Relationships between Hair Melanization, Glutathione Levels, and Senescence in Wild Boars

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ABSTRACT

The synthesis of melanins, which are the most common animal pigments, is influenced by glutathione (GSH), a key intracellular antioxidant. At high GSH levels, pheomelanin (the lightest melanin form) is produced, whereas production of eumelanin (the darkest melanin form) does not require GSH. Oxidative damage typically increases with age, and age-related decreases in GSH have accordingly been found in diverse organisms. Therefore, there should be positive associations between the capacity to produce eumelanic traits, GSH levels, and senescence, whereas there should be negative associations between the capacity to produce pheomelanic traits, GSH levels, and senescence. We explored this hypothesis in a free-ranging population of wild boars *Sus scrofa* of different ages. As expected from the fact that pheomelanogenesis consumes GSH, levels of this antioxidant in muscle tended to be negatively related to pheomelanization and positively related to eumelanization in pelage, and the degree of pelage pheomelanization was positively related to oxidative damage as reflected by levels of thiobarbituric-acid-reactive substances (TBARS), which is consistent with the hypothesis that pheomelanin synthesis has physiological costs. In our cross-sectional sample, GSH levels did not show senescence effects, and we did not detect senescence effects in pelage melanization. Prime body condition and low TBARS levels were also associated with hair graying, which is attributable to a loss of melanin produced by oxidative stress, thus raising the possibility that hair graying constitutes a signal of resistance to oxidative stress in wild boars. Our results suggest that the degree of melanization is linked to GSH levels in wild boars and that their antioxidant damage shows senescence effects.

Introduction

Physiological mechanisms underlying pigment acquisition and production in animals are the subject of intense research that aims at finding a general framework to explain the evolution of color traits, which frequently have a role in communication by acting as signals of diverse information (e.g., Mougeot et al. 2009; Helfenstein et al. 2010). Melanins are the most common animal pigments and fulfill roles as diverse as protection against mechanical abrasion and damaging radiation (Tadokoro et al. 2003; Bortolotti 2006), immunomodulation (Sugumaran 2002), and signaling of individual quality (Bortolotti et al. 2006; Hoi and Griggio 2008). Melanins are synthesized by animals in a process (melanogenesis) that is intrinsically related to oxidative stress (i.e., the imbalance between the production of oxidizing compounds and the availability of antioxidant substances, tipped toward the former; Galván and Solano 2009). Because environmental variability may strongly influence oxidative stress in animals (e.g., Metcalfe and Alonso-Alvarez 2010), we can hypothesize that the expression of melanic traits, despite being under a relatively well-known genetic control (e.g., Hoekstra 2006; Mundy 2006), can be fully understood only with the additional consideration of factors affecting endogenous and exogenous oxidative stress.

The influence of oxidative stress on melanogenesis is exerted through the differential presence of antioxidants in the two main pathways of the process. Both pathways, which lead to the synthesis of eumelanin (the darkest form of melanin) or pheomelanin (the lightest form of melanin), share the first chemical steps, which end with the hydroxylation of the amino acid tyrosine by the enzyme tyrosinase to produce dopaquinone in specialized cells called melanocytes. Dopaquinone can then react with thiol groups to synthesize pheomelanin or, in the absence of thiol groups, undergo a cyclization that leads to the synthesis of eumelanin (García-Borrón and Olivares Sánchez 2011). Thiol groups that react with dopaquinone are provided by the amino acid cysteine or by the cysteine-containing tripeptide glutathione (GSH; Potterf et al. 1999), which also acts

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as the main physiological reservoir of cysteine (Benedetto et al. 1981). GSH is also the most important intracellular antioxidant (Anderson 1998; Wu et al. 2004). Thus, cysteine and GSH levels must be high to induce the synthesis of pheomelanin, but these values are reduced during the process, because cysteine is incorporated into the pheomelanogenesis pathway. This means that pheomelanogenesis consumes cysteine and GSH, whereas eumelanogenesis requires low GSH levels to be triggered (Meyskens et al. 1999; Galván and Alonso-Alvarez 2008; Galván and Solano 2009; Galván and Møller 2011; Galván et al. 2011, 2012; Pavel et al. 2011). Indeed, pheomelanin may have evolved because cysteine, which can be toxic at very high levels, is removed from cells during pheomelanin production (Galván et al. 2012). Despite differences between vertebrates and invertebrates with respect to the enzymes that control melanogenesis, the basic components of this metabolic pathway are similar among animal groups (Sugumaran 2002; Nappi and Christensen 2005). Indeed, the influence of GSH on the expression of melanic traits is the same in animals that are as phylogenetically distant as insects (Clark et al. 2010), mammals (Benedetto et al. 1981), and birds (Galván and Alonso-Alvarez 2008; Hõrak et al. 2010). Therefore, a correct understanding of the evolution of melanic traits in animals must consider the role of GSH and oxidative stress on melanogenesis.

One of the processes related to oxidative stress is senescence, which is the decrease in fecundity and survival that organisms experience with age and which is normally accompanied by a deterioration of the phenotype (Finch and Kirkwood 2000). Free radicals produced as a consequence of normal cellular respiration and the associated oxidative damage that accumulates with age cause oxidative stress, which is the main mechanism responsible for senescence (Finch and Kirkwood 2000; Finkel and Holbrook 2000; however, see Buffenstein et al. 2008 and Speakman and Selman 2011 for reviews showing evidence against this theory). There is ample evidence that GSH undergoes an age-related decrease in diverse organisms, from mosquitoes to humans (reviewed in Bains and Shaw 1997). Thus, the capacity and costs of producing melanins may change as a consequence of senescence, which, in the case of melanic traits that evolve as signals, may lead to changes in signal content and reliability (traits must be differentially costly to produce for low- and high-quality individuals to evolve as honest indicators of quality; Hasson 1997). The interaction between senescence, oxidative stress, and melanization has been investigated only in the particular case of human hair graying (i.e., loss of melanization capacity with age; Arck et al. 2006), in which melanogenesis is blocked by the inhibition of the activity of tyrosinase as a consequence of oxidative stress produced by H_2O_2 (Wood et al. 2009). This has led to the free radical theory of graying, according to which humans showing grayer hairs should also present higher oxidative stress levels (Arck et al. 2006). However, the relationship between age-related changes in GSH levels and differential expression of eumelanic and pheomelanic traits with age has never been investigated.

An exploration of the interaction between senescence, GSH, oxidative damage, and melanization may shed light on our understanding of the evolution of melanic traits. In many animals, melanic traits have a role in communication by acting as signals of quality, an issue that has been studied mainly in birds (reviewed in McGraw 2008). It has been proposed that the honesty in the expression of melanic traits may be related to the influence of GSH on melanogenesis, so that only high-quality individuals may be able to produce large amounts of pheomelanin under conditions of relatively high oxidative stress or large amounts of eumelanin under conditions of relatively low oxidative stress (Galván and Alonso-Alvarez 2009; Galván and Solano 2009). Because the effects of oxidative damage accumulate with age (Finch and Kirkwood 2000; Kirkwood and Austad 2000; but see Buffenstein et al. 2008 and Speakman and Selman 2011), senescence may be viewed as a process in which endogenous oxidative stress increases with age. Thus, the older the individual, the higher the costs of producing pheomelanin. This means that, if GSH levels actually decrease with age and oxidative damage levels increase with age, and if the production of melanin is coupled to these changes, then the reliability of pheomelanic traits that evolve as signals of quality would increase with age. This would explain why the expression of some pheomelanic traits that are under sexual selection (i.e., traits for which females prefer males who produce large amounts of pheomelanin) apparently do not show senescence effects but increase with age (Galván and Møller 2009; Lifjeld et al. 2011). From another point of view, this age-related increase in the expression of quality signals may be an adaptive strategy followed by older individuals to improve their reduced residual reproductive value (i.e., chance of future reproduction; Evans et al. 2011).

The aim of this study is to investigate whether age-related effects on the production of melanins are coupled with age-related effects on levels of GSH and oxidative damage of cells. As a model, we use a free-ranging population of wild boars *Sus scrofa* presenting a large variability in levels of hair melanization. In animals ranging in age from a few months to almost 6 yr, the content of melanins in the pelage, the proportions of eumelanic and pheomelanic hair, and the presence of hair graying were determined. In muscle samples, we determined the total levels of GSH and, as two indices of oxidative damage, the levels of oxidized GSH (GSSG) and lipid peroxidation (thiobarbituric-acid-reactive substances [TBARS]). We predicted that, as has been previously found in different animal species, GSH levels should decrease with age (Bains and Shaw 1997) in a linear or curvilinear fashion, as has been frequently observed in senescence effects on phenotypes (Møller and de Lope 1999). The effects of this decrease in GSH levels with age should be reflected in the oxidative damage of cells, and thus GSSG and TBARS levels should increase with age, as has been previously found in other mammals and birds (Alonso-Alvarez et al. 2010 and references cited therein). Moreover, because pheomelanogenesis consumes GSH (Meyskens et al. 1999; Galván and Solano 2009; Galván and Møller 2011; Galván et al. 2011, 2012; Pavel et al. 2011), there should be a negative relationship between GSH levels and pheomelanin-based color expression, and wild boars that present a high degree of hair pheomela-

nization could pay a cost in terms of increased levels of oxidative damage. Additionally, following the free radical theory of graying (Arck et al. 2006; Wood et al. 2009), which has never been tested in wild animals (with humans being the only exception), we predicted that the presence of hair graying in wild boars should be positively related to oxidative damage levels. Finally, we explored possible associations between the expression of the melanic characters of wild boar pelage and a potential signal of quality to determine whether melanic characters may be part of a multiple signal of quality. We used the size of mandibular lower canines as a potential signal of quality, because it is a sexually dimorphic trait, with males having larger canines than females, and because male wild boars use these teeth during aggressive interactions to get access to females and to mark trees (Fernández-Llario and Mateos-Quesada 2003). A summary of predictions is shown in table 1.

Material and Methods

Field Methods

Fieldwork was conducted in Doñana National Park, located in southwestern Spain, during February–April 2008. One hundred European wild boars were sampled within the park by shooting as part of a larger study on the epidemiology of tuberculosis in the ungulates living in the park (Gortázar et al. 2011), which required the collection of internal organs and therefore the killing of the animals. The sampling scheme included an even number of individuals from different areas (northern, central, and southern portions of the park) to minimize potential pseudoreplication caused by genetic relatedness within family groups. The culling of wild ungulates was approved by the Research Commission of Doñana National Park in accordance with management rules established by the Autonomous Government of Andalucía. Sampling was performed according to European (86/609) and Spanish (RD 223/1988; RD1021/2005) laws and current guidelines for ethical use of animals in research (ASAB, 2006) and the Universidad de Castilla-La Mancha animal experimentation committee.

The body length and the thoracic perimeter of wild boars were measured with a ruler to the nearest 0.5 cm, and these measurements were then multiplied to obtain an index of body size. The maximum length and width of the mandibular lower canines were measured with a digital calliper to the nearest 0.01 cm. The sex of wild boars was determined visually in the field, as was the prevalence of tuberculosis-like lesions (Vicente et al. 2006). Two digital photographs were taken of each wild boar, one of the forehead (face) and another of the lateral side (fig. 1). Finally, 1 cm^3 of the sternocleidomastoid muscle of each of the wild boars was taken and immediately stored in liquid nitrogen until analyses of antioxidants and oxidative damage were made.

Age Determination

The age of wild boars was estimated by examining the pattern of teeth growth, which allowed us to determine the age with a precision of 1 mo up to an age of 4 yr (Sáenz de Buruaga et al. 1991). The age of older individuals was determined by radiographic examination of the first incisor tooth using a Sedecal (Algete, Spain) high-frequency x-ray generator (125 KVp, 320 mA). Images were developed using a Konica Minolta (Tokyo) Regius model 110 high-performance single-bay computed radiography system. These images were used to calculate the ratio P/T, where P is the width of the tooth's pulp cavity measured at the point of widest pulpar cavity and T is the width of the tooth (Sáez-Royuela et al. 1989). The regression equation calculated by Sáez-Royuela et al. (1989) for samples from a wild boar population from central Spain was used to estimate the age of our samples. Two old wild boars for which we could not obtain the incisor teeth were assigned the average age of the rest of the old individuals (i.e., 50 mo). The results of the analyses did not change when these individuals were not considered (results not shown).

Wild Boar Morphs

In our study population, different phenotypes (color morphs) coexist with the common black morph observed in Eurasian

Table 1: Summary of predictions and results for age-related variation in antioxidant and oxidative damage levels in muscle cells and in pelage melanization levels of wild boars

	Predicted variation with age		
Trait	Traits with no effects on reproductive value	Traits with effects on reproductive value	Evidence of age-related variation
tGSH	Decrease	Increase	No age-related variation
GSSG	Increase	Decrease	First increase, then decrease
TBARS	Increase	Decrease	First increase, then decrease
Eumelanin-based color	Increase	Decrease	No age-related variation
Pheomelanin-based color	Decrease	Increase	No age-related variation
Hair graying	Increase	Increase	No age-related variation

Note. The predictions are divided into those that assume that traits have no effects on the reproductive value of individuals and those that assume that traits may have effects on reproductive value (i.e., ornaments or signals of quality). Evidence for age-related variation is also indicated. GSSG = oxidized glutathione; TBARS = lipid peroxidation; tGSH = total glutathione.

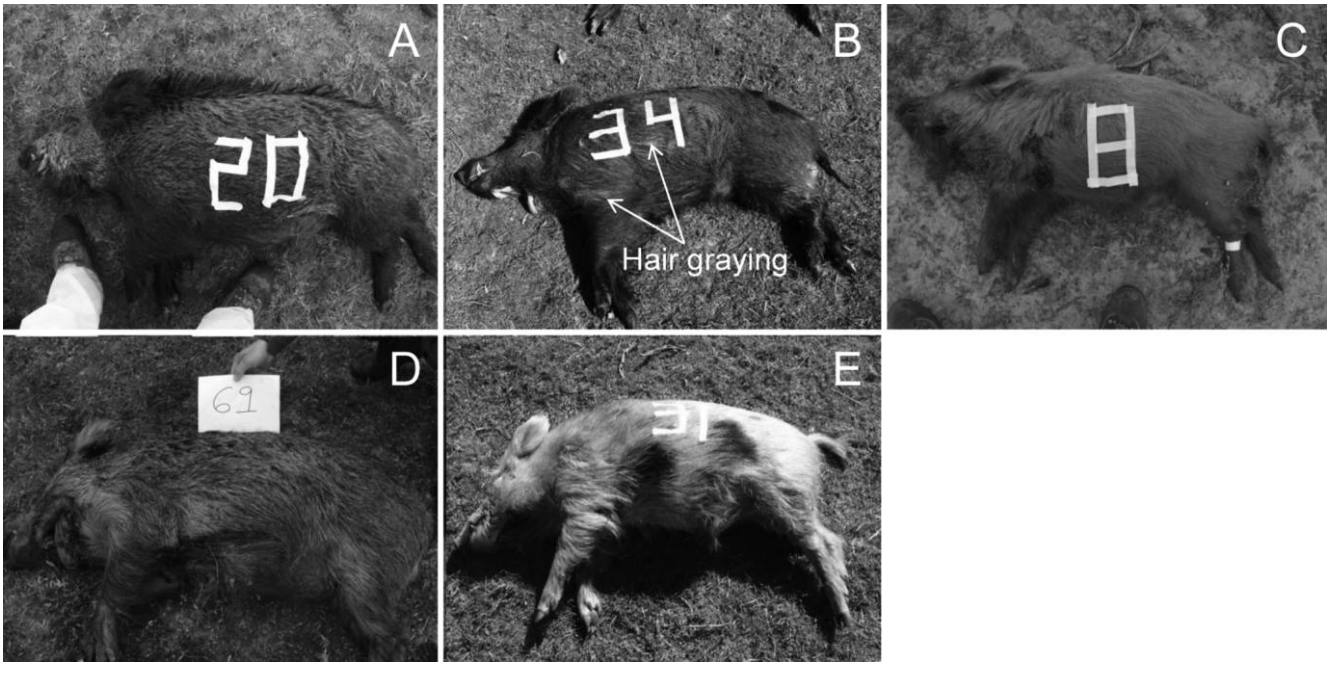

Figure 1. Wild boars of different color morphs used in this study. The specimen shown in *A* corresponds to the black morph shown by a majority of European wild boars. Eumelanic and pheomelanic color scores assigned to the specimens shown here are as follows (eumelanic score–pheomelanic score): *A*, 4.00–1.25; *B*, 4.50–0.75; *C*, 0.00–5.00; *D*, 1.75–3.00; *E*, 0.75–4.25. The specimen shown in *B* presents hair graying in the body. A color version of this figure appears in the online edition of *Physiological and Biochemical Zoology*.

wild boars (fig. 1). The widespread black type is related to only two alleles in the melanocortin 1 receptor locus (Fang et al. 2009). Because these morphs greatly differ in pelage coloration and the pattern of pelage melanization (fig. 1), the wild boars used in this study were separated in morphs, and morph identity was then included in the statistical analyses. We distinguished five different morphs of wild boars (fig. 1). One of us (I.G.) and three independent observers blinded to the aims of the study assigned a morph to the wild boars by examining the photographs taken of the boars before laboratory analyses. This classification of morphs was repeatable between observers ($r = 0.37$, $F_{3,396} = 24.59$, $P < 0.0001$). We used the mean of the morph values assigned by the four observers in subsequent analyses.

Body Condition

The body condition of wild boars was estimated by using morphometric measurements, which allowed us to indirectly estimate the kidney fat index (KFI). The KFI, calculated as the weight of perirenal fat divided by kidney weight and multiplied by 100, is the most used index of physical condition in wild boars and other ungulates (Finger et al. 1981; Vicente et al. 2004). We estimated KFI by a regression equation obtained from 377 wild boars from central Spain with both known KFI and morphological measurements (J. Vicente, unpublished data). In that sample, the body length and thoracic perimeter were regressed against the average KFI for both kidneys. Thoracic perimeter was the only significant predictor of KFI. To estimate the KFI of the wild boars used in this study, separate regression equations for males (KFI $= -2.657 + 0.844 \times$ throracic perimeter; $F_{1,170} = 12.13$, $P < 0.001$, $R^2 = 0.067$) and females (KFI $= -62.245 + 1.727 \times$ throracic perimeter; $F_{1,203} = 49.47$, $P < 0.0001$, $R^2 = 0.196$) were used.

Melanic Portions of Hair

Ten hairs chosen at random were removed from the face area of each wild boar. Up to three portions were distinguished for each hair: a black portion composed mainly of eumelanin, a reddish-brown portion composed mainly of pheomelanin, and a white portion devoid of melanic pigment. Not all hairs presented all three portions. Using a light microscope and a digital calliper, we measured the length of these portions in each hair to the nearest 0.1 mm. These measurements were repeatable among hairs of the same wild boars (eumelanic portion: $r = 0.55$, $F_{68,593} = 12.52$, $P < 0.0001$; pheomelanic portion: $r = 0.67$, $F_{60,256} = 11.16$, $P < 0.0001$; white portion: $r = 0.69$, $F_{46,200} = 26.59$, $P < 0.0001$). We then calculated the average length of the different portions for each individual wild boar. It was not possible to collect hairs from all wild boars, because the face area of some animals had deteriorated after being shot.

Melanin Content of Hair

Wild boar hairs were processed in the Chemical Ecology Laboratory at Doñana Biological Station, following a patented procedure (p200703395 in the European Union) that can be summarized as follows: three to nine hairs from the face of each wild boar chosen at random, or ca. 5 mg per individual, were subjected to an alkaline digestion by adding 1 mL 20% NaOH in an Eppendorff tube. The solutions were sonicated in a water bath at 60°C for 15 min and later centrifuged at 13,000 rpm for 15 min at 4°C. After centrifugation, we obtained a brownish supernatant containing soluble pheomelanin and a black pellet containing eumelanin. The pheomelanin-containing supernatant was directly measured in a UV-VIS spectrophotometer at 450 nm. The eumelanin pellet was resuspended in 1 mL 20% NaOH and 20 μL of 30% H_2O_2. This suspension was sonicated in a water bath at 60°C for 15 min and later centrifuged at 13,000 rpm for 5 min at 4°C. The peroxidized eumelanin in the resulting solution was immediately measured spectrophotometrically at 450 nm. This method is repeatable, as deducted from hair samples from 10 wild boars that were measured twice (eumelanin concentration: $r = 0.84$, $F_{9,10} = 11.73$, $P < 0.001$; pheomelanin concentration: $r = 0.41$, $F_{9,10} = 2.41$, $P = 0.093$). All samples contained both pheomelanin and eumelanin in variable proportions. The relative concentration of both melanin types, calculated as absorbance units (Au) divided by hair mass, was estimated spectrophotometrically as in Toral et al. (2008), Negro et al. (2009), and Galván et al. (2010*a*).

Melanin-Based Color Extension

In addition to the melanin content of hairs, we measured the extension of pelage covered by melanin-based coloration as a complementary measurement of the degree of melanization of wild boars. Eumelanic and pheomelanic traits are generally of distinctive colors, the former being responsible for black and gray colors and the latter for yellowish, reddish, chestnut, and brown colors (Toral et al. 2008). Eumelanin and pheomelanin normally occur simultaneously in the tissues (García-Borrón and Olivares Sánchez 2011), but the darker colors conferred by eumelanin (Toral et al. 2008) make evident the lower pigment content in chestnut and brown colors, compared with black and gray colors (Galván and Alonso-Alvarez 2009). Therefore, we considered that black and gray pelage colors were predominantly generated by eumelanin, whereas chestnut and brown colors were predominantly generated by pheomelanin. Thus, we quantified the proportion of body covered by eumelanic and pheomelanic pelage coloration by examining the dorsal views of wild boars provided by the pictures taken in the field. A method similar to that used by Beauchamp and Heeb (2001) and Galván (2008) in birds was followed to obtain estimates of the proportion of eumelanic and pheomelanic color present in the pelage of each wild boar, and scores were assigned that ranged from 0 (total lack of melanic color) to 5 (all melanic). Scores were assigned by one of us (I.G.) and three independent observers blinded to the aims of the study before laboratory analyses. This scoring method was repeatable between observers (eumelanic color score: $r = 0.42$, $F_{99,300} = 3.93$, $P < 0.0001$; pheomelanic color score: $r = 0.45$, $F_{99,300} = 6.00$, $P < 0.0001$). We used the mean of the color scores assigned by the four observers in subsequent analyses.

The eumelanic color score was positively correlated with the length of the eumelanic (i.e., black) portion of hairs ($r = 0.43$, $n = 73$, $P < 0.001$), and the pheomelanic color score was positively correlated with the length of the pheomelanic (i.e., brownish) portion of hairs ($r = 0.40$, $n = 73$, $P < 0.001$). The eumelanic color score was also positively correlated with the eumelanin content of hair ($r = 0.56$, $n = 73$, $P < 0.0001$), but the pheomelanic color score was not correlated with the pheomelanin content of hair ($r = -0.14$, $n = 73$, $P = 0.221$). This indicates that, in the case of eumelanin, measures of melanin content of hair and melanin-based color scores provide approximately similar information about the degree of melanization, but the information provided by measures of pheomelanin content of hair and pheomelanic color scores slightly differs, in that measures of pheomelanin content are related to the intensity of pheomelanization, whereas pheomelanic color scores are related to the extent of this pigment on the body. Therefore, we used both measures of melanin content of hair and melanin-based color scores in the analyses as complementary measures of the degree of melanization of wild boars. The length of eumelanic and pheomelanic portions of hairs was not used in the analyses, because it was highly correlated with the melanin-based color scores, which were available for a greater number of wild boars; therefore, color scores were used instead.

Hair Graying

The presence or absence of hair graying in the pelage of wild boars was determined by examining the photographs taken of the facial area and the lateral body side (fig. 1*B*). One of us (I.G.) determined whether there was the presence of white unmelanized hairs in the face and the body separately. The distinction between face and body was made because face movements are part of aggressive displays in wild boars (Andersen et al. 2000), so the pattern of hair coloration of the face (i.e., graying) may be involved in such displays. Scores (presence or absence) for hair graying were assigned by one of us (I.G.) and three independent observers blinded to the aims of the study before laboratory analyses. The scores assigned by the different observers were correlated among each other (Kendall tau correlation; face: $\tau = 0.30$, 0.29, and 0.25, $n = 100$, $P < 0.001$; body: $\tau = 0.26$, 0.19, and 0.21, $n = 100$, $P < 0.003$), indicating that the scoring method for hair graying is repeatable between observers. We used the mean of the color scores assigned by the four observers in subsequent analyses.

Total GSH (tGSH) in Muscle

The tGSH levels in muscle cells were determined by following the method described by Tietze (1969) and Griffith (1980) with some particular modifications. Briefly, the blood muscle sam-

ples were thawed and immediately diluted (1 : 10 w/v) and homogenized in a stock buffer (0.01 M phosphate-buffered saline and 0.02 M EDTA), always working on ice to avoid oxidation. Three working solutions were made up in the same stock buffer as follows: (1) 0.3 mM NADPH, (2) 6 mM DTNB, and (3) 50 U GSH reductase/mL. An aliquot (0.5 mL) of homogenate of muscle cells was vortexed with 0.5 mL of diluted trichloroacetic acid (10% in H_2O) three times during 5 s each bout within a 15-min period. In the meantime, samples were removed from light and refrigerated to prevent oxidation. Afterward, the mixture was centrifuged (1,125 *g*, 15 min, 6°C), and the supernatant was removed. The next steps were performed in an automated spectrophotometer (A25-Autoanalyzer; Biosystems, Barcelona, Spain). Solutions 1 and 2 were mixed at 7 : 1 volume, respectively, and 160 μL of this new mixture was automatically added to 40 μL of sample (supernatant) in a cuvette. Afterward, 20 μL of solution 3 was added after 15 s, and the absorbance at 405 nm was monitored after 30 and 60 s. The change in absorbance was used to determine the tGSH concentration in muscle cells by comparing the output with the results from a standard curve generated by serial dilution of GSH from 1 to 0.031 mM. High repeatability values of this method have been published elsewhere (e.g., Alonso-Alvarez et al. 2010). Concentration is presented as micromoles of GSH per gram of muscle.

Oxidative Damage in Muscle

To determine GSSG levels in muscle cells, an aliquot (400 μL) of the supernatant obtained for tGSH assessment was adjusted to a pH of 7.5 by adding 6 N NaOH. Afterward, 2-vinylpyridine (8 μL) was added to the aliquot, and the mixture was vigorously shaken at ambient temperature in the dark to promote GSH derivatization. The mixture was then centrifuged (3,500 rpm for 10 min), and the change in absorbance of the supernatant was assessed at 405 nm as described for the tGSH assay. High repeatability values of this method have been published elsewhere (e.g., Alonso-Alvarez et al. 2010). Seven values deviated strongly from the linear standard curve (highest values). This potential source of variation in the data was considered by adding a factor with this information to the statistical analyses.

We also measured lipid peroxidation levels as a complementary index of oxidative damage in muscle cells. The test is based on the principle that most tissues contain a mixture of TBARS, including lipid hydroperoxides and aldehydes, whose concentrations increase as a result of oxidative stress. We followed the protocol described in Aust (1985). First, 1 mL of the homogenate used for the tGSH analysis was mixed with 2 mL of a solution of 15% trichloroacetic acid, 0.77% hydrochloric acid, and 0.375% thiobarbituric acid and with 20 μL of 2% 2,6-di-tertbutyl-4-methylphenol (BHT) in ethanol in a closed glass tube. Tubes were then heated for 30 min at 90°C and then cooled in ice-cold water for 10 min. The mixture was centrifuged at 2,025 *g* for 15 min, and the absorbance of the supernatant was read at 535 nm. The concentration of peroxidized lipids was determined in reference to a standard curve with 0, 1.25, 2.5, and 5 nmol/mL of malondialdehyde (MDA) in H_2O (i.e., the end product of lipid peroxidation) and presented as nanomoles of MDA per gram of muscle. High repeatability values of this method have been published elsewhere (e.g., Alonso-Alvarez et al. 2010).

Statistical Analyses

Because eumelanic and pheomelanic color scores for a given individual always summed to five, they were perfectly and negatively correlated. Thus, only one color score was used in the analyses (pheomelanic color score), and the meaning of any effect for this variable can be interpreted as the opposite of the effect of the eumelanic color score. The concentrations of eumelanin and pheomelanin pigments in the hair of wild boars were not significantly correlated ($r = 0.22$, $n = 70$, $P = 0.071$), so these variables were both used in the analyses.

Potential effects of age on tGSH, GSSG, and TBARS levels were evaluated by means of general linear models with these response variables and with age and the quadratic effect of age (i.e., age^2) as covariates. Because the antioxidant machinery may differ between the different morphs of wild boars considered here (e.g., Galván et al. 2010*b*), morph identity was added to the models as a five-level fixed factor (fig. 1). The body condition of wild boars may also affect their antioxidant profile, so KFI was added to the models as a covariate. Additional covariates were sex and prevalence of tuberculosis (because decreases in tGSH levels and increases in GSSG and lipid peroxidation levels have been associated with tuberculosis infection in other mammals; Palanisamy et al. 2011), which were added to the models as dummy variables with the values 0 (male or absence of tuberculosis, respectively) and 1 (female or presence of tuberculosis, respectively). The interaction between sex and KFI was also considered to control for a possible differential influence of body condition on the antioxidant profile of the different sexes. tGSH and the dummy variable indicating the seven highest GSSG values (above) were also controlled for in the model for GSSG as additional covariates. Finally, because the production of pheomelanin consumes GSH and can thus generate oxidative damage (Meyskens et al. 1999; Galván and Solano 2009; Galván and Møller 2011; Galván et al. 2011, 2012; Pavel et al. 2011), the pheomelanic color score and the pheomelanin content of hair were added as additional covariates to the models.

Similar general linear models were run to evaluate the potential variation of the pheomelanic color score and eumelanin and pheomelanin content of hair with age and tGSH, adding morph as a fixed factor and prevalence of tuberculosis, sex, body condition, and the interaction between sex and body condition as covariates, for the reasons explained above. Age (and age^2) and tGSH were added as additional covariates to the models. Other models were run to evaluate the potential variation of prevalence of hair graying in the face area and the body with age. To evaluate the potential variation of prevalence of hair graying with variation in tGSH and oxidative damage, a separated (because oxidative damage levels were related to

age; see "Results") general linear model was used with tGSH, GSSG, and TBARS added as covariates.

General linear models were also used to evaluate potential associations of the pheomelanic color score, the content of eumelanin and pheomelanin of hair, and the prevalence of hair graying with the average size of the lower canines of male wild boars (length and width of canines as two predictor variables). These analyses were controlled for the effect of body size, which was also added as a covariate to the models.

Nonsignificant terms were removed from the models (except the effect of tGSH values in the model for GSSG, which was not removed), which established a P value of 0.1 to abandon the model. The exploration of the distribution of residuals from the models confirmed that the normality assumption was fulfilled.

Results

A summary of results is shown in table 1. We found evidence of age-related variation in oxidative damage levels but not in antioxidant (tGSH) or pelage melanization levels (table 1).

Age-Related Variation in tGSH

The model obtained to explore the variation in muscle tGSH levels in wild boars of different ages was significant ($F_{1,64} = 4.40$, $P = 0.040$). However, the effects of age and age^2 were nonsignificant. The model included only the effect of sex, because males presented higher tGSH levels than females (table 2).

Age-Related Variation in Oxidative Damage

A significant model ($F_{5,51} = 5.94$, $P < 0.001$) resulted in a marginally nonsignificant and negative effect of age^2 on GSSG levels (table 2), which indicated that oxidative damage as reflected by GSSG tended to increase with age, although some decrease is observed in the oldest individuals (fig. 2*B*). Other terms remaining in the model were the effect of low GSSG levels, tGSH, and pheomelanin concentration of hair, although the latter two were not significant (table 2).

The pattern followed by oxidative damage as reflected by lipid peroxidation confirmed the tendency observed in GSSG levels, because the model ($F_{3,62} = 8.63$, $P < 0.0001$) for TBARS levels included a significant negative effect of age^2 (table 2), which indicated that lipid peroxidation levels are lowest in the oldest individuals and highest in individuals of medium age (fig. 2*C*). This model also included a positive effect of the pheomelanin color score (table 2), which indicated that lipid peroxidation levels increased with the extent of pelage covered by pheomelanin-based color and decreased with the extent of pelage covered by eumelanin-based color.

Age- and GSH-Related Variation in Melanin-Based Color and Melanin Content

The pheomelanic color score of wild boars differed between morphs but did not vary with age (table 3), as indicated by a significant model ($F_{5,60} = 5.74$, $P < 0.001$). A marginally nonsignificant effect of tGSH levels (table 3) indicated that the extent of pelage covered by pheomelanin-based color tended to decrease with the levels of tGSH, whereas the extension covered by eumelanin-based color tended to increase (fig. 3*A*). This model also included the effects of morph, sex, and KFI and the interaction between sex and KFI (table 3).

The pheomelanin content of the hair of wild boars did not vary with either age or tGSH, and only the effect of morph remained in the model for this variable (table 3). The eumelanin content of hair covaried neither with age nor with tGSH but was negatively related to body condition (table 3; fig. 3*B*). This model was significant ($F_{3,69} = 8.23$, $P < 0.0001$; table 3).

Variation in Hair Graying with Age, tGSH, and Oxidative Damage

The prevalence of hair graying in the face area of wild boars was not associated with age or tGSH, but it was positively associated with KFI, and there was a nonsignificant tendency to decrease with oxidative damage as reflected by TBARS (table 4). The interaction between sex and KFI was also significant, because the slope of the regression between hair graying prevalence and KFI was significant in males ($b = 0.014$, $t = 2.92$, $P = 0.004$) but not in females ($b = 0.003$, $t = 1.18$, $P = 0.242$). This model was significant ($F_{2,63} = 3.60$, $P = 0.033$). This indicates that the presence of hair graying in the face was associated with high body condition in wild boars, but only in males.

The models run for prevalence of hair graying on the body corroborated the results of the models for hair graying in the face. Thus, there was a marginally nonsignificant positive effect of KFI (table 4, pt. A) and a significant negative effect of TBARS (table 4, pt. B) on hair graying prevalence in the body. Therefore, hair graying was associated with good condition, particularly in males, and low oxidative damage as reflected by lipid peroxidation in wild boars (fig. 4).

Variation in Melanin-Based Color, Melanin Content, and Hair Graying with Canine Size

None of the measures of canine size (width and length) was related to either the pheomelanic color score (width: $F_{1,30} = 1.93$, $P = 0.175$; length: $F_{1,30} = 1.63$, $P = 0.211$; body size: $F_{1,30} = 0.35$, $P = 0.559$) or the pheomelanin content of hair (width: $F_{1,23} = 1.97$, $P = 0.174$; length: $F_{1,23} = 1.88$, $P = 0.184$; body size: $F_{1,23} = 2.14$, $P = 0.157$). However, the eumelanin content of hair was positively related to canine width ($b = 0.01$, $F_{1,25} = 5.20$, $P = 0.031$; length: $F_{1,25} = 1.35$, $P = 0.257$; body size: $F_{1,25} = 0.58$, $P = 0.454$).

The prevalence of hair graying in the face area of wild boars

Table 2: Results of general linear models testing for associations between age and total glutathione (tGSH), oxidized glutathione (GSSG), and lipid peroxidation (TBARS) levels in muscle of wild boars

	tGSH (μmol/g)					GSSG (μmol/g)					TBARS (nmol MDA/g)				
Effect	df	*b*	*F*	*P*	Ex. seq.	df	*b*	*F*	*P*	Ex. seq.	df	*b*	*F*	*P*	Ex. seq.
Age	1, 62	1.72×10^{-4}	.55	.463	5	**1, 51**	**.33**	**1.86**	**.179**	**...**	**1, 62**	**.01**	**5.96**	**.017**	**...**
Age^2	1, 61	-1.70×10^{-5}	1.20	.278	4	**1, 51**	**-6.6×10^{-3}**	**3.46**	**.069**	...	**1, 62**	**-1.97×10^{-4}**	**4.42**	**.039**	**...**
Pheomelanin conc.	...	...	...	...	...	**1, 51**	**−69.78**	**2.87**	**.096**	...	1, 49	−.93	.60	.441	3
Pheomelanic score	...	...	...	...	...	1, 48	.57	.39	.535	4	**1, 62**	**.09**	**22.92**	**<.0001**	**...**
Morph	4, 57	...	1.04	.395	3	1, 50	−1.25	1.14	.290	6	1, 48	**.01**	.04	.838	2
Sex	**1, 64**	**...**	**4.40**	**.040**	**...**	1, 47	...	.17	.685	3	1, 61	...	.33	.565	6
KFI	1, 56	1.05×10^{-4}	.09	.766	2	1, 46	5.4×10^{-3}	.00	.950	2	1, 60	8.85×10^{-4}	.19	.666	5
Sex × KFI	1, 55	...	.04	.842	1	1, 45	...	.21	.651	1	1, 59	...	1.17	.284	4
TB	1, 63	−.01	1.94	.169	6	1, 49	...	.85	.362	5	1, 47	...	.03	.866	1
tGSH	...	...	...	...	...	**1, 51**	**40.52**	**1.89**	**.175**	...	...	...	...	...	...
High GSSG value	...	...	...	...	...	**1, 51**	**9.73**	**12.30**	**.001**	**...**	...	...	...	...	...

Note. The analyses are controlled for the effects of pheomelanization level (pheomelanin concentration of hair and pheomelanic color score), morph, sex, body condition (kidney fat index [KFI]), prevalence of tuberculosis (TB), and tGSH and high GSSG values (the latter two terms only in the model for GSSG). For each term, the degrees of freedom, the fitted regression coefficient (*b*), and the *F* ratio and its corresponding significance are provided. Terms remaining in the final models are marked in bold. The exclusion sequence (ex. seq.) is given for the terms that were removed from the models. conc. = concentration.

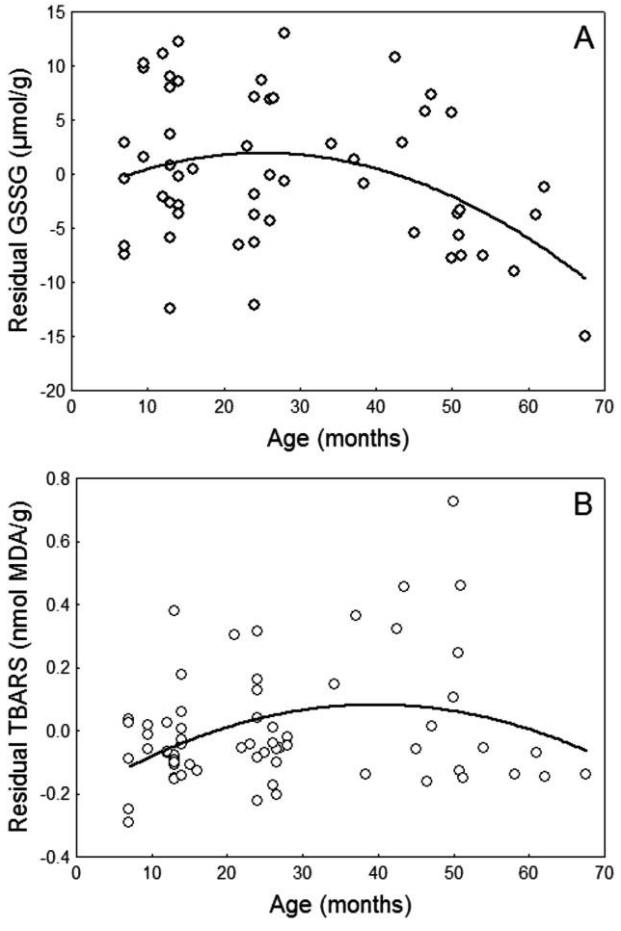

Figure 2. Relationship between age and oxidized glutathione (GSSG; *A*) and lipid peroxidation (TBARS; *B*) levels in muscle of wild boars. The residual values of the response variables are shown (i.e., partial effects after applying the final models in table 2 without age). The lines are the regression lines.

was positively related to canine length ($b = 0.10$, $F_{1,30} = 12.69$, $P = 0.001$; fig. 5*A*) and negatively related to canine width ($b = -0.13$, $F_{1,30} = 5.14$, $P = 0.031$; body size: $F_{1,30} = 0.10$, $P = 0.753$). The prevalence of hair graying in the body was also positively related to canine length ($b = 0.07$, $F_{1,30} = 9.23$, $P = 0.005$; width: $F_{1,30} = 2.83$, $P = 0.102$; body size: $F_{1,30} = 0.50$, $P = 0.484$; fig. 5*B*).

Discussion

GSH- and Oxidative-Damage-Related Variation in Melanic Traits

The pheomelanic and eumelanic pelage of wild boars was partially explained by variability in tGSH level in muscle cells. Pheomelanic scores were negatively related to tGSH levels, whereas the relationship was positive for eumelanic scores, although the relationships were marginally nonsignificant. This was as predicted, given the role fulfilled by GSH during melanogenesis; high levels of cysteine (GSH is the main reservoir of cysteine; Benedetto et al. 1981) promote pheomelanogenesis, a pathway to which cysteine is incorporated (García-Borrón and Olivares Sánchez 2011). This influence of GSH on the expression of melanic traits has previously been found in other mammals (Benedetto et al. 1981) and also in birds (Galván and Alonso-Alvarez 2008; Hõrak et al. 2010) and insects (Clark et al. 2010). This study thus supports the hypothesis that the influence of GSH on the expression of melanic traits is a general pattern, at least among higher vertebrates. Interestingly, we also found that wild boars with high pheomelanic color scores (i.e., with low eumelanic color scores) had higher levels of oxidative damage in cells (TBARS) than did wild boars with lower color scores. The fact that the cysteine contained in GSH molecules is used for the synthesis of pheomelanin polymers means that pheomelanogenesis consumes GSH (Meyskens et al. 1999; Pavel et al. 2011), which decreases the antioxidant capacity of cells, as shown by an increased cancer risk in humans with high contents of pheomelanin in cells (Hill and Hill 2000; Simon and Peles 2010; Pavel et al. 2011). Thus, the negative tendency of wild boars with high pheomelanin-based color scores to have low tGSH levels and the positive association between pheomelanin-based color scores and TBARS levels supports the fact that pheomelanogenesis consumes GSH and that it has an oxidative cost. Indeed, recent studies involving birds suggest that the production of pheomelanin imposes physiological constraints, as derived from negative relationships between pheomelanic plumage color scores and brain size (Galván and Møller 2011) and capacity to resist the effects of ionizing radiation across species (Galván et al. 2011). It has also been reported that more pheomelanic individual barn owls *Tyto alba* are more sensitive than less pheomelanic birds to physiological stress caused by corticosterone (Almasi et al. 2008) and that tawny owls *Strix aluco* belonging to the pheomelanic morph have lower viability during adverse environmental conditions than conspecifics belonging to the eumelanic morph (Karell et al. 2011). Our results do not contradict recent findings in another bird species (the great tit *Parus major*; Galván and Alonso-Alvarez 2008) that show that GSH levels can be negatively related to the expression of an eumelanic (but not pheomelanic) plumage trait, because it was the result of an experimental inhibition of GSH synthesis, and the present results are correlative, thus revealing the consumption of GSH for pheomelanogenesis. Thus, overall, our results support the dependence of the expression of pheomelanic traits on GSH levels and that this has physiological effects that are related to increased oxidative damage.

However, our measurements of hair melanin content, unlike melanic color scores, were not predicted by tGSH levels, even when the eumelanin content of hair was positively correlated with the eumelanic color score. Here we must first consider that melanic color scores are based on most of the pelage of wild boars, whereas melanin quantifications were performed on a limited area of the body (the face). The tGSH levels in muscle cells would mostly represent systemic levels instead of those local levels potentially detected in hair melanocytes. We must also note that the pheomelanic color scores of wild boars

Table 3: Results of general linear models testing for associations between pheomelanic color score (mean score between observers) and pheomelanin and eumelanin concentration of hair and age and total glutathione (tGSH) levels of muscle in wild boars

	Pheomelanic color score					Pheomelanin concentration (Au/mg)					Eumelanin concentration (Au/mg)				
Effect	df	*b*	*F*	*P*	Ex. seq.	df	*b*	*F*	*P*	Ex. seq.	df	*b*	*F*	*P*	Ex. seq.
Age	1, 59	-5.48×10^{-3}	.27	.602	3	1, 49	4.80×10^{-5}	.05	.818	2	1, 67	2.40×10^{-4}	.80	.374	4
Age^2	1, 58	5.4×10^{-4}	.72	.398	2	1, 48	-6.00×10^{-6}	.17	.685	1	1, 49	-7.00×10^{-6}	.10	.756	2
tGSH	**1, 60**	**−7.93**	**3.13**	**.082**	...	1, 53	−.13	2.11	.152	6	1, 50	−.08	.29	.591	3
Morph	**1, 60**	**.61**	**21.93**	**<.0001**	**...**	**1, 71**	$\mathbf{-7.40 \times 10^{-3}}$	**6.89**	**.011**	**...**	**1, 69**	**−.02**	**20.71**	**<.0001**	**...**
Sex	**1, 60**	**...**	**4.63**	**.035**	**...**	1, 70	...	2.05	.157	7	**1, 69**	**...**	**2.90**	**.093**	**...**
KFI	**1, 60**	**.01**	**.95**	**.334**	**...**	1, 51	6.90×10^{-5}	.12	.726	4	**1, 69**	$\mathbf{-5.94 \times 10^{-4}}$	**4.96**	**.029**	**...**
Sex × KFI	**1, 60**	**...**	**3.21**	**.078**	**...**	1, 50	...	.50	.483	3	1, 68	...	1.44	.234	5
TB	1, 57	...	.01	.910	1	1, 52	3.36×10^{-4}	.83	.365	5	1, 48	...	.02	.877	1

Note. The analyses are controlled for the effects of morph, sex, body condition (kidney fat index [KFI]), and prevalence of tuberculosis (TB). For each term, the degrees of freedom, the fitted regression coefficient (*b*), and the *F* ratio and its corresponding significance are provided. Terms remaining in the final models are marked in bold. The exclusion sequence (ex. seq.) is given for the terms that were removed from the models.

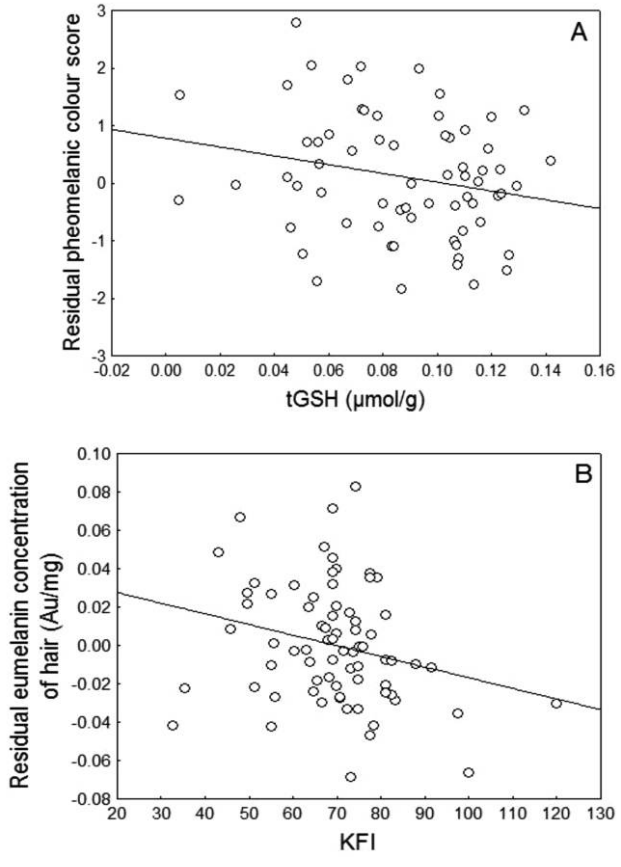

Figure 3. Relationship between pheomelanic color score and total glutathione (tGSH) levels in muscle of wild boars (*A*) and relationship between the eumelanin concentration of hair and body condition as reflected by the kidney fat index (KFI) in wild boars (*B*). The residual values of the response variables are shown (i.e., partial effects after applying the final models in table 3 without tGSH and without KFI in *A* and *B*, respectively). The lines are the regression lines.

were correlated with the length of the pheomelanic portion of hairs but not with the pheomelanin content of hair. All this suggests that the pheomelanic color score is an index of melanization that is qualitatively different from the pheomelanin hair content. Pheomelanin concentration in an individual hair should have been predicted by tGSH levels in the pigmentary units of that follicle. Our measurements of eumelanin content of hair were nonetheless correlated with the eumelanic color score, so the former could also have been related to tGSH levels in muscles like the eumelanic color score. However, in the models, body condition was a stronger predictor of the eumelanin content of hair than were tGSH levels, which may explain why the effect of tGSH did not remain in the models. It must also be considered that sample size was lower for measures of tGSH levels than for measures of hair melanization, which may prevent us from finding an association between the two traits.

Indeed, the highest values of eumelanin concentration in hair were found in the wild boars in poorest body condition, although this was not coupled to variation in tGSH levels. The ultimate reason for the negative relationship between eumelanin hair content and body condition may be that the degree of pelage unmelanization is a signal of quality in wild boars. This would be in agreement with our results for hair graying (i.e., prevalence of unmelanized hairs). First, wild boars with hair graying had lower levels of oxidative damage, as reflected by lipid peroxidation, than did wild boars without hair graying. This apparently contradicts the free radical theory of graying, which hypothesizes that this phenomenon is caused by the blocking of hair melanization by oxidative stress (Arck et al. 2006; Wood et al. 2009), because this predicts that oxidative damage should be greater in wild boars with hair graying. However, this finding may be explained in the context of quality signals, because wild boars with hair graying may be signaling to conspecifics their capacity to avoid, at a systemic level, the oxidative damage that their hair melanocytes might experience (Arck et al. 2006; Wood et al. 2009). That hair graying in wild boars is a signal of quality is further supported by the positive association between hair graying and body condition, particularly in males, and between hair graying and the length of male lower canines, which probably represents an intersexual signal of quality (Fernández-Llario and Mateos-Quesada 2003) and which was greater in males with hair graying. Therefore, hair graying seems to be a secondary sexual trait in male wild boars. Although our study is entirely correlational, and thus our results should be taken with caution, future studies should pay attention to the signaling potential of hair graying in wild boars and other mammals, which is an issue that, to our knowledge, has thus far been overlooked.

Age-Related Variation in GSH and Oxidative Damage

The tGSH levels in muscle cells were not associated with the age of wild boars. This was not as predicted, because age-related decreases in GSH levels have been observed in a variety of organisms (reviewed in Bains and Shaw 1997). By contrast, our measurements of oxidative damage (GSSG and TBARS) showed a curvilinear pattern of variation with age, with levels being highest in wild boars of medium age and lowest in older individuals. Increases in the lipid peroxidation levels of cells with age have also been found in birds (Alonso-Alvarez et al. 2010). In accordance with the free radical theory of aging, this is probably a result of diminished antioxidant capacity because of the accumulation of oxidative damage with age as a consequence of the free radical production during normal cellular respiration (Finch and Kirkwood 2000; Finkel and Holbrook 2000). Such a diminished antioxidant capacity was not reflected in the GSH levels of wild boars, but the possibility that other antioxidants are more sensitive to senescence effects should not be discarded. However, the levels of oxidative damage decreased again in the oldest wild boars. Although senescence effects are normally reflected by a deterioration of the phenotype at old ages, either after a progressive deterioration or after an initial improvement in performance, signals of quality (i.e., traits that improve reproductive success) seem to be exceptions to that

Table 4: Results of general linear models testing for associations between prevalence of hair graying (mean score between observers) in face and body and age (A) or total glutathione (tGSH) and oxidative damage levels in muscle (B) in wild boars

	Hair graying in face					Hair graying in body				
Effect	df	*b*	*F*	*P*	Ex. seq.	df	*b*	*F*	*P*	Ex. seq.
A:										
Age	1, 94	3.40×10^{-3}	1.84	.179	3	1, 93	7.31×10^{-4}	.12	.727	2
Age^2	1, 92	-1.53×10^{-4}	.82	.368	1	1, 92	-7.80×10^{-5}	.31	.580	1
Morph	1, 93	−.04	1.30	.256	2	1, 95	−.03	1.05	.309	4
Sex	**1, 95**	**...**	**4.24**	**.042**	...	1, 97	...	.02	.879	6
KFI	**1, 95**	**3.04×10^{-4}**	**9.82**	**.002**	...	**1, 98**	**3.61×10^{-3}**	**3.86**	**.052**	**...**
Sex × KFI	**1, 95**	**...**	**4.11**	**.045**	...	1, 96	...	1.63	.204	5
TB	**1, 95**	**...**	**3.37**	**.069**	...	1, 94	...	.13	.721	3
B:										
tGSH	1, 61	1.59	1.30	.258	5	1, 58	.50	.23	.630	2
GSSG	1, 56	10^{-4}	.00	.987	1	1, 56	-1.01×10^{-3}	.05	.820	1
TBARS	**1, 63**	**−.32**	**2.68**	**.100**	**...**	**1, 64**	**−.37**	**6.77**	**.011**	**...**
Morph	1, 58	−.03	.35	.558	2	1, 60	−.05	2.08	.155	4
Sex	1, 62	...	1.36	.247	6	1, 62	...	.07	.784	6
KFI	**1, 63**	**6.29×10^{-3}**	**5.49**	**.022**	**...**	1, 63	3.16×10^{-3}	2.60	.111	7
Sex × KFI	1, 60	...	.70	.406	4	1, 61	...	2.33	.132	5
TB	1, 59	...	.45	.506	3	1, 59	...	.57	.455	3
High GSSG value	1, 56	−2.20	1.43	.237	1	1, 56	−.17	1.97	.166	1

Note. The analyses are controlled for the effects of morph, sex, body condition (kidney fat index [KFI]), prevalence of tuberculosis (TB), and high GSSG values. For each term, the degrees of freedom, the fitted regression coefficient (*b*), and the *F* ratio and its corresponding significance are provided. Terms remaining in the final models are marked in bold. The exclusion sequence (ex. seq.) is given for the terms that were removed from the models. GSSG = oxidized glutathione; TBARS = lipid peroxidation.

rule, because their expression may improve instead of deteriorate with increasing age as a consequence of the increased reliability of the signals with increased age (Galván and Møller 2009; Galván and Moreno 2009) or as a consequence of an attempt by older individuals to increase their reduced residual reproductive value (Evans et al. 2011). Of course, levels of oxidative damage cannot be considered a part of a sexual ornament, but reproduction may generate oxidative stress (Alonso-Alvarez et al. 2004; Metcalfe and Alonso-Alvarez 2010), and then an improvement in the antioxidant performance may increase the residual reproductive value of older individuals, which may explain why we found a decrease in oxidative damage in the oldest wild boars. However, it must also be considered that the free radical theory of aging has been challenged by recent evidence showing in different organisms that oxidative damage levels may not be associated with longevity (Buffenstein et al. 2008; Speakman and Selman 2011). Therefore, the absence of senescence effects in GSH levels and the decrease in oxidative damage levels in the oldest wild boars found here as well as the previous studies showing a lack of senescence effects in the expression of sexually selected traits (see above) may be indicative that the free radical theory of aging is actually not valid, at least for some organisms. The correlational nature of our study limits our capacity to test the free radical theory of aging in wild boars, but future ecological studies should consider the body of accumulating evidence against this theory (Buffenstein et al. 2008; Speakman and Selman 2011) to explain the evolution of life-history traits.

Alternatively, the fact that the oldest wild boars in our sample showed lower levels of oxidative damage could be a consequence of our cross-sectional sample (e.g., Forstmeier 2002; Nussey et al. 2008). Therefore, we cannot discard the possibility that oxidative damage of the oldest individuals was even lower at younger ages, and this might explain why they survived better than other individuals. Different U-shaped trends in antioxidant and oxidative damage levels with age have been reported in other cross-sectional studies. However, the most common pattern is one that shows an increase and then a decrease in antioxidant levels with age and a decrease and then an increase in oxidative damage with age (e.g., birds: Alonso-Alvarez et al. 2010; reptiles: Isaksson et al. 2011). However, in some cross-sectional samples of humans (Kasapoglu and Özben 2001; Rizvi and Maurya 2007), it has been shown that TBARS levels in blood increase and then decrease with age, such as in our wild boars, although the authors did not statistically test the quadratic pattern (but see Gil et al. 2006 for a simple positive relationship with age). However, Jones et al. (2002) described a U-shaped pattern between GSSG and age in humans, thus contrasting with our negative quadratic curve. Although longitudinal sampling is the optimal procedure, it is particularly difficult to perform when studying wild populations, because recapture is required.

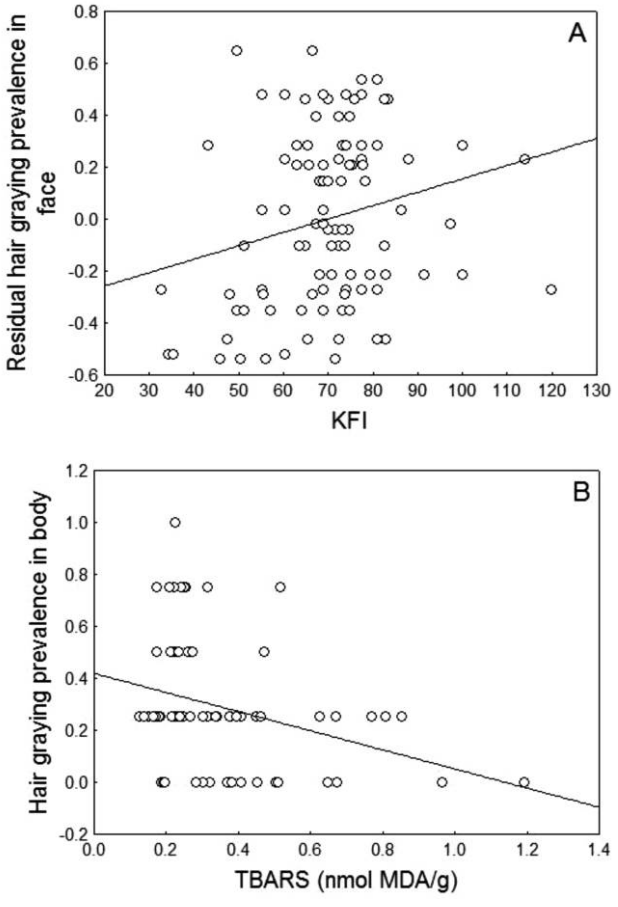

Figure 4. Relationship between prevalence of hair graying (mean value of the scores assigned by four different observers) in face and body condition as reflected by the kidney fat index (KFI; *A*) and between prevalence of hair graying in body and lipid peroxidation (TBARS) levels in muscle of wild boars (*B*). The residual values of the response variable are shown in *A* (i.e., partial effects after applying the final model in table 4 without sex and prevalence of tuberculosis). The lines are the regression lines.

Age-Related Variation in Melanic Traits

We found no association between any measure of hair melanization (i.e., melanin-based color scores and melanin content of hair) and age. Therefore, there was no evidence of senescence effects in the melanization patterns of wild boars. Thus, it seems that the lack of senescence effects on tGSH levels parallels a lack of senescence effects on melanization. However, melanization seems to be dependent on GSH levels (here shown by a tendency for tGSH levels and melanin-based color scores to covary). Similar to the explanation for the decrease in oxidative damage at the oldest ages, if we assume that melanization in hair plays a role in mate choice or intrasexual competition (see Galván and Møller 2009 and Lifjeld et al. 2011 for examples of sexual selection on pheomelanic traits in birds), the lack of senescence effects on pelage melanization could be attributable to an attempt of individuals to maintain their residual reproductive value (Galván and Møller 2009; Evans et al. 2011). However, pheomelanic color scores were positively related (which implies that the eumelanic color score was negatively related) to oxidative damage levels. Thus, it is possible that the avoidance of senescence effects in the pheomelanin-based hair color by wild boars incurs a physiological cost, because maintaining GSH above a certain age-dependent level to produce pheomelanin (García-Borrón and Olivares Sánchez 2011) may be costly, even though GSH levels did not decrease with age. Indeed, there was evidence of an oxidative cost of aging in wild boars, because oxidative damage levels increased until certain ages. Therefore, wild boars may be avoiding senescence effects in their pheomelanic color of hair to increase their residual reproductive value but doing so at the expense of an oxidative cost. The cross-sectional and correlative design of our study does not allow us to establish a cause-effect relationship between oxidative damage levels and the absence of senescence effects in melanin-based color, so we call for future studies to

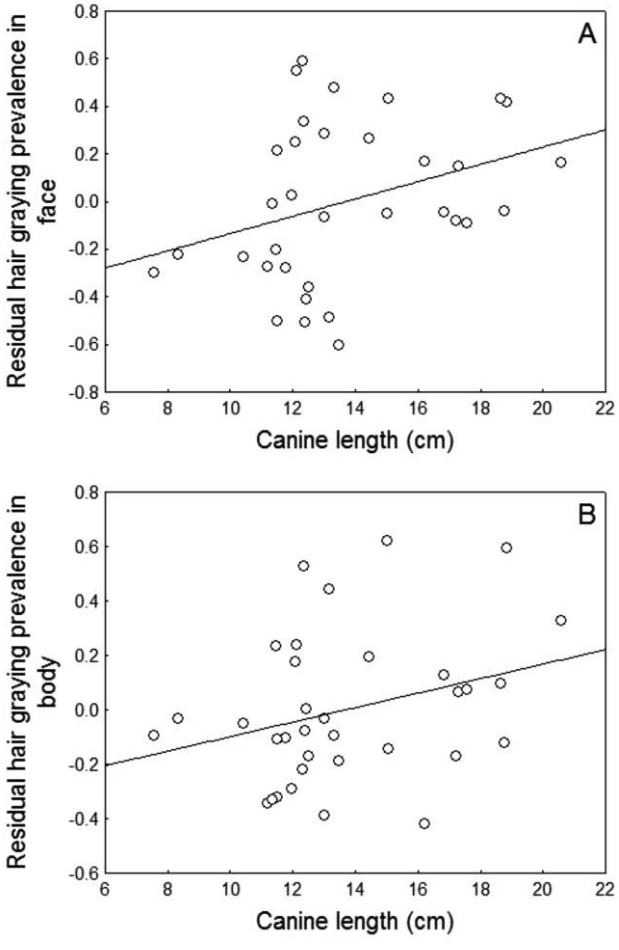

Figure 5. Relationship between prevalence of hair graying (mean value of the scores assigned by four different observers) in face (*A*) and body (*B*) and length of mandibular lower canines in male wild boars. The residual values of the response variables are shown (i.e., partial effects after controlling for the effects of canine width and body size). The lines are the regression lines.

pay attention to the relationship between antioxidants, oxidative damage, and melanization patterns, which is relevant for a proper understanding of the evolution of senescence.

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