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RESEARCH ARTICLE

Short-term and long-term effects of transient exogenous cortisol manipulation on oxidative stress in juvenile brown trout

Kim Birnie-Gauvin[1,2,3,*], Kathryn S. Peiman[1], Martin H. Larsen[3,4], Kim Aarestrup[3], William G. Willmore[2] and Steven J. Cooke[1]

ABSTRACT

In the wild, animals are exposed to a growing number of stressors with increasing frequency and intensity, as a result of human activities and human-induced environmental change. To fully understand how wild organisms are affected by stressors, it is crucial to understand the physiology that underlies an organism's response to a stressor. Prolonged levels of elevated glucocorticoids are associated with a state of chronic stress and decreased fitness. Exogenous glucocorticoid manipulation reduces an individual's ability to forage, avoid predators and grow, thereby limiting the resources available for physiological functions like defence against oxidative stress. Using brown trout (*Salmo trutta*), we evaluated the short-term (2 weeks) and long-term (4 months over winter) effects of exogenous cortisol manipulations (versus relevant shams and controls) on the oxidative status of wild juveniles. Cortisol caused an increase in glutathione over a 2 week period and appeared to reduce glutathione over winter. Cortisol treatment did not affect oxidative stress levels or low molecular weight antioxidants. Cortisol caused a significant decrease in growth rates but did not affect predation risk. Over-winter survival in the stream was associated with low levels of oxidative stress and glutathione. Thus, oxidative stress may be a mechanism by which elevated cortisol causes negative physiological effects.

KEY WORDS: Antioxidants, *Salmo trutta*, Glucocorticoids, Glutathione, Oxidative ecology, Reactive oxygen species

INTRODUCTION

Wild animals are constantly exposed to intrinsic and extrinsic stressors, which arise from anthropogenic (e.g. climate change, habitat disturbances) and natural (e.g. predation, social interactions, disease and nutritional limitations) sources (Johnstone et al., 2012; Boonstra, 2013b), and challenge their homeostatic balance. To fully appreciate how organisms in the wild are affected by stressors, it is crucial to understand the physiology that underlies an organism's response (Baker et al., 2013; Dantzer et al., 2014). Although there has been much work on this topic, most has been done in laboratory settings (reviewed in Barton and Iwama, 1991; Barton, 2002; Sopinka et al., 2016), and much less is known about how wild animals in their natural environment respond to different stressors (Boonstra, 2013a). The neuroendocrine system is responsible for translating environmental signals into physiological and behavioural responses (Denver et al., 2009). Understanding these basic underpinning concepts is crucial to discern the links between stressors and their impacts on behaviour, survival and life-history trade-offs (Ball and Balthazart, 2008; Denver et al., 2009).

Stressful events lead to the activation of the hypothalamic-pituitary–interrenal (HPI) axis in fish. Circulating levels of glucocorticosteroid (GC) hormones such as cortisol increase rapidly, followed by the mobilization of fatty acids and liver glycogen to provide energy resources to cope with the stressor (Barton, 2002; Mileva et al., 2009). The purpose of this complex cascade of events is to re-establish homeostasis and it initiates both physiological and behavioural responses (Barton, 2002). However, prolonged elevation of cortisol can have detrimental effects, such as the suppression of the immune response (reviewed in Sapolsky et al., 2000; Tort, 2011), and make individuals more susceptible to predation (Tort, 2011). Cortisol causes the diversion of resources from other activities to enable individuals to focus on survival, having important consequences on behaviour and, ultimately, life-history trade-offs (Wingfield et al., 1998).

Stressful conditions generate important ecological pressures, modulating adaptive responses in natural populations (Romero, 2004). In recent years, much attention has been given to the role of redox chemistry in the context of life-history theory (Metcalfe and Alonso-Alvarez, 2010; Speakman et al., 2015), with growing interest in the study of oxidative stress in an ecological context (i.e. oxidative ecology; Beaulieu et al., 2013). Oxidative stress occurs as a result of an imbalance between pro-oxidants and antioxidants (Sies, 1991). Reactive oxygen species (ROS) are pro-oxidants that are produced as a result of aerobic metabolism and are normally quenched by antioxidant defences. When this does not happen, the remaining ROS negatively impact the cell and result in oxidative stress (Monaghan et al., 2009; Metcalfe and Alonso-Alvarez, 2010). If maintained, this imbalance leads to oxidative damage, including severe damage to most macromolecules (i.e. DNA, RNA, proteins and lipids; Asada and Takahashi, 1987), decreased fertility (Halliwell and Gutteridge, 1999) and accelerated cellular ageing, all of which are accompanied by a decrease in survival probability (Haenold et al., 2005; Monaghan et al., 2009). We used the oxidation of thiols in glutathione as a measurement of oxidative stress levels. Though this is not a measure of oxidative damage, it provides a measurement of the occurrence of oxidative stress (Jones, 2006; Sohal and Orr, 2012). ROS production and an animal's capacity to fight oxidative stress vary depending on developmental stage, ecological conditions and life-history strategy, making the study of oxidative stress, in an ecological context, highly relevant (reviewed in Metcalfe and Alonso-Alvarez, 2010; Beaulieu et al., 2013; Costantini et al., 2008a,b; Trivelpiece et al., 2011).

[1]Fish Ecology and Conservation Physiology Laboratory, Department of Biology and Institute of Environmental Science, Carleton University, 1125 Colonel By Drive, Ottawa, ON, Canada K1S 5B6. [2]Department of Biology and Institute of Biochemistry, Carleton University, 1125 Colonel By Drive, Ottawa, ON, Canada K1S 5B6. [3]DTU AQUA, National Institute of Aquatic Resources, Section for Freshwater Fisheries Ecology, Technical University of Denmark, Vejlsøvej 39, Silkeborg 8600, Denmark. [4]Danish Centre for Wild Salmon, Brusgårdsvej 15, Randers 8960, Denmark.

*Author for correspondence (kbir@aqua.dtu.dk)

K.B., 0000-0001-9242-0560

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Glucocorticoid circulation may change depending on an individual's current life-history trajectory, as animals energetically invest more in aspects of their life histories that contribute most to fitness (Ricklefs and Wikelski, 2002). Elevated levels of GCs can reduce an individual's ability to forage, avoid predators and grow (Wingfield et al., 1998), therefore limiting the energetic resources available for defence against and repair of oxidative damage. Studies that manipulate circulating levels of GCs via exogenous manipulations are becoming increasingly common, not only to understand fundamental aspects of organismal function but to also understand the ecology of stress in wild animals (Sopinka et al., 2015; Crossin et al., 2016). Elevated levels of GCs have been suggested to increase oxidative stress via an elevation in metabolic rate, which causes an increased flux of electrons at the level of the electron transport chain (Wingfield et al., 1998; Roussel et al., 2004). GC administration also causes increased lipid peroxidation and decreased total antioxidant capacity (Behl et al., 1997; Orzechowski et al., 2002). Because of the increased catabolic activity, uncoupling and proton leak that may result from GC manipulation (Wingfield et al., 1998; Roussel et al., 2004), ROS production and oxidative stress levels should increase when GCs are manipulated (reviewed in Costantini et al., 2011). Additionally, these effects are dependent on the duration of treatment, as long-term GC manipulation generally showed larger effects on oxidative stress (Costantini et al., 2011). To date, however, only a few studies have investigated the link between GC manipulation and oxidative status, and they focused on avian and mammalian taxa (reviewed in Costantini et al., 2011) over short-term periods (days to weeks; Alonso-Alvarez et al., 2004; Costantini, 2008). Fewer studies have focused on oxidative stress in wild fish (Taylor et al., 2015).

Brown trout (*Salmo trutta*) are a semi-anadromous salmonid species native to many regions of Europe (MacCrimmon et al., 1970). Groups of brown trout form two subpopulations: anadromous (i.e. migratory; sea trout) and resident (i.e. non-migratory) individuals, both originating from the same parents (Jonsson and Jonsson, 1993). Brown trout can be implanted with small passive integrated transponders (PIT tags) to uniquely identify recaptured individuals to enable repeat sampling of individuals (e.g. for growth or oxidative status), or to estimate mortality (see Gibbons and Andrews, 2004) by scanning bird colonies, for example. During early life stages (e.g. juveniles), when all fish are in stream environments, fish can be easily captured with electrofishing as part of mark–recapture protocols. Here, we used an experimental approach, comparing oxidative status, growth and survival among a control group, a sham group and a group that received an intracoelomic injection of cortisol. The protocol we used consisted of implanting a cortisol-bearing vehicle to transiently elevate cortisol levels, an approach commonly used in fish (Gamperl et al., 1994; Sopinka et al., 2015). Though a single (transient) exogenous manipulation of cortisol is a common method for studying 'stress', it fails to fully emulate a stress response per se in that it does not include the process of the organism perceiving a stressor and the associated neuroendocrine cascade (Sopinka et al., 2015). Nonetheless, this approach does have merit for testing the effects of experimental elevation of GCs on organismal biology (Sopinka et al., 2015; Crossin et al., 2016). Given that increased GC levels have been shown to increase metabolic rate and may reduce the availability of resources to fight oxidative stress, we predicted that growth rate will be lower and predation higher in fish manipulated with cortisol relative to control and sham treatment groups. The intrinsic energetic reserves of an individual should affect their ability to deal with pro-oxidants or maintain antioxidants (e.g. Morales et al., 2004; Pedernera et al., 2010), and so we predicted a negative relationship between condition/growth rate and oxidative stress and a positive relationship between condition/growth rate and antioxidants. We also predicted that oxidative stress levels and antioxidant capacity will be higher in fish injected with cortisol relative to the control and sham treatment groups in the short term (herein defined as 2 weeks). We further predicted that these effects will not be maintained in the long term (herein defined as 4 months), given that the manipulation we used results in a transient increase in circulating plasma cortisol levels. This study is among the first of its kind to explore the link between cortisol and oxidative stress in a wild population of fish.

MATERIALS AND METHODS

Study location

The Gudsø stream is located in east-central Jutland, Denmark. The stream runs through agricultural land over approximately 16 km, and several tributaries flow into the main stem, before reaching the sea at Kolding Fjord (Fig. 1). The stream has natural populations of semi-anadromous brown trout (*Salmo trutta* Linnaeus), eel (*Anguilla anguilla*) and lamprey (*Lampetra planeri*).

Fish sampling and tagging

Fish were captured in the main stem of the Gudsø stream, starting 2000 m from the mouth of the stream and continuing upstream for approximately 2500 m (Fig. 1A, inset) from 20 to 25 October 2015. All trout greater than 120 mm in length were captured using single-pass electrofishing gear (Stampes Elektro A/S, Ringkøbing, Denmark) and placed in a 60 l container of fresh stream water (~50 fish per container for less than 1 h). The water was changed continually (approximately every 15–20 min) until processing. A total of 793 juvenile brown trout were captured. Fish were placed in a solution of 0.03 g l^{-1} benzocaine until their opercular rate had slowed significantly and they were unresponsive to external stimuli

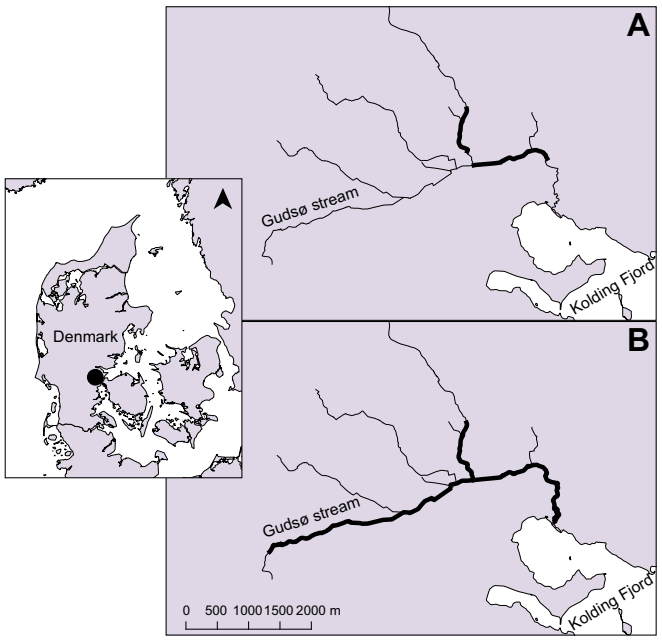

Fig. 1. Map of Gudsø stream, Denmark. The location of the stream is indicated by a black circle in the inset. Sampling locations are highlighted by the thick black trace (A) for initial and 2 week capture locations in October and November 2015, and (B) for overwinter capture locations in February/March 2016.

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(usually less than 3 min). Total length (±1 mm) and wet mass (±0.1 g) were measured for individual fish. Fish were then tagged with a 23 mm PIT tag (RI-TRP-RRHP, 134 kHz, 0.6 g mass in air; Texas Instruments, Plano, TX, USA) inserted into their body cavity. Larsen et al. (2013) demonstrated that the retention of these tags in Atlantic salmon (*Salmo salar*) was 97% with no effects on mortality rate and growth. A condition factor (K) was calculated using Eqn 1.

$$K = \left(\frac{\text{mass}}{\text{length}^3}\right) \times 100. \quad (1)$$

Blood sampling and cortisol treatment

Blood samples of 0.1 ml were obtained from the caudal vasculature of individual fish using a 1.5 inch 25 gauge heparinized needle. Within 10 min of sampling, blood was centrifuged at 6000 rpm for 2 min in the field (samples were kept on ice meanwhile), after which plasma was separated from red blood cells (RBCs). RBCs were flash-frozen in liquid nitrogen and later placed at −80°C. Fish were then randomly assigned to control (n=426), sham (n=282) or cortisol (n=276) treatment groups. Cortisol fish received an intracoelomic injection of hydrocortisone 21-hemisuccinate (Sigma-Aldrich, St Louis, MO, USA) suspended in vegetable shortening (100% vegetable shortening, Crisco, OH, USA) using a dosage of 100 mg kg^{-1}. This dosage has been validated to elevate circulating baseline plasma cortisol levels in juvenile brown trout for at least 9 days post-treatment: at day 3, levels are approximately 900 ng ml^{-1}; at day 6, they decrease to approximately 400 ng ml^{-1}; and at day 9, levels are approximately 200 ng ml^{-1} – all of which were higher than those in controls and shams (K.B.-G., K.S.P., M.H.L., K. M. Gilmour, K. Aarestrup and S.J.C., in review). Though cortisol values at day 3 were beyond the physiological levels seen in fish, average values were within the range of stress-induced responses (20–500 ng ml^{-1}) by day 6 (Gamperl et al., 1994). The validation study used the same population and the same products (cortisol and vehicle) as we used here, and similar methods for elevating cortisol have been used in several other studies of the same trout population (Midwood et al., 2014, 2015, 2016). We therefore did not measure individual cortisol levels in the current study as the objective was to investigate average treatment effects. Sham fish received the same injection of vegetable shortening, with no cortisol; control fish were left undisturbed. Fish from all treatments were allowed to recover in a 60 l container of fresh stream water, where cortisol fish were kept separate from control and sham fish. Fish were then released at their site of capture within the stream. These standardized techniques were approved by the Danish Animal Experiments Inspectorate (licence number: 2012-DY-2934-00007).

Resampling of fish

To evaluate the short-term effects of cortisol, fish were resampled from Gudsø stream from 5 to 7 November 2015 using the same techniques as described above. All captured fish were scanned for PIT tags. A total of 80 controls, 95 shams and 99 cortisol-treated fish were recaptured and resampled, after which sampling efforts were stopped. Tagged trout were placed in a 60 l container of fresh stream water until processing. Total length and mass were measured; the mass of the PIT tag (0.6 g) was subtracted from the overall wet mass. A blood sample was obtained from recaptured trout (as above). The same methodology was applied for recaptures from 29 February to 2 March 2016 to evaluate the long-term effects of cortisol, where 34 control fish (9.50%), 18 sham fish (7.69%) and 4 cortisol fish (1.70%) were recaptured. The resampling started 750 m downstream and ended 1600 m upstream of our initial sampling locations (Fig. 1B). Growth rate was determined in terms of both mass and length for the short-term and long-term effects of treatments. A condition factor (K) was also determined using these measurements.

Choice of oxidative stress assays

We opted to measure glutathione (GSH), given that it is the most abundant antioxidant in eukaryotic cells (millimolar concentrations in tissues), and that it is critical to protect vital organs against oxidative damage (Owen and Butterfield, 2010). GSH not only is the primary antioxidant in cells but also is involved in detoxification (through glutathione *S*-transferases) and the protection of proteins from oxidative damage (through glutathionylation). Metabolically, generating GSH is costly, and hence the molecule is not typically broken down. For these reasons, it is useful when measuring effects over a longer time scale. We also chose to measure oxidative stress levels via the ratio of oxidized (glutathione disulfide, GSSG) to reduced glutathione (GSSG:GSH), which provides an indication of the redox status of the cell (Owen and Butterfield, 2010). Additionally, we opted for the oxygen radical absorbance capacity (ORAC) as a second method to measure overall antioxidant capacity of low molecular weight antioxidants because it is one of the few methods that takes the quenching reaction of ROS to completion. In essence, it combines both the time and percentage of ROS quenching by antioxidants, and converts it into a single quantity (Cao and Prior, 1999).

GSH antioxidant and oxidative stress levels (GSSG:GSH)

All RBC samples were ground and homogenized on ice in non-denaturing lysis buffer (20 mmol l^{-1} Tris-HCl, 137 mmol l^{-1} NaCl, 1% NP-40, 10% glycerol, 2 mmol l^{-1} EDTA and 100 µmol l^{-1} phenylmethylsulfonyl fluoride in isopropanol), and centrifuged at 13,500 rpm for 10 min at 4°C (Hermle Labnet Z216MK, Mandel Scientific Inc., Guelph, ON, Canada). Supernatants were further homogenized (1:5) in 5% sulfosalicylic acid solution (bubbled with N_2 gas). Sample lysates were centrifuged at 13,500 rpm for 10 min at 4°C. Supernatants were used to assess total glutathione (TGSH), oxidized glutathione (GSSG) and reduced glutathione (GSH). The last of these is measured indirectly using the following equation: TGSH=GSH+2GSSG. Glutathione assays were performed using an Epoch microplate reader with Gen5 data analysis software (2.00.18, BioTek Instruments Inc., Winooski, VT, USA) and clear 96-well Costar microplates. Glutathione assays were performed by following the rate of reduction of 5,5′-dithiobis(2-nitrobenzoic acid, DTNB) by GSH at 412 nm compared to a standard curve of GSH.

To measure TGSH, the reaction media contained 20 µl of sample, 5 IU ml^{-1} glutathione reductase, 0.5 mol l^{-1} potassium phosphate buffer (pH 7.0), 0.3 mmol l^{-1} NADPH and 60 mmol l^{-1} DTNB. The reduction was read for 30 min and compared with a GSH standard curve (0–4 mmol l^{-1}). To quantify only GSSG, 50 µl of the initial supernatant and the GSSG standards (0–0.5263 µmol l^{-1}) was treated with 44.7 mmol l^{-1} 2-vinylpyridine and 227.27 mmol l^{-1} KPi in a total volume of 110 µl and allowed to incubate at room temperature for 90 min to derivatize the GSH. Once complete, the GSSG was measured in the same manner as TGSH using the methods described above. GSH values were calculated using the equation described above. All samples were run in duplicates (mean values were calculated and used for analysis), with an inter-assay variation of 3.74%.

Low molecular weight antioxidants (ORAC)

Samples of RBCs were homogenized on ice (1:5) in lysis buffer (20 mmol l^{-1} Tris-HCl, 137 mmol l^{-1} NaCl, 1% NP-40, 10%

glycerol, 2 mmol l^{-1} EDTA) using a hand-held Tissue Master 125 (Omni International, Kennesaw, GA, USA). Lysates were centrifuged at 13,000 rpm for 5 min at 4°C in a Hermle Labnet Z216MK and supernatants were stored at −80°C until the ORAC assay was performed (as described in Wilson et al., 2012). ORAC analyses were performed using a Cytation 5 microplate reader (BioTek Instruments Inc., Winooski, VT, USA) and black 96-well Costar microplates. Fluorescence was measured with an excitation wavelength of 485 nm and emission of 520 nm. Gen5 data analysis software (2.07.17, BioTek Instruments Inc.) was used to analyse the fluorescence data.

Each reaction well contained 20 µl of sample, blank (75 mmol l^{-1} potassium phosphate, pH 7.4) or standard [0–400 µmol l^{-1} 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox)], and 3.82 µmol l^{-1} fluorescein in 75 mmol l^{-1} potassium phosphate (pH 7.4). The plate was incubated at 37°C for 20 min before rapidly adding the free radical generator 2,2″-azobis(2-amidinopropane) dihydrochloride (AAPH) to a final concentration of 79.83 mmol l^{-1}. The plate was placed immediately in the microplate reader and the fluorescence was read every 80 s for 90 min. The area under the fluorescence decay curve was determined for the samples and Trolox standards to determine the Trolox equivalency. Total protein of the samples was determined using the BioRad assay and final antioxidant capacity values are reported in Trolox equivalents (TE) per µg total protein. All samples were run in duplicate (mean values were calculated and used for analysis), with an inter-assay variation of 2.34%.

Evaluation of predation

Two cormorant (*Phalacrocoracidae* sp.) colonies are located near the Gudsø stream. During the same time frame as long-term fish resampling was conducted (14 and 15 March), each colony was scanned by two people to detect excreted PIT tags, each person sweeping the entire area of the colonies once. Scanned PIT tags allowed us to determine which fish had died from cormorant predation.

Statistical analysis

To assess short-term changes, 20 recaptured individuals were randomly chosen within each treatment group to assay TE/protein, GSH and GSSG:GSH. All long-term overwinter recaptures were used for these assays. We then ran these assays on the initial samples from these same individuals, thus forming two different initial groups. As long-term recaptures may not be representative of the whole population in the autumn (as these individuals survived and were still in the stream, meaning they were either late migrators or had chosen the residency strategy), we used one-way ANOVA to compare treatments at each time point (short and long term) separately.

To determine whether long-term survivors differed from the individuals randomly chosen for the short-term group, we used a one-way ANOVA to evaluate differences between the initial fish used in these two analyses. We noted that seven individuals were used in both ‘initial’ analyses (2 control, 4 sham and 1 cortisol) as they were long-term recaptures but also randomly selected for short-term analysis; otherwise, the two groups represent unique individuals.

GSSG:GSH contained true zero values and was highly skewed, so we used non-parametric Wilcoxon tests, which precludes testing for interactions, and so for this metric we analysed treatment separately at each time point (short-term initial sample, long-term initial sample, short-term sample and long-term sample) and used the Steel–Dwass method for analysing which treatments differed.

Changes in GSH, protein and TE/protein due to treatment, time point (initial versus short term, or initial versus long term) and their interaction were analysed using two-way ANOVA with individual ID as a random effect, with a Tukey *post hoc* test to determine which groups differed.

We also calculated specific (daily) growth rate for mass and length using the equation $(\log Y_2-\log Y_1)/(t_2-t_1)$, where Y_1 is the length or mass at the time of tagging (t_1) and Y_2 is the length or mass at the time of recapture (t_2). Specific growth rate and specific size were analysed using one-way ANOVA to test for treatment effects for short-term and long-term groups separately. A Kolmogorov–Smirnov test was performed to determine whether the data were normally distributed. Mass, length, GSH, protein and TE/protein were log transformed to achieve normality.

To explore relationships between growth rate/condition factor and GSH, GSSG:GSH or TE/protein, we used Pearson correlations within each time point (initial, short-term and long-term) and treatment.

A Pearson’s chi-square analysis was performed to evaluate whether mortality as a result of cormorant predation differed among treatment groups, and to evaluate whether the percentage of overwinter recaptures differed among treatment groups. Statistical analyses were conducted using JMP v12.1.0.

RESULTS

Though we present results testing for long-term effects of cortisol treatment, we note that we only recaptured four individuals for this category, resulting in imprecise estimates for all long-term metrics in this treatment, and so those results should be interpreted with caution.

Specific growth rate was lower in cortisol-treated fish than in control or sham fish in the short term ($F_{2,56}$=5.70, P=0.0056; Fig. 2A) and long term ($F_{2,53}$=13.90, P<0.0001; Fig. 2B), where control fish had higher growth rate than sham fish, which had higher growth rate than cortisol-treated fish. Specific size was not affected by short-term treatment (P=0.30) or long-term treatment ($F_{2,52}$=0.47, P=0.63; Table S1).

In the short term, cortisol fish showed higher levels of reduced glutathione (GSH) levels while sham and control fish did not show a change (treatment×time interaction, $F_{2,113}$=3.51, P=0.033; Fig. 3A). Oxidative stress levels (GSSG:GSH) decreased in the short term in all treatments (χ^2=7.59, P=0.022; Fig. 3C) and there were no initial differences among treatments (P=0.94). Protein concentration increased overall in the short term ($F_{1,109}$=4.82, P=0.030) but did not differ among treatments ($F_{2,109}$=0.5, P=0.61). Low molecular weight antioxidants (TE/protein) also decreased in the short term ($F_{1,109}$=45.5, P<0.0001; Fig. 3E) but did not differ among treatments ($F_{2,109}$=2.87, P=0.06).

In the long term (over winter), GSH decreased in cortisol-treated fish while control and sham fish did not show a change (treatment×time interaction, $F_{2,100}$=3.75, P=0.027; Fig. 3B). Protein concentration decreased in the long term ($F_{1,101}$=14.48, P=0.0002) but did not differ among treatments ($F_{2,101}$=2.97, P=0.056). Neither oxidative stress levels (GSSG:GSH; P>0.37; Fig. 3D) nor low molecular weight antioxidants (TE/protein; P>0.26, Fig. 3F) were affected by treatment or time.

The two initial groups used for the short-term and long-term studies differed from each other: both glutathione (GSH; $F_{1,30}$=17.66, P=0.0002; Fig. 3A,B) and oxidative stress levels (GSSG:GSH; χ^2=66.35, P<0.0001; Fig. 3C,D) were higher in the random group selected for short-term analysis than in the overwinter long-term group. Protein (P=0.99), TE/protein (P=0.99), mass (P=0.36), length (P=0.26) and condition factor (P=0.067) did not differ between these initial groups (Table S1).

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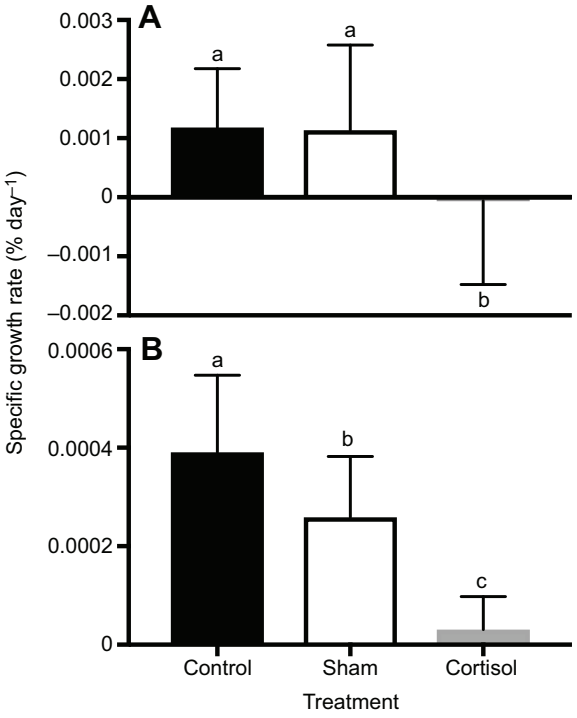

Fig. 2. Specific growth rate of brown trout following exogenous cortisol treatment. The figure shows changes in mass for (A) the 2 week (short-term) study and (B) the overwinter (long-term) study following cortisol treatment versus control and sham conditions. Means±s.d. are presented (2 week study: n=20 control, n=20 sham and n=20 cortisol; overwinter study: n=34 control, n=18 sham and n=4 cortisol). Different letters denote significant differences between groups (Tukey *post hoc* test, P<0.05).

At initial (pre-treatment) sampling, all fish showed a negative relationship between condition and oxidative stress (GSSG:GSH; control: n=50, R^2=0.09, P=0.032; sham: n=33, R^2=0.23, P=0.0047; cortisol: n=23, R^2=0.18, P=0.042). Cortisol-treated fish showed a negative relationship between GSH and growth rate (mass) in the short term (n=19, R^2=0.25, P=0.03). Control fish showed a positive relationship between growth rate (mass) and low molecular weight antioxidants (n=32, R^2=0.17, P=0.0183) in the long term. No other oxidative metric was related to condition or growth rate at any other time point or in any other treatment (all P>0.07).

The proportion of mortality as a result of cormorant predation did not differ among treatments (Pearson's χ^2=0.10, d.f.=2, P=0.995). In total, 12 control fish were predated, six sham fish and six cortisol-treated fish. However, the overwinter recapture rates were lower for cortisol-treated fish (1.70%) than for control (9.50%) and sham (7.69%) fish (Pearson's χ^2=12.629, d.f.=2, P=0.002).

DISCUSSION

It has been hypothesized that prolonged secretion of GCs results in increased oxidative stress levels (Agostinho et al., 2010), and thus oxidative stress may provide a potential mechanism for the costs associated with chronic stress (Costantini et al., 2011). The ratio of oxidized (GSSG) to reduced (GSH) glutathione is commonly used as a measure of oxidative stress, where a larger ratio represents a redox imbalance in favour of pro-oxidants. We found that cortisol manipulation did not increase oxidative stress levels in the short term, but it did increase the reduced form of glutathione (GSH), an important antioxidant in fish, suggesting that the increase in GSH potentially counteracted ROS production. Hence, cortisol may protect against, rather than generate, oxidative stress, and may upregulate antioxidant defences via genomic pathways as well as affect other mechanisms that limit the production of pro-oxidants such as ROS (Costantini et al., 2011). This is supported by the negative relationship between GSH and short-term growth rate that was only found in cortisol-treated fish. However, we found that in the long term, cortisol appeared to cause a decrease in GSH, though this result is tempered by the low sample size. This indicates that the increased GSH in the short term could not be maintained, and that cortisol may have caused the diversion of resources away from GSH production, probably to counteract other cortisol-induced effects (e.g. increased susceptibility to disease; Wingfield et al., 1998). GSH produced early after the cortisol manipulation may have been utilized to combat chronic effects of cortisol later on, which is surprising considering these long-term effects were seen 4 months after a transient cortisol elevation.

The effects of short-term administration of GCs on oxidative status have been studied in various taxa. In broiler chickens (*Gallus gallus domesticus*), a 14 day corticosterone diet manipulation led to elevated lipid peroxidation and plasma antioxidant activity (Lin et al., 2004). A similar study in captive kestrels (*Falco sparverius*) showed that corticosteroid administration through diet increased reactive oxygen metabolites, but did not impact total antioxidant capacity or oxidative stress levels (Costantini et al., 2008a,b). In rats (*Rattus norvegicus*), cortisol treatment did not affect the rate of ROS production in liver, but did increase DNA oxidative damage (Caro et al., 2007). We found that cortisol induced an increase in GSH in the short term and a decrease in GSH in the long term, but did not affect oxidative stress levels or low molecular weight antioxidants. These findings suggest that the effects of GCs on oxidative stress processes vary between species, and remain poorly understood. Furthermore, it appears that many of the GC-induced oxidative stress changes are tissue specific (e.g. McIntosh et al., 1998; Costantini et al., 2011). Our longitudinal sampling approach did not involve lethal sampling to examine heart, liver and brain tissue, therefore limiting us to the use of blood samples. The results suggest that the mechanisms by which cortisol affects oxidative status are complex and may differ by tissue type, taxa and duration.

Initially, all fish regardless of subsequent treatment showed the predicted negative relationship between oxidative stress levels and body condition, supporting the hypothesis that higher levels of oxidative stress divert resources away from body maintenance and possibly other physiological functions. Interestingly, a decrease in oxidative stress levels and low molecular weight antioxidants was observed in the short term in all treatments and the negative relationship between oxidative stress levels and condition was lost. It appears that resources were diverted from the production of low molecular weight antioxidants to generate other forms, such as enzymatic antioxidants, through unknown mechanisms. There are two possible causes for this: (1) seasonal variation (i.e. winter conditions such as lower temperatures and higher predation from cormorants); or (2) the potential stress of handling. The latter has important implications for future studies that aim to measure oxidative stress parameters and their relationship with condition metrics shortly after animals have been handled. Moreover, in the long term, control fish were able to re-establish the predicted relationship between growth rate and low molecular weight antioxidants. The lower growth rates in sham and cortisol-treated fish may have precluded even the faster growing fish from allocating resources toward this form of antioxidants, suggesting that these may be costly to generate.

Only fish that survived and stayed in the stream over winter could be used to evaluate the long-term effects of treatments on oxidative

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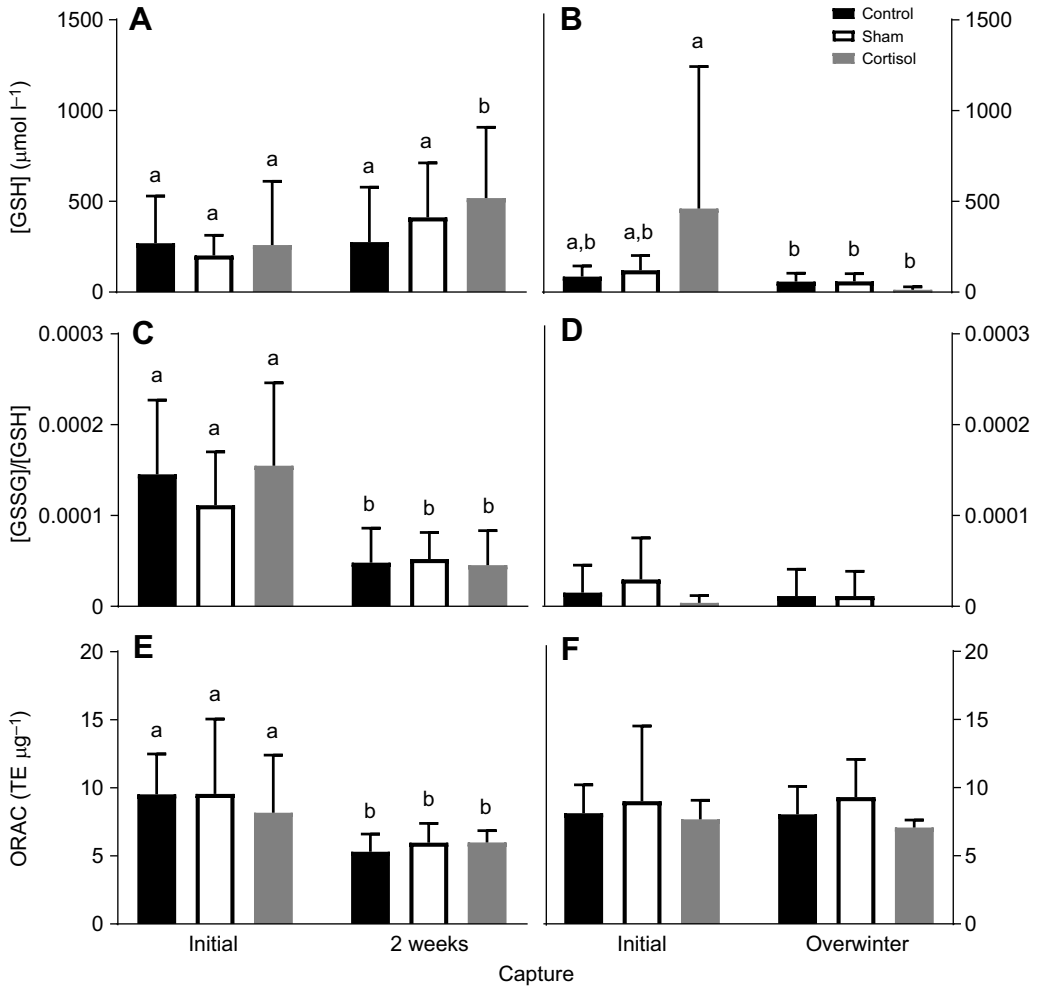

Fig. 3. Oxidative and antioxidant parameters. (A,B) Glutathione concentration (GSH) at (A) initial and 2 week recapture, and (B) initial and overwinter recapture. (C,D) Oxidized glutathione to reduced glutathione ratio (GSSG:GSH; oxidative stress) at (C) initial and 2 week recapture, and (D) initial and overwinter recapture (note that the ratios for cortisol-treated fish were 0 for all overwinter recaptures). (E,F) Low molecular weight antioxidants [oxygen radical absorbance capacity (ORAC); in Trolox equivalents (TE) per µg protein], at (E) initial and 2 week recapture, and (F) initial and overwinter recapture. Means±s.d. are presented (2 week study: *n*=20 control, *n*=20 sham and *n*=20 cortisol; overwinter study: *n*=34 control, *n*=18 sham and *n*=4 cortisol). Different letters denote significant differences between groups (Tukey *post hoc* test for GSH and ORAC, Steel–Dwass *post hoc* test for GSSG:GSH, $P<0.05$). Absence of letters in D and F indicates no groups differed from each other.

status. To our surprise, these overwinter fish initially had different oxidative statuses from randomly chosen fish irrespective of experimental treatment: overwinter fish initially had lower GSH antioxidant and oxidative stress (GSSG:GSH) levels than the general population (Fig. 3A,B). This suggests that individuals that survive over winter and/or migrate later are already physiologically different from other fish in the autumn. Bize et al. (2008) showed that in the alpine swift (*Apus melba*), males with higher resistance to oxidative stress tended to survive to the next season. Taken together, these findings suggest that lower oxidative stress levels may promote survival in wild organisms.

Although the evaluation of survival was not the focus of this study and the ultimate fate of each individual cannot be known for certain, past research has shown that overwinter mortality is highly variable in brown trout (Elliot, 1993). Additionally, exogenous cortisol manipulation causes increased overwinter mortality in brown trout of the same stream (Midwood et al., 2015). In general, high GC levels are associated with decreased fitness (Romero and Wikelski, 2001). However, the level of known predation at two cormorant colonies did not differ among treatment groups, suggesting that cortisol manipulation did not make fish more susceptible to predation by cormorants. Nonetheless, cortisol-treated fish showed significantly lower recapture rates, which may be attributed to other causes of death, such as decreased foraging ability (Wingfield et al., 1998) and decreased immunity/increased susceptibility to disease (Davis et al., 2008).

Glucocorticoid manipulation may affect body mass through its role in the hormonal control of appetite and food intake (Friedman and Halaas, 1998). Both baseline and acute GCs negatively co-vary with body mass (e.g. Schoech et al., 1997). Growth depression is a common observation following such GC manipulation in birds, reptiles, fish and mammals (Davies et al., 2013; Cote et al., 2010; O'Connor et al., 2011; Brooks and Mateo, 2013). We found that cortisol manipulation caused a decrease in growth rate (mass) over 2 weeks and despite a low sample size it appeared that cortisol also caused a decrease in growth rate (mass) and lower growth rate (length) over the long term. The reduced growth rate observed in cortisol-treated fish may be the result of a reduction in food intake (Morales et al., 2004) and decreased foraging ability (Wingfield et al., 1998). Caloric restriction can increase the expression of heat

shock proteins, which have the ability to quench ROS (Sørensen, 2010) and may explain why we did not observe an increase in oxidative stress levels in those same fish. Alternatively, it is also possible that cortisol-treated fish became less active after receiving the treatment, and thus may have a decreased metabolic rate. If this is the case, both ROS production and food consumption would have decreased, resulting in lower oxidative stress levels and lower growth rates in cortisol-treated fish. To date, no studies have made such observations. In either case, it appears that the link between GCs and oxidative stress is still poorly understood in fish, and may be more complex than first thought.

Conclusions

This study demonstrates that exogenous cortisol manipulation does not change oxidative stress levels but does affect antioxidant capacity, though these patterns differ with time, providing evidence that cortisol has different short- and long-term effects in fish. We also provide the first evidence that overwinter survival may be associated with low oxidative stress levels and low antioxidant capacity in the autumn. This may have important implications for the survival of hatchery-reared salmonids that are released in the wild before winter. Ensuring low oxidative stress levels in those fish, potentially through antioxidant supplementation in the diet, may provide them with better chances of survival over winter, though this would have to be balanced with our other results that suggest low levels of low molecular weight antioxidants are linked to survival over winter. Alternatively, those fish could be released after winter. This study also emphasizes the need to measure indicators of both oxidative stress levels and antioxidant capacity when studying oxidative stress, as their interactions remain largely unpredictable. This underlines the need for more manipulative studies of oxidative stress in wild organisms across different time scales in their natural environment.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

K.B.-G., K.S.P., M.H.L., K.A., W.G.W. and S.J.C. planned the experimental design. K.B.-G., K.S.P. and M.H.L. completed all the field work. K.B.-G. analysed all the samples. K.B.-G., K.S.P. and W.G.W. interpreted the data. K.B.-G. wrote the manuscript, with comments and feedback from K.S.P., M.H.L., K.A., W.G.W. and S.J.C.

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Data availability

Data are available from figshare: https://figshare.com/s/9a5dcbeeecc7779b9ce0

Supplementary information

Supplementary information available online at
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