RESEARCH ARTICLE

Supraphysiological Cortisol Elevation Alters the Response of Wild Bluegill Sunfish to Subsequent Stressors

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ABSTRACT

Wild fish are frequently exposed to multiple stressors, but the influence of previous or ongoing stress on an animal's subsequent response is poorly understood. Using wild-caught bluegill sunfish (*Lepomis macrochirus*) as a model, we used exogenous hormone implants to experimentally raise circulating cortisol in a group of fish for ~10 days. We also maintained sham-treated and control groups of fish. We subjected all animals to a secondary stressor in the form of either a heat challenge or fasting challenge. We compared survival, body condition, and plasma-borne indicators of physiological status among cortisol-treated, sham-treated, and control groups following the secondary stressor. In order to compare short- and long-term effects of cortisol treatment, we initiated the secondary stressor either 4 or 30 days following initial cortisol treatment. Cortisol-treated fish succumbed to the fasting challenge sooner than sham-treated and control fish at both 4 and 30 days. Interestingly, cortisol-treated fish lost equilibrium sooner than sham-treated and control fish during the heat challenge when conducted at 30 days, but not at 4 days. These results demonstrate that multiple simultaneous stressors have cumulative effects on bluegill sunfish. Furthermore, these results demonstrate that supraphysiological cortisol doses alter the long-term responses of bluegill sunfish to additional challenges, even after apparent recovery. Such cumulative and long-term effects may be an important factor in mediating the response of wild animals to natural and anthropogenic stressors, and should be considered in ecological studies. *J. Exp. Zool. 317:321–332, 2012.* © 2012 Wiley Periodicals, Inc.

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Wild fish encounter many challenges that elicit a stress response throughout their lifetime. Some challenges are natural, such as scarcity of food resources (Dutil and Lambert, 2000; Barcellos et al., 2010), thermal variability (Shrimpton et al., 2007), disease (Barton et al., '86; Barton, 2002; Lepak and Kraft, 2008), or predation attempts (Pfeiffer, '62; Fraser and Gilliam, '92). Other challenges are anthropogenic, including urbanization (Wang et al., 2000), contaminants (Marentette et al., 2010), agricultural development (Stauffer et al., 2000), dams and turbines (Anderson

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et al., 2006; Murchie et al., 2008), or exposure to fishing practices (e.g., Davis, 2002; Cooke and Suski, 2005; Arlinghaus et al., 2007). Given the pervasive nature of these challenges in the wild and the potential for these challenges to influence survival and fitness, there is an explicit need to elucidate the long-term consequences of physiological stress on wild fish.

Given that physiological challenges do not occur in isolation, it is also necessary to understand the impacts of multiple simultaneous or sequential stressors. For the purposes of this paper, we adopt Barton and Iwama's ('91) definition of the stress response: the reaction by fish to a stimulus or stressor that alters the fish's homeostatic state. Here, we focus on the elevation of circulating glucocorticoid (i.e., cortisol) concentrations and the corresponding changes in physiology and behavior as a response to a stressor. Results from studies investigating the physiological and whole-body responses to multiple stressors have been varied and context-dependent. For example, Barton et al. ('86) noted that the physiological stress response accumulates in an additive fashion. Conversely, fish residing in polluted systems exhibit an impaired stress response when challenged with an acute stressor (Hontela et al., '97; Vijayan et al., '97). Basu et al. (2002) also observed that fish exposed to chronic stressors displayed an impaired stress response at the cellular (i.e., heat shock proteins) and organismal (i.e., cortisol) level, and Barton et al. ('87) determined that the continuous feeding of cortisol to juvenile fish for a 10-week period eliminates cortisol elevation following acute stress. Therefore, it is known that the cortisol response is impaired when fish experiencing a chronic cortisol elevation are exposed to acute stress (Barton, 2002).

Nevertheless, these studies do not explicitly incorporate recovery time into an ecologically relevant context, where fish may be subjected to environmental stressors during or after the period of recovery from cortisol elevation. Also, almost all studies involving experimental cortisol elevation have been conducted in the laboratory and on domesticated fish (Vijayan et al., '91; Morgan and Iwama, '96; Gregory and Wood, '99; Wang et al., 2005). Few studies have evaluated the long-term consequences of chronic stress and multiple stressors in wild fish (see O'Connor et al., 2010), although there have been several relevant studies on wild birds. For example, a study involving the Eurasian kestrel (*Falco tinnunculus*) and barn owl (*Tyto alba*) revealed that resting and handling-induced corticosterone (the main glucocorticoid in birds) levels return to untreated control values 20 days following corticosterone implantation (Müller et al., 2009). Goutte et al. (2010) observed decreased survival over a 2-year period in black-legged kittiwakes (*Rissa tridactyla*) implanted subcutaneously with corticosterone. The present study will provide some clarification to the inconsistencies seen in fish-related literature by using wild fish and time scales similar to the bird studies discussed above.

Injections of cortisol are an established method of raising circulating cortisol in a controlled manner, and are associated with the same secondary and tertiary changes as a natural stressor (Gamperl et al., '94; Barton, 2002). We subjected wild bluegill sunfish (*Lepomis machrochirus*), immediately after capture, to an intraperitoneal injection of cortisol in cocoa butter in order to address several questions. First, we examined if fish treated with cortisol show cumulative effects of stress when subjected to secondary stressors during the period of cortisol elevation (i.e., several days after experimental cortisol elevation). Second, we examined if fish treated with cortisol exhibit long-term effects of stress when subjected to a temporally separated secondary stressors after cortisol recovery (i.e., when cortisol levels reduce to preinjection levels).

MATERIALS AND METHODS

All fish were sampled under an Ontario Ministry of Natural Resources Scientific Collection Permit and handled in accordance with the guidelines of the Canadian Council on Animal Care (Carleton University, B09–11). Experimentation took place at Queen's University Biological Field Station in eastern Ontario, Canada (44°31′N, 76°20′W). Adult wild bluegill sunfish (total length > 150 mm) were used as a model species, because they are abundant and can easily be captured and held in captivity. In May and June, 2009, fish were captured from Lake Opinicon by angling and were then held in 1,000-L outdoor tanks supplied with flow-through lake water (~18°C). Fish were randomly distributed into three groups: cortisol treatment, sham treatment, and control. Cortisol-treated fish received an intraperitoneal injection of 50 mg kg^{-1} cortisol (hydrocortisone 21-hemisuccinate; Sigma H4881, Sigma-Aldrich, Oakville, Ontario, Canada) emulsified in pure cocoa butter (0.005 mL g^{-1}). Sham-treated fish received only the cocoa butter vehicle, while control fish were not injected. All fish were identified by individual and treatment type using anchor tags. We conducted two distinct experiments, each using these initial cortisol treatments. Both experiments consisted of applying a secondary stressor at either 4 or 30 days following the initial treatment. All fish were fed pellets (2% adjusted body weight; Martin Mills, Floating Feed, 3 mm) twice-daily during the 4- or 30-day holding periods, and were monitored closely at regular intervals for loss of condition (e.g., emaciation) leading to mortality and/or abnormal behavior (e.g., loss of equilibrium).

Time Course of Cortisol Effects

To provide context for the elevation of cortisol used in the experiments, we conducted a time-course experiment in which cortisol, glucose, hematocrit, and condition values were measured over a 30-day period. In the spring of 2010, groups of 60 fish ($n = 20$ per treatment) were angled, randomly subjected to one of our three treatments, and placed in a 1,000-L outdoor

tank. Fish were fed twice-daily and monitored closely for 24 hr, 4 days, 10 days, or 30 days. At the end of the monitoring period, a subset of fish ($n = 10$ per treatment) was removed from the tank and individually placed into individual opaque sensory deprivation chambers (~2 L) supplied with a constant flow of lake water for 24 hr. Fish were then removed from the chambers individually and blood sampled by caudal puncture, using lithium-heparinized 1-mL syringes and 25 gauge, 38-mm needles. Blood samples were collected within 2 min of removing fish from the chambers or samples were excluded from analysis. Previous research by our group has revealed that acute cortisol responses for bluegill are still below 170 ng mL^{-1} at 10 min and peak at 40 min post-stressor (Cook, 2011), which makes a 2-min sample appropriate as a nonstressed value. In addition, a separate group of fish ($n = 20$) was sampled immediately after angling from the lake for baseline field values, an approach that has previously used to obtain baseline values for wild fish (O'Connor et al., 2009). Whole blood was analyzed for glucose concentration, measured on 10 μL of whole blood with a hand-held glucose meter (ACCU-CHEK glucose meter; Roche Diagnostics, Basel, Switzerland), a device previously validated for use on fish (Cooke et al., 2008). Hematocrit values were determined using microhematocrit tubes centrifuged for 5 min (CritSpin-Micro-Hematocrit Centrifuge, Norwood, MA, USA). The remaining blood was centrifuged (Fisher Scientific Micro-Fuge, Toronto, Ontario, Canada) at 2,000 × g for 5 min. Plasma samples were frozen immediately in liquid nitrogen and then transferred to a −80°C freezer and stored until cortisol analysis (see below). Fish were euthanized by cerebral percussion and dissected, collecting information relevant to condition and health of the individual.

We used a chasing protocol to compare our exogenous cortisol elevation to endogenous cortisol elevation caused by stress in bluegill sunfish. A group of fish ($n = 10$) was obtained from the lake and fish were chased to exhaustion (loss of equilibrium, ~5 min; Kieffer, 2000). Once equilibrium was lost, fish were held in isolation chambers for ~40 min to ensure fish were sampled as cortisol peaked, (Cook, 2011) and blood samples were taken and processed as previously described.

Experiment 1: Heat Challenge (Short- and Long-Term)

For the short-term heat challenge, 75 fish ($n = 25$ per treatment) were placed together into a 1,000-L tank after being exposed to the above three treatments. Four days after treatment, the tank was outfitted with a temperature logger and two electrical water heaters that increased water temperature by approximately 1°C per hour until all fish had lost equilibrium (range: 15–34C). Fish were monitored closely during the heat challenge and were removed from the tank immediately following loss of equilibrium. The time and temperature at removal were documented and the fish was sampled for ~1 mL of blood. Blood samples were held in an ice-water slurry for no more than 1 hr until they were processed as described above. We recognized that the netting of moribund fish from the tank had the potential to induce stress and thus elevate cortisol. However, we only netted fish once they lost equilibrium that was associated with them appearing at the water surface, which enabled rapid collection with a small net. We feel that this approach minimized disturbance on conspecifics still remaining in the tank.

For the long-term challenge, 75 fish ($n = 25$ per treatment) were placed together into a 1,000-L tank after being exposed to one of the three treatments. Fish were fed twice-daily and monitored closely for a 30-day period. After 30 days, the heat challenge protocol described above was carried out. Mortality rates were quantified during the 30-day holding period; any fish that exhibited endpoints characterized as loss of equilibrium and reflex responsiveness (considered a humane and appropriate endpoint) was netted and considered a mortality.

Experiment 2: Fasting Challenge (Short- and Long-Term)

For the short-term challenge, 75 fish ($n = 25$ per treatment) were placed together into a 1,000-L tank after being exposed to the above three treatments. After 4 days, fish were fasted for up to 50 days. Fish were removed from the experiment if loss of equilibrium occurred and the day of removal was noted. Upon removal or at the end of the experiment (after 50 days), fish were euthanized using cerebral percussion and the following measures were collected: hepato-somatic index (HSI), splenic index (SSI), condition factor (K), and several condition indices modified from the health assessment index (HAI) as described in Adams et al. ('93). HSI (HSI = [wet liver mass/wet body mass]) × 100%) is expected to be correlated with the nutritional state of the fish (Busacker et al., '90). The splenic index (SSI = [wet spleen mass/wet body mass] × 100%) enabled us to evaluate splenic enlargement, which is an indicator of disease (Adams et al., '93). Condition factor (K = mass × fork length^{-3}) was measured because it is useful in reflecting the nutritional state of fish (Busacker et al., '90). The HAI is a rapid and inexpensive method used to evaluate the effects of stress on the health of fish populations. It is a quantitative index where necropsy observations are given numerical values so that statistical comparisons can be made among experimental groups. We modified the index to focus on three main variables expected to reveal differences in health and immune function among our treatments: total parasite load (each organ affected was given a score of 5 = low, 10 = moderate, 15 = high), gill condition (level of gill fray [10 = low, 20 = moderate, 30 = high], necrotic gill tissue present [5 = low, 10 = moderate, 15 = high], and parasite load [5 = low, 10 = moderate, 15 = high]), and fungus covering the fish's skin (measured as percent [%] cover).

For the long-term challenge, 75 fish ($n = 25$ per treatment) were placed together into a 1,000-L tank after being exposed to one of the three treatments. Fish were fed twice daily and monitored closely for a 30-day period. After 30 days, the fasting

challenge protocol described above was carried out. Mortality rates were quantified during holding.

Cortisol Analysis

Plasma cortisol concentration was determined using a commercial radioimmunoassay kit (ImmunoChem Cortisol $_{125}$I RIA kit, MP Biomedicals, Orangeburg, NY) previously validated for teleost fish (Gamperl et al., '94) and a Cobra Auto-Gamma (Hewlett-Packard, Palo Alto, CA). All samples were run in a single assay, and intra-assay variability (% CV) was 7.9%. The cortisol levels we measured for bluegill are comparatively high relative to other teleost fish (see Mommsen et al., '99), however, it is worth noting that our cortisol values are comparable to those generated by independent laboratories that have characterized bluegill stress responses (see Magee et al., 2006). Moreover, the same RIA methods we used in the present study have been validated for use for bluegill (Cook et al., 2011), as well as for a variety of other centrarchid fish (e.g., largemouth bass [*Micropterus salmoides*], O'Connor et al., 2010; smallmouth bass [*M. dolomieu*], Dey et al., 2010) and yielded cortisol levels much lower than those observed. Such results emphasize that the values that we present, although relatively high, are valid and likely reflect interspecific variation.

Statistical Analyses

For the 24-hr, 4-day, 10-day, and 30-day time-course analysis, two-way ANOVAs were performed for all parameters to determine whether there were any treatment effects across the time series. Mortality for each experiment was expressed as a percentage (%) of dead fish to original fish among the groups was compared using Pearson chi-square tests. Log-rank survival analyses to 50% equilibrium loss were conducted separately for all of the short- and long-term portions of both the heat and fasting challenges. Survival analyses for the heat challenge were performed in terms of temperature at loss of equilibrium. Survival analyses for the fasting challenge were performed in terms of date of removal from the experiment. For the short- and long-term heat challenges, two-way ANOVAs were carried out on blood parameters (plasma cortisol concentration, whole-blood glucose concentration, and hematocrit), to determine differences among treatment groups and experiment timing. The assumptions of equality of variances and normal distribution were tested for all analyses and relevant transformations applied where assumptions could not be met. For those analyses not able to be transformed, nonparametric, log-rank analyses were performed. Tukey–Kramer HSD tests were used for post-hoc tests for the two-way ANOVAs when significant differences were found. All analyses were conducted using JMP 8.0.2 software (SAS institute, Cary, NC). The significance level was set at $\alpha = 0.05$, except when Bonferroni-corrected significance levels were used to address the problem of multiple comparisons (noted as BF α).

RESULTS

Time Course of Cortisol Effects

After 24 hr, bluegill sunfish injected with cortisol had cortisol levels that averaged 1804 ± 118 ng mL^{-1} and were significantly higher than both sham (102 ± 129 ng mL^{-1}) and control fish (44 ± 118 ng mL^{-1}; $F = 70.0$, df = 2, $P < 0.001$; BF α 0.006). At 4 days, cortisol levels in cortisol-treated fish (662 ± 112 ng mL^{-1}) still remained significantly higher than both shams (21 ± 89 ng mL^{-1}) and controls (77 ± 89 ng mL^{-1}; $\lambda^2 = 17.6$, df = 2, $P < 0.001$; BF α 0.006). At 10 days, cortisol values remained significantly elevated (953 ± 96 ng mL^{-1}) relative to shams (119 ± 92 ng mL^{-1}) and controls (36 ± 92 ng mL^{-1}; $\lambda^2 = 14.4$, df = 2, $P = 0.0008$; BF α 0.006). At 30 days, cortisol values for sham-, cortisol-treated, and control fish did not differ significantly from one another (246 ± 89, 325 ± 78 ng mL^{-1}, and 79 ± 81 ng mL^{-1}, respectively; $F = 4.34$, df = 2, $P = 0.02$; BF α 0.006; Fig. 1A). Cortisol-treated fish displayed significantly higher blood glucose concentrations at 24 hr when compared to control and sham-treated fish ($F = 21.6$, df = 2, $P < 0.001$; BF α 0.006) (Fig. 1B). Condition parameters decreased throughout the 30-day period with little differences among treatment groups at all sampling points (Figs. 2A–F). Mortality did not differ significantly among treatment groups prior to the 30-day sampling period ($\chi^2 = 2.9$, df = 2, $P = 0.2$). Mortality rates were 24, 36, and 48% for controls, sham-, and cortisol-treated fish, respectively.

Plasma cortisol values for bluegill sunfish captured from the lake and sampled within 2 min (representative of baseline values for wild fish) were on average 47.7 ± 34 ng mL^{-1} ($n = 10$) with a range of 0.3–552.9 ng mL $^{-1}$. Values measured in bluegill sunfish that were chased to exhaustion and allowed to recover for ~40 min ($n = 15$) averaged 560 ± 69 ng mL^{-1} (range: 248–1172 ng mL^{-1}).

Experiment 1: Heat Challenge (Short- and Long-Term)

For the short-term heat challenge, no mortalities occurred in the 4 days prior to the challenge (i.e., between cortisol treatment and the secondary challenge). During the secondary heat challenge, a log-rank survival analysis to 50% mortality ($\lambda^2 = 2.0$, df = 2, $P = 0.4$) revealed no differences among treatment groups for the rate of equilibrium loss as temperatures increased (Fig. 3A). When sampled, cortisol-treated fish exhibited higher plasma cortisol levels than both control and sham-treated groups, but there were no differences in plasma glucose levels or hematocrit among the groups (see Figs. 4A–C).

Thirty days prior to the long-term heat challenge, 56% of cortisol-treated fish were removed from the experiment, and 8% of both sham-treated and control fish were removed. Mortality rates differed significantly among treatment groups prior to the long-term heat challenge ($\chi^2 = 21.1$, df = 2, $P < 0.001$). During the heat challenge at day 30, cortisol-treated fish lost

equilibrium at lower temperatures compared to the other treatment groups (log-rank survival analysis to 50%; $\lambda^2 = 40.7$, df = 2, $P < 0.001$; Fig. 1B). For the long-term heat challenge, cortisol-treated fish displayed higher cortisol levels than controls and sham-treated fish (treatment effect, $F = 9.5$, df = 2, $P = < 0.001$; Fig. 2D). Cortisol-treated fish also displayed significantly lower glucose values (experiment effect, $F = 5.0$, df = 2, $P = 0.03$; treatment effect, $F = 13.0$, df = 2, $P < 0.001$; interaction effect, $F = 17.4$, df = 2, $P < 0.001$) than control and sham-treated fish (Fig. 2B). Overall for the heat challenge experiment, cortisol values decreased in the long-term (experiment effect, $F = 8.8$, df = 2, $P = 0.004$) with no interaction effect ($F = 0.1$, df = 2, $P = 0.9$). Hematocrit values did not differ significantly among treatment groups (experiment effect, $F = 5.1$, df = 2, $P = 0.04$; treatment effect, $F = 3.9$, df = 2, $P = 0.03$, interaction effect, $F = 3.9$, df = 2, $P = 0.03$; BF α 0.02; Fig. 4E).

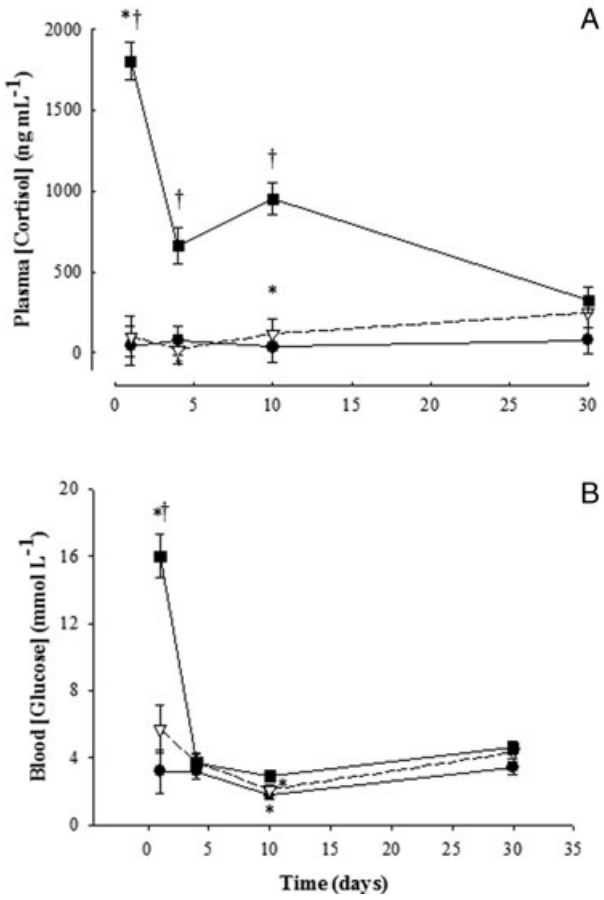

Figure 1. (A and B) Concentrations of plasma cortisol (A), and blood glucose (B) for *Lepomis macrochirus* after 24 hr, 4 days, 10 days or 30 days of treatment with a cortisol-impregnated intraperitoneal implant (–■–, cortisol), vehicle alone (–▽– sham), or no treatment (–●–control). Sample sizes were as follows: 24 hr (control = 12, sham = 10, cortisol = 13), 4 days (control = 11, sham = 11, cortisol = 8), 10 days (control = 12, sham = 12, cortisol = 11), and 30 days (control = 12, sham = 11, cortisol = 13). An asterisk (*) signifies a significant difference within a treatment group from the 30-day value, whereas a dagger (†) indicates a difference from the control group at a given time point. Data were log-transformed and analyses were Bonferroni corrected at $\alpha = 0.006$.

Experiment 2: Fasting Challenge (Short- and Long-Term)

There were no mortalities during the 4 days prior to the short-term fasting challenge. During the fasting challenge itself, cortisol-treated fish were removed from the experiment at a higher rate than sham-treated and control fish (log-rank survival analysis to 50% mortality; $\lambda^2 = 12.0$, df = 2, $P = 0.002$; Fig. 5A). There were no differences in any condition variables for fish that were removed from the experiment, regardless of treatment, before or after the 50-day period was completed (Table 1).

Prior to the long-term fasting challenge (i.e., during the 30 days between cortisol treatment and the onset of the secondary stressor), 56% of cortisol-treated fish, 32% of sham-treated fish, and 12% of control fish were removed from the experiment. Mortality rates differed significantly among treatment groups prior to the long-term fasting challenge ($\chi^2 = 9.4$, df = 2, $P = 0.009$). Fish removed prior to the secondary challenge did not differ among treatment groups for any condition parameters except HSI, where cortisol-treated fish had lower HSI values than sham-treated and control fish ($F = 4.1$, df = 2, $P = 0.04$). There were no differences in the rate of removal from the experiment (i.e., during the fasting challenge) among the three treatment groups (log-rank survival analysis to 50% mortality; $\lambda^2 = 1.6$, df = 2, $P = 0.4$; Fig. 3B). No condition parameters differed among treatment groups for fish removed during or at the end of the 50-day fasting period (Table 2).

DISCUSSION

We found that short-term cortisol elevation (~10 days) is associated with short- and long-term costs. In the short-term, we found that fish treated with cortisol implants succumb to a fasting challenge earlier than sham-treated and control fish. In the long-term, we found that fish treated with cortisol implants succumb faster to a fasting challenge and to a heat challenge than other treatment groups. These results demonstrate that the physiological changes associated with cortisol elevation carry significant costs that compromise the ability of bluegill sunfish to adequately deal with short- or long-term nutritional limitations. Results also emphasize that the physiological changes associated with cortisol elevation can cause long-term effects and influence the ability of bluegill sunfish to respond to secondary environmental challenges, such as elevated thermal conditions.

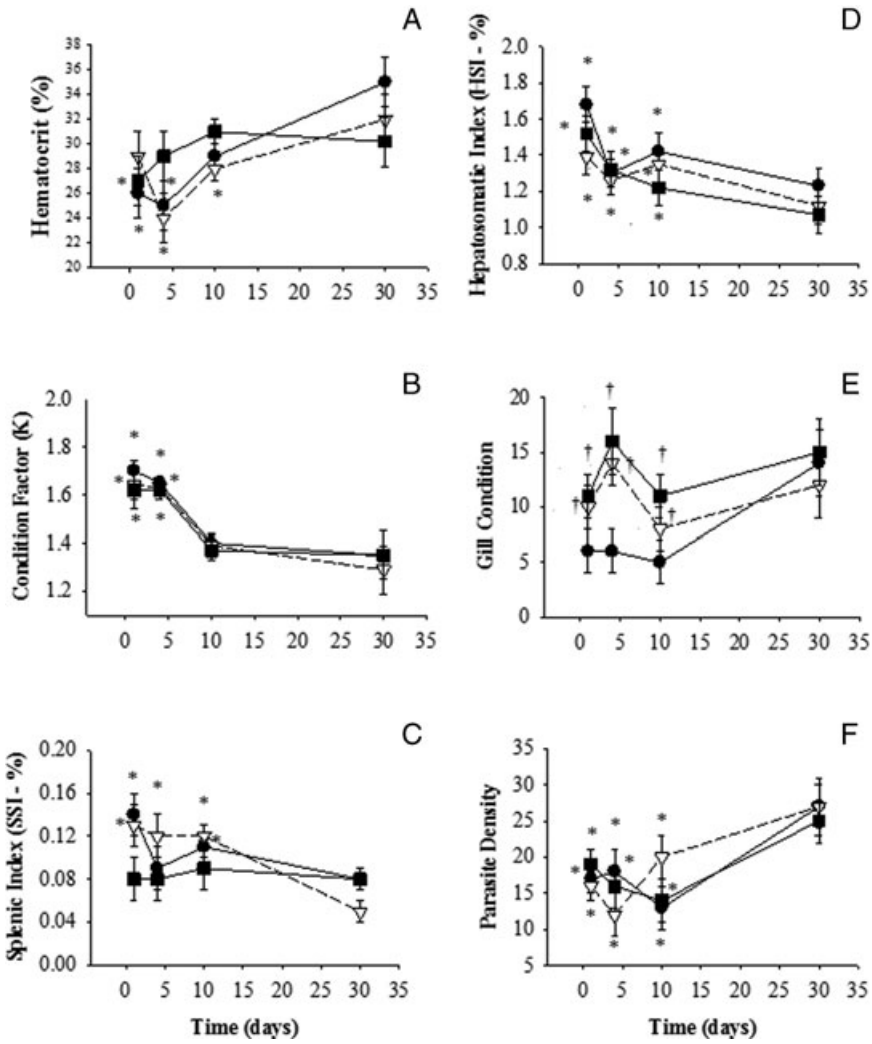

Figure 2. (A–F) Hematocrit (A), condition factor, (B), splenic index (C), hepatosomatic index (D), gill condition (E), and parasite density (F) for *Lepomis macrochirus* after 24 hr, 4 days, 10 days, or 30 days of treatment with a cortisol-impregnated intraperitoneal implant (–■–, cortisol), vehicle alone (–▽–sham), or no treatment (–●– control). Sample sizes were as follows: 24 hr (control = 12, sham = 10, cortisol = 13), 4 days (control = 11, sham = 11, cortisol = 8), 10 days (control = 12, sham = 12, cortisol = 11), and 30 days (control = 12, sham = 11, cortisol = 13). An asterisk (*) signifies a significant difference within a treatment group from the 30-day value, whereas a dagger (†) indicates a difference from the control group at a given time point. Data were log-transformed and analyses were Bonferroni corrected at $\alpha = 0.006$. All parameters indicated as percentages were analyzed as (ArcSin(Square Root(value)).

Collectively, these results indicate that ecologically relevant stressors are modulated by both previous and simultaneous stressors, a finding that has rarely been observed in the wild. These results have implications for understanding the full suite of biological consequences associated with anthropogenic stressors that are increasingly common for wild fish and other animals.

How Do Cortisol Implants Influence the Response to Simultaneous Secondary Stressors?

During the short-term heat challenge, we observed no differences between cortisol-treated fish and the other treatment groups in how individuals responded to the challenge. It is likely that no difference occurred, because other than high circulating cortisol levels (attributable to the implant and not the

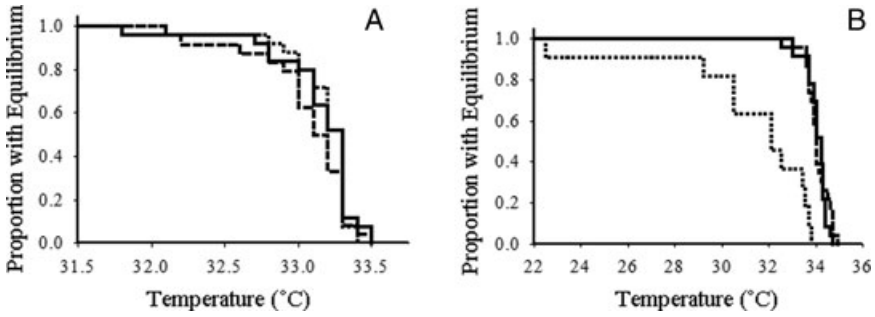

Figure 3. (A and B) Log-rank survival analyses to 50% mortality for *Lepomis macrochirus* during the short- (A) and long-term (B) heat challenges. Treatment groups: Control (), sham (– –), cortisol-treated (. . . .). Sample sizes are as follows: short-term; control = 25, sham = 24, cortisol = 25. long-term; control and sham = 23, cortisol = 11.

secondary challenge), all other physiological parameters were similar among treatment groups. It is worth noting that we observed differences in cortisol values among treatment groups despite the fact that they were netted from the same stock tank and that there were no differences in the time of equilibrium loss. As such, even if our netting procedure (which involved collecting fish once they lost equilibrium and appeared at the water surface and was thus unobtrusive to fish remaining at the bottom of the tank) did induce stress for those fish still in the tank, we would not have expected to see differences in cortisol values among treatments. Some secondary (e.g., osmoregulatory disturbances and changes in immune function) and most tertiary effects (e.g., changes in growth and behavioral modifications) of cortisol elevation may not have manifested themselves in the cortisol-treated fish by 4 days (Barton, 2002). It seems as though in the short-term, fish are responding maximally to the implant, and there is either no benefit to mounting a further response, or they are unable to respond further to an additional challenge. However, the short-term fasting experiment yielded results that suggest fish responding to a chronic elevation of cortisol are less likely to survive a fasting event neither in the short-term nor in the long-term. This result could have been observed for a number of reasons, mainly having to do with a decrease in energy stores. For example, cortisol elevation can cause an increase in swimming activity (largemouth bass, O'Connor et al., 2010) and a decrease in feeding activity as a result of social subordination (rainbow trout, Gilmour et al., 2005). Cortisol elevation can also increase catabolism leading to decreases in the hepatosomatic index (Barton et al., '87), as seen in the long-term fasting challenge (up to 30 days after injection). Our results are consistent with previous studies and suggest that decreased available energy as a result of elevated circulating cortisol led to the documented pattern of mortality when faced with a fasting challenge.

How Do Cortisol Implants Influence the Response to Sequential Secondary Stressors?

The long-term heat challenge reveals that cortisol-treated fish have a suppressed glucose response when faced with a secondary challenge, while in the short-term this effect is not present. Increases in plasma glucose during a glucocorticoid stress response is the result of mobilization of carbohydrate energy stores, and these resources are finite (Mommsen et al., '99; Barton, 2002). Therefore, we can speculate that cortisol-treated fish had lower carbohydrate stores, and therefore, displayed reduced circulating glucose levels, in response to the secondary challenge. In general, physiological processes adapt to compensate for extreme stress, but there are limits in this response (Schreck et al., 2001). It is also plausible that cortisol-treated fish had altered physiological thresholds, limiting their ability to maintain homeostasis during the experimental challenge (Schreck et al., 2001).

Chronically increased plasma cortisol levels are likely causing physiological factors (other than energy storage) to compromise resistance to ecologically relevant stressors, even after the artificial elevation of cortisol has expired. Further study is required to determine what factors are contributing in the context of this study. For example, cortisol-treated fish may be more susceptible to thermal stress. Basu et al. (2001) determined that thermal stress and an increase in circulating cortisol values reduce heat shock protein number 70 levels in liver and gill tissue in fish. Heat shock proteins have been indicated as having a protective role in fish during stress in terms of cellular "housekeeping" (Basu et al., 2001). Since heat shock proteins aid in stress recovery but may be negatively affected by stress events, it may be useful to investigate heat shock protein expression during multiple stress events in cortisol-treated fish.

It is unknown how an individual exposed to one stress event will respond to a secondary stressor, temporally separated from the initial stressor. In the ecological literature, such temporally

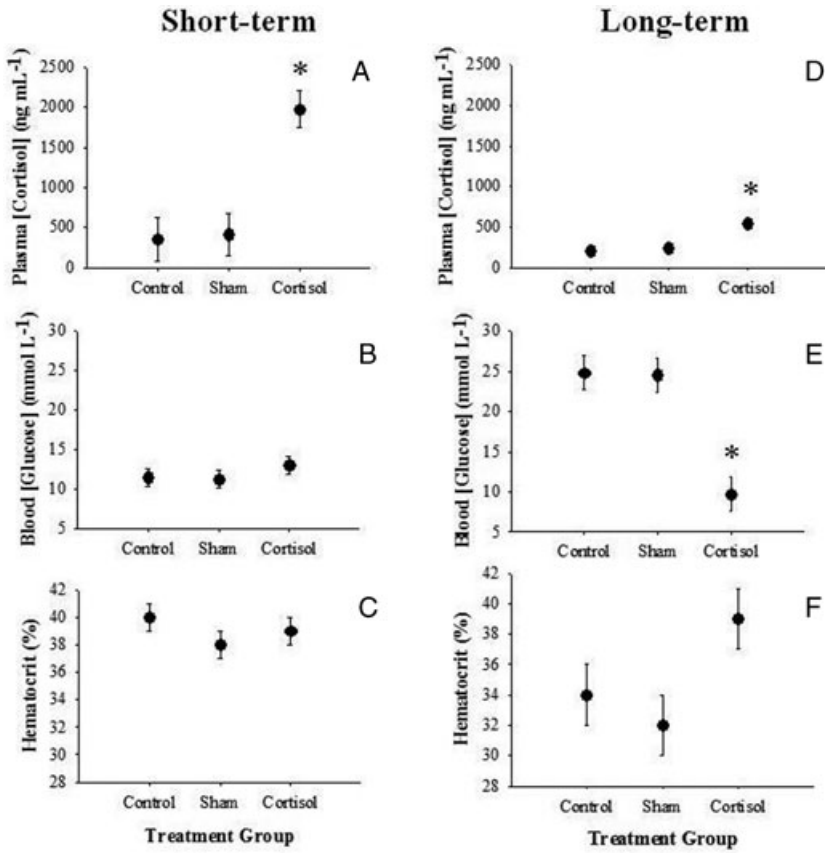

Figure 4. (A–F) Concentrations of plasma cortisol (A and D), blood glucose (B and E), and hematocrit (C and F; percent packed red blood cell volume) for control, sham-treated, and cortisol-treated *Lepomis macrochirus* during the short- (A through C) and long-term (D through F) heat challenge experiments. An asterisk (*) denotes a significant difference from the control treatment group (Tukey–Kramer HSD test, $P < 0.017$). Sample sizes are as follows: short-term cortisol (control = 11, sham = 12, cortisol = 15), glucose and hematocrit ($N = 25$ control, cortisol, sham = 23); long-term cortisol (control = 18, sham = 19, cortisol = 8), glucose ($N = 22$ control, sham, cortisol = 10), hematocrit (control = 22, sham = 21, cortisol = 9). Glucose for the long-term challenge was analyzed using Wilcoxon Rank-Sum test. Short-term cortisol and glucose data were log-transformed. Long-term cortisol data were Square root transformed. Data were analyzed using Bonferroni corrected $\alpha = 0.02$. All parameters indicated as percentages were analyzed as (ArcSin(Square Root(value)).

separated effects of stressors on responses to subsequent stressors are referred to as "carryover effects" (Norris and Marra, 2007; Harrison et al., 2010). This concept has rarely been explored in fishes (see O'Connor et al., 2010), but it is likely that sublethal stressful events modulate how a fish can respond to subsequent stressors after physiological recovery from the initial stressor. In the framework of carryover effects, the results of this study suggest that our experimental cortisol manipulations had sublethal effects that appeared at a temporal scale removed from the stress event (O'Connor et al., 2010). The time-course experiment revealed that after 30 days, baseline physiology and condition values for cortisol-treated fish were similar to control and sham-treated fish. However, the experimental stress events revealed that cortisol-treated fish were less able to respond appropriately in the long-term. For example, decreases in feeding and changes in social behavior caused by the chronic cortisol elevation may have contributed to a decrease in the energy stores required for the proper physiological and behavioral response to stress in the long-term (Gregory and Wood, '99; Schreck et al., 2001).

Although we observed no decreases in condition, our measures may not have been at the appropriate scale, and the fish could have had reduced energetic stores (e.g., reserved fat stores, liver glycogen, etc.). Sapolsky et al. (2000) suggest that chronically elevated cortisol levels cause muscle wastage and fat redistribution, which provides another mechanism for

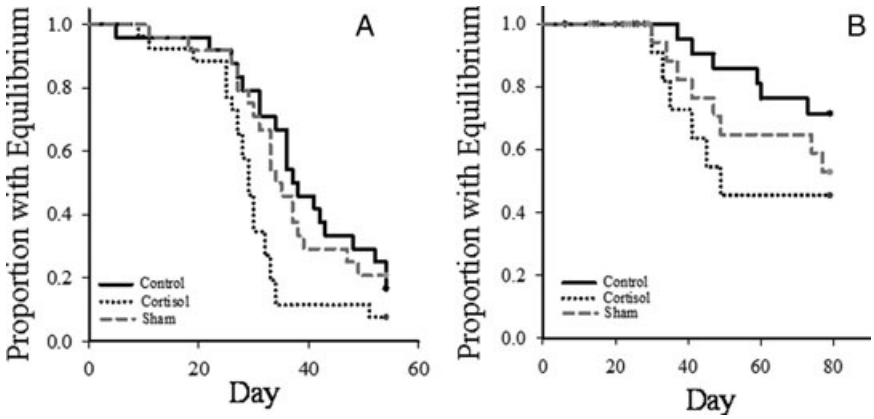

Figure 5. (A and B) Log-rank survival analyses to 50% mortality for *Lepomis macrochirus* during the short- (A) and long-term (B) fasting challenges. Treatment groups: Control (—), sham (– –), cortisol-treated (. . . .). Sample sizes are as follows: Short-term; control = 18, sham = 19, cortisol = 24. Long-term; control and cortisol = 6, sham = 8.

Table 1. Summary of *Lepomis macrochirus* condition variables for the short-term fasting challenge.

Variable	Treatment			Statistic	*P* value
	Control (mean ± SE)	Sham (mean ± SE)	Cortisol (mean ± SE)		
Fish removed during 50-day fasting period					
Fungal Cover (%)†	37 ± 5	21 ± 5	27 ± 5	2.57	0.086
Gill Condition (Score out of 60)†	32 ± 4	32 ± 4	33 ± 3	0.008	0.992
Parasite Load (Score out of 105)†	39 ± 4	39 ± 4	43 ± 3	0.522	0.596
HSI (% Body Weight)	0.96 ± 0.1	1.03 ± 0.1	0.97 ± 0.1	0.371	0.692
Condition Factor (K)	1.53 ± 0.04	1.51 ± 0.04	1.53 ± 0.03	0.054	0.947
Fish removed following 50-day fasting period					
Gill Condition (Score out of 60)†	31 ± 4	35 ± 4	43 ± 5	1.94	0.199
Parasite Load (Score out of 105)†	31 ± 7	35 ± 7	40 ± 9	0.341	0.720
HSI (% Body Weight)	0.56 ± 0.03	0.51 ± 0.03	0.70 ± 0.04	5.91	0.023
Condition Factor (K)	1.43 ± 0.1	1.30 ± 0.1	1.37 ± 0.1	0.542	0.600

Analyses were separated by fish that succumbed to the challenge during the 50-day fasting period and by fish that remained in the experiment for the full 50-day period. Sample sizes are as follows: fish removed during: control = 17, sham = 17, cortisol = 22; fish removed afterwards: control = 5, sham = 4, cortisol = 3. "†" refers to treatments whose data were log-transformed. No fungal cover was seen in any fish in the "fish removed following the 50-day fasting period" group. All comparisons were analyzed using one-way ANOVAs. Bonferroni corrections include, $\alpha = 0.01$ for fish removed during fasting period and $\alpha = 0.013$ for fish removed following 50-day fasting period.

decreased condition. A recent study by Goutte et al. (2010) determined that black-legged kittiwakes (*R. tridactyla*) exposed to a chronic elevation in cortisol displayed decreased survival over a 2-year period when compared to sham-treated birds. It is suggested that experimental manipulation of cortisol values down-regulated the endogenous secretion of corticosterone through a prolonged negative feedback loop, altering the bird's ability to trigger an appropriate stress response during subsequent stress events, decreasing the probability of survival (Goutte et al., 2010). Similarly, O'Connor et al. (2010) detected the occurrence of carryover effects in largemouth bass exposed to chronic cortisol elevations. Largemouth bass exposed to cortisol during the spring displayed increased activity levels over the subsequent year and died earlier during a winter-kill event when compared

Table 2. Summary of *Lepomis macrochirus* condition variables for the long-term fasting challenge.

Variable	Treatment Control (mean ± SE)	Sham (mean ± SE)	Cortisol (mean ± SE)	Statistic	*P* value
Fish removed before challenge (during the 30-day period)					
Fungal Cover (%)	12 ± 8	13 ± 7	17 ± 4	0.044	0.958
Gill Condition (Score out of 60)	12 ± 13	28 ± 11	26 ± 6	1.68	0.432
Parasite Load (Score out of 105)	20 ± 8	35 ± 7	33 ± 4	2.55	0.279
HSI (% Body Weight)	1.66 ± 0.2^{A}	1.32 ± 0.2^{AB}	1.14 ± 0.1^{B}	4.10	**0.037**
Condition Factor (K)	1.78 ± 0.1	1.67 ± 0.1	1.60 ± 0.1	1.28	0.303
Fish removed during 50-day fasting period					
Fungal Cover (%)	7 ± 5	8 ± 5	20 ± 5	2.45	0.128
Gill Condition (Score out of 60)	47 ± 5	40 ± 5	29 ± 5	3.66	0.057
Parasite Load (Score out of 105)	28 ± 7	26 ± 7	26 ± 7	0.024	0.976
HSI (% Body Weight)	1.01 ± 0.3	1.47 ± 0.4	1.21 ± 0.3	0.382	0.691
Condition Factor (K)	1.33 ± 0.2	1.50 ± 0.2	1.45 ± 0.2	0.228	0.799
Fish removed following 50-day fasting period					
Gill Condition (Score out of 60)	23 ± 2	16 ± 3	24 ± 4	2.41	0.109
Parasite Load (Score out of 105)	24 ± 2	27 ± 3	30 ± 4	2.62	0.270
HSI (% Body Weight)	0.79 ± 0.04	0.74 ± 0.1	0.76 ± 0.1	0.436	0.652
Condition Factor (K)	1.41 ± 0.1	1.47 ± 0.1	1.29 ± 0.1	0.547	0.586

Analyses were separated by fish that were removed during the 30-day holding period, removed during the 50-day fasting period and those that completed the 50-day fasting period. Sample sizes are as follows: fish removed before: control = 3, sham = 4, cortisol = 14; fish removed during: $N = 5$, all groups; fish removed after: control = 16, sham = 9, cortisol = 5. No fungal cover was seen in any fish in the "fish removed following the 50-day fasting period" group. All comparisons were analyzed using one-way ANOVAs. Bonferroni corrections include $\alpha = 0.04$ for fish removed before and during fasting period and $\alpha = 0.02$ for fish removed following 50-day fasting period. Dissimilar letters denote significant differences among treatment groups (Tukey–Kramer HSD test, $P < 0.01$ and $P < 0.01$.

with sham-treated and control fish (O'Connor et al., 2010). In short, it seems as though chronic cortisol elevations decrease the amount of stored energy available for subsequent response to stressors later in an animal's life. In addition, continual inter-renal activity will downregulate the HPI axis, which can attenuate the stress response during later stress-events (Barton, 2002) and can cause impaired inter-renal functioning (Hontela et al., '92; Laflamme et al., 2000). Further studies could reveal more insight to the role of chronic stress in HPI axis suppression, and the resulting physiological and energetic consequences.

Study Limitations

Cortisol implants elevated circulating cortisol to supraphysiological levels for at least 24 hr (i.e., maximum value in the wild: 1,172 ng mL^{-1}; maximum value after 24 hr: 2,834 ng mL^{-1}), but concentrations decreased to levels observed in fish chased to exhaustion by 4 days. Circulating cortisol concentrations decreased to baseline values by 30 days. Therefore, at 24 hr, our dose was verging on the high end of the physiological response to a stressor typical of bluegill sunfish. The cortisol concentrations used in this study followed doses used in a confamilial, the smallmouth bass (Dey et al., 2010). It appears that responses to cortisol dosages vary even among confamilial species. While supraphysiological, the results from this study provide insight into the physiological consequences of experimentally elevated cortisol levels and how those consequences manifest themselves following additional stressors throughout a fish's lifetime. This research revealed that there were indeed negative consequences associated with exposing fish to supraphysiological concentrations of cortisol. Further studies using physiologically relevant dosages would therefore be useful to determine whether a similar physiological stress response would yield similar results.

Fish condition decreased over 30 days in all treatment groups suggesting that holding wild bluegill sunfish in captivity was stressful in itself. Considerable mortality was noted in groups of fish held for 30 days, including control fish. Temperature data collected throughout the time-course and long-term experiments revealed that mortality began to occur around 16°C. Elevated water temperatures are generally associated with stress and incidences of parasites and pathogens (Gilhousen, '90; Harvell et al., 2002). However, we were still able to compare within experiments given that all fish in each experiment were exposed to

similar conditions and we did see differences among our treatment groups that were consistent among experiments.

Summary

In summary, our study can be viewed as an experimental manipulation set out to determine how fish respond to multiple stressors following a chronic cortisol elevation. The results from this study show that chronic cortisol elevation carries immediate (i.e., glucose elevation) and long-term physiological (i.e., lowering of energy stores) costs that make fish less able to withstand subsequent stressors. As a whole, these results suggest that fish exposed to sublethal stressors may experience lower survival in the short- and long-term if they encounter other challenges prior to full recovery or when recovery is apparent based on the metrics used to evaluate recovery. In the context of our work, the fish had apparently recovered (or at least cortisol was no longer elevated), which suggests that we observed a carryover effect (i.e., Harrison et al., 2010), a phenomenon that has rarely been explored or documented in fish. These findings clearly emphasize the importance of considering multiple and long-term effects of stress in wild fish populations, especially in the context of anthropogenic stressors, which are increasing. In other words, when fish are exposed to anthropogenic stressors such as a fisheries interaction or pollution, it is not possible to assume that the consequences of such stressors are immediately apparent, which has significant ramifications for the conservation and management of fish and other wild animals. However, additional work is needed to understand the potential compensatory mechanisms that may exist in wild populations in their natural environments that could moderate or obfuscate the effects of stressors.

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