Melanin-based coloration predicts body mass and antioxidant capacity in the brown trout (*Salmo trutta*)

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Abstract

In many vertebrate species, individuals exhibit large variation in the degree of melanin-based coloration on their body. Dark and pale individuals differ in diverse physiological and behavioral traits, suggesting that melanic coloration may reveal individual quality. However, the relationships between physiological and livery traits, in terms of melanin-based coloration, in wild fish is very scant. The present correlative study aimed at investigating the relationships between physiology and melanin-based coloration of the livery of free-living brown trouts (*Salmo trutta*). We scrutinized the relationships between body condition (body mass and Fulton's K condition factor), oxidative status (plasmatic total antioxidant capacity and amount of pro-oxidant molecules) and the degree of melanin-based coloration assessed by digital photography and image analysis. We showed a significant covariation between body mass and melanin-based coloration, suggesting that, independently of age and sex, darker individuals grow more than paler conspecifics. Moreover, a significant covariation between plasmatic total antioxidant capacity and melanic coloration was noted. Our results demonstrated that in the brown trout melanin-based coloration may serve as a reliable signal to communicate good body condition and a better antioxidant defense to conspecifics.

Keywords: melanin-based coloration, body mass, antioxidant capacity, brown trout

1. Introduction

The coloration of fish skin is a distinctive phenotypic trait that has attracted the interest of ecologists and evolutionary biologist for a long time. Fish display a remarkable diversity of colors and patterns adorning their livery, which depends on five main types of specific cells named chromatophores specialized in the storage and/or in the synthesis of light-absorbing pigments or light-reflecting structures (Leclerq et al., 2010). The colorations of the skin of fish mainly depend on the morphology, density, distribution and interactions of specific chromatophores within the integument (Leclerq et al., 2010). This variety of colors has been individuated to play a pivotal role in diverse intra- and inter-specific interactions, including predator avoidance (e.g. camouflage, signaling of palatability), predation (e.g. camouflage and aggressive mimicry), and communication with conspecifics (e.g. mating and agonistic signalling; see Cheney et al., 2008; Mills and Patterson, 2009).

Carotenoids (causing yellow-orange or red hues) and melanin (causing brown, black and grey coloration) are the two pigment classes adorning the fish body that are mainly involved in social interactions and intra-specific communication. Carotenoids are pigments that cannot by synthesized *de novo* by animals but are exclusively obtained from the diet (Olson and Owens, 1998). As carotenoids can be a limiting resource and have diverse effects on individual physiology, they are assumed to serve as a reliable signal of health, vigour and genetic quality in a number of vertebrate taxa (e.g., Olson and Owens, 1998; Møller et al., 2000). Differently from carotenoids, melanins can be synthesized by the animal itself in specialized organelles named melanosomes (Sugimoto, 2002) and their synthesis is under strong genetic control (Majerus and Mundy, 2003). Since condition-dependent traits are reliable signals of individual quality because only high-quality individuals can afford the expression and the maintenance of a costly trait (Andersson 1994), within-species variation in a melanin-based trait is a less obvious signal of inter-individual quality (Badyaev and Hill 2000, Wedekin et al., 2008). However, some studies of vertebrates have shown relationships between melanin-based coloration and health, vigour (Roulin, 2004a; McGraw, 2005) and diverse fitness traits (Roulin, 2004b; Bize et al., 2006, Fargallo et al., 2007; Roulin, 2007). For instance, variation in the degree of melanin-based coloration has been associated with sexual behavior, aggressiveness, maintenance of energy homeostasis, resistance to stressors and body mass, whereby darker individuals are more sexually active, aggressive, efficient to maintain homeostasis and resistant to stress than paler ones (Ducrest et al. 2008 and references therein). Thus, these findings support the idea that melanin-based coloration can be considered an honest signal of individual quality (McGraw, 2008), although this information in fish is very scant.

Salmonid fish display wide intra- and inter-specific variation in skin coloration and pigment patterning, which are completely established only at the adult stage (Djurdjevic et al., 2016). Such variation occurs among species and life-stages, and depends on differences in morphology, density, distribution and arrangement of skin chromatophores (Djurdjevic et al., 2016). The brown trout (*Salmo trutta*) is a salmonid fish with external fertilization and no paternal care, whose livery is dappled with brown, black and red spots on the body sides and the adipose fin. The red spots contain large quantities of different carotenoids (Steven, 1948), while the black spots are formed by specialized chromatophores (melanophores) producing the dark pigment eumelanin (Bagnara, 1998). The great variation in terms of both carotenoid- and melanin-based coloration that distinguishes the brown trout livery at individual and population levels (Blanc et al., 1982, 1994; Aparicio et al., 2005; Bud et al., 2009; Kocabas et al., 2011) makes this salmonid species an excellent model to investigate the role of livery traits as a signals of individual quality in fish. In a recent study we have demonstrated positive correlations between non-enzymatic and enzymatic activity and the intensity of the ventral carotenoid-based coloration, suggesting the role of this livery trait in signaling individual quality in terms of antioxidant defenses (Parolini et al., submitted). In spite of these findings, the relationships between life-history traits and melanin-based coloration have been poorly. A single study of the brown trout has demonstrated the role of melanin-based coloration in spawning displays and in influencing reproductive success, whereby darker males (displaying a more marked melanin-based coloration) sired more viable offspring (Wedekind et al., 2008). However, no studies have focused on the relationships between melanin-based coloration and physiological traits in this salmonid species.

Thus, the present study was aimed at investigating if physiological traits (body mass, body condition factor and oxidative status) are associated with melanin-based coloration in brown trout individuals from a resident population of a stream within the Gran Paradiso National Park (Northwestern Italy). As this population has not been supplemented by hatchery fish and/or deprived by fishery activity for a long time, and does not undergo direct anthropic pressure, we had a unique opportunity to study the relationships between physiological and livery traits under a natural selection regime. Melanin-based coloration of brown trout livery was assessed by digital photography and image analysis. Standard length and body mass were measured as morphometric traits and were used to calculate the Fulton's condition factor (K-factor). K-factor is based on length–weight relationships and rests on the assumption that the heavier a fish is for a certain body length, the greater its energy reserves and, consequently, the better its condition are (Bolger and Connolly, 1989). For this reason, it is considered a simple proxy of energy reserves in fish body (Mozsar et al., 2015). The total antioxidant capacity (TAC) and the total amount of pro-oxidants (TOS) were measured in plasma as oxidative status markers. Since in wild vertebrates melanin-based coloration is often associated with energy homeostasis (Ducrest et al., 2008 and references therein), we expected a positive covariation of the extent of melanin-based coloration with body mass and K-factor. Lastly, considering the antioxidant activity of melanin (McGraw, 2005) and the prediction that darker melanic individuals should have better resistance to oxidative stress than paler ones (see Ducrest et al., 2008), we expected that melanin-based coloration covaried positively with TAC and negatively with TOS.

2. Materials and Methods

2.1 Sampling survey

Brown trout individuals were collected by electrofishing every ten days in the period ranging between the 6th of May and the 28th of July 2015 in the Piantonetto stream (Gran Paradiso National Park; Northwestern Italy). Six linear transects each about 100 meters long were travelled twice about 30 minutes apart along the course of the streams to catch as many individuals as possible. All captured fish were immediately transferred to a perforated drum kept into the stream. At the end of the sampling operation, trouts were transferred to a 100 L tank crossed by a constant flow of water located within the aquaculture facility set up by the Gran Paradiso National Park at Ghiglieri (Noasca, TO). The substrate of the tank was similar to that of the riverbed to allow a gradual acclimatization of trout, preventing any physiological variation in the color of their livery due to stress and/or substrate variation (Westley et al., 2013). Trouts were kept in this tank for about 1 hour and then they were photographed, measured (standard length and body mass) and then sacrificed in accordance with the current animal welfare regulations. In the lab, each trout was gutted and weighed again. The sex of each individual was determined by evaluating the maturation stage of the gonads, while the age was assigned according to the standard growth curves for the model species. The Fulton's condition factor (hereafter K-factor) was calculated according to the formula: K-factor = wet mass (g)/standard length (mm)^3 × 100 (Mozsar et al., 2015). We relied on 143 brown trout individuals grouped into four classes of age as follows: age 1+ = 19 individuals (6 females and 13 males); age 2+ = 64 individuals (24 females and 40 males); age 3+ = 47 individuals (28 females and 19 males); age 4+ = 11 individuals (6 females and 5 males). The study was carried out under permission of the Gran Paradiso National Park, which allowed the sampling and the euthanasia of fish, *a latere* of a Life+ project (BIOAQUE) aimed at the conservation of the marble trout (*Salmo marmoratus*) in the rivers of the park.

2.4 Oxidative status markers

The non-enzymatic total antioxidant capacity (TAC) was measured in plasma of each single brown trout according to a colorimetric method developed by Erel (2004). The color of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{*+}$) bleaches depending on the concentration of plasmatic antioxidants. The reaction is monitored spectrophotometrically and the final absorbance is inversely related to TAC of the sample. The assay was calibrated using a standard curve with serial dilutions of Trolox and the results were expressed as mM Trolox equivalent. The total amount of pro-oxidant molecules (hereafter TOS) was measured according to a colorimetric method developed by Erel (2005). The oxidant molecules in the plasma oxidize the ferrous ion-o-dianisidine complex to the ferric ion, which reacting with xylenol orange gives a blue complex. The reaction was monitored by a spectrophotometer at $\lambda = 535$ nm and the absorbance is proportional to the oxidant molecules in the plasma. The assay was calibrated by using a standard curve made with hydrogen peroxide (H_2O_2).

2.5 Analysis of brown trout livery

Although melanin-based coloration of salmonids has been investigate by the count of the number or density of black spots dappling the body (e.g. Wedekind et al., 2008; Kittilsen et al., 2009), we decided to use an approach based on digital photography and image analysis because it allows to avoid over- or under-estimation of the number of black spots due to spot miscount Moreover, considering that 1) melanophores are not only present in black spots dappling the skin but also in association with iridophores (i.e. melano-iridophore complexes), and their aggregation state can contribute to skin darkening (Fujii, 2000), and 2) dorsal-lateral area is also spotted by red spots that vary in number and dimension, the image analysis allows to assess the whole melanic coloration of the trout taking into consideration all the potentially confounding factors mentioned above.

Photographs were taken after the acclimation period with a Canon EOS 450D digital camera and transferred to a computer for further analyses of livery. Prior to photography, each fish was placed on a board equipped with a ruler at millimeter resolution to allow an accurate estimation of the fish body size. Photographs were taken in a standardized position and each image included a Munsell X-rite color checker card (X-rite, Inc., Grand Rapids, MI, USA), which was used to correct for any subtle difference in lighting or exposure (Westley et al., 2013). We handled all individuals in a similar way to minimize any potential bias resulting from the process. Melanin-based coloration was assessed using digital photography and image analysis. First, photographs were prepared for analyses in Adobe Photoshop CS3® (Adobe Systems Incorporated, San Francisco, CA, USA). Specifically, we cropped each standardized photograph from dorsal-lateral body area (Figure S1) to assess melanin-based coloration. The white vignette of the Munsell X-rite color checker card was cropped and digitized as a red-green-blue (RGB) color array using the Pictocolor plugin. Color calibration coefficients were calculated for each cropped vignette as the percentage difference between the average value of each RGB spectrum and the corresponding Munsell set point for the white vignette. Then, calibration coefficients were used to calibrate each image to a common standard (Stevens et al., 2007). The median of these color parameters was calculated for each trout within the area of interest. The darkness of the skin of the trout dorsal-lateral area was measured as the mean gray values, calculated as the mean of the color channels RGB, of the body side, according to Wilkins et al. (2017). The lower is the mean RGB value the higher is the melanin-based coloration of the dorsal-lateral body area of the trout.

2.5 Statistical analysis

The effect of age and sex, as well as of their interaction, on body mass, K-factor, TAC and TOS of the brown trout was investigated by means of Generalized Linear Models (GLMs). Since the sampling spanned over two months, we first included the date of sampling as a covariate in the models. However, as the effect of the date of sampling was always non-significant, we then excluded it from all the models. To investigate the covariation between physiological traits and melanin-based coloration we included the darkness as a covariate in separate models. The analyses were run using SPSS 21.0 statistical package.

3. Results

The sex ratio of our sample was balanced (females 64/143 = 0.45; males 79/143 = 0.55; $\chi^2_1 = 0.287$; P = 0.592). The analysis of the dorsal-lateral darkness did not show significant differences between the left and the right side (paired t-test; t = -1.893; P = 0.205). Thus, to investigate the differences in darkness of livery due to individual sex and age, as well as the relationships with physiological traits (body mass, K-factor and oxidative status markers), we considered the mean value of the two body sides. A significant effect of age ($F_{1,135} = 3.713$; P = 0.013), but not of sex ($F_{3,135} = 0.515$; P = 0.474) and their interaction ($F_{3,135} = 0.428$; P = 0.734), was noted on dorsal-lateral darkness, with older individuals being darker than their younger conspecifics. As expected, the GLMs showed a significant effect of the age on the body mass, but no effect of sex and age × sex interaction was found (Table 1). K-factor did not depend on age or on sex of the fish (Table 1). When we included the mean RGB in the models as a covariate, a significant negative covariation between body mass ($F_{1,135}$ = 11.437, P = 0.001, coefficient: -712 (SE: 210)), but not K-factor, and the mean RGB coloration was found, with individuals showing paler darkness of their dorsal-lateral area being heavier compared to brighter ones (Table 1; Figure 1a). No significant effect of age, sex and their interaction on plasmatic TAC and TOS was found, with the exception of a significant positive covariation between TAC and age (Table 1). A significant negative covariation between the mean RGB and TAC was noted $F_{1,135}$ = 3.960, P = 0.049, coefficient: -0.21 (SE: 0.10), with darker individuals having a higher antioxidant capacity than paler ones (Table1; Figure 1b)

Discussion

The results of this cross-sectional, correlative study of free-living brown trout showed that dorsal-lateral darkness significantly negatively covaried with body mass, suggesting that, independently of age and sex, darker individuals grow more than their paler conspecifics. Moreover, melanin-based coloration significantly covaried with plasmatic total antioxidant capacity, with darker individuals having a higher antioxidant capacity than paler ones.

The livery of the brown trout shows great phenotypic variation in terms of pigmentation and pattering of melanin-based coloration, which is particularly evident in the number of black spots dappling the skin (Blanc et al., 1982, 1994; Aparicio et al., 2005; Bud et al., 2009; Kocabas et al., 2011). Although we did not consider the mere number or density of black spots as proxy of melanic coloration (see above), we found a notable variation of skin melanin-based coloration, which depended on the age of individuals, with older trouts being darker than younger ones. Although in salmonids melanin-based coloration is mainly involved in cryptic camouflage (e.g. Donnelly and Whoriskey, 1991), the present results suggest that this phenotypic trait covaries with physiological, morphological and behavioural traits and may be implicated in social communication as a reliable signal of individual quality. A significant negative covariation between body mass and dorsal-lateral darkness was noted in our population, indicating that darker individuals grow more compared to paler ones (Figure 1a). The large body mass achieved by darker individuals, independently of their age and sex, may be due to their higher food intake with respect to paler conspecific. Although higher food intake was noted in rainbow trout spotted group compared to the non-spotted one (Kittilsen et al., 2009), previous studies of wild fish have shown no associations between eumelanin pigmentation and the regulation of feed intake (see Ducrest et al., 2008 and references therein). Darker individuals can achieve a large body mass because of better capacity of maintaining energy homeostasis, in terms of the balance between food intake and energy expenditure, than paler ones. In fact, melanin has been recognized as a reliable indicator of homeostasis because of its role in diverse developmental pathways underlying various functions (Badyaev and Young, 2004; Roulin, 2004a). Since we did not find a significant covariation between the K-factor, a simple proxy of energy reserves in fish body (Mozsar et al., 2015), and dorsal-lateral darkness, this hypothesis should be confirmed by a further investigation on energy homeostasis. In addition to a role in revealing a better body condition, melanin-based coloration may be also involved in mate choice. In fact, considering the covariation between body mass and melanin-based coloration and that heavier and larger brown trout males are more successful in intra-sexual competition than lighter and smaller ones (Jacob et al., 2007), we may speculate that, independently of sex, darker and heavier individuals are preferred in mate choice. Unfortunately, this relationship has never been investigated in any study but it should deserve attention to understand the role of melanic displays in mate choice decisions.

A proximate physiological explanation for the covariation between body mass, K-factor and melanin-based coloration may consist in the pleiotropic effects of melanocortin system (Ducrest et al. 2008). Melanocortins are bioactive peptides, encoded by the proopiomelanocortin (*POMC*) gene, which exert their neuroendocrine and paracrine functions by binding to five tissue-specific receptors ($MC_{1\text{-}5}$; see Ducrest et al., 2008; Roulin and Ducrest, 2011). Through the binding with MC_1, melanocortins regulate melanogenesis, while by binding to $MC_{3\text{-}4}$ they regulate energy homeostasis (Ducrest et al., 2008), as demonstrated also in a salmonid fish (Schjolden et al. 2009). In addition, through the binding with MC_4, melanocortins can regulate and enhance the resistance to oxidative stress, as demonstrated by a previous study of human dermal fibroblasts exposed to UV radiation (Bohm et al., 2005). Thus, considering both the intrinsic antioxidant property of melanin (McGraw, 2005) and the role of the melanocortin system, darker individuals may be more resistant to oxidative stress compared to paler conspecifics (Roulin and Ducrest, 2011). According to our expectations, plasmatic TAC covaried with melanin-based coloration, suggesting that this livery trait reflects the antioxidant capacity of individuals. A previous study of brown trout from the same population demonstrated that also carotenoid-based coloration can be considered a reliable signal of antioxidant defense. In fact, individuals displaying an intense ventral carotenoid-based coloration had higher total antioxidant capacity both in the plasma and in the liver, as well as a higher hepatic activity of the antioxidant enzymes superoxide dismutase and catalase, with respect to their paler conspecifics (Parolini et al., submitted). Thus, these findings suggest that carotenoids and melanins are not mutually exclusive in revealing the individual antioxidant capacity to conspecifics, and either might cooperate in preventing an oxidative stress situation.

In conclusion, this correlative study demonstrated for the first time in a free-living salmonid population that melanin-based coloration covaries with physiological traits and may signal a good body condition and a better antioxidant capacity of the bearer. However, because of the cross-sectional sampling design of the study, the relationships we showed have to be considered with caution and should be confirmed by a longitudinal study. Thus, this study contributes to enlarge the limited knowledge regarding the relationships between melanin-based coloration and physiological traits in wild animals, suggesting the importance to scrutinize the pleiotropic role of melanocortin system in determining the covariation among melanic coloration and other phenotypic traits.

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Table 1: Generalized Linear Models (GLM) of body mass, Fulton's body condition factor (K-factor), plasmatic total antioxidant capacity (TAC) and amount of pro-oxidant molecules (TOS) in relation to sex and age of brown trout individuals. Melanin-based coloration (as dorsal-lateral darkness) was included in the models as a covariate. The interaction terms are reported in the table but excluded from the final models because they were always non-significant. Significant effects are reported in bold.

	F	**d.f.**	**P**
Body mass			
Sex	0.804	1,135	0.371
Age	196.539	3,135	**<0.001**
Melanin-based coloration	11.437	1,135	**<0.001**
Excluded term			
Sex × age	0.325	3,132	0.807
K-factor			
Sex	0.158	1,135	0.692
Age	0.837	3,135	0.476
Melanin-based coloration	0.593	1,135	0.443
Excluded term			
Sex × age	1.616	3,132	0.189
TAC			
Sex	0.034	1,135	0.853
Age	4.884	3,135	**<0.001**
Melanin-based coloration	3.960	1,135	**0.049**
Excluded term			
Sex × age	0.179	3,132	0.911
TOS			
Sex	0.588	1,135	0.445
Age	1.402	3,135	0.245
Melanin-based coloration	1.963	1,135	0.164
Excluded term			
Sex × age	0.156	3,132	0.926

Figure 1: covariation between body mass (a), plasmatic total antioxidant capacity (TAC expressed as mM Trolox equivalent; b) and darkness of dorsal-lateral body area of the brown trout.

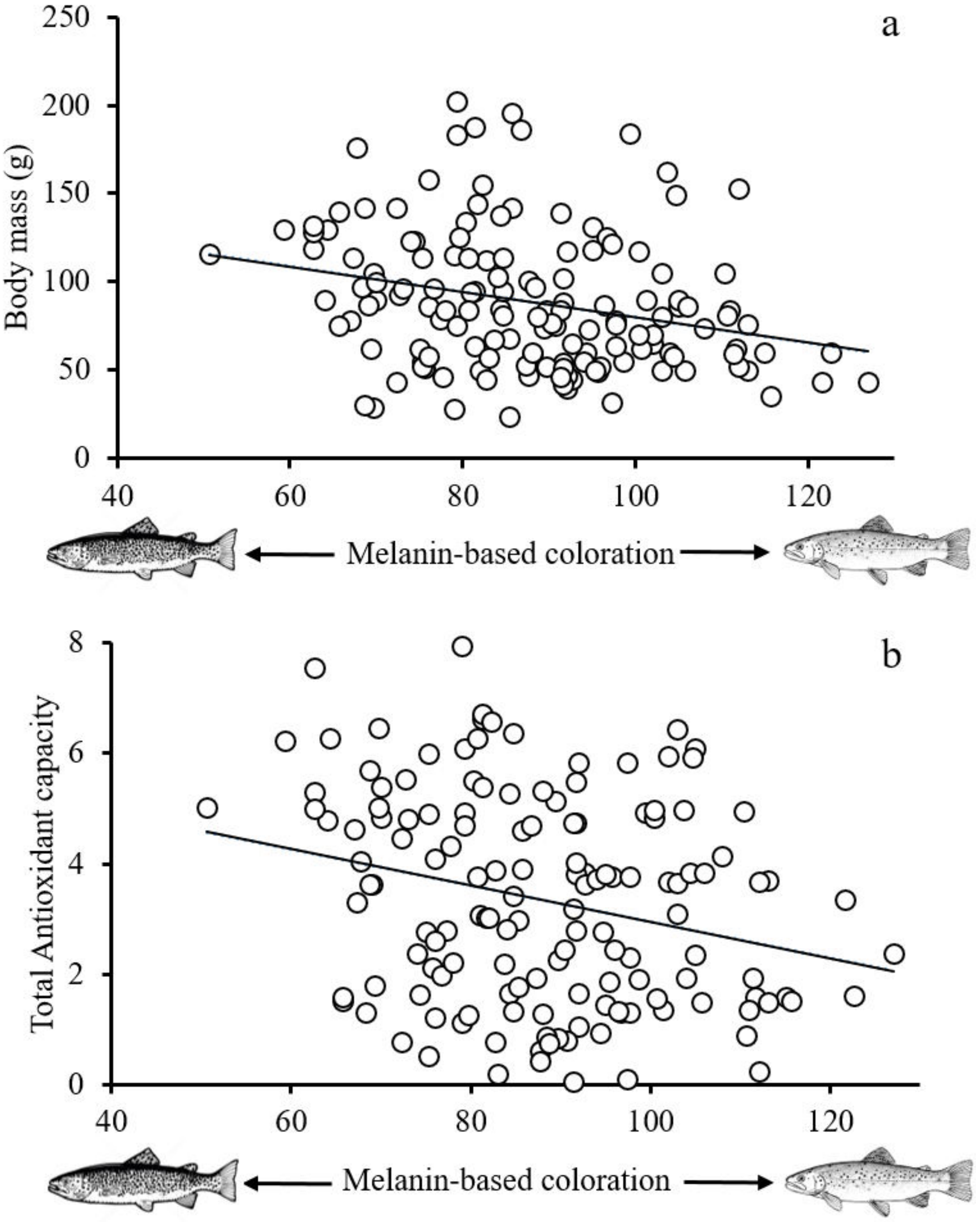

Figure S1: Photograph of a brown trout individual used to assess livery melanin-based coloration. Photograph was analyzed in Adobe Photoshop CS3® and we assessed melanin-based coloration of dorsal-lateral body area (darkness; indicated by dotted black polygon).