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Age- and sex-dependent variation in the activity of antioxidant enzymes in the brown trout (*Salmo trutta*)

Marco Parolini[1], Rocco Iacobuzio[1], Bruno Bassano[2], Roberta Pennati[1], Nicola Saino[1]

1. *Department of Environmental Science and Policy, University of Milan, via Celoria 2, I-20133 Milano, Italy*

2. *Alpine Wildlife Research Centre, Gran Paradiso National Park, Degioz, Valsavarenche (AO), Italy*

*Correspondence should be addressed to:

Marco Parolini (marco.parolini@unimi.it)

Department of Environmental Science and Policy, University of Milan, via Celoria 26, I-20133 Milano, Italy

Abstract

In natural environments, organisms experience diverse stressful conditions, ultimately leading to the production of reactive oxygen species (ROS) and the consequent onset of oxidative stress, which is one of the main driving force of aging. To counteract the harmful effects of oxidative stress, organisms evolved a complex antioxidant systems. According to the free radical theory of ageing, while the production of ROS increases with age, the antioxidant defenses decline. Although this relationship has been elucidated in diverse vertebrate *taxa*, the information in fish is scant and inconsistent, particularly for populations in the wild. Thus, the aim of the present study was the investigation of age- and sex-related changes of the antioxidant enzymes activity in free-living individuals of the brown trout (*Salmo trutta*). We measured the activity of the main enzymes involved in antioxidant protection, namely superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione S-transferase (GST), in the gills and the liver dissected from brown trouts (1+ to 9+ year-old). A significant age-dependent variation in the activity of antioxidant enzymes was noted, with the exception of CAT. GPx activity followed a significant increasing trend with age in both the organs, while SOD increased in the gills but decreased in the liver. Increased GST activity was found only in gills. SOD and CAT showed sex-dependent differences in the liver of brown trouts, with males showing lower enzymatic activity than females. Our data contribute to fill the gap of knowledge on the relationship between antioxidant enzyme activity and aging in fish.

Keywords: Antioxidant enzymes, aging, brown trout, oxidative stress

1. Introduction

In recent years, a great interest has arisen on the role of oxidative stress in ecological context (Beaulieu et al., 2013; Speakman et al., 2015). Oxidative stress is defined as the imbalance of the equilibrium between pro-oxidant and antioxidant molecules in favor of the former (Finkel and Holbrook, 2000). Organisms may suffer an oxidative stress situation because of an overproduction of pro-oxidants (i.e. reactive oxygen species; ROS) due to diverse stressors and/or a deficiency of antioxidant molecules. In animals, ROS are produced endogenously by diverse physiological processes, including changes in metabolism, somatic growth (Rollo et al. 1996; Alonso-Alvarez et al. 2007), seasonal changes in environmental conditions (e.g. temperature, oxygen and food availability) and reproduction (Alonso-Alvarez et al., 2004; Martinez-Alvarez et al., 2005; Birnie-Gavin et al., 2017), as well as intra- and inter-specific competition (Boonstra, 2013). Moreover, exogenous generation of ROS can originate by the exposure to diverse environmental pollutants, which exert their toxicity through the onset of oxidative stress (Luschak, 2011; Birnie-Gavin et al., 2017). The ROS originated by all the factors mentioned above can interact with cellular macromolecules and cause oxidation of membrane lipids, proteins and DNA, leading to cell senescence, cell death and organism aging (Banudevi et al., 2006). In fact, oxidative stress has been individuated as one of the major causes of physiological aging (Harman, 2003), which is defined as the progressive accumulation of deleterious changes in cells and tissues enhancing the risk of disease and death with increasing age (Harman, 2001). Thus, to prevent the detrimental consequences of oxidative stress, organisms have evolved diverse antioxidant mechanisms to counteract ROS generation and to repair oxidative damage (e.g. Metcalfe and Monaghan, 2013; Costantini, 2014). The first antioxidant defenses rely on non-enzymatic molecules that counteract ROS toxicity through the scavenging action of glutathione (GSH), non-protein thiols and other low molecular weight molecules (e.g. vitamins, carotenoids; Modesto and Martinez, 2010). In addition, ROS can be detoxified by a complex enzymatic defense system, including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), which act according to a cascade mechanism to counteract the toxicity of ROS produced by both intrinsic and extrinsic factors. SOD is the first enzyme of the antioxidant shield against ROS and catalyzes the disproportionation of superoxide radicals ($O2^{\cdot-}$) to hydrogen peroxide, which is then removed by the concomitant activity of CAT and GPx. Moreover, the glutathione S-transferase (GST) is involved in antioxidant defenses by catalyzing the conjugation of reduced glutathione to diverse substrates, including breakdown products of lipid peroxidation, making them more hydrophilic and ease to be excreted (Ketterer et al., 1983).

Like all the aerobic organisms, fish are extremely susceptible to the attack of ROS, so they have evolved an efficient antioxidant machinery to prevent the onset of oxidative stress. Many studies of diverse fish species have been performed to investigate the modulation of enzymatic antioxidants due to diverse environmental stressors, including xenobiotic exposure, nutrient deprivation, and reproduction (Martinez-Alvarez et al., 2005; Birnie-Gavin et al., 2017 and references therein), showing that failure of these defenses can lead to a detrimental oxidative stress situation. However, although some studies on fish have demonstrated age-related changes in different physiological and biochemical endpoints (e.g. Barciela et al., 1993; Patnaik et al., 1994), there is a dearth of information concerning the susceptibility and the effects of age on the activity of antioxidant enzymes in this *taxon* (Birnie-Gavin et al., 2017). In addition, the few studies investigating this issue have neglected the differences related to the sex of individuals, although it has been demonstrated that the activity of antioxidant enzymes can differ between males and females (Sobocanec et al., 2003).

To fill this gap of knowledge, the present study aimed at investigating age- and sex-dependent changes in the activity of antioxidant enzymes in free-living individuals of the brown trout (*Salmo trutta*). A previous study of brown trout showed that glutathione levels change as the fish grow in farmed individuals of age ranging between 5 and 3 year-old (Almroth et al., 2010). Nevertheless, to date there is a dearth of information on the effects of age, as well as of sex, on antioxidant enzyme activity in trouts older than 3 years, both in captivity and in a natural selection regime. Thus, we measured the activity SOD, CAT, GPx and GST, in both the gills and the liver of brown trout individuals (age range 1+ to 9+ year-old) from two streams of the Gran Paradiso National Park (Northwestern Italy). As both these streams have not been supplemented by hatchery fish and/or deprived by fishing for a long time (about 30 years) and do not suffer of any direct anthropic pressure, resident brown trout population represents a unique opportunity to study the age-related variation of antioxidant enzymes in the wild. Enzyme activity was measured in gills because they are the first organ to be affected by environmental stressors due to their delicate structure and multiple functions, as well as their direct and continuous contact with water (Evans, 1987). We also focused on the liver because in fish it is a crucial organ for systemic regulation and detoxification processes, acting to eliminate pathogens, toxic substances and metabolic byproducts and maintain normal physiological functions of other organs (de Andrade et al., 2015). Moreover, as the liver is particularly prone to ROS production because of its high metabolic activity, it is a reservoir of non-enzymatic and enzymatic antioxidants contributing to prevent the onset of oxidative stress. According to the free radical theory of ageing postulating that the production of ROS increases with age and the antioxidant defenses decline (Harman, 1956), we expected a progressive decline of antioxidant activity with age. However, although this link has been elucidated in humans, contrasting and non-conclusive evidence

115 is available for fish (see Martinez-Alvarez et al., 2005). Lastly, since changes in antioxidant enzyme
116 activities can be sex-specific (Ehrenbrink et al., 2006), we expect that the enzyme activity varies
117 between sexes, but we have no *a priori* expectation on the sex with the highest enzymatic antioxidant
118 defenses because of the inconsistency of the data on fish.
119

120 ## 2. Materials and Methods

121 Brown trout individuals were sampled by electrofishing in two streams in the Gran Paradiso National
122 Park, namely Valsoera and Piantonetto. Sampling operations were performed every ten days and
123 spanned over the period ranging between the 6th of May and the 28th of July 2015. Brown trouts were
124 sampled early in their pre-reproductive period to prevent potential confounding factors of food
125 scarceness and reproduction on enzyme activity. In fact, both food deprivation (Robinson et al., 1997)
126 and high metabolic rates experienced by individuals during the reproductive period may cause ROS
127 overproduction and, consequently, changes in antioxidant activity and oxidative damage in fish
128 (Birnie-Gavin et al., 2017). Ten linear transects (about 100 meters each) were travelled twice about
129 30 minutes apart along the course of the streams (2 transects in the Valsoera; 8 transects in the
130 Piantonetto stream). All the sampled brown trouts were immediately transferred to a perforated drum
131 kept into the stream and then, at the end of the sampling operation, they were transferred to a 100 L
132 tank crossed by a constant waterflow located within the aquaculture facility of the Gran Paradiso
133 National Park at Ghiglieri (Noasca, TO). Trouts were maintained in this tank for about 1 hour and,
134 after measuring body length and total weight, they were sacrificed in accordance with the current
135 animal welfare regulations. In the lab, each individual was dissected to isolate the liver and the gills,
136 which were maintained at -20 °C until the analyses of enzyme activity. The sex of each trout was
137 determined by assessing the maturation stage of the gonads, while age was assigned according to the
138 standard growth curves for the model species. We relied on 98 brown trout individuals sampled in
139 Valsoera (n = 12) and Piantonetto (n = 86) streams. Individuals of each sex were grouped in seven
140 classes of age as follows: age 1+ = 12 individuals (5 females; 7 males); age 2+ = 32 individuals (14
141 females; 18 males); age 3+ = 27 individuals (16 females; 11 males); age 4+ = 16 individuals (8
142 females; 8 males); age 5+ = 9 individuals (2 females; 7 males); age 7+ = 1 individual (male) and age
143 9+ = 1 individual (male). The present study was performed under the permission of the Gran Paradiso
144 National Park, which allowed both the sampling and the euthanasia of fish, *a latere* of a Life+ project
145 (BIOAQUE) aimed at the conservation of the marble trout (*Salmo marmoratus*) in the aquatic
146 ecosystems of the park.

147 *2.3 Antioxidant enzyme activity in gills and liver*

148 The activity of antioxidant (SOD, CAT and GPx) and detoxifying (GST) enzymes was measured in
149 triplicate in the cytosolic fraction extracted from homogenates of gills and liver dissected by brown
150 trout individuals. An appropriate amount of gills and liver (≈ 1 g fresh weight) was homogenized in
151 100 mM phosphate buffer (pH 7.4) with the addition of 100 mM KCl and 1 mM EDTA, specific
152 protease inhibitor cocktail (1:10 v/v) and 100 mM dithiothreitol. Sample homogenates were
153 centrifuged at 45.000 *g* for 1 hour at 4 °C. The supernatant was collected and immediately processed
154 for the determination of protein content according to the Bradford method (1976), using bovine serum
155 albumin (BSA) as a standard. Enzyme activities were assessed spectrophotometrically as described
156 by Parolini et al. (2010). Briefly, the SOD activity was assessed by measuring the inhibition in the
157 reduction of cytochrome c (10 μM) at λ = 550 nm caused by the superoxide anion generated by the
158 xanthine oxidase (1.87 mU/mL)/hypoxanthine (50 μM) reaction. The enzyme activity is reported in
159 SOD units (a SOD unit corresponds to the 50% inhibition of the xanthine oxidase reaction). The CAT
160 activity was determined by measuring the consumption of 50 mM H_2O_2 at λ = 240 nm. The GPx
161 activity was assessed by monitoring the consumption of NADPH at λ = 340 nm using 0.2 mM H_2O_2
162 as a substrate in 50 mM phosphate buffer (pH 7) added with glutathione (2 mM), sodium azide (NaN_3;
163 1 mM), glutathione reductase (2 U/mL) and NADPH (120 μM). Lastly, the GST activity was
164 measured by adding 1 mM reduced glutathione (GSH) and 1-chloro-2,4 dinitrobenzene (CDNB) in
165 phosphate buffer (pH 7.4) to the cytosolic fraction; the resulting reaction was monitored for 1 min at
166 λ = 340 nm.

167 *2.5 Statistical analysis*

168 The effect of age and sex, as well as of their interaction, on the activity of antioxidant enzymes
169 measured in the gills and the liver was investigated by means of Linear Mixed Models (LMMs),
170 including the stream as a random factor in all the models. Interaction terms between age and sex were
171 excluded from the final models because they were always non-significant. Since the sampling
172 spanned over two months, we first included the date of sampling as fixed-effect variable in the
173 models. However, because the effect of the date of sampling was always non-significant, we excluded
174 it from all models. Because of the limited number of very old individuals (>5+ year-old) we also run
175 the analysis excluding the two oldest males (7+ and 9+ year-old) to confirm the age- and sex-
176 dependency of antioxidant enzyme activity in both the gills and the liver. In a separate LMM
177 including organ as a fixed factor, we also investigated if the activity of enzymes differed between the
178 considered organs. All the analyses were performed using SPSS 21.0 statistical package.

179

180 ## 3. Results

Overall, the sex ratio of the sample was balanced (females 45/98 = 0.46; males 53/98 = 0.54; χ^2_1 = 0.287; P = 0.592). The LMMs did not show any significant effect of sex and age × sex interaction for all the enzyme activities measured in gills. A significant effect of the age was found on the activity of SOD, GPx and GST in gills, but not of CAT (Table 1), with older individuals showing higher activity compared to the younger ones (Figure 1). The hepatic activity of SOD and GPx showed a significant age-dependent trend (Table 2). However, although the activity of GPx increased with the age of fish according to the same trend as in gills, the activity of SOD decreased with the age of the trout, showing an opposite trend compared to the gills (Figure 2). No significant effect of age was noted for CAT, although females showed a significantly higher activity compared to males. In contrast to results obtained on gills, hepatic GST activity did not depend on age or on sex.

Regardless of the effect of trout sex and age, the activity of antioxidant enzymes was higher in the liver compared to the gills for all the enzymatic activities we monitored ($F > 5.351$; $P < 0.022$ for all enzymes). The LMMs run after the exclusion of the oldest individuals (see Statistical analysis) confirmed the results obtained on the whole sample for both the gills and the liver, with the exception of SOD activity in the gills, which did not significantly vary with age (Table S1 and S2).

4. Discussion

This cross-sectional, correlative study showed a significant age-dependent variation in the activity of enzymes involved in defenses against oxidative stress in both gills and liver of brown trout individuals from a natural population. Whilst GPx activity showed a significant increase with age in both the gills and the liver, SOD showed an opposite trend in either organ, showing an increasing trend in the gills and a decreasing one in the liver. Increased GST activity was noted only in the gills, while CAT activity did not show any significant age-dependency. In addition, sex-dependent activity of SOD and CAT was found in the liver of brown trouts, with males showing lower enzymatic activity than females.

The imbalance in the delicate equilibrium between the production of ROS and the antioxidant defenses results in adverse consequences for physiological functions and it has been individuated as the major cause of senescence (Murphy et al., 2011). The functional deterioration associated with age derives from an accumulation of oxidative damage on lipids, proteins and nucleic acids inflicted by ROS that antioxidants do not efficiently scavenge. In fish, the limited number of studies investigating the effects of age on antioxidant defenses returned inconsistent patterns. For instance, Wdzieczak and coauthors (1982) showed that younger fish of diverse species had higher antioxidant capacity than

older fish. Similarly, Otto and Moon (1996) found that the activity of SOD and glutathione reductase (GR) declined with age in the liver and extrahepatic tissues of the rainbow trout (*Oncorhynchus mykiss*) and black bullhead (*Ameiurus melas*) from two age classes (1+ and 3+ year-old), while no age-dependency was observed for GPx and CAT. Despite these findings, our results showed a contrasting situation. Although no age-dependent trend was noted for CAT activity neither in the gills nor in the liver, a significant age-dependent decrease was found for hepatic SOD (Figure 2). This specific trend may be due to the intense hepatic metabolism that could overproduce ROS causing a physiological deterioration of antioxidant enzymes designated to their detoxification. These results are consistent with those from previous studies of fish (Wdzieczak et al. 1982; Otto and Moon, 1996) and support the free radical theory of ageing (Harman, 1956), which postulates a decline of antioxidant defenses with age due to the increase of ROS production. The excess of ROS may consequently cause the oxidation of cellular macromolecules and the accumulation of unrepaired structural damage to cells, which disrupts cellular functions. The age-dependent decrease of SOD may result in a reduced capacity of the whole antioxidant defense in counteracting ROS toxicity. However, the increase of hepatic GPx activity in liver may be interpreted as a necessary physiological adjustment that trouts activate to maintain a balanced redox status and to prevent the onset of an oxidative stress situation. In contrast to our expectations and to the enzyme activity trends found for the liver, gill activity of SOD, GPx and GST significantly increased with the age of the trouts (Figure 1). The discrepancy in age-related trends of antioxidant enzymes between organs was shown in diverse fish species (see Martinez-Alvarez et al., 2005) and suggests that different organs may differentially respond and/or have a different susceptibility to ROS. In fact, according to other studies of fish, the hepatic enzymatic activity was higher than that measured in the gills, revealing that the liver has higher antioxidant capacity compared to other organs (Perez-Campo et al., 1993; Otto and Moon, 1996). The cause of such differences could be the high rate of ROS generation by metabolic activities in the liver compared to the gills (Gomez et al., 2010). The lower metabolism of the gills may produce a lower amount of ROS that do not affect the functionality of antioxidant enzymes during senescence. According to our findings, the activity of CAT, GPx and GST in gills from older (3+ year-old) rainbow trout individuals was higher than that of younger ones (1+ year-old; Otto and Moon, 1996). In addition, despite GPx activity decreases in the brain and in the liver during the maturation phase (young versus middle-aged), an increase of hepatic activity of this enzyme with age was found in the freshwater murrel (*Channa punctatus*; Nayak et al., 1999). Similarly, an age-dependent increase of GPx and CAT activity was found in the plasma and erythrocytes of the Adriatic sturgeon *Acipenser naccarii* (Sanz et al., 2001). These results suggest that some fish species showing gradual senescence may have evolved an increment of their antioxidant capacity with age (Birnie-

247 Gavin et al., 2017). To explain this particular trend, we may speculate that under favorable conditions
248 in terms of food availability, trouts acquire a great amount of non-enzymatic antioxidants via the diet
249 (i.e. vitamins and carotenoids), which can serve to counteract ROS production and to protect the
250 functionality of enzymatic antioxidants, preventing their deterioration with age. Lastly, besides age-
251 dependent relationships, we found sex-dependent activity of SOD and CAT in the liver of brown
252 trouts, with female having higher activity levels of both the enzymes compared to males. Our findings
253 are not in agreement with those from previous studies of fish, which reported diverse results about
254 the sex-related differences of antioxidant enzymes. For instance, whilst Otto and Moon (1996) did
255 not find any sex-related difference in antioxidant enzyme activity in the rainbow trout or in black
256 bullhead, a study of Nile tilapia (*Oreochromis niloticus*) showed that sex has an effect in SOD and
257 GST activities, with males having higher activity than females (Figueiredo-Fernandes et al., 2006).
258 Despite these discrepancies, studies of vertebrates have described that both the expression and the
259 activity of antioxidant enzymes are overexpressed in females compared to males, mainly in species
260 in which females live longer than males (Borras et al., 2003; Viña et al., 2003), because of the
261 hormonal regulation exerted by estrogens (Viña et al., 2005). Thus, having an elevated antioxidant
262 activity may be an advantage for trout females because it may allow an efficient protection against
263 ROS generated by both endogenous metabolism and exogenous stressors (i.e. exposure to
264 environmental pollutants, changes in environmental conditions). This could be particularly true
265 during reproductive and/or food deprivation periods. In fact, reproduction is a highly demanding
266 activity characterized by an elevated metabolic rate enhancing ROS production (Alonso-Alvarez et
267 al., 2004). In addition, food scarceness during winter may result in a lower dietary uptake of non-
268 enzymatic antioxidants and in a wide range of physiological injuries (e.g. accelerated aging,
269 susceptibility to chemical toxicity), most of which can be related to ROS production (Robinson et al.,
270 1997). Thus, females having high levels of SOD and CAT can counteract more efficiently than males
271 adverse situations boosting the production of ROS that they have to face during their lifespan.

272 In conclusion, this correlative study showed for the first time in free-living individuals of the brown
273 trout that the enzymatic antioxidant defenses differ between organs and depend on age and sex.
274 Interestingly, most of the age-dependent trends we found were opposite to the expectation based on
275 the free radical theory of aging, suggesting the necessity of further studies to elucidate the changes of
276 antioxidant defenses in wild organisms, as well as the implication of intrinsic (i.e. metabolism) and
277 extrinsic factors (e.g. seasonal changes, pollutant exposure) modulating their activity during lifespan.
278 However, these relationships have to be considered with caution because of the cross-sectional nature
279 of the study and should have to be confirmed by a longitudinal study. Moreover, antioxidant activity
280 differed between sexes, suggesting that females may counteract more efficiently than males the toxic

effects of ROS, preventing the onset of oxidative stress situation and potentially slowing down the aging process. Thus, our data contribute to fill the gap of knowledge on the relationship between antioxidant enzyme activity and aging in fish, suggesting that studies of oxidative stress due to both intrinsic and extrinsic factors should consider age of the individuals as a potentially confounding factor.

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406

407 Table 1: Linear mixed models of the activity of defense enzymes (SOD, CAT, GPx and GST) in gills
408 from brown trout individuals (1+ to 9+ year-old), with stream as a random factor. Interaction terms
409 were included in the table but they were excluded from the final models because always non-
410 significant. Significant effects are reported in bold.

411

412

	F	df	P
SOD			
Sex	0.606	1,89	0.438
Age	8.198	6,89	**0.000**
Excluded terms			
Sex × age	0.559	4,81	0.693
CAT			
Sex	0.041	1,90	0.841
Age	0.800	6,90	0.573
Excluded terms			
Sex × age	0.797	4,86	0.530
GPx			
Sex	0.009	1,90	0.924
Age	4.616	6,90	**0.000**
Excluded terms			
Sex × age	1.790	4,81	0.139
GST			
Sex	1.484	1,89	0.226
Age	9.224	6,89	**0.000**
Excluded terms			
Sex × age	2.398	4,85	0.056

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Table 2: Linear mixed models of the activity of defense enzymes (SOD, CAT, GPx and GST) in liver from brown trout individuals (1+ to 9+ year-old), with stream as a random factor. Interaction terms were included in the table but they were excluded from the final models because always non-significant. Significant effects are reported in bold.

	F	df	P
SOD			
Sex	8.635	1,89	**0.004**
Age	4.355	6,77	**0.001**
Excluded terms			
Sex × age	1.956	4,73	0.112
CAT			
Sex	5.214	1,89	**0.025**
Age	1.323	6,83	0.256
Excluded terms			
Sex × age	2.051	4,82	0.095
GPx			
Sex	1.782	1,89	0.185
Age	3.498	6,74	**0.004**
Excluded terms			
Sex × age	1.280	4,76	0.287
GST			
Sex	0.865	1,90	0.355
Age	1.570	6,90	0.165
Excluded terms			
Sex × age	0.807	4,86	0.524

Figure 1: age-dependent variation in the activity of SOD (a), CAT (b), GPx (c) and GST (d) in gills dissected by brown trout individuals (1+ to 9+ year-old). Solid lines show significant relationships, while dashed lines show non-significant relationships.

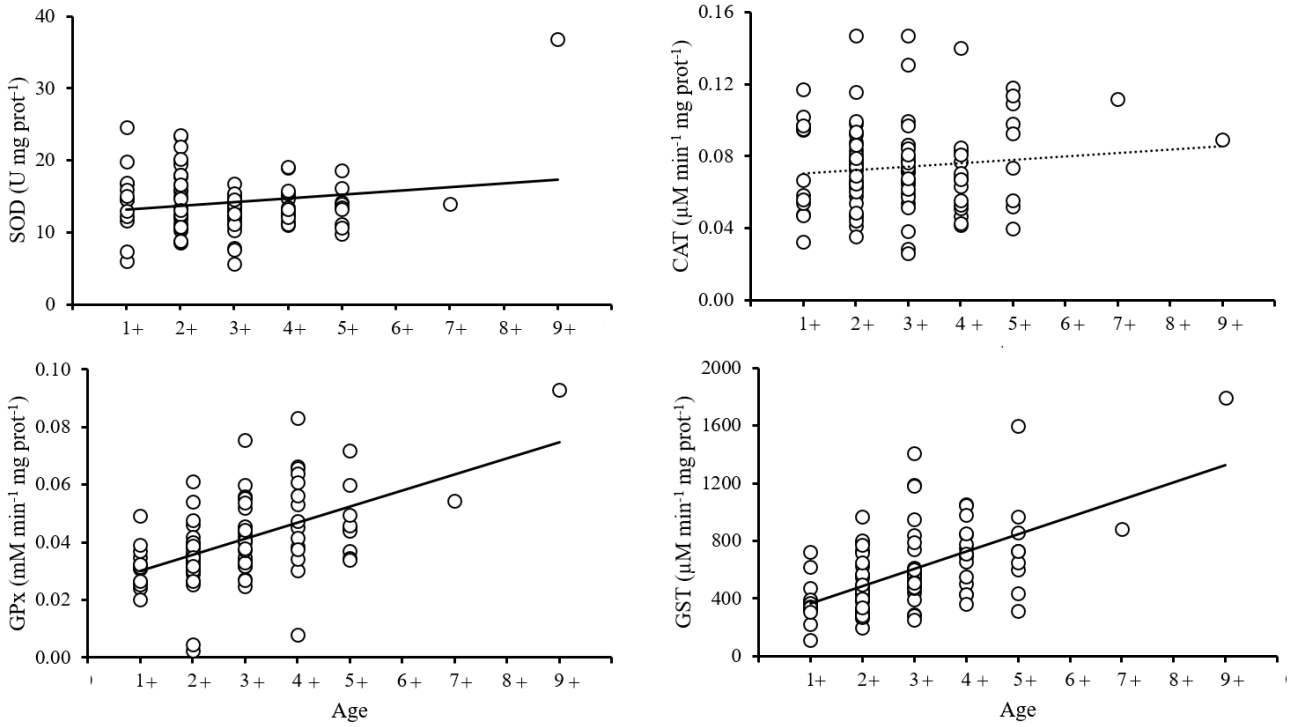

Figure 2: age-dependent variation in the activity of SOD (a), CAT (b), GPx (c) and GST (d) in liver dissected by brown trout individuals (1+ to 9+ year-old). Solid lines show significant relationships, while dashed lines show non-significant relationships.

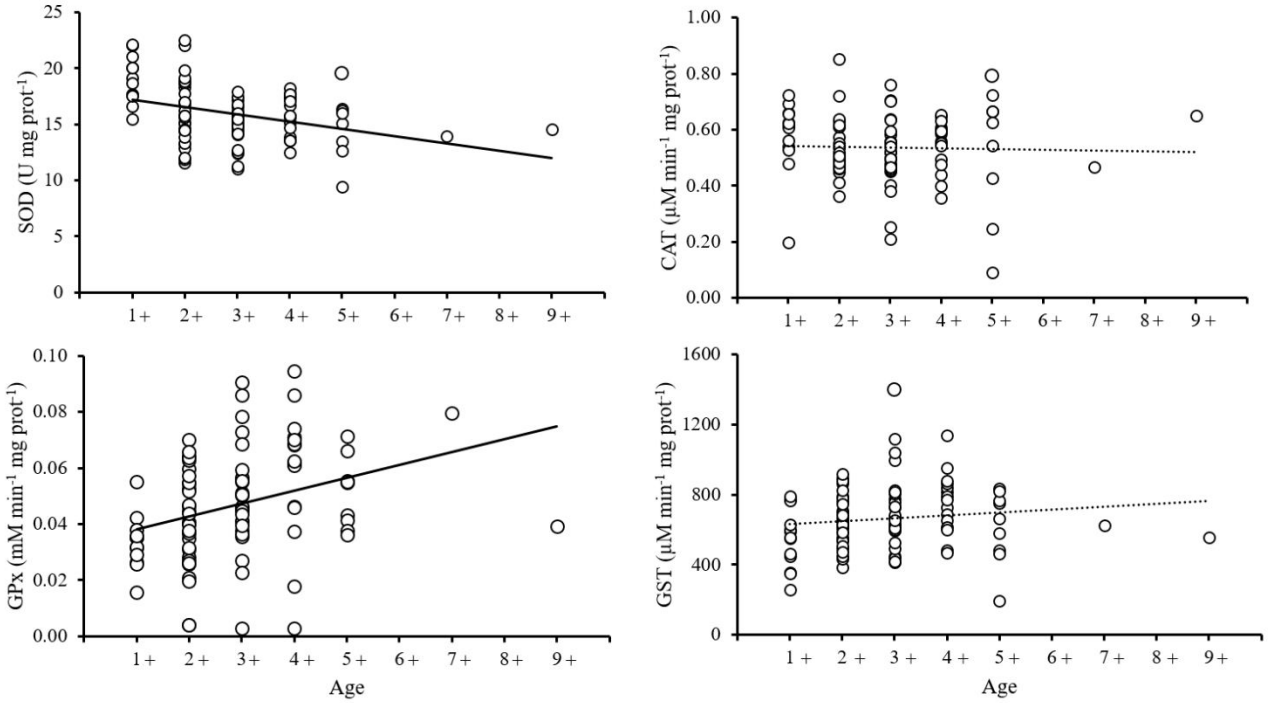

Table S1: Linear mixed models of the activity of defense enzymes (SOD, CAT, GPx and GST) in gills from brown trout individuals (1+ to 5+ year-old), with stream as a random factor. Interaction terms were excluded from the final models when they were non-significant. Significant effects are reported in bold.

	F	df	P
SOD			
Sex	0.606	1,89	0.438
Age	1.144	4,89	0.341
Excluded terms			
Sex × age	0.559	4,81	0.693
CAT			
Sex	0.041	1,90	0.841
Age	0.591	4,90	0.670
Excluded terms			
Sex × age	0.797	4,86	0.530
GPx			
Sex	0.016	1,86	0.899
Age	5.239	4,86	**0.001**
Sex × age	2.589	4,86	**0.042**
GST			
Sex	1.484	1,89	0.226
Age	7.083	4,89	**0.000**
Excluded terms			
Sex × age	2.398	4,85	0.056

480 Table S2: Linear mixed models of the activity of defense enzymes (SOD, CAT, GPx and GST) in
481 liver from brown trout individuals (1+ to 5+ year-old), with stream as a random factor. Interaction
482 terms were excluded from the final models when they were always non-significant. Significant effects
483 are reported in bold.

	F	df	P
SOD			
Sex	19.397	1,86	**0.000**
Age	7.939	4,72	**0.000**
Sex × age	5.493	4,63	**0.001**
CAT			
Sex	5.214	1,89	**0.025**
Age	1.545	4,83	0.197
Excluded terms			
Sex × age	2.051	4,82	0.095
GPx			
Sex	1.782	1,89	0.185
Age	4.229	4,75	**0.004**
Excluded terms			
Sex × age	1.280	4,66	0.287
GST			
Sex	0.865	1,90	0.355
Age	2.227	4,90	0.072
Excluded terms			
Sex × age	0.807	4,86	0.524

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