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Effects of an experimental short-term cortisol challenge on the behaviour of wild creek chub *Semotilus atromaculatus* in mesocosm and stream environments

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The consequences of stress on the behaviour of wild creek chub *Semotilus atromaculatus* outside the reproductive period were studied using a single intra-coelomic injection of cortisol, suspended in coconut butter, to experimentally raise plasma cortisol levels. Behaviour between cortisol-treated, sham-treated (injected with coconut butter) and control *S. atromaculatus* was compared in a mesocosm system, using a passive integrated transponder array, and in a natural stream system (excluding shams), using surgically implanted radio transmitters. While laboratory time-course studies revealed that the cortisol injection provided a physiologically relevant challenge, causing prolonged (*c*. 3 days) elevations of plasma cortisol similar to that achieved with a standardized chasing protocol, no differences in fine-scale movements were observed between cortisol-treated, sham-treated and control *S. atromaculatus* nor in the large-scale movements of cortisol-treated and control *S. atromaculatus*. Moreover, no differences were observed in diel activity patterns among treatments. Differential mortality, however, occurred starting 10 days after treatment where cortisol-treated *S. atromaculatus* exhibited nearly twice as many mortalities as shams and controls. These results suggest that, although the experimental manipulation of cortisol titres was sufficient to cause mortality in some individuals, there were compensatory mechanisms that maintained behaviours (*i.e*. including activity and movement) prior to death. This study is one of the first to use experimental cortisol implants outside a laboratory environment and during the non-reproductive period and yields insight into how wild animals respond to additional challenges (in this case elevated cortisol) using ecologically meaningful endpoints.

Key words: corticosterone; movement; stream fishes.

INTRODUCTION

As the world's human population expands, increased demands on natural resources will lead to the degradation of ecological functions and conditions of natural landscapes (Vitousek, 1997; Foley *et al*., 2005). In the case of aquatic ecosystems,

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changes in adjacent land-use, particularly in the riparian zone, can alter watershed hydrology and biology, exposing wild fishes to a wide range of anthropogenic stressors known to influence fish distribution, abundance and community structure (Allan, 2004). Underlying changes in fish distribution and abundance is the assumption that environmental conditions have changed beyond some threshold that exceeds environmental tolerances and physiological capacity for a given species (Fry, 1947). As such, research has assessed, and will continue to evaluate, the physiological and endocrine responses of fishes when exposed to a variety of environmental stressors (Barton & Iwama, 1991; Wendelaar-Bonga, 1997).

In general, existing literature has broadly categorized fish stress responses into primary, secondary and tertiary responses. While primary responses mark the recognition of a stressor by the central nervous system and the subsequent neuroendocrine responses, secondary responses occur as a consequence of neuroendocrine responses which mediate alterations in metabolic pathways (Mazeaud *et al*., 1977). Although tertiary responses do not always occur in response to a stressor, these responses involve changes to whole-body activity and performance (Schreck, 1990). Up to a point, fish stress responses are thought to be adaptive and enable the animal to cope with imposed challenges (Schreck *et al*., 1997). While much is known about primary and secondary stress responses in artificial laboratory conditions, less is known about tertiary responses or the ecology of stress in wild fishes. In an effort to assess the whole-body effects of stress on fishes, research has begun to use the monitoring of an individual's physiological metrics (*e.g*. energy transfer, nutritional condition and metabolism) as indicators of an organism's performance within an environment in the context of environmental monitoring and assessment (Doherty *et al*., 2005; Gray & Munkittrick, 2005; Peterson *et al*., 2011). Physiological metrics are useful tools in assessing environmental effects on species as they control the degree to which an organism can acclimate to an environment and respond to various environmental challenges (Ricklefs & Wikelski, 2002; Wikelski & Cooke, 2006; Cooke & O'Connor, 2010). As natural systems become more degraded or threatened, monitoring physiological variables presents an opportunity to assess the effects of inhabiting an environment as well as to assess current and future rehabilitation and restoration efforts (Cooke & Suski, 2008).

A commonly used physiological metric when evaluating fish stress response is the measurement of circulating plasma cortisol concentrations (Wendelaar-Bonga, 1997). Attributed to the stimulation of the hypothalamic–pituitary–interrenal (HPI) axis, cortisol is a valuable indicator of stress in fishes as its release is triggered within minutes of an individual being exposed to a stressor, as a primary response, and cortisol can also be associated with immune function impairments following chronic elevation (Barton, 2002). Many studies interested in the consequences of stress in fishes have used experimental exogenous manipulation of cortisol titres, accompanied with monitoring of physiological variables, to assess how fishes respond to the challenge (Vijayan *et al*., 1996; DiBattista *et al*., 2005). In particular, the use of a single intra-coelomic or intra-muscular injection of cortisol in a coconut butter vehicle has been found to act as an effective method to evaluate chronic levels of plasma cortisol in fishes (Gamperl *et al*., 1994). As cortisol and coconut butter implants function by releasing hormone over an extended period (usually 3–5 days; Gamperl *et al*., 1994), challenging fishes with a physiologically relevant dosage of cortisol serves as a controlled means of examining how fishes respond to stress in the

environment. Although there have been many studies that have used experimental cortisol manipulations to study fish responses (Gamperl *et al*., 1994), there are few that have occurred outside the confines of a laboratory.

Outside laboratory experiments, exogenous manipulations of cortisol titres have been useful in assessing the effects of a short-term stressor on the growth and survival of wild fishes (O'Connor *et al*., 2011), the role of stress during parental care (O'Connor *et al*., 2009; Dey *et al*., 2010) and the carry-over effects of stress on wild fishes (O'Connor *et al*., 2010). Although cortisol manipulations have been limited in their field applications, the marriage between field manipulations of cortisol titres and effects-based assessment models provides a good opportunity to investigate the implications that stress may have on the ecology of wild fishes. Used to gain insight on the effects of land use on the physiological function of an organism (Doherty *et al*., 2005; Gray & Munkittrick, 2005), effects-based assessments can also be useful in assessing spatial and temporal movements of fishes. While variations in factors such as fish size, body condition and growth rate have been shown to affect fish mobility (Belica & Rahel, 2008), research has yet to examine the effects of an elevated cortisol challenge on the movements of wild stream fishes. In fact, behaviour (*e.g*. movement rates and activity levels) has rarely been used as an endpoint in examining how fishes respond to stressors in the wild, although in a laboratory context, there are a number of studies that have documented altered behaviour in fishes challenged *via* exogenous cortisol manipulation (Øverli *et al*., 2002; DiBattista *et al*., 2005; Schjolden *et al*., 2009) or exposure to acute or chronic stressors (Mesa, 1994; Schreck *et al*., 1997). Studies where cortisol titres have been experimentally manipulated in the wild typically have been performed during the reproductive period and have failed to observe alterations in behaviour (O'Connor *et al*., 2009; Dey *et al*., 2010; McConnachie *et al*., 2012). From a life-history theory perspective, it would be predicted that baseline behaviour would be maintained during reproduction in the face of stress to facilitate reproductive success at least for animals with few reproductive opportunities (*e.g*. short-lived with few spawning attempts or semelparous species; Sapolsky *et al*., 2000). Only one study, as far as is known, has evaluated the behaviour of fishes in the wild after cortisol injection outside of the reproductive period (O'Connor *et al*., 2010) and this study also failed to demonstrate differences in behaviour, except in the event of a secondary stressor (*i.e*. winter hypoxia) where cortisol-treated bass *Micropterus salmoides* (Lacépède 1802) exhibited less activity than control and sham-treated *M. salmoides* immediately prior to death. Movement studies play an important role in fish ecology as they enable researchers to gain insight on the spatial ecology of their focal species which can influence resource utilization (Lucas & Baras, 2000). Studies of this nature are valuable as they can be applied to a variety of systems and can provide understanding around the functional interactions between organisms and landscapes. Interestingly, fish movement and activity as ecologically relevant endpoints have rarely been used when studying fish stress unlike birds for which there is a rich literature on the topic (Wingfield, 2003).

This study sought to determine whether a short-term experimental cortisol challenge can influence the behaviour of wild fishes. A single exogenous cortisol implant was administered to experimentally raise circulating plasma cortisol values and mimic the cortisol-dependent physiological effects of the stress response observed in teleosts. It is important to note that such manipulations do not mimic the complete

stress response which in normal situations would be initiated after sensory perception of a perceived stressor and associated activation of the HPI axis (Wendelaar-Bonga, 1997). Similar to Fitzgerald *et al*. (1999), who used creek chub *Semotilus atromaculatus* (Mitchill 1818) as a sentinel species, this study used *S. atromaculatus* as a model species because they are robust, satisfied size requirements, occur in high numbers within the system, lack major fishing pressures, have easily measured life-history characteristics and exhibit rapid growth and maturation. Fish behaviour was evaluated using a complementary set of mesocosm and field experiments. The null hypothesis that there would be no difference in the behaviour of control, cortisol and sham-treated *S. atromaculatus* was tested. A mesocosm approach was used to investigate fine-scale movements (*e.g*. diel activity patterns) using a passive integrated transponder (PIT) antenna array (Binder & McDonald, 2008), while a field component with radio telemetry was used to assess large-scale movements of wild *S. atromaculatus*. Because past studies have related elevated cortisol levels to increases in variables such as blood pressure, energy use, food intake and immune function in the laboratory (Gregory & Wood, 1999; Barton, 2002; Lankford *et al*., 2005), and have identified that stress alters behaviour (Schreck, 1990), although typically not during the reproductive period (O'Connor *et al*., 2009; Dey *et al*., 2010), it was predicted that administering a short-term cortisol challenge outside the reproductive period would cause alterations in *S. atromaculatus* behaviour relative to shams and controls. This experimental approach to challenging fishes will help to elucidate the potential mechanisms by which stress can influence the ecology of stream fishes and to understand and predict the consequences of environmental change on wild animals (Tuomainen & Candolin, 2011).

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS AND CORTISOL TREATMENTS

Sampling of *S. atromaculatus* was performed under an Ontario Ministry of Natural Resources Scientific Collection Permit (Licence Number: 1061994). *Semotilus atromaculatus* were processed with adherence to the guidelines set out by the Canadian Council on Animal Care as issued by Carleton University (B10-9). All *S. atromaculatus* were captured using standard electrofishing techniques, with a battery-powered backpack electrofisher (Halltech Aquatic Research Inc.; model Ht-2000; www.htex.com). Pulsed-DC electrofishing is considered to be a safe methodological tool for studying *S. atromaculatus* and results by Gatz & Linder (2008) suggest this practice is unlikely to have meaningful biological effects on *S. atromaculatus* condition, growth and movements. As *S. atromaculatus* generally spawn in the spring, beginning at temperatures of 12·8° C (Scott & Crossman, 1973), sampling was undertaken outside this range to avoid any confounding effects of the reproductive period. *Semotilus atromaculatus* used in laboratory and mesocosm studies were transported by lorry in a 66 l cooler, equipped with four battery-powered aerators.

For all experiments, cortisol-treated *S. atromaculatus* were given a single intra-coelomic injection of $10\,\mathrm{mg\,ml^{-1}}$ of cortisol (hydrocortisone; Sigma H2882, Sigma-Aldrich; www.sigmaaldrich.com) suspended in coconut butter at $0{\cdot}005\,\mathrm{ml\,g^{-1}}$ body mass (Gamperl *et al*., 1994). Sham-treated *S. atromaculatus* were given a single intra-coelomic injection of coconut butter at $0{\cdot}005\,\mathrm{ml\,g^{-1}}$ body mass. Control *S. atromaculatus* received no injections. *Semotilus atromaculatus* were then separately placed in aerated recovery bins, according to their treatment and the experimental protocol. Anaesthesia was only used for implantation of radio transmitters and for cortisol injection of telemetered *S. atromaculatus*. No anaesthesia was used for PIT tag injection or cortisol (or sham) injection of *S. atromaculatus* that were

not being anaesthetized for transmitter implantation as per Canadian Council for Animal Care Guidelines.

LABORATORY STUDY

Blood sampling and cortisol assay

Blood (*c*. 0·2–0·4 ml) was collected *via* caudal puncture, using sodium-heparinized 1 ml syringes. Blood samples were preserved in water-ice slurries until centrifuged at 10 000 *g* for 6 min (Compact II Centrifuge, Clay Adams; www.bd.net). The resultant plasma samples were flash frozen in liquid nitrogen and stored at −80° C until further analysis. Concentrations of plasma cortisol were determined using a commercially available enzyme-linked immunosorbent assay kit in accordance with manufacturer specifications (Assay Designs, Kit #900-071; www.enzolifesciences.com). Sink *et al*. (2008) recommended this particular kit for cortisol detection relative to other commercially available brands.

Cortisol dosage validation

On 18 and 21 July 2010, blood cortisol levels for baseline *S. atromaculatus* at resting state were obtained by lethal blood sampling of wild *S. atromaculatus* ($n = 18$; total length, $L_T = 133{\cdot}7 \pm 5{\cdot}4$ mm; $27{\cdot}2 \pm 3{\cdot}3$ g; mean ± S.D.) within 2–3 min of capture *via* electrofishing from local area streams, using techniques described above. On 27 May 2011, plasma cortisol for individual *S. atromaculatus* exposed to a common stressor was determined by exposing wild *S. atromaculatus* outside of the reproductive period ($n = 8$; $131{\cdot}1 \pm 5{\cdot}7$ mm; $22{\cdot}0 \pm 3{\cdot}8$ g; mean ± S.D.) to a combination of chasing to exhaustion (*c*. 5 min) and air exposure (*c*. 30 s). Once they exhibited a loss of equilibrium and colour change (*i.e*. became pale), *S. atromaculatus* were placed in an aerated cooler for 30 min and terminally sampled for blood following cerebral percussion.

Using the mean maximal cortisol values generated *via* exhaustive exercise and chasing as a benchmark, *in vivo* laboratory experiments were subsequently employed to ensure that the cortisol manipulation elicited a response within the natural physiological range of stressed individuals. The cortisol dosage was validated by subjecting 36 *S. atromaculatus*, previously acclimated to an outdoor holding tank [*c*. 4290 l, for 1 week at Queen's University Biological Field Station in eastern Ontario, Canada (44° 31′ N; 76° 20′ W)], to one of the three cortisol treatments [*i.e*. control ($n = 12$; $137 \pm 32{\cdot}1$ mm; $32{\cdot}1 \pm 21{\cdot}7$ g; mean ± S.D.), sham ($n = 12$; $146{\cdot}5 \pm 40{\cdot}9$; $38{\cdot}8 \pm 29{\cdot}6$ g) and cortisol ($n = 12$; $132{\cdot}2 \pm 38{\cdot}3$ mm; $29{\cdot}5 \pm 22{\cdot}5$ g)]. *Semotilus atromaculatus* were fed (Nutrafin Max Sinking Pellets, Rolf C. Hagen Inc.; http://ca-en.hagen.com/Nutrafin/) daily and debris was removed from the tank using a siphon in an effort to maintain water quality prior to implantation. Following implantation, *S. atromaculatus* were individually placed in opaque experimental chambers (*c*. 10 l), receiving a constant flow of fresh Lake Opinicon water. After 3 days, the blood of *S. atromaculatus* was sampled as described above. As experiments were conducted outside, water temperatures fluctuated between 17 and 26° C.

MESOCOSM STUDY

Thirty *S. atromaculatus* that were captured from local area streams were acclimated to a *c*. 4290 l tank as above. On 22 June 2011, after the reproductive period, acclimated *S. atromaculatus* were assigned to one of the three treatment groups: (1) control ($n = 10$; $155{\cdot}9 \pm 6$ mm; $38{\cdot}9 \pm 4{\cdot}1$ g; mean ± S.D.), (2) sham ($n = 10$; $154{\cdot}5 \pm 6{\cdot}1$ mm; $38{\cdot}9 \pm 3{\cdot}6$ g) and (3) cortisol ($n = 10$; $150 \pm 6{\cdot}7$ mm; $36{\cdot}6 \pm 5$ g; no size differences among treatments, $P < 0{\cdot}05$). Without anaesthesia, *S. atromaculatus* were implanted with a uniquely coded PIT tag (Texas Instruments Radio Frequency Identification; www.ti.com; 23 mm, 0·6 g). Individuals were not anaesthetized for PIT tagging as the *S. atromaculatus* were gently restrained in the water (which itself had a calming effect) and a purpose-built PIT injector was used which enabled rapid tagging (5 s). Tags were injected into the coelom with a 10 gauge PIT injector needle off the ventral midline of each *S. atromaculatus* (Binder & McDonald, 2008). Movements were

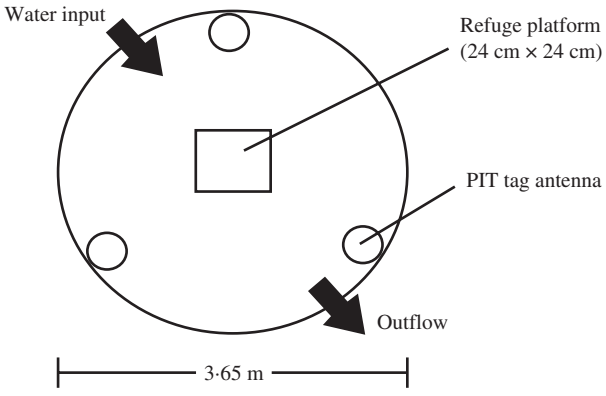

FIG. 1. Schematic diagram of the outdoor tank used for monitoring *Semotilus atromaculatus* during the mesocosm experiment. Three passive integrated transponder tag antennas, positioned equidistant apart, monitored small-scale movements around the tank and centralized refuge platform constructed of a raised patio block.

monitored for 20 days using three PIT antennas, equidistant apart, in an outdoor tank (3·65 m diameter, 41 cm depth, *c*. 4290 l; Fig. 1). The tank had a centralized refuge (24 cm × 24 cm) constructed from a single patio block while the substratum of the tank was covered in crushed white gravel. Gravel was present to simulate substrata commonly found in local streams to minimize the stress of being in an unfamiliar environment. Water temperature was monitored every hour using two iButton DS1921Z thermal loggers (Maxim Integrated Products, Inc.; www.maximintegrated.com; factory-stated resolution $\pm 0{\cdot}1^{\circ}$ C, accuracy $\pm 1^{\circ}$ C) encased in an inert synthetic plastic coating (Plasti Dip International; http://www.plastidip.com/) and was $21{\cdot}3 \pm 1{\cdot}4^{\circ}$ C (mean ± S.D.; range 17·9–26·4° C). Fresh water from Lake Opinicon was pumped into the tank daily for *c*. 15 min through a garden hose. In attempts to minimize human disturbance near the tank during experiments, *S. atromaculatus* were fed (Nutrafin Max Sinking Pellets) daily at approximately the same time and location although variations did occur. As the tank was located in a field, far from regular human activity, minimal effort was necessary to control for human disturbances.

Semotilus atromaculatus movements were quantified by the number of times an individual passed between two different PIT tag antennas (Binder & McDonald, 2008). Radio frequency identification (RFID) Half Duplex Reader with Antenna Multiplexer (Oregon RFID; www.oregonrfid.com) was used to record the date, time, antenna number (1–3) and PIT tag number for each *S. atromaculatus* passing an antenna. A Palm Pilot (model no. m505; Palm; www.hpwebos.com) was used to transfer data to a computer. Prior to analysis, data collected during the first 12 h of the study were removed in attempts to reduce the effects of the handling and tagging procedure.

FIELD STREAM TELEMETRY STUDY

Study site

Experimental procedures were conducted in Mosquito Creek (45° 16′ N; 75° 40′ W), a tributary of the Rideau River, located in Ottawa, Ontario, Canada (Fig. 2). While initially flowing through agricultural fields, Mosquito Creek travels alongside a developing suburban area where all sampling and tracking took place. The reach includes several road crossings, culverts and storm water inputs. For purposes of this study, land use within the study reach was classified as a developing urban area using ArcView GIS (version 10, Environmental Systems Research Institute; www.esri.com) and geospatial data mapping land use patterns of the National Capital Commission Region (City of Ottawa, 2005). Using a 50 m buffer and

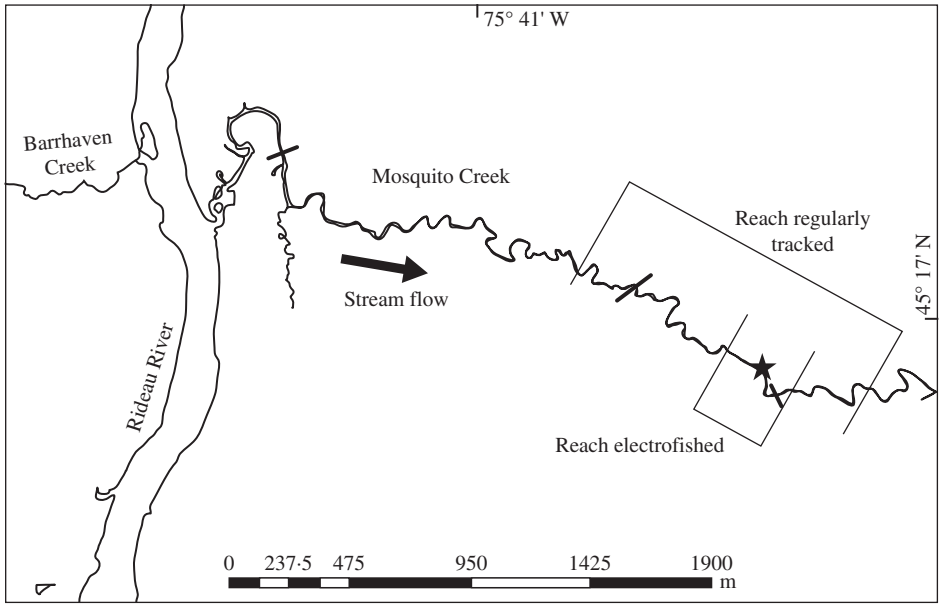

FIG. 2. Location of study site, Mosquito Creek (45° 16′ N; 75° 40′ W) in Ottawa, Ontario, Canada. ▬ intersecting the creek indicate roadways where water is passed through culverts. ★, where radio-telemetered *Semotilus atromaculatus* were released.

taking into account more recent developments not incorporated into data files, land uses within the reach were estimated to be: *c*. 57% urban or recreational, *c*. 28% agriculture, *c*. 9% forest and *c*. 6% other. Common riparian vegetation identified within the study area included sugar maple *Acer saccharum*, basswood *Tilia americana*, white ash *Fraxinus americana*, American elm *Ulmus americana*, ironwood *Carpinus caroliniana*, herbaceous vegetation and grasses. Along the entire reach, riparian vegetation alternated between areas dominated by deciduous trees, herbaceous vegetation and grasses.

Implantation of radio transmitters

On 1 and 2 November 2010, 15 and eight *S. atromaculatus* were captured, respectively, at Mosquito Creek at water temperatures of *c*. 5° C. *Semotilus atromaculatus* were individually anaesthetized in an induction bath of 70 ppm clove oil emulsified in ethanol (one part clove oil to 10 parts ethanol; Anderson *et al*., 1997). Once unresponsive, *S. atromaculatus* were placed on a moist C-notch sponge surgery platform in a supine position, where the gills were irrigated continuously with a recirculating maintenance bath of 20 ppm of clove oil in creek water. A small (15–20 mm) incision was made posterior of the pelvic girdle, slightly off the ventral midline, to implant a pulsed radio transmitter (Sigma Eight Inc.; www.grant.ca; model: Renata, 14 mm × 7 mm × 6 mm, 0·8 g, range of 1–3% of body mass, antenna length 15 cm, total pulse duration 5 ms, burst interval 3 s, life expectancy 42 days, frequency 148·32–149·08 Hz) into the coelomic cavity. Prior to surgery, tags were programmed to transmit between 0600 and 1800 h (*i.e*. daylight tracking hours) to maximize battery life. From an anterior direction, a 20 gauge needle was externally inserted in the right side of the posterior end of the incision to create a separate exit hole that the antenna wire was fed through (as described in Cooke *et al*., 2003). A blunt probe was used to ensure that there was no damage to the viscera upon insertion of the needle. Incisions were closed with two simple interrupted sutures, using monofilament absorbable suture material (Ethicon 3-0 PDS II, Johnson and Johnson; www.ethicon.com). All instruments, including radio transmitters, were disinfected using iodine solution. All surgery was completed within 5 min by the same

surgeon. While under anaesthesia, L_T and mass of all radio-tagged *S. atromaculatus* was taken to the nearest mm and g (no size difference among treatments, $P > 0{\cdot}05$) and for treatment, the cortisol was injected while the individual was anaesthetized. A roughly equal number of *S. atromaculatus*, from both sampling days, were assigned to one of the two treatment groups: (1) control ($n = 12$; $161{\cdot}9 \pm 5{\cdot}4$ mm; $40{\cdot}6 \pm 4$ g; mean $\pm$ S.D.) and (2) cortisol ($n = 11$; $160{\cdot}4 \pm 5{\cdot}6$ mm; $40{\cdot}6 \pm 5{\cdot}1$ g). Sham *S. atromaculatus* were excluded from this experiment as control *S. atromaculatus* also experienced surgery which was a necessary component of the study but which itself represented a short-term stressor (Lower *et al*., 2005). All *S. atromaculatus* were released in a central location along the sampling reach (45° 17′ 0·3·14″ N; 75° 40′ 0·8·55″ W).

Tracking methods

Portable receivers (SRX 600 and Biotracker, Lotek Wireless Inc.; www.lotek.com) were used to manually track radio-tagged *S. atromaculatus* from 3 to 30 November 2010. Tracking was conducted every second day by walking along the bank of the creek while scanning for all frequencies. When found, signal strength and gain reduction methods were used for fine-scale positioning of each *S. atromaculatus* (to calculate movement distances). Once positioned, Universal Transverse Mercator (UTM) co-ordinates (etrex, Garmin; www.garmin.com) were recorded for every individual. Detailed temporal positioning of *S. atromaculatus* occurred twice, on 22 and 30 November 2010. On both occasions, a sub-set of the same radio-tagged *S. atromaculatus* ($n = 13$; six controls and seven cortisol-treated) was positioned once between each of following time intervals: 0600–0900, 0900–1200, 1200–1500 and 1500–1800 hours.

STATISTICAL ANALYSIS

Prior to field application, a one-way ANOVA was used to compare plasma cortisol concentrations of treatments within the cortisol validation study against exhausted, air-exposed and baseline, resting state *S. atromaculatus*. Levene's test was used to evaluate equality of error variance and Kolmogorov–Smirnov tests to evaluate normality. Circulating plasma cortisol concentrations were square-root transformed to meet such assumptions (Zar, 1984). Type IV sum-of-squares methods were employed to account for the unequal sample sizes between treatments ($n_{control} = 8$, $n_{cortisol} = 9$, $n_{sham} = 9$, $n_{exhausted} = 7$ and $n_{baseline} = 18$; Shaw & Mitchell-Olds, 1993, Hanson *et al*., 2008). Differences among treatment groups were assessed using Tukey HSD *post hoc* tests of multiple comparisons to determine statistical significance between means.

During the field mesocosm study, *S. atromaculatus* movements were categorized as day or night-time movements using a government database on regional sunrise and sunset (http://www.nrc-cnrc.gc.ca/eng/services/hia/sunrise-sunset.html/). Daytime movements were characterized as occurring 1 h post-sunrise to 1 h pre-sunset, whereas the remaining movements were classified as night-time movements. Two-way repeated measures ANOVAs (main effects: treatment, average detections per time period, treatment × average detections per time period) were performed for the first 3 days post-injection and over the entire study period (*i.e*. 23–29 June 2011), taking into account variations in individual mortality rates (continuous covariate). Data from individuals dying within 7 days of the experiment were excluded from analysis. One-way ANOVAs followed by Tukey HSD *post hoc* tests were used to test for statistical significance between factors and interactions. Additionally, a Kaplan–Meier survival analysis log-rank test was performed to assess whether survivorship differed between treatment groups.

In the field telemetry study, distances travelled for each *S. atromaculatus* were calculated using a Network Analyst OD cost matrix in ArcMap (version 10, Environmental Systems Research Institute). Movements were tested for normality and homogeneity of variance using Kolmogorov–Smirnov and Levene's tests, respectively. The total distance travelled and total linear range were compared between treatment groups using general linear one-way ANOVAs, taking into account variability in detection rates across individuals (continuous covariate). As total distance travelled and total linear range did not meet the assumptions of normality, values were $\log_{10}$ and square-root transformed prior to analysis, respectively. Because the

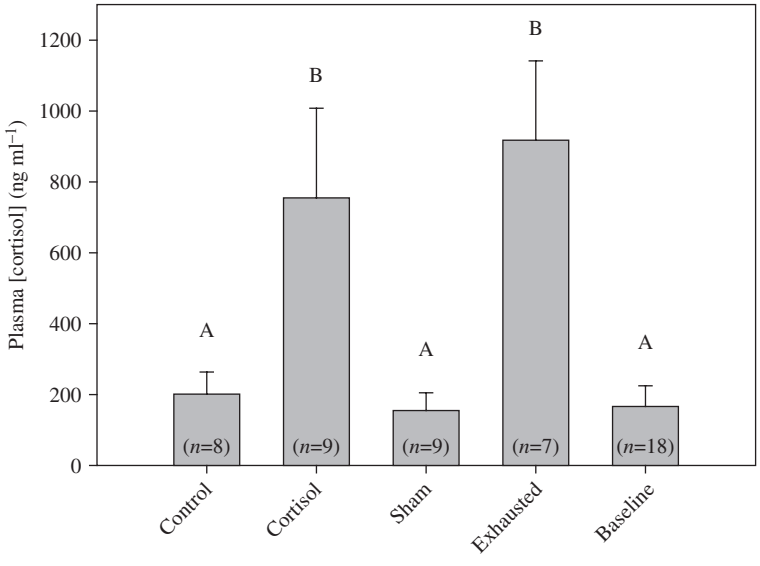

FIG. 3. Plasma cortisol concentrations (mean ± S.E.) of 3 day trial *Semotilus atromaculatus* (*i.e.* post-control, cortisol and sham treatments) compared to *S. atromaculatus* subjected to a standardized stress protocol (labelled as exhausted) and baseline *S. atromaculatus*. One-way ANOVAs, with square-root transformations were used to assess differences between treatment groups. Different upper-case letters between treatment groups indicate statistical difference ($\alpha = 0{\cdot}05$; n = sample size).

above cortisol validation study found that the time course of plasma cortisol elevation peaked at *c*. 3 days post-injection, non-parametric tests were performed to compare the total linear range during the first 3 days post-injection for each treatment. Additionally, non-parametric tests were used to evaluate the total distances travelled during the two 12 h tracking periods, for both cortisol-treated and control *S. atromaculatus*.

Statistical analysis was carried out using IBM SPSS Statistics 19.0 (IBM Corporation; www.ibm.com) with the exception of Kaplan–Meier survival analysis which was performed using JMP, version 7.0.1 (SAS Institute; www.sas.com). Statistical significances for all analyses were set at $\alpha = 0{\cdot}05$.

RESULTS

CORTISOL VALIDATION

During the three-day trial, cortisol injection had an effect on individual circulating plasma cortisol concentrations (Fig. 3 and Table I; ANOVA, $F_{4,50} = 9{\cdot}3$, $P < 0{\cdot}001$). More specifically, three days following cortisol injection, the plasma cortisol concentration of *S. atromaculatus* was similar to that of *S. atromaculatus* subjected to a standardized stress protocol (Tukey HSD, $P > 0{\cdot}05$), while cortisol-treated *S. atromaculatus* had significantly higher plasma cortisol concentrations than sham-treated (Tukey HSD, $P < 0{\cdot}05$), control (Tukey HSD, $P < 0{\cdot}05$) and baseline *S. atromaculatus* (Tukey HSD, $P < 0{\cdot}05$). Hence, an ecologically relevant physiological challenge was generated using the cortisol injection to mimic cortisol-dependent processes associated with the stress response.

TABLE I. ANOVAs results testing the effects of treatment on cortisol concentration (as part of the validation study) and behaviour (*i.e.* activity and movement) of *Semotilus atromaculatus*

		Time period							
		Three days				Entire study period			
Experiment	Factors	d.f.	SS	*F*	*P*	d.f.	SS	*F*	*P*
Cortisol validation	Whole model	4	2753·9	9·3	<0·001				
	Treatment	4	2753·9	9·3					
	Error	50	3681·0						
Mesocosm study	Average detections per time period	1	307·9	0·9	>0·05	1	26691·4	82·6	<0·01
	Average detections per time period × treatment	2	696·8	1·0	>0·05	2	630·4	1·0	>0·05
	Error	19	6678·4			19	6136·5		
Telemetry field study, total distance travelled	Whole model	2	3·6	7·0	<0·01	2	3·5E6	2·5	>0·05
	Number of detections	1	3·5	13·2	<0·01	1	2·4E5	4·2	0·05
	Treatment	1	0·07	0·2	>0·05	1	1·3E7	0·9	>0·05
	Error	17	4·4			18			
Telemetry field study, total linear range	Whole model					2	496·6	1·2	>0·05
	Number of detections					1	253·0	1·2	>0·05
	Treatment					1	167·5	0·8	>0·05
	Error					20	4275·4		

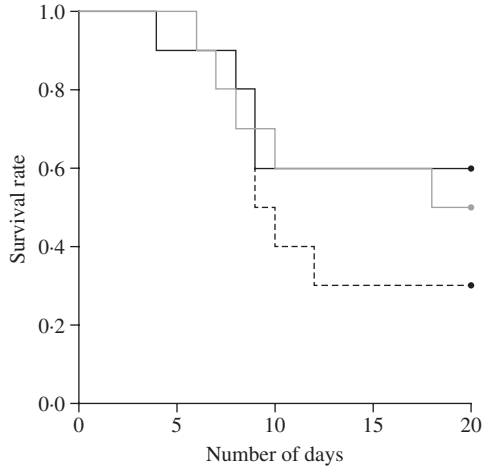

FIG. 4. Surviving *Semotilus atromaculatus* from control (——), cortisol (- - -) and sham (——) treatments during the mesocosm experiment between 22 June and 11 July 2011. There was no statistical effect of treatment on survival rate.

MESOCOSM STUDY

Throughout the 20 day monitoring period, 16 of the 30 tagged *S. atromaculatus* died. By the end of the experiment, mortality rates for the cortisol-treated *S. atromaculatus* were nearly twice that of the sham-treated or control *S. atromaculatus*, but there was no significant treatment effect on fish mortality rates (Fig. 4; survival analysis: log-rank test, $\chi^2 = 1{\cdot}373$, d.f. $= 3$, $P > 0{\cdot}05$). During the entire study period (*i.e.* 23–29 June 2011), while all *S. atromaculatus* were found to exhibit significantly more activity during daytime rather than night-time (Fig. 5 and Tables I and II; repeated-measures ANOVA, $F_{1,19} = 82{\cdot}6$, $P < 0{\cdot}05$), there was no effect of treatment on activity (Table II; repeated-measures ANOVA, $F_{2,19} = 1{\cdot}77$, $P > 0{\cdot}05$). Conversely, 3 days post-injection (*i.e.* 23–26 June 2011), *S. atromaculatus* activity was found to be consistent across diel periods (Table I; repeated measures ANOVA, $F_{1,19} = 0{\cdot}9$, $P > 0{\cdot}05$) and treatments (Table I; repeated measures ANOVA, $F_{2,19} = 1{\cdot}0$, $P > 0{\cdot}05$).

FIELD STREAM TELEMETRY STUDY

Cortisol treatment did not have any effects on the large-scale movements of adult *S. atromaculatus*. Over the course of the study (*i.e.* 3–29 November 2010), no significant difference was found between the total distances travelled (Fig. 6 and Table I; ANOVA, $F_{2,18} = 2{\cdot}50$, $P > 0{\cdot}05$) or the total linear range (Fig. 6 and Table I; ANOVA, $F_{2,20} = 1{\cdot}16$, $P > 0{\cdot}05$) of control and cortisol-treated *S. atromaculatus*, when using the number of detections as a covariate. Furthermore, treatment had no effect on the total distance travelled (Table I; ANOVA, $F_{1,17} = 6{\cdot}99$, $P > 0{\cdot}05$) or the total linear range (Kolmogorov–Smirnov Z, $D = 0{\cdot}418$, $P > 0{\cdot}05$) during the first two tracking sessions (*i.e.* first three days post-cortisol manipulation), when using the number of detections as a covariate. During the two 12 h tracking sessions, no difference was found between the total distance travelled for cortisol-treated and control

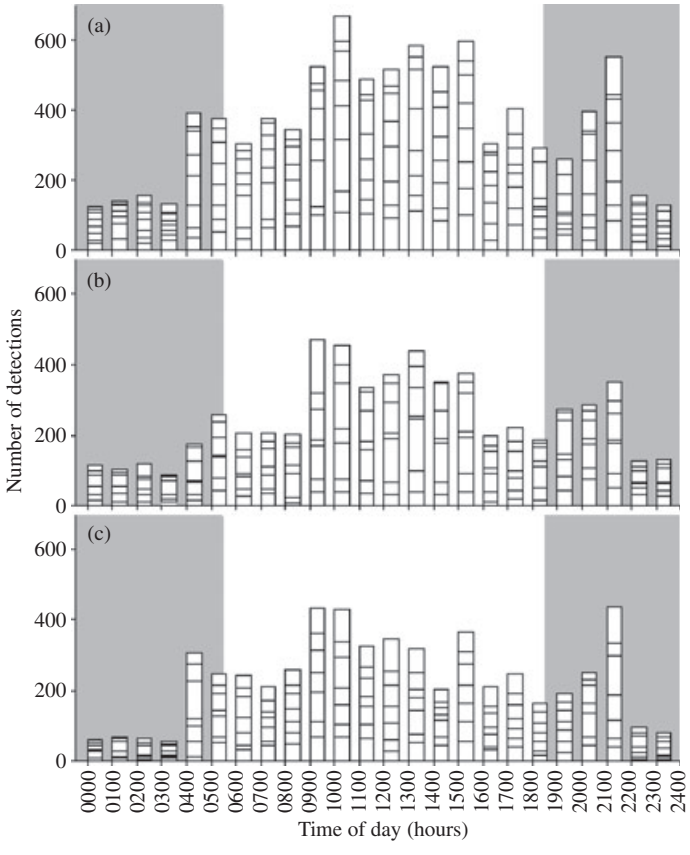

FIG. 5. Diel activity patterns of (a) control, (b) cortisol and (c) sham-treated *Semotilus atromaculatus* during mesocosm experiments between 23 and 29 June 2011. Sample sizes for each treatment group are included in Table I. Stacked bars indicate the individual contribution of each *S. atromaculatus* at each time period. Light and dark shading represents day and night-time, respectively.

S. atromaculatus on 22 November (Kolmogorov–Smirnov Z, $D = 0{\cdot}621$, $P > 0{\cdot}05$) or on 30 November 2010 (Kolmogorov–Smirnov Z, $D = 0{\cdot}833$, $P > 0{\cdot}05$). Of the *S. atromaculatus* positioned five or more times, control *S. atromaculatus* exhibited upstream movement (31%), downstream movement (38%) and non-movement (31%) at frequencies similar to cortisol-treated *S. atromaculatus* (upstream 34%, downstream 35% and non-movement 30%).

DISCUSSION

This study used mesocosm and field telemetry methods to examine the effects of an experimental short-term cortisol challenge on the behaviour of wild *S. atromaculatus* outside of the reproductive period. The use of the two approaches for studying behaviour enabled examination of both small-scale movements (*i.e.* activity patterns) under controlled conditions and large-scale movements within an urban stream, to provide a more complete picture of the behaviour of *S. atromaculatus*

TABLE II. Number of detections (mean ± S.E.) for control ($n = 8$), cortisol- ($n = 7$) and sham-treated ($n = 7$) *Semotilus atromaculatus* within the fine-scale, mesocosm study. *Semotilus atromaculatus* that died prior to 30 June 2011 were not included in these calculations

	Control		Cortisol		Sham	
	Day	Night	Day	Night	Day	Night
23 June 2011	63·1 ± 19·6	87·1 ± 17·5	35·7 ± 11·5	45·4 ± 19·8	33·4 ± 10·3	41·3 ± 13·9
24 June 2011	96·5 ± 19·0	61·5 ± 12·0	48·0 ± 10·3	47·7 ± 8·7	44·7 ± 12·0	46·1 ± 12·0
25 June 2011	121·7 ± 14·0	84·7 ± 12·2	97·9 ± 21·6	81·3 ± 17·7	61·3 ± 11·7	59·4 ± 3·7
26 June 2011	177·7 ± 19·2	49·1 ± 12·9	161·4 ± 39·7	44·3 ± 13·2	130·1 ± 19·2	34·3 ± 8·5
27 June 2011	166·9 ± 30·4	22·2 ± 3·5	141·4 ± 31·3	25·6 ± 5·3	151·7 ± 13·7	28·4 ± 6·1
28 June 2011	81·2 ± 19·0	16·4 ± 2·9	57·0 ± 10·4	17·3 ± 2·1	62·9 ± 4·5	19·1 ± 3·3
29 June 2011	55·7 ± 10·8	16·4 ± 4·2	47·3 ± 22·7	12·7 ± 5·8	66·7 ± 11·1	20·4 ± 5·2

when exposed to a stressor. Overall, exogenous manipulations of plasma cortisol concentrations did not alter *S. atromaculatus* locomotor activity relative to controls, despite the fact that these manipulations significantly raised circulating plasma cortisol concentrations during experiments that validated cortisol dosage. These results indicate that while cortisol manipulation was effective at emulating the cortisol-dependent aspects of a primary stress response among studied *S. atromaculatus*, it was unable to produce a detectable tertiary stress response within the study variables (*i.e.* behaviour), emphasizing the complicated relationship between physiological stress responses and behaviour. Moreover, there was no statistical evidence of treatment on mortality rate, which could further indicate that there were little to no long-term tertiary effects of the cortisol treatment. Mortality rates, however, were generally high for cortisol-treated *S. atromaculatus* (*c.* 70% mortality after 3 weeks in the mesocosm experiment), and nearly twice that observed for sham-treated and control *S. atromaculatus*.

INTRA-COELOMIC CORTISOL INJECTIONS, VALIDATIONS AND LIMITATIONS

The cortisol dose used to experimentally raise circulating plasma cortisol concentrations was based on values commonly used to induce a stress response in teleosts (10 mg ml^{-1} of cortisol, suspended in coconut butter, at 0·005 ml g^{-1} body mass; Gamperl *et al.*, 1994, although O'Connor *et al.*, 2009 used a low-dose cortisol treatment for *M. salmoides*). Analysis of pilot samples found this dosage to provide a physiologically relevant cortisol challenge as experimentally stressed *S. atromaculatus* had mean cortisol values similar to those *S. atromaculatus* chased to exhaustion. It is recognized that the post-injection and chase-induced cortisol values reported here for *S. atromaculatus* are five to 10 times higher than typical post-stress cortisol values for teleosts such as salmonids (Mommsen *et al.*, 1999; Barton, 2002). The confamilial and closely related European chub *Leuciscus cephalus* (L. 1758), however, also exhibited a similar massive cortisol elevation in response to standardized stressors (Pottinger *et al.*, 2000). Moreover, other work by the authors on *S. atromaculatus* from Illinois revealed similar post-stress values using the same assay as this (C. Suski, unpubl. data). The assay used in this study has been validated for other

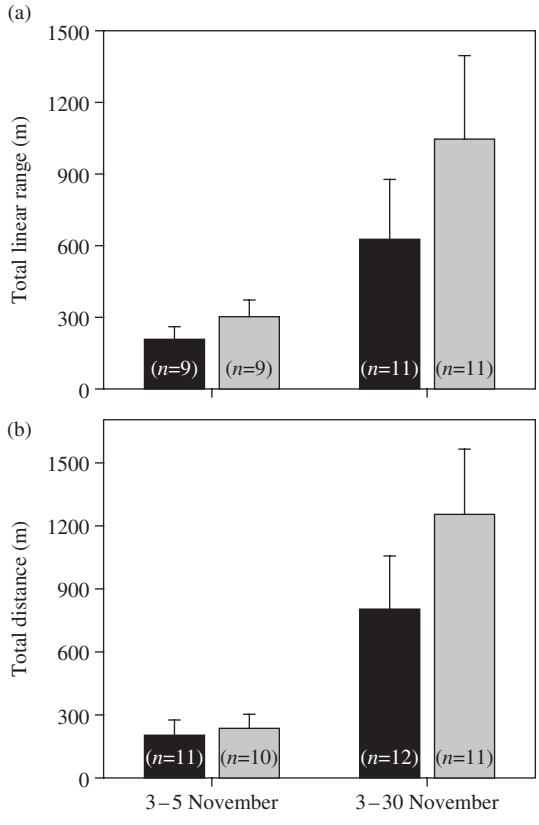

FIG. 6. Mean ± S.E. (a) total linear range and (b) total distances for telemetered *Semotilus atromaculatus* from control (■) and cortisol (□) groups 3 days post-injection (3–5 November 2010) and for the accumulative study period (3–30 November 2010). *n*, the number of individuals contributing to the mean of each distance. No significant differences were found between treatments.

teleosts (such as *M. salmoides*) which yielded values similar to those observed in *M. salmoides* from other laboratories (Gingerich & Suski, 2012). Also, the cortisol assays were run with appropriate intra and inter-assay controls to ensure accuracy and precision. It is therefore believed that the cortisol injection emulated a semi-chronic (*c*. 3–5 days) stress response (*i.e*. the cortisol-dependent component) similar to that expected during ecologically relevant events such as a drought, flood or periods of starvation. While sham treatments were used to account for the stress of cocoa butter injections and the associated immune response with the addition of foreign matter in the body cavity, Hoogenboom *et al*. (2011) report that cocoa butter implants can act as a stressor, reducing the growth, egg and hatching size in female brown trout *Salmo trutta* L. 1758. Unfortunately, sham treatments for the field telemetry component were not possible. Control *S. atromaculatus*, however, were also exposed to surgery (including anaesthesia, laparotomy and wound closure) which is more invasive than the injection. As such, the only difference between controls and treatments in the telemetry study was the presence of the cocoa butter and cortisol rather than the injection procedure itself.

Mortality was associated with all treatments in the mesocosm study and, more so than observed, in the field study. Although efforts were made to minimize handling stress, experiments were conducted on wild *S. atromaculatus* that were captured and transported to a research facility. Moving wild *S. atromaculatus* to captivity is not easy and fungus was evident on *S. atromaculatus* in the mesocosm. O'Connor *et al*. (2009) noted that saprolegnian fungal infections were greater in cortisol-treated *M. salmoides*. Although not statistically different, the fact that cortisol-treated *S. atromaculatus* had mortality levels nearly twice that of control and sham *S. atromaculatus* indicates that the cortisol treatment did appear to increase mortality. Moreover, most *S. atromaculatus* that died did exhibit signs of saprolegnian infections suggesting the potential that mortality was mediated by immune function impairments. Nevertheless, while these experimental limitations must be acknowledged, the results obtained from this study provide an accurate assessment of the long-term cortisol-dependent effects of cortisol administration on the behaviour of wild stream fishes.

It is important to recall that although administration of cortisol mimics the cortisol-dependent processes associated with stress response, it does not mimic the sensory aspects or the neuro-endocrine activation of the HPI axis. For that reason, interpretation must focus on the cortisol-dependent processes and recognize that other components of the response that are probably not influenced by experimental cortisol manipulations (*e.g*. adrenalin; Reid *et al*., 1998) are not addressed by this study design. Nonetheless, this approach has and continues to be widely used for the study of stress in animals, particularly fishes (Gamperl *et al*., 1994), with the recognition that some important components of the stress response are not integrated into such experimental approaches. In this study, however, adrenaline levels were presumably not increased through the administration of cortisol, beyond acute capture- and handling-induced stressors applied to all treatments, which may have failed to account for an important mediator of locomotor activity. Adrenaline is well known to influence locomotion both in an acute sense (fright or flight) and chronically (Reid *et al*., 1998). The one fish study that has examined behaviour of *M. salmoides* following administration of cortisol in the wild noted some differences in locomotor activity relative to control and sham-treated fish but only when exposed to an environmental challenge (O'Connor *et al*., 2010). In the laboratory using hatchery-reared rainbow trout *Oncorhynchus mykiss* (Walbaum 1792), Øverli *et al*. (2002) noted that short-term cortisol administration (*via* feed) resulted in elevated locomotor activity while longer-term treatments inhibited locomotion. Although cortisol itself may influence behaviour even in the absence of a traditional stress response beginning with the sensory system and the release of adrenaline and a suite of other neuro-endocrine changes, the findings of O'Connor *et al*. (2010) do support the notion that a complete normal activation of the HPI may be needed to elicit a change in behaviour. Conversely, the findings from Øverli *et al*. (2002) suggest that such neuro-endocrine pathways are not required to elicit locomotor alterations. Clearly additional research is needed on this topic. Although the exact mechanisms by which cortisol may alter fish locomotion are poorly understood, there is evidence from other taxa that locomotion is indeed influenced by cortisol manipulation (*e.g*. rats, Sandi *et al*., 1996; birds, Breuner *et al*., 1998). For now, and given the outcome of this study, it is suggested that the relationship between cortisol elevation and locomotion requires further testing and that overall there is rather small burden of evidence on which to base interpretation.

FINE-SCALE ACTIVITY WITHIN MESOCOSM

In general, there were no meaningful differences in locomotor activity among control, cortisol and sham-treated *S. atromaculatus*. During the first 3 days of the experiment, however, individuals from all treatment groups exhibited altered feeding patterns, as *S. atromaculatus* previously observed to readily feed during the pre-treatment acclimation period had reduced food intakes. As the experiment progressed, the number of *S. atromaculatus* observed feeding increased, although whether treatment affected the rate in which an individual returned to regular feeding behaviour was untested. Similar short-term behaviours shared among treatment groups may suggest that these results more accurately represent the effects that tag insertion and its associated handling stress have on wild *S. atromaculatus* and consequently mask the short-term effects of each treatment. Furthermore, as anaesthetic was not administered prior to treatment and tag insertion, handling procedures would have activated the HPI axis and caused the subsequent release of cortisol prior to the onset of treatment effect (Small, 2003). Anaesthesia is itself not without physiological consequences (Pirhonen & Schreck, 2003) and given the warm water conditions at the time of PIT tagging, there was concern regarding post-anaesthetic recovery. Handling and injection were quite rapid (<30 s total) and were conducted in water, an approach used in other tagging studies to minimize handling and tagging-related stress (Cooke *et al*., 2005). Most importantly, cortisol injection would have elevated cortisol titres in cortisol-treated *S. atromaculatus* which would have been consistently high and less transient than the stress associated with short-term handling.

LARGE-SCALE ACTIVITY

Exogenous cortisol manipulations, with paralleled control groups, were found to have no effect on the large-scale movements of wild *S. atromaculatus*, in an urban stream, as evidenced by treatment groups moving similar distances throughout the study. Owing to the *a priori* variability of cortisol responses found among sham-treated *S. atromaculatus* within the mesocosm study, sham treatments were not included within this portion of experimentation. Similarly, DiBattista *et al*. (2005) argued that sham treatments have a tendency to produce unpredictable cortisol responses and, as a consequence, create complicated interpretations as the distinction of cortisol values between treatment groups become less definitive. Nevertheless, the lack of variation between the large-scale movements of cortisol-treated and control *S. atromaculatus* was surprising as past research has found that elevated blood cortisol values significantly increase blood pressure and energy usage as well as reducing food intake and immune function (Wendelaar-Bonga, 1997). Although only loose connections have been suggested between the effects of blood pressure, energy usage, food intake and immune function on fish movements, it was expected that these effects would have a large enough effect to reduce the mobility of cortisol-treated *S. atromaculatus*. The equivocal results may suggest an adaptive benefit of being able to modulate behavioural responses to physiologically and ecologically relevant levels of stress. Thresholds and feedbacks that determine when physiological stress will elicit a behavioural response are currently unclear and require further study.

As found in other studies (Skalski & Gilliam, 2000; Belica & Rahel, 2008), *S. atromaculatus* exhibited leptokurtic movement distributions, high turnover rates (*i.e*. proportion of population that moves) and low displacement distances

(*i.e.* distance that each individual moves). As characteristic of leptokurtosis, a large proportion of *S. atromaculatus*, from both treatment groups, were found to remain within particular sections of the creek at relatively close proximity (*c.* 600 m) to the release site. Occasionally, individuals moved larger distances (*c.* 1–2 km) between consecutive tracking periods; however, few *S. atromaculatus* were found to be consistently mobile. Thus, *S. atromaculatus* behaviour (of both control and cortisol-treated *S. atromaculatus*) was apparently normal, although leptokurtotic movements may have impeded detection of treatment-level effects. As such, use of other model species including those that are consistently highly mobile as well as those that are consistently highly sedentary (*i.e.* rarely if ever engaging in the movements observed in this study) would be useful for understanding the consequences of stressors on behaviour. Another approach would be to conduct experiments in a before-after-control-impact design where individual behavioural modes (*e.g.* movers *v.* stayers) are measured prior to manipulating treatment fishes. Observationally, *S. atromaculatus* locations independent of treatment were closely associated with patches of habitat offering refuge in the form of backwater, plunge and lateral scour pools, containing structures such as woody debris, in-stream rock and macrophyte beds, consistent with previous studies of *S. atromaculatus* habitat use (Belica & Rahel, 2008). Differences in habitat use among treatments were not quantitatively explored in this study but this would be worthwhile in the future.

FUTURE DIRECTION

As far as is known, this study represents the first attempt to test whether an experimental short-term cortisol challenge has an effect on the small and large-scale movements of wild, adult stream fishes. Overall, these results provide evidence indicating that exogenous cortisol manipulations do not significantly affect behaviours related to locomotor activity, providing further support to the idea that the relationship between physiological stressors and behaviour is complex (Cabanac, 2011) and responses are often non-intuitive (Wingfield, 2003; Tuomainen & Candolin, 2011) in vertebrates (mostly birds and mammals). This finding is particularly interesting, given that there are few studies of how experimental cortisol elevation influences behaviour of fishes outside of the reproductive period. All known studies that involve cortisol manipulation prior to or during reproduction have failed to document behavioural differences relative to controls (O'Connor *et al.*, 2009; Dey *et al.*, 2010). Only a study by O'Connor *et al.* (2010) noted reductions in movement following cortisol injection, but that occurred in *M. salmoides* in the winter, well outside of the reproduction period, and immediately prior to total mortality associated with a winter-kill event. The absence of variation between control, cortisol and sham-treated *S. atromaculatus* in this study may suggest that compensatory mechanisms exist that enable cortisol-treated *S. atromaculatus* to behave similarly to their experimental counterparts, even outside the reproductive period. That finding is somewhat notable as life-history theory would predict that fishes that are reproductively active should maintain behaviour in the face of stressors (Sapolsky *et al.*, 2000), but not so for fishes outside the reproductive period. While compensatory mechanisms can be adaptive in that they can help an individual operating with an allostatic load to survive a stressful event, they can also be maladaptive as they tend to have negative implications on longer-term necessary life processes (Schreck *et al.*, 2001). In

particular, a potential trade-off between reproductive success, in terms of gamete or progeny quality, for potential survival has long been identified as a possible consequence of compensatory mechanism (Pickering *et al*., 1987; Schreck *et al*., 2001; Ostrand *et al*., 2004). While this study did not assess the fecundity of treatment in *S. atromaculatus*, it is important to note that, although exogenous manipulations did not affect behaviour, other whole-body effects of cortisol elevations are possible (*e.g*. metabolic rate, immune function and reproductive endpoints). In addition, it is acknowledged that there are other behavioural endpoints beyond those used here including feeding and predator avoidance that also deserve study in the context of organismal stress. Given the short-term basis of this study and the single stressor, clearly, there is a need for additional research that extends across multiple seasons and years, with a particular focus on understanding the consequences of single *v*. multiple stressors (both sequential and simultaneous) and their interactions (*e.g*. synergistic *v*. cumulative).

As indicated by their ability to inhabit streams throughout a range of different landscape patterns, *S. atromaculatus* are known to be relatively robust, with a wide distribution (Scott & Crossman, 1973). The results speak for the resilience of *S. atromaculatus* as it was found that their behaviour and movement remain unaffected when given an additional exogenous cortisol challenge. Considerable mortality was observed for cortisol-treated *S. atromaculatus* in the mesocosm study, however, suggesting that there was a cost associated with the cortisol treatment. While the mean circulating plasma cortisol concentrations for stress-induced (cortisol treatment and chasing) and baseline *S. atromaculatus* were similar to values reported in *L. cepalus*, cortisol values in this study were found within the range known to cause immunosuppression as well as inhibition of growth and reproduction in salmonids (Pottinger *et al*., 2000). Comparative analyses performed by Pottinger *et al*. (2000) indicate that while the number of total binding sites in gill tissue are similar between Salmonidae and *L. cepalus*, the affinity of binding sites for cortisol are eight-fold higher in Salmonidae. This low cortisol receptor affinity in *L. cepalus* may help to explain the lack of response found among the exogenous cortisol manipulations. Future experiments should focus on evaluating other sublethal metrics (*e.g*. metabolic rate, energy metabolism and lymphocytes) related to organismal condition and in understanding the mechanisms that led to mortality. There is a need for additional studies that use ecologically relevant endpoints such as behaviour and habitat use to study the effects of stressors on wild fishes and other vertebrates.

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