

Environmental stress affects the expression of a carotenoid-based sexual trait in male zebra finches

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Summary

Abiotic factors including thermal stress are suggested to exert constraints on sexual ornaments through trade-offs between sexual displays and physiological functions related to self-maintenance. Given the health properties of carotenoid pigments, carotenoid-based ornaments offer a relevant context in which to investigate the effect of environmental stress, such as ambient temperature, on the production and maintenance of secondary sexual traits and, also, to explore the proximate mechanisms shaping their expression. In this study, we exposed male zebra finches (*Taeniopygia guttata*) to environmental stress by exposing them to two temperature regimes (6 and 26°C) over a 4 week period. Simultaneously, half of the males in each temperature group were supplemented with carotenoids, whereas the other half were not. The expression of a carotenoid-based sexual trait (bill colour) and the amount of circulating carotenoids were assessed before and at the end of the experiment. Carotenoid-supplemented males developed a redder bill, but the effect

of supplementation was reduced under cold exposure. However, we found evidence that birds facing a cold stress were carotenoid limited, since supplemented males developed redder bills than the non-supplemented ones. Interestingly, while cold-exposed and non-supplemented males developed duller bills, they circulated a higher amount of carotenoids at the end of the experiment compared to the pre-experimental values. Together, these results suggest that ambient temperature might contribute to the modulation of the expression of carotenoid-based ornaments. Our findings suggest that carotenoids are a limiting resource under cold exposure and that they might be prioritized for self-maintenance at the expense of the ornament. The physiological functions related to self-maintenance that might have benefited from carotenoid saving are discussed.

Key words: carotenoids, environmental stress, self-maintenance, sexual traits, trade-offs, *Taeniopygia guttata*.

Introduction

Males are assumed to signal their high phenotypic and/or genetic value through exaggerated sexual ornaments or vigorous courtship behaviour. As the cost of production and maintenance increases with the intensity of the signal (Parsons, 1995), it has been suggested that only high quality males could afford to allocate resources to produce and maintain extravagant traits. This idea is central to the handicap hypothesis of sexual selection, and mechanisms ensuring the honesty of sexually selected traits have been extensively investigated (reviewed in Ligon, 1999). Interestingly, classic examples of sexually selected traits involve bird ornaments that are expressed or exaggerated during the mating/breeding season only. The loss of breeding plumage in male ducks (Andersson, 1994) and the loss of orange bill colouration in American goldfinch (McGraw,

2004) after the breeding season are among well known examples in the class Aves. This widespread phenomenon reveals the phenotypic plasticity of sexual traits and emphasizes the role of forces other than sexual selection in their expression (Wiens, 2001). For instance, it has been suggested that the environment might exert substantial selective pressure on sexual traits, acting upon the balance between costs and benefits associated with their expression (reviewed in Wiens, 2001). In particular, when animals restrict sexual displays to the mating/breeding season only, it is thought that benefits associated with mate attraction or competitor eviction during this season would be outweighed by costs from environmental factors during other times of the year (Andersson, 1994) (but see McGraw, 2004). Resource availability, predation risk and habitat characteristics acting upon a signal are usually proposed

as important environmental factors shaping the expression of sexual traits (Andersson, 1994; Wiens, 2001). Interestingly, besides these effects, ambient temperature was also recently shown to be a proximate factor significantly affecting sexual traits in males (West and Packer, 2002).

Among plausible hypotheses to explain how ambient temperature may affect the expression of an ornament, it has been suggested that a thermal stress might constrain ornamental displays through trade-offs between sexual displays and physiological functions related to self-maintenance and survival (West and Packer, 2002). In homeothermic organisms, the loss of sexual ornaments at a time when individuals have to fuel thermoregulatory functions might suggest that the expression of sexual traits is modulated by ambient temperature through resource-based trade-offs. In support of this, experimentally induced food deprivation was shown to decrease the expression of coloured sexual ornaments in male House finches (Hill, 2000). However, while these results would intuitively suggest that the crucial limiting resource could be energy (Hill, 2000), other key resources in thermal-stressed individuals might also serve as the basis for allocation trade-offs between sexual signalling and physiological functions related to self-maintenance.

Because, in some species, carotenoid pigments are shared between different physiological functions and colourations on a daily basis, carotenoid-based signals offer a relevant context for investigating the effect of environmental stress, such as ambient temperature, on the production and maintenance of coloured secondary sexual traits, and for exploring the proximate mechanisms shaping their expression. Carotenoids are pigments that are widely used in animals to colour ornamental features yellow, orange or red. Carotenoid-based sexual traits are usually more elaborated in males and several studies have found that females mating with more coloured males experienced direct and/or indirect benefits (Ligon, 1999). Accordingly, it has been suggested that these traits would signal the phenotypic/genetic quality of their bearers (Endler, 1980; Lozano, 1994; Olson and Owens, 1998; McGraw and Hill, 2000; Hill et al., 2002; Faivre et al., 2003a; Faivre et al., 2003b; Alonso-Alvarez et al., 2004a; Hōrak et al., 2004). In addition, carotenoid-based pigmentation is hypothesized to incur important nutritional/energy costs, since several steps of the colouration process (absorption, metabolic conversion and transport of pigments) are thought to be energy demanding (Hill, 2000; McGraw et al., 2005). In line with this hypothesis, recent findings show that the absorption of dietary pigments is constrained by the nutritional/energy state of individuals (Hill, 2000; McGraw et al., 2005). Additionally, carotenoids have antioxidant properties (Møller et al., 2000; Krinsky, 2001; Blount et al., 2002; Stahl and Sies, 2003; Sahin et al., 2006) (but see Costantini et al., 2006; Costantini et al., 2007; Hōrak et al., 2006; Tummeleht et al., 2006) and, consequently, pigments invested in a sexual ornament are depleted for antioxidant defences. Relatedly, carotenoid-based sexual traits have been supposed to reflect the antioxidant status of an individual (von Schantz et al., 1999). Therefore, thermal stress might shape the expression of carotenoid-based sexual ornaments through allocation trade-offs, the key resource being energy and/or antioxidant pigments *per se* since thermal stress was shown to

elicit an increased susceptibility to oxidative stress in several bird species (Sahin et al., 2002; Lin et al., 2006).

To our knowledge, the sensitivity of carotenoid-based sexual ornaments to ambient temperature has never been investigated. The aim of this study was to partially fill this gap. We used male zebra finches (*Taeniopygia guttata*) as suitable models since these birds present useful biological traits. Firstly, males express orange/red bills whose colouration relies on carotenoid pigments (McGraw et al., 2002). Secondly, the bill colour has been shown to be the target of female preference (Burley and Coopersmith, 1987; Blount et al., 2003) (but see Collins and ten Cate, 1996). Thirdly, males always bear a coloured bill, with colours ranging from orange to dark red. Finally, whatever the bill colour expressed by males, this colouration requires the allocation of carotenoids on a daily basis, as shown by the short term variations detected with experiments controlling access to carotenoids (Alonso-Alvarez et al., 2004a). Interestingly, if this contrasts with other carotenoid-based sexual ornaments such as feathers, which require the allocation of pigments only during the moult, this also suggests that males may have to face continuous trade-offs. Practically, we submitted birds to a range of temperature regimes that individuals may experience under natural conditions. For instance, zebra finches may breed on the New England Tableland (Australia), much of which is above 1000 m elevation, and up to 50 days of frost are expected each year (Zann, 1996). In addition, this species was also recorded as breeding during the winter, when mean daily minimum temperature is 4°C (Zann, 1996). Accordingly, one group of birds was submitted to a cold stress (6°C) for 4 weeks. This treatment was supposed to increase metabolic rate and concomitant energy consumption compared to that of a second group of birds that was kept within the thermoneutral zone (26°C). In addition, half of the individuals in each treatment were provided with supplementary carotenoids in the drinking water. If birds facing a thermal stress give priority to self-maintenance at the expense of the sexual signal, we predicted that males under cold stress should develop duller bills. In addition, if energy is the only key resource implicated in a trade-off between bill colour and self-maintenance, we predicted that males under cold stress should be energy but not carotenoid limited and that carotenoid supplementation would not allow them to exhibit redder bills because energy limitation should compromise pigment allocation to the colouration process.

Materials and methods

General experimental design

Ninety-three male zebra finches (*Taeniopygia guttata* Vieillot 1817) were housed indoors (13 h:11 h L:D) in individual cages. Birds were fed *ad libitum* with a commercial birdseed mix and grit. Males were randomly assigned to four groups and acclimatized for 2 weeks in two rooms (temperature=21°C) before the start of the experiment. After this period and over 7 days, the temperature was gradually increased in one room to reach the value of 26°C, while it was reduced in the second room to reach the value of 6°C. Since the metabolic rate of zebra finches linearly increases as ambient temperature drops from 26°C to 6°C (Gavrilov and Dolnik, 1985; Sedunova et al., 1998), using data from Gavrilov and Dolnik (Gavrilov and Dolnik, 1985) we estimated that males

sustained a daily energy expenditure of 58.8 kJ day⁻¹ and 25.3 kJ day⁻¹ when cold exposed and maintained at 26°C, respectively. After this 7 day period, two groups (one in each temperature treatment) were provided with extra carotenoids. Lutein and zeaxanthin are the most abundant carotenoids in the diet of captive zebra finches (McGraw et al., 2002). Accordingly, supplemented males received a mix of lutein and zeaxanthin [(ratio lutein/zeaxanthin=20:1, w:w) OroGlo liquid, Kemin France SRL, Nantes, France] in the drinking water. The concentration in water (110 mg l⁻¹) was defined following Alonso-Alvarez et al. (Alonso-Alvarez et al., 2004a). Controls received tapwater only. Overall, our experimental design consisted of four groups: non-supplemented males at 6°C ($N=23$) and 26°C ($N=23$), and supplemented males at 6°C ($N=24$) and 26°C ($N=23$). Water, carotenoids and food were dispensed every 2 days, and each individual was carefully checked for any signs of sickness. Birds were maintained under these conditions for 4 weeks. Only two rooms were available for the experiment. Consequently, to minimize the risk that differences between temperature regimes were due to a room effect, birds were transferred between the two rooms, at the mid-point of the experiment (2 weeks after the start of the carotenoid supplementation), while room temperature was quickly reversed to maintain birds at the same temperature regime. Since birds were maintained under the same conditions after the transfer, any changes in fast dynamic variables related to physiological and behavioural traits (between the end of the first 2 week period and the end of the last 2 week period) would reflect changes in environmental conditions experienced by birds due to a room effect. To detect a potential room effect, an additional measurement of body mass and food intake (at day 21) was included in the protocol described below. Therefore, body mass (taken as an integrative measure of physiological condition) and food intake (taken as a measure of behaviour) were measured immediately before and after each period elapsed in the two rooms, and changes observed for the two variables were compared between the two periods. For each of the experimental groups, neither change in body mass nor change in food intake was affected by room change (paired *t*-tests; food intake: all *P* values ≥ 0.16 , body mass: all *P* values ≥ 0.24). In addition, similar results were found when investigating a room effect within each temperature treatment (paired *t*-tests; food intake: all *P* values ≥ 0.29 , body mass: all *P* values ≥ 0.42). We are therefore confident that environmental conditions experienced by birds in the two rooms were similar and did not produce biased results.

Before the experiment, at the end of the 2 week acclimation period at 21°C (day 0), we measured mass, food intake, bill colour and circulating carotenoids in plasma. The same variables were recorded at the end of the experiment (day 35). Birds were weighed using an electronic balance (± 0.1 g). Food was dispensed in a large plastic cup (height: 12 cm, diameter: 10 cm), so that seeds that were not eaten did not spill on the floor but remained within the cup, allowing us to assess food intake by weighing (± 0.1 g) the amount of seeds ingested from 09:00 h to 19:00 h. A blood sample (100–150 μ l) was collected from the brachial vein on day 0 and day 35 using heparinized microcapillary tubes. Blood was centrifuged at 1800 *g* for 15 min and the plasma was stored at -80°C until carotenoid

analysis. No birds showed any sign of sickness during the course of the experiment.

Carotenoid analysis

Total plasma carotenoids were assessed by spectrophotometry following Alonso-Alvarez et al. (Alonso-Alvarez et al., 2004a). Briefly, carotenoids were extracted by diluting 10 μ l of plasma in 190 μ l of absolute ethanol. The solution was vortexed for 1 min and centrifuged for 10 min at 1500 *g* to precipitate the flocculent proteins. 100 μ l of the supernatant was then added in ELISA plates and the optical density was read with a microplate reader device at 450 nm. Carotenoid concentration was determined from a standard curve of lutein of known concentration (700 μ g ml⁻¹). The standard curve was achieved by serially diluting lutein in absolute ethanol as follows: 0, 0.1, 0.31, 0.625, 1.25, 2.5, 5 and 10 μ g ml⁻¹. For a subset of individuals ($N=32$) high-performance liquid chromatography (HPLC) analyses were also performed, allowing us to test for the reliability of colorimetric measurements. The total amount of plasma carotenoids given by colorimetric measurements was highly correlated with the sum of the four major carotenoids (lutein, zeaxanthin, anhydrolutein, β -cryptoxanthin) determined by HPLC (Pearson correlation coefficient; $r=0.80$, $P<0.0001$, $N=32$).

Assessment of bill colouration

Bill colour was scored under standardized conditions by reference to a DuluxTM Trade Colour chart (Dulux, Asnières, France) as described previously (Blount et al., 2003; Bertrand et al., 2006). In accordance with Blount et al. (Blount et al., 2003), the following specific scale was used, with scores ranging from 1 (light orange) to 9 (dark red): 1 (69YR 34/780), 2 (56YR 28/778), 3 (44YR 26/756), 4 (34YR 20/708), 5 (31YR 18/648), 6 (16YR 16/594), 7 (19YR 13/558), 8 (09YR 11/475), 9 (14YR 10/434); where the alphanumeric code denotes the hue, the numerator is the brightness and the denominator indicates the saturation. Bill colour was always scored by the same observer (B.F.) in an independent room under constant light conditions. At each time, two males were randomly selected in each treatment group (by G.D.) and given to the observer who was therefore blind regarding the treatment group. Colour scores were highly repeatable both between and within observers (intraclass correlation coefficient: $R=0.96$, $P<0.0001$, $N=39$; $R=0.93$, $P<0.0001$, $N=64$). We also checked the relevance of the scores obtained using the colour chart by comparing them with the hue values provided by image analysis software (LUCIA G 4.81 Software, Nikon, Champigny-sur-Marne, France). To do this, we scored the bill colour of 15 males using the colour chart and took a digital photograph of their bills under standardized light conditions. The pictures were analysed to obtain a hue value for each of them. The colour scores and the hue values were strongly negatively correlated (Spearman's rank correlation, $r_s=0.886$, $P<0.001$, $N=15$) demonstrating that the scores used in this study provided an objective and reliable measure of variation in hue (a decrease in hue value provided by the software indicated an increase in red colouration). At day 0, bill score and plasma carotenoids were positively correlated (Spearman's rank correlation, $r_s=0.328$, $P<0.002$, $N=93$). As expected, males circulating higher carotenoid levels displayed

redder bills (see also Blount et al., 2003; McGraw and Ardia, 2003).

Statistical analyses

When necessary, data were log transformed to achieve the normality of residuals and homogeneity of variances. The effects of treatments on change in mass and food intake over the course of the experiment (from day 0 to day 35) were analysed using repeated measurements general linear models (GLMs) and individual contrasts. A similar analysis was performed to investigate the effect of experimental treatments on the change in plasma carotenoid levels. Since bill colour was recorded on an ordinal scale ranging from 1 to 9, the effect of treatments on bill colour was investigated using a GLM with a logit link and an ordinal error distribution (see Bertrand et al., 2006). The GLM was fitted with change in bill colour as the dependent variable [post-experimental score (day 30) minus pre-experimental score (day 0)], treatments and their interactions as fixed effects, and initial bill score as covariate. Analyses were carried out using Statistica 7.0 (StatSoft, Maisons-Alfort, France) and JMP 5.1 (SAS Institute, Cary, NC, USA) software packages. *P* values were two-tailed and the level of significance was set to 0.05. Means are given \pm 1 s.e.m.

Results

Effect of cold exposure on change in food intake and body mass

Change in food intake and body mass throughout the experimental period did not differ between carotenoid supplementation regimes (repeated GLMs: supplementation; food intake, $F_{1,89}=2.76$, $P=0.10$; body mass, $F_{1,89}=2.56$, $P=0.113$). On the other hand, changes in food intake and body mass were significantly affected by temperature treatment (repeated GLMs: temperature; food intake, $F_{1,89}=161.94$, $P<0.0001$; body mass, $F_{1,89}=4.37$, $P=0.039$). Indeed, (i) food consumption was clearly increased in cold-exposed males and slightly decreased in males kept at a warm temperature (Fig. 1A) and (ii) body mass slightly increased in cold-exposed males whereas it slightly decreased in males kept at a warm temperature (Fig. 1B). We found no evidence that the effect of temperature treatment differed between carotenoid supplementation regimes (repeated GLMs: temperature \times supplementation; respectively, $F_{1,89}=0.003$, $P=0.956$ and $F_{1,89}=0.291$, $P=0.591$).

Overall, these results show that the metabolic activity of males was increased under cold exposure, but importantly that these males remained in energy balance.

Effect of cold exposure on change in circulating carotenoids

As expected, carotenoid supplementation strongly affected the amount of circulating carotenoids in the plasma, with supplemented males showing a greater increase in plasma carotenoids than non-supplemented ones (repeated GLM; supplementation: $F_{1,89}=70.30$, $P<0.0001$, Fig. 1C). Values for supplemented birds were similar to those reported by Alonso-Alvarez et al. (Alonso-Alvarez et al., 2004a). On average, supplemented birds showed a 2.6-fold increase (s.e.m. ± 0.14) in their circulating carotenoid levels between day 0 and day 35. Furthermore, changes in plasma carotenoid level also differed

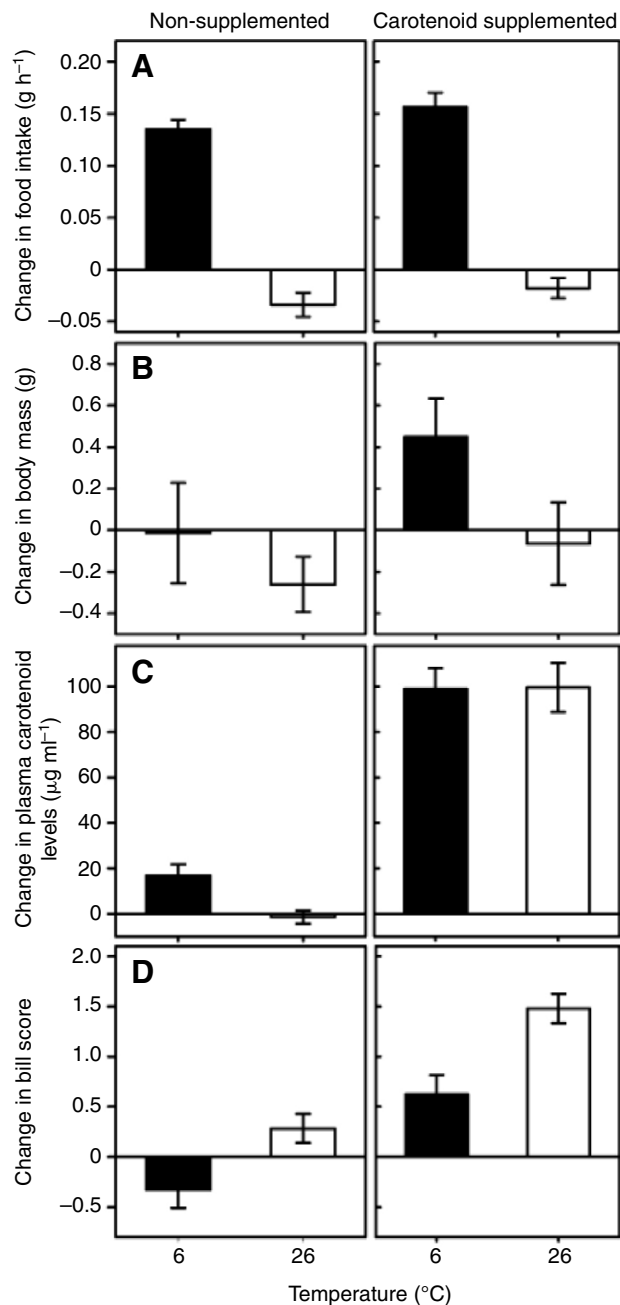


Fig. 1. Change in: (A) food intake (g h⁻¹), (B) body mass (g), (C) plasma carotenoid levels (μg ml⁻¹) and (D) bill colour over the course of the experiment for the four experimental groups (carotenoid-supplemented vs non-supplemented males and cold-exposed vs warm-exposed males). Values are means \pm s.e.m. ($N=23$ for all bars except 6°C carotenoid supplemented, where $N=24$).

between the two temperature regimes (repeated GLM; temperature: $F_{1,89}=5.48$, $P=0.021$). However, while the effect of temperature did not significantly interact with the supplementation regime (repeated GLM; temperature \times supplementation: $F_{1,89}=1.42$, $P=0.236$), analysis revealed that the effect of temperature relied mainly on the contrast existing between the two non-supplemented groups (individual contrast; $F_{1,89}=6.175$, $P=0.015$) rather than on the contrast existing

between the supplemented groups (individual contrast; $F_{1,89}=0.665$, $P=0.417$, Fig. 1C). Interestingly, in the absence of carotenoid supplementation, cold-exposed males showed a greater increase in circulating carotenoids than males housed at 26°C (Fig. 1C), and this difference persisted after including food intake in the model (individual contrast; $F_{1,88}=5.060$, $P=0.027$).

Effect of cold exposure on change in bill colouration

After controlling for initial bill score (Wald statistic; covariate: $\chi^2=12.06$, $P<0.001$), the change in bill colour throughout the experiment was found to be significantly affected by the temperature regime (Wald statistic; temperature: $\chi^2=16.48$, $P<0.001$). Following the prediction that males facing a thermal stress give priority to self-maintenance at the expense of the sexual signal, we found that in the absence of carotenoid supplementation, cold-exposed males developed duller bills than males housed at a warm temperature (Fig. 1D). Similarly, carotenoid-supplemented and cold-exposed males developed less red bills than their conspecifics kept at a warm temperature (Fig. 1D). In addition, we found that carotenoid supplementation significantly affected the change in bill colour (Wald statistic; supplementation: $\chi^2=32.77$, $P<0.001$). Supplemented males developed redder bills than the non-supplemented ones, even under cold exposure (Wald statistic; temperature \times supplementation: $\chi^2=0.21$, $P<0.604$; Fig. 1D), suggesting that birds facing a cold stress were also carotenoid limited.

Discussion

Environmental factors may exert substantial selective pressure on secondary sexual traits by outweighing the benefits associated with their expression (Wiens, 2001). For instance, a recent study has shown that, among abiotic environmental factors, ambient temperature may constrain the expression of sexual ornaments presumably through trade-offs between sexual traits and physiological functions related to self-maintenance (West and Packer, 2002). To date, few studies have experimentally investigated the plasticity of sexually selected traits in response to abiotic environmental stressors (West and Packer, 2002). In this study, we explored the effect of environmental stress, such as ambient temperature, on the production and the maintenance of carotenoid-based sexual ornaments in male zebra finches. Male zebra finches were simultaneously exposed to variable regimes of ambient temperature and carotenoid availability to experimentally investigate the allocation of resources to two competing functions, sexual display (bill colour) and self-maintenance. We found that cold-exposed males reduced the expression of the carotenoid-based sexual trait, suggesting that ambient temperature shaped its expression. But, interestingly, carotenoid supplementation had an additive effect, enabling supplemented males to express redder bills than their counterparts.

Integumentary carotenoid-based colouration, such as red bill in zebra finches, develops *via* a multistep process. These steps include the absorption of carotenoid pigments from the diet, their incorporation into and their transport through the blood stream *via* lipid carriers (lipoproteins), their metabolic conversion into red ketocarotenoids *via* oxidation reactions and,

finally, their deposition into integuments (Parker, 1996). Pigmentation is therefore hypothesized to incur important nutritional/energetic costs since metabolic conversion of yellow carotenoids and lipoprotein assemblages are thought to be energy demanding (Hill, 2000; McGraw et al., 2005). Accordingly, one hypothesis that might explain the reduced expression of bill colour in cold-exposed birds is that thermoregulatory functions have sequestered energy needed for the pigmentation process. Following this hypothesis, if absorption of carotenoids and bill colouration process were impaired due to energetic constraints only, cold-stressed birds would have taken up lower amounts of pigments from their diet and/or would have shown a reduced expression of their bill colour irrespective of carotenoid availability. At first sight, one part of our results might be consistent with this hypothesis because non-supplemented and cold-exposed males had a depressed bill colour but higher circulating carotenoids at the end of the experiment compared to the pre-experimental values (which could correspond to accumulated unconverted yellow carotenoid precursors in the plasma). However, several other results suggest that this explanation is unlikely. Firstly, cold-stressed birds compensated by increasing their food intake, and their body mass slightly increased during the experiment, suggesting that cold-stressed birds remained in energy balance. Secondly, carotenoid levels were significantly increased in a similar fashion in the two supplemented groups, indicating that absorption of dietary pigments was not limited by the amount of energy used to fuel the thermoregulatory functions. Thirdly, even under cold exposure, supplemented males developed a redder bill than non-supplemented ones, suggesting that (i) the pigmentation process was not suppressed for cold tolerance and importantly that (ii) cold-stressed birds were carotenoid limited.

Carotenoid pigments invested in a sexual ornament are depleted for other functions, and in male zebra finches there is evidence that the development and the maintenance of a colourful bill involves the mobilization of a huge amount of carotenoids as shown by the high carotenoid concentration required to achieve the reddest bills (Alonso-Alvarez et al., 2004a). Accordingly, among alternative hypotheses to that of energy limitation is that the down-regulation of carotenoid allocation to the sexual signal in cold-exposed males saves carotenoids to store them or up-regulate some physiological functions related to their self-maintenance. Our findings that carotenoids were limiting resources under cold exposure and that non-supplemented and cold-exposed males have depressed bill colour while circulating more carotenoids are supportive of this hypothesis. In addition, the fact that cold-exposed and supplemented males were unable to develop and maintain bill colour in a similar fashion to warm-exposed and supplemented ones, notwithstanding a similar increase in circulating carotenoids within these two groups, also supports the view that cold-exposed, supplemented males also had to save carotenoids and prioritize self-maintenance.

Among physiological functions related to self-maintenance that might have benefited from carotenoid saving, one could first mention the antioxidant barriers (but see Hörak et al., 2006; Tummeleht et al., 2006; Costantini et al., 2007). It is well known that thermal stress causes high tissue oxygen consumption and oxidative stress (Selman et al., 2000; Lin et al., 2006; Sahin et

al., 2002; Sahin et al., 2006). Therefore, it might have been crucial for cold-exposed birds to up-regulate their antioxidant barriers to counter the increase in free radical production during this period of high energy turnover (Jenkins et al., 1988; Scandalios, 2002). Interestingly, recent studies suggest that carotenoids might contribute to the maintenance of redox homeostasis in birds facing a thermal stress. Indeed, in agreement with previous studies showing that carotenoid-supplemented black-backed gulls (*Larus fuscus*) elicited a higher plasma antioxidant activity (Blount et al., 2002), Sahin et al. (Sahin et al., 2006) have shown that supplementation with lycopene (a member of the carotenoid family) attenuated the increase in biomarkers of oxidative stress such as malondialdehyde and homocysteine in thermal-stressed quails (*Coturnix c. japonica*). Additionally, Alonso-Alvarez et al. (Alonso-Alvarez et al., 2004a) have reported that male zebra finches with the highest increase in plasma carotenoids showed the highest resistance to a free radical attack. However, the hypothesis that cold-stressed zebra finches have upregulated their antioxidant barriers contrasts sharply with recent experiments showing that antioxidant defences are compromised during other metabolic workload [i.e. breeding (Alonso-Alvarez et al., 2004b; Wiersma et al., 2004)]. Nevertheless, these findings might be reconciled with the well known versatility of the antioxidant system (Levine and Kidd, 1996). Indeed, antioxidant defences have been shown to respond very differently according to the pattern of metabolic workload (Ji et al., 1998). For instance, while acute and strenuous physical exercise can substantially impair the effectiveness of antioxidant defences (Robertson et al., 1991; Aslan et al., 1998; Leeuwenburg and Heinecke, 2001), regular physical exercise has been shown to prevent the accumulation of oxidative damage by up-regulating the antioxidant system *via* an increase of both enzymatic (Robertson et al., 1991; Aslan et al., 1998; Brites et al., 1999; Liu et al., 2000) and non-enzymatic components (Brites et al., 1999; Hübner-Wo'zniak et al., 1994; Kitamura et al., 1997; Cooper et al., 2002; Pincemail et al., 2002). Interestingly, long term cold exposure was also shown to elicit an upregulation of the antioxidant system through an increase in antioxidant enzyme activities (Selman et al., 2000).

Among other physiological functions related to self-maintenance that might have also benefited from carotenoid saving, one could mention the immune defences. Indeed, cold stress has also been shown to exert variable effects on immune functions. Although some studies suggest a negative effect of cold exposure on immune performance (Svensson et al., 1998; Cichoń et al., 2002), other studies suggest that cold stress might enhance different components of the immune system [humoral immunity (Subba Rao and Glick, 1977); cell-mediated immunity (Hangalapura et al., 2004; Van Loon et al., 2004)]. Besides their antioxidant properties, carotenoid pigments are also potent immunostimulants in male zebra finches (Blount et al., 2003; McGraw and Ardia, 2003). Accordingly, a non-mutually exclusive hypothesis might be that cold-exposed males have saved carotenoids to up-regulate immune functions. Nevertheless, further work is clearly needed to tease apart these two hypotheses, as well as to investigate how non-enzymatic antioxidants such as carotenoids react to long term cold exposure in other species.

The expression level of an ornament is a product of the balance between the costs and benefits associated with its development and maintenance. Accordingly, an alternative explanation for our findings could be that by cold-exposing males, we might have changed the benefits associated with investment in the sexual ornament, not the costs of its development and maintenance. More specifically, birds might have used low temperature as a cue with regard to the probability of a successful breeding event within a short time period (Hau, 2001). This probability being presumably reduced under cold conditions (but see Zann, 1996; Alonso-Alvarez et al., 2006), birds might have reduced their investment in bill colour. In the same way, since our males were housed in the absence of females, they might not have been stimulated to invest carotenoids in the sexual signal to get a mate. According to these scenarios, we might have expected the reduction in ornament development to occur independently of any resource limitation. However, our results showing that cold-stressed birds, as well as males kept at a warm temperature, developed redder bills when provided with extra carotenoids, suggest that males were not reluctant to invest carotenoids under cold exposure or when housed in the absence of females (see also Alonso-Alvarez et al., 2004a). Rather our findings suggest that thermal stress might have affected the cost of maintenance of the sexual signal and that carotenoids are among plausible resources that might translate this cost.

In conclusion, the present study provides new insights into the plasticity of carotenoid-based colour displays in response to abiotic environmental stress and the potential role of resource allocation trade-offs in shaping their expression. In addition, our results suggest that resources other than energy may be prioritized for self-maintenance at the expense of sexual signalling. For instance, our findings would suggest that carotenoid pigments might serve as the basis for a resource-based trade-off between sexual ornaments and physiological functions related to self-maintenance under environmental stress. Moreover, the down-regulation of bill colouration in carotenoid-supplemented and cold-exposed males would suggest that this mechanism would be achieved through a differential allocation pathway rather than the mobilization of keratinized pigments from the bill to the plasma. However, the physiological functions related to self-maintenance that might have benefited from carotenoid saving are currently equivocal. Although the antioxidant system is among the candidates, further studies are definitely needed since there is evidence, at least for some species, that carotenoids do not contribute to modulating the level of oxidative damage (Costantini et al., 2007).

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