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Relationship between oxidative stress and circulating testosterone and cortisol in pre-spawning female brown trout

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ABSTRACT

Reproduction in vertebrates is an energy-demanding process that is mediated by endogenous hormones and potentially results in oxidative stress. The primary aim of this study was to quantify the relationship between oxidative stress parameters (antioxidant capacity and levels of reactive oxygen metabolites) and circulating testosterone and cortisol in a common and widespread teleost fish, the brown trout (*Salmo trutta*, L.). Results show that trout with higher testosterone levels prior to spawning have higher levels of oxidative damage at the time that they spawn (although by the time of spawning testosterone levels had dropped, leading to a negative relationship between testosterone and oxidative damage at that time). Cortisol levels were not directly related to oxidative damage or antioxidant capacity, but concentrations of this hormone were positively related to levels of fungal infection, which was itself associated both with lower antioxidant capacity and lower levels of oxidative damage. These results highlight the complexity of interactions between different components of the endocrine system and metabolism and suggest that caution be used in interpreting relationships between a single hormone and indicators of oxidative balance or other fitness proxies.

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1. Introduction

The production of reactive oxygen compounds is an inevitable consequence of aerobic metabolism. In the presence of oxygen, the reactions of metabolic processes generate superoxide and other reactive oxygen species that can be biologically toxic and cause damage to proteins, lipids and nucleic acids (Halliwell and Gutteridge, 2007; Hulbert et al., 2007). To avoid such damage, living cells use a suite of antioxidants, such as antioxidant enzymes, thiols, or vitamins, that act to neutralise reactive oxygen species before they interfere with cell function (Ames et al., 1993; Halliwell and Gutteridge, 2007). Generation of biomolecular oxidative damage (for definitions see Sies, 1991; Halliwell and Gutteridge, 2007), disruption of redox signalling and control (Jones, 2006) and over-oxidation of thiols (Sohal and Orr, 2012) reflect a status of oxidative stress.

Oxidative stress has important implications for the biology and ecology of organisms throughout their lifecycle (Costantini, 2008, 2010; Metcalfe and Alonso-Alvarez, 2010). For instance, juvenile finches that display accelerated growth during their early development have blood cells with a decreased ability to resist damage from free radical attack (Alonso-Alvarez et al., 2007b). Later in life,

and particularly for females, reproduction is a highly energy demanding process (Zera and Harshman, 2001), and causes an increase in individual metabolic rate (Speakman et al., 2004). Associated with these metabolic demands, changes in oxidative damage and antioxidant status have been recorded during the reproductive period for several species (Alonso-Alvarez et al., 2004; Aras et al., 2009). In addition to the effects of increased metabolic rate, levels of ROS can vary seasonally (e.g. with temperature), and can be elevated by infection, activity of detoxifying enzyme systems and presence of pollutants (Costantini, 2008; Metcalfe and Alonso-Alvarez, 2010). In summary, both endogenous and extrinsic processes contribute to variation in the levels of oxidative stress experienced by individuals during their ontogeny.

Maturation and reproduction in vertebrates are associated with an increase in the concentrations of circulating hormones (Squires, 2010). There are a range of costs and benefits associated with these increases in hormone levels: elevating testosterone in female birds can increase social status and mating success but can also delay the onset of laying and suppress the immune system (reviewed by Veiga and Polo, 2008). Similarly, elevated testosterone levels in males of some species of lizards enhance mating success at the cost of reduced individual growth (Cox and John-Adler, 2005). Changes in circulating hormone levels during maturation are also pronounced in freshwater fish such as brown trout *Salmo trutta* and Atlantic salmon *Salmo salar*. For males of these species, plasma concentrations of 11-ketotestosterone vary up to 10-fold during the months prior to spawning (Kime and Manning, 1982; Pottinger and Pickering, 1985).

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For female salmonids concentrations of cortisol, estradiol and testosterone increase strongly throughout the egg production period (Pickering and Christie, 1981, Fig. 1), with the latter reaching peak levels approximately one month prior to spawning (Slater et al., 1994).

There are numerous known interactions between the endocrine system and metabolism (Norris and Hobbs, 2006; Moore and Hopkins, 2009). First, several hormones, including testosterone (Sangiao-Alvarellos et al., 2006) and estradiol (Irwin et al., 2008), directly affect energy metabolism and, correspondingly, oxidative balance (Norris and Hobbs, 2006). Glucocorticoids are involved in the mobilisation of energy during stress (e.g., Moore and Hopkins, 2009) and administration of these hormones affects the activity of important antioxidant enzymes such as superoxide dismutase (SOD, Goncharova et al., 2006) and, depending on the duration of treatment, can have significant effects on oxidative stress (Costantini et al., 2011). Despite detailed evidence in

the literature regarding changes in hormone levels during reproduction of vertebrates in general (Feoktistova et al., 2010; Horton et al., 2010; Mull et al., 2010) and salmonid fish in particular (Fig. 1), the consequences of hormone variation for oxidative stress remain poorly understood. The primary aim of this study was to investigate the relationship between oxidative stress parameters (plasma non-enzymatic antioxidant capacity and levels of hydroperoxides) and circulating testosterone and cortisol in a common and widespread teleost fish, the brown trout; a species for which hormone profiles during reproduction are well documented (see Fig. 1). Our study focused on female trout because, for these fish, reproduction is highly energetically expensive due to their production of thousands of eggs over a protracted oogenesis period of more than 4 months, and because their circulating hormone levels change dramatically during reproduction. Specifically, we hypothesised that pronounced changes in testosterone and cortisol levels experienced by female brown trout during their pre-spawning phase are associated with increased

Fig. 1. Synthesis of literature data describing variation in circulating concentrations of four hormones for female brown trout over the course of an annual cycle. Time values (x-axis) as expressed as days prior to (negative values) or after (positive values) spawning (at time = 0). Solid bars represent median values, boxes represent inter-quartile range, whiskers represent standard deviation, circles represent outliers and numerals above each box represent sample sizes.

Data are sourced from Lambert et al. (1978); Scott et al. (1980); Pickering and Christie (1981); van Bohemen et al. (1981); Bromage et al. (1982); Scott et al. (1982); Scott and Sumpter (1983); Scott et al. (1983); Whitehead et al. (1983); Ueda et al. (1984); Dye et al. (1986); Fitzpatrick et al. (1986); Liley et al. (1986); Truscott et al. (1986); Pottinger and Pickering (1987); Norberg et al. (1989); Slater et al. (1994); Tveiten et al. (1998); Kubokawa et al. (1999); Lokman et al. (2002); Estay et al. (2003).

oxidative stress. Our expectation was that increases in testosterone and cortisol would be accompanied by an increase in hydroperoxides (i.e., intermediate oxidative damage molecules) that might lead to a condition of oxidative stress depending on the capacity of individual trout to produce antioxidants.

2. Materials and methods

2.1. Animal husbandry

Mature, 3 year old, female brown trout were transferred from Howietoun Fishery (University of Stirling, Scotland) to the Marine Scotland Science freshwater hatchery facility at Almondbank (Perthshire, Scotland) in August 2009. The fish ($N=72$) ranged in size from 32–42 cm (fork length, average 35 cm) and weighed 380–860 g (average 528 g). They were maintained in six tanks (12 individuals per 2 m diameter tank) that were supplied with a continuous flow of river water. Throughout the experiment, commercial salmonid pellet food was supplied *ad libitum* by automated feeders although the fish were largely not feeding during the period prior to spawning. All experimental work was conducted in accordance with the Animals (Scientific Procedures) Act 1986, under UK Home Office Project Licence PPL 60/3625.

2.2. Experimental design

To manipulate circulating hormone levels, cocoa butter implants were inserted into the intra-peritoneal cavity of the trout (e.g., [Camperl et al., 1994](#)). Hormone implants were prepared by dissolving pure, crystalline testosterone (Fluka, 99% T) and cortisol (Sigma, hydrocortisone 98%) in 5 mL of ethanol and homogenising with molten cocoa-butter (Mycryo, Callebaut) at 40 °C using a temperature-controlled stirring plate. Subsequently, temperature was increased to 75 °C and the suspension stirred continuously to evaporate the ethanol. A fixed volume (0.5 mL) of molten hormone suspension was drawn into sterile 1 mL syringes, allowed to harden into pellets, and stored at –20 °C prior to use. Five different implant types were prepared; a sham implant containing only cocoa butter, two cortisol implants with doses corresponding to 30 mg/kg and 50 mg/kg and two testosterone implants with doses corresponding to 2 mg/kg and 5 mg/kg. Hormone doses were chosen to elevate circulating hormones within the physiologically relevant range based on published studies on fish ([Pickering et al., 1989](#); [Sangiao-Alvarellos et al., 2006](#)). Our intention in introducing the hormone implants was to elevate hormone levels in addition to natural increases during the pre-spawning period to experimentally test for an effect of hormone levels on oxidative balance parameters. In addition to the 5 groups of treated fish, we included an untreated control group of fish that were maintained under identical conditions to the treated fish but were not given a hormone dose. The control group was specifically included to allow us to detect whether the hormone implants enhanced circulating hormone levels, and to enable investigation of fish with naturally varying hormone levels. We elected to focus on cortisol and testosterone because both of these hormones are elevated in female trout prior to spawning ([Fig. 1](#)). Cortisol is not typically considered to be an element of the reproductive endocrine system but is integral to the neuroendocrine stress response and is known to affect metabolism and, hence, potentially influences oxidative balance. We focused on testosterone in place of estradiol because, although the latter promotes the production of highly-peroxidizable egg-yolk lipoproteins, for female brown trout estradiol production peaks 60–90 days prior to spawning compared to ~30 days prior to spawning for testosterone ([Fig. 1](#)). We reasoned that the substantially longer offset in the timing of estradiol production compared to testosterone production could potentially obscure any relationship between estradiol concentration and oxidative balance measured at spawning.

Fish were randomly allocated between the 6 treatments (12 individuals per treatment) and given the appropriate hormone implant

2 months prior to the expected date of spawning, corresponding with the period of maximum yolk production ([van Bohemen et al., 1981](#); [Norberg et al., 1989](#)). After fish had been anaesthetised in benzocaine, implants were surgically inserted into the intraperitoneal cavity. To do this, a small ventral incision was made posterior to the pelvic girdle, the syringe plunger was used to extrude the solid implant from the syringe, and the incision was closed with 2 nylon stitches and an antibiotic wound adhesive. At the time of implantation, fish were measured for length and weight, photographed, and tagged with a passive integrated transponder (PIT) inserted in the same incision as the hormone implant. Fish in the no implant control treatment were anaesthetised, handled and measured in the same manner as implant fish, and were similarly implanted with a PIT tag in the intraperitoneal cavity, but were not given an implant. Following implantation, all fish were re-allocated to the 6 holding tanks such that there were 2 fish per treatment in each tank. No mortality was observed in the week following the procedure.

2.3. Sample collection

Blood samples were taken from 7 to 12 of the experimental fish in each treatment at 4 and 7 weeks post-implantation ('interim' samples) and again at the termination of the experiment ('spawning' samples). The interim samples were timed to capture the period of peak testosterone levels during the reproductive cycle, which occurs approximately 1 month prior to spawning in trout ([Slater et al., 1994, Fig. 1](#)). Circulating cortisol levels are highly labile and increase above baseline levels within 4–6 min following the onset of a stressor such as capture and handling ([Laidley and Leatherland, 1988](#); [Grutter and Pankhurst, 2000](#)). Therefore, to quantify baseline cortisol levels for all of the sampled fish we developed a technique to minimise stress associated with capture, anaesthesia and handling during blood sampling. Adult brown trout prefer to take up station in locations that provide overhead cover ([Bachman, 1984](#)). Three days prior to sampling, 12 sections (40 cm long, 12 cm inner diameter) of opaque plastic drain pipe, with a small metal plate attached to the base to prevent movement within tanks, were introduced into each tank as 'sampling-shelters'. The fish rapidly took up position in these shelters and could not then see people approaching the tank. During blood sampling, shelters containing fish were gently sequentially removed from the tanks, fish were quickly transferred into anaesthetic (benzocaine), and a 300 μ L blood sample was taken laterally from the caudal vein generally within 2 min of capture (range 78–189 s). Fish in the remaining shelters appeared undisturbed by this process, and subsequent analyses of cortisol showed no effect on B levels of either capture sequence (Pearson's $R=0.17$, $p=0.21$, $df=56$) or time under anaesthesia (Pearson's $R=-0.08$, $p=0.56$, $df=56$), indicating that the technique was successful. Blood samples were collected in heparinised syringes to prevent clotting, transferred to 1.5 mL eppendorf tubes and centrifuged for 10 min to precipitate the red blood cells before the plasma was transferred into a fresh eppendorf tube and frozen at –20 °C. No damage to the caudal fin was observed to result from the blood sampling. The cortisol sampling scheme could not be used at the termination of the experiment because egg samples were taken at this time for a related study on the effects of egg hormone content on juvenile development. As a result of the procedure required to obtain the egg samples, blood samples could not be collected within 2 min of capture and reliable cortisol data could not be obtained for these samples.

Fish were removed from the experiment, and a terminal blood sample for measuring oxidative stress was taken, following ovulation when eggs become loose within the body cavity in preparation for spawning. Approaching the expected spawning time (mid-November), ovulation status was checked twice per week by treating all fish within each tank with a very mild anaesthetic, netting them with hand nets and gently massaging their abdomen to detect loose eggs. The fish did not ovulate simultaneously and terminal samples were therefore collected in three

batches (on 30/11/09, 9/12/09 and 21/12/09) hereafter referred to as spawning groups. There was no indication that spawning time depended upon the initial hormone treatment: each spawning group contained individuals from each of the treatment groups (cortisol-implanted, testosterone-implanted, sham-implanted and not-implanted). Salmonid fish are highly susceptible to fungal infections during their pre-spawning period (Pickering and Christie, 1980). To account for any effects of infection on oxidative balance, the intensity of fungal infection experienced by each fish at the time of spawning was scored by visual estimation ($\pm 5\%$) of the proportion of the fish's caudal, dorsal and pectoral fins that were affected. Fish were not noticeably infected when the interim blood samples were taken so fungal infection was only noted at the termination of the experiment.

2.4. Hormone assays

Plasma concentrations of cortisol, the predominant glucocorticoid steroid in fish, and testosterone were quantified by radioimmunoassay (RIA). To extract the hormones, approximately 300 mg of plasma was weighed (accuracy 0.001 g), 200 μL of MilliQ filtered water was added and 50 μL of ^3H -labelled cortisol was added to trace the recovery of extracted hormones during the extraction procedure. This solution was incubated for 15 min at 37 °C before being extracted in 2 mL of diethyl ether/petroleumbenzene (DEE/PB, 70/30 v/v) by vortexing for 60 s. Extracts were centrifuged at 600 g for 3 min (4 °C) to separate the ether/hormone phase, then snap-frozen and decanted into a fresh tube. The extraction procedure was repeated with an additional 2 mL of DEE/PB, vortexed for 30 s, and the extracts combined and dried under nitrogen. Hormone extracts were rinsed in 2 mL of 70% methanol, centrifuged, decanted into a fresh tube, re-dried under nitrogen and stored at -20 °C. This methanol step was included to precipitate lipids because the samples were noticeably cloudy due to the presence of these lipids.

Subsequently, extracts were thawed and dissolved in 100 μL of phosphate-buffered-saline with gelatine (PBSG). From this solution, 20 μL was mixed with scintillation cocktail (Ultima Gold, Perkin Elmer) and radioactivity counted on a liquid scintillation counter. Subsequently, 20 μL of sample was used for cortisol (B) determination using kits purchased from Orion Diagnostica ('Spectria', Espoo, Finland) and 20 μL was diluted in 500 μL PBSG ($\times 26$ dilution) and 50 μL of the diluted sample was used for testosterone (T) determination using kits purchased from Beckman Coulter GmbH ('DSL-4000', Sinsheim, Germany). Standards were prepared using dilution series from pre-prepared stock and ranged from 4 to 500 ng mL^{-1} for B and 0.04–20 ng mL^{-1} for T. Recoveries were calculated by comparison to non-extracted labelled hormone and averaged 64% ($\pm$ s.d. 9%). 'Pools' of plasma were used as external controls and intra-assay CVs (4 RIAs) were 1.3% and 2.7% for B and T respectively, while inter-assay CVs were 5.6% for B and 12.9% for T.

2.5. Oxidative damage assay

We measured plasma hydroperoxides using the d-ROMs test (specificity over 90%, Alberti et al., 2000; Diacron International srl, Grosseto, Italy), according to established protocols for fish (Bagni et al., 2007) with minor modifications. Hydroperoxides are intermediate peroxidation products of biological macromolecules, such as lipids, proteins and nucleic acids, and are precursors of end-products of lipid peroxidation, such as malondialdehyde, hydroxynonenal and isoprostanes. The plasma (8–16 μL) was incubated with 200 μL of a solution containing 0.01 M acetic acid/sodium acetate buffer (pH 4.8) and N,N-diethyl-*p*-phenylenediamine as chromogen for 75 min at 37 °C. Preliminary analyses showed the presence of a precipitate on the bottom of wells. To reduce measuring error associated with this precipitate the reaction was incubated in small tubes, the solutions were subsequently centrifuged (16,000 g for 2 min) and the

supernatant (190 μL) was pipetted into a fresh plate. The same procedure was applied to the reference standard and blank. The absorbance was read with a Thermo Scientific Multiskan Spectrum (ThermoFisher, Vantaa, Finland) at a wavelength of 490 nm. Measurements are expressed as mM of H_2O_2 equivalents. Analyses were run in duplicate and the mean CV was 5.63%. Plasma hydroperoxides ('ROMs') level in six samples was undetectable; hence they were assigned a nominal value of 0.087 mM (half the lowest point of the calibration curve).

2.6. Plasma antioxidant capacity assay

We measured the non-enzymatic antioxidant capacity ('OXY'), of plasma using the OXY-Adsorbent test (Diacron International, Grosseto, Italy), implemented according to established protocols for fish (Bagni et al., 2007). This assay quantifies the overall ability of antioxidant compounds present in a solution to cope with the in vitro oxidant action of hypochlorous acid (HOCl; an endogenously-produced oxidant). Compared to other assays of general antioxidant capacity, it does not overemphasise the contribution of any particular antioxidant molecule, such as uric acid (Costantini, 2011). Plasma was diluted 1:100 with distilled water and a 200 μL aliquot of HOCl solution was incubated with 5 μL of the diluted plasma for 10 min at 37 °C. The same relative volumes were used for reference standards and blanks. At the end of incubation, 5 μL of chromogen N,N-diethyl-*p*-phenylenediamine was added. In this reaction an alkyl-substituted aromatic amine dissolved in the chromogen is oxidised by the residual HOCl and transformed into a pink derivative. The intensity of the coloured complex is inversely related to the antioxidant capacity of the sample. The absorbance was read with a Thermo Scientific Multiskan Spectrum at a wavelength of 490 nm. Analyses were run in duplicate and the mean CV was 5.66%. Measurements are expressed as mM of HOCl neutralised because the assay tests an in vitro reaction of plasma antioxidants with HOCl. A standard serum (whose OXY capacity is known) is used as a reference (positive control) together with a blank (negative control). OXY capacity is calculated according to Costantini (2011) and the use of a standard and blank gives the same results as those obtained using a calibration curve. Both ROMs and OXY were analysed in blood instead of in other tissues to enable direct comparison with circulating hormone levels, and because blood is sensitive to oxidative challenge (Veskoukis et al., 2009).

2.7. Data analysis

Variation in oxidative status (hydroperoxides and OXY) was analysed using general linear models (GLM) with one fixed factor (spawning group, G) differentiating individuals based on date of ovulation, and five covariates and/or categorical factors; treatment group (X), testosterone at time of spawning (T_2), fungal infection intensity (F), interim testosterone (T_1), interim cortisol level (B) and body mass at the time of spawning (W). This multiple-regression approach quantified how much of the variation in OXY and hydroperoxides was attributable to each of the explanatory variables, and identified variables with statistically significant effects on oxidative balance. Interim hormone levels ranged from 3.0 to 65 ng mL^{-1} for T (average $T_1 = 35$) and from 4 to 243 ng mL^{-1} for B (average $B = 76$), whereas spawning T levels ranged from 0.3 to 78 ng mL^{-1} (average $T_2 = 19$). These concentrations are consistent with other studies of hormone variation for female brown trout; for this species B ranges up to 275 ng mL^{-1} (Lokman et al., 2002) and T up to 52 ng mL^{-1} (Pottinger and Pickering, 1987, Fig. 1). The hormone treatments were ineffective in elevating circulating hormone levels (one-way ANOVA on log- or square root-transformed data, $p > 0.4$ for each of B, T_1 and T_2) and, likewise, did not affect oxidative balance (one-way ANOVA on log-transformed data, $p > 0.6$ for each of ROMs and OXY) and were therefore not retained in the minimal models

($p > 0.35$). The ineffectiveness of the hormone implants was most likely due to the unusually cold water temperatures ($\sim 3^\circ\text{C}$) experienced during the experimental period which prevented dissolution of the cocoa-butter implants and, therefore, the release of hormones into circulation. There was also no difference in circulating hormone levels among fish sampled either at 4 weeks or 7 weeks post implantation (one-way ANOVA, $F_{1,37} = 0.17$ for cortisol and 2.3 for testosterone, $p = 0.68$ and 0.14 respectively, Fig. 2), so the timing of these interim samples was not included as a separate factor in the subsequent analyses. We note that the failure of the hormone implants to generate a statistically significant increase in circulating hormone levels meant that our analyses focussed upon the relationships between among-individual variations in circulating hormone levels and oxidative balance, rather than analysing a specific effect of experimentally enhanced hormone levels.

A backward-deletion approach was used to identify the most parsimonious model based on likelihood ratio tests of sequentially simplified models. The 'full' model was specified as:

$$y = W + F + X + G + B + T_1 + T_2 + B * T_1 + G * T_2 + T_1 * T_2 \quad (1)$$

where y (the dependent variable) is either oxidative damage (hydroperoxides) or plasma non-enzymatic antioxidant capacity (OXY). Trout frequently display high mortality rates associated with reproduction (Campbell, 1971) and despite all due care, by the end of the experiment, incidental mortality primarily due to untreatable fungal infection had reduced our sample size to $N = 39$ fish. This restricted the number of GLM parameters that could be estimated from the data and, correspondingly, the 'full model' included only the main effects and the plausible two-way interactions. In the case of the latter, we expected that levels of testosterone mid-way through the experiment (T_1) might potentially depend upon levels of cortisol (B) and with testosterone levels at spawning (T_2) despite the fact that pairwise correlations between these variables were not statistically significant (Pearson's $r = -0.23$, $p > 0.17$). In addition, we tested whether the relationship between T_2 and oxidative balance was consistent among the different spawning groups, due to the potential correlation between testosterone and metabolic rate (e.g., Ros et al., 2004; Moore and Hopkins, 2009), and observations in birds that elevated T leads to a delay in oviposition and egg laying (Goerlich et al., 2010). The variance inflation factor was less than 4.0 for each of the predictor variables, confirming that our results are not sensitive to correlations among the independent variables.

3. Results

Hydroperoxides (i.e., intermediate oxidative damage molecules) and plasma non-enzymatic antioxidant capacity (OXY) were related both to concentrations of circulating testosterone and to the intensity of fungal infection (Table 1). On the other hand, circulating concentrations of cortisol measured mid-way through the experimental period did not affect oxidative balance parameters: this variable was not included in the minimal models for either OXY or hydroperoxide values (Table 1). However, cortisol content from samples taken mid-way through the experimental period was positively correlated with the intensity of fungal infection at the time of spawning (Pearson's $R = 0.42$, $t_{37} = 2.78$, $p < 0.01$).

Plasma antioxidant capacity was significantly associated with the intensity of fungal infection, spawning group, circulating testosterone concentration at spawning and a significant interaction between spawning batch and testosterone concentration (Table 1, multiple $R^2 = 0.49$). The group by testosterone interaction was manifest as a positive relationship between OXY and testosterone for group 1, no relationship between these two measures for group 2 and a negative relationship for group 3 (Fig. 3). Overall, these results indicate that factors that influence the timing of spawning of female trout mediate the way in which steroid hormones stimulate or suppress plasma antioxidant capacity. The minimal model for circulating levels of hydroperoxides explained 36% of the variation in the data (multiple R^2) and included the variables describing fungal infection intensity together with both interim and spawning levels of testosterone. That is, fish that had high testosterone levels prior to spawning tended to have a higher hydroperoxide content at spawning (Fig. 4A) whereas those with high testosterone at spawning had low hydroperoxide content at that time (Fig. 4B).

Female brown trout with higher intensity of fungal infection (*Saprolegnia* spp.) had both reduced antioxidant potential (Fig. 5A) and, generally, lower levels of reactive oxygen metabolites (Fig. 5B). With respect to hydroperoxide levels, our results suggest some non-linearity in the relationship between infection and content of hydroperoxides (dashed line in Fig. 5B), with hydroperoxides increasing slightly with low levels of infection (between 0 and 30% fin cover). However, while a non-linear regression explained an additional 5% of the variation in the data, model selection techniques supported the retention of the simpler (linear) model (Akaike weight 81% for the linear regression and 19% for the nonlinear regression shown in Fig. 5B).

Fig. 2. Levels of circulating testosterone (A) and cortisol (B) from interim plasma samples taken from female brown trout.

Table 1

Analysis of variance of the effects of plasma hormone concentrations on plasma non-enzymatic antioxidant capacity (OXY) and levels of oxidative damage (hydroperoxides). Results show the treatment effects retained in minimal general linear models identified by backward deletion of non-significant terms. Goodness of fit statistics of the minimal models were $R^2 = 0.36$ for antioxidant potential (OXY) and $R^2 = 0.49$ for oxidative damage (hydroperoxides).

Factor	DF	F	p
<i>Antioxidant potential (OXY)</i>			
Infection intensity	1, 32	11	<0.01
Spawning group	2, 32	4.9	<0.05
Spawning testosterone	1, 32	0.2	0.7
Spawning group × spawning testosterone	2, 32	5.0	<0.05
<i>Oxidative damage (hydroperoxides)</i>			
Infection intensity	1, 35	8.1	<0.01
Interim testosterone	1, 35	4.8	<0.05
Spawning testosterone	1, 35	7.1	<0.05

4. Discussion

Contrary to our expectation that increases in steroid hormone concentrations would be accompanied by an increase in levels of hydroperoxides, our results demonstrate that levels of hydroperoxides are negatively associated with circulating testosterone at the time of spawning for female brown trout. Moreover, the direction and magnitude of the relationship between antioxidant capacity and circulating testosterone depended upon the timing of spawning: fish that spawned early had higher antioxidant capacity with increasing testosterone levels whereas the converse was true for those that spawned late. Concentrations of cortisol were only indirectly related to antioxidant capacity and levels of hydroperoxides, with these two variables being more strongly related to the intensity of fungal infection experienced by female trout than they were to cortisol levels (although cortisol levels and infection intensity were themselves correlated).

Our results indicate that factors that influence the timing of spawning of female trout potentially mediate the way in which steroid hormones influence plasma non-enzymatic antioxidant capacity. There are three likely explanations for the among-group (i.e., spawning group) variation in the direction and magnitude of the relationship

between antioxidant capacity and testosterone observed in this study. First, and despite the fact that the population of female trout used in this study were maintained under identical conditions for 1 year prior to the experiment, the difference in ovulation times meant that the implantation, tagging and weighing procedures associated with the initiation of the experiment fell within a different portion of the egg production cycle for fish in the different spawning groups. Egg production is energetically expensive and moving fish among tanks earlier in the vitellogenic cycle might have been inherently more challenging for the antioxidant status of late-spawning trout. Second, the timing of ovulation might have been related to the social status of individual trout. We were unable to assess social status and, because social dominance is related to metabolic rate in salmonids (Metcalf et al., 1995), it is conceivable that some component of behaviour that is correlated with early maturation contributed to this result, or that dominant fish mature earlier and are better able to maintain oxidative balance throughout egg development. We propose that further studies into the relationship between social status, maturation date and levels of hormone precursors of testosterone will be a profitable avenue of research. Finally, differences in the range of T levels observed among individuals within groups may have altered the direction and magnitude of the relationship between circulating testosterone and antioxidant capacity. In fact, our data suggest the presence of a non-linear relationship between T and antioxidant capacity, such that OXY is maximised at intermediate levels of plasma testosterone. Unfortunately we could not control the timing of ovulation or the circulating testosterone levels of the trout and this meant that mean T levels were higher, and the range of observed values greater, in spawning group 3 compared to the other two groups. In birds, increased levels of T delay oviposition (e.g., Goerlich et al., 2010) and our results suggest that a similar process might affect spawning of female trout. Nevertheless, if the overall relationship between T and OXY is indeed non-linear, different ranges of testosterone levels among the different spawning groups could cause the effect of T on OXY to differ among-groups.

Our results imply that attributes of female trout that cause them to have higher testosterone levels in the period leading up to spawning also reduce their capacity to manage oxidative stress at the time of spawning. Although relationships between testosterone and oxidative stress are poorly studied in fish, androgen hormones have been demonstrated to cause oxidative stress in other organisms including rats (Iliescu et al., 2007) and birds (Alonso-Alvarez et al., 2007a). High levels of androgen hormones are typically associated with aggressive behaviour which, for salmonids, is in turn correlated with metabolic rate (Metcalf et al., 1995). If maintained throughout the reproductive period, inflated basal energetic costs plus additional activity costs associated with aggression might reduce energy available for mediating oxidative stress and drive a general increase in levels of hydroperoxides at spawning. The positive relationship between interim testosterone and hydroperoxides at spawning could also reflect carry-over effects of the increase in metabolic rate generated by the testosterone itself (Buchanan et al., 2001). Conversely, levels of reactive oxygen metabolites at spawning were negatively associated with circulating testosterone levels measured at the same time. This finding is unexpected because data on three sub-species of wild female trout show that lipid peroxidation levels and antioxidant enzyme activities in the liver coincide with the peak in gonado-somatic index just prior to spawning (Aras et al., 2009). We propose that this result might be driven by the fact that levels of testosterone are in decline at spawning for female brown trout (Norberg et al., 1989) and the cessation of endogenous T production may alter blood redox balance. In addition, we cannot exclude the possibility that declining T levels correspond with increasing concentrations of other hormones. Testosterone is readily converted into estradiol through the enzyme aromatase, and estradiol can affect levels of hydroperoxides (Brambilla et al., 2003) and other redox components (Costantini, 2010). As has been observed for some species of birds (Williams et al., 2004), estradiol production is associated with

Fig. 3. Plasma non-enzymatic anti-oxidant capacity in relation to plasma testosterone concentrations at spawning. Symbols represent individual trout from three spawning groups (based upon timing of ovulation) as described in the legend. Lines are trend-lines depicting the slopes of the relationship within each group ($2.4 \pm \text{s.e. } 1.3$, $0.5 \pm \text{s.e. } 2.9$, and $-4.4 \pm \text{s.e. } 1.6$ for groups 1 through 3 respectively). The minimal analysis of variance model for these data is described in Table 1.

Fig. 4. Relationship between hydroperoxides and plasma testosterone concentration at time of sampling mid-way through the experimental period (A) and time of spawning (B). Data points are values for individual females and dashed lines are trend-lines depicting statistically significant relationships identified using analysis of variance (Table 1), with slopes equal to $0.007 \pm \text{s.e. } 0.003$ in (A) and $-0.008 \pm \text{s.e. } 0.003$ in (B).

vitellogenesis and levels of this hormone in salmonid fish peak prior to ovulation and decline during the final phase of egg development. For salmonids, a pregnen hormone ($17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one) is produced in very high levels immediately prior to ovulation, and stimulates spawning (Slater et al., 1994). It therefore seems unlikely that aromatase production, and conversion of testosterone to estradiol, was a strong driver of the patterns of oxidative stress observed in this study.

The results of this study are consistent with the general literature showing an association between stress, as indicated by elevated concentrations of corticosteroids, and susceptibility to infection (Pickering and Duston, 1983; Norris and Hobbs, 2006). Overall, our results indicate that the positive association between cortisol and infection is initiated several weeks prior to the time at which fungal growth becomes visually apparent. In wild populations of salmonid fish, these fungal infections, typically associated with a condition known as ulcerative dermal necrosis, can affect up to 30% of individuals (Roberts, 1993), and are estimated to have caused mortality of up to 6% of the total population of sea trout in Scotland during the late 1960s (Munro, 1970). Consistent with our finding that plasma antioxidants decrease linearly with fungal cover, one previous study has demonstrated that dermal infection resulted in oxidative stress in brown trout due to the inhibition of antioxidant mechanisms (Kurhalyuk et al., 2010). The OXY decrease in trout with low levels of infection is also in agreement with the observed depletion of antioxidants during the oxidative burst associated with immune stimulation in birds (Costantini and Moller, 2009). More broadly, patterns of

variation observed here for both oxidative damage and antioxidant capacity are compatible with a stimulation of immune response at low fungal load but a suppression of normal immune activity at high fungal load. Studies on mammals show that stimulation of blood leucocyte activity enhances the blood levels of hydroperoxides (Sirak et al., 1991). Similarly, studies on birds show that plasma hydroperoxides and plasma antioxidant capacity significantly increase and decrease respectively after stimulation of the immune system (Costantini and Dell'Omo, 2006; Costantini and Moller, 2009). The increase in plasma hydroperoxides is compatible with the slight increase we observed in female trout whose fins were 20–30% covered in fungus (see dashed line in Fig. 5B, and Results section above). The continuous decrease in OXY we observed in trout with stronger infection might therefore reflect a passive adjustment of antioxidant levels to the decrease in pro-oxidant production, rather than a state of oxidative stress in the blood.

Changes in circulating hormone levels are intrinsically linked with reproduction but the effects of these changes on oxidative balance are poorly understood. This study investigated the hypothesis that increasing concentrations of testosterone and cortisol observed during reproduction of female brown trout would increase the risk of oxidative stress. We have shown that increased testosterone levels in the months prior to spawning lead to higher levels of reactive oxygen metabolites at spawning, suggesting that fish with elevated testosterone during vitellogenesis have a higher risk of oxidative damage. Cortisol levels were not directly related to oxidative damage

Fig. 5. Relationship between intensity of fungal infection (% cover of fins) and plasma non-enzymatic antioxidant capacity (A) and content of hydroperoxides (B). Data points represent individual trout sampled at the time of spawning and lines. Solid line indicates linear regression (slopes are $-123 \pm \text{s.e. } 32$ and $-0.39 \pm \text{s.e. } 0.014$ in A and B respectively), based on the minimal models described in Table 1. Dashed line represents fit of the equation $y = a + (b - e^{-\alpha x})e^{-\beta x}$ with parameters estimated as $a = 0.2$, $b = 1.7$, $\alpha = 0.04$ and $\beta = 0.02$.

or antioxidant capacity, but concentrations of this hormone were positively related to levels of fungal infection, which was itself associated both with lower antioxidant capacity and lower levels of oxidative damage. The value of our study lies in the fact that so few other studies have measured both hormone levels and oxidative stress in vertebrates during reproduction. We suggest that future studies assessing how different hormones (e.g. testosterone and estradiol) interact to determine rates of energy metabolism, and measuring multiple pathways of energy mobilisation (e.g. blood glucose and end-products of lipid peroxidation like malondialdehyde) would be a fruitful area of future research. Our results highlight the complexity of interactions between different components of the endocrine system and metabolism and suggest that caution be used in interpreting relationships between a single hormone and indicators of oxidative balance or other fitness proxies.

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