

Birds receiving extra carotenoids keep singing during the sickness phase induced by inflammation

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Abstract Life history theory predicts that individuals have to trade-off resources between diverse energy-demanding activities, such as mounting an immune response and performing advertisement behaviour. The availability of immunomodulatory micronutrients can affect this trade-off. Carotenoids can upregulate both the humoral and cell-mediated immune response, but little is known about their effect on behavioural traits during the sickness phase induced by a common inflammation. To investigate whether dietary carotenoids can mitigate the severity of the sickness syndrome and promote fitness-related traits, we studied how the song rate of captive male European starlings (*Sturnus vulgaris*, Linnaeus 1758) receiving dietary carotenoids and coping with a challenge (lipopolysaccharide, LPS) mimicking a bacterial infection varied during the sickness phase and the subsequent recovery phase. We found that birds not provisioned with carotenoids and injected with LPS sang less than control birds during the sickness phase, but not during the recovery phase. Conversely, birds provided access to a carotenoid-enriched diet never decreased their song rate. Our results show that immune-challenged birds have to trade-off between mounting an immune response and advertising only when their access to dietary carotenoids is limited. No differences in song rate were observed between treatments during the recovery phase. Our study is the first to investigate the role of dietary carotenoids

on a behavioural syndrome-like sickness and to show that providing a carotenoid-rich diet can alleviate the social costs associated with the occurrence of an inflammation.

Keywords Lipopolysaccharide (LPS) · Dietary carotenoid · Lutein · Birdsong · Sickness behaviour · Bacterial infection · Acute phase response · Fitness-related traits

Introduction

Life history theory predicts that organisms face competing demands of limited resources that have to be carefully traded-off in accordance with the context (Zera and Harshman 2001; Stearns 2008). Sexually selected traits have been intensively studied within this theoretical framework because they are costly to produce and mirror individual condition (Zahavi 1975; Hamilton and Zuk 1982). Avian song represents a good example in this respect, and several studies have reported an association between the condition of the immune system and song expression (Saino et al. 1997; Møller et al. 2000; Garamszegi et al. 2004). More specifically, it has been shown that an immune activation can negatively affect song performance (Owen-Ashley et al. 2006; Munoz et al. 2010; Lopes et al. 2012). For example, Munoz et al. (2010) reported that white-crowned sparrows (*Zonotrichia leucophrys oriantha*, Oberholser, 1932) decreased their singing output and shifted their vocal performances from a structured song to a more simple alarm call when the immune system was activated.

It is widely acknowledged that mounting an immune response is a resource-demanding process (Norris and Evans 2000; Zera and Harshman 2001; Zuk and Stoehr 2002; Lochmiller and Deerenberg 2000), and among diverse immune activations, the acute phase response can be considered

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as the most energetically demanding one (Bonneaud et al. 2003; Koutsos et al. 2003; Hasselquist and Nilsson 2012). In vertebrates, including birds, the acute phase response is an example of an immuno-neuro-endocrine circuit characterized by both physiological and behavioural patterns (Owen-Ashley and Wingfield 2012). The physiological responses involved are non-specific, being activated by a diverse array of pathogens, from viruses to multicellular pathogens. These responses are generally represented by the secretion of cytokines from the immune cells, the upregulation of hepatic acute phase proteins (e.g. haptoglobin), the suppression of hepatic albumin and lipoproteins, an unbalance of body thermoregulation and the secretion of glucocorticoids (Hart 1988; Owen-Ashley and Wingfield 2007).

The expression of these inflammatory processes is energetically expensive (Hasselquist and Nilsson 2012) and is usually accompanied by a behavioural syndrome of sickness characterized by a diminishing of general activity (resting, sleeping behaviour, anorexia and limitation of social interactions) that enables the host to redirect resources to both inflammatory and recovery processes (Hart 1988). Although sickness behaviour is, in this sense, adaptive, it also represents a further detrimental effect of an infection that can have great repercussions on individual fitness (Owen-Ashley and Wingfield 2012). For example, sick individuals usually cease to perform crucial activities such as foraging, mating, defending the territory or keeping their social rank by interacting with conspecifics (Owen-Ashley and Wingfield 2006). Therefore, when infected individuals have the chance to mitigate the severity of sickness to accomplish more urgent functions they usually do so (Owen-Ashley and Wingfield 2012). However, the physiological mechanisms that enable bypassing the sickness phase and the evolutionary implications of the variation in expressing sickness behaviour are far from being clear (Adelman and Martin 2009). For example, in a context of sexual signalling, we can expect that birds that can bypass a sickness syndrome, keeping singing also when their immune system has been challenged, will benefit by maintaining their territory, their mate or their social rank.

Intriguingly, it seems that the immunological resource requirements are not just energetic (calories) but qualitative as well (Hasselquist and Nilsson 2012). For example, the environmental conditions determining the availability of dietary micronutrients with immunomodulatory effects can alleviate the cost of mounting an immunoresponse. Carotenoids are a clear example in this respect (Blount et al. 2003; McGraw and Ardia 2003; Smith et al. 2007; Pap et al. 2009; Sepp et al. 2011; McGraw and Toomey 2013). These pigments have attracted the attention of ecologists because of their dual role. On the one hand they are involved in health-related functions being immunoregulators (Blount 2004; Svensson and Wong 2011), antioxidants (Pérez-Rodríguez 2009) and regulators of the metabolism of proteins and lipids (Zhang et al. 1992;

Wang et al. 2007). On the other hand they promote the expression of sexual traits as pigments (Svensson and Wong 2011) or enhance the expression of non-visual traits, such as song (Van Hout et al. 2011, Casagrande et al. 2014). Importantly, carotenoids are limited resources for animals (Lozano 1994; Blount 2004; Svensson and Wong 2011), which can be acquired solely through consumption of food containing carotenoids, except for extremely rare exceptions (Altincicek et al. 2012).

Although the role of carotenoids in benefiting immune functions is relatively well studied (see below), less is known about the differential effect that these pigments can have on the different components of the immune response. For example, while it is well established that they upregulate both the humoral and cell-mediated immune response (Blount et al. 2003; McGraw and Ardia 2003; Chew and Park 2004a; Pérez-Rodríguez et al. 2008), little is known about the effect of dietary carotenoids on the acute phase syndrome, except for limited studies in poultry science (Koutsos and Kasing 2001; Koutsos et al. 2006; Selvaraj et al. 2006; Shanmugasundaram and Selvaraj 2011). These studies show that carotenoids can have an anti-inflammatory action. Koutsos et al. (2006), for example, carried out a physiological study in growing chickens (*Gallus gallus domesticus*, Linnaeus 1758) where birds were exposed to different concentrations of carotenoids, both in ovo and in posthatch phases. They found that higher concentrations of carotenoids decrease the inflammatory response, assessed by measuring the concentration of circulating haptoglobin, an acute phase protein. Although behavioural traits were not considered in their study, a consequent mitigation of an energy-saving behaviour, such as sickness, can be predicted. Nevertheless, independently from carotenoids, it is not so evident that a suppression of inflammation is always translated in suppression of sickness behaviour and vice versa (Lopes et al. 2013).

Several studies have described the physiological effects of carotenoids on humoral or cell-mediated responses (Svensson and Wong 2011), but the behavioural response associated with inflammation has largely been neglected. In this study, we have investigated the possibility that dietary carotenoids alleviate the intensity of sickness behaviour enabling the birds to continue to perform singing behaviour. We immunized birds with a bacterial lipopolysaccharide (LPS), a non-pathogenic endotoxin that elicits solely an immune response, but without further complications as likely to result from the introduction of a pathogen (Munoz et al. 2010). LPS triggers both the acute phase response and the humoral immune response, by mimicking an actual bacterial infection. Although bacterial infections are probably the most common challenges for vertebrates, it is still not clear how individuals cope with this challenge at the behavioural level, by controlling the severity of the sickness syndrome and, consequently, the effect on their fitness. In accordance with the existing literature, we firstly predict that if energy and resources to generate an immune

response are acquired at the expense of song behaviour (trade-off between mounting an immune response and singing), then we expect that LPS-treated individuals would reduce their song rate. Secondly, since carotenoids can mitigate the acute phase response of inflammation, we expect that, within immunized birds, those provided with carotenoid (Caro birds) do not lower the song rate during the sickness phase, as foreseen in controls (Cont birds).

We used male European starlings, *Sturnus vulgaris*, to test these hypotheses. They are a highly suitable model species because their complex song is sexually selected (Eens 1997), and song performance has been shown to mirror their immune competence (Duffy and Ball 2002). Moreover, it has recently been demonstrated that song rate is upregulated by carotenoids in this species, showing that carotenoids are a limited resource that has to be traded-off between different functions (Van Hout et al. 2011; Casagrande et al. 2014).

Material and methods

Housing conditions and experimental design

This study used 56 adult European starling males that were captured as juveniles in the Antwerp region in 2010 and kept in large outdoor aviaries at the campus of the University of Antwerp (GPS coordinates: N 51° 13' 11.9532", E 4° 23' 46.4532"). The European starling is not an endangered or protected species and has been shown to easily adapt to captivity and to show normal social behaviour in these conditions (Eens et al. 1990, 1991; Van Hout et al. 2011). All males were kept under similar captive conditions until the experiment started. One month before the experiment, the males were randomly assigned to four outdoor aviaries. Aviaries hosting Caro and Cont groups alternated with each other, and birds of each aviary were in visual and acoustic contact with birds of other aviaries. The aviaries, measuring ($L \times W \times H$) 27.0 × 7.0 × 2.75 m each, were equipped with 14 nestboxes. Each nestbox contained a singing perch and other perches were located at several places in the aviary as described in Van Hout et al. (2011). Food and water were provided ad libitum. All starlings were marked with a unique combination of coloured bands, which allowed individual recognition from a remote hidden observation. Twenty-eight birds received food enriched with carotenoids (Caro group) for 30 days before they were immunized on the 6th of December, (Fig. 1) while the other 28 males were kept under a standard maintenance diet (Cont group). Each dietary treatment was represented by two aviaries (two Caro and two Cont) of 14 birds. Seven birds per cage were randomly immunized and four groups were formed: Cont-Cont (birds that did not receive extra carotenoids and were not immunized), Cont-LPS (birds that did not receive extra carotenoids and were immunized), Caro-Cont (birds that

received extra carotenoids and were not immunized) and Caro-LPS (birds that received extra carotenoids and were immunized). The experiment was carried out from the 6th to the 12th of December 2012; thus, the birds were exposed to a natural decreasing photoperiod.

Birds belonging to the Caro group were kept on a carotenoid-rich diet starting 30 days before the immunization (Fig. 1; see also below). Before the dietary treatment, there was no difference in song rate between Caro and Cont males (Casagrande et al. 2014). We registered a positive effect of carotenoid treatment on song performance after 30 days (Casagrande et al. 2014) in accordance with previous results (Van Hout et al. 2011). Therefore, Caro males sang at higher song rates than Cont males at the onset of the present experiment (Fig. 2).

Carotenoid supplementation

Dietary carotenoid supplementation was performed in accordance to the previous studies (Van Hout et al. 2011; Casagrande et al. 2014). Briefly, we mixed 50 g of ORO GLO™ (Kemin Industries Inc., Iowa, USA; extracted from marigolds—*Tagetes erecta*, Linnaeus 1753—and containing 15.0 g kg⁻¹ of lutein) per 1 kg of the starlings' standard feed (mixed 1:3 Orlux Uni Patee, Orlux, Belgium and 2:3 Merelkorrel Speciaal, Nifra, Van Camp, Belgium). Lutein content is not reported in these products; nevertheless, in previous studies (Van Hout et al. 2011; Casagrande et al. 2014), we ascertained that the provided dose of lutein is effective in increasing the concentration of circulating lutein to the levels observed in free-ranging males of the closely related spotless starling *Sturnus unicolor* during the reproductive season (mean ± standard error (se), 9.20 ± 0.64 µg mL⁻¹; Navarro et al. 2010). As reported in Casagrande et al. (2014), indeed, on the day of immunization, the concentration of circulating lutein in Caro birds (10.16 µg mL⁻¹) was very close to the one registered in the spotless starling. Diversely, Cont birds showed a significantly lower concentration (3.23 µg mL⁻¹, $p=0.005$, Casagrande et al. 2014), suggesting that the access to dietary lutein was limited in this group. Since plasma carotenoids usually fluctuate over time, with a maximum peak during the reproductive season and a minimum peak during the non-reproductive season (Casagrande et al. 2011), working in winter time enabled us to obtain two groups (high and low circulating carotenoids), both having circulating lutein within a natural range.

Sampling and immunization

Birds were immunized with bacterial LPS, a component of the bacterial membrane known for being pro-inflammatory and for causing a humoral response with a first peak of anti-LPS production after 7 days (Parmentier et al. 2008). In birds, the

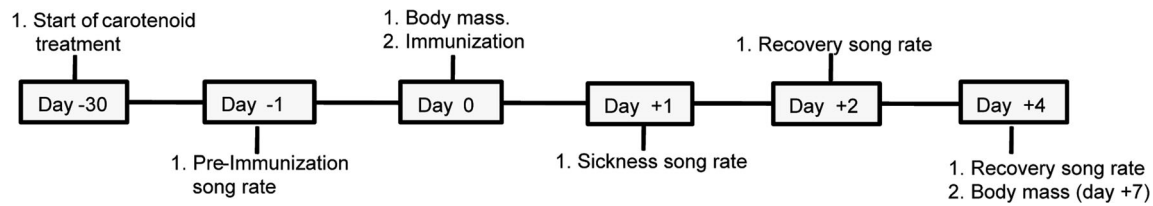


Fig. 1 Design of the experiment and summary of all activities made on each sampling day

acute phase response usually appears within 3 h from immunization and lasts 24–48 h (Owen-Ashley et al. 2006; Burness et al., 2010; Lopes et al. 2012). All birds were captured to measure the body mass (to the nearest 1 g) as an indicator of condition (Van Hout et al. 2012) immediately prior to immunization and 7 days later. Just before releasing the birds in their cage, we subcutaneously injected seven birds per cage in accordance to the treatment they were randomly assigned to (immunized or

control). LPS birds received 0.1 mL of a sterile solution of LPS derived from *Escherichia coli* (serotype 055:B5; cat no. L4005, Sigma-Aldrich) and phosphate buffer saline (PBS 0.01 M, 7.4 pH) resulting in a dose of 2 mg/kg tissue, while controls were injected solely with the saline solution of equal volume. The dose was chosen in accordance with data from the literature (Koutsos et al. 2006). After performing a trial in two birds and one control bird, a few days prior to the experiment, we ascertained the

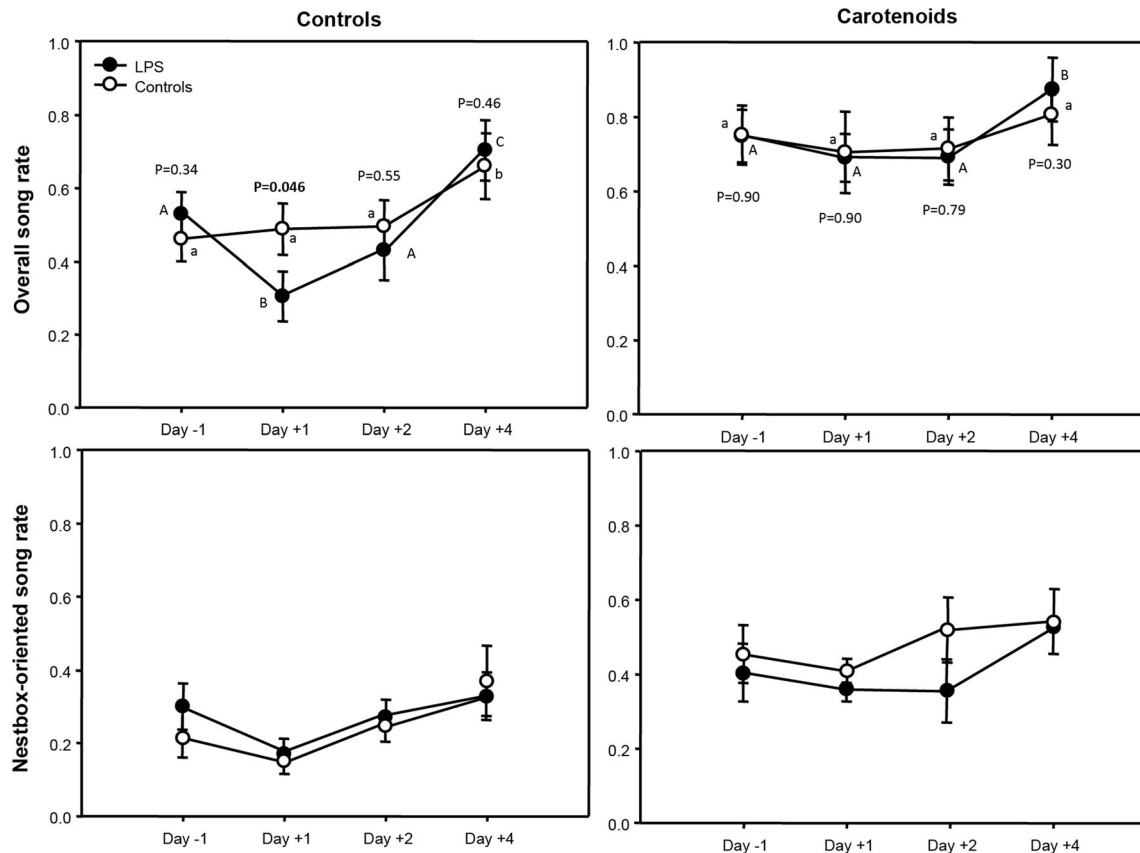


Fig. 2 Overall song rate and nestbox-oriented song rate from 1 day before immunization to 4 days after immunization in controls (Cont-) (left) and carotenoid-treated (Caro-) male European starlings. Letters (capital for immunized birds) refer to post hoc analyses within each group in relation to day -1 (different letters are reported when post hoc analysis resulted in $p < 0.05$). p values refer to post hoc analysis between immunization treatments within the same day. Day +1 represents the sickness phase, while days +2 and +4 the recovery phase. Between dietary treatments within day post hoc (not reported in the figure): day

-1, Cont-Cont vs. Caro-Cont: $p = 0.009$, Cont-LPS vs. Caro-LPS: $p = 0.047$; day +1, Cont-Cont vs. Caro-Cont: $p = 0.05$, Cont-LPS vs. Caro-LPS: $p = 0.002$; day +2, Cont-Cont vs. Caro-Cont: $p = 0.047$, Cont-LPS vs. Caro-LPS: $p = 0.02$; day +4, Cont-Cont vs. Caro-Cont: $p = 0.11$, Cont-LPS vs. Caro-LPS: $p = 0.06$. Although we did not find any significant effect of the three-way interaction for nestbox-oriented song rate, we checked post hoc to exclude any significant difference between Caro-Cont and Caro-LPS, finding all $p > 0.17$

occurrence of sickness syndrome (resting, sleeping) 3 h after injection (data not shown).

Behavioural observations

We simultaneously monitored the singing behaviour of all the starlings hosted in one aviary, using a point sampling technique, with an interval of 1 min (Van Hout et al. 2011; Casagrande et al. 2014). We performed one observation session of 45–60 min per day per aviary between 0900 h and 1300 h 1 day before immunization, and, respectively, 1, 2 and 4 days after immunization. Indeed, it is known that the inflammation caused by LPS can affect birdsong also 24 h after the challenge (Owen-Ashley et al. 2006; Munoz et al. 2010). Overall song rate was defined as the proportion of samples during which a male was singing (in any and all locations) compared to the total number of samples. As nestbox-oriented song is more linked to the reproductive state and more influenced by the endocrine status than overall song rate (Van Hout et al. 2011), we also determined the proportion of the time spent performing nestbox-oriented song rate, which only includes song production inside or on the top of the nestbox or on the perch connected to the nestbox (Pinxten et al. 2002).

We described sickness behaviour 24 h (day +1) after the treatment in accordance with other studies aimed at evaluating the effect of LPS on birdsong (Owen-Ashley et al. 2006; Munoz et al. 2010).

Data analysis

Given our experimental design, we predicted that male song rate honestly signals the condition of the bearer and that, in immunized birds, it decreases in controls, but not in carotenoid-treated birds. We tested this prediction both during the sickness phase (approximately 24 h after immunization—day +1) and during recovery (2 and 4 days after immunization) by using linear mixed models with a repeated measures design. Response variables were nestbox-oriented song rate, overall song rate and body mass. In each model, we included dietary treatment (Caro and Cont), immunization (LPS and Cont) and time (day −1, day +1, day +2, day +4 to analyse song behaviour; day −1 and day +7 to analyse body mass) and their interaction as fixed factors. Individual (nested within cage) and cage were included as random factors to control for the non-independence of multiple measures from the same individual and from individuals sharing the same cage. Post hoc comparisons between and within groups were performed using pairwise differences of least square means. Statistical analyses were performed using SAS 9.3 (Cary, NC, USA). Response variables were transformed when necessary to achieve normality of residuals (arcsine square root

transformation of overall song rate and nestbox-oriented song rate). Values reported in the text and graphs are means±se

Results

On the day of the immunization, Caro birds had been receiving extra dietary carotenoids during the preceding 30-day period. We found a positive effect of carotenoid treatment on song performance following these 30 days of dietary treatment (Casagrande et al. 2014), in accordance with previous published results (Van Hout et al. 2011). Therefore, at the onset of the present experiment, Caro males sang at higher song rates than Cont males ($p<0.001$; Fig. 2; Casagrande et al. 2014).

Song behaviour variation between sickness phase and the recovery phase: comparison between 1 day before and 4 days after injection

The overall song rate varied during time in a different way for the four groups (immunization×dietary treatment×time, $F_{(10, 156)}=1.88$, $p=0.05$; immunization $F_{(1,156)}=0.02$, $p=0.89$; dietary treatment $F_{(1,156)}=14.95$, $p=0.0002$; time $F_{(3,156)}=16.95$, $p<0.0001$). Specifically, the overall song rate of Cont-LPS birds significantly decreased 1 day after immunization ($p=0.0002$) while song rate of Caro-LPS did not ($p=0.40$; Fig. 2) decrease. All the other differences within groups between different days were not significant with the exception of day +4, when both Cont-LPS and Cont-Cont birds sang more compared to day −1 ($p=0.008$ and $p=0.04$, respectively). Comparisons within dietary groups and between immunization treatments showed that Cont-LPS sang less than Cont-Cont birds 24 h after the immunization ($p=0.046$; Fig. 2). All the other comparisons proved not significant (Fig. 2).

Nestbox-oriented song rate did not vary during the two treatments (immunization×dietary treatment×time, $F_{(10, 156)}=0.53$, $p=0.87$; LPS $F_{(1,156)}=1.16$, $p=0.69$). In general, birds that received carotenoids showed a higher rate of nestbox-oriented song (0.24 ± 0.03) than controls (0.12 ± 0.02) (dietary treatment $F_{(1,156)}=8.29$, $p=0.0045$). All birds decreased nestbox-oriented song rate 1 day after injection (day −1, 0.17 ± 0.02 ; day +1, 0.14 ± 0.02) and increased it over the following days (day +2, 0.17 ± 0.02 ; day +4, 0.25 ± 0.03 ; time $F_{(3,156)}=6.51$, $p=0.0004$) (see also Fig. 2).

Body mass

Body mass tended to vary only in accordance with time ($F_{(1,52)}=3.89$, $p=0.053$), with all birds losing weight from day −1 (83.38 ± 0.67 g) until day +7 (82.23 ± 0.53 g). All the other factors and interactions were not significant (dietary treatment, $F_{(1,52)}=0.43$, $p=0.52$;

LPS, $F_{(1,52)}=1.87$, $p=0.18$; dietary treatment \times immunization \times time $F_{(4,52)}=0.82$, $p=0.52$).

Discussion

Twenty-four hours after injection, corresponding to the sickness phase of the immunoresponse (Owen-Ashley et al. 2006), birds injected with LPS sang less than birds injected with PBS, when they did not receive carotenoids in their food. By contrast, birds receiving carotenoids in their diet did not decrease their song rate during the sickness phase. This result supports both our first and second hypothesis because it shows that immune-challenged birds have to trade-off between mounting an immune response and singing. It also shows that carotenoids are able to downregulate the behavioural component of an inflammatory response (i.e. sickness behaviour) enabling the normal exhibition of a fitness-related behaviour. From the second day of the immunization onwards, with the attenuation of the inflammation, there was no longer a difference in song performance between control and immunized birds within each dietary group. To the best of our knowledge, our study is the first to show that carotenoids provided with the diet can alleviate the costs associated with the occurrence of a sickness syndrome, benefiting the birds in their singing performance.

We found that an inflammatory stimulus, such as LPS, can cause a significant decrease in song rate, in accordance with previous results obtained in other species (Owen-Ashley et al. 2006; Munoz et al. 2010; Lopes et al. 2013). Since the activation of the immunoresponse is expensive (see “Introduction”), a negative relationship between the expression of a demanding trait, such as birdsong, and an inflammatory response was expected (Hasselquist and Nilsson 2012). Within the immune response, the activation of sickness behaviour is particularly costly for an individual. For example, individuals are more exposed to predation due to diminished activity, while the homeostasis of the organism can be compromised because food and water intake is suppressed, and stored resources are mobilized to fuel the inflammatory processes (Adelman and Martin 2009). In addition, the onset of resting and sleeping activities that are registered during the sickness phase interferes with several fitness-related behaviours such as territorial activity, sexual behaviour and parental care (Owen-Ashley and Wingfield 2006; Adelman and Martin 2009). This implies that this component of the immune response can induce significant costs and that individuals that are able to minimize these costs can benefit in terms of fitness. Male starlings exhibit a high song rate throughout the year without a significant difference between the reproductive and non-reproductive season (Eens 1997; Van Hout et al. 2009). During the non-reproductive season, the main function of singing is to maintain a proper social status and to signal the

occupancy of a nest territory (Eens 1997; Spencer et al. 2004). Therefore, it is plausible that birds that cope better with the infection maintain their social status and nesting territory more easily. This type of benefit was evident 24 h after the immunization, when the song rate of immunized Caro birds was similar to the song rate registered before immunization, while this was not the case for immunized Cont birds, which decreased their song rate over the same time period.

Our results appear to indicate that one way to minimize the costs associated with sickness is to consume food rich in carotenoids. This study, to the best of our knowledge, is the first to indicate that carotenoids can alleviate the cost of an inflammation by reducing the adverse effects of sickness behaviour on the expression of birdsong. However, we know from a previous study on chickens (Koutsos et al. 2006) that carotenoids have a clear role in controlling the inflammation processes by diminishing the concentration of circulating haptoglobin.

We did not measure the physiological biomarker of inflammation because it would have been too stressful for the birds to be recaptured after 24 h from the injection, but we can briefly speculate about the potential physiological mechanisms underlying our results. It is possible that the mitigated sickness syndrome mirrored the suppression of the immune response expressed at the physiological level and that starlings fed with extra carotenoids could have switched from a costly inflammatory response to a highly specific, but less costly, antibody production (Lee 2006), accelerating the recovery processes. Nevertheless, another scenario is also possible as the behavioural symptoms of sickness induced by LPS can be suppressed without altering the inflammatory response (Lopes et al. 2013). Indeed, Lopes and colleagues detected the presence of specific inflammatory interleukins both when birds displayed sickness behaviour and when they did not. Another possibility is that carotenoids play a role in the promotion of specific lipids and proteins that could have provided additional resources used to perform an energy-demanding activity such as birdsong. Indeed, carotenoids can promote the synthesis of hepatic proteins and lipids (Zhang et al. 1992; Hininger et al., 2001; Ben-dor et al., 2005; Casagrande et al. 2014) while inflammatory processes tend to suppress them (see “Introduction”) and, moreover, can substantially alter nutrient partitioning directly affecting lipid and protein metabolism (Koutsos et al. 2003). A further explanation is that lutein acted as an antioxidant or as a promoter of the antioxidant network controlling the production of reactive species, which are by-products of inflammation (Costantini and Møller 2009). This may be particularly important because oxidative damage can limit the expression of song behaviour (Casagrande et al. 2014).

Our experimental design enabled us to control for specific factors affecting sickness behaviour. For example, high levels of testosterone usually suppress sickness behaviour, as

observed in the seasonally breeding Gambel's white-crowned sparrow males (Ashley et al. 2009; Lopes et al. 2012). Testosterone can directly inhibit cytokine-producing immune cells via androgen receptors or indirectly promote a redistribution of energetic resources that penalizes the onset of the acute phase response (Ashley et al. 2009). We studied male starlings of the same age, which were kept in a single sex group when they were reproductively inactive, under a short, declining photoperiod, and denied the possibility to see females. Under these conditions, male starlings have very low doses of circulating testosterone (Riters et al. 2002; Van Hout et al. 2009) and they are more prone to exhibit sickness behaviour (Owen-Ashley et al. 2009). A final consideration about the involvement of hormones can be made considering that we also quantified nestbox-oriented song, which is known to be related to circulating testosterone (see Introduction). We did not observe any change in nestbox-oriented song after immunization; most probably because it was at very low levels during this phase of the year, following the hormonal status of singing males (Riters et al. 2002; Casagrande et al. 2014). The lack of variation of this song trait also allows us to exclude a suppression of androgens caused by the activation of the immune system as observed in another study (Casagrande and Groothuis 2011).

Conclusions

While many studies have focused on the effects of carotenoids on humoral or cell-mediated responses of the immune system, by considering what happens at the physiological level, the behavioural response to inflammation has largely been neglected. Bacterial infections are probably the most common challenges that a vertebrate's immune system faces. However, we still do not clearly know how individuals cope with this challenge at the behavioural level. Here, we show for the first time that birds can alleviate the social costs represented by sickness, by consuming a carotenoid-rich diet. This finding has important implications for a better understanding of the ecological, evolutionary and physiological pressures acting on sickness behaviour.

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

Compliance with ethical standards The capture of wild European starlings *S. vulgaris* and their housing in captivity for scientific purpose was approved by the Agency for Nature and Forest (ANB) of the Flemish government (permit number: 08-11344). This study was performed in Antwerp in conformity with the Belgian and Flemish legislation and was approved by the ethical committee for animal experiments (ECD) of the University of Antwerp (ID number: 2011-32). This article does not contain any studies with human participants performed by any of the authors.

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