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Original Article

Context-dependent effects of carotenoid supplementation on reproduction in zebra finches

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Carotenoid-dependent sexual coloration is one of the best-studied sexual signals, but how the honesty of such signals is maintained remains uncertain. The main hypotheses focus on acquisition limits and physiological use of carotenoids in immune function and regulating oxidative stress. A hypothesis that has received less attention states that carotenoids can also be detrimental, depending on an animal's state. Hence, carotenoid-dependent signals may be a handicap, signaling the ability to evade or tolerate detrimental effects of carotenoids. To investigate this hypothesis, we examined the effects of carotenoid supplementation on subsequent reproduction in zebra finches in 2 different foraging environments ("easy" and "hard"), thereby generating variation in physiological state. We find support for context-dependent negative effects of carotenoid supplementation on subsequent laying latency and on total number of eggs laid: carotenoids had a detrimental effect in the "easy" conditions and a beneficial effect in the "hard" conditions. Thus, our results support the hypothesis that carotenoids can have context-dependent detrimental effects. Dissecting the relative contribution of the different mutually nonexclusive honesty mechanisms—acquisition, physiological benefits, and context-dependent detrimental effects of carotenoids—maintaining carotenoid-dependent signal honesty will be an exciting challenge.

Key words: **bird, carotenoids, coloration, honest signaling, ornament, sexual selection.**

INTRODUCTION

A common form of sexual signaling (Andersson and Iwasa 1996), especially in birds (Olson and Owens 2005), is carotenoid-dependent coloration (Olson and Owens 1998; Kemp et al. 2012; Simons, Cohen, et al. 2012). This usually yellowish to reddish coloration is often assumed to act as a sexual signal and indeed for several species mate choice for the extent of carotenoid-dependent signaling has been demonstrated (Künzler and Bakker 2001; Pike et al. 2007; Simons and Verhulst 2011; Toomey and McGraw 2012). Hypotheses concerning the honesty of carotenoid-dependent traits stretch from acquisition of the pigment, because carotenoids can only be exclusively derived from the diet, to physiological roles of carotenoids in supporting immune functioning and regulating oxidative stress state via its antioxidant potential (Olson and Owens 1998; Pérez-Rodríguez 2009; Simons, Cohen, et al. 2012). A strikingly different hypothesis (Hartley and Kennedy 2004) was inspired by a study in humans in which β-carotene supplementation to smokers increased risks to develop lung cancer (Omenn et al. 1996). Could carotenoids (not only β-carotene) actually be detrimental in specific physiological circumstances, for example, under oxidative stress (Hartley and Kennedy 2004; Bertrand et al. 2006; Svensson and Wong 2011; Beamonte-Barrientos et al. 2013)? In such a scenario, signal expression of carotenoid-dependent coloration may actually be a handicap (Zahavi 1975; Grafen 1990), showing the ability to evade and/or tolerate carotenoid's detrimental effects, rather than its presumed benefits to physiological functioning.

All other hypotheses concerning carotenoid-dependent signaling have in common the assumption that carotenoids are expected to have either a positive effect or a null effect, for example, when an honesty mechanism is based solely on pigment acquisition or when carotenoids are not important mediators of physiological trade-offs. There is evidence that carotenoids enhance immune and oxidative stress state, supporting the hypotheses that assume that the honesty of carotenoid-dependent traits is due to its function in physiological processes. However, effect sizes are low, which could suggest that other honesty mechanisms are also operating (meta-analysis in Simons, Cohen, et al. 2012). If (detrimental) effects of carotenoids are context dependent, heterogeneity in contexts may explain 1) why only sometimes positive effects of carotenoids are found and 2) why the overall effect sizes of these positive effects are

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so low. To test context-dependent effects of carotenoids, we investigated the effects of carotenoid supplementation in 2 contrasting experimental high- and low-cost foraging conditions (De Coster et al. 2011; Koetsier and Verhulst 2011) on subsequent reproduction in zebra finches.

In the context of carotenoid-dependent sexual signaling, the zebra finch is a relevant study species (Blount et al. 2003). Both male and female zebra finches express carotenoid-dependent bill coloration (McGraw et al. 2003) that signals survival and reproduction (Simons, Briga, et al. 2012). Female choice for male bill coloration has been demonstrated (meta-analysis in Simons and Verhulst 2011), but male choice for female bill coloration is not well studied. Choice for females is, however, probable with male choice for redder bills predicted to yield benefits: female bill redness is positively associated with longevity (Simons, Briga, et al. 2012), fledgling production (Simons, Briga, et al. 2012), and carotenoid deposition in eggs, which has been associated with fitness benefits to the embryo (McGraw et al. 2005). Also in a species without carotenoid-dependent signaling, detrimental effects of carotenoid would not be expected, as the positive effects of carotenoids are not exclusive to species exhibiting a carotenoid-dependent trait (Simons, Cohen, et al. 2012). Thus, any context-dependent, especially detrimental, effects of carotenoids on physiological functioning will support a potential role for these context-dependent effects in maintaining honesty signaling of carotenoid-dependent sexual traits, widening the scope of honesty mechanisms and maintaining carotenoid-dependent signal honesty, beyond the physiological beneficial actions of carotenoids. Here, we present evidence for such a context-dependent effect of carotenoid supplementation, using reproduction as a proxy of physiological functioning.

METHODS

From our colony in Groningen, the Netherlands, we randomly selected 60 males and 60 females (5 months < age < 19 months) and housed these birds in 4 roofed outdoor sex-separated aviaries (L × H × W, 310 × 210 × 150 cm) in which tropical seed mix, cuttlebone, water, sand, and grit were provided ad libitum. For an experimental timeline, please see Figure 1. All birds were trained to "work" for their food within 2.5 weeks (in November 2012), by gradually shortening perches from a food box suspended from the aviary roof with holes from which seeds could be obtained. Any spillage of seeds was collected by a reception device effectively forcing the birds to forage by hovering in front of the food box ("hard" treatment) (Koetsier and Verhulst 2011).

After the training period, the sexes were mixed and 2 of the 4 aviaries had sitting perches reinserted in their food boxes ("easy" treatment), effectively creating 2 environments with differential foraging costs (Koetsier and Verhulst 2011). Nest-boxes (15 per aviary) were provided 11 days later to all 4 aviaries to induce reproduction, but all eggs were continuously removed within 6 days of laying. Birds in both treatments built nests with the provided hay (ad libitum), but strikingly not a single egg was found in the hard treatment, contrary to the easy treatment in which a total of 142 eggs were collected during the outdoor period in which nest-boxes were available (51 days).

All 4 groups were balanced for body mass, bill coloration, and age and this resulted in nonsignificant differences in these measures among groups (combined statistics for both sexes tested separately; mass: $F_{3,49} = 0.17{:}1.12$, $P = 0.35{:}0.91$; bill color: $F_{3,49} = 0.35{:}0.66$, $P = 0.59{:}0.79$; age [rank test]: $\chi^2(3) = 0.99{:}3.32$, $P = 0.35{:}0.80$). Bill coloration was measured using digital photography (Stevens et al. 2007) with fixed camera settings (Sony α-200) in a controlled lighting environment (Kaiser Photography table with 4 Philips Photocrescenta 150 W light bulbs). RGB (Red, Green, Blue) values obtained from JPEG images were converted (Stigell et al. 2007) to simulated spectra (using known spectra and photographs of Munsell glossy finish color patches) and we determined the inflection point of this spectrum as a measure of hue. We have earlier verified the precision of this method in a subset of zebra finches using spectrophotometry (Simons, Briga, et al. 2012).

Three weeks after introduction of the nest-boxes, carotenoid supplementation commenced. To allow for a fully crossed design of carotenoid supplementation without pseudoreplication of aviary, carotenoid supplementation and control treatment were performed within each aviary (half of the females and half of the males assigned at random) via individual oral pipetting (using a pipetman p20) at the same time (between 11:50 and 14:45) each treatment day. Both males and females received carotenoids 3 times a week for 4 consecutive weeks (till the birds were moved indoors for breeding measurements). Note that because the birds were communally housed, we could not ascertain which females laid eggs and, hence, could not investigate carotenoid supplementation effects during this phase of the experiment.

A dose consisted of a 10 µL mixture of FloraGLO (Kemin) and refined sunflower oil (controls received sunflower oil only). FloraGLO was extracted from commercially available lutein/zeaxanthin (20:0.86) softgels for human use (Proviform, Bergen op Zoom, the Netherlands). Both the distributor (Proviform) and manufacturer (Kemin) stated its purity, that is, void of other antioxidants and/or carotenoids. The supplement was diluted in oil such that one dosage equaled 262.5 µg of carotenoids per day [(262.5 × 3)/7 = 112.5 µg/day]. This FloraGLO mix was prepared fresh every day to avoid oxidation. In addition, birds could extract carotenoids from the pure tropical seed diet, which provides birds with approximately 30 µg of carotenoids a day (McGraw and Ardia 2003). We did not provide egg food, rich in carotenoids, at any time during the experiment, although this is part of the standard diet in our colony.

It is difficult to relate the dosage to dietary intake of carotenoids in the wild, especially because zebra finches are known to also occasionally consume fresh plant material and insects (Zann 1996), which are much higher in carotenoid content than seeds (Catoni et al. 2008). However, the dosage we gave is close to earlier supplementation studies that found positive physiological effects of carotenoids (≈125 µg/day, Blount et al. 2003; Alonso-Álvarez et al.

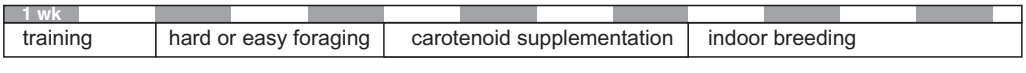

Figure 1
Timeline of the 4 different phases of the experiment. Each line segment (gray and empty alternating) indicates 1 week.

2004) in which carotenoids were supplemented to drinking water (assuming 2.5 mL water intake per day, McGraw et al. 2004). We are, therefore, confident that within the scope of our experimental aims, we provided the birds with a relevant dose.

After the carotenoid treatment, birds were randomly paired in indoor cages (L × H × W: 80 × 40 × 40 cm, 2 perches and an open nest-box, with treatments divided equally across 2 separate rooms). In total, 53 pairs were formed (per treatment: 14 control—"easy," 13 carotenoids—"easy," 14 control—"hard," 12 carotenoids—"hard"), which is less than 60 due to some mortality outdoors and shortly after moving indoors, unbiased with respect to treatment. Birds were paired with an opposite sex bird that had received the same carotenoid and foraging treatment, but paired birds came from different aviaries, to avoid possible confounding effects of previous pair formation (Balzer and Williams 1998). Tropical seed mix, cuttlebone, and grit were provided ad libitum. Fluorescent tubes provided a long photoperiod (16:8 h light:dark) and the room was maintained at 22 °C and a relative humidity of 50%. Nests were checked daily to establish laying latency and all eggs were marked with small dots of ink using a small brush and left in the nest to count total eggs produced.

After 31 days, the indoor experiment was terminated at which time more than 80% of the pairs had commenced laying. We chose this time point because egg production was declining (Figure 2) and to avoid the possibility that pairs would start relaying or that eggs from clutches would hatch, further confounding our measures of latency to lay and number of eggs laid. This confounding between latency to lay and number of eggs laid is difficult to overcome because the completion of a clutch in zebra finches is sometimes difficult to judge. Eggs are not always laid on subsequent days and birds, especially first time breeders as used here, can break eggs, presumably by accident (hence the ink marking of eggs). We do present both measures, although correlated they provide the most complete picture of how our treatments affected short-term breeding performance.

Laying interval was analyzed with Cox proportional hazard models (Cox 1972), and pairs that had not produced eggs were censored in these analyses. In brief, these models analyze the time it takes for an event, in this case egg laying, to occur, and by censoring the nonlayers these pairs could be retained in the analysis despite the absence of a known laying interval. All statistical analyses were performed in SAS JMP 7.

Blood was taken from all birds when the birds were moved inside for breeding and at the end of the experiment to evaluate plasma carotenoids. Total carotenoids were analyzed by spectrophotometer to a lutein standard (Sigma), sensu Alonso-Álvarez et al. (2004). In females, plasma carotenoids were elevated by carotenoid supplementation ($F_{1,50} = 5.31$, $P = 0.025$) irrespective of time point or foraging cost treatment (Supplementary Figure S1, $P > 0.12$; estimates ± standard error per time point, prior: 5.2 ± 2.7, after: 3.7 ± 2.6 µg total carotenoids/mL plasma, a 18–22% increase), and plasma carotenoid levels were lower after indoor breeding than before ($F_{1,52} = 17.11$, $P = 0.0001$, all analyses in a mixed model context including bird ID as random term). In males, supplementation

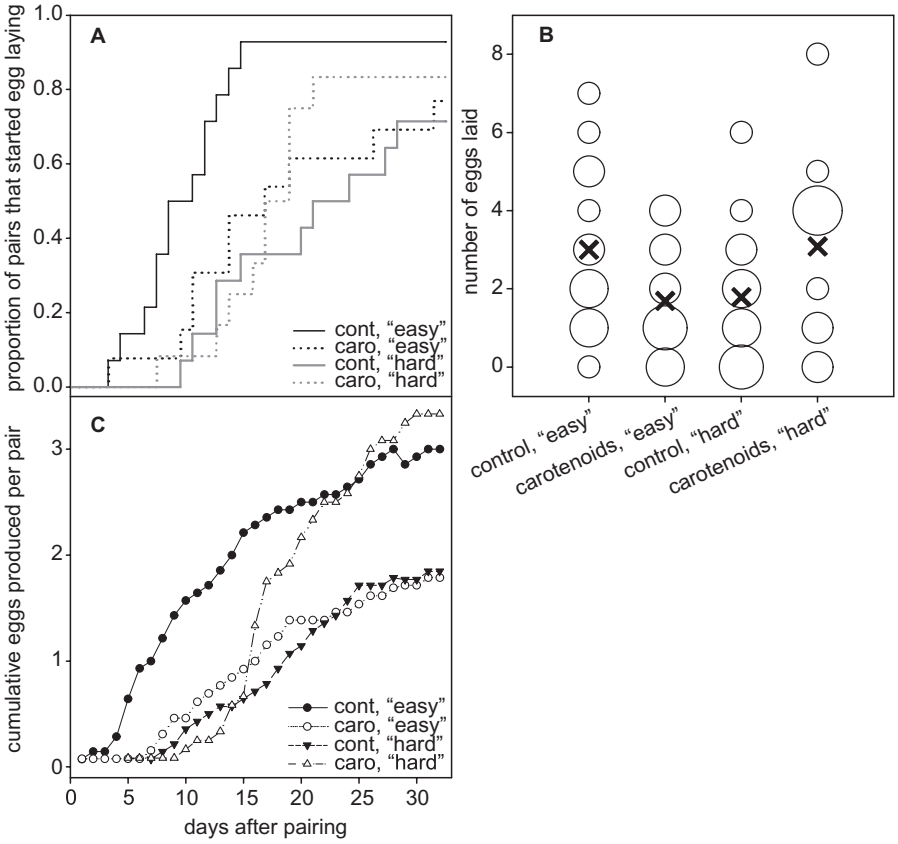

Figure 2

(A) Proportion of females that had laid their first egg. (B) Total number of eggs laid during indoor breeding. Bubble area indicates the number of individuals per dot, with the smallest bubble area indicating 1 individual pair. Crosses indicate the raw averages per experimental group. (C) Cumulative eggs produced during the experiment per pair.

did not increase plasma carotenoid levels, neither prior nor after the indoor phase of the experiment ($F_{1,51}$ = 1.49, P = 0.23; prior: −1.4 ± 2.3, after: −3.9 ± 2.2 μg total carotenoids/mL plasma, a 4–10% decrease), possibly due to sex-specific carotenoid allocation strategies (Supplementary Figure S1).

This research was carried out under the approval of the Animal Experimentation Ethical Committee of the University of Groningen, license 5150D.

RESULTS

Carotenoid supplementation and foraging treatment interacted to determine laying interval after having been pair housed indoors (Cox proportional hazards, $\chi^2(1)$ = 5.39, P = 0.020). Among non-carotenoid-supplemented birds, birds that had experienced the easy foraging treatment started laying sooner than birds from the hard foraging treatment. However, as indicated by the significant interaction, this effect was negated by carotenoid supplementation. Birds from the easy foraging environment that received carotenoids subsequently delayed laying (Table 1 and Figure 2A), whereas no such effect was seen in the hard foraging environment (Table 1 and Figure 2A).

With respect to total egg production, we also found a significant interaction between carotenoid supplementation and foraging treatment (generalized linear model [exponential − log link], $\chi^2(1)$ = 5.87, P = 0.015, Figure 2B and Table 1). Carotenoid supplementation almost significantly reduced total egg production in the easy foraging environment but tended to enhance egg production in the hard foraging environment (Table 1). Thus, the combined effect on egg laying interval and total eggs laid resulted in opposite effects of carotenoids on reproductive output depending on the foraging environment in which it was supplemented (Figure 2C).

We also examined effects of carotenoid supplementation and foraging treatment on bill coloration and body condition (mass change). First, as expected, carotenoid concentrations were positively related to bill hue (redness) in both sexes (measured prior to pair formation; males: r = 0.44, n = 52, P = 0.001; females: r = 0.38, n = 52, P = 0.005; Supplementary Figure S2). One female data point of carotenoid levels is missing due to blood sampling failure. One male bill hue data point was removed from the analysis as an outlier due to a bill malformation. Supplementation of carotenoids did not significantly increase bill coloration in either sex (Table 2). Foraging treatment significantly reduced bill coloration of males but not of females (Table 2). Females lost mass during the course of experiment and carotenoid supplementation partially but significantly compensated this loss and these patterns were absent for males (Table 2). No significant interaction between foraging treatment and carotenoid supplementation was detected for either variable (P > 0.39).

DISCUSSION

Living with hard foraging conditions reduced current (outdoor) and subsequent (indoor under ad libitum conditions) reproduction, and the latter is in line with earlier findings in this species (Wiersma and Verhulst 2005). We assume that the pattern during the outdoor treatment period reflects resource allocation away from reproduction toward somatic maintenance (Kirkwood and Holliday 1979; Wiersma and Verhulst 2005). But, despite higher reproductive effort in the easy treatment, a negative effect of the foraging costs manipulation on physiological state remained, given that reproduction after the treatment period was also affected by prior foraging treatment. This suggests carryover effects of hard work that are not purely energetic (Veasey et al. 2001; Nilsson 2002), given that there were no treatment effects on mass at the time the birds were brought indoors (Table 2). These "hidden" costs could, for example, have contributed to changes in body composition (Veasey et al. 2001) and oxidative stress (Wiersma et al. 2004; Monaghan et al.

Table 1

Between-treatment cell comparisons following from significant interactions between foraging treatment and carotenoid supplementation on laying interval (above the diagonal) and total egg production (beneath the diagonal)

		Compared with			
		Control/"easy"	Control/"hard"	Carotenoids/"easy"	Carotenoids/"hard"
Focal group	Control/"easy"		1.25 ± 0.44, P = 0.005	0.94 ± 0.44, P = 0.031	1.20 ± 0.45, P = 0.008
	Control/"hard"	−0.52 ± 0.32, P = 0.11		−0.24 ± 0.44, P = 0.59	−0.43 ± 0.46, P = 0.35
	Carotenoids/"easy"	−0.57 ± 0.30, P = 0.06	−0.05 ± 0.36, P = 0.88		−0.05 ± 0.45, P = 0.91
	Carotenoids/"hard"	0.027 ± 0.29, P = 0.92	0.55 ± 0.35, P = 0.12	0.60 ± 0.32, P = 0.06	

Directions of effects are expressed in the direction compared with the groups in the column. Hazards indicate egg laying start, that is, positive estimates indicate shortened intervals. Cox proportional hazard models estimate the probability of an event to occur relative to a data-estimated baseline. This probability, or hazard ratio, is expressed as an exponential coefficient in a regression model. A hazard ratio (exp(coef)) of 1 implies no effect, whereas a hazard ratio of, for example, 1.2 means that 1 group took on average 20% shorter than the baseline laying intervals.

Table 2

General linear models (per sex) of the effects of carotenoid supplementation and the foraging treatment (both left in the model) on mass and bill hue change between the start of the experiment and the start of indoor breeding (Figure 1)

	Intercept	Carotenoid supplementation (control = 0, carotenoids = 1)	Foraging treatment ("easy" = 0, "hard" = 1)
Female mass	−1.01 ± 0.18, P < 0.01	0.44 ± 0.21, P = 0.04	0.12 ± 0.21, P = 0.56
Female bill color	0.43 ± 0.63, P = 0.49	1.18 ± 0.75, P = 0.12	0.34 ± 0.74, P = 0.65
Male mass	−0.01 ± 0.15, P = 0.92	−0.20 ± 0.18, P = 0.27	−0.18 ± 0.18, P = 0.34
Male bill color	0.66 ± 0.41, P = 0.11	−0.29 ± 0.49, P = 0.55	−1.30 ± 0.49, P = 0.01

2009) and may also have caused the reduction of bill coloration of males in the hard foraging environment compared with the easy foraging environment.

Carotenoid supplementation in the "hard" environment had positive effects on subsequent reproduction. In contrast, carotenoid supplementation in the easy environment negatively affected subsequent reproduction. This suggests that context-dependent detrimental physiological effects of carotenoids are affecting reproduction in a way that is likely to affect fitness, and, because more ornamented individuals have higher carotenoid levels in the plasma (Simons, Cohen, et al. 2012), this effect could play a role in maintaining honesty of carotenoid-dependent signals. Moreover, it suggests that carotenoid supplementation affected physiological state differentially in both environments, reducing subsequent reproduction, despite the fact that carotenoids were not supplemented when measuring reproduction indoors.

Note, however, that we cannot exclude that the interaction between carotenoid supplementation and foraging treatment affected male attractiveness, in turn affecting female breeding decisions, instead of an effect on female physiology. Our design, providing both sexes within the pair with the same treatment, did not allow us to distinguish between these possibilities. We chose to maximize our statistical power toward detecting any context-dependent detrimental effect of carotenoids, and further considered that context-dependent negative effects of carotenoids would support the Hartley and Kennedy (2004) hypothesis that context-dependent detrimental effects of carotenoids could maintain signal honesty regardless of how they come about. It is also worth noting that in the analyses of 2 possible proxies of male attractiveness we measured, bill color and body mass, we found no interactions between the foraging treatment and carotenoid supplementation. We, therefore, tentatively conclude that the interaction between carotenoid supplementation and foraging treatment on reproduction affected female physiology directly, rather than being mediated via male attractiveness, but this awaits further study.

The specific physiological context in the easy foraging environment inducing detrimental effects of carotenoids could be related to heightened oxidative stress compared with the hard foraging condition (Briga M, Verhulst S, unpublished data). Reasoning from these unpublished data under long-term hard foraging conditions (De Coster et al. 2011; Koetsier and Verhulst 2011), counterintuitively, the hard foraging environment may create a situation of dietary restriction (Wiersma and Verhulst 2005) that compromises reproduction but, in addition, reduces oxidative stress (Walsh et al. 2013). Possibly in line with this interpretation is the robust reduction in body mass observed under long-term "hard" foraging conditions (Briga et al., unpublished), although our relatively short-term exposure to the "hard" foraging environment did not affect body mass (Table 2). Yet, effects of short- and long-term dietary restriction on the transcriptome are relatively similar (Cao et al. 2001), and although a reduction in body mass might indicate dietary restriction, mass loss itself might not be necessary for its physiological effects. Another possibility is that birds regulated their antioxidant machinery and food intake differentially in the 2 foraging treatments, and hence, the supplementation of carotenoids had differential effects. Recent work on self-medication with antioxidants in birds have highlighted that antioxidant intake is not always maximized and depends on the context (Beaulieu and Schaefer 2013; Beaulieu et al. 2014). This also highlights that supplementation of antioxidants, including carotenoids, might not always be beneficial but might also have potentially detrimental effects. Carotenoid availability may therefore not be limiting (Hill and Johnson 2012), but instead its uptake may be constrained by context-dependent detrimental effects of carotenoids. Therefore, detrimental effects of carotenoids may only become apparent when individuals are shifted away from their optimal carotenoid intake by forced supplementation or because it is a prerequisite of increased sexual signaling. Detrimental effects may, therefore, constitute the actual physiological "handicap" of tolerating context-dependent detrimental effects of carotenoids or can signal the optimization of carotenoid intake to avoid the risk of detrimental effects of carotenoids. Examining which of these scenarios is operating will be challenging and will benefit from mechanistic insight into the causes of context-dependent detrimental effects of carotenoids, currently hypothesized to be related to oxidative stress (Hartley and Kennedy 2004).

Experimentally linking the detrimental effects of carotenoids and oxidative stress will require specific manipulations and/or measurements of oxidative stress state, which are both difficult (Meitern et al. 2013), and hence, environmental manipulations as we employed here may be preferred, potentially in combination with a different antioxidant treatment or antioxidant self-medication (Beaulieu and Schaefer 2013; Beaulieu et al. 2014) to experimentally link negative effects of carotenoids to changes in oxidative stress state. Alternatively, a sudden drop in carotenoid availability during indoor breeding might have differential and also detrimental effects if rates of carotenoids acquisition differ. Our data on plasma carotenoids do not support this hypothesis because we found no effects of foraging treatment on plasma carotenoids, but it remains a possibility.

We did not find context-dependent detrimental effects of carotenoids on bill coloration, the sexual signal. The fitness trade-offs involved with context-dependent detrimental effects of carotenoids we detected might still underlie honest signaling and cannot exclude that effects on bill coloration would become apparent in the longer term. A further complication of the trade-offs underlying carotenoid-dependent signaling is the benefit of carotenoid deposition in eggs by females (McGraw et al. 2005) and this might explain the sex-specific effects of carotenoid supplementation on plasma carotenoid levels and of the foraging treatment on bill coloration we detect.

The evidence we present here further complicates our understanding of how honesty of carotenoid-dependent signals is maintained. Separate but not mutually exclusive mechanisms—acquisition, resource based, and context-dependent detrimental effects—may operate in synergy or the relative contribution of these separate honesty maintaining mechanisms may differ between species (meta-analysis in Simons, Cohen, et al. 2012). Context-dependent effects of carotenoids may also explain why support for the various honesty mechanisms proposed is mixed because they may depend on the context they are studied in (Simons, Cohen, et al. 2012). Dissecting the common ground between these mechanisms and discerning the aspects of physiology maintaining honesty will be an exciting future direction and will likely reveal underappreciated aspects of physiology while at the same time enhancing our understanding of sexual selection.

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SUPPLEMENTARY MATERIAL

Supplementary material can be found at http://www.beheco.oxfordjournals.org/

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