

## Environmental constraints for plumage melanization in the northern goshawk *Accipiter gentilis*

Ismael Galván, Rob G. Bijlsma, Juan J. Negro, Manuel Jarén and Juan Garrido-Fernández

I. Galván (*ism.galvan@gmail.com*), Dept of Evol. Ecol., Natl. Mus. of Nat. Sci. (CSIC), José Gutiérrez Abascal 2, ES-28006 Madrid, Spain. Present address for IG: Dept. of Evol. Ecol., Doñana Biol. Station (CSIC), Avda. Americo Vespucio s/n, ES-41092 Seville, Spain. – R. G. Bijlsma, Anim. Ecol. Group, Centre for Ecol. and Evol. Stud., Univ. of Groningen, PO Box 14, NL-9750 AA Haren, the Netherlands. – J. J. Negro, Dept. of Evol. Ecol., Doñana Biol. Station (CSIC), Avda. Americo Vespucio s/n, ES-41092 Seville, Spain. – M. Jarén and J. Garrido-Fernández, Food Biotechnology Dept., Inst. de la Grasa (CSIC), Avda. Padre García Tejero 4, ES-41012 Seville, Spain.

Although it is recognized that certain environmental factors are important determinants of the expression of melanin-based traits, their influence in wild populations of animals is poorly known. One of these factors is the availability of amino acids that serve as precursors of melanins. Here we measured eumelanin and pheomelanin content in feathers of northern goshawk *Accipiter gentilis* nestlings, hypothesizing that, if the availability of melanin precursors is related to food abundance and habitat quality, plumage melanization should be affected by those variables. Although the eumelanin content increased with food abundance as predicted, the levels of this variable were higher in low-quality habitats (homogeneous coniferous forests) and in nestlings in poor condition, and the pheomelanin content and eumelanin:pheomelanin ratio were lower and higher, respectively, in subpopulations where nestlings were in poorer condition. Therefore, environmental availability of melanin precursors seems to determine plumage melanization in goshawks, but our findings may also be explained by the differential effects of environmental oxidative stress on both forms of melanin, as eumelanin and pheomelanin production are favoured under high and low levels, respectively, of oxidative stress.

Melanin is the most common pigment of the animal integuments, where it fulfills a variety of functions that ranges from thermoregulation and protection against ultraviolet radiation and mechanical abrasion to inter- and intraspecific signaling roles like camouflage and sexual attraction (Galván and Alonso-Alvarez 2008). In vertebrates, this pigment is produced in two forms in melanosomes, the specialized organelles of melanocytes, often simultaneously in the same cells but one form usually prevailing over the other (Ozeki et al. 1997). The two forms of melanin are eumelanin, a polymer of dihydroxyindole and carboxylic acid units, and pheomelanin, produced by benzothiazine derivatives (Prota 1992). These pigments are synthesized de novo by the organisms from the amino acid tyrosine, which is oxidized to dopaquinone by the enzyme tyrosinase. Dopaquinone acts as an intermediate for the production of eu- and pheomelanin, but eumelanogenesis occurs under high tyrosinase activity and low cysteine levels, while opposite conditions lead to the alternative pathway of pheomelanogenesis (Ozeki et al. 1997, Benathan et al. 1999). These biochemical differences between eumelanin and pheomelanin are also reflected in the appearance of the traits coloured by them, eumelanic traits being responsible for black and grey (i.e. darker) colours and pheomelanic traits for yellowish, reddish, chestnut and

brown (i.e. lighter) colours (Toral et al. 2008). These differences in colour make that variations in the ratio eumelanin:pheomelanin are reflected in variations in the brightness of the coloured traits (Solano et al. 2006).

The fact that melanins can be synthesized de novo, as opposed to other pigments like carotenoids that must be ingested in the diet, has led to the assumption that the expression of most melanic traits is strictly under genetic control, which has represented a difficulty for the understanding of the evolution of those melanic traits acting as signals of quality (Griffith et al. 2006). This is because any reliable signal needs to be costly to produce or maintain, and these costs are generated by environmental factors (Hasson 1997). Nonetheless, a growing number of studies have begun to show a high phenotypic plasticity in these traits.

Firstly, the availability of some amino acids in the diet, particularly tyrosine and phenylalanine (which serves as a precursor of tyrosine), has been proven to determine the melanin-based colour intensity of mammal coat and bird plumage in different species (reviewed by McGraw 2008), meaning that the availability of melanin precursors represents a limiting factor for these pigments. Secondly, the availability of calcium, a metal playing a crucial role in regulating melanin transfer to keratinocytes (Joshi et al.

2007) and melanin production by acting as a cofactor for the enzyme responsible for the synthesis of tyrosine from phenylalanine (i.e. phenylalanine hydroxylase; Bush and Simon 2007), determines the expression of a melanin plumage trait in zebra finches *Taeniopygia guttata* (McGraw 2007). Lastly, in great tits *Parus major*, the cysteine-containing tripeptide glutathione (GSH), which is the most important intracellular antioxidant (Anderson 1998, Wu et al. 2004), limits the production of eumelanin (Galván and Alonso-Alvarez 2008). This means that the expression of eumelanin traits is only possible for individuals presenting low levels of GSH, and that these individuals must counteract this paucity by mobilizing alternative antioxidants, which could represent a handicap for the production of eumelanin-based signals (Galván and Alonso-Alvarez 2008). Given that the production of GSH is genetically controlled (Soltaninassab et al. 2000) but also affected by environmental factors promoting oxidative stress such as thermal stress, UV radiation, food scarcity or certain pro-oxidative pollutants (Cho et al. 1981, Lautier et al. 1992, Ohtsuka et al. 1994, Sewalk et al. 2001), the influence of GSH on the expression of melanic traits is also environmentally controlled (Galván and Alonso-Alvarez 2009).

In summary, the argument that the expression of melanic traits is weakly influenced by environmental factors because these pigments can be synthesized by the organisms is increasingly being rejected (Griffith et al. 2006, Galván and Alonso-Alvarez 2008, 2009, Galván and Solano 2009). In addition, it has been suggested that melanins could be even more costly to produce than other pigments (i.e. their production is not so tightly controlled by genes) because their precursor amino acids must experience a series of transformations before melanin polymers are synthesized (Stoehr 2006), while other pigments like carotenoids do not experience a synthesizing process once ingested (but see del Val et al. 2009).

Our aim has been to assess a possible influence of food abundance and habitat quality on the intensity of plumage melanization in nestlings of the northern goshawk *Accipiter gentilis*, a medium-sized forest raptor. The mantle feathers in this species are dark grey and, although the exact function of this colouration has not been investigated in goshawks, it is likely that it functions in making the birds cryptic, as melanin-based colouration seems to do in most raptors (Ferguson-Lees and Christie 2001, Negro 2008, Negro et al. 2009). We extracted and quantified eumelanin and pheomelanin from goshawk feathers, which permitted us to make a further step in our understanding of environmental influences on the expression of melanic traits, as quantitative measurements of both types of melanin have never before been related to environmental variables in free-ranging animals. We determined food abundance in territories where goshawk nestlings had been reared, assuming that, as carnivorous animals like northern goshawks (this species' biology is intimately linked to the availability of avian prey; Rutz 2005, Rutz et al. 2006a) ingest a high content of proteins in their diet (Robbins et al. 2005), low food abundance indicates a low general availability of amino acids (i.e. melanin precursors). Although northern goshawks are generalist predators, it has been shown that food abundance is actually limiting goshawk population

increase in the Netherlands (Rutz and Bijlsma 2006, Rutz et al. 2006a). Therefore, we predicted that, if melanin production depends on environmental availability of melanin precursors, nestlings from territories with low food abundance should present a lower content of both eumelanin and pheomelanin in their feathers (Fig. 1).

We also measured the proportion of forested area surrounding the nests as an indicator of habitat quality, potentially influencing the effect of food abundance on the plumage melanization of goshawk nestlings, as this variable has been shown to influence the effect of food supplementation on the reproductive performance of goshawks in Finland (Byholm and Kekkonen 2008). These authors found that artificial food supplementation increased the reproductive performance of goshawks only in low-quality breeding territories, that is, territories with low presence of good habitat (i.e. forest). In the Netherlands, a mixture of forest with farmland is the best habitat for northern goshawks in terms of density and reproductive success (Rutz et al. 2006b). Therefore, we predicted that nestling goshawk melanization would increase with the proportion of forested area surrounding nests (Fig. 1). Additionally, we determined habitat type (homogeneous coniferous vs mixed coniferous–deciduous woodland) as another indicator of habitat quality, because homogeneous coniferous forests represent the poorest habitat for goshawks in the Netherlands (Rutz and Bijlsma 2006, Rutz et al. 2006b), predicting lower melanin contents in this habitat type. We also predicted an interaction between food abundance and habitat type indicating a limiting effect of food availability on plumage melanization and food abundance in poor habitats (i.e. homogeneous coniferous forest) only.

Finally, we searched for possible differences in nestling body condition between three different areas (subpopulations; Methods) in which goshawk territories were located. As poor body condition is first and foremost due to low food availability (Bradbury and Blakey 1998, Alonso-Alvarez and Tella 2001), we predicted that the lowest values of plumage melanin content should be found in areas with nestlings in poor condition.

Although we predicted the same effect of food abundance and habitat quality on both forms of melanin (because the influence of amino acids (tyrosine) occurs in

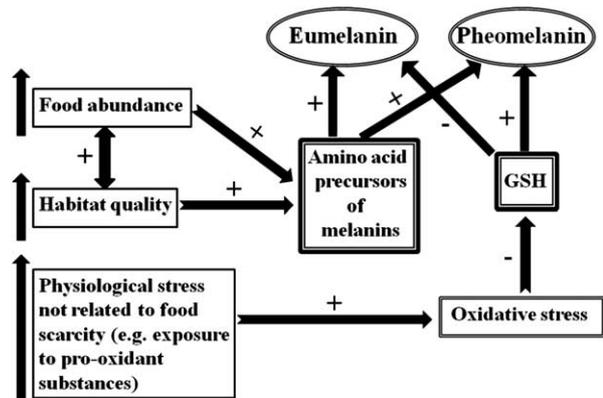


Figure 1. Scheme showing predictions for the influence of an increase in the magnitude of environmental factors (food abundance, habitat quality and physiological/oxidative stress) on the synthesis of eumelanin and pheomelanin. GSH: glutathione.

the first step of the melanogenesis pathway, which is common to eu- and pheomelanogenesis; Ozeki et al. 1997, Benathan et al. 1999), it is possible that measurements of habitat quality (i.e. forested area proportion and habitat type) are indeed indicators of agents generating stress to goshawks not related to food scarcity (Fig. 1). For example, low forested values and homogeneous coniferous forests could cause physiological stress (i.e. physiological, hormonal or behavioural changes that occur as a response to unpredictable and noxious stimuli; Romero 2004) on goshawks, which in turn could increase their corticosterone levels (Suorsa et al. 2003) and thus induce oxidative stress (Lin et al. 2004). As it has recently been proposed that the expression of eu- and pheomelanin traits respond differently to environmental oxidative stress (Galván and Alonso-Alvarez 2009, Galván and Solano 2009), we considered these ideas when searching for possible differences between eumelanin and pheomelanin content of feathers in the influence of habitat quality (Fig. 1).

## Methods

### Field methods

The study was conducted during June 2006 in three different areas (subpopulations) that pertain to a northern goshawk population in the Netherlands: Drenthe, Veluwe and Het Gooi (hereafter referred to as area 1, area 2 and area 3, respectively). Veluwe and Het Gooi are 40 km apart and both these regions lie 100 km to the south of Drenthe.

The study area in Drenthe (52°51'N, 6°19'E) lies on fine and loamy fine sand overlying glacial till (6–12 m above sea level) in the northern Netherlands. The landscape is dominated by woodland (64%), heaths and small-scale farmland. The woodland is mostly coniferous with Scots pine, Norway spruce and a larch hybrid *Larix* sp. as dominant tree species (planted from the 1940s onwards). Crop husbandry on farmland bordering the study area is mostly restricted to green maize, potatoes, some spring barley and rye, and increasingly the cultivation of lilies. Human settlements are few and small (<3000 inhabitants per village).

Veluwe constitutes large uninterrupted woodland on coarse sandy soil between 10 and 100 m a.s.l. The study area covers 100 km<sup>2</sup> in the southwestern part of Veluwe near Ede (52°03'N, 5°40'E). The planted forests consist mostly of Scots pine *Pinus sylvestris* on coarse sandy soil, with some larch *Larix* sp., Norway spruce *Picea abies*, Douglas fir *Pseudotsuga menziesii*, beech *Fagus sylvatica* and oak *Quercus robur*. The forests are interspersed with *Calluna*-heaths, sand drift relicts and arable fields. The study area is partly fringed by a string of towns and villages with 5000–60 000 inhabitants each, and bordered by the river Rhine with its forelands in the south, by agri-industry in the Gelderse Vallei in the west, and by woodland and heaths in the north and east.

The coarse and fine loamy sand of Het Gooi (52°14'N, 5°10'E), between 1 and 15 m a.s.l., is wedged in between peat lands in the north and marshes in the south. Situated close to Amsterdam, the region is heavily urbanised and

used for recreational activities. The undulating landscape is covered with coniferous and mixed woodlands, park land and remnants of heaths and sand drifts. Compared to Veluwe and Drenthe, this area has a higher diversity in forest structure and age of stands.

In each study plot, all Goshawk nests are routinely located each year, as part of an ongoing study on the population dynamics of this species in the Netherlands (Rutz et al. 2006b). Trees with nests were climbed several times in the breeding season to assess clutch size, to ring and measure nestlings and to collect data on food. Chick age was back-calculated from sex-specific growth curves of wing length, based on 624 measurements of chicks on 50 nests which were visited 2–41 times each (Bijlsma 1997). During the visits in 2006, 4–5 mantle feathers were collected from 78 goshawk nestlings that had been reared in 35 different nests located in the three areas (area 1: 19 nestlings from 8 nests, area 2: 16 nestlings from 6 nests, area 3: 43 nestlings from 21 nests). The nestlings were between 21 and 39 days old (mean ± SE: 28.2 ± 0.5 days) when taken. The collected feathers are juvenal and would naturally be moulted after one year.

The percentage of woodland was calculated for a 5 × 5 km square surrounding the nest, which was considered to include the larger part of a particular pair's home range (Kenward 2006). The woodland was categorized as coniferous or deciduous when >75% of the 5 × 5 km around the focal point consisted of either forest type; coniferous woodland often consisted of a single tree species (usually Scots pine *Pinus sylvestris*). Mixed woodland consisted of a variety of coniferous and deciduous stands, neither exceeding 75% of the total surface area.

Food abundance was determined for each nest site, using relative densities of the four main prey species (wood pigeon *Columba palumbus*, feral/racing pigeon *Columba livia*, starling *Sturnus vulgaris* and Eurasian jay *Garrulus glandarius*; Rutz and Bijlsma 2006). For each km-square presence and absence of these species have been assessed for 12 random neighbouring km-squares within a 25 km radius (74% within 4 km). Kriging was used to calculate relative densities, ranging between 0 and 1. This density figure denotes the detectability of a species during two 1-h visits in a km-square (SOVON Vogelonderzoek Nederland 2002). Although the relative densities are based on field work in 1998–2000, we consider the data representative for later years as well (Rutz and Bijlsma 2006, van Dijk and Bijlsma 2006).

### Pigment analyses

Northern goshawk feathers were processed in our Lab of Chemical Ecology at Doñana Biological Station, following a patented procedure (p200703395 in the European Union) that can be summarized as follows: feather vanes with no shafts (4–5 feathers or 1.1–2.7 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  $\mu$ l of 30% H<sub>2</sub>O<sub>2</sub>. 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. All samples contained both pheomelanin and eumelanin in variable proportions. The relative concentration [absorbance units (Au)/feather mass] of both melanin types was estimated spectrophotometrically as in Toral et al. (2008) and Negro et al. (2009).

## Statistical analyses

General linear mixed models (GLMMs) were used to investigate the factors that explained variance in the eumelanin and pheomelanin content of goshawk nestling feathers and in the eumelanin:pheomelanin ratio. As nestling characteristics potentially affecting those variables (all log-transformed), sex was included as a fixed factor and nestling age (in days) and body condition as covariates. Body condition for each nestling was calculated as the percentage of deviation from the average body mass (100% = expected) for the given age and sex. This average body mass was calculated from a growth curve made on the basis of 1141 measurements of nestlings on 53 nests in several Dutch populations over a 20-year period for which accurate ages were known and multiple measurements per breeding season were available, including 10 nests for which nestlings were measured daily from hatching until fledging (Bijlsma 1997). Additionally, brood size was included as a covariate in the models because oxidative stress levels of nestlings increase with brood size (Costantini et al. 2006, Alonso-Alvarez et al. 2007), and oxidative stress has a direct role in determining plumage melanization (Galván and Alonso-Alvarez 2008, 2009, Galván and Solano 2009).

Environmental variables potentially affecting the melanin content of feathers were also included in the models. Habitat type (coniferous vs mixed woodland) in which the nests were located was entered as a fixed factor. Percentage of woodland in a 25 km<sup>2</sup> area centred on the nest was entered as a covariate. Lastly, for food abundance scores, a linear contrast table was used, ordering the averaged relative abundance for the four main prey species in three categories: good food abundance (factor score 3; relative abundance 0.71–1.00), moderate abundance (2; relative abundance 0.41–0.70), poor abundance (1; relative abundance 0.10–0.40). The interaction between habitat type and food abundance scores was also included in the models in order to investigate whether the possible effect of food abundance was only significant in a particular habitat or whether the sign of the relationship differed between habitat types. The area (i.e. subpopulation) to which the nests pertained was included as a fixed factor (area 1, 2 or 3) in the models.

Finally, nest identity was set as a random variable in order to control for the fact that the same growth environment is shared by all nestlings in a brood. Starting from saturated models, a backward stepwise procedure was

used to remove non-significant terms, establishing a probability of 0.1 to abandon the model and not removing a term until all interactions it contained had been removed previously. Inspections of residuals from the models confirmed that the normality assumption was fulfilled. Pairwise comparisons were made by means of Tukey tests. Only the effects remaining in the models after the stepwise procedure are shown. Analyses were performed using SAS software (Littell et al. 2006).

The same procedure described above was used to investigate factors that affected variation in nestling body condition. To this aim we used a more extensive data set comprised of information on 149 goshawk nestlings and 56 nests from four different breeding seasons (2005–2008). In terms of laying date, clutch and brood size and nest success, neither of these years was an outlier within the period concerned (Bijlsma 2009). Only breeding pairs that were different from the ones used for melanin content analyses or, when the same, had another pair composition, were included in this data set. Sex (fixed factor), age and brood size (covariates) were included in the model, as well as the area to which the nests pertained (fixed factor). Nest identity was introduced as a random factor. Information on habitat type, food abundance and woodland area proportion was not available for this extended data set, but are likely within the range of the primary data set (same regions, methods and researchers).

## Results

In all cases, the eumelanin content of feathers was higher than the pheomelanin content, with a mean ( $\pm$  SE) eumelanin:pheomelanin ratio of  $2.96 \pm 0.14$ . In each sample, eumelanin and pheomelanin contents were not correlated (Pearson correlation test:  $r = 0.17$ ,  $n = 78$ ,  $p = 0.129$ ).

The model for eumelanin content explained 24% of variance in that variable (Table 1). The sex and age of nestlings had marginally significant and significant effects, respectively, with males tending to have higher eumelanin content ( $0.41 \pm 0.01$  Au mg<sup>-1</sup>) than females ( $0.37 \pm 0.02$  Au mg<sup>-1</sup>), and older nestlings having lower content ( $\beta = -0.26$ ,  $p = 0.028$ ). The eumelanin content also decreased with nestling body condition ( $\beta = -0.27$ ,  $p = 0.019$ ) and brood size ( $\beta = -0.25$ ,  $p = 0.031$ ). On the other hand, the environmental variables explaining a significant portion of the variation in eumelanin plumage content were habitat type, with nestlings reared in coniferous woodland having higher values ( $0.40 \pm 0.01$  Au mg<sup>-1</sup>) than those from coniferous–deciduous mixed woodland ( $0.38 \pm 0.01$  Au mg<sup>-1</sup>), and the interaction between habitat type and food abundance scores, with a negative but non-significant relationship between eumelanin content and food abundance in homogeneous coniferous woodland ( $\beta = -0.39$ ,  $p = 0.133$ ) and a significant positive relationship in mixed woodland ( $\beta = 0.50$ ,  $p = 0.018$ ; Fig. 2).

The model for pheomelanin content explained 26% of the variance. There was a negative, marginally significant relationship between the pheomelanin content of feathers and brood size ( $\beta = -0.18$ ,  $p = 0.089$ ). The area was the other term that remained in the model, pheomelanin content increasing from area 1 to area 3 (Fig. 3a). Pairwise

Table 1. Results of the linear models obtained to investigate the factors that explained variance in the amount of eumelanin and pheomelanin in back feathers of northern goshawk nestlings and in eumelanin:pheomelanin ratio. Proportion of forested area and nest identity are not shown because they were removed from all the models. \* $R^2 = 0.24$ , \*\* $R^2 = 0.26$ , \*\*\* $R^2 = 0.29$ .

Effect	Eumelanin content ( $\text{Au mg}^{-1}$ )*				Pheomelanin content ( $\text{Au mg}^{-1}$ )**				Eumelanin: Pheomelanin ratio***			
	DF	MS	F	p	DF	MS	F	p	DF	MS	F	p
Sex	1	0.023	3.15	0.080	—	—	—	ns	—	—	—	ns
Age	1	0.037	5.06	0.028	—	—	—	ns	—	—	—	ns
Body condition	1	0.041	5.72	0.019	—	—	—	ns	—	—	—	ns
Brood size	1	0.035	4.82	0.031	1	0.071	2.98	0.089	—	—	—	ns
Habitat	1	0.047	6.51	0.013	—	—	—	ns	1	0.071	2.74	0.102
Food abundance	1	0.004	0.50	0.482	—	—	—	ns	1	0.000	0.00	0.960
Food abundance × Habitat	1	0.056	7.70	0.007	—	—	—	ns	1	0.080	3.10	0.082
Area	—	—	—	ns	2	0.268	11.17	<0.0001	2	0.200	7.76	0.001
Error	69	0.007	—	—	74	0.024	—	—	72	0.026	—	—

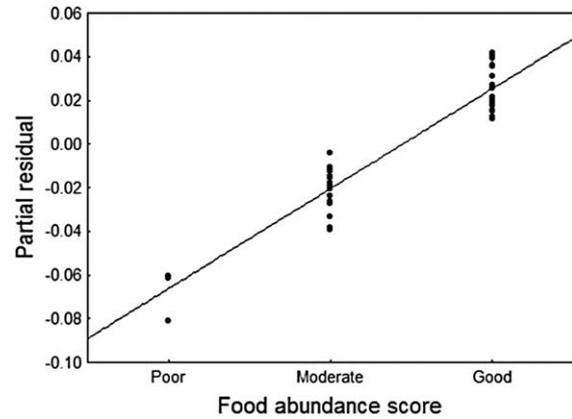


Figure 2. Relationship between eumelanin content (log-transformed) of northern goshawk nestling feathers and food abundance scores in coniferous–deciduous mixed woodlands after controlling for nestling sex, age and body condition and for the brood size in which they were reared. The residual figure of the response variable is shown (i.e. partial effects after applying the full model in Table 1 without food abundance and habitat type). The line is the regression line.

comparisons showed significant differences between areas 1 and 3 ( $p < 0.001$ ) and between areas 2 and 3 ( $p = 0.043$ ), but not between areas 1 and 2 ( $p = 0.226$ ).

The model for eumelanin:pheomelanin ratio explained 29% of the variance. The effects of food abundance and habitat type were not significant, but their interaction was marginally significant because of the same tendency observed for eumelanin content, i.e. a negative relationship between eumelanin:pheomelanin ratio and food abundance in coniferous woodland ( $\beta = -0.34$ ,  $p = 0.211$ ) and a positive relationship in mixed woodland ( $\beta = 0.25$ ,  $p = 0.199$ ), though none of the slopes were significantly different from zero. The effect of area was, however, strong, eumelanin:pheomelanin ratio decreasing from area 1 to area 3 (Fig. 3b). Pairwise comparisons showed significant differences between areas 1 and 3 ( $p < 0.001$ ) and between areas 2 and 3 ( $p = 0.019$ ), but not between areas 1 and 2 ( $p = 0.222$ ).

In the models for eu- and pheomelanin content and eumelanin:pheomelanin ratio the effect of woodland area proportion was not significant, and the same applied to the random effect of nest identity, so these terms were not retained in any model (Table 1).

On the other hand, the model for nestling body condition with the data set comprising information from 2005 to 2008 included nest identity ( $Z = 8.46$ ,  $p < 0.001$ ), sex (males:  $105.13 \pm 0.86\%$ , females:  $102.15 \pm 0.85\%$ ;  $F_{1,149} = 7.88$ ,  $p = 0.006$ ) and area ( $F_{2,149} = 9.38$ ,  $p < 0.001$ ), with body condition values increasing from area 1 to area 3 (Fig. 3c). Pairwise comparisons showed significant differences in nestling body condition between areas 1 and 3 ( $p < 0.001$ ) and between areas 1 and 2 ( $p = 0.004$ ), while differences between areas 2 and 3 were not significant ( $p = 0.753$ ).

## Discussion

The eumelanin content in the plumage of northern goshawk nestlings increased with food abundance in the

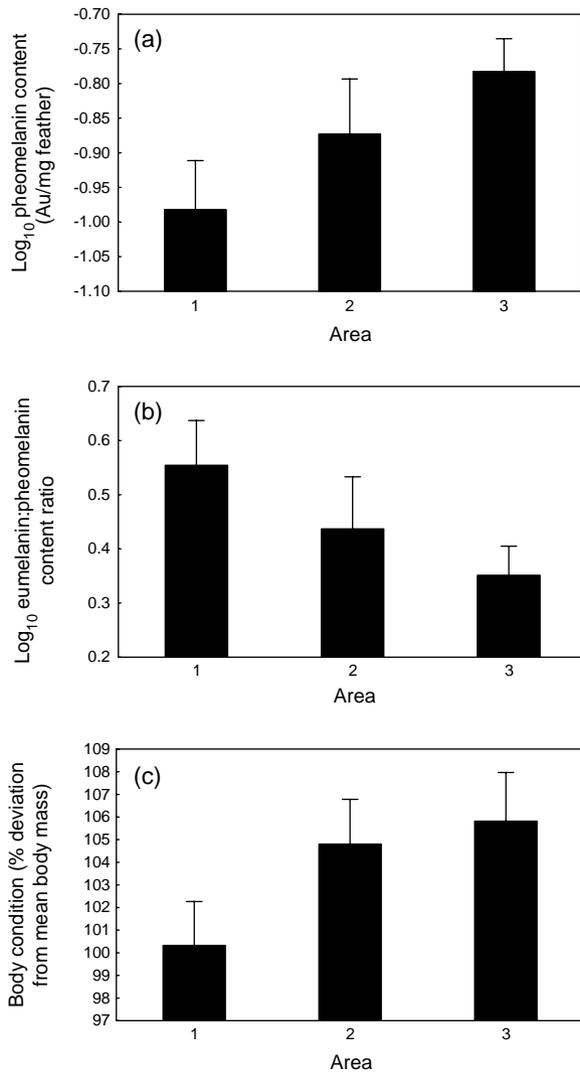


Figure 3. Pheomelanin content in feathers (a), eumelanin:pheomelanin ratio (b) and body condition (c) in northern goshawk nestlings reared in three different areas (subpopulations) of the Netherlands. Bars represent the values of least squares means obtained from the models performed for those variables +95% confidence interval.

territories where they had been reared, as predicted. This suggests that food abundance may be limiting the availability of amino acids precursors of melanins (i.e. phenylalanine and tyrosine) to growing goshawks. Other authors have previously shown a dependence of the development of melanic traits on diet composition in birds and mammals. In particular, availability of dietary amino acids seems to determine the melanin-based colour intensity of cat coat, human hair and plumage of different species of birds (reviewed by McGraw 2008). Perhaps the most detailed of those studies is that of Poston et al. (2005), who experimentally manipulated the diet content in phenylalanine and tyrosine of captive house sparrows *Passer domesticus* and found a decrease in brightness of the eumelanin bib patch in birds fed with diets poor in those amino acids. Our study also suggests an environmental limitation to plumage melanization related to food abundance or quality, and constitutes the first report that relates a detailed

quantification of melanins to habitat quality variables in a wild animal population.

The positive relationship between the eumelanin content of feathers and food abundance was only apparent, however, in nestlings reared in territories located in coniferous–deciduous mixed forests. This result was contrary to expectations, as we predicted that food abundance would be especially limited in low-quality habitats (homogeneous coniferous forests in our population; Rutz and Bijlsma 2006, Rutz et al. 2006b), as shown by Byholm and Kekkonen (2008) for fitness-related variables in a Finnish population of goshawks. In our case, the eumelanin content of goshawk feathers increased with food abundance in the preferred habitat (coniferous–deciduous mixed woodland) only, which may suggest that food quality is so low in homogeneous coniferous forests (van den Burg 2009) that any increase in its abundance does not correspond to an increase in the availability of melanin precursors. Indeed, it is possible that poor habitats for goshawks are poor habitats for their prey too (Strong and Sherry 2000, Bearhop et al. 2004), so that food abundance no longer reflects amino acid availability if this latter variable depends on the physical condition of individual prey and this is, in turn, bad in homogeneous coniferous forests. By contrast, the same effect was not found in our separate measurements of pheomelanin either.

Unexpected results were also obtained for the effect of habitat type per se, as we predicted higher melanin contents in nestlings from territories located in favourable habitats (coniferous–deciduous mixed woodland), while the highest eumelanin contents corresponded to nestlings from homogeneous coniferous forests (i.e. poor habitat). However, our predictions were made on the basis that the availability of melanin precursors should limit plumage melanization, but recent ideas that relate melanin production to oxidative stress should be also considered (Galván and Alonso-Alvarez 2008, 2009, Galván and Solano 2009). This model predicts that the production of eumelanin will be favoured under environmental conditions generating high oxidative stress (i.e. the imbalance between production of reactive oxygen species and availability of antioxidant compounds) because under those conditions GSH levels are forced to be low, while pheomelanin production will prevail under more favourable environments (i.e. when GSH and thus cysteine levels can be high; Galván and Solano 2009; Fig. 1). Poor rearing conditions can generate oxidative stress because of factors different from diet quality, for example as a consequence of physiological stress experienced in those areas, which increases corticosterone levels in birds (Suorsa et al. 2003) and can be thus accompanied by oxidative stress (Lin et al. 2004). Therefore, it seems that the effect of habitat on the production of eumelanin in goshawk nestlings may depend more on environmental oxidative stress than on availability of melanin precursors. Accordingly, a negative relationship between nestling body condition and eumelanin feather content was found inside subpopulations. By contrast, the production of pheomelanin did not depend on habitat type, which supports the view that eu- and pheomelanogenesis are independent processes, the former being favoured under

conditions generating high oxidative stress levels (Galván and Solano 2009). In fact, eumelanin and pheomelanin contents were not correlated in each feather sample.

There are several particular factors that may affect the levels of exogenous oxidative stress experienced by goshawks, and thus, their melanization levels. For example, diquat dibromide is a pro-oxidant substance commonly used as an aquatic herbicide in the Netherlands and other European countries (de Jong and de Snoo 2002), and has been shown to increase the expression of eumelanin traits and to decrease that of pheomelanin traits in the plumage of red-legged partridges *Alectoris rufa* through a negative effect on GSH levels (Galván and Alonso-Alvarez 2009). Similarly, exposure to heavy metals, which decreases GSH levels (Congiu et al. 2000), is known to increase the expression of eumelanin traits in great tits (Dauwe and Eens 2008). Therefore, differences in the use of diquat or in heavy metal pollution between goshawk territories may lead them to consume prey exposed to different levels of herbicides and heavy metals, which in turn, would create variability in the levels of diquat and metals to which goshawks are exposed and in their melanization levels. Another environmental factor, that affects the capacity to produce melanins through a decrease in GSH levels and a subsequent increase in oxidative stress, is thermal stress caused by temperatures that are limited or exceed the tolerance range of a particular species during a given time period (Ohtsuka et al. 1994). Thus, goshawk nestlings reared in territories differing in thermal characteristics would suffer from different levels of oxidative stress and would vary in their capacity to produce melanins. The same applies to the increase in melanization levels caused by oxidative stress generated by ultraviolet (UV) radiation (Tran et al. 2008) and a potential effect of habitat characteristics creating a variability in the levels of UV exposure by goshawks reared in different territories. Future studies should explore these possibilities.

It is interesting that the ratio eumelanin:pheomelanin followed a similar pattern as that observed in the pheomelanin content, i.e. increasing from area 1 to area 3. This means that the variability in the eumelanin:pheomelanin ratio was mainly driven by a variability in pheomelanin content. Eumelanin content in goshawk feathers was three times higher than the pheomelanin content, which indicates that pheomelanin may be of biological importance for birds even at low relative concentrations, when its colour in feathers is not apparently perceived as the darker colour conferred by eumelanin dominates (Toral et al. 2008). This also suggests that, as eu- and pheomelanogenesis are independent processes (Galván and Solano 2009), the production of eumelanin and pheomelanin may be differentially affected by environmental factors. For example, eumelanin may be more dependent upon availability of amino acid precursors than pheomelanin, which may be mainly affected by environmental oxidative stress. Perhaps in accordance with this, it has been experimentally shown that cats only develop eumelanin hair if fed with diets with relatively high content of phenylalanine and tyrosine, while pheomelanin hair is developed with lower contents of these amino acids (Anderson et al. 2002). Under this scenario, we should thus expect that, with equal contents of dietary amino acids, variability in stressful conditions create greater

variability in pheo- than in eumelanin content, so that low oxidative stress levels tilt the eumelanin:pheomelanin ratio towards pheomelanin and high oxidative stress levels toward eu- or pheomelanin depending on the particular availability of precursor amino acids (Anderson et al. 2002). As both genetic and environmental factors affect melanization levels (Galván and Alonso-Alvarez 2009), the magnitude of this potential variability would be limited by the genetic control determining which pigment prevails in the eumelanin:pheomelanin ratio. This opens new questions for future studies.

On the other hand, we found differences in the body condition of nestlings from three different areas (subpopulations) of our population, with values increasing from area 1 to area 3. As predicted on the basis that these differences in condition were probably due to differences in food abundance and/or quality, levels of pheomelanin also increased from area 1 to area 3.

Two main environmental agents seem to constrain melanin production in animals: availability of melanin precursors and environmental oxidative stress. Both factors may well be correlated, as low availability of precursors due to low-quality diets may increase oxidative stress levels (Monaghan et al. 2009). This fact may prevent us from disentangling which of these factors is actually limiting melanin production, by searching for associations between environmental variables and melanization levels, but predictions indeed differ between melanin types. Thus, for the availability of melanin precursors we should expect that a high availability is reflected in a higher production of both eumelanin and pheomelanin, as the amount of available phenylalanine and tyrosine affects the initial step of the biochemical pathway, which is common to eu- and pheomelanogenesis (Galván and Solano 2009). However, oxidative stress determines whether the pathway is directed towards eu- or pheomelanogenesis, and thus we should expect that high oxidative stress enhances eumelanin production, but inhibits pheomelanin production (Galván and Solano 2009). It is not likely that the variation in melanin content found here is due to genetic differentiation between our goshawk subpopulations, as raptors in general (Alcaide et al. 2009), and northern goshawks in particular, do not tend to present genetically structured populations (Takaki et al. 2009), especially if it is considered that the subpopulations studied here are separated by only 50–100 km. In any case, this could easily be determined by cross-fostering experiments (Bortolotti et al. 2000, Laaksonen et al. 2008).

In conclusion, the eumelanin content of nestling goshawk feathers is positively associated to food abundance and poor habitat type, and negatively associated to body condition within subpopulations, while the pheomelanin content is positively associated to body condition between subpopulations. Therefore, it seems that both environmental factors (i.e. melanin precursors availability and oxidative stress) affect the production of feather melanins. Future studies should disentangle the differential importance of those factors and investigate if a different investment in eumelanin and pheomelanin production is translated into differential effects on fitness.

*Acknowledgements* – The feathers from goshawk nestlings in Het Gooi were collected by Hanneke Sevink and her coworkers. IG benefited from a FPI grant from the Spanish Ministry of Science and Innovation (formerly Ministry of Education and Science). Vereniging Natuurmonumenten and State Forestry Service allowed RGB free access in their forests. JJN was supported by Research Project CGL2006-07481 of the Spanish Ministry of Science and Technology. Melanin concentration was determined at the Lab of Chemical Ecology at Estación Biológica de Doñana by Rosario Quintero. We thank J. Poston and an anonymous referee for useful comments on the manuscript.

## References

- Alcaide, M., Serrano, D., Tella, J. L. and Negro, J. J. 2009. Strong phyloptry derived from capture-recapture records does not lead to fine-scale genetic differentiation in lesser kestrels. – *J. Anim. Ecol.* 78: 468–475.
- Alonso-Alvarez, C. and Tella, J. L. 2001. Effects of experimental food restriction and body-mass changes on the avian T-cell-mediated immune response. – *Can. J. Zool.* 79: 101–105.
- Alonso-Alvarez, C., Bertrand, S., Faivre, B. and Sorci, G. 2007. Increased susceptibility to oxidative damage as a cost of accelerated somatic growth in zebra finches. – *Funct. Ecol.* 21: 873–879.
- Anderson, M. E. 1998. Glutathione: an overview of biosynthesis and modulation. – *Chem. Biol. Interact.* 111–112: 1–14.
- Anderson, P. J. B., Rogers, Q. R. and Morris, J. G. 2002. Cats require more dietary phenylalanine or tyrosine for melanin deposition in hair than for maximal growth. – *J. Nutr.* 132: 2037–2042.
- Bearhop, S., Hilton, G. M., Vorier, S. C. and Waldron, S. 2004. Stable isotope ratios indicate that body condition in migrating passerines is influenced by winter habitat. – *Proc. R. Soc. Lond. B* 271: S215–S218.
- Benathan, M., Virador, V., Furumura, M., Kobayashi, N., Panizzon, R. G. and Hearing, V. J. 1999. Co-regulation of melanin precursors and tyrosinase in human pigment cells: roles of cysteine and glutathione. – *Cell. Mol. Biol.* 45: 981–990.
- Bijlsma, R. G. 1997. Handleiding veldonderzoek Roofvogels – KNNV Uitgeverij, Utrecht.
- Bijlsma, R. G. 2009. Trends and breeding success of raptors in the Netherlands in 2008. – *De Takkeling* 17: 7–50, in Dutch, with English summary.
- Bortolotti, G. R., Tella, J. L., Forero, M. G., Dawson, R. D. and Negro, J. J. 2000. Genetics, local environment and health as factors influencing plasma carotenoids in wild American kestrels *Falco sparverius*. – *Proc. R. Soc. Lond. B* 267: 1433–1438.
- Bradbury, R. B. and Blakey, J. K. 1998. Diet, maternal condition and offspring sex ratio in the zebra finch, *Poephila guttata*. – *Proc. R. Soc. Lond. B* 265: 895–899.
- Bush, W. and Simon, J. D. 2007. Quantification of Ca<sup>2+</sup> binding to melanin supports the hypothesis that melanosomes serve a functional role in regulating calcium homeostasis. – *Pigment Cell Res.* 20: 134–139.
- Byholm, P. and Kekkonen, M. 2008. Food regulates reproduction differently in different habitats: experimental evidence in the goshawk. – *Ecology* 89: 1696–1702.
- Cho, E. S., Sahyoun, N. and Stegink, L. D. 1981. Tissue glutathione as a cyste(e)ine reservoir during fasting and refeeding of rats. – *J. Nutr.* 111: 914–922.
- Congiu, L., Chicca, M., Pilastro, A., Turchetto, M. and Tallandini, L. 2000. Effects of chronic dietary cadmium on hepatic glutathione levels and glutathione peroxidase activity in starlings *Sturnus vulgaris*. – *Arch. Environ. Con. Tox.* 38: 357–361.
- Costantini, D., Casagrande, S., de Filippis, S., Brambilla, G., Fanfani, A., Tagliavini, J. and dell’Omo, G. 2006. Correlates of oxidative stress in wild kestrel nestlings *Falco tinnunculus*. – *J. Comp. Physiol. B* 176: 329–337.
- Dauwe, T. and Eens, M. 2008. Melanin- and carotenoid-dependent signals of great tits *Parus major* relate differently to metal pollution. – *Naturwissenschaften* 95: 969–973.
- de Jong, F. M. W. and de Snoo, G. R. 2002. A comparison of the environmental impact of pesticide use in integrated and conventional potato cultivation in the Netherlands. – *Agric. Ecosyst. Environ.* 91: 5–13.
- del Val, E., Senar, J. C., Garrido-Fernández, J., Jarén, M., Borràs, A., Cabrera, J. and Negro, J. J. 2009. The liver but not the skin is the site for conversion of a red carotenoid in a passerine bird. – *Naturwissenschaften* 96: 797–801.
- Ferguson-Lees, J. and Christie, D. A. 2001. Raptors of the world. – Helm.
- Galván, I. and Alonso-Alvarez, C. 2008. An intracellular anti-oxidant determines the expression of a melanin-based signal in a bird. – *PLoS ONE* 3: e3335.
- Galván, I. and Alonso-Alvarez, C. 2009. The expression of melanin-based plumage is separately modulated by exogenous oxidative stress and a melanocortin. – *Proc. R. Soc. Lond. B* 276: 3089–3097.
- Galván, I. and Solano, F. 2009. The evolution of eu- and pheomelanin traits may respond to an economy of pigments related to environmental oxidative stress. – *Pigment Cell Melanoma Res.* 22: 339–342.
- Griffith, S. C., Parker, T. H. and Olson, V. A. 2006. Melanin-versus carotenoid-based sexual signals: is the difference really so black and red? – *Anim. Behav.* 71: 749–763.
- Hasson, O. 1997. Towards a general theory of biological signaling. – *J. Theor. Biol.* 185: 139–156.
- Joshi, P. G., Nair, N., Begum, G., Joshi, N. B., Sinkar, V. P. and Vora, S. 2007. Melanocyte-keratinocyte interaction induces calcium signalling and melanin transfer to keratinocytes. – *Pigment Cell Res.* 20: 380–384.
- Kenward, R. E. 2006. The goshawk. – Poyser.
- Laaksonen, T., Negro, J. J., Lyytinen, S., Valkama, J., Ots, I. and Korpimäki, E. 2008. Effects of experimental brood size manipulation and gender on carotenoid levels of Eurasian kestrels *Falco tinnunculus*. – *PLoS ONE* 3: e2374.
- Lautier, D., Luscher, P. and Tyrrell, R. M. 1992. Endogenous glutathione levels modulate both constitutive and UVA radiation/hydrogen peroxide inducible expression of the human heme oxygenase gene. – *Carcinogenesis* 13: 227–232.
- Lin, H., Decuypere, E. and Buyse, J. 2004. Oxidative stress induced by corticosterone administration in broiler chickens *Gallus gallus domesticus*: 2. Short-term effect. – *Comp. Biochem. Physiol. B* 139: 745–751.
- Littell, R. C., Milliken, G. A., Stroup, W. W. and Wolfinger, R. D. 2006. SAS for mixed models. – SAS Institute, Cary, CA, USA.
- McGraw, K. J. 2007. Dietary mineral content influences the expression of melanin-based ornamental coloration. – *Behav. Ecol.* 18: 137–142.
- McGraw, K. J. 2008. An update of the honesty of melanin-based color signals in birds. – *Pigment Cell Melanoma Res.* 21: 133–138.
- Monaghan, P., Metcalfe, N. B. and Torres, R. 2009. Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. – *Ecol. Lett.* 12: 75–92.
- Negro, J. J. 2008. Two aberrant serpent-eagles may be visual mimics of bird-eating raptors. – *Ibis* 150: 307–314.
- Negro, J. J., Bortolotti, G. R., Mateo, R. and Garcia, I. M. 2009. Porphyrins and pheomelanins contribute to the reddish

- juvenile plumage of black-shouldered kites. – *Comp. Biochem. Physiol. B* 153: 296–299.
- Ohtsuka, Y., Yabunaka, N., Fujisawa, H., Watanabe, I. and Agishi, Y. 1994. Effect of thermal-stress on glutathione metabolism in human erythrocytes. – *J. Appl. Physiol.* 68: 87–91.
- Ozeki, H., Ito, S., Wakamatsu, K. and Ishiguro, I. 1997. Chemical characterization of pheomelanogenesis starting from dihydroxyphenylalanine or tyrosine and cysteine. Effects of tyrosinase and cysteine concentrations and reaction time. – *Biochem. Biophys. Acta* 1336: 539–548.
- Poston, J. P., Hasselquist, D., Stewart, I. R. K. and Westneat, D. F. 2005. Dietary amino acids influence plumage traits and immune responses of male house sparrows, *Passer domesticus*, but not as expected. – *Anim. Behav.* 70: 1171–1181.
- Prota, G. 1992. Melanins and melanogenesis. – Academic Press.
- Robbins, C. T., Felicetti, L. A. and Sponheimer, M. 2005. The effect of dietary protein quality on nitrogen isotope discrimination in mammals and birds. – *Oecologia* 144: 534–540.
- Romero, L. M. 2004. Physiological stress in ecology: lessons from biomedical research. – *Trends Ecol. Evol.* 19: 249–255.
- Rutz, C. 2005. The northern goshawk: population dynamics and behavioural ecology. PhD thesis. – Univ. of Oxford.
- Rutz, C. and Bijlsma, R. G. 2006. Food-limitation in a generalist predator. – *Proc. R. Soc. Lond. B* 273: 2069–2076.
- Rutz, C., Whittingham, M. J. and Newton, I. 2006a. Age-dependent diet choice in an avian top predator. – *Proc. R. Soc. Lond. B* 273: 579–586.
- Rutz, C., Bijlsma, R. G., Marquiss, M. and Kenward, R. E. 2006b. Population limitation in the northern goshawk in Europe: a review with case studies. – *Stud. Avian Biol.* 31: 158–197.
- Sewalk, C. J., Brewer, G. L. and Hoffman, D. J. 2001. Effects of diquat, an aquatic herbicide, on the development of mallard embryos. – *J. Toxicol. Env. Health A* 62: 33–45.
- Solano, F., Briganti, S., Picardo, M. and Ghanem, G. 2006. Hypopigmenting agents: an updated review on biological, chemical and clinical aspects. – *Pigment Cell Res.* 19: 550–571.
- Soltaninassab, S. R., Sekhar, K. R., Meredith, M. J. and Freeman, M. L. 2000. Multi-faceted regulation of  $\gamma$ -glutamylcysteine synthetase. – *J. Cell Physiol.* 182: 163–170.
- SOVON Vogelonderzoek Nederland. 2002. Atlas van de Nederlandse Broedvogels 1998–2000. Nederlandse fauna 5. – Nationaal Natuurhistorisch Museum Naturalis, KNNV Uitgeverij and European Invertebrate Survey-Nederland, Leiden.
- Stoehr, A. M. 2006. Costly melanin ornaments: the importance of taxon? – *Funct. Ecol.* 20: 276–281.
- Strong, A. M. and Sherry, T. W. 2000. Habitat-specific effects of food abundance on the condition of ovenbirds wintering in Jamaica. – *J. Anim. Ecol.* 69: 883–895.
- Suorsa, P., Huhta, E., Nikula, A., Nikinmaa, M., Jäntti, A., Helle, H. and Hakkarainen, H. 2003. Forest management is associated with physiological stress in an old-growth forest passerine. – *Proc. R. Soc. Lond. B* 270: 963–969.
- Takaki, Y., Kawahara, T., Kitamura, H., Endo, K.-I. and Kudo, T. 2009. Genetic diversity and genetic structure of northern goshawk *Accipiter gentilis* populations in eastern Japan and central Asia. – *Conserv. Genet.* 10: 269–279.
- Toral, G. M., Figuerola, J. and Negro, J. J. 2008. Multiple ways to become red: pigment identification in red feathers using spectrometry. – *Comp. Biochem. Physiol. B* 150: 147–152.
- Tran, T. N., Schulman, J. and Fisher, D. E. 2008. UV and pigmentation: molecular mechanisms and social controversies. – *Pigment Cell Melanoma Res.* 21: 509–516.
- van den Burg, A. B. 2009. Limitations of owl reproduction in the wild: is there a role for food quality besides quantity? – *Ardea* 97: 609–614.
- van Dijk, A. J. and Bijlsma, R. G. 2006. Long-term changes in the breeding bird fauna of Wapserveld-Berkenheuvel. – *Drentse Vogels* 20: 1–25, in Dutch, with English summary.
- Wu, G., Fang, Y. Z., Yang, S., Lupton, J. R. and Turner, N. D. 2004. Glutathione metabolism and its implications for health. – *J. Nutr.* 134: 489–492.