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ORIGINAL PAPER

The presence of females modulates the expression of a carotenoid-based sexual signal

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Abstract Several environmental factors have been shown to shape the pattern of investment of carotenoids into the expression of sexual signals. Surprisingly, the impact of the social environment has been neglected. If a carotenoid-based sexual trait is used by females to choose a mate or by males to assess the quality of potential competitors for mates, males, in the presence of females, should upregulate expression of the trait. We tested this hypothesis in male zebra finches that were housed either with females or in a male-only social environment. Additionally, to investigate whether the social modulation of the expression of a sexual trait depends on the physiological need of carotenoids, we challenged half of the males with *Escherichia coli* lipopolysaccharide (LPS). We found that the social environment modulated the expression of bill color, with males kept in the presence of females harboring a redder bill at the end of the 3 weeks that the experiment lasted. Males injected with LPS showed duller bill color regardless of the presence of females, and social interactions with females result in upregulated bill color similarly for phosphate-buffered saline (PBS) and LPS males. Thus, social environment and immune activation had an additive effect on the expression of bill color. The effect of social environment on plasma carotenoids was less clear. Indeed, a first replicate of the entire experiment showed that both immune challenge and social context affected bill color, with a negative effect of immune challenge and a positive effect of the presence of females on circulating carotenoids. However, a second replicate of the experiment showed only a negative effect of the immune challenge. These results, therefore, suggest that the social environment can affect the expression of carotenoid-based sexual traits under both benign and carotenoid-demanding conditions. Whatever the signaling function of bill color (female mate choice or male–male competition for mates), the observed flexibility may be adaptive because the expression of the signal can be modulated depending on the expected rewards or costs associated with the presence or absence of females. Nevertheless, the mechanisms underlying such an effect are still unknown.

Keywords Social environment · Carotenoids · Bill color · Immune challenge · Sexual signal

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Introduction

In a variety of taxa, secondary sexual traits are often based on carotenoids, a family of pigments produced by a wide range of plants. Since the seminal work on guppies (*Poecilia reticulata*; Endler 1980, 1983, 1990), carotenoid-based sexual traits have been shown to be involved in the mate-choice process in many species (Kodric-Brown 1985; Burley and Coopersmith 1987; Hill 1990; Milinski and Bakker 1990; Zuk et al. 1990; Blount et al. 2003). The

mechanisms ensuring the honesty of carotenoid-based sexual traits have also been extensively studied. Two main hypotheses have been put forward. First, carotenoid-based signals might indicate the ability of individuals to find, gather, absorb, and process this supposedly limiting resource (Kodric-Brown 1989; Hill 1991; McGraw 2005). Second, it has been suggested that carotenoid-based signals might convey information on the efficiency of the immune and detoxification system of their bearers (Lozano 1994; Von Schantz et al. 1999). In agreement with this hypothesis, experimental activation of the immune system reduced carotenoid-based bill color in two bird species, suggesting a shift in the allocation of carotenoids between sexual signaling and the immune system (Faivre et al. 2003; Alonso-Alvarez et al. 2004). However, other studies that focused on development of colored feathers have found little support for this trade-off (Navara and Hill 2004). More experimental work is, therefore, needed to improve our picture on the information content of carotenoid-based signals.

Whatever the information content of carotenoid-based signals, the previous results unambiguously show that the allocation of carotenoids to colored traits plastically respond to environmental variation. However, the importance of the social environment in the expression of carotenoid-based traits has been largely neglected. This is surprising because the social environment may act on signals either through the presence or absence of stimulating factors as well as through the presence or absence of receivers of the signal. From a general point of view, if a signal is costly to produce and/or maintain and if it can rapidly respond to an environmental stimulus, one should expect a downregulation of the signal in the absence of receivers or in the absence of the environmental stimulus; conversely, in the presence of receivers and of the environmental stimulus, one should expect the upregulation of the signal, at the expense of other functions. Although previous studies have shown that social interactions between males may influence the expression of secondary sexual traits in birds, probably through androgen production (Zuk and Johnsen 2000; Parker et al. 2002; McGraw et al. 2003; see also Blas et al. 2006), and that females' behavior may affect the development of males' signals (West and King 1998; Freeberg 2004), the influence of presence vs. absence of females on male signals remains poorly investigated.

Here, we investigated whether the social environment (presence vs. absence of females) modulates the expression of a carotenoid-based sexual trait in male zebra finches (*Taeniopygia guttata*) when facing (1) benign and (2) carotenoid-demanding conditions. Male zebra finches exhibit a colored bill that ranges from orange to dark red, and females prefer males with redder bills (Burley and Coopersmith 1987; Blount et al. 2003; but see also Collins and Ten Cate 1996). In zebra finches, bill color is a carotenoid-dependent trait (Blount et al. 2003; McGraw and Ardia 2003; Alonso-Alvarez et al. 2004) whose expression is affected by the activation of the immune system (Alonso-Alvarez et al. 2004). Finally, it has recently been reported that male zebra finches use information provided by conspecifics to adjust their behavior towards female partners, reinforcing the idea that the social context is an important environmental component for this species (Vignal et al. 2004). We predict that, if the sexual advertisement is important in the process of mate choice or in male–male competition, males kept in the presence of females should upregulate the expression of the signal under both benign and stressful conditions.

Material and methods

General experimental design and rearing conditions

Adult zebra finches were housed in wire cages (0.6×0.4×0.4 m) separated in two compartments, under controlled daily light cycle (13L: 11D) and temperature (22±2°C). Food (a commercial seed mix mostly containing white, red, and golden yellow millet, canary grass seed, and niger seed) and water were provided ad libitum. Before the experiment, males were caged individually during 4 weeks (the time needed for bill color to stabilize) in rooms housing only males. To start the experiment, males were randomly assigned to one of the two social environments. In the mixed social environment, each male was caged in one compartment with a female in the other one. Males kept in the male-only environment were caged in one compartment with a male as a neighbor. In addition, the cages were placed so that each male was in visual and acoustic contact with several other birds (males + females, or males only). Each social environment treatment was replicated twice (i.e., two rooms with a mixed social environment, $n=2\times12$ tested males, and two rooms with a male-only social environment, $n=2\times12$ tested males; the four rooms were of equal size resulting in equal densities, i.e., 24 birds per room). Because our experimental conditions are not natural because groups of male zebra finches do not occur without females in nature (Zann 1996), our results cannot be directly applied to wild populations. However, experimental approaches require narrow conditions to investigate accurately the influence of specific factors. Variable sex ratios between experimental groups would have been, for instance, a possible refinement of the design applied here. However, because we did not know how males may change their behavior with female relative abundance (gradually or with thresholds), we have chosen this simpler design.

Challenge to immune system

Carotenoid-demanding conditions were created by challenging individuals with an inert antigen (lipopolysaccharide, LPS) known to use up circulating carotenoids (Allen 1997; Alonso-Alvarez et al. 2004). Half of the males in each social environment were injected intraperitoneally with 100 μl of an LPS solution (0.01 mg/0.1 ml; lipopolysaccharide of *Escherichia coli*, serotype O55: B5). The other half was injected intraperitoneally with 100 μl of phosphate-buffered saline (PBS) for control. Birds were injected weekly during the course of the experiment that lasted 3 weeks. Body mass was measured before and at the end of the experiment, using an electronic balance (+0.1 g).

The entire experiment was fully replicated a few weeks later, using different individuals (overall initial sample size= 96). However, three males died in each of the two experiments (over the period including acclimatization phase and experiment itself), reducing the overall sample size to 90 birds.

Measurement of bill color

Bill color was assessed, before and at the end of the experiment, using a Dulux Trade Color chart (Dulux) under the same light conditions. A specific scale, ranging from less red to redder colors was used: 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 first number and letters indicate the hue, the numerator is the brightness, and the denominator is the saturation (Blount et al. 2003). Bill color was scored blindly with respect to experimental conditions. One person captured the birds arbitrarily and brought them to a separate room where they were scored independently by three measurers. We also checked the relevance of the scores obtained using the color chart by comparing them with the hue values provided by image analysis software (LUCIA G 4.81 Finale Software, Nikon, France). To do this, we scored bill color of 15 males using the color chart, and we took a digital photograph of their bill under standardized light conditions. The pictures were analyzed to obtain a hue value for each of them. The color scores and the hue values were strongly negatively correlated (Pearson correlation coefficient, $r=-0.867$, $P<0.0001$, $n=15$), showing that the scores we used in this study reliably captured variation in hue. This color-scoring method is unable to capture ultraviolet (UV) component that may be associated to carotenoid-based secondary sexual traits in birds (Mougeot et al. 2005; Mougeot and Arroyo 2006). However, to our knowledge, only one recent study has reported a UV component in the bill color of zebra finches that might be involved in sexual signaling (Bolund et al. 2007).

Initial values of bill color scores were homogeneous (mean + SE=6.87+0.067; lower 95% confidence limit= 6.74, upper 95% confidence limit=7.01; $n=90$) and did not differ between treatments (ordinal analysis of variance (ANOVA) with multinomial distribution, social environment, $\chi^2_1=0.07$, $P=0.7858$; immune treatment, $\chi^2_1=0.02$, $P=0.8787$). The scores were highly repeatable, both within and between measurers (intraclass correlation coefficient, $R=0.96$, $n=39$, $P<0.0001$, $R=0.93$, $n=64$, $P<0.0001$, respectively).

Measurement of plasma carotenoids

Carotenoid concentration was assessed from individual plasma samples. Blood samples (100 to 150 μl) were collected from the brachial vein using sterile needles and heparinized capillaries. Blood was immediately centrifuged (4,000 rpm, 4°C, 15 min) and plasma was stored at −80°C before analysis. Carotenoids were extracted in a two step process. First, 15 μl of plasma were added to 200 μl of pure methanol plus an internal standard (astaxanthin) before centrifugation (4,000 rpm, 4°C, 10 min), and the supernatant was recovered. Second, the remaining residual obtained was added to 200 μl of MTBE (Methyl Tertio Buthyl Ether) before a second centrifugation (4,000 rpm, 4°C, 10 min). The supernatant was recovered and added to the supernatant from the first extraction step before complete evaporation under nitrogen. Extracted carotenoids were diluted in 50 μsl of MTBE-methanol (*v*/*v*, 50/50) and the solution was analyzed using high performance liquid chromatography (HPLC). HPLC separations were performed on a 250× 4.6-mm stainless steel ProntoSIL C30 reversed-phase column (Bischoff, Leonberg, Germany). Separations of carotenoid extracts were carried out using a mixture of MTBE, methanol and deionized water as mobile phase. Absorption spectra were collected from 250 to 600 nm with a Waters 996 photodiode array detector. Chromatograms were plotted at 450 nm, and peak identification was effected by comparing elution order, retention times, and absorption properties (λ_{max} values) with pure standards. We assessed levels of four carotenoids, lutein, zeaxanthin, anhydrolutein, and b-cryptoxanthin, which appeared highly correlated (all $P<0.001$), as previously shown in other studies (McGraw and Ardia 2004). Thus, we considered only total concentration of circulating carotenoids in the statistical analyses. For technical reasons, the HPLC was not available to measure circulating carotenoids during the second experiment. We, therefore, used a colorimetric technique that has been shown to provide values highly correlated to those obtained by HPLC (Alonso-Alvarez et al. 2004). Briefly, 20 μl of plasma were diluted in 180 μl of absolute ethanol. The dilution was vortexed and flocculent proteins were precipitated by centrifuging the sample at

1,500×*g* for 10 min. We examined the supernatant in a spectrophotometer and determined the optical density of the carotenoid peak at 450 nm. Carotenoid concentration was determined from a standard curve of lutein of known concentration.

Statistical analyses

Because bill color score is an ordinal variable, we used an ordinal ANOVA with multinomial distribution and a cumulative logit link function (PROC GENMOD, SAS 2001) to assess the effect of treatments on changes of bill color during the course of the experiment. The difference in post- and pre-experimental bill color scores was used as the dependent variable in the model. We fitted a model where changes in bill color scores were function of the two treatments (social environment and immune activation) and their interaction. We also took into account the effect of the rooms where the birds were maintained (nested within the social environment treatment), and we checked whether the effect of the treatments differed between the two replicates of the experiment.

Changes in body mass and plasma carotenoids were analyzed using nested ANOVA models (PROC GLM, SAS 2001). All data conformed to the assumptions of parametric statistics for these analyses. These models were fitted using the same factors as for bill color.

Results

Both the social environment and the immune treatment affected bill color, whereas the social environment by immune treatment interaction was not significant (Table 1). Therefore, the two treatments had an additive effect on the expression of the signal. As predicted, males upregulated the expression of the signal (i.e., harbored a redder bill) when kept in the presence of the females (Fig. 1), whereas immune activation depressed the expression of the trait (i.e., bill became duller; Fig. 1). The two replicated experiments provided similar results as shown by the nonsignificant replicate × immune treatment and replicate × social environment interactions (Table 1). Similarly, the effect of the rooms where the birds were housed was not significant (Table 1).

Males in the mixed social environment harbored a redder bill at the end of the experiment when injected with PBS (Signed Rank, $S=64$, $P=0.0077$) but not when challenged with LPS (Signed Rank, $S=11$, $P=0.55$). Conversely, males in the male-only social environment harbored a less red bill at the end of the experiment in the LPS challenged group

Table 1 Effect of the social environment (presence vs. absence of females) and immune activation (injections of LPS vs. PBS) on change in bill color of male zebra finches

Sources of variation	*df*	χ^2	*P*-value
Social environment	1	6.27	0.0123
Immune activation	1	18.82	<0.0001
Room (social environment)	2	0.52	0.7695
Replicate	1	0.32	0.5730
Social environment × immune activation	1	0.03	0.8669
Immune activation ×replicate	1	1.13	0.2872
Social environment ×replicate	1	0.01	0.9362

The results of an ordinal analysis of variance with a multinomial distribution and a cumulative logit link function. The effect of the rooms where the birds were housed nested within the social environment, as well as the two replicates of the experiment were included into the model

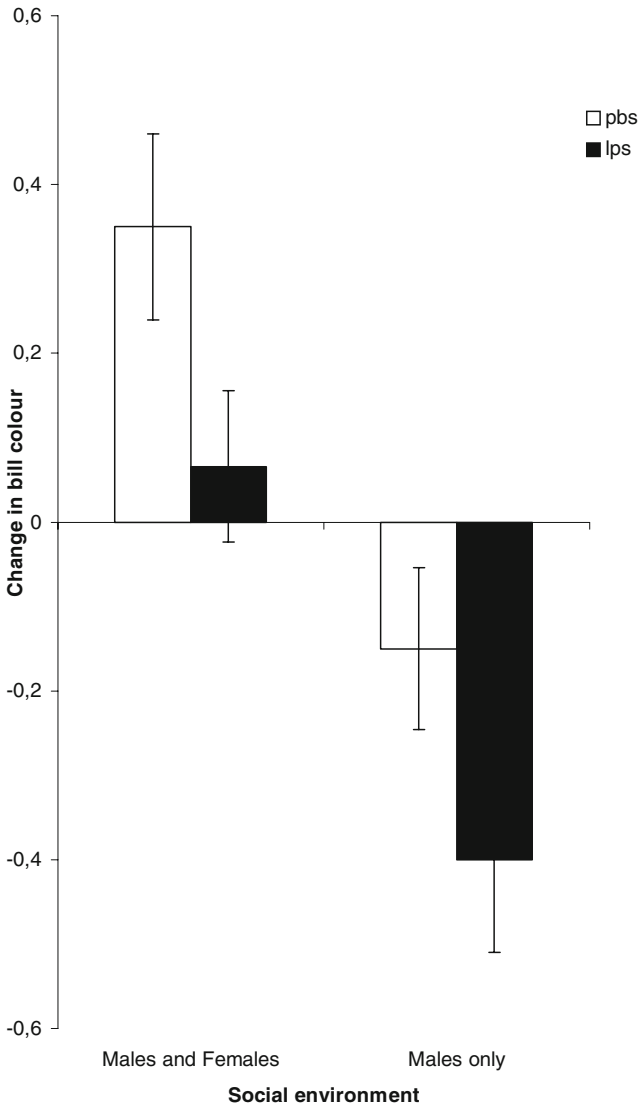

Fig. 1 Change in bill color (mean + SE; post-experimental minus pre-experimental values) of male zebra finches kept in a mixed or a male-only social environment. *Empty* and *black bars* refer to individuals injected with PBS and LPS, respectively

(Signed Rank, $S=-73$, $P=0.0016$) but not in the control group (Signed Rank, $S=-31.5$, $P=0.1064$).

A more complex pattern was found for plasma carotenoids because the two replicated experiments provided, to some extent, different results. This heterogeneity between replicates translated into a statistically significant replicate by immune treatment interaction and replicate by social context interaction (Table 2). We, therefore, decided to analyze the two experiments separately. The analysis of the first experiment showed an additive effect of immune treatment ($F_{1,39}=20.45$, $P<0.0001$) and social environment ($F_{1,39}=12.19$, $P=0.0012$; Fig. 2a) on the amount of circulating carotenoids. The analysis of the second experiment revealed that the immune treatment affected the change in the amount of circulating carotenoids, with LPS-injected birds having significant less carotenoids than PBS individuals ($F_{1,29}=7.96$, $P=0.0085$, Fig. 2b); however, contrary to the first experiment, the social context did not affect the change in the amount of circulating carotenoids ($F_{1,29}=1.44$, $P=0.2406$, Fig. 2b).

Finally, the two treatments (the social environment and the immune activation) did not affect the temporal variation in body mass during the course of the experiment (Table 3).

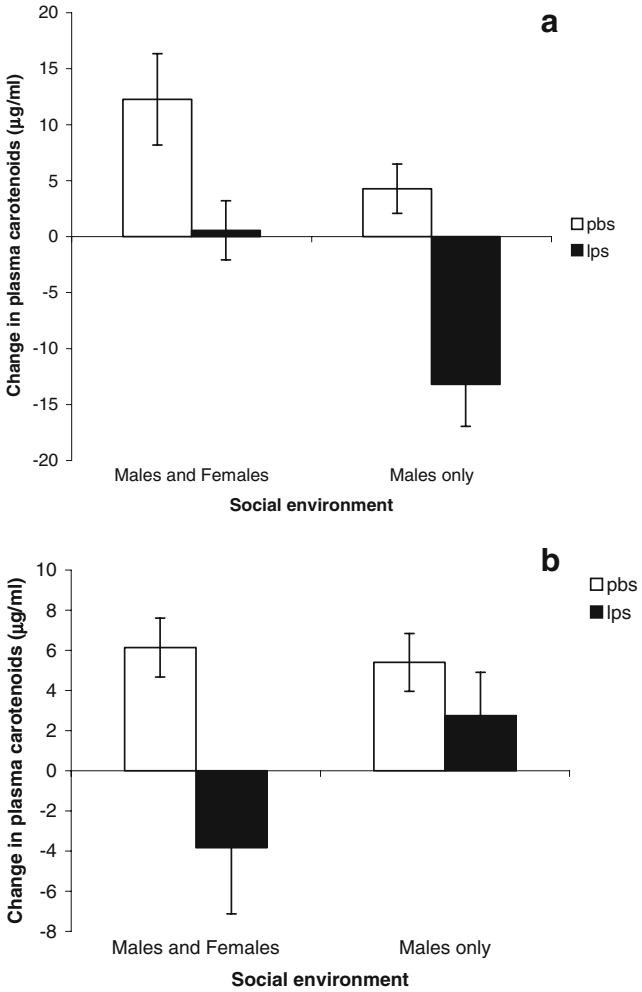

Fig. 2 Change in plasma carotenoids (mean + SE; post-experimental minus pre-experimental values) of male zebra finches kept in a mixed or a male-only social environment. *Empty* and *black bars* refer to individuals injected with PBS and LPS, respectively. **a** and **b** refer to the two replicates of the experiment

Discussion

A signal can be defined as a trait produced by one individual to influence the behavior of another individual (Maynard Smith and Harper 2003). In this context, it is intuitive to expect that signal production and regulation should be modulated by the relative presence of receivers. In agreement with this view, there exist several examples where the expression and intensity of signaling depend on the presence of receivers. For instance, young of birds and mammals often use noisy vocalizations and costly behaviors, collectively called begging display, to signal their need for food to parents. Although young can beg when the parents are away in the search of food, begging is, by far, the most intense when parents are at nest (Budden and Wright 2001; Leonard and Horn 2001). Similarly, the male Siamese fighting fish (*Betta splendens*) increased the intensity of conspicuous displays when females were present (Doutrelant et al. 2001), and male starlings (*Sturnus vulgaris*) sang more often in the presence of females than in the presence of males (Gwinner et al. 2002). In addition, West and King (1988) showed that, in the Cowbird (*Molothrus ater*), male's song was modulated by female presence and behavior and also by social context (Freeberg 2004). In this species, specific vocalizations that trigger copulatory postures in females were incorporated in males' song when young males were reared with females. Finally, male bowerbirds (*Ptilonorhynchus violaceus*) adjusted their behavioral displays in response to female behavior

Table 2 Effect of the social environment (presence vs. absence of females) and immune activation (injections of LPS vs. PBS) on change in circulating carotenoids (µg/ml) of male zebra finches

Sources of variation	*df*	*F*	*P*-value
Social environment	1	3.80	0.0553
Immune activation	1	23.75	< 0.0001
Room (social environment)	2	5.22	0.0077
Replicate	1	1.09	0.3010
Social environment × immune activation	1	0.00	0.9705
Immune activation ×replicate	1	4.13	0.0458
Social environment ×replicate	1	10.49	0.0018

The results of an analysis of variance; the effect of the rooms where the birds were housed, nested within the social environment, as well as the two replicates of the experiment were included into the model

Table 3 Effect of the social environment (presence vs. absence of females) and immune activation (injections of LPS vs. PBS) on change in body mass (g) of male zebra finches

Sources of variation	*df*	*F*	*P*-value
Social environment	1	1.97	0.1644
Immune activation	1	0.06	0.8092
Room (social environment)	2	0.34	0.7142
Replicate	1	4.50	0.0370
Social environment ×immune activation	1	0.29	0.5923
Immune activation ×replicate	1	2.74	0.1016
Social environment ×replicate	1	0.82	0.3686

The results of an analysis of variance; the effect of the rooms where the birds were housed, nested within the social environment, as well as the two replicates of the experiment were included into the model

during courtship, i.e., they increase their display intensity in response to an increase in female crouching (Patricelli et al. 2002, 2006). These examples illustrate how signalers modulate the expression of costly signals depending on the reward they expect to receive. However, although behaviors can be instantaneously modulated according to the social stimuli, morphological traits usually do not exhibit this plasticity. Nevertheless, at a longer temporal scale, the expression of morphological sexual signals can also change depending on the expected reward. For instance, males of many organisms develop particular traits during the breeding season with the aim of attracting a partner and/or competing with rivals. These sexual traits usually regress or disappear once the breeding season is over. The seasonal fluctuation in the expression of sexual signal is likely to be the consequence of the cost of maintaining these traits throughout the year, when females and/or males are no longer receptive; therefore, there is no reward to the signaler (Andersson 1994).

Here, we showed that a carotenoid-based ornamental trait can rapidly respond to a social stimulus. Previous studies have convincingly shown that social rank may affect secondary sexual traits in males. For example, comb size is positively related to dominance rank in male red jungle fowls (*Gallus gallus*; Zuk and Johnsen 2000; Parker et al. 2002). Similarly, in male mandrills (*Mandrillus sphinx*), the red coloration of males depends on social rank (Setchell and Dixson 2001), and dominant male house sparrows (*Passer domesticus*) grew larger badges (a melanin-dependent trait) than subordinates (McGraw et al. 2003). In the present study, we found, for the first time, that the presence of females upregulated the expression of bill color in male zebra finches. Interestingly, males upregulated their signal in the presence of females both when facing benign environmental conditions as well as when facing stressful conditions (an antigenic stress) reducing the availability of carotenoids. Signals based on the deposition of carotenoids into keratinized structures or into the skin have been shown to vary rapidly and respond to environmental conditions. For instance, carotenoid supplementation is known to enhance bill color of zebra finches within few weeks (Blount et al. 2003; McGraw and Ardia 2003; Alonso-Alvarez et al. 2004), and activation of the immune system can reduce the expression of bill color in passerine species (Faivre et al. 2003; Alonso-Alvarez et al. 2004). These results, in conjunction with those reported in this study, show that carotenoid allocation is a plastic trait and can be adaptively adjusted depending on the social environment.

Signaling theory is based on the assumption that honest signals should be costly. The cost of carotenoid-based sexual traits has been frequently reported. In agreement with previous studies (Blount et al. 2003; Faivre et al. 2003; Alonso-Alvarez et al. 2004), our results suggest a trade-off in carotenoid allocation between sexual signal and immune defenses because the antigenic stress faced by LPS-injected males produced a lightening of the bill color. They also support the idea that sexual signals mirror the health status of the bearer (Lozano 1994). Because carotenoids are thought to play an important role in self-maintenance (Mortensen et al. 1997; Lozano 2001; Chew and Park 2004), carotenoid-based sexual signals have been frequently reported as costly because pigments may be sequestered in the signal at the expense of other functions (Lozano 1994, 2001). Therefore, the "short term" flexibility of this trait may be particularly important to avoid wasting carotenoids. As bill color is used by females as a cue to choose a mate (Burley and Coopersmith 1987; Blount et al. 2003; but see also Collins and Ten Cate 1996), the observed flexibility may be adaptive because (1) the presence of females can indicate a close opportunity to mate and breed and (2) in the absence of the receivers, it does not pay to overexpress a costly signal. However, we may also hypothesize that males, themselves, are the receivers of the signal which reveals competitive abilities in males, as it has been shown in the Blackbird (*Turdus merula*), for instance (Bright and Waas 2002). In this case, females played as a stimulus, and males were less stimulated to compete when females were absent and, thus, exhibited less red bills. In addition, and independently of the social context, less red bills of challenged males might have signaled their reduced abilities to compete with other males. However, a recent study on the role of bill color in intrasexual competition in zebra finches seems indicated that this trait is unlikely to be a signal of dominance (Bolund et al. 2007).

Although this study shows that the presence of potential partners affects bill color, the precise mechanism underlying this effect is currently unknown. Circulating carotenoids are usually interpreted as a proxy for the amount of pigments that can be allocated to different functions. Our

results show that the link between bill color and circulating carotenoids can be more complex than previously thought. By replicating the experiment with different individuals, we found that both experimental treatments affected the amount of circulating carotenoids in a nonparallel way. Although in both replicates, activation of the immune system reduced the amount of circulating carotenoids (a result that has already been reported in a number of previous studies), the effect of the social environment was statistically significant in only one of the two replicates (see Fig. 2). We cannot fully exclude that this discrepancy originates from the difference between the methods used to quantify carotenoids in the two replicates of the experiment. However, it remains unlikely because both methods are known to provide reliable quantifications. Therefore, our results underline the complexity of links between circulating carotenoids and secondary sexual traits.

The upregulation of bill color observed in males that were maintained in the presence of females opens the question of the source of the pigments and the physiological processes leading to the modulation of the signal. All individuals used in this experiment had access to the same food; therefore, the environmental availability of carotenoids did not differ among males. Several hypotheses may be put forward. First, males in the mixed environment might allocate more carotenoids to bill color at the expense of other functions or might improve their efficiency during the process of carotenoid assimilation, transport, and conversion. For instance, recent work has shown that testosterone upregulates the production of lipoproteins that carry carotenoids to peripheral tissue and enhance bill color in zebra finches (McGraw 2006; McGraw et al. 2006; see also Blas et al. 2006: in red-legged partridges). Because testosterone has been shown to enhance bill color in male zebra finches (McGraw 2006; McGraw et al. 2006), we may hypothesize that this hormone had a key role in our study: its secretion might have been influenced by the presence of females, with consequences on bill color. However, further studies are required to investigate this hypothesis. Second, once ingested and absorbed, carotenoids can be stored in tissues, such as the liver, and can be released from tissues back into the plasma (Furr and Clark 2004). Therefore, it is possible that males maintained in the presence of females released stored carotenoids to use them for the expression of the signal. Third, we cannot rule out that blood flow under keratinized layers of the bill was stimulated in males kept with females. Nevertheless, further works are required to elucidate the biochemical control that governs the release of stored carotenoids or increases blood flow under keratinized tissue of the bill. Finally, males housed with females may have increased their feeding rate and ingested more carotenoids, then deposited in higher amount into bill tissues. Again, exact mechanisms remain unknown, but as in above hypotheses, male sexual hormones (androgens) are potential candidates for this role as they are rapidly produced in response to sexual stimuli. It would be interesting to explore this avenue in the near future.

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