish after 133 days at large in the ponds was approximately 83% (60 of 72), and there was no evidence that survival in either treatment group differed from control (GLM, df = 71, z = 0.85, −0.70, p = 0.48, 0.39 for sham and cortisol fish, respectively). Only 40% of fish were recovered from pond 6, but this was due to the aforementioned stranding incident that occurred when the ponds were drained.

Externally, there was no evidence of a difference in condition among treatment groups at the end of the experiment. On average control, sham, and cortisol fish recovered from the two ponds increased in mass by 23.4 ± 4.2%, 15.6 ± 1.6%, and 20.8 ± 2.5%, respectively. Larger fish gained significantly more mass than smaller fish (GLMM, t = 2.183, p = 0.03), but there was no significant difference among groups (GLMM and Tukey's test, $p > 0.25$ for all comparisons). Change in Fulton's condition factor ($F$) also did not differ significantly among the three treatment groups (Fig. 2; GLMM and Tukey's test, $p > 0.77$ for all comparisons). Mean condition factor increased slightly over the experimental period in all three groups (0.025 ± 0.023, 0.027 ± 0.021, 0.016 ± 0.022 for control, sham, and cortisol, respectively); however, the increase was not significantly different from zero (one-sample *t*-test, df = 84, t = 1.45, p = 0.15).

Despite the absence of differences in external condition among treatment groups, there was physiological evidence for effects of cortisol implants on condition 133 days after initial exposure. Liver size was smaller in cortisol-exposed fish than in control and sham fish. Hepatosomatic index (HSI) in cortisol fish was 23% lower than in control fish (Fig. 2; GLMM and Tukey's HSD, p = 0.02) and 18% lower than in sham fish (GLMM and Tukey's HSD, p = 0.09). There was, however, no difference in white muscle water content (Fig. 2; GLMM and Tukey's HSD, $p > 0.05$ for all comparisons) or white muscle lipid content (Fig. 2; GLMM and Tukey's HSD, $p > 0.97$ for all comparisons) among treatment groups. HSI (GLMM, t = 0.300, p = 0.76), white muscle water content (GLMM, t = 1.909, p = 0.06), and white muscle lipid content (GLMM, t = −1.199, p = 0.23) were not related to post-experiment mass of the fish.

We saw no evidence of effects of cortisol implants on immune function by 133 days post-exposure to elevated cortisol. Spleen somatic index (SSI) was significantly greater in smaller individuals than larger individuals (GLMM, t = −3.294, $p < 0.01$), but did not differ among the three treatment groups (Fig. 2; GLMM and Tukey's HSD, $p > 0.41$ for all comparisons). Moreover, while parasite load varied greatly among fish, with combined parasite scores ranging from 1 to 20 (out of a possible 30), there was no difference in parasite load among treatment groups (Fig. 2; Kruskal–Wallis test, df = 2, chi-squared = 0.73, p = 0.69).

## 4. Discussion

Winter is inherently challenging for temperate zone fish. As such, we hypothesized that if fish were exposed to an additional physiological stressor immediately prior to winter, those individuals would exhibit reduced survival as well as exhibit a variety of sublethal physiological and energetic consequences relative to conspecifics not exposed to the stressor. In general, we failed to find support for our hypothesis, aside from reductions in HSI among cortisol-treated fish.

Over-winter mortality in juvenile largemouth bass, as well as other centrarchids, has been well studied (reviewed in Devries et al., 2009; Suski and Ridgway, 2009). Winter can be a period of low food availability and high predation, with over-winter survival predicted to be positively related to body size (i.e., larger individuals more likely to survive; Post et al., 1998; Miranda and Hubbard, 1994; Ludsin and Devries, 1997). Interestingly, we failed to observe a relationship between size and mortality, despite using a wide range of juvenile body size. Size-related differences in over-winter mortality may be most severe at northern latitudes and should vary with factors like predation risk and degree of competition for resources (Fullerton et al., 2000; Garvey et al., 2004). The ponds used in this study were subject to avian predation during non-ice covered periods, but there were no larger piscine predators aside from conspecific juveniles that may have

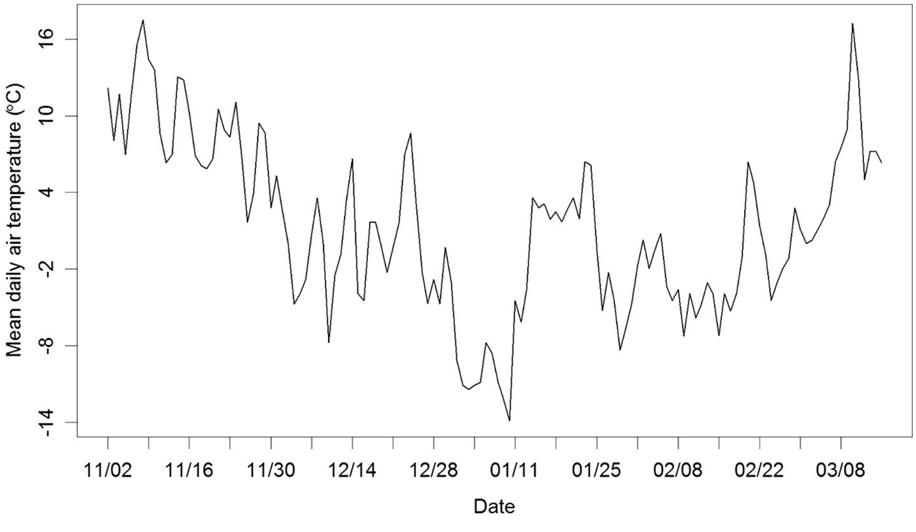

**Fig. 1.** Mean daily air temperature (°C) between 02 November 2009 and 15 March 2010. Temperature ranged between 17.5 °C and −13.9 °C and averaged 1.3 °C over the duration of the study.

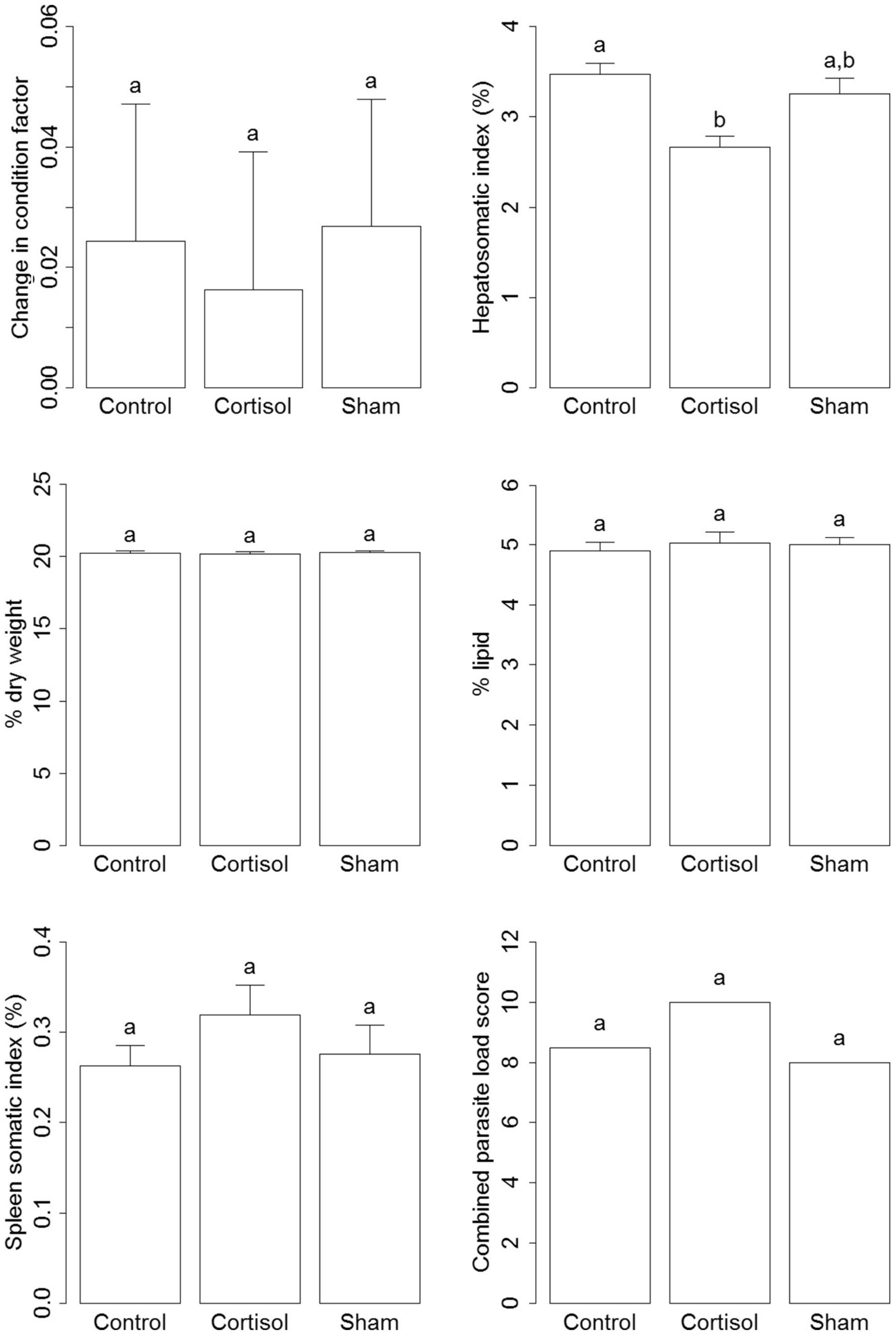

**Fig. 2.** Comparison of health and condition among control, sham and cortisol-implanted juvenile largemouth bass (*Micropterus salmoides*) released into one of two 400 $m^2$ outdoor experimental ponds and retrieved 133 days later. Plots display mean ± standard error (S.E.), except the combined parasite load plot, which displays median combined parasite scores. Letters above each plot indicate statistical significance. Values with different letters differ significantly from one another ($\alpha = 0.05$).

been cannibalistic (e.g., Keast and Eadie, 1985). No supplemental food was added to the ponds during the experiment. Nonetheless, fish in all treatments grew in mass between 16 and 23%, with no treatment-level differences observed, suggesting that food was not limited. The ponds were naturally colonized by diverse invertebrate assemblages, which were apparently sufficient to yield positive growth over a period where mortality is often observed. Presumably, cortisol-treated fish would have had to increase their over-winter foraging activity to compensate for increased energy expenditure as a result of being stressed at the onset of the study, although it is also possible that compensatory foraging occurred in late spring, after ice cover had left the ponds. Therefore, the observed effect of the cortisol implants may have been greater if we had ended the experiment earlier, or if the experimental ponds had contained piscine predators, as increased foraging would have come at the expense of increased risk of predation (Garvey et al., 2004). Alternatively, the lack of obvious treatment effects could be due to the fact that the winter period experienced by fish in this study may simply not have been as "challenging" as those in previous studies. For example, the carryover effects (O'Connor et al., 2014) found in the O'Connor et al. (2011) study were driven by a sustained hypoxic event in a lake in eastern Ontario near the northern extent of their range. In comparison, the pond site in Illinois is centrally located (latitudinally) within the largemouth bass distributional range. Populations are generally well-adapted to winter conditions in their region (Fullerton et al., 2000), so significant over-winter mortality is likely to occur mainly in years with harsher than average conditions.

For the purpose of this study, we used exogenous cortisol injection in the coelom to elevate cortisol levels, a common approach in studies of fish physiology (Gamperl et al., 1994). Based on previous studies using similar protocols (O'Connor et al, 2009; McConnachie et al., 2012), we estimate that cortisol elevation persisted in our experimental fish for between one and two weeks, simulating a semi-chronic stress event. The administration of cortisol mimics the cortisol-dependent processes associated with stress response, but it does not mimic the sensory aspects or the neuroendocrine activation of the HPI axis (Nagrodski et al., 2013). As such, it is possible that some important components of the stress response were not integrated into our experimental approach. However, research on wild adult largemouth bass has demonstrated seasonal carryover effects associated with fall cortisol injection (O'Connor et al., 2011), and summer growth impairments associated with summer cortisol injection (O'Connor et al., 2010). In the current study, however, the only significant difference noted among treatments was a significantly lower HSI in cortisol-treated bass. HSI is a commonly used indicator of energy reserves in fishes (e.g., Htun-Han, 1978; Jensen, 1979; Goede and Barton, 1990; Chellappa et al., 1995) including largemouth bass (Adams et al., 1982; Adams and McLean, 1985; Brown and Murphy, 2004). In general, a decrease in HSI, particularly when overall body condition does not differ among treatments (e.g., Brown and Murphy, 2004), is an indicator of chronic stress (e.g., Lee et al., 1983). We observed that HSI was lowest among the cortisol-treated fish, while other indicators of condition and somatic energy reserves were similar among groups. Brown and Murphy (2004) reported that in sub-adult largemouth bass (~125–199 mm), HSI values were generally low in November through February relative to other seasons and then increased starting in March concurrent with increases in the values of other condition indices. It is possible that the cortisol-treated fish in our study either failed to acquire the same resources over the fall and winter period or utilized hepatic energy stores at higher levels compared to sham and control fish, or a combination of those two scenarios. It is also possible that the difference was driven by increases in HSI (similar to those reported in Adams et al., 1982 and Brown and Murphy, 1994) in the control and sham fish in March, while the cortisol fish were somewhat delayed. Interestingly, O'Connor et al. (2013) found that female largemouth bass treated with cortisol several weeks prior to reproduction displayed a decrease in overall mass, and a corresponding increase in HSI, relative to control fish when sampled during the reproductive period. The specific mechanism for differences in HSI remains unclear but in some way cortisol (presumably acting through chronic stress; Lee et al., 1983; Barton, 2002) is driving the difference.

The present study is consistent with a growing body of literature on glucocorticoid manipulations in wild fish where the findings are sometimes counter-intuitive. Based on our conceptual understanding of multiple stressors, either in the context of allostasis (McEwen and Wingfield, 2003), or perhaps more appropriately the “reactive scope model” (Romero et al., 2009), stressors applied during already challenging periods such as winter have the potential to induce homeostatic overload which can have pathological consequences (including mortality; Romero et al., 2009). Interestingly, we failed to observe strong evidence of homeostatic overload here, in that there were no differences in immune function (based on parasite burdens and spleen size), body condition (aside from HSI) or mortality between cortisol-treated and control or sham fish. Much of the early work involving the negative consequences of cortisol manipulations was done in laboratory environments (reviewed in Gamperl et al., 1994; also see Barton, 2002). Field environments differ from controlled laboratory environments in that there may be more opportunities to compensate for different stressors given heterogeneous conditions and the ability of an individual fish to select conditions (e.g., physical habitat, thermal environment, food resources) that may mediate the effects of a given stressor (Huey, 1991). For example, effects may have been less pronounced in our study because the cold temperatures and consequent decrease in basal metabolism may have helped to mitigate the energetic and immune burden of elevated cortisol (Barton and Schreck, 1987). Moreover, biotic interactions like predator–prey dynamics and intraspecific competition could also be confounding factors. There is need for additional research to understand the mechanisms by which wild fish compensate for stressors in the wild, as well as how various biotic and abiotic environmental factors interact to influence the onset, intensity, and duration of the glucocorticoid-mediated stress response.

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