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PHYSIOLOGICAL ECOLOGY – ORIGINAL RESEARCH

Gyrfalcons *Falco rusticolus* adjust *CTNS* expression to food abundance: a possible contribution to cysteine homeostasis

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Abstract Melanins form the basis of animal pigmentation. When the sulphurated form of melanin, termed pheomelanin, is synthesized, the sulfhydryl group of cysteine is incorporated to the pigment structure. This may constrain physiological performance because it consumes the most important intracellular antioxidant (i.e., glutathione, GSH), of which cysteine is a constitutive amino acid. However, this may also help avoid excess cysteine, which is toxic. Pheomelanin synthesis is regulated by several genes, some of them exerting this regulation by controlling the transport of cysteine in melanocytes. We investigated the possibility that these genes are epigenetically labile regarding protein intake and thus contribute to cysteine homeostasis. We found in the Icelandic population of gyrfalcon *Falco rusticolus*, a species that pigments its plumage with pheomelanin, that the expression of a gene regulating the export of cystine out of melanosomes (*CTNS*) in feather melanocytes of developing nestlings increases with food abundance in the breeding territories where they were reared. The expression of other genes regulating pheomelanin synthesis by different mechanisms of influence on cysteine availability (*Slc7a11* and *Slc45a2*) or by other processes (*MC1R* and *AGRP*) was not affected by food abundance. As the gyrfalcon is a strict carnivore and variation in food abundance mainly reflects variation in protein intake, we suggest that epigenetic lability in *CTNS* has evolved in some species because of its potential benefits contributing to cysteine homeostasis. Potential applications of our results should now be investigated in the context of renal failure and other disorders associated with cystinosis caused by *CTNS* dysfunction.

Keywords *CTNS* · Cysteine homeostasis · Food abundance · Gyrfalcon · Pheomelanin

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Introduction

Vertebrate pigmentation mainly depends on several genes that regulate the synthesis, structure and transport of melanins in melanocytes (Hubbard et al. 2010; Hearing 2011). During the synthesis of the sulphurated form of melanin (i.e., pheomelanin), which is responsible for orange-reddish animal coloration, the sulfhydryl group of the amino acid cysteine is incorporated to the pigment structure (García-Borrón and Olivares Sánchez 2011). As pheomelanin is then transferred to inert integumentary structures of the skin, feathers or hairs, cysteine used for pheomelanin synthesis is no longer available as an antioxidant as part of the tripeptide glutathione (GSH). As a consequence, pheomelanin synthesis represents a consumption of the most important intracellular antioxidant (Panzella et al. 2014). This consumption of cysteine/GSH contributes to some disorders, including melanoma and malignant glioma in humans (Panzella et al. 2014; Robert et al. 2015; Wang et al. 2016) and constraints to physiological performance in animals exposed

to environmental sources of oxidative stress (Karell et al. 2011; Galván et al. 2011, 2014). However, cysteine is toxic if in excess; thus, its consumption during pheomelanogenesis may also be advantageous under environmental conditions leading to excess cysteine, which may indeed explain the evolution of pheomelanin-based pigmentation (Galván et al. 2012).

The expression of genes regulating pheomelanin synthesis should, therefore, be a key in determining the double-sided nature of pheomelanin-based pigmentation, in interaction with environmental factors. Some of us have recently found that zebra finches *Taeniopygia guttata* developing feathers pigmented by pheomelanin downregulate the expression of the gene *Slc7a11* (solute carrier family 7 member 11), which codes for the plasma membrane cystine/glutamate exchanger xCT that transports cystine (the cysteine dimer) to melanocytes to produce pheomelanin (Chintala et al. 2005), under exposure to a pro-oxidant substance (Galván et al. 2017). As this downregulation of *Slc7a11* avoided cellular oxidative stress exerted by the pro-oxidant, it means that some species have evolved an epigenetic lability in *Slc7a11* that reduces cysteine consumption for pheomelanin production under stressful environmental conditions, when cysteine/GSH are most required to combat oxidative stress (Galván et al. 2017). Similar to this influence of environmental oxidative stress on gene expression, epigenetic permeability of genes regulating pheomelanin synthesis to environmental conditions that produce excess cysteine should also be physiologically advantageous. However, this possibility has never been explored under natural variations in dietary cysteine levels (i.e., food abundance) as a potential adaptive strategy.

Cysteine is a semi-essential amino acid; thus, diet is an important source of cysteine for animals (Klasing 1998; Stipanuk et al. 2006). Excess cysteine can occur when its content in diet is higher than needed for protein synthesis (Stipanuk et al. 2006). Under such conditions, the toxicity of excess cysteine is produced by its autoxidation to cystine, which generates hydrogen peroxide, hence decreasing GSH levels (Viña et al. 1983) and causing oxidative DNA damage (Park and Imlay 2003). This causes several diseases in mammals and metabolic acidosis and disorders such as thinning of egg shells and poor growth in birds (Klasing 1998). However, cysteine is essential for protein structure, hence the importance of maintaining cysteine homeostasis. The enzyme cysteine dioxygenase (CDO) catalyzes the addition of molecular oxygen to the sulfhydryl group of cysteine to form less toxic products such as sulfate and taurine (Stipanuk et al. 2006). Nevertheless, the capacity of CDO to maintain cysteine homeostasis is probably limited, as derived from the fact that cystinosis, a disease characterized by the intralysosomal accumulation of cystine, is only explained by dysfunction in the cystine/H^+ symporter (cystinosin) that exports cystine out of lysosomes (Chiaverini et al. 2012). Pheomelanin synthesis may also affect, together with CDO, cysteine homeostasis (Galván et al. 2012). In fact, cystinosin and pheomelanin are intrinsically associated, as the gene encoding cystinosin (*CTNS*) is expressed in the organelles where melanin synthesis takes place in melanocytes (i.e., melanosomes) and, thus, inhibits pheomelanogenesis by promoting the efflux of cystine (Chiaverini et al. 2012).

Potential epigenetic mechanisms effective in avoiding excess cysteine may, therefore, be related to changes in the expression of *Slc7a11* and *CTNS*. Here we test these possibilities in a wild population of gyrfalcons *Falco rusticolus* exposed to different food regimes in Iceland, as these birds synthesize pheomelanin to pigment the feathers of their first plumage (Galván and Jorge 2015). As the gyrfalcon is a strict carnivore, variation in the food abundance to which nestlings are exposed during growth mainly reflects variation in protein availability in the diet (Klasing 1998). We also considered the gene *Slc45a2* (solute carrier family 45 member 2), which has also been suggested to affect pheomelanin synthesis by transporting cysteine into melanosomes (Xu et al. 2013). Lastly, we considered the melanocortin-1 receptor (*MC1R*) and agouti-related protein (*AGRP*) genes, which affect pheomelanin synthesis without influencing cysteine/GSH availability (Nadeau et al. 2008). We, thus, predicted that if epigenetic sensitivity to protein availability has evolved in *Slc7a11* and *CTNS* (and possibly *Slc45a2*) as an adaptive mechanism to avoid excess cysteine, the expression of these genes should be adjusted to food abundance while *MC1R* and *AGRP* should not be affected.

Methods

Field methods

The study was conducted during two consecutive breeding seasons (June 2015 and 2016) in 16 gyrfalcon territories in Iceland. Nests were entered when the nestlings were 16–35 days old. Nestlings were ringed, weighed and the following morphometrics taken: flat chord of the wing, culmen and length of central tail feathers. Sexing was based on morphometrics. Ageing was based on length of central tail feather (Ó.K. Nielsen, unpublished data). Body condition was calculated as the residuals of body mass regressed against wing chord, culmen length and age. The study comprised a total of 47 gyrfalcon nestlings (Online Resource 1).

One or two growing body feathers were plucked from the back of nestling gyrfalcons and immersed in RNAlater solution (Ambion, Thermo Fisher Scientific, Waltham, MA, USA) to stabilize and protect RNA, and stored at −20 °C until the analyses. These feathers are dark brown with rusty-yellowish patches (Fig. 1). The yellowish coloration

Fig. 1 Gyrfalcon nestlings in a nest surrounded by prey remains in Iceland (credit: Daniel Bergmann). Insert photograph shows details of the melanin-based pigment pattern of tail feathers

is produced by pheomelanin, while the brown coloration is produced by the non-sulphurated form of melanin (i.e., eumelanin; Galván and Jorge 2015).

Estimation of food abundance

Two or three collecting trips were made to each nest during the season, the last after the young had fledged. During each visit all remains were collected, including all bones and pellets, and also some feathers. For the analysis, remains were arranged according to species, age groups and type, from which we estimated the number and biomass of prey [see Nielsen (2003) for details]. Number and biomass of prey were thus considered as two indices of food abundance to which nestling gyrfalcons were exposed during growth.

Gene expression in melanocytes

During feather development, melanocytes at the dermal papillae show intense melanogenesis (Lin et al. 2013). Therefore, the bottommost portion of the feather follicles corresponding to the melanin unit, which represents an important reservoir of melanocytes like in hair follicles (Mohanty et al. 2011), was isolated. RNA obtained from these samples, therefore, corresponds to melanocytes to a large extent. All follicular melanin units obtained from each gyrfalcon nestling were pooled. Total RNA was extracted using TRI Reagent (Ambion), and residual genomic DNA carry over was removed using the TURBO DNA-free kit (Ambion). Complementary DNA (cDNA) was prepared from total RNA using RevertAid Reverse Transcriptase provided in the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, Thermo Fisher Scientific). qPCR was thus performed on cDNA for the genes *CTNS*, *Slc7a11*, *Slc45a2*, *MC1R* and *AGRP*. Reactions were performed using SYBR Green I Master in a LightCycler 480 System (Roche, Basel, Switzerland). The housekeeping glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used for normalization of cDNA samples. Cycle threshold (C_t) levels were used as a measure of gene expression, with lower C_t values indicating higher mRNA levels and higher gene expression levels. Two replicates were obtained for each sample, and the mean value was used in the statistical analyses. We did not obtain any amplification of genes from eight nestlings in the case of *CTNS*, from 6 nestlings in the case of *Slc7a11*, from seven nestlings in the cases of *Slc45a2*, *MC1R* and *GAPDH*, and from ten nestlings in the case of *AGRP* (Online Resource 1).

Gene primers were designed based on *Falco cherrug* refseq sequences (GenBank) using the Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The oligonucleotide primer sets used were as follows: 5′-GCT GGGGAATTGCTGCTTT-3′ and 5′-TGCAAC GAAGAA CATCCTGG-3′ for *Slc7a11*; 5′-CATGCCATCCCACTA TCGCT-3′ and 5′-GAGGCGCATAAGGACTACCC-3′ for *Slc45a2*; 5′-GGGAGAAGCCGTGAGCTTT-3′ and 5′-CAG TGAGTCCATCACCAGGC-3′ for *CTNS*; 5′-CTCCTTCCT GGGGGTCATC-3′ and 5′-TGATGAAGATGGTGCTGG

AGA-3′ for *MC1R*; 5′-GAAGCCAACGGTGACCTCAT-3′ and 5′-AAGGCATTGAAGAAGCGGCA-3′ for *AGRP*; and 5′-GGACCAGGTTGTTTCCTGTG-3′ and 5′-TCCTTGGATGCCATGTGGA-3′ for *GAPDH*.

Statistical analyses

We tested if gene expression levels (C_t) changed with food abundance using linear mixed-effect models (LMM) fit with restricted maximum likelihood (REML) estimation in R environment (R Core Team 2017). As the two indices of food abundance (i.e., number and biomass of prey) were strongly correlated ($r = 0.98$, $N = 19$, $P < 0.0001$), we conducted separate models for these variables. LMM's thus included number or biomass of prey, in addition to sex and body condition, as fixed terms. Territory identity was added as a random factor to account for the common origin of gyrfalcon nestlings belonging to the same nests. Different models were fit for each gene. Effect sizes (b) were calculated with the library *lme4*, and P values were calculated from the analysis of deviance of the models using the library *car*. All continuous variables were $\log_{10}$-transformed prior to analyses to achieve normality assumptions.

Results

The effects of sex and body condition were not significant in explaining variation in the expression of any gene (sex: $0.09 < \chi^2_1 < 1.16$, $0.282 < P < 0.763$; condition: $0.00 < \chi^2_1 < 1.61$, $0.205 < P < 0.998$), and were thus removed from the models. There was a strong significant and negative effect of the number of prey on C_t for the gene *CTNS* ($b = -0.11$, $\chi^2_1 = 9.42$, $P = 0.002$), and a similar effect of the biomass of prey ($b = -0.11$, $\chi^2_1 = 8.38$, $P = 0.004$). This means that *CTNS* expression increased with the number of prey and with the biomass of prey available to gyrfalcon nestlings (Fig. 2). These significant effects did not change if sex and condition were kept in the models. In contrast, no index of food abundance was significant in the models for the other genes (*Slc7a11*: $0.48 < \chi^2_1 < 1.11$, $0.292 < P < 0.489$; *Slc45a2*: $0.39 < \chi^2_1 < 0.80$, $0.372 < P < 0.531$; *MC1R*: $0.01 < \chi^2_1 < 0.35$, $0.553 < P < 0.914$; *AGRP*: $2 \times 10^{-4} < \chi^2_1 < 0.01$; $0.942 < P < 0.990$).

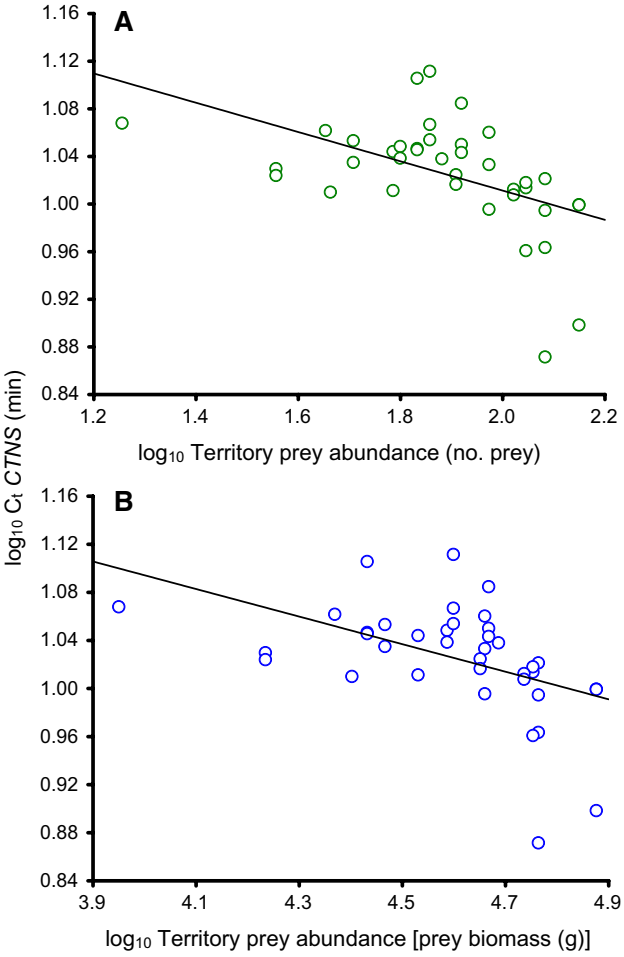

Fig. 2 Relationship between *CTNS* expression in feather melanocytes of gyrfalcon nestlings and two estimates of food abundance in the territories where they were reared: number of prey (**a**) and biomass of prey (**b**). Lower C_t values indicate higher gene expression levels. The lines are the best-fit lines

Discussion

Although modulation of *CTNS* expression in response to intracellular cysteine levels has been shown in vitro in human kidney cells (Bellomo et al. 2010), our findings represent the first evidence of epigenetic lability in the gene *CTNS* as response to a natural variation in a dietary source of cysteine. Diet constitutes an important source of cysteine, and consequently elicits the modulation of the enzyme CDO to maintain cysteine homeostasis (Stipanuk et al. 2009). Protein intake, therefore, affects overall cysteine levels in the organism (Stipanuk et al. 2009), especially in strict carnivores like the gyrfalcon. *CTNS* expression was highest in gyrfalcon nestlings reared in territories with highest food abundance, meaning that this gene is epigenetically labile in gyrfalcons. We thus suggest that epigenetic sensitivity to protein intake in *CTNS* may contribute to cysteine homeostasis, because a high expression of this gene avoids cystine accumulation in melanosomes (Chiaverini et al. 2012).

CTNS affects pheomelanin synthesis, whose induction may represent a mechanism to remove excess cysteine (Galván et al. 2012). However, *CTNS* only affects pheomelanogenesis under high systemic cysteine levels in the organism as derived from a comparison between levels of cystine

in mouse blood (50 µM) and in B16 cell culture medium (380 µM), suggesting the existence of a threshold cysteine/cystine concentration below which cystinosin activity and pigmentation are unaffected (Chiaverini et al. 2012). While the threshold value will have to be investigated in birds, this may explain why *CTNS* expression increased and not decreased with food abundance in gyrfalcons. The possible physiological benefits derived from the epigenetic lability of *CTNS* may, therefore, be related to cysteine efflux instead of pheomelanin synthesis. This may, thus, contribute to cysteine homeostasis without affecting pigmentation. In fact, although we could not characterize the plumage pigmentation of nestling gyrfalcons, it is known that a single plumage color variant that is explained by certain *MC1R* alleles exists in the Icelandic population of this species (Johnson et al. 2012). In other words, Icelandic gyrfalcons show variation in the expression of *CTNS* in feather melanocytes according to food abundance in the breeding territories, but little variation in plumage pigmentation.

The expression of other genes that regulate pheomelanogenesis by influencing cysteine availability (*Slc7a11* and *Slc45a2*) was not affected by food abundance. Interestingly, however, *Slc7a11* can be downregulated under environmental conditions that generate oxidative stress (Conrad and Sato 2012; Galván et al. 2017). Therefore, epigenetic lability in *Slc7a11* and *CTNS* may have evolved because of the physiological benefits of modulating their expression according to the double-sided nature of pheomelanin, which depends on environmental conditions (Galván et al. 2012; Fig. 3). Thus, conditions generating oxidative stress may downregulate *Slc7a11* expression, which avoids cysteine transport to melanocytes, while conditions generating excess cysteine may upregulate *CTNS* expression, which avoids cysteine accumulation in melanosomes. Given the intrinsic association between these processes and pheomelanin synthesis, the pigmentation phenotype of animals may also be affected in some cases (Galván et al. 2017), and either oxidative stress or excess cysteine derived from protein intake would lead to an inhibition of the expression of pheomelanin-based pigmentation (Fig. 3). As melanin-based pigmentation contributes to several adaptive benefits, ranging from protection against mutagenic ultraviolet (UV) radiation to visual communication (Galván and Solano 2016), it is expected that the evolution of pigmentation phenotypes closely interacts with the physiological need of animals for modulating *Slc7a11* and *CTNS* expression in response to environmental factors.

However, it is not expected that all species have evolved the capacity to adjust *CTNS* expression to dietary influences. In this regard, *CTNS* dysfunction causes cystinosis in humans and other animals, an autosomal recessive disease that has renal failure as the most severe symptom (Chiaverini et al. 2012). A specific gene therapy has proved successful at decreasing cystine levels in *CTNS*-deficient cells in

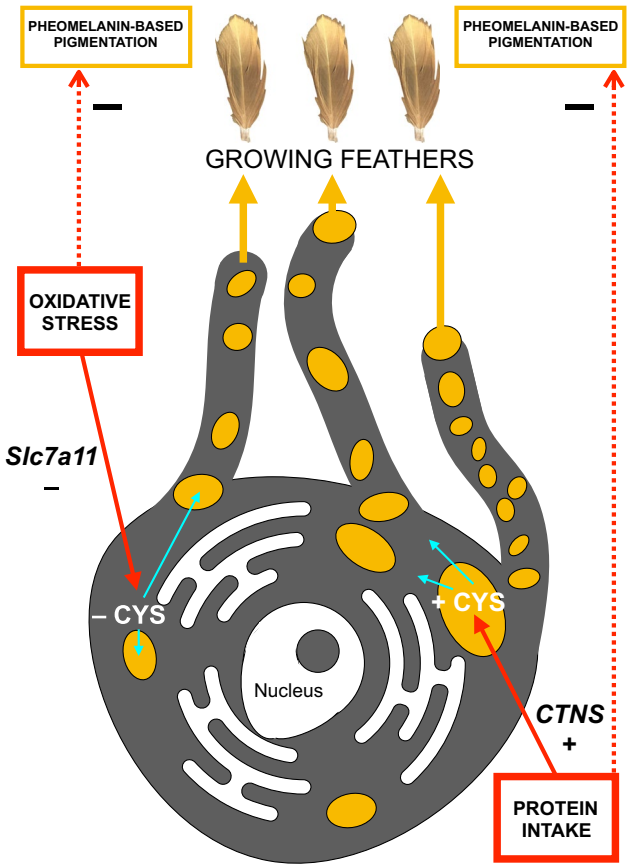

Fig. 3 Schematic representation of a melanocyte with potentially adaptive epigenetic lability in response to two environmental factors. On one hand, protein intake can increase systemic levels of cysteine, thus potentially increasing its levels inside melanocytes. The gene *CTNS* can be upregulated in response to this, pumping cysteine out of melanosomes (*yellow* granules) and thus avoiding its accumulation. As pheomelanin synthesis takes place inside pheomelanosomes, *CTNS* upregulation can also inhibit pheomelanin-based pigmentation in skin and associated structures such as feathers and hairs, although this inhibition only occurs above a threshold of systemic cysteine concentration. On the other hand, oxidative stress produced by environmental factors can induce the downregulation of the gene *Slc7a11*, thus decreasing the transport of cysteine within melanocytes and the availability of this amino acid to pheomelanosomes, again inhibiting the expression of pheomelanin-based pigmentation (color figure online)

mice (Harrison et al. 2013). This response would possibly be enhanced by an additional inducement of *CTNS* expression, which may be achieved by a mechanism similar to that allowing gyrfalcons to increase *CTNS* expression in response to food abundance. Although to our knowledge, no study has addressed the possibility that exposure to solar (UV) radiation affects the expression of pigmentation genes in birds, this is known to occur in humans regarding the *MC1R* gene (Scott et al. 2002); thus, the potential influence of UV exposure on *CTNS* should be considered together with diet. Future studies should investigate this potential applicability

of *CTNS* epigenetic lability to avoid the physiological consequences of cystinosis in animals including humans.

It could also be suggested that the potential lack of dietary influence on *CTNS* expression in humans may represent a physiological cost for those producing large amounts of pheomelanin (i.e., associated with low *CTNS* expression; Chiaverini et al. 2012) such as humans with the red hair phenotype (Ito et al. 2011), thus contributing to some damaging consequences associated with this phenotype like a higher resistance to analgesics and an increased tendency to bruising (Liem et al. 2006). Some evidence suggests that Neanderthal hominids had a red hair phenotype (Lalueza-Fox et al. 2007), and it is known that their diet was heavily based on meat in at least some populations (Weyrich et al. 2017), thus allowing to speculate that a low *CTNS* expression with no epigenetic lability may have contributed to their extinction. Our study should stimulate research on the new field on the interaction between epigenetic lability and pigmentation phenotype in a variety of study areas.

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Author contribution statement IG conceived and designed this study, conducted laboratory work, analyzed the data and wrote the manuscript. ÂI designed gene primers and participated in laboratory work. ÓKN conducted fieldwork.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Statement of animal rights All applicable institutional and/or national guidelines for the care and use of animals were followed.

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