Oxidative stress and partial migration in brown trout (Salmo trutta)

Kim Birnie-Gauvin, Kathryn S. Peiman, Martin H. Larsen, Henrik Baktoft, Kim Aarestrup, William G. Willmore, and Steven J. Cooke

Abstract: During migration, animals are typically limited by their endogenous energetic resources that must be allocated to the physiological costs associated with locomotion, as well as avoiding and (or) compensating for oxidative stress. To date, there have been few attempts to understand the role of oxidative status in migration biology, particularly in fish. Semi-anadromous brown trout (Salmo trutta L., 1758) exhibit partial migration, where some individuals smoltify and migrate to sea, and others become stream residents, providing us with an excellent model to investigate the link between oxidative stress and migration. Using the brown trout, we obtained blood samples from juveniles from a coastal stream in Denmark in the fall prior to peak seaward migration that occurs in the spring, and assayed for antioxidant capacity (oxygen radical absorbance capacity) and oxidative stress levels (ratio of oxidized to reduced glutathione). We found that individuals that migrated had higher antioxidant capacity than residents and that future migration date was negatively correlated with both antioxidant capacity and body length in the fall. This study provides the first evidence that oxidative status is associated with migration strategy and timing, months in advance of the actual migration, and provides insight into the role of oxidative status in animal migration.

Key words: brown trout, Salmo trutta, antioxidant, partial migration, resident, migrant, oxidative stress.

Introduction

Migrations represent some of the most fascinating and energy-demanding phenomena in the animal world and are often typified by prolonged elevation in metabolic rate associated with high levels of locomotor activity (Leffler 1993; Jonsson et al. 1997). The idea that elevated metabolism leads to increased production of reactive oxygen species (ROS) via an increased flux of electrons at the level of the electron transport chain is widespread in the literature (Wingfield et al. 1998), though not well supported empirically (Salin et al. 2015 and references therein). An increase in ROS can result in an imbalance between ROS and antioxidants (molecules that delay or inhibit oxidation; Halliwell and Gutteridge 2007) and can lead to oxidative stress and the damage of macromolecules (Asada and Takahashi 1987). When organisms allocate their limited resources to enhance their antioxidant capacity and resist oxidative stress, energy is diverted from other activities such as growth, locomotion, immunity, and avoiding predators (Ball and Balthazart 2008; Denver et al. 2009).

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The relationship between oxidative stress and life-history traits is still poorly understood (Speakman et al. 2015). The majority of studies have focused on immediate and short-term effects of ROS, but there is increasing realization that oxidative stress will impact life histories over longer time scales (Monaghan et al. 2009; Birnie-Gauvin et al. 2017a). For example, Costantini et al. (2008) provided evidence that homing pigeons (Columba livia Gmelin, 1789) undergo longer flights experience greater oxidative stress than individuals that undergo shorter flights, as well as control individuals (no flight). The few studies published to date that investigated the link between oxidative stress parameters and life-history strategies are often limited to birds (Costantini 2014 and references therein), therefore restricting the generality of those conclusions to other taxa (but for a review see Birnie-Gauvin et al. 2017a). As a result of elevated metabolism and high energetic demands during migration, we would expect migratory species to be better equipped to deal with oxidative stress than non-migratory species (Costantini 2008). The same pattern may exist between migratory and resident individuals within a species (i.e., when a species exhibits partial migration; Chapman et al. 2011). No studies that we know of have investigated the potential role of oxidative status as a determinant of partial migration strategy.

Semi-anadromous brown trout (Salmo trutta L., 1758) undergo partial migration, where migratory and resident individuals coexist within the same population (Jonsson and Jonsson 1993). The decision to smoltify and migrate to sea or to assume residency in their native stream is a complex interaction between environment and physiology that is particularly sensitive to stressful conditions (Thorpe et al. 1992; Metcalfe 1998; Cucherousset et al. 2005; Dingle and Drake 2007; Boel et al. 2014). The decision to migrate or not is thought to be made at the end of the summer before spring migration (Metcalfe 1998). We used blood samples collected in the fall to determine whether oxidative stress was associated with migration strategy and migration timing in the spring. Consequently, our goal was to evaluate the link between oxidative stress and partial migration in wild juvenile brown trout in relation to within-year variation in timing of migration and migration strategy (migratory versus resident), and not related to whether migratory individuals undertake migration in one year versus the next year (between-year variation). Larger individuals are more constrained by low food availability in stream environments than smaller individuals and are thus more likely to migrate to marine environments, where food availability is greater (Ökland et al. 1993; Thorpe et al. 1998). Additionally, larger individuals often have faster growth rates and higher metabolic rates (Ökland et al. 1993; Thorpe et al. 1998), and thus may have higher levels of oxidative stress than smaller individuals. Consequently, we predicted that (i) larger individuals will migrate; (ii) individuals with higher levels of oxidative stress and lower levels of antioxidants will migrate; (iii) within migratory individuals, larger individuals will migrate sooner; and (iv) individuals with higher levels of oxidative stress and lower levels of antioxidants will migrate sooner.

Materials and methods

Study location

The Gudsø stream (mean width ~2 m) is located in east-central Jutland, Denmark (Figs. 1A, 1B). The stream runs over approximately 16 km and is surrounded by agricultural land. Several tributaries flow into the main stem, before reaching the northwest Baltic Sea at Kolding Fjord. The stream supports natural populations of semi-anadromous brown trout, common eel (Anguilla anguilla (L., 1758)), and European brook lamprey (Lampetra planeri (Bloch, 1784)). Two passive integrated transponder (PIT) reading stations (PIT stations 1 and 2) are located approximately 1 km from the outflow of the stream into the fjord record the passage of PIT-tagged fish (Figs. 1A, 1B). The distance between the two PIT reading stations is 150 m. Each reading station consists of two loop-shaped antennas spaced 5 m apart, each covering the entire cross-section of the stream. This allowed us to determine the swimming direction of migrating trout. We evaluated the tag detection efficiency of PIT station 1 using the formula described in Zydlewski et al. (2006), and found it to be 88%.

**Fish sampling and tagging**

Fish were captured in the main stem of the Gudsø stream, approximately 2 km upstream of the entrance to the fjord (Figs. 1A, 1B), from 20 to 25 October 2015. Additional fish were captured from a tributary on 2 and 3 November 2015. All trout greater than 120 mm in length were captured using single-pass electrofishing gear (Stampes Elektro AS, Ringkøbing, Denmark) and placed in a 60 L container of fresh stream water. The water was changed continuously to provide freshly oxygenated water. Fish were placed in a 0.03 g/L benzocaine solution until their opercular rate had slowed and fish were unresponsive to external stimuli (usually less than 4 min). Total length (±1 mm) and wet mass (±0.1 g) were measured for individual fish. A relative condition factor (K) was calculated using eq. 1 (Bolger and Connolly 1989). Fish were then tagged with a 23 mm PIT tag (Texas Instruments model No. RJ-TRP-RRHP, 134 kHz, 0.6 g mass in air; Texas Instruments, Plano, Texas, USA) inserted into their body cavity. Larsen et al. (2013) demonstrated that the retention of these tags in Atlantic salmon (Salmo salar L., 1758) was 97% with no effects on mortality or growth.

\[ K = \left( \frac{\text{mass}}{\text{length}^3} \right) \times 100 \]

**Blood sampling**

Blood samples of 0.1 mL were obtained from the caudal vascularity of individual fish using a heparinized 1.5 inch (1 inch = 2.54 cm) 25-gauge needle. Within 10 min of sampling, blood was centrifuged at 6000 rev/min (2000g) for 2 min, after which plasma was separated from red blood cells (RBCs). RBCs were flash-frozen in liquid nitrogen and then stored at −80 °C. Fish were allowed to recover in a 60 L container of fresh stream water and were released near their site of capture. These standardized techniques were approved by the Danish Animal Experiments Inspectorate (license number 2013-15-2934-00808).

**Antioxidant capacity**

Samples of RBCs were homogenized on ice in 1:5 lysis buffer (20 mmol/L Tris-HCl, 137 mmol/L NaCl, 1% NP-40, 10% glycerol, 2 mmol/L EDTA) using a handheld Tissue Master 125 (Omi International, Kennesaw, Georgia, USA). Lysates were centrifuged at 13 000 rev/min (11000g) for 5 min at 4 °C in a Hermle Labnet Z216MK (Mandel, Guelph, Ontario, Canada). Supernatants were separated into two: one half for the antioxidant capacity assay and the other half for the oxidative stress level assay (see below). Samples were stored at −80 °C until the oxygen radical absorbance capacity (ORAC) assay was performed (as described in Wilson et al. 2012). ORAC assays were performed using a Cytation 5 microplate reader (BioTek Instruments Inc., Winooski, Vermont, USA) and black 96-well Costar microplates. Fluorescence was measured with an excitation wavelength of 485 nm and emission of 520 nm. Gen5 version 2.07.17 data analysis software (BioTek Instruments Inc., Winooski, Vermont, USA) was used to analyze the fluorescence data.

Each reaction well contained 20 μL of each sample, blank (75 mmol/L potassium phosphate; pH 7.4), or standard (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox); 0–400 μmol/L) and 3.82 μmol/L fluorescein in 75 mmol/L 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 to a final concentration of 79.83 mmol/L. The plate was placed immediately in the microplate reader and the fluo-
rescence was read every 80 s for 90 min. The area under the fluorescence decay curve was determined for the samples and Trolox standards to determine the Trolox equivalency, commonly used as a benchmark for antioxidant capacity. Total protein of samples was determined using the BioRad assay and final values are reported in Trolox equivalents (TE) per microgram of total protein. All samples were run in duplicates, and the mean of both values was used as the final parameter value.

The ORAC assay provides a measure of low molecular weight antioxidants and is one of the few techniques that takes the reaction of ROS to completion (Cao and Prior 1999). Like most antioxidants, low molecular weight antioxidants are typically derived from diet, and are therefore expected to remain relatively consistent through time, provided that an individual continues to feed on the same food sources.

Oxidative stress levels

Following the homogenization of the RBC samples in lysis buffer described above, supernatants (half of the total amount) were further homogenized in 5% sulfosalicylic acid solution (1:5; bubbled with N₂ gas). Sample lysates were centrifuged at 13 500 rev/min (11000 g) for 10 min at 4 °C. Supernatants were used to assess total glutathione (TGSH), reduced glutathione (GSH), and oxidized glutathione (GSSG) (where TGSH = GSH + 2GSSG). Glutathione assays were performed using an Epoch microplate reader with Gen5 version 2.07.17 data analysis software (Biotek Instruments, Winooski, Vermont, 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 with a standard curve of GSH.
For the measurement of TGSH, the reaction media contained 20 μL of sample, 5 IU/mL glutathione reductase, 0.5 mol/L potassium phosphate buffer (pH 7.0), 0.3 mmol/L nicotinamide adenine dinucleotide 2’-phosphate (NADPH), and 60 mmol/L DTNB, and was read for 30 min and compared with a GSH standard curve (0–8 μmol/L). To quantify only GSSG, 50 μL of the initial supernatant and the GSSG standards (0–0.5263 mmol/L) were treated with 44.7 mmol/L 2-vinylpyridine and 227.27 mmol/L KPi in a total volume of 110 μL and allowed to incubate at room temperature for 90 min to derivatize the GSH. Once complete, the GSSG was measured in the same manner as TGSH using the methods described above. GSH values were calculated using the equation described above. Final values are reported in concentrations of millimole per litre. All samples were run in duplicates, and the mean of both values was used as the final parameter value.

We opted to measure glutathione because it is the most abundant antioxidant in eukaryotic cells and is critical for the protection against oxidative damage (Owen and Butterfield 2010). Moreover, glutathione is costly to generate and is therefore seldom broken down, making it useful to investigate effects over longer time scale (i.e., tends to remain fairly constant over time within an individual).

Evaluation of predation and migration

Two cormorant (family Phalacrocoracidae) colonies are located approximately 2 and 5 km away from the Gudsø stream. Each colony was scanned on 14–15 March 2016 by two people, each sweeping the entire area of the colonies once to detect excreted PIT tags. Scanned PIT tags allowed us to determine which fish had died from cormorant predation.

The main stem and one tributary of the Gudsø stream were resampled entirely between 29 February and 2 March 2016 to evaluate residency. Recaptured fish were assumed to be residents unless the fish was later detected at the PIT reading stations. PIT data were downloaded from 27 October 2016, past the peak migration period for smolts, which occurred in March–April 2016. All fish detected at the PIT antennas were considered to be migrants. Fish that were neither detected at the PIT antennas, recaptured in the spring, nor found in the cormorant colonies were defined as unknown.

Statistical analysis

Statistical analyses were conducted using R version 3.2.3 (R Core Team 2015; nlme and AICcmodavg packages by Pinheiro et al. 2016). To explore potential relationships between individual life-history strategy (migratory versus resident) and oxidative stress levels (OSL) or antioxidant capacity (AOX) obtained for each fish, we fit the following generalized linear model, with length and condition (K) as covariates:

\[
\text{strategy}_i \sim \text{bin}(n_i, 1) \\
\text{E(strategy)}_i = \pi_i \\
\text{var(strategy)}_i = \pi_i(1 - \pi_i) \\
\logit(\pi_i) = \eta_i \\
\eta_i = \beta_1 + \beta_2 \cdot \text{length}_i + \beta_3 \cdot K_i + \beta_4 \cdot \text{OSL}_i + \beta_5 \cdot \log(\text{AOX})_i
\]

The model states that the life-history strategy of fish \(i\) follows a binomial distribution with probability parameter \(\pi_i\) and \(n = 1\), i.e., a Bernoulli distribution. \(\pi_i\) is specified through a logit-link by the predictor function \(\eta_i\) to be a linear function of the included covariates (\(\text{length}_i, K_i, \text{OSL}_i, \) and \(\text{AOX}_i\)).

For the migratory fish, we modelled day of migration (DOM; unit: day of year, where day 300 = 28 October 2015) as a function of individual oxidative stress metrics and day of tagging (DOT; unit: day of year) by the following model fitted using generalized least squares:

\[
\text{DOM}_i = N(\mu_i, \sigma^2 \cdot \text{length}^2, r^2) \\
\epsilon(\text{DOM})_i = \mu_i \\
\text{var(DOM)}_i = \sigma^2 \cdot \text{length}^2 \\
\mu_i = \beta_1 + \beta_2 \cdot \text{length}_i + \beta_3 \cdot K_i + \beta_4 \cdot \text{OSL}_i + \beta_5 \cdot \log(\text{AOX}) + \beta_6 \cdot \text{DOM}_i
\]

The model assumes DOM of fish \(i\) follows a Gaussian distribution with mean \(\mu_i\) specified as an identity-linked predictor function of the included covariates (\(\text{length}_i, K_i, \text{OSL}_i, \text{AOX}_i, \) and \(\text{DOM}_i\)). A covariate variance structure incorporating length, was used to accommodate variance heterogeneity.

For both models, OSL was transformed to a categorical variable representing whether or not individual OSL values were zero (i.e., true zeros) or not. Additionally, AOX was log-transformed because preliminary analysis indicated that the models would otherwise violate underlying assumptions.

We tested whether length was correlated with OSL or AOX using a linear regression independently within migratory and resident individuals.

Results

A total of 414 juvenile brown trout were initially captured, tagged, and sampled in the fall. Of those fish, 24 were recaptured in the spring, of which only 13 (3.1%) were not detected at the PIT antennas, and are therefore assumed to be residents. We found that 147 (35.5%) of all tagged individuals were known migrants, 11 (2.7%) were predated by cormorants, and 241 (58.2%) had an unknown fate (presumably mortalities). Of the 147 migratory individuals from the study, a subsample of 48 was randomly chosen to perform oxidative stress assays (with some individuals randomly chosen within specific time intervals to ensure coverage across the migratory season), while all 13 residents were assessed. Two of the migratory individuals were considered to be outliers and therefore removed from subsequent analyses (\(n = 46\)).

In the fall, migratory individuals had a higher antioxidant capacity than resident individuals (GLM, \(Z = -2.05, P = 0.042\); Fig. 2). No significant relationships were detected between life-history strategy and oxidative stress levels (\(Z = -0.688, P = 0.49\), length \(Z = -1.63, P = 0.10\), or condition \(Z = 0.25, P = 0.80\). For all parameter values see Table 1.

Within migratory individuals, day of migration was negatively correlated with antioxidant capacity (\(t = -2.20, P = 0.0393\); Fig. 3A) and body length (\(t = -3.81, P = 0.0005\); Fig. 3B), but was not associated with oxidative stress levels (\(t = -1.35, P = 0.18\) or condition (\(t = 0.64, P = 0.52\)).

Within migratory individuals, body length was positively correlated with oxidative stress levels (\(t = 2.36, P = 0.023\), but not antioxidant capacity (\(t = -1.89, P = 0.065\)). Within resident individuals, body length was not correlated with oxidative stress levels (\(t = -0.35, P = 0.73\) or antioxidant capacity (\(t = -0.68, P = 0.51\)).

Discussion

Migration is an energetically demanding activity, which involves physiological costs such as a consistently elevated metabolic rate, thus depleting finite resources more rapidly and potentially increasing the production of ROS compared with non-migratory individuals (Lefler 1993; Jonsson et al. 1997; Costantini et al. 2007). Migratory individuals must therefore have the ability to cope with the increased ROS production that migration induces, which implies having sufficient repair mechanisms and antioxidants (Costantini 2008). Here, we tested whether this overall greater ability to deal with high levels of ROS production is apparent long before migration actually occurs.

Migrant and resident brown trout show differences in morphological (e.g., color and body form) traits, which could play a role in forging an individual’s oxidative status. For example, resident individuals display yellow bellies and red spots on their sides.
ualswould increase their levels of antioxidants as they approached migration). This also predicts that later migrating individuals may invest their resources into building antioxidant capacity to prepare for migration, such as silvering in colour and increased sodium potassium ATPase activity in the gills (Hoar 1988; Aarestrup et al. 2000; Nielsen et al. 2004), but there is no indication that they divert antioxidant resources to do so. We found that antioxidant capacity, measured as ORAC (i.e., low molecular weight antioxidants), was higher in migratory individuals than in resident individuals. These antioxidants were elevated days to months in advance of migration. It is possible that our findings reflect other physiological differences such as differences in growth rate or standard metabolic rate (SMR) among individuals, which may make an individual more likely to adopt one strategy over the other. Specifically, individuals with higher growth rates and metabolic rates may have higher antioxidant capacities to compensate for higher metabolic demands, and may be more likely to migrate because these individuals are more constrained by low food availability in freshwater stream environments.

We observed that larger individuals migrated sooner, which is a well-supported pattern in the literature (Metcalfe et al. 1990; Bohlman et al. 1996). Because fish of larger size may have higher growth rates and higher metabolic rates than their smaller counterparts (Økland et al. 1993; Thorpe et al. 1998), we would predict that larger individuals have higher levels of oxidative stress. Our data suggests that this is the case only within migrants, where oxidative stress levels were positively correlated with length, possibly emphasizing that larger individuals are more constrained by low food availability in freshwater stream environments.

It is possible that our findings reflect other physiological differences such as differences in growth rate or standard metabolic rate (SMR) among individuals, which may make an individual more likely to adopt one strategy over the other. Specifically, individuals with higher growth rates and metabolic rates may have higher antioxidant capacities to compensate for higher metabolic demands, and may be more likely to migrate because these individuals are more constrained by low food availability. For example, Sloat and Reeves (2014) showed that rainbow trout (Oncorhynchus mykiss (Walbaum, 1792)) with high SMR were more likely to smoltify and migrate than those with lower SMR. As such, the higher antioxidant capacities that we observed in migrant individuals in the present study may reflect a compensatory mechanism rather than individual readiness to migrate. Future studies should consider measures of SMR and oxidative stress indices in the context of partial migration to answer this question, though this may represent a challenge in field studies.

We cannot exclude the possibility that sex and age played a role in the patterns in oxidative parameters observed in this study. Although it has been reported that females tend to migrate more often than males (reviewed in Jonsson and Jonsson 1993), most studies of partial migration do not sex juvenile fish (e.g., Morinville and Rasmussen 2003) because this process requires lethal sampling or expensive laboratory assays. However, even if the observed resident–migrant differences were due to sex, this itself is an intriguing possibility as no other studies that we know of have documented differences in oxidative parameters due to sex in immature wild fish. Similarly, age was not determined for these fish and may have affected oxidative status. Though the link between oxidative stress markers and age has been established in humans (Harman 1956), we know very little about fish in that

### Table 1. Measured variables for migratory and resident brown trout (Salmo trutta).

<table>
<thead>
<tr>
<th>Strategy</th>
<th>Migrants</th>
<th>Residents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass (g)</td>
<td>28.6±16.3</td>
<td>25.1±8.9</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>14.3±1.9</td>
<td>13.6±1.3</td>
</tr>
<tr>
<td>Condition</td>
<td>0.9±0.08</td>
<td>1.0±0.08</td>
</tr>
<tr>
<td>TGSH (mmol/L)</td>
<td>250.7±177.0</td>
<td>75.6±45.8</td>
</tr>
<tr>
<td>GSH (mmol/L)</td>
<td>250.7±177.9</td>
<td>75.6±45.8</td>
</tr>
<tr>
<td>GSSG (mmol/L)</td>
<td>0.006±0.01</td>
<td>0.002±0.004</td>
</tr>
<tr>
<td>GSSG/GSH</td>
<td>0.00002±0.00003</td>
<td>0.00002±0.00003</td>
</tr>
<tr>
<td>ORAC (TE/µg protein)</td>
<td>13.0±9.7</td>
<td>8.5±2.2</td>
</tr>
<tr>
<td>Protein (µg)</td>
<td>3.0±1.2</td>
<td>5.5±1.2</td>
</tr>
</tbody>
</table>

Note: All values are presented as mean ± SD for migrants (n = 46) and residents (n = 13). TGSH, total glutathione; GSH, reduced glutathione; GSSG, oxidized glutathione; ORAC, oxygen radical absorbance capacity (Trolox equivalents (TE) per microgram of protein).
Fig. 3. Migration timing of brown trout (*Salmo trutta*). (A) Migration day (day 300 = 28 October 2015) as a function of antioxidant capacity (oxygen radical absorbance capacity (ORAC)), which is measured in Trolox equivalents (TE) per microgram of protein (GLM, $P = 0.0393$, $n = 46$). (B) Migration day as a function of body length, which is measured in centimetres (GLM, $P = 0.0005$, $n = 46$). Solid lines represent model predictions, whereas broken lines indicate 95% confidence intervals.

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