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Sex differences in carotenoid status and immune performance in zebra finches

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ABSTRACT

Sex differences in immunity are common throughout the animal kingdom, with males typically showing reduced immune capacity compared with females due to the immunosuppressive action of androgens like testosterone. However, in animals (e.g. birds, fishes) in which immunostimulatory carotenoid pigments are used to develop colourful traits, males tend to circulate higher concentrations of these immunomodulators to become more colourful than females. In these instances, it is uncertain how differences in carotenoid status might affect sex-specific patterns of immune function. We tested the relationship between carotenoid status and two measures of immunocompetence in male and female zebra finches (*Taeniopygia guttata*). Non-breeding males circulated a higher concentration of carotenoids through blood than females. Females mounted a significantly higher cell-mediated immune response (to phytohaemagglutinin) than males, but males mounted a significantly higher humoral response (to sheep red blood cells) than females. When supplemented with dietary carotenoids, males showed significant improvements in both cell-mediated and humoral immune performance, while females only showed elevated humoral responsiveness. These results generally support the immunostimulatory role of carotenoids in colourful male and female birds, and indicate that males may gain certain offsetting, carotenoid-facilitated immunological benefits relative to females. It appears that shunting carotenoids to the humoral arm of the immune system – perhaps a more costly yet more effective means of fighting off pathogens – may take priority over elevating cell-mediated immunity in both sexes.

Keywords: carotenoids, immunocompetence, sex differences in immunity, sexual selection, *Taeniopygia guttata*.

INTRODUCTION

Immune defence is generally thought to be an energetically demanding physiological activity (Martin *et al.*, 2002) that has quite severe consequences for the health, life-history

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decisions, reproductive efforts and survival of animals (Zuk, 1996; Rolff, 2002; Zuk and Stoehr, 2002). Males and females often differ in immune performance, with males mounting lower immune responses than females in a variety of species, including several birds (e.g. Magellenic penguins [*Spheniscus magellanicus*]: Moreno *et al.*, 2001; red-winged blackbirds [*Agelaius phoeniceus*]: Hasselquist *et al.*, 1999; red junglefowl [*Gallus gallus*]: Zuk, 1996) and mammals (e.g. voles, mice, rats; Klein and Nelson, 1997, 1998; Rivero *et al.*, 2002). The primary explanation for this sex difference has been the presence of higher testosterone concentrations in males, which directly suppress immune function (see Folstad and Karter, 1994, and references therein). Several other factors may also influence the strength of immune defence in the two sexes, including the amount of mating (Klein and Nelson, 1999; Klein, 2000) or reproductive (Adamo *et al.*, 2001) effort expended, the intensity of aggressive encounters (Klein *et al.*, 1997) and body condition (Yourth *et al.*, 2002).

Although males appear to suffer immunologically due to several different circumstances, there may be other environmental or physiological conditions that boost immunity in males relative to females. One such factor may be carotenoid pigments. Carotenoids are colourful red, orange and yellow molecules that are produced by photosynthetic organisms like plants and algae and acquired by animals through the diet (Olson and Owens, 1998). Many male birds and fishes use carotenoids to colour their integument (e.g. skin, feathers) and become sexually attractive (Hill, 1999a). In these situations, males typically circulate higher levels of carotenoids through the body than do females (Hill, 1995; Bortolotti *et al.*, 1996; Figuerola and Gutierrez, 1998; Negro *et al.*, 1998). Circulating carotenoids can also serve a number of physiological functions, including scavenging potentially damaging free-radicals as well as stimulating the immune system (Bendich, 1989; Lozano, 1994; Vershinin, 1999; Møller *et al.*, 2000; Hughes, 2001). Thus, because of sex differences in the concentrations of carotenoid immunostimulants, males in certain species may actually experience immunological benefits compared with females. Previous studies that have tested for sex differences in immunity were performed with species that are generally carotenoid-deficient (e.g. mammals: Hill, 1999b) or under conditions where carotenoid status was not taken into account.

In this study, we experimentally investigated the immuno-enhancing properties of carotenoids in male and female zebra finches (*Taeniopygia guttata*). Zebra finches are sexually dichromatic passerines, with males developing a bright red, carotenoid-pigmented beak (McGraw *et al.*, 2002). Females also incorporate carotenoids into the beak, but at a lower concentration, resulting in a less colourful, orange appearance (McGraw *et al.*, 2003). Zebra finches acquire carotenoids from the diet of weed and grass seeds that they consume throughout the year (Zann, 1996). Males also circulate a higher concentration of carotenoids through the bloodstream than females (McGraw *et al.*, 2003). This led us to predict that male zebra finches would not show universally depressed immune systems relative to females. To test this idea, we measured blood carotenoid concentrations and immune performance in 10 captive pairs of male and female zebra finches. We measured both cell-mediated and humoral immunity, using standard phytohaemagglutinin (PHA) skin tests (Smits *et al.*, 1999) and a sheep red blood cell haemagglutination assay (SRBC) (Higgins, 1996), respectively. We also conducted an experiment in which we supplemented 10 different male–female pairs of finches with a dose of dietary carotenoids, to determine whether carotenoids can boost cell-mediated and humoral immune function in this species.

METHODS

Housing conditions

Twenty male–female pairs of zebra finches with wild-type plumage were housed in small wire cages (0.6 m long × 0.4 m wide × 0.4 m tall) on a 14 : 10 h light/dark cycle in an animal-approved indoor room on the campus of Cornell University. Males used in this study were the same as those from our previous work on carotenoids and health in zebra finches (McGraw and Ardia, 2003). All birds were in non-breeding condition, housed without nest material or cups, and had similar breeding experience. We fed the finches an *ad libitum* diet of tap water, crushed oystershells and Kaytee® Forti-Diet™ finch blend (Kaytee Products Inc., Chilton, WI) (see McGraw *et al.*, 2002, for components of this seed mix).

Correlational study of immune performance

For 10 unmanipulated pairs of zebra finches, we compared cell-mediated and humoral immunity between the sexes. We subjected each bird to a PHA skin test, which assays an individual's mitogenic, T-lymphocyte responsiveness to a foreign plant protein (Smits *et al.*, 1999), and the SRBC assay, which measures the humoral response to T-dependent antigens (Bacon, 1992; Higgins, 1996). Both tests have been used previously and effectively in assaying cell-mediated and humoral immunity in zebra finches (e.g. Deerenberg *et al.*, 1997; Birkhead *et al.*, 1998; Ewenson *et al.*, 2001), in other songbirds by the authors (e.g. tree swallows, *Tachycineta bicolor*: Ardia *et al.*, 2003), and in relation to carotenoid supplementation in humans (Kramer and Burri, 1997) and mice (Jyonouchi *et al.*, 1994).

To conduct the PHA test, we measured the right wing web of each bird three times with a digital micrometer (to the nearest 0.05 mm) to obtain an average pre-swelling measurement and then injected this area with 0.15 mg of PHA-P (Sigma Chemical Co., St. Louis, MO) in 30 μl of phosphate-buffered saline (PBS) (Hõrak *et al.*, 1999). The birds were immediately placed back in their housing cages and we returned 24 h later to measure the swollen area. We present results as the difference between mean post-injection swelling and mean pre-injection-swelling (*sensu* Smits *et al.*, 1999). Within-individual repeatability of wing-web swelling measurements was high (pre-injection: $R_i = 0.55$, $F_{19,20} = 4.73$, $P = 0.04$; post-injection: $R_i = 0.62$, $F_{19,20} = 3.65$, $P = 0.05$), as measured by the intraclass correlation coefficient (Lessells and Boag, 1987).

To perform the SRBC assay, we first drew 50–100 μl of whole blood from each bird through the alar vein into heparinized microcapillary tubes and the plasma was centrifuged off and saved in 1.5 ml Eppendorf tubes at −80°C for analysis (see also carotenoid analyses below). From 20 μl plasma we determined background, pre-exposure levels to this antigen. We then injected each bird intra-abdominally with 5×10^7 sheep red blood cells (ICN Biomedicals, Aurora, OH) suspended in 100 μl PBS (*sensu* Deerenberg *et al.*, 1997). Nine days later, we returned to draw blood again from the finches to determine post-exposure antibody titres in plasma. We waited 9 days post-immunization to sample blood because a previous study with zebra finches showed that responsiveness to this antigen peaked at this time (Birkhead *et al.*, 1998). We determined antibody concentrations from 20 μl plasma using a base-2 serial dilution haemagglutination test (*sensu* Roitt *et al.*, 2001). For each plate, we ran both a positive and negative control. Assays were run in duplicate and we report averages here. Again, repeatabilities were high for both sets of

measurements (pre-exposure: $R_i = 0.60$, $F_{19,20} = 3.91$, $P = 0.03$; post-exposure: $R_i = 0.65$, $F_{19,20} = 4.65$, $P = 0.01$).

Although we had determined in a previous study that male zebra finches circulate a higher concentration of carotenoids through blood than females (McGraw *et al.*, 2003), we wanted to be sure that this was true in this sample of birds as well. Thus, following the methods of McGraw *et al.* (2002), we extracted carotenoids from plasma and used high-performance liquid chromatography to quantify the types and amounts present in birds at the time immune performance was measured.

Carotenoid-supplementation experiment

To determine if carotenoids do in fact elevate immunocompetence in these birds, we performed an experiment in which we added carotenoids to the diet of 10 different male–female pairs of finches, for comparison with the 10 control pairs described above. In an earlier study of food intake, we determined that individual birds consume a range of 2–4 g of seeds per day (McGraw *et al.*, 2003), which amounts to 20–40 μg of carotenoids daily. Ninety-five per cent of all carotenoids in this seed mix are made up of the two major plant xanthophylls, lutein (78%) and zeaxanthin (17%) (McGraw *et al.*, 2002). Thus, we designed our experiment to manipulate the concentration of these two primary dietary carotenoids following these relative and absolute amounts, using water-dispersable lutein and zeaxanthin beadlets kindly supplied by Roche Vitamins Inc. (Parsippany, NJ).

These carotenoid-provisioned birds received 9 μg lutein and 2 μg zeaxanthin per millilitre of drinking water (whereas control birds received no carotenoids in water). In a pilot study, we found that birds drank 2–4 ml of water per day; thus, the high-carotenoid group was receiving a dose approximately twice that of daily carotenoid intake. Because circulating blood carotenoid concentrations in provisioned zebra finches remained within the physiological limits of non-provisioned finches during our study (see Results), this proved to be a reasonable dose. Supplementation continued for 5 weeks, which is sufficient time for carotenoid enrichment to take effect in a host of animals (e.g. humans: Ringer *et al.*, 1991; domestic chickens [*Gallus domesticus*]: Bauernfiend, 1981), and was terminated on the day when immune measures were completed (day 9 of the SRBC assay). We performed the procedures described previously for control birds to determine plasma carotenoid concentration and cell-mediated and humoral immunocompetence. We timed all bleeding events (pre-supplementation, 4 weeks after supplementation but just prior to immunization, and at the end of the study, which was on day 9 of the SRBC assay) and immunological assays so that they occurred on the same days for control and treatment groups.

Statistical analyses

All data conformed to the assumptions of parametric statistics (e.g. normality, homoscedascity), so we used analyses of variance to compare immune performance and blood carotenoid concentrations between the sexes and in relation to carotenoid treatment.

RESULTS

Correlational study of immune performance

Plasma carotenoid concentrations

Total plasma carotenoid concentrations were significantly higher in control, non-breeding males than in females (Fig. 1). Males circulated just under twice as many plasma carotenoids (range 70–85% more) than females in all three blood-sampling periods: at the beginning of the study (ANOVA, $F_{1,18} = 15.2$, $P < 0.001$), on the day before immunization ($F_{1,18} = 11.5$, $P = 0.003$) and 9 days post-immunization ($F_{1,18} = 7.8$, $P < 0.01$). Males circulated higher concentrations of each of the four plasma carotenoids than did females (Fig. 1; $P < 0.03$ for all individual sampling periods), but the sexes did not differ in the relative proportions of the different pigments (ANOVA, $P > 0.07$ for all sampling periods; as in McGraw *et al.*, 2003). It should be pointed out that overall carotenoid concentrations in these captive birds (means 13–40 $\mu g \cdot ml^{-1}$) were comparable to (e.g. American kestrels [*Falco sparverius*]: Bortolotti *et al.*, 2000) or even higher than (e.g. barn swallow [*Hirundo rustica*]: Saino *et al.*, 1999; lesser black-backed gull [*Larus fuscus*]: Blount *et al.*, 2002) those found in wild, carotenoid-pigmented birds (see also Tella *et al.*, 2004), indicating that these finches did not suffer from a carotenoid-deprived diet.

Immune activity

Females mounted a significantly higher cell-mediated immune response (to PHA) than did males (Fig. 2A). Males, however, produced significantly more antibodies

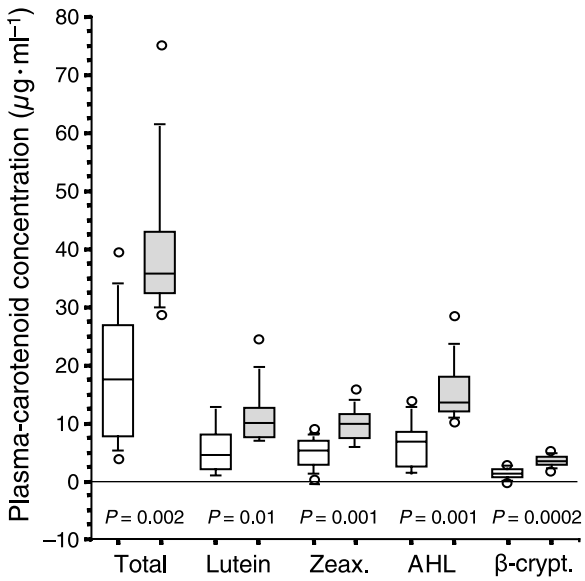

Fig. 1. Plasma-carotenoid concentrations of 10 non-breeding adult male–female pairs of zebra finches that partook in the correlational portion of this study (mean ± standard error of the mean). Shaded boxes denote males, open boxes denote females. Data analysed for each sampling period show the same pattern (see Results). *Abbreviations*: Zeax. = zeaxanthin; AHL = 2′,3′-anhydrolutein; *β*-crypt. = *β*-cryptoxanthin.

in response to our humoral immune challenge (sheep red blood cells) than did females (Fig. 2B).

Carotenoid supplementation experiment

Plasma carotenoid concentrations

Male and female finches supplemented with the dietary carotenoids lutein and zeaxanthin for 5 weeks showed dramatic increases in plasma carotenoid concentrations. On average, females showed a nearly 8 $\mu g \cdot ml^{-1}$, or 75%, increase in total blood carotenoid concentration in response to our treatment (Fig. 3). For comparison, plasma carotenoids in our 10 control

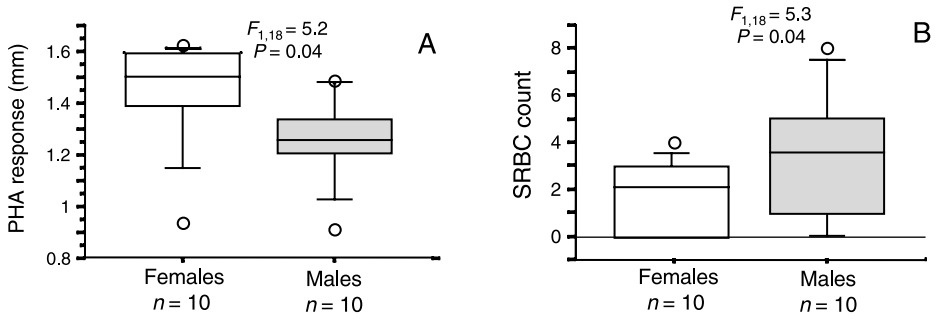

Fig. 2. Responses of male and female zebra finches to (A) cell-mediated and (B) humoral immune challenges. Cell-mediated immunity was measured as the change in wing-web swelling when challenged with a foreign plant protein (PHA) during a 24-h period. Humoral immunity was determined using the SRBC haemagglutination assay. Values are presented as the log of the reciprocal of the last dilution showing agglutination (*sensu* Birkhead *et al.*, 1998).

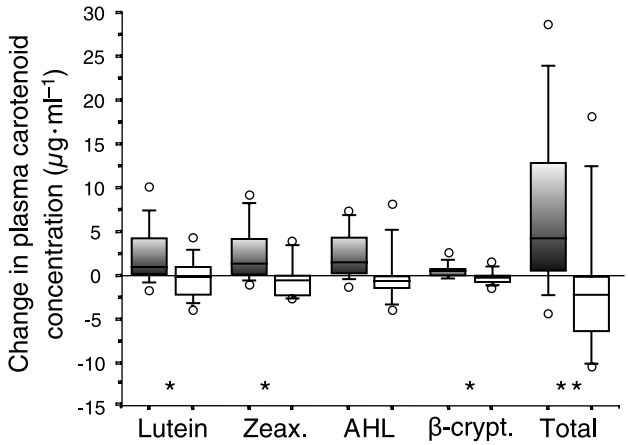

Fig. 3. Bar chart showing the mean (± standard error of the mean) change in plasma-carotenoid concentrations over the 4 weeks of our carotenoid-supplementation experiment. Shaded boxes indicate carotenoid-supplemented females ($n = 10$), whereas open boxes denote control females ($n = 10$). Note the significant increases in carotenoid concentrations in provisioned females: $*P < 0.05$; $**P < 0.01$. Similar results were obtained for males (see McGraw and Ardia, 2003). *Abbreviations*: Zeax. = zeaxanthin; AHL = 2′,3′-anhydrolutein; *β*-crypt. = *β*-cryptoxanthin.

females showed a small but statistically insignificant (paired t-test, $t_9 = -0.43$, $P = 0.68$) decrease ($-1.1\ \mu g \cdot ml^{-1}$, or an 8% loss) during this same period. In a previous study (McGraw and Ardia, 2003), we showed that pigment-provisioned males showed a similar ($8\ \mu g \cdot ml^{-1}$, or 22% increase) and statistically significant increase in blood carotenoid concentration as well, while those in our 10 control males decreased slightly ($-2\ \mu g \cdot ml^{-1}$, or a 6% loss), as occurred in females. It is worth noting that males circulated higher concentrations of carotenoids than did females when treated with carotenoids as well (at 4 weeks: $F_{1,18} = 14.6$, $P < 0.001$; at the end of the study: $F_{1,18} = 4.2$, $P < 0.05$).

Immune activity

Carotenoid-supplemented males showed significantly elevated cell-mediated and humoral immune responses compared with control males (see McGraw and Ardia, 2003). Carotenoid-provisioned females mounted higher humoral responses than controls, but we found no significant difference in cell-mediated immune performance between treatment and control females (Fig. 4).

Because humoral immunity responded positively to carotenoid enrichment in both sexes, the sex difference in antibody production to SRBC assays was retained in this experiment, with post-treatment males still producing more than post-treatment females (*post-hoc* Fisher's PLSD test, $P = 0.04$). However, because cell-mediated immune responses were elevated only in males after carotenoid supplementation, the prior sex difference in PHA responsiveness was eliminated, and nearly reversed, with males mounting (although not significantly so) stronger responses than females (*post-hoc* Fisher's PLSD test, $P = 0.11$).

DISCUSSION

The basic notion that carotenoids are immunostimulatory molecules has been confirmed in a number of mammals (e.g. humans: Gerster, 1993; cats: Kim *et al.*, 2000a; dogs: Kim *et al.*, 2000b; rats: Zhao *et al.*, 1998; mice: Chew *et al.*, 1996; Jyonouchi *et al.*, 1996), certain fishes (e.g. rainbow trout: Amar *et al.*, 2000, 2001; parrotfish: Tachibana *et al.*, 1997), and recently in colourful male birds. Two previous studies (Blount *et al.*, 2003a; McGraw and Ardia, 2003) demonstrated experimentally that carotenoids boost immunity in male zebra finches. Here we show that these immunological benefits of carotenoid accumulation can be extended to female birds with ornamental colours. Males

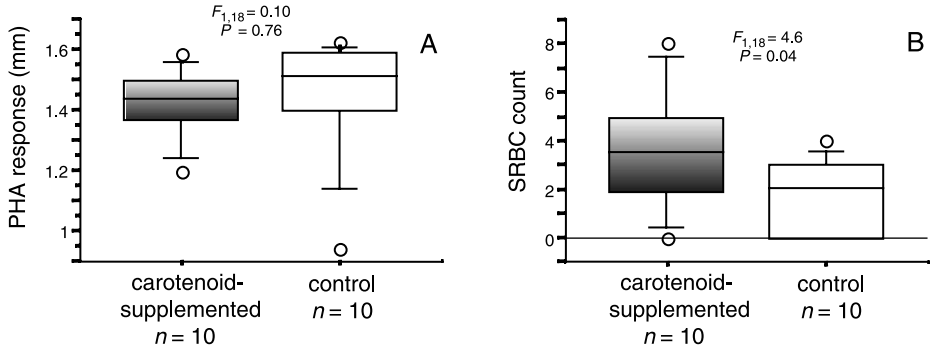

Fig. 4. Effect of carotenoid enrichment on (A) cell-mediated and (B) humoral immune performance in female zebra finches (mean ± standard error of the mean). See Fig. 2 legend for more details. Comparable data for males are presented in McGraw and Ardia (2003).

and females of many avian species, including zebra finches (Burley and Coopersmith, 1987; Houtman, 1992; Blount *et al.*, 2003a; but see Collins and ten Cate, 1996), also benefit by accumulating high concentrations of these compounds for use as integumentary colorants that attract mates (reviewed in Hill, 1999a). Our results indicate that elevated carotenoid status in both sexes may improve their health and make them particularly attractive, high-quality individuals with which to mate.

The focal premise of this study was the idea that, while male animals typically suffer immunologically compared with females, the presence of high concentrations of carotenoid pigments in males may dampen or negate such an immunocompromised state. Here, we found that male zebra finches circulated higher concentrations of immunostimulatory carotenoids than did females under several different conditions (e.g. during non-breeding periods, whether immunologically challenged or not). This sex difference in carotenoid status has been observed in several other wild birds (e.g. house finch [*Carpodacus mexicanus*]: Hill, 1995; cirl bunting [*Emberiza cirlus*]: Figuerola and Gutierrez, 1998). Thus, males of these species may not be expected to mount universally lower immune responses than females. In fact, we found that the humoral immune responses of unmanipulated male zebra finches were superior to those of females. However, we also found that females performed better than males in our cell-mediated immune challenge. A previous study of immunity in captive and wild zebra finches similarly found that females had a higher total leukocyte count than males (Ewenson *et al.*, 2001).

Although several studies have stressed the importance of considering different branches of the immune system because they may respond differently to environmental or physiological stressors (e.g. Zuk and Johnsen, 1998; Norris and Evans, 2000; Blount *et al.*, 2003b), it is often argued that males show reduced cell-mediated *and* humoral immunoresponsiveness compared with females (e.g. Grossman *et al.*, 1991; Klein, 2000). Here, we found the sexes to differ in the relative strength of these two arms of the immune system. It is tempting to speculate that the abundance of carotenoids in male zebra finches specifically aided the humoral arm of the immune system. We are aware of only one other case where male birds appeared to perform better immunologically than females (in European starlings [*Sturnus vulgaris*]: Duffy *et al.*, 2000), and it is curious that this species also uses carotenoids as colorants in sexual displays (e.g. yellow-pigmented beak). We realize, however, that these data are only correlational and that there remain more conclusive tests that could be done to evaluate this hypothesis. For example, assuming that testosterone concentrations primarily mediate immunosuppression in males, one could concurrently manipulate testosterone and carotenoid concentrations in males and females to determine whether sex-related immune superiority could be reversed. Also, the immune responses of closely related species that differ in carotenoid content (one showing a sex difference, the other not) could be compared to establish whether in fact males are immunocomprised relative to females when they do not circulate more blood carotenoids.

When supplemented with carotenoids, males showed elevated cell-mediated and humoral immunoresponsiveness (McGraw and Ardia, 2003), but only the humoral arm of the immune system was enhanced in females. This again indicates something particularly unique or valuable about the humoral arm of the immune system (over cell-mediated responsiveness) in relation to carotenoids (recall that males had unexpectedly higher humoral responses than females). Humoral immunity involves the *de novo* production of antibody-producing lymphocytes (Higgins, 1996), whereas a cell-mediated immune response to PHA involves a non-specific and transient response of circulating T lymphocytes to a mild antigenic stimulus

(Smits *et al.*, 1999). It has been argued that humoral immunity is a more costly yet more effective way of fending off pathogens (Roitt *et al.*, 2001; Moret, 2003; Schmid-Hempel and Ebert, 2003) and, at the molecular level, it is possible that carotenoids might more directly (or preferentially) promote antibody proliferation by protecting immune cells from free-radical damage, as opposed to aiding the recruitment of lymphocytes already present in the circulation.

This study is not the first to suggest that carotenoids may relax male-typical immunosuppressive conditions in animals. Royle *et al.* (2001) discussed a similar idea for the co-existence of carotenoids and androgens in bird eggs, suggesting that carotenoids may be present in high concentrations in yolks that are also provisioned with high levels of steroids, so that carotenoids can counteract the immunocompromising action of testosterone. However, in their study of lesser black-backed gulls, they found that testosterone concentrations were not positively – but instead were inversely – related in egg-yolks, and interpreted this result as a mechanism for complementary, not offsetting, maternal effects. From these and our results, it is clear that more studies of the relationship between carotenoids and immunocompetence in male and female animals are needed. Because so many environmental, physiological and life-history parameters affect the expression of these two traits, we are likely to elucidate several, quite complex interrelationships between antioxidants and immunity in animals.

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