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

[1] Fish Ecology and Conservation Physiology Laboratory, Department of Biology and Institute of Environmental Science, Carleton University, 1125 Colonel By Drive, Ottawa, ON, Canada K1S 5B6

[2] Department of Biology and Institute of Biochemistry, Carleton University, 1125 Colonel By Drive, Ottawa, ON, Canada K1S 5B6

[3] DTU AQUA, National Institute of Aquatic Resources, Section for Freshwater Fisheries Ecology, Technical University of Denmark, Vejlsøvej 39, 8600 Silkeborg, Denmark

[4] Danish Centre for Wild Salmon, Brusgårdsvej 15, 8960 Randers, Denmark.

[†] Author for Correspondence: kbir@aqua.dtu.dk
+45 35 88 31 25

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Abstract

During migration, animals are typically limited by their endogenous energetic resources which 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*, Linnaeus 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 which 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*, Linnaeus 1758), 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).

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 timescales (Monaghan et al. 2009; Birnie-Gauvin et al. in press). For example, Costantini et al. (2008) provided evidence that homing pigeons (*Columba livia*, Gmelin 1789) which undergo longer flights experience greater oxidative stress than individuals which 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 see Birnie-Gauvin et al. in press for review). 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* (Linnaeus 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 (Økland et al. 1993; Thorpe et al. 1998). Additionally, larger individuals often have faster growth rates and higher metabolic rates

(Økland et al. 1993; Thorpe et al. 1998), and thus may have higher levels of oxidative stress than smaller individuals. Consequently, we predicted that: (1) larger individuals will migrate, (2) individuals with higher levels of oxidative stress and lower levels of antioxidants will migrate, (3) within migratory individuals, larger individuals will migrate sooner, and (4) 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 (Figure 1). 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 (*Salmo trutta*), eel (*Anguilla anguilla*) and lamprey (*Lampetra planeri*). Two passive integrated transponder (PIT) reading stations (PIT stations 1 and 2) located approximately 1 km from the outflow of the stream into fjord record the passage of PIT tagged fish (Figure 1). 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 (Figure 1) from October 20 to October 25, 2015. Additional fish were captured from a tributary on November 2 and November 3, 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 60L container of fresh stream water. The water was changed continuously to provide freshly oxygenated water. Fish were placed in a 0.03g L^{-1} benzocaine solution until their opercular rate had slowed and fish were unresponsive to external stimuli (usually less than 4 minutes). Total length (±1 mm) and wet mass (±0.1 g) were measured for individual fish. A relative condition factor (K) was calculated using equation 1 (Bolger and Connolly 1989). Fish were then tagged with a 23mm 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*, Linnaeus 1758) was 97% with no effects on mortality or growth.

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

Blood sampling

Blood samples of 0.1mL were obtained from the caudal vasculature of individual fish using a heparinized 1.5 inch 25-gauge needle. Within 10 minutes of sampling, blood was centrifuged at 6,000 rpm for 2 minutes, 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 60L 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).

158 *Antioxidant capacity*

159 Samples of RBCs were homogenized on ice in 1:5 lysis buffer (20mM Tris-HCl, 137mM NaCl,
160 1% NP-40, 10% glycerol, 2mM EDTA) using a handheld Tissue Master 125 (Omni
161 International, Kennesaw, GA). Lysates were centrifuged at 13,000 rpm for 5 minutes at 4°C in a
162 Hermle Labnet Z216MK (Mandel, Guelph, ON). Supernatants were separated into two: one half
163 for the antioxidant capacity assay, and the other half for the oxidative stress level assay (see
164 below). Samples were stored at -80°C until the Oxygen Radical Absorbance Capacity (ORAC)
165 assay was performed (as described in Wilson et al. 2012). ORAC assays were performed using a
166 Cytation 5 microplate reader (BioTek Instruments Inc., Winooski, VT, USA) and black 96-Well
167 Costar microplates. Fluorescence was measured with an excitation wavelength of 485 nm and
168 emission of 520 nm. Gen5 data analysis software (2.07.17 BioTek Instruments Inc., Winooski,
169 VT, USA) was used to analyze the fluorescence data.

170 Each reaction well contained 20 µL of either sample, blank (75 mM potassium phosphate
171 (pH 7.4)), or standard (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox); 0-400
172 µM), and 3.82 µM fluorescein in 75 mM potassium phosphate (pH 7.4). The plate was incubated
173 at 37°C for 20 min before rapidly adding the free radical generator 2,2"-azobis (2-
174 amidinopropane) dihydrochloride to a final concentration of 79.83 mM. The plate was placed
175 immediately in the microplate reader and the fluorescence was read every 80 seconds for 90
176 minutes. The area under the fluorescence decay curve was determined for the samples and
177 Trolox standards to determine the Trolox equivalency, commonly used as a benchmark for
178 antioxidant capacity. Total protein of samples was determined using the BioRad assay and final
179 values are reported in Trolox equivalents/µg total protein. All samples were run in duplicates,
180 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 which 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 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), reduced glutathione (GSH) and oxidized glutathione (GSSG) [TGSH = GSH + 2GSSG]. Glutathione assays were performed using an Epoch microplate reader with Gen5 data analysis software (Biotek Instruments, 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 412nm compared to a standard curve of GSH.

For the measurement of TGSH, the reaction media contained 20µL of sample, 5 IU/mL glutathione reductase, 0.5M potassium phosphate buffer (pH 7.0), 0.3 mM nicotinamide adenine dinucleotide 2'-phosphate (NADPH), and 60 mM DTNB and reduction was read for 30 minutes and compared to a GSH standard curve (0-8 µM). 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. Final values are reported in mM concentrations. All samples were run in duplicates, and the mean of both values was used as the final parameter value.

We opted to measure glutathione as it is the most abundant antioxidant in eukaryotic cells and 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 timescale (i.e., tends to remain fairly constant over time within an individual).

Evaluation of predation and migration

Two cormorant (*Phalacrocoracidae* sp., Linnaeus 1758) colonies are located approximately 2 and 5 km away from the Gudsø stream. Each colony was scanned on March 14-15, 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 February 29 and March 2, 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 on October 27th, 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 or 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 and Mazerolle 2016, respectively). To explore potential relationships between individual life history strategy (migratory vs. 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:

$$\text{strategy}_i \sim \text{Bin}(\pi_i, 1)$$

$$E(\text{strategy}_i) = \pi_i$$

$$\text{var}(\text{strategy}_i) = \pi_i * (1 - \pi_i)$$

$$\text{logit}(\pi_i) = \eta_i$$

$$\eta_i = \beta_1 + \beta_2*\text{length}_i + \beta_3*K_i + \beta_4*OSL_i + \beta_5*\log(AOX_i)$$

The model states that the life history strategy of fish *i* follows a binomial distribution with probability parameter π_i and n = 1, i.e. a Bernoulli distribution. π_i is specified through a logit-link by the predictor function η_i to be a linear function of the included covariates (length_i, K_i, OSL_i and AOX_i).

For the migratory fish, we modelled day of migration (DOM, unit: day of year, where Day 300 = October 28, 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:

$$DOM_i \sim N(\mu_i, \sigma^2 \text{ x } \text{length}_i^{2 \text{ x } \delta})$$

$$E(DOM_i) = \mu_i$$

$$\text{var}(DOM_i) = \sigma^2 \text{ x } \text{length}_i^{2 \text{ x } \delta}$$

$$\mu_i = \beta_1 + \beta_2*\text{length}_i + \beta_3*K_i + \beta_4*OSL_i + \beta_5*\log(AOX_i) + \beta_6*DOT_i$$

The model assumes DOM of fish i follows a Gaussian distribution with mean μ_i specified as an identity linked predictor function of the included covariates (length_i, K_i, OSL_i, AOX_i and DOT_i).

A covariate variance structure incorporating $length_i$ 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 as preliminary analysis indicated 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 as outliers and therefore removed from subsequent analyses ($n = 46$).

In the fall, migratory individuals had a higher antioxidant capacity than resident individuals (GLM, $Z = -2.05$, $P = 0.042$; Figure 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 ($Z = 0.25$, $P = 0.80$). See Table 1 for all parameter values.

Within migratory individuals, day of migration was negatively correlated with antioxidant capacity (t = -2.20, P = 0.0393, Figure 3A) and body length (t = -3.81, P = 0.0005, Figure 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 reactive oxygen species (ROS) compared to non-migratory individuals (Leffler 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, both of which can result from the presence of carotenoids, an important source of antioxidants (Youngson et al. 1997). Smolts in contrast, undergo massive physiological and morphological changes to prepare for

296 migration, such as silvering in colour and increased sodium potassium ATPase activity in the
297 gills (Hoar 1988; Aarestrup et al. 2000; Nielsen et al. 2004), but there is no indication that they
298 divert antioxidant resources to do so. We found that antioxidant capacity, measured as the
299 oxygen radical absorbance capacity (i.e., low molecular weight antioxidants), was higher in
300 migratory individuals than in resident individuals. These antioxidants were elevated days to
301 months in advance of migration. It is possible that residents deflect resources from building
302 antioxidant capacity to invest in coloration (i.e., carotenoids in this case). In contrast, migratory
303 individuals may invest their resources into building antioxidant capacity to deal with the
304 demands of migration. This hypothesis is further supported by our finding that migrants with
305 higher antioxidant capacity migrate earlier, perhaps as a sign that fish are ready to migrate (i.e.,
306 physiologically prepared to deal with oxidative stress during migration). This also predicts that
307 later migrating individuals would increase their levels of antioxidants as they approached their
308 migration date, an intriguing area for future study.

309 We observed that larger individuals migrated sooner, which is a well-supported pattern in
310 the literature (Metcalfe et al. 1990; Bohlin et al. 1996). Because fish of larger size may have
311 higher growth rates and higher metabolic rates than their smaller counterparts (Økland et al.
312 1993; Thorpe et al. 1998), we would predict that larger individuals have higher levels of
313 oxidative stress. Our data suggests this is the case only within migrants, where oxidative stress
314 levels were positively correlated with length, possibly emphasizing that larger individuals are
315 more constrained by low food availability in freshwater stream environments.

316 It is possible that our findings reflect other physiological differences such as differences
317 in growth rate or standard metabolic rate (SMR) among individuals, which may make an
318 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 as these individuals are more constrained by 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 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. While 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) as 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 impacted 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 aspect (Martinez-Alvarez et al. 2005), which would provide an interesting avenue for future studies. Nonetheless, only fish between 12 and 20 cm were used, which are typically thought to be 16 to 18 months of age (personal observation, K. Aarestrup; Jonsson 1985) and so it is unlikely that the relationships between oxidative parameters and migratory strategy can be attributed to age. Furthermore, because some

individuals may migrate after 3 years of stream residency (Økland et al. 1993), we cannot say for certain that individuals identified as residents in this study will always remain residents; they may in fact migrate during the following year. However, the factors affecting migration between years may be different than those affecting migration within any given year and thus, our study focused on the potential physiological aspects that underpin partial migration within one year.

Conclusion

To better understand the physiological factors that may promote the evolution of partial migration in fish, we examined oxidative stress markers in both migrant and resident individuals of brown trout. During migration, these fish must distribute their limited resources toward swimming efforts, immunity, and predator avoidance among other physiological demands, in addition to coping with the elevated production of ROS. We show that for migrant fish, these resources are also invested toward building antioxidant capacity days to months before migration occurs. Our study suggests that antioxidant capacity is associated with migratory status and migratory timing in brown trout: migrants have higher antioxidant capacity than residents, and within migrants those with higher antioxidant capacity migrate sooner. This has important ecological implications: (1) increased antioxidant capacity may be a component of smoltification for migratory fish, and (2) fish exposed to stressful conditions may be less able to invest resource in antioxidants, which may delay fish in their migration, and impact population dynamics (Theriault et al. 2008). Our findings support the hypothesis that migrants have mechanisms to cope with the added ROS production induced by a sustained increase in metabolic rate during migration.

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Figure Captions

Figure 1. Map of the Gudsø stream, Jutland, Denmark. Inserted circle shows location of the stream in Denmark. Sampling locations are highlighted in the thick black trace, A) for the fall sampling, and B) for the spring sampling (adapted with permission from Birnie-Gauvin et al. 2017, J. Exp. Biol. 220: 1693-1700).

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Figure 2. Antioxidant capacity. Antioxidant capacity (ORAC; Oxygen Radical Absorbance Capacity) in Trolox equivalents per μg protein for migrants (n = 46) and resident individuals (n = 13). There is a significant association between life-history strategy and antioxidant capacity (GLM, $P < 0.05$).

Figure 3. Migration timing. (A) migration day (Day 300 = October 28, 2015) as a function of antioxidant capacity (ORAC; Oxygen Radical Absorbance Capacity) in Trolox equivalents per μg protein (n = 46, GLM, P = 0.0393). (B) migration day as a function of body length (n = 46, GLM, P = 0.0005). Lines represent model predictions, dotted lines indicate 95% confidence intervals.

546 **Table 1. Measured variables for migratory and resident individuals.** All values presented are
547 mean (±SD) for migrants (n = 46) and residents (n = 13).

	Strategy	
	Migrants	**Residents**
Mass (g)	28.6±16.3	25.1±8.9
Length (cm)	14.3±1.9	13.6±1.3
Condition	0.9±0.08	1.0±0.08
TGSH (mM)	250.7±178.0	75.6±45.8
GSH (mM)	250.7±177.9	75.6±45.8
GSSG (mM)	0.006±0.01	0.002±0.004
GSSG/GSH	0.00002±0.00003	0.00002±0.00003
ORAC (TE/μg protein)	13.0±9.7	8.5±2.2
Protein (μg)	3.0±1.2	5.5±1.2

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