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

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Summary Statement

Transient exogenous cortisol administration to brown trout caused an increased glutathione in the short-term, but this increase was not maintained in the long-term, and overwinter survival was associated with low levels of oxidative stress and glutathione.

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 the defence against oxidative stress. Using the brown trout (*Salmo trutta*), we evaluated the short-term (2 weeks) and long-term (4 months over winter) effects of exogenous cortisol manipulations (as well as relevant shams and controls) on the oxidative status of wild juveniles. Cortisol caused an increase in glutathione over a two-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 consequences.

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Introduction

Wild animals are constantly exposed to intrinsic and extrinsic stressors that challenge their homeostatic balance, 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). To fully understand 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. in press), 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

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resources from other activities to cause 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 defenses. 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 aging, 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). Reactive oxygen species (ROS) production and an animal's capacity to fight oxidative stress varies 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., 2008; Trivelpiece et al., 2011).

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 to the defence and repair against 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). Due to 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 were 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, 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). Their populations consist of 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 individuals that are recaptured 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 approach 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 approach 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 the control and sham 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 predict 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 groups in the short-term (herein defined as two weeks). We further predicted that these effects will not be maintained in the long-term (herein defined as four 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 (Figure 1). The stream has natural populations of semi-anadromous brown trout (*Salmo trutta*), eel (*Anguilla anguilla*) and lamprey (*Lampetra planeri*).

Fish sampling and tagging

Fish were captured in the main stem of the Gudsø stream, starting 2,000 m from the mouth of the stream and continuing upstream for approximately 2,500 m (indicated by insert in Figure 1) from October 20 to October 25, 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 hour). The water was

changed continually until processing (water was changed approx. every 15-20 minutes). A total of 793 juvenile brown trout were captured. Fish were placed in a solution of 0.03 $g \cdot l^{-1}$ of benzocaine until their opercular rate had slowed significantly and fish were unresponsive to external stimuli (usually less than 3 minutes). 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 (Texas Instruments, RI-TRP-RRHP, 134 kHz, 0.6g mass in air, 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 of 97% with no effects on mortality rate and growth. A condition factor (K) was calculated using equation (1).

$$K = \left(\frac{mass}{length^3}\right)(100) \qquad (1)$$

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 minutes of sampling, blood was centrifuged at 6,000 rpm for 2 minutes 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 $mg \cdot kg^{-1}$. 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 $ng \cdot ml^{-1}$; at day 6, they decrease to approximately 400 $ng \cdot ml^{-1}$; and at day 9, levels are approximately 200 $ng \cdot ml^{-1}$;

all of which were higher than controls and shams (Birnie-Gauvin et al., *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 to 500 $ng \cdot ml^{-1}$) 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. Fish from all treatments were allowed to recover in a 60 L container of fresh stream water, where cortisol fish were 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 (License Number: 2012-DY-2934-00007).

Resampling of fish

To evaluate the short-term effects of cortisol, Gudsø stream was resampled from November 5 to November 7, 2015 using the same techniques 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 per above description). The same methodology was applied for recaptures from February 29 to March 2, 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 1,600 m upstream of our initial sampling locations. A growth rate was determined both in terms of 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). Glutathione is not only the primary antioxidant in cells, but is also involved in detoxification (through the glutathione S-transferases) and protection of proteins from oxidative damage (through glutathionylation). Metabolically, generating glutathione is costly, and hence the molecule is not typically broken down. For these reasons, it is useful when measuring effects over a longer timescale. We also chose to measure oxidative stress levels via the ratio of oxidized 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).

Glutathione antioxidant (GSH) and oxidative stress levels (GSSG:GSH)

All RBC samples were ground and homogenized on ice in non-denaturing lysis buffer (20 mM Tris-HCl, 137mM NaCl, 1% NP-40, 10% glycerol, 2 mM EDTA and 100 µM phenylmethylsulfonyl fluoride (PMSF) in isopropanol), and centrifuged at 13,500 rpm for 10 minutes at 4°C in a Hermle Labnet Z216MK (Mandel Scientific Inc., Guelph, ON). Supernatants were further homogenized in 1:5 5% sulfosalicylic acid solution (bubbled with N_2 gas). Sample lysates were centrifuged at 13,500 rpm for 10 minutes at 4°C. Supernatants were used to assess total glutathione (TGSH), oxidized glutathione (GSSG) and reduced glutathione (GSH). The latter 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 glutathione reductase, 0.5 M potassium phosphate buffer (pH 7.0), 0.3 mM nicotinamide adenine dinucleotide 2'-phosphate (NADPH), and 60 mM DTNB. The reduction was read for 30 minutes and compared to a GSH standard curve (0-4 mM). To quantify only GSSG, 50 µL of the initial supernatant and the GSSG standards (0-0.5263 µM) were treated with 44.7 mM 2-vinylpyridine and 227.27 mM KPi in a total volume of 110 µL and allowed to incubate at room temperature for 90 minutes 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 in 1:5 lysis buffer (20 mM Tris-HCl, 137 mM NaCl, 1% NP-40, 10% glycerol, 2 mM EDTA) using a handheld Tissue Master 125 (Omni International, Kennesaw, GA). Lysates were centrifuged at 13,000 rpm for 5 minutes 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., Winooski, VT, USA) was used to analyze the fluorescence data.

Each reaction well contained 20 μL of either sample, blank (75 mM potassium phosphate (pH 7.4)), or standard (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox); 0-400 μM), and 3.82 μM fluorescein in 75 mM 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 mM. The plate was placed immediately in the microplate reader and the fluorescence was read every 80 seconds for 90 minutes. The area under the fluorescence decay curve (AUC) was determined for the samples and Trolox standards to determine the Trolox equivalency. Total protein of 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 duplicates (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 (March 14 and 15), each colony was scanned to detect excreted PIT tags by two people, each 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, twenty 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 fall (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 ANOVAs 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 7 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 nonparametric Wilcoxon tests, which precludes testing for interactions, and so for this metric we

analyzed 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 analyzing which treatments differed.

Changes in GSH, protein, and TE/protein due to treatment, time point (initial vs short-term, or initial vs long-term) and their interaction were analyzed using two-way ANOVAs 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 analyzed using one-way ANOVAs to test for treatments effects for short-term and long-term groups separately. A Kolmogorov-Smirnov test was performed to determine if 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 4 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 control or sham in the short-term ($F_{2,56}$=5.70, p=0.0056, Fig. 2A) and long-term ($F_{2,50}$=7.53, p=0.0014, Fig. 2B). Specific size was not affected by short-term treatment (p=0.30) but was also lower in cortisol fish than control or sham in the long-term ($F_{2,51}$=6.58, p=0.0029, Table A1).

In the short-term, cortisol fish showed higher levels of reduced glutathione (GSH) levels while sham and control fish did not change (treatment X 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 change (treatment X 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 the long-term studies differed from each other: both glutathione (GSH) ($F_{1,30}=17.66$, $p=0.0002$, Fig. 3A and B) and oxidative stress levels (GSSG:GSH) ($\chi^2=66.35$, $p<0.0001$, Fig. 3C and D) were higher in the random group selected for short-term analysis than 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 A1).

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: $n=2$, $R^2=0.18$, $p=0.042$). Cortisol-treated fish showed a negative relationship between glutathione (GSH) and growth rate in the short term ($n=19$, $R2=0.25$, $p=0.03$). 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 $\chi^2 = 0.10$, df = 2, $P = 0.995$). In total, 12 control fish were predated, 6 sham fish, and 6 cortisol-treated fish. However, the overwinter recapture rates were lower for cortisol-treated fish (1.70%) than control (9.50%) and sham (7.69%) (Pearson's $\chi^2 = 12.629$, df = 2, $P = 0.002$).

Discussion

It has been hypothesized that prolonged secretion of GCs result 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 defenses 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, likely to counteract other cortisol-induced effects (e.g., increase 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., 2008). In rats (*Ratus 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, 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-caused oxidative stress changes are tissue-specific (e.g., McIntosh et al., 1998; Costantini, 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. This suggests 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 (e.g., 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.

Only fish that survived and stayed in the stream over winter could be used to evaluate the long-term effects of treatments on oxidative status. To our surprise, these over winter fish initially had different oxidative statuses than randomly-chosen fish irrespective of experimental treatments: over winter fish initially had lower GSH antioxidants and oxidative stress levels (GSSG:GSH) levels than the general population (Figure 3A,B). This suggests that individuals that survive over winter and/or migrate later are already physiologically different from other fish in the fall. 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 over winter mortality is highly variable in brown trout (Elliot, 1993). Additionally, exogenous cortisol manipulation causes increased over winter 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 entrained by 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 covary 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., 2006; O'Connor et al., 2011; Brooks and Mateo, 2013). We found that cortisol manipulation caused a decrease in growth rate (mass) over two 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 a 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 decreased metabolic rates. 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 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 over winter survival may be associated with low oxidative stress levels and low antioxidant capacity in the fall. 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 emphasizes the need for more manipulative studies of oxidative stress in wild organisms across different time scales in their natural environment.

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Author Contributions

KBG, KSP, MHL, KA, WGW and SJC planned the experimental design. KBG, KSP and MHL completed all the field work. KBG analyzed all the samples. KBG, KSP and WGW interpreted the data. KBG wrote the manuscript, with comments and feedback from KSP, MHL, KA, WGW and SJC.

Data availability

Data will be made available in Dryad upon acceptance of the manuscript.

References

Alonso-Alvarez, C., Bertrand, S., Devevey, G., Prost, J., Faivre, B., Sorci, G. (2004). Increased susceptibility to oxidative stress as a proximate cost of reproduction. Ecol. Lett. **7**, 363-368

Asada, K. and Takahashi, M. (1987). Production and scavenging of active oxygen in photo-synthesis. In: Kyle, D.J., Osmond, C.B., Arntzen, C.J. (Eds.), Photoinhibition, Elsevier, Amsterdam, pp. 228–87.

Baker, M.R., Gobush, K.S., & Vynne, C.H. (2013). Review of factors influencing stress hormones in fish and wildlife. J. Nat. Conserv. **21**, 309-318.

Ball, G.F. and Balthazart, J. (2008). Individual variation and the endocrine regulation of behavior and physiology in birds: a cellular/molecular perspective. Philos. Trans. R. Soc. Lond. B Biol. Sci. **363**, 517-525.

Barton, B. (2002). Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. Integr. Comp. Biol. **42**, 517-525

Barton, B.A., Iwama, G.K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Annu. Rev. Fish Dis. **1**, 3-26.

Beaulieu, M., Thierry, A-M., González-Acuña, D., Polito, M.J. (2013). Integrating oxidative ecology into conservation physiology. Conserv. Physiol. **1**, 1-6

Behl, C., Lezoualch, F., Trapp, T., Widmann, M., Skutella, T., Holsboer, F. (1997). Glucocorticoids enhance oxidative stress-induced cell death in hippocampal neurons *in vitro*. Endocrinol. **138**, 101-106.

Binder, T.R., O'Connor, C.M., McConnachie, S.H., Wilson, S.M., Nannini, M.A., Wahl, D.H. and Cooke, S.J. (2015). Is winter worse for stressed fish? The consequences of exogenous cortisol manipulation on over-winter survival and condition of juvenile largemouth bass. Comp. Biochem. Physiol. A **187**, 97-102

Bize, P., Devevey, G., Monaghan, P., Doligez, B., Christe, P. (2008). Fecundity and survival in relation to resistance to oxidative stress in a free-living bird. Ecol. **89**, 2584-2593.

Boonstra, R. (2013a). The ecology of stress: a marriage of disciplines. Funct. Ecol. **27**, 7-10.

Boonstra, R. (2013b). Reality as the leading cause of stress: rethinking the impact of chronic stress in nature. Funct. Ecol. **27**, 11-23.

Brooks, K.C., Mateo, J.M. (2013). Chronically raised glucocorticoids reduce innate immune function in Belding's ground squirrels (*Urocitellus beldingi*) after an immune challenge. Gen. Comp. Endocr. **193**, 149-157.

Bruton, M.N. (1995). Have fishes had their chips? The dilemma of threatened fishes. Environ. Biol. Fish. **43**, 1-27.

Cadenas, E. (1989). Biochemistry of oxygen toxicity. Annu. Rev. Biochem. **58**, 79– 110

Cao, G., Prior, R.L. (1999). [5] Measurement of oxygen radical absorbance capacity in biological samples. Methods Enzymol. **299**, 50-62.

Caro, P., Gomez, J., Sanz, A., Portero-Otin, M., Pamplona, R., Barja, G. (2007). Effect of graded corticosterone treatmenton aging-related markers of oxidative stress in rat liver mitochondria. Biogerontol. **8**, 1-11.

Costantini, D. (2008). Oxidative stress in ecology and evolution: lessons from avian studies. Ecol. Lett. **11**, 1238-1251.

Costantini, D., Dell'Ariccia, G. and Lipp, H-P. (2008). Long flights and age affect oxidative status of homing pigeons (*Columba livia*). J. Exp. Biol. **4**, 377–381.

Costantini, D. Fanfani, A., Dell'Omo, G. (2008). Effects of corticosteroids on oxidative damage and circulating carotenoids in captive adult kestrels (*Falco tinnunculus*). J. Comp. Physiol. B **178**, 829-835.

Costantini, D., Marasco, V., Møller, A.P. (2011). A meta-analysis of glucocorticoids as modulators of oxidative stress in vertebrates. J. Comp. Physiol. B **181**, 447-456.

Cote, J., Meylan, S., Clobert, J., Voituron, Y. (2010). Carotenoid-based coloration, oxidative stress and corticosterone in common lizards. J. Exp. Biol. **213**, 2116-2124.

Crossin, G.T., Love, O.P., Cooke, S.J., Williams, T.D. (2016). Glucocorticoid manipulations in free-living animals: considerations of dose delivery, life-history context and reproductive state. Funct. Ecol. **30**, 116-125.

Dantzer, B., Fletcher, Q. E., Boonstra, R., Sheriff, M.J. (2014). Measures of physiological stress: a transparent or opaque window into the status, management and conservation of species? Conserv. Physiol. **2**, cou023.

Davies, S., Rodriguez, N.S., Sweazea, K.L., Deviche, P. (2013). The effect of acute stress and long-term corticosteroid administration on plasma metabolites in an urban and desert songbird. Physiol. Biochem. Zool. **86**, 47-60.

Davis, A.K., Maney, D.L., Maerz, J.C. (2008). The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. Funct. Ecol. **22**, 760-772.

Denver, R., Hopking, P., McCormick, S., Propper, C., Riddiford, L., Sower, S., Wingfield, J. (2009). Comparative endocrinology in the 21st century. Integr. Comp. Biol. **49**, 339-348.

Dey, C.J., O'Connor, C.M., Gilmour, K.M., Van Der Kraak, G., Cooke, S.J. (2010). Behavioral and physiological responses of a wild teleost fish to cortisol and androgen manipulation during paternal care. Horm. Behav. **58**, 599-605.

Dudgeon, D., Arthington, A.H., Gessner, M.O., Kawabata, Z.I., Knowler, D.J., Lévêque, C., Naiman, R.J., Prieur-Richard, A.H., Soto, D., Stiassny, M.L.J., Sullivan, C.A. (2006). Freshwater biodiversity: importance, threats, status and conservation challenges. Biol. Rev. **81**, 163-182.

Elliot, J.M. (1993). A 25-year study of production of juvenile sea-trout, *Salmo trutta* an English lake district stream. In: Gibson, R.J., Cutting, R.E. (Eds.), Production of juvenile Atlantic salmon, *Salmo salar*, in natural waters. Can. Spe. Publi. Fish Aquat. Sci. pp. 109-112.

Finkel, T., Holbrook, N.J. (2000). Oxidants, oxidative stress and the biology of ageing. Nature **408**, 239-247.

Friedman, J.M., Halaas, J.L. (1998). Leptin and the regulation of body weight in mammals. Nature **395**, 763-770.

Fridovich, I. (1998). Oxygen toxicity: a radical explanation. J. Exp. Biol. **201**, 1203–1209.

Gamperl, A.K., Vijayan, M.M., Boutilier, R.G. (1994). Experimental control of stress hormone levels in fishes: techniques and applications. Rev. Fish Biol. Fish. **4**, 215-255.

Gibbons, W.J., Andrews, K.M. (2004). PIT tagging: simple technology at its best. Biosci. **54**, 447-454.

Haenold, R., Wassef, D.M., Heinemann, S.H., Hoshi, T. (2005). Oxidative damage, aging and anti-aging strategies. Age **27**, 183–199.

Halliwell, B., Gutteridge, J.M.C. (1999). Free Radicals in Biology and Medicine. Oxford University Press, New York, pp. 936.

Holmlund, C.M., Hammer, M. (1999). Ecosystem services generated by fish populations. Ecol. Econ. **29**, 253-268.

Johnstone, C.P., Reina, R. D., Lill, A. (2012). Interpreting indices of physiological stress in free-living vertebrates. J. Comp. Physiol. B **182**, 861-879.

Jones, D.P. (2006). Redefining Oxidative Stress. Antiox. Redox Signal. **8**, 1865-1879.

Larsen, M.H., Thorn, A.N., Skov, C., Aarestrup, K. (2013). Effects of passive integrated transponder tags on survival and growth of juvenile Atlantic salmon *Salmo salar*. Anim. Biotelem. **1**, 19-25.

Lin, H., Deculypere, E., Buyse, J. (2004). Oxidative stress induced by corticosterone admisnitration in broiler chickens (*Gallus gallus domesticus*) – 1. Chronic exposure. Comp. Biochem. Physiol. B **139**, 737-744.

McIntosh, L.J., Cortopassi, K.M., Sapolsky, R.M. (1998). Glucocorticoids may alter antioxidant enzyme capacity in the brain: kainic acid studies. Brain Res. **791**, 215–222.

Metcalfe, N.B., Alonso-Alvarez, C. (2010). Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. Funct. Ecol. **24**, 984-996

Meylan, S., Belliure, J., Clobert, J., de Fraipont, M. (2002). Stress and body condition as prenatal and postnatal determinants of dispersal in the common lizard (*Lacerta vivipara*). Horm. Behav. **42**, 319-326.

Morales, A.E., Perez-Jimenez, A., Hidalgo, M.C., Abellan, E., Cardenete, G. (2004). Oxidative stress and antioxidant defenses after prolonged starvation in *Dentex dentex* liver. Comp. Biochem. Physiol. C **139**, 153-161.

Midwood, J.D., Larsen, M.H., Boel, M., Jepsen, N., Aarestrup, K., Cooke, S.J. (2014). Does cortisol manipulation influence outmigration behaviour, survival and growth of sea trout? A field test of carryover effects in wild fish. Mar. Ecol. Prog. Series **496**, 135-144.

Midwood, J.D., Larsen, M.H., Boel, M., Aarestrup, K., Cooke, S.J. (2015). An experimental field evaluation of winter carryover effects in semi-anadromous brown trout (*Salmo trutta*). J. Exp. Zool. **323A**, 645-654.

Midwood, J.D., Larsen, M.H., Aarestrup, K., Cooke, S.J. (2016). Stress and food deprivation: linking physiological state to migration success in a teleost fish. J. Exp. Biol. In press.

Mileva, V., Fitzpatrick, J., Marsh-Rollo, S., Gilmour, K.M., Wood, C., Balshine, S. (2009). The stress response of the highly social African cichlid *Neolamprologus pulcher*. Physiol. Bioche. Zool. **82**, 720-729.

Monaghan, P., Metcalfe, N.B., Torres, R. (2009). Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. Ecol. Lett. **12**, 75–92.

Morales, A.E., Pérez-Jiménez, A., Carmen Hidalgo, M., Abellan, E., Cardenete, G. (2004). Oxidative stress and antioxidant defenses after prolonged starvation in *Dentez dentex* liver. Comp. Biochem. Physiol. Part C. **139**, 153-161.

Nicol, S., Clarke, J., Romaine, S.J., Kawaguchi, S., Williams, G. and Hosie, G.W. (2008). Krill (*Euphausia superba*) abundance and Adélie penguin (Pygoscelis adeliae) breeding performance in the waters off the Béchervaise Island colony, East Antarctica in 2 years with contrasting ecological conditions. Deep Sea Res. Pt II **55**, 540–557.

O'Connor, C.M., Gilmour, K.M., Arlinghaus, R., Hasler, C.T., Philipp, D.P., Cooke, S.J. (2010). Seasonal carryover effects following the administration of cortisol to a wild teleost fish. Physiol. Biochem. Zool. **83**, 950-957

O'Connor, C.M., Gilmour, K.M., Arlinghaus, R., Matsumura, S., Suski, C.D., Philipp, D.P., Cooke, S.J. (2011). The consequences of short-term cortisol elevation on individual physiology and growth rate in wild largemouth bass (*Micropterus salmoides*). Can. J. Fish. Aquat. Sci. **68**, 693-705.

Orzechowski, O., Ostaszewski, P., Wilczak, J., Jank, M., Balasinska, B., Wareski, P., Fuller, J. Jr. (2002). Rats with a glucocorticoid-induced catabolic states show symptoms of oxidative stress and spleen atrophy: the effect of age and recovery. J. Vet. Med. A **49**, 256-263.

Owen, J.B., Butterfield, D.A. (2010). Measurement of oxidized/reduced glutathione ratio. Methods Mol. Biol. **648**, 269-277.

Pedernera, M., Celi, P., Garcia, S.C., Salvin, H.E., Barchia, I., Fulkerson, W.J. (2010). Effect of diet, energy balance and milk production on oxidative stress in early-lactating dairy cows grazing pasture. Vet. J. **186**, 352-357.

Ricklefts, R.E., Wikelski, M. (2002). The physiology/life-history nexus. Trends Ecol. Evol. **17**, 462-468.

Romero, L.M. (2004). Physiological stress in ecology: lessons from biomedical research. Trends Ecol. Evol. **19**, 249-255.

Romero, L.M., Wikelski, M. (2001). Corticosterone levels predict survival probabilities of Galapagos marine iguanas during El Niño events. Proc. Natl. Acad. Sci. USA **98**, 7366-7370.

Roussel, D., Dumas, J.F., Simard, G., Malthiery, Y., Ritz, P. (2004). Kinetics and control of oxidative phosphorylation in rat liver mitochondria after dexamethasone treatment. Biochem. J. **382**, 491–499.

Sapolsky, R.M., Romero, L.M., Munck, A.U. (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrin. Rev. **21**, 55-89.

Sala, O.E., Chapin, F.S. III., Armesto, J.J., Berlow, E., Bloomfields, J., Dirzo, R., HuberSanwald, E., Huenneke, L.F., Jackson, R.B., Kinzig, A., et al. (2000). Global biodiversity scenarios for the year 2100. Sci. **287**, 1770-1774.

Schoech, S.J., Mumme, R.L., Wingfield, J.C. (1997). Corticosterone, reproductive status and body mass in a cooperative breeder, the Florida scrub-jay (*Aphelocoma coerulescens*). Physiol. Zool. **70**, 68-73.

Sies, H. (1991). Oxidative Stress II. Oxidants and Antioxidants. Academic Press, London.

Sohal, R.S., Orr, W.C. (2012). The redox stress hypothesis of aging. Free Rad. Biol. Med. **52**, 539-555.

Sopinka, N.M., Patterson, L.D., Redfern, J.C., Pleizier, N.K., Belanger, C.B., Midwood, J.D., Crossin, G.T., Cooke, S.J. (2015). Manipulating glucocorticoids in wild-animals: basic and applied perspectives. Cons. Physiol. **3**, cov031.

Sopinka, N.M., Donaldson, M.R., O'Connor, C.M., Suski, C.D., Cooke, S.J. In press. Stress indicators in fish. In *Fish Physiology*, *Biology of Stress in Fish*, Vol. 35 (Schreck, C.B., Tort, L., Farrell, A.P., Brauner, C.J., eds.). Amsterdam: Academic Press.

Sørensen, J. (2010). Application of heat shock protein expression for detecting natural adaptation and exposure to stress in natural populations. Curr. Zool. **56**, 703-716.

Speakman, J.R., Blount, J.D., Bronikowski, A.M., Buffenstein, R., Isaksson, C., Kirkwood, T.B.L., Monaghan, P., Ozanne, S.E., Beaulieu, M., Briga, M., et al. (2015). Oxidative stress and life histories: unresolved issues and current needs. Ecol. Evol. **5**, 5745-5757.

Spée, M., Marchal, L., Lazin, D., Le Maho, Y., Chastel, O., Beaulieu, M., Raclot, T. (2011). Exogenous corticosterone and nest abandonment: a study in a long-lived bird, the Adélie penguin. Horm. Behav. **60**, 362-370.

Taylor, J.J., Wilson, S.M., Sopinka, N.M., Hinch, S.G., Patterson, D.A., Cooke, S.J., Willmore, W.G. (2015). Are there intergenerational and population-specific effects of oxidative stress in sockeye salmon (*Oncorhynchus nerka*)? Comp. Biochem. Physiol. A **184**, 97-104.

Tierney, M., Emmerson, L., Hindell, M. (2009). Temporal variation in Adélie penguin diet at Béchervaise Island, east Antarctica and its relationship to reproductive performance. Marine Biol. **156**, 1633–1645

Tort, L. (2011). Stress and immune modulation in fish. Dev. Comp. Immunol. **35**, 1366-1375.

Trivelpiece, W.Z., Hinke, J.T., Miller, A.K., Reiss, C., Trivelpiece, S.G. and Watters, G.M. (2011). Variability in krill biomass links harvesting and climate warming to penguin population changes in Antarctica. Proc. Natl. Acad. Sci. USA **108**, 7625–7628

Wikelski, M., Cooke, S.J. (2006). Conservation Physiology. Trends Ecol. Evol. **21**, 38-46

Wilson, S.M., Gravel, M-A., Machie, T.A., Willmore, W.G., Cooke, S.J. (2012). Oxidative stress asscoaited with paternal care in smallmouth bass (*Micropterus dolomieu*). Comp. Biochem. Physiol. A. **162**, 212-218.

Wingfield, J., Maney, D., Breuner, C., Jacobs, J., Lynn, S., Ramenosky, M., Richardson, R. (1998). Ecological bases of hormone-behaviour interactions: the "emergency life history stage". Amer. Zool. **38**, 191-206.

Zydlewski, G.B., Horton, G., Dubreuil, T., Letcher, B., Casey, S. (2006). Remote monitoring of fish in small streams: a unified approach using pit tags. Fish. Res. **31**, 492-502.

Figures

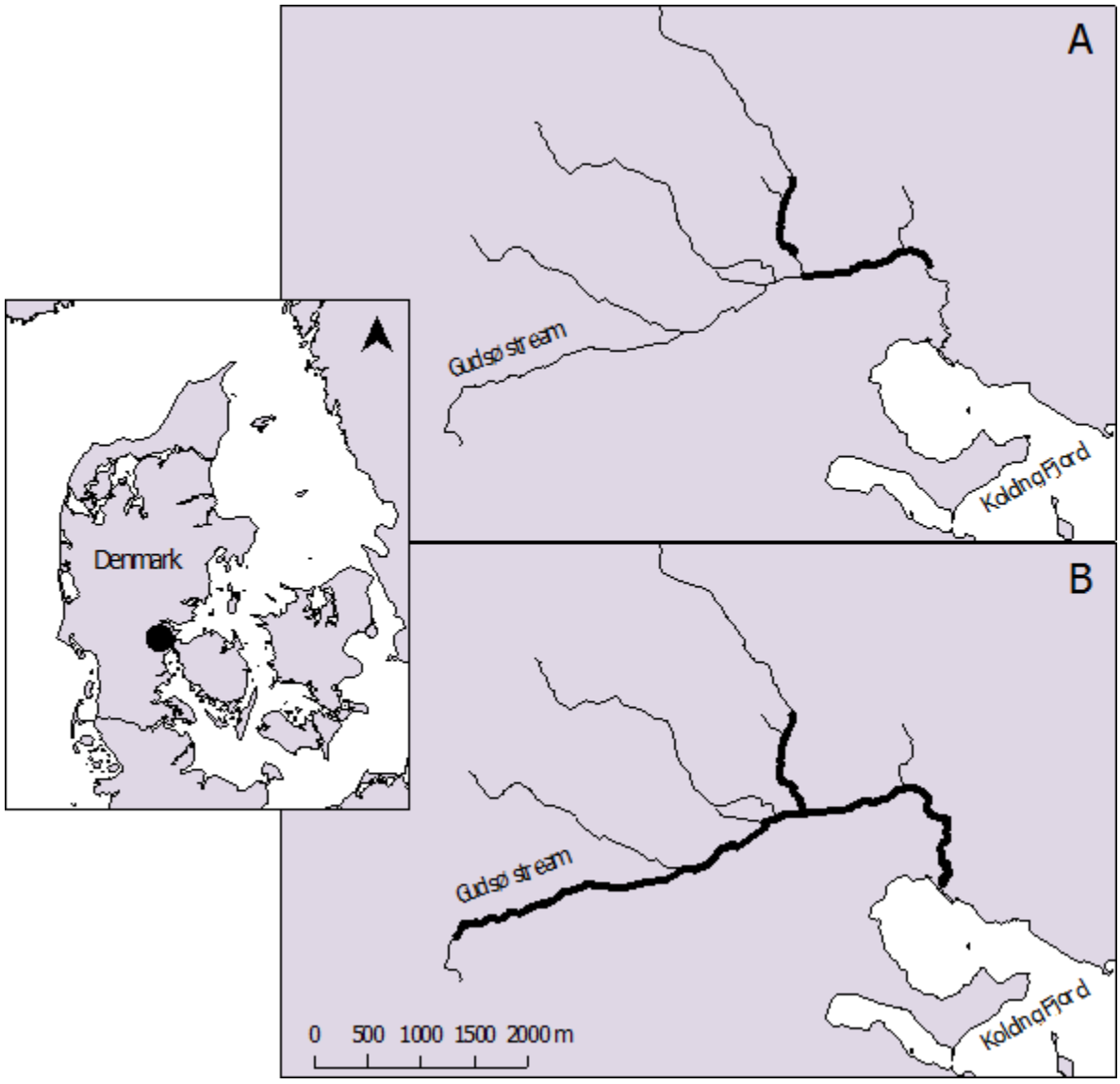

Figure 1. Map of the Gudsø stream, Denmark. A black circle shows location of the stream in Denmark. Sampling locations are highlighted in the thick black trace, (A) for initial and two-week capture locations in October and November 2015, and (B) for overwinter capture locations in February/March 2016.

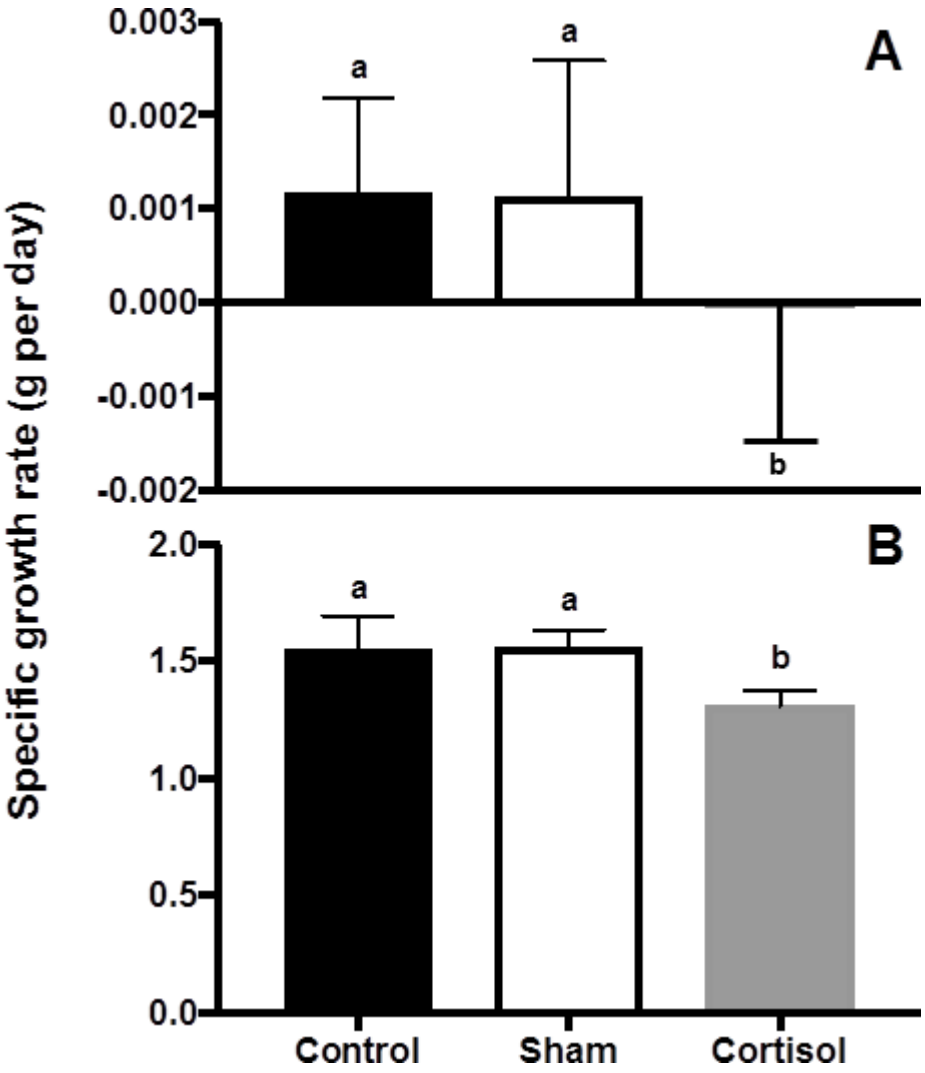

Figure 2. Specific growth rate for (A) two-week changes in mass (g per day), (B) overwinter changes in mass (g per day). Two-week study used *n* = 20 (control), *n* = 20 (sham), and *n* = 20 (cortisol); overwinter study used *n* = 34 (control), *n* = 18 (sham), and *n* = 4 (cortisol). Mean values are presented with standard deviations. Dissimilar letters (a, b) denote significant differences between groups (Tukey *post-hoc* test $P < 0.05$).

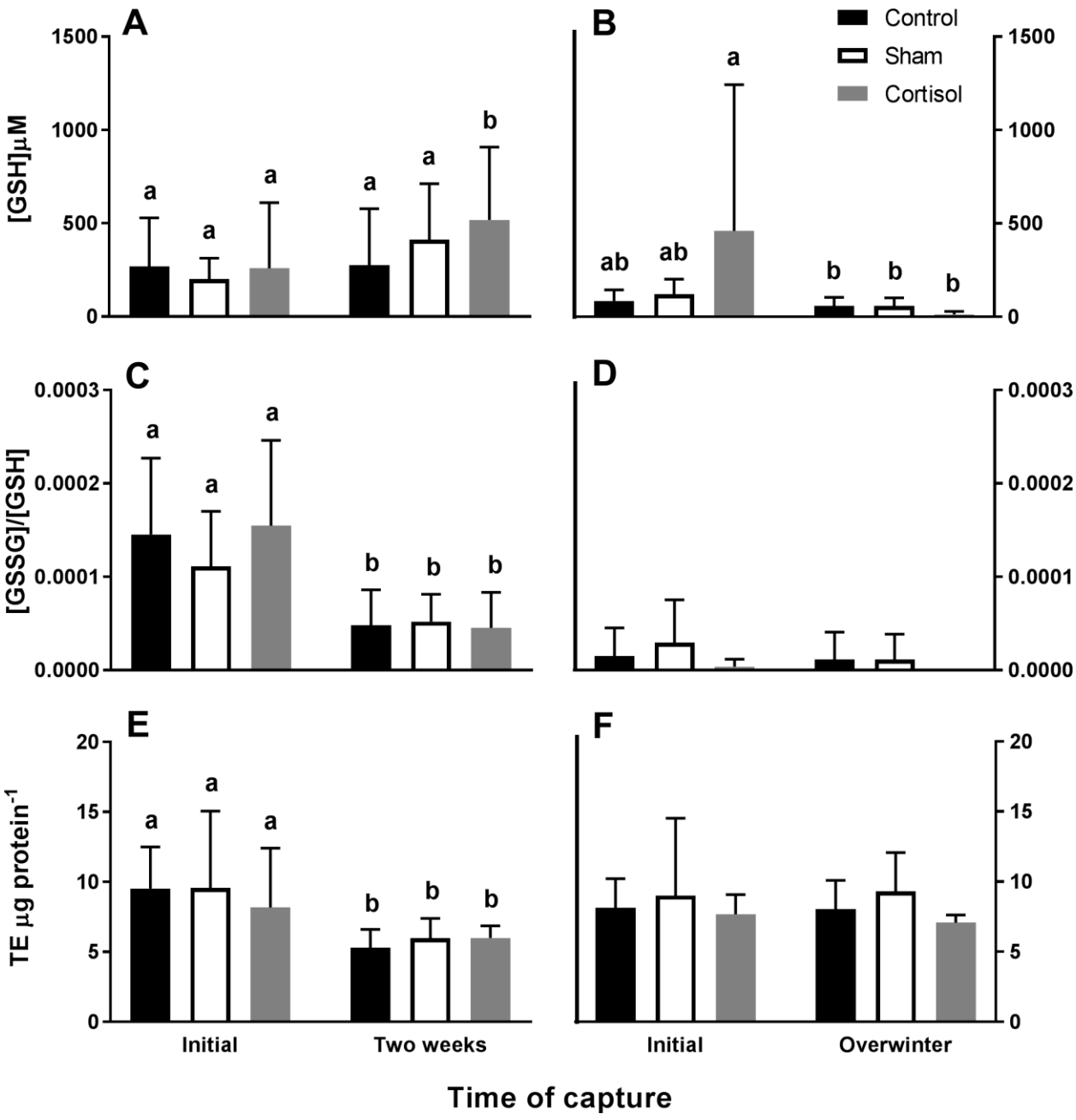

Figure 3. Oxidative and antioxidant parameters. Glutathione concentration (GSH, mM) at (A) initial and two-week recapture, and (B) initial and over winter recapture. Oxidized to reduced

glutathione ratio (GSSG:GSH; oxidative stress) at (C) initial and two-week recapture, and (D) initial and over winter recapture (note that the ratios for cortisol-treated fish were 0 for all over winter recaptures). Low molecular weight antioxidants (ORAC) in Trolox equivalents per μg protein, at (E) initial and two-week recapture, and (F) initial and overwinter recapture. Two-week study used n = 20 (control), n = 20 (sham), and n = 20 (cortisol); overwinter study used n = 34 (control), n = 18 (sham), and n = 4 (cortisol). Mean values are presented with standard deviations. Dissimilar letters (a, b) denote significant differences between groups (Tukey *post-hoc* test for GSH and ORAC, Steel-Dwass *post-hoc* test for GSSG:GSH, $P < 0.05$). No letters are provided for D and F as no groups differed from each other.

Appendix

Table A1. Summary data (mean ± SD).

	Timing					
	Initial			Two weeks		
	Control	Sham	Cortisol	Control	Sham	Cortisol
Number	20	20	20	20	20	20
Mass (g)	26.57±3.85	28.19±4.38	28.86±3.48	27.62±4.05	29.28±4.66	28.84±3.62
Length (cm)	14.4±0.89	14.7±0.87	14.7±0.74	14.2±0.90	14.5±0.90	14.6±0.75
Condition factor (K)	1.06±0.06	1.04±0.06	1.02±0.05	0.96±0.01	0.96±0.01	0.93±0.01
Growth rate (g per day)	N/A	N/A	N/A	0.074±0.060	0.076±0.10	-0.0029 ±0.094
GSH (μM)	269.72 ±259.34	201.48 ±111.71	259.05351.32	275.49 ±302.02	411.52 ±300.16	473.80 ±354.48
GSSG/GSH	0.00015 ±0.000082	0.00011 ±0.000059	0.00016 ±0.000090	0.000048 ±0.000038	0.000052 ±0.000029	0.000045 ±0.000040
ORAC (TE per μg protein)	9.53±2.97	9.60±5.66	8.22±4.35	5.30±1.30	5.97±1.42	5.99±0.87
	Initial			Overwinter		
	Control	Sham	Cortisol	Control	Sham	Cortisol
Number	29	18	4	29	18	4
Mass (g)	26.30±8.30	29.22±5.33	21.40±2.39	36.45±8.78	37.64±5.97	21.05±3.14
Length (cm)	13.9±1.32	14.4±1.09	13.0±0.26	15.7±1.39	15.6±0.91	13.1±0.50
Condition factor (K)	0.94±0.08	0.94±0.06	0.97±0.05	0.93±0.08	0.98±0.08	0.93±0.06
Growth rate (g per day)	N/A	N/A	N/A	0.081±0.035	0.065±0.032	-0.0027 ±0.0064
GSH (μM)	89.23±57.05	120.43±80.90	460.41±782.29	61.14±47.73	59.55±41.73	13±15.78
GSSG/GSH	0.000017 ±0.000032	0.000030 ±0.000046	0.0000039 ±0.0000079	0.000013 ±0.000031	0.000011 ±0.000027	0.00 ±0.00
ORAC (TE per μg protein)	8.12±2.10	9.00±5.52	7.68±1.40	8.04±2.04	9.31±2.76	7.08±0.54

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