The Importance of Carotenoid Dose in Supplementation Studies with Songbirds

Rebecca E. Koch[1,*]
Alan E. Wilson[1,2]
Geoffrey E. Hill[1]
[1]Department of Biological Sciences, 331 Funchess Hall, Auburn University, Auburn, Alabama 36849; [2]School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University, Auburn, Alabama 36849

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ABSTRACT

Carotenoid coloration is the one of the most frequently studied ornamental traits in animals. Many studies of carotenoid coloration test the associations between carotenoid coloration and measures of performance, such as immunocompetence and oxidative state, proceeding from the premise that carotenoids are limited resources. Such studies commonly involve supplementing the diets of captive birds with carotenoids. In many cases, however, the amount of carotenoid administered is poorly justified, and even supposedly carotenoid-limited diets may saturate birds' systems. To quantify the relationships among the amount of carotenoids administered in experiments, levels of circulating carotenoids, and quantities of carotenoids deposited into colored ornaments, we performed a meta-analysis of 15 published studies that supplemented carotenoids to one of seven songbird species. We used allometric scaling equations to estimate the per-gram carotenoid consumption of each study's subjects, and we used meta-regression to evaluate the effects of this carotenoid dose on differences in coloration and plasma carotenoid levels between supplemented and control groups of birds. After accounting for supplementation duration and species, we observed a significant positive correlation between carotenoid intake and response of plasma carotenoid level to supplementation. The presence of supplemental carotenoids also tended to increase the expression of ornamental coloration, but the magnitude of the carotenoid dose did not significantly affect how strongly coloration changed with supplementation. Further, coloration effect sizes had no significant relationship with plasma carotenoid effect sizes. We also found significant heterogeneity in responses among studies and species, and the parameters used to measure color significantly affected response to supplementation. Our results emphasize the importance of performing dosage trials to determine what supplementation levels provide limited versus surplus carotenoids and of measuring the natural level of carotenoid intake by the study species to validate the appropriateness of supplementation levels for a particular study species and experimental design.

Keywords: carotenoid supplement, ornamental coloration, plasma carotenoids, plumage coloration, bill coloration.

Introduction

Carotenoid-based ornaments in birds have drawn substantial attention as indicator traits because numerous studies have reported correlations between the expression of carotenoid coloration and aspects of male quality, including fat reserves, basal metabolic rate, effectiveness of immune response (immunocompetence), and oxidative state (reviewed in Hill 2002, 2006; Svensson and Wong 2011). Carotenoid pigments are responsible for most of the vibrant red, orange, and yellow coloration of the feathers and soft parts of birds (McGraw 2006), and they may also play important physiological roles as vitamin A precursors, boosters of the immune system, and antioxidants (Mougeot et al. 2010; Pérez-Rodríguez et al. 2010; Hill and Johnson 2012). Because these pigments cannot be synthesized in the bodies of animals and must be acquired from the diet (Goodwin 1984), carotenoids are often considered limited resources such that only birds in the best condition can afford to allocate carotenoid pigments toward colored ornaments rather than retain them for potential internal benefit; thus, carotenoid resource trade-offs have been hypothesized to maintain signal honesty in these traits (Møller et al. 2000; Alonso-Alvarez et al. 2004).

Numerous studies of carotenoid ornamentation aim to establish and clarify whether this hypothesized carotenoid resource trade-off may explain the condition dependence of carotenoid coloration in birds by validating that (1) higher levels of circulating carotenoids improve immune function and/or oxidative stress maintenance, (2) restricted dietary intake limits the quantity of circulating carotenoids, and (3) generation of a high-quality ornament sequesters circulating carotenoids such that colored traits impose a cost on other processes that utilize carotenoids (von Schantz et al. 1999; Møller et al. 2000; Alonso-Alvarez et al. 2008). Fundamental to testing these predictions of

*Corresponding author; e-mail: rek0005@auburn.edu.

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the trade-off hypothesis are experiments that manipulate carotenoid availability and measure the effect of carotenoid dose on both ornamentation and physiology. In laboratory settings, researchers commonly supplement or restrict dietary carotenoid levels and evaluate the resulting effects on various measures of ornamentation and internal condition (Hill 2006). However, the results of these studies are often inconclusive, and the importance of allocation trade-offs to carotenoid-based signal honesty as well as the physiological functions of carotenoid themselves remain debated (Hill 1994, 1999, 2011, 2014; Hudon 1994; Hartley and Kennedy 2004; Hadfield and Owens 2006; Costantini and Møller 2008).

One critical but often overlooked complication of carotenoid manipulation studies is the biological relevance of the quantities of carotenoids that are administered to test animals. Commonly, supplemental carotenoids are provided ad lib. in food or water without an assessment of the amounts of pigments that are actually ingested and without proper consideration for how levels of supplemental carotenoids compare with quantities ingested by birds under natural conditions. Moreover, the quantitative relationship between the amount of carotenoids ingested and quantities of circulating carotenoids is usually not measured in either lab or field systems, so it is difficult to judge the results of carotenoid supplementation. For example, the quantity of ingested carotenoids may greatly exceed that which is present in the plasma if birds rapidly transport consumed carotenoids to storage in fat, ornamentation, or other tissues; therefore, birds with vastly different carotenoid access may have the same levels of plasma carotenoids if the bird with greater consumption allocates his excess carotenoids outside of circulation. For this reason, comparing plasma carotenoid levels of captive birds to wild conspecifics is insufficient to justify that the captive supplementation dose mimics the levels of carotenoids available to wild birds. Because the differential allocation of limited carotenoids is key to the resource trade-off hypothesis, it is essential to better track carotenoid usage through quantifying the relationships among amounts ingested, circulated, and deposited in ornaments.

Several studies have addressed this issue by using dosage trials to compare supplementation levels to levels of circulating carotenoids in order to identify doses that do not saturate their subjects' systems (e.g., Alonso-Alvarez et al. 2004; Aguilera and Amat 2007). Too often, however, the carotenoid supplementation regimens used in avian studies are based on methods developed for other species or from studies of different dietary carotenoids (e.g., Navara and Hill 2003; Baeta et al. 2008); carotenoid consumption and absorption varies markedly across species with different masses and life histories (Tella et al. 2004; McGraw 2005), so extrapolating carotenoid doses among species with no validation could lead to experiments that provide carotenoid doses that are too high or too low to yield meaningful results.

Because the focus of most studies utilizing carotenoid supplementation is testing for trade-offs in the use of limited carotenoid resources for ornamentation versus body maintenance, poorly controlled dosing undermines the goals of the research. For a study of resource limitation or trade-off to be meaningful, then the resource must be provided at a level below saturation. If the lowest supplementation level provides sufficient carotenoids for both body maintenance and ornament production, then studying the effects of dose becomes meaningless. As fundamental as these ideas appear to be, many studies proceed on the unstated assumption that supplementation levels are below saturation.

To better quantify the effects of supplementation on circulating carotenoid availability and carotenoid-based ornamentation, we performed a meta-analysis of 15 published studies that include groups of both carotenoid supplemented and unsupplemented birds and that report the resulting plasma carotenoid levels and/or ornamental color of each group. A previous meta-analysis investigated correlations among these variables, but it grouped studies as either supplemented or unsupplemented without including supplementation dose as a cofactor (Simons et al. 2012), missing a critical source of variation among studies. In our analysis, we built on the existing literature by first using published levels of carotenoid supplementation and allometric scaling equations to estimate individual consumption of carotenoids. We then modeled how variation in intake between supplemented and control groups affected the relationships between circulating carotenoids and allocation to ornamentation in songbirds. By quantifying the physiological responses to varying levels of carotenoid ingestion in different studies and seven different songbird species, we provide a foundational model for predicting the biological relevance of particular carotenoid supplementation regimens and can assess the variables that modulate response to carotenoid intake.

Methods

Literature Search

We surveyed the existing carotenoid literature using the Web of Science database on March 23, 2014, using the keywords "carotenoid*" AND "supp*" AND "bird" OR "avian." We included only studies (1) reporting the level of carotenoid supplementation as well as the food source provided; (2) including data on both carotenoid-supplemented and control groups of individuals; (3) reporting the values of plasma carotenoid levels and/or coloration; (4) not repeating measures on the same group of birds that were reported in a study already incorporated into the meta-analysis (a potential source of pseudoreplication); (5) testing adult male birds rather than nestlings (in which both carotenoid physiology and ornamental function differ greatly from sexually reproducing adult birds, and the quantity of carotenoids acquired from egg yolk or parental provisioning is often unknown; Hill and McGraw 2006); and (6) supplementing with only the carotenoids lutein and/or zeaxanthin, the most prevalent carotenoid pigments in the avian diet (McGraw 2006). With the exception of one study supplementing with only lutein (Stirnemann et al. 2009), all studies included in our meta-analysis supplemented primarily with

lutein and trace amounts of zeaxanthin (e.g., 20 : 1 ratio of lutein : zeaxanthin; Blount et al. 2003; Hõrak et al. 2007; Karu et al. 2007; Baeta et al. 2008; Sild et al. 2011; Sepp et al. 2011).

This latter point is important because most terrestrial birds consume diets containing primarily these two yellow and structurally similar carotenoid pigments, which many species must then metabolize into red pigments (in species with red coloration) or ornamental yellow pigments (e.g., canary xanthophylls). Critically, chemical properties and therefore potential physiological functions vary across these dietary and ornamental pigments, and the costs of converting dietary to ornamental pigments may play a key role in the honesty of carotenoid-based coloration (Hill 1996; Hill and Johnson 2012; Johnson and Hill 2013). Studies supplementing with other pigments, particularly the red carotenoids at the end points of these carotenoid conversion pathways (such as canthaxanthin; e.g., McGraw et al. 2002; Smith et al. 2007), bypass some of the mechanisms relating coloration to physiology that may be important to carotenoid signal honesty, so such studies are not appropriate for this analysis.

Despite the extensive literature on carotenoid ornamentation (more than 300 results to our initial keyword search), only 19 studies met our criteria of providing measurable carotenoid supplementation quantities to adult birds. Because 16 of 19 studies investigated songbird species (order Passeriformes), we excluded one study of red junglefowl (*Gallus gallus*; McGraw and Klasing 2006), one study of mallards (*Anas platyrhynchos*; Butler and McGraw 2013), and one study of kestrels (*Falco tinnunculus*; Costantini et al. 2007) to capture the majority of available data while avoiding comparing data from phylogenetically distant taxa with different physiologies. We also excluded one study on society finches (*Lonchura striata domestica*; McGraw et al. 2006) because this species lacks carotenoid-based ornamentation and so is not subject to the potential costs of allocating carotenoids as colorants. We performed our analysis on the remaining 15 studies of seven songbird species with carotenoid-based ornaments: the American goldfinch (*Carduelis tristis*) with yellow plumage and pink-red bill ornamentation, the house finch (*Haemorrhous mexicanus*) with red plumage ornamentation, the zebra finch (*Taeniopygia guttata*) with red bill ornamentation, the diamond firetail (*Stagonopleura guttata*) with red bill ornamentation, the great tit (*Parus major*) with yellow plumage ornamentation, the Eurasian blackbird (*Turdus merula*) with red-orange bill ornamentation, and the European greenfinch (*Carduelis chloris*) with yellow plumage ornamentation.

Carotenoid Supplementation Calculations

Most experiments supplemented carotenoids to the main food or water supply and reported doses as the concentration of carotenoids added per unit food or water. One study by Peters et al. (2011) quantified daily carotenoid intake of individuals during the experiment, so these values were used in our analysis. For all other studies, we estimated the quantity of carotenoids consumed by each bird by first calculating the average daily food or water intake of an individual of the focal species, using allometric scaling equations to account for the nonlinear relationship between species size and consumption. When carotenoids were supplemented in the water supply, we estimated daily water intake using the mass of the study species and the scaling equation for passerines reported by Calder and Braun (1983). When a study supplemented carotenoids in the food supply, we estimated the daily food intake of the study's focal species by using the energy content of the food provided (often, millet or sunflower seeds; Caraco et al. 1980; Hõrak et al. 2003) and a scaling equation for passerines that predicts the consumption needed to meet daily energetic requirements (Nagy et al. 1999). When the exact mass of individuals included in the study was not reported, we estimated the average mass of the species from the *Handbook of the Birds of the World* (del Hoyo 2010). From our estimates of daily food or water intake, we then used each study's published details on the concentration of carotenoids supplemented to calculate the quantity of carotenoids ingested along with food or water. We also calculated the carotenoid content of the basic diet provided to both control and supplemented birds, using reported carotenoid content values or published measurements of the content of the seeds supplied (McGraw et al. 2001; Peters et al. 2008) to account for dietary carotenoids acquired independently of supplementation (app. A, available online).

To standardize levels of supplemental carotenoids ingested in species of varying body sizes, we divided daily carotenoid consumption amount by species mass in grams. We then calculated the difference in carotenoid intake between supplemented and control groups for each study (carotenoid intake difference). Most often, this measure of intake difference was nearly identical to the actual intake of the supplemented group, since most control groups acquired negligible levels of carotenoids.

Effect Size Calculations

We calculated the natural log response ratio and its variance from reported means and standard deviations of control and supplemented groups according to the formulas outlined by Koricheva et al. (2013); the response ratio allows for the standardization of measurements across studies by converting each measured effect into a unitless ratio of the mean response of the supplemented group to the mean response of the control group. When other experimental manipulations were present in a study, we used data from the otherwise unaltered control groups that varied only in carotenoid supplementation. We calculated two types of effect sizes per study, when possible, to measure the effects of supplementation on plasma carotenoid levels and ornamental coloration. When necessary, we extracted means and errors from figures using either ImageJ (Rasband 1997–2014) or WebPlotDigitizer v. 2.6 (Rohatgi 2013). When mean values were not published in text or figures, we contacted authors to retrieve the raw data and calculate mean values. Along with effect size, we recorded each study's focal species and the number of days that supplementation was

provided. If a study reported multiple response values over time, we recorded only values from before supplementation and at the end of supplementation for consistency among studies. We included multiple effect sizes for one study only if each differed in a key variable, such as a different carotenoid supplementation dose or ornament measured. In addition, because the color of feathers is determined only during molt when carotenoids are actively deposited in growing feathers (Hill 2002), we extracted plumage color effect sizes only from studies of molting individuals; we calculated effect sizes from nonmolting birds with plumage ornaments only for the relationship between carotenoid intake and plasma carotenoid concentration. The color of a soft part, such as the bill, can change rapidly during any season (Rosenthal et al. 2012), so we could extract both coloration and plasma carotenoid level effect sizes from studies of these ornaments, regardless of molt status.

The means of assessing ornamental coloration is important to consider in our analysis because color is generally quantified along one or more of three main axes: hue, or the shade of the color (e.g., red, orange, yellow); chroma, or the intensity of the color (also called saturation); and brightness, or the lightness/darkness of the color. In addition, principle component analysis can be used to create a composite metric directly from the reflectance spectrum of a color (Montgomerie 2006). Each of these axes of color tends to relate to different properties of the colored ornament itself. For example, chroma may be a good generalization of pigment density, while hue may be more representative of the proportion of red to yellow pigments in a carotenoid-colored ornament (Inouye et al. 2001; Hill and McGraw 2006). The choice of color parameter used in a particular study is therefore important to include in our analysis because it may affect study conclusions by representing different properties of the ornament measured.

Statistical Analyses

We performed all analyses using the metafor package (ver. 1.9–7; Viechtbauer 2010) in R (ver. 3.2.1; R Core Team 2015). We ran two separate overall meta-analyses, one for plasma carotenoid levels and a second for ornamental coloration. Both analyses used meta-regression to estimate the dose-dependent effect of carotenoid intake difference (between supplemented and unsupplemented groups) on response, also including species, supplementation duration, and measure type (for color measurements) as moderators and including a random effect of study (to control for multiple effect sizes from one experiment). Each model took within-study variation—or the error around each effect size—into account when estimating overall effects.

After initial investigation, we discovered that one study in our plasma carotenoid content analysis (Peters et al. 2011) had a modest effect size but an order of magnitude larger daily carotenoid consumption per individual than any other study, so we ran a separate meta-regression omitting this outlying data point to better model the patterns in the remaining studies. We also performed two subgroup analyses for studies of the plasma content of greenfinches and zebra finches, which had the greatest number of individual effect sizes (nine and seven, respectively) and allowed an opportunity to specifically assess the relationships among parameters in these species. We also performed a separate analysis of zebra finches for the relationship of supplementation to coloration. We ran several further subgroup analyses to better parse the effects of particular model variables when significant sources of heterogeneity were held constant (e.g., on data with only hue or only chroma color parameters). Last, to examine whether the effects of supplementation on coloration depend on plasma carotenoid content, we performed an additional meta-regression to investigate the effects of species, carotenoid intake difference, color parameter measured, duration of supplementation, and plasma effect size on coloration effect size; this analysis was performed on the subset of studies that measured both plasma carotenoid content and the color of ornaments.

We investigated the extent of publication bias in the main plasma carotenoid content and coloration data sets using funnel plots of effect size versus standard error, a measure of study precision, according to the guidelines of Koricheva et al. (2013). Along with a visual examination of plots, we statistically tested for funnel plot asymmetry using a regression test (Viechtbauer 2010). To estimate the impact of study heterogeneity on meta-regression results, we calculated Q values, which test whether there was significant residual heterogeneity in effect sizes that could not be attributed to variation in carotenoid consumption level and other moderators (Viechtbauer 2010; Koricheva et al. 2013).

Results

Overall, we calculated 40 effect sizes from 15 studies of the seven focal species. Among the studies we assessed, carotenoid intake between supplemented and control groups of birds differed by an average of 19.9 $\pm$ 15.1 μg/d/g body mass and ranged from 0.01 (Stirnemann et al. 2009) to 432.2 μg/d/g body mass (Peters et al. 2011). Duration of supplementation was similarly variable, with an average of 39.0 $\pm$ 4.70 d and a range from 7 (Karu et al. 2007) to 84 d (Navara and Hill 2003; figs. B1, B2; app. A; figs. B1–B3 and app. B available online).

Effect of Carotenoid Supplementation on Plasma Carotenoid Levels

We calculated 21 effect sizes for plasma carotenoid content from 13 studies of six species (all focal species except the American goldfinch; figs. 1, B1, app. A). When we included all data, no variable significantly predicted the effect of supplementation on plasma carotenoid response (all $P > 0.12$; table 1); however, when we omitted one effect size from a study on great tits that featured an exceptionally large daily carotenoid intake (Peters et al. 2011), we found that carotenoid intake difference between supplemented and unsupplemented groups had a significant effect on plasma carotenoid content response ratio (table 1). Subgroup models where only greenfinches or zebra

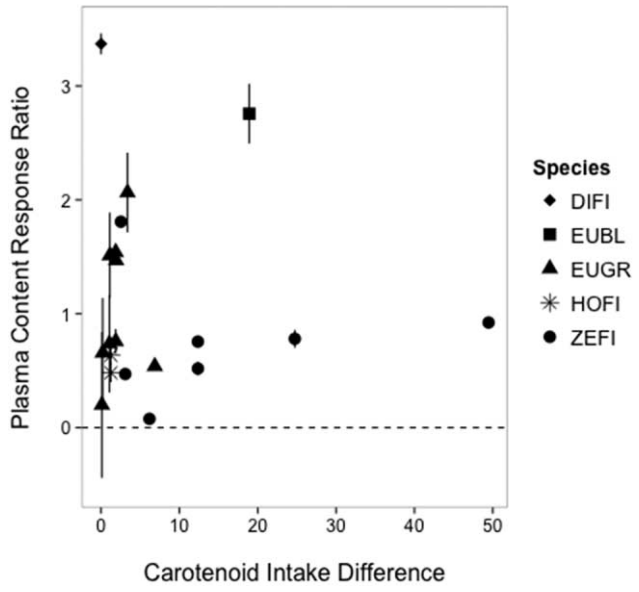

Figure 1. Plasma carotenoid content response ratio (±SE) relative to the difference in carotenoid intake between supplemented and unsupplemented groups. Points with no visible error bars represent errors less than the diameter of the point. The dashed line represents an effect size of zero, or no difference in plasma carotenoid content between supplemented and control groups. Species codes are as follows: DIFI, diamond firetail (*Stagonopleura guttata*); EUBL, Eurasian blackbird (*Turdus merula*); EUGR, European greenfinch (*Carduelis chloris*); HOFI, house finch (*Haemorhous mexicanus*); ZEFI, zebra finch (*Taeniopygia guttata*). Not pictured is the effect size from Peters et al. (2011), an outlier excluded from main analyses.

finches were included also revealed either a trend (greenfinch) or a significant effect (zebra finch) of carotenoid intake on the response of plasma carotenoid levels to supplementation, though the slope of this relationship differed between the two species: greenfinches exhibited a larger increase in coloration with increasing carotenoid intake, on average, than zebra finches (table 1; fig. 2).

In addition, we found that duration of supplementation had a significant negative effect on response in the greenfinch subgroup, indicating that increasing the number of days of supplementation tended to decrease the effect of supplementation on the response of plasma carotenoid levels in this species (table 1). This relationship appeared driven by a single study with a 60-d duration (Peters et al. 2008) and a comparatively low effect size relative to carotenoid intake difference, so we ran an additional meta-regression on a data set excluding this data point and found that the negative effect of supplementation duration was no longer significant, while the difference in daily carotenoid intake continued to trend toward significance ($P = 0.054$; table 1). Interestingly, the study of Peters et al. (2008) was exceptional not only in its long duration but also in that it was the single study of greenfinch plasma carotenoid levels performed while the birds were undergoing molt (app. A); if the process of depositing carotenoids in the growing feathers significantly altered plasma carotenoid levels, then molt (rather than supplement duration) could be responsible for the lower effect size relative to carotenoid intake observed in this study.

Significant residual heterogeneity remained in the full data set model, the full model excluding the Peters et al. (2011) outlier (described above), and the model including only the zebra finch data but not in the models containing only greenfinch data (both with and without Peters et al. 2008; table 1).

Effect of Carotenoid Supplementation on Coloration

We extracted 19 coloration effect sizes from eight studies of five species: the American goldfinch, diamond firetail, European greenfinch, Eurasian blackbird, and zebra finch (figs. 3, B2; app. A). Meta-regression indicated that only the type of measurement used to quantify coloration (i.e., hue, chroma, principle component analysis) was significant in predicting the magnitude of the effect of supplementation on coloration. While the presence of supplementation increased coloration in most studies (fig. 3), neither increasing the difference in carotenoid intake between supplemented and unsupplemented birds nor increasing the duration of supplementation affected the difference in color between experimental and control groups of birds (table 1). Carotenoid intake continued to have no significant relationship with effect size, even in subgroup models isolating studies measuring only the parameters of hue or chroma ($P > 0.4$), indicating that variation in color measurement was not obscuring effects of variation in carotenoid intake in the overall model (table 1).

The separate analysis of zebra finch data also revealed a significant effect of only measurement type on the response of coloration to supplementation (table 1). Performing an additional analysis of the zebra finch data set comprising only effect sizes measured with hue (excluding one effect size of principle component analysis; McGraw and Ardia 2003) did not alter the significance of other model variables; neither days of supplementation nor the magnitude of carotenoid intake had a significant effect on the color difference between experimental and control groups of zebra finches (table 1).

When we examined the relationship between the responses of coloration and plasma carotenoid content, we found no significant effect of any model parameter. Incorporating the plasma carotenoid content effect size in the coloration model did reduce the effects of residual heterogeneity from highly significant in the overall model to nonsignificant (table 1), indicating that variation in plasma carotenoid content likely caused some of the variation in effect sizes present in the overall coloration data set. Visual inspection of the plotted relationship between plasma content and coloration effect sizes revealed that most points fell below the 1 : 1 line (fig. 4), so the effect of supplementation on coloration tended to be smaller than the effect of the same supplementation regimen on plasma carotenoid content.

Significant residual heterogeneity remained in the overall model and the models of only hue or chroma but not in the zebra finch or plasma carotenoid content models (table 1).

Table 1: Meta-regression model and test of residual heterogeneity results: model effect estimates ± SE

Model and subgroup	No. effect sizes	Intercept	Carotenoid intake difference	Supplementation duration	Species	Measurement type	Plasma effect size	Cochran's Q
Plasma carotenoid content:								
Overall	21	2.47 ± .65***	.0001 ± .002	−.0065 ± .17	−.28 ± .18	NA	NA	112.3**
Overall, without outlier	20	2.59 ± .58***	.014 ± .005***	−.015 ± .016	−.24 ± .16	NA	NA	117.1***
ZEFI	7	1.01 ± 2.27	.014 ± .005***	−.004 ± .05	NA	NA	NA	46.0***
EUGR	9	1.61 ± .18***	.29 ± .16*	−.047 ± .018***	NA	NA	NA	4.36
EUGR, without outlier	8	.90 ± .55	.44 ± .23*	−.03 ± .02	NA	NA	NA	3.33
Coloration:								
Overall	19	.12 ± .45	.0050 ± .011	−.0004 ± .0057	.11 ± .09	−.14 ± .050***	NA	197.0***
ZEFI	7	−2.22 ± 1.14*	.011 ± .012	.001 ± .008	NA	1.15 ± .39***	NA	.9
ZEFI, hue only	6	.098 ± .52	.011 ± .012	.0013 ± .0089	NA	NA	NA	.9
Hue	10	−.038 ± .084	.0081 ± .010	−.0002 ± .0008	.046 ± .038	NA	NA	149.9***
Chroma	8	.33 ± .44	.036 ± .081	−.0013 ± .0044	−.097 ± .19	NA	NA	11.3**
Color vs. plasma	11	−2.80 ± 1.81	.0016 ± .011	.0036 ± .011	.55 ± .44	.005 ± .48	.50 ± .47	9.35

Note. NA, not applicable (moderators that were not included in the given model).

*$P < 0.10$.

**$P < 0.05$.

***$P < 0.01$.

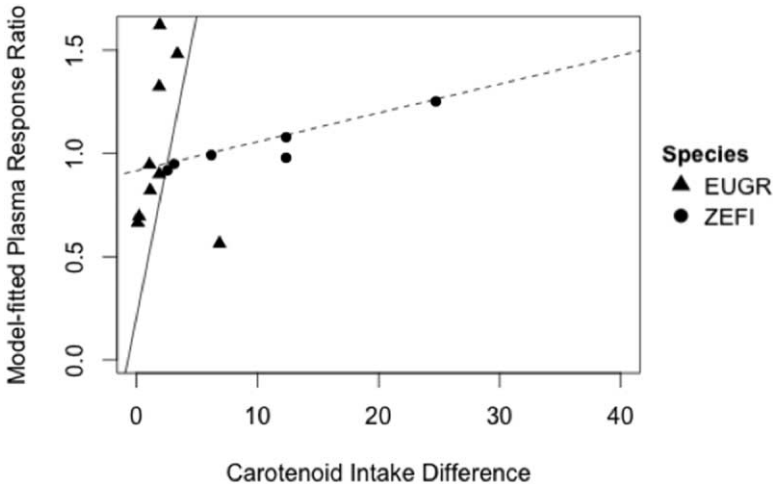

Figure 2. Model-fitted plasma carotenoid response ratios relative to the difference in carotenoid intake between supplemented and unsupplemented groups for two subgroup models comprising data from only zebra finches (ZEFI; circles) or greenfinches (EUGR; triangles). Lines indicate the model-predicted slope of the response of zebra finches (dashed line) or greenfinches (solid line) to increasing carotenoid intake, assuming a constant supplementation duration of 25 d. The greenfinch point with the highest value for carotenoid intake difference represents the effect size from Peters et al. (2011), the only plasma carotenoid content measurement for this species that was taken during molt.

Publication Bias

Visual inspection of funnel plots indicates little bias in the studies examined in our analyses (fig. B3), though many effect sizes were positive; this is not unexpected, given the predicted physiological relationships between carotenoid intake, plasma carotenoid content, and coloration. Regression analyses of funnel plot asymmetry indicated no significant bias in either of the two sets of data (plasma: $z = -0.83, P = 0.40$; color: $z = 1.03$, $P = 0.30$).

Discussion

For studies to meaningfully test differential allocation of a limited pool of carotenoid resources acquired from the diet, they must provide experimental subjects with biologically relevant carotenoid doses. Saturating the diets of birds with carotenoids will obscure physiological trade-offs that may occur between carotenoid absorption, circulation, and use for ornamentation. However, little justification is generally given for the dosage and experimental design used in studies that aim to test for carotenoid trade-offs. To assess the effect that carotenoid dose has on the physiological responses of birds, we performed meta-regressions on data extracted from 15 published studies of seven songbird species. Not surprisingly, and as demonstrated in a previous meta-analysis (Simons et al. 2012), the presence of carotenoid supplementation tended to increase plasma carotenoid levels and the expression of carotenoid-based coloration. However, we found that supplementing an experimental group of birds for a longer period of time or with a larger dose of carotenoids did not increase the difference in color between control and supplemented birds.

Because all carotenoids in the system of an adult bird are derived from the diet, both plasma carotenoid levels and the expression of carotenoid-based coloration are often assumed to directly reflect carotenoid intake (Hill et al. 2002; McGraw 2005). The results of our meta-regression of plasma carotenoid levels indicate that this assumption is correct across the range of supplementation doses provided in studies of captive birds, although the number of days of supplementation did not affect the response. A dose- but not time-dependent effect of supplementation on plasma carotenoid response suggests that the presence of supplementary carotenoids causes an increase in plasma carotenoid levels to a stable level that varies according to the dose offered but that does not continue to increase over the duration of the experiment; supplementation appears to cause the same pattern of increase followed by stabilization in the expression of ornamental coloration, though this effect is not dose dependent. While it is always important to validate whether these trends hold true in a particular study system before applying them to other experimental designs, our results indicate that future studies need not supplement birds for long periods of time in order to collect meaningful data on either plasma carotenoid levels or coloration.

Interestingly, we found a more strongly positive relationship between increasing supplementation dose and increasing plasma carotenoid content in greenfinches than in zebra finches. A fundamental difference between these two species is that greenfinches have yellow feathers that are colored only during the annual molt, while zebra finches have red bills that can be rapidly colored or recolored at any time of the year (Hill and McGraw 2006; Rosenthal et al. 2012). The different patterns of carotenoid absorption and circulation in these two species may reflect the different physiological requirements for pigmenting feathers versus bare parts. Specifically, most greenfinches were not undergoing molt at the time of plasma carotenoid content measurement in the studies we examined, so it is possible that they retained higher levels of ingested carotenoids than zebra finches, which may have been actively depositing carotenoids in their bill ornaments at the time of measurement. The single data point

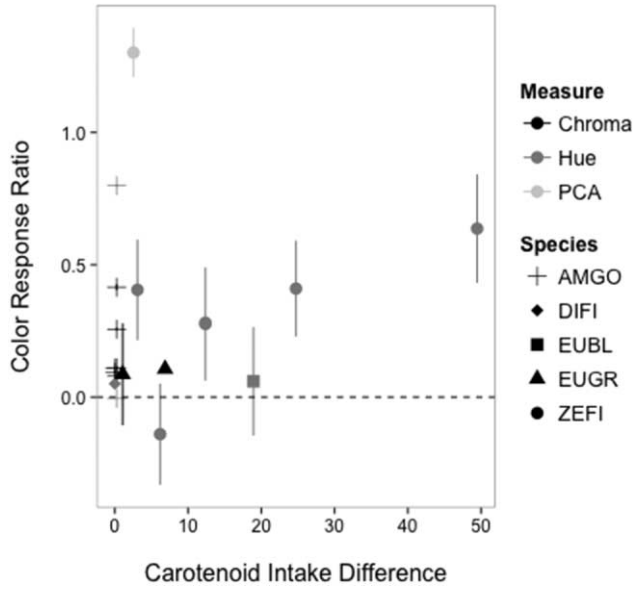

Figure 3. Ornamental coloration response ratio (±SE) relative to carotenoid intake. Symbols with no visible error bars represent errors less than the diameter of the point. Shading indicates the aspect of color that was measured. The dashed line represents an effect size of zero, or no difference in coloration between supplemented and control groups. Species codes are as follows: AMGO, American goldfinch; DIFI, diamond firetail; EUBL, Eurasian blackbird; EUGR, European greenfinch; ZEFI, zebra finch.

from molting greenfinches (Peters et al. 2008) showed the lowest plasma content effect size for its given supplementation regimen, which may have been a consequence of the active deposition of carotenoids into feathers. Unfortunately, the range of studies available in the literature for our analysis did not have the breadth required for separate investigations of whether carotenoid metabolism to produce ornamental pigments from dietary pigments (e.g., to produce red vs. yellow coloration) also affected the relationship between carotenoid intake and plasma content (Hill and Johnson 2012).

One study of great tits (Peters et al. 2011) had a supplementation dose that was orders of magnitude larger than that of the other studies included in this analysis; however, the plasma carotenoid levels measured in this experiment were within the range of those of other studies. One explanation for this finding is that the great tits in this study may have been at the point of maximal carotenoid absorption from their diet such that even their exceptionally large consumption did not cause a corresponding increase in circulating carotenoids (the point of physiological carotenoid saturation). It is also possible that the insect-rich diet of great tits, as opposed to the seed-based diet of many of the finch species in our analysis, necessitates corresponding differences in both carotenoid access and metabolism; however, both the supplemental carotenoid dose and the plasma effect size of another insect-eating species, the Eurasian blackbird, was more similar to the finch species in our study than to these measurements of the great tit. Further examination of the dose-dependent responses of adult great tits to carotenoid supplementation as well as measurement of the quantity of these carotenoids that are allocated to ornamentation will be essential to extricating how this species makes use of dietary carotenoids and how it may differ from the cardueline finches commonly studied in analyses of carotenoid-based ornamentation.

In contrast to the strong positive relationship between levels of carotenoid supplementation and levels of circulating carotenoids, we found that, while the presence of supplementation tended to enhance ornamental coloration, increasing the dose used in supplementation did not cause a corresponding increase in the response of ornamental coloration. Moreover, the only significant predictor of how strongly color responded to supplementation was the parameter used to quantify coloration. These results call into question the general and perhaps overly simplistic assumption that greater carotenoid intake should inexorably lead to showier coloration. The complexity of physiological systems involved in carotenoid coloration (Hill and Johnson 2012) and the links between carotenoid coloration and metabolism (Johnson and Hill 2013; Hill 2014) make simple associations between intake and coloration unlikely, since the expression of coloration is dependent on a variety of physiological variables beyond carotenoid availability alone. In fact, our observation that the response of plasma carotenoid content to supplementation tended to exceed that of coloration indicates that the levels of carotenoids present in circulation

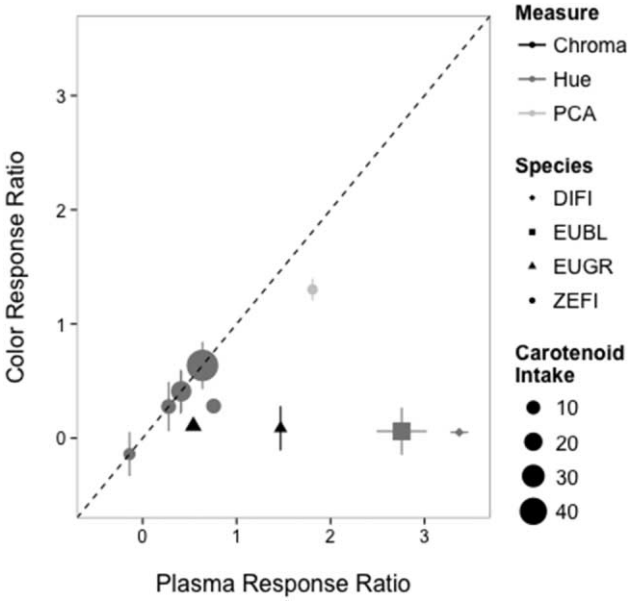

Figure 4. Ornamental coloration response ratio (±SE) relative to plasma carotenoid content response ratio (±SE). Symbols with no visible error bars represent errors less than the diameter of the point. The dashed line represents a 1 : 1 relationship between the two effect sizes. The size of each symbol represents the magnitude of the carotenoid intake difference between supplemented and unsupplemented birds for the given effect size. Species codes are as follows: DIFI, diamond firetail; EUBL, Eurasian blackbird; EUGR, European greenfinch; ZEFI, zebra finch.

were more than adequate for expressing colorful ornaments in the species examined, so factors other than carotenoid limitation appear responsible for the variation in coloration responses observed.

Even after accounting for the effects of moderators, many of our models contained significant residual heterogeneity that could not always be eliminated in subgroup analyses by species or by measurement. The persistent variation in effect sizes within each model emphasizes the unpredictability of response to supplementation among studies, even within one species and controlling for variation in carotenoid dose, supplementation duration, and measurement type. Our results substantiate the importance of validating that a particular supplementation regimen is appropriate for a particular experimental design, perhaps through dosage trials, which are currently used in only a minority of studies (Alonso-Alvarez et al. 2004; Aguilera and Amat 2007).

An additional source of variation in our meta-analysis may be our estimates of carotenoid intake, which are calculated from predicted food or water intake based on the diet and mass of each focal passerine species. In fact, while our analyses were limited to an average measure of consumption for a particular species, an important consideration for future supplementation experiments is how food or water intake may vary among individuals or among treatment groups. The possibility that birds may use behavioral changes to alter physiological carotenoid access remains largely unexplored (but see Hill 1995; McGraw et al. 2003; Peters et al. 2011) and poses a challenge to detecting internal resource trade-offs. Incorporating measures of water or food intake with analyses of circulating carotenoids and ornamental coloration is a simple but highly valuable step to understand the true magnitude—and, consequently, biological relevance—of supplementation.

Despite the large number of studies that have tested the physiological effects of carotenoids on ornamentation, only a small sample of studies performed controlled supplementation of adult birds with carotenoid-based coloration. Although this small sample size necessarily limits the breadth of the inferences that can be drawn from our study, we found some intriguing patterns that are not necessarily intuitive. Our ultimate goal is to emphasize important methodological and theoretical considerations for future studies using carotenoid supplementation to assess the condition dependence of carotenoid-based ornaments. Improving the clarity of the relationships between carotenoid intake, circulation, and deposition in ornamentation in a variety of species will be an important step to better understanding the size and function of the pool of dietary carotenoids available to songbirds and may reduce ambiguity in the results of studies searching for carotenoid allocation trade-offs.

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Literature Cited

Aguilera E. and J.A. Amat. 2007. Carotenoids, immune response and the expression of sexual ornaments in male greenfinches (*Carduelis chloris*). Naturwissenschaften 94:895–902.

Alonso-Alvarez C., S. Bertrand, G. Devevey, M. Gaillard, J. Prost, B. Faivre, and G. Sorci. 2004. An experimental test of the dose-dependent effect of carotenoids and immune activation on sexual signals and antioxidant activity. Am Nat 164:651–659.

Alonso-Alvarez C., L. Perez-Rodriguez, R. Mateo, O. Chastel, and J. Vinuela. 2008. The oxidation handicap hypothesis and the carotenoid allocation trade-off. J Evol Biol 21:1789–1797.

Baeta R., B. Faivre, S. Motreuil, M. Gaillard, and J. Moreau. 2008. Carotenoid trade-off between parasitic resistance and sexual display: an experimental study in the blackbird (*Turdus merula*). Proc R Soc B 275:427–434.

Blount J.D., N.B. Metcalfe, T.R. Birkhead, and P.F. Surai. 2003. Carotenoid modulation of immune function and sexual attractiveness in zebra finches. Science 300:125–127.

Butler M.W. and K.J. McGraw. 2013. Immune function is related to adult carotenoid and bile pigment levels, but not to dietary carotenoid access during development, in female mallard ducks. J Exp Biol 216:2632–2640.

Calder W. and E.J. Braun. 1983. Scaling of osmotic regulation in mammals and birds. Am J Physiol 244:R601–R606.

Caraco T., S. Martindale, and T.S. Whittam. 1980. An empirical demonstration of risk-sensitive foraging preferences. Anim Behav 28:820–830.

Costantini D., C. Coluzza, A. Fanfani, and G. Dell'Omo. 2007. Effects of carotenoid supplementation on colour expression, oxidative stress and body mass in rehabilitated captive adult kestrels (*Falco tinnunculus*). J Comp Physiol B 177: 723–731.

Costantini D. and A.P. Møller. 2008. Carotenoids are minor antioxidants for birds. Funct Ecol 22:367–370.

del Hoyo J., A. Elliott, J. Sargatal, and J. Cabot. 2010. Handbook of the birds of the world. Lynx, Barcelona.

Goodwin T.W. 1984. The biochemistry of the carotenoids. Springer, Berlin.

Hadfield J.D. and I.P.F. Owens. 2006. Strong environmental determination of a carotenoid-based plumage trait is not mediated by carotenoid availability. J Evol Biol 19:1104–1114.

Hartley R.C. and M.W. Kennedy. 2004. Are carotenoids a red herring in sexual display? Trends Ecol Evol 19:353–354.

Hill G.E. 1994. House finches are what they eat: a reply to Hudon. Auk 111:221–225.

———. 1995. Seasonal variation in circulating carotenoid pigments in the house finch. Auk 112:1057–1061.

———. 1996. Redness as a measure of the production cost of ornamental coloration. Ethol Ecol Evol 8:157–175.

———. 1999. Is there an immunological cost to carotenoid-based ornamental coloration? Am Nat 154:589–595.

———. 2002. A red bird in a brown bag: the function and evolution of colorful plumage in the house finch. Oxford University Press, Oxford.

———. 2006. Environmental regulation of ornamental coloration. Pp. 507–560 in G.E. Hill and K.J. McGraw, eds. Bird coloration: mechanisms and measurements. Harvard University Press, Cambridge, MA.

———. 2011. Condition-dependent traits as signals of the functionality of vital cellular processes. Ecol Lett 14:625–634.

———. 2014. Cellular respiration: the nexus of stress, condition, and ornamentation. Integr Comp Biol 54:645–657.

Hill G.E., C.Y. Inouye, and R. Montgomerie. 2002. Dietary carotenoids predict plumage coloration in wild house finches. Proc R Soc B 269:1119–1124.

Hill G.E. and J.D. Johnson. 2012. The vitamin A–redox hypothesis: a biochemical basis for honest signaling via carotenoid pigmentation. Am Nat 180:E127–E150.

Hill G.E. and K.J. McGraw. 2006. Bird coloration: mechanisms and measurements. Harvard University Press, Cambridge, MA.

Hõrak P., L. Saks, I. Ots, T. Kullissaar, H. Kollist, and M. Zilmer. 2003. Physiological effects of immune challenge in captive greenfinches (*Carduelis chloris*). Can J Zool 81:371–379.

Hõrak P., L. Saks, M. Zilmer, U. Karu, and K. Zilmer. 2007. Do dietary antioxidants alleviate the cost of immune activation? an experiment with greenfinches. Am Nat 170:625–635.

Hudon J. 1994. Showiness, carotenoids, and captivity: a comment on Hill (1992). Auk 111:218–221.

Inouye C.Y., G.E. Hill, R.D. Stradi, and R. Montgomerie. 2001. Carotenoid pigments in male house finch plumage in relation to age, subspecies, and ornamental coloration. Auk 118:900–915.

Johnson J.D. and G.E. Hill. 2013. Is carotenoid ornamentation linked to the inner mitochondria membrane potential? a hypothesis for the maintenance of signal honesty. Biochimie 95:436–444.

Karu U., L. Saks, and P. Horak. 2007. Carotenoid coloration in greenfinches is individually consistent irrespective of foraging ability. Physiol Biochem Zool 80:663–670.

Koricheva J., J. Gurevitch, and K. Mengersen. 2013. Handbook of meta-analysis in ecology and evolution. Princeton University Press, Princeton, NJ.

McGraw K. 2005. Interspecific variation in dietary carotenoid assimilation in birds: links to phylogeny and color ornamentation. Comp Biochem Physiol B 142:245–250.

McGraw K.J. and D.R. Ardia. 2003. Carotenoids, immunocompetence, and the information content of sexual colors: an experimental test. Am Nat 162:704–712.

McGraw K.J., O.L. Crino, W. Medina-Jerez, and P.M. Nolan. 2006. Effect of dietary carotenoid supplementation on food intake and immune function in a songbird with no carotenoid coloration. Ethology 112:1209–1216.

McGraw K.J., A.J. Gregory, R.S. Parker, E. Adkins-Regan, and R. Prum. 2003. Diet, plasma carotenoids, and sexual coloration in the zebra finch (*Taeniopygia guttata*). Auk 120:400–410.

McGraw K.J., G.E. Hill, R. Stradi, and R.S. Parker. 2001. The influence of carotenoid acquisition and utilization on the maintenance of species-typical plumage pigmentation in male American goldfinches (*Carduelis tristis*) and northern cardinals (*Cardinalis cardinalis*). Physiol Biochem Zool 74:843–852.

———. 2002. The effect of dietary carotenoid access on sexual dichromatism and plumage pigment composition in the American goldfinch. Comp Biochem Physiol B 131:261–269.

McGraw K.J. and K.C. Klasing. 2006. Carotenoids, immunity, and integumentary coloration in red junglefowl (*Gallus gallus*). Auk 123:1161–1171.

Møller A.P., C. Biard, J.D. Blount, D.C. Houston, P. Ninni, N. Saino, and P.F. Surai. 2000. Carotenoid-dependent signals: indicators of foraging efficiency, immunocompetence or detoxification ability? Avian Poult Biol Rev 11:137–159.

Montgomerie R. 2006. Analyzing colors. Pp. 90–147 in G.E. Hill and K.J. McGraw, eds. Bird coloration: mechanisms and measurements. Harvard University Press, Cambridge, MA.

Mougeot F., J. Martinez-Padilla, J.D. Blount, L. Perez-Rodriguez, L.M.I. Webster, and S.B. Piertney. 2010. Oxidative stress and the effect of parasites on a carotenoid-based ornament. J Exp Biol 213:400–407.

Nagy K.A., I.A. Girard, and T.K. Brown. 1999. Energetics of free-ranging mammals, reptiles, and birds. Annu Rev Nutr 19:247–277.

Navara K.J. and G.E. Hill. 2003. Dietary carotenoid pigments and immune function in a songbird with extensive carotenoid-based plumage coloration. Behav Ecol 14:909–916.

Pérez-Rodríguez L., F. Mougeot, and C. Alonso-Alvarez. 2010. Carotenoid-based coloration predicts resistance to oxidative damage during immune challenge. J Exp Biol 213:1685–1690.

Peters A., K. Delhey, S. Andersson, H. van Noordwijk, and M.I. Foerschler. 2008. Condition-dependence of multiple carotenoid-based plumage traits: an experimental study. Funct Ecol 22:831–839.

Peters A., S. Magdeburg, and K. Delhey. 2011. The carotenoid conundrum: improved nutrition boosts plasma carotenoid levels but not immune benefits of carotenoid supplementation. Oecologia 166:35–43.

Rasband W.S. 1997–2014. ImageJ. National Institutes of Health, Bethesda, MD.

R Development Core Team. 2015. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna.

Rohatgi A. 2013. WebPlotDigitizer. http://arohatgi.info/WebPlotDigitizer.

Rosenthal M.F., T.G. Murphy, N. Darling, and K.A. Tarvin. 2012. Ornamental bill color rapidly signals changing condition. J Avian Biol 43:553–564.

Sepp T., U. Karu, E. Sild, M. Manniste, and P. Horak. 2011. Effects of carotenoids, immune activation and immune suppression on the intensity of chronic coccidiosis in greenfinches. Exp Parasitol 127:651–657.

Sild E., T. Sepp, M. Manniste, and P. Horak. 2011. Carotenoid intake does not affect immune-stimulated oxidative burst in greenfinches. J Exp Biol 214:3467–3473.

Simons M.J., A.A. Cohen, and S. Verhulst. 2012. What does carotenoid-dependent coloration tell? plasma carotenoid level signals immunocompetence and oxidative stress state in birds: a meta-analysis. PLoS One 7:e43088.

Smith H.G., L. Raberg, T. Ohlsson, M. Granbom, and D. Hasselquist. 2007. Carotenoid and protein supplementation have differential effects on pheasant ornamentation and immunity. J Evol Biol 20:310–319.

Stirnemann I., G. Johnston, B. Rich, J. Robertson, and S. Kleindorfer. 2009. Phytohaemagglutinin (PHA) response and bill-hue wavelength increase with carotenoid supplementation in diamond firetails (*Stagonopleura guttata*). Emu 109:344–351.

Svensson P.A. and B.B.M. Wong. 2011. Carotenoid-based signals in behavioural ecology: a review. Behaviour 148:131–189.

Tella J.L., J. Figuerola, J.J. Negro, G. Blanco, R. Rodriguez-Estrella, M.G. Forero, M.C. Blazquez, A.J. Green, and F. Hiraldo. 2004. Ecological, morphological and phylogenetic correlates of interspecific variation in plasma carotenoid concentration in birds. J Evol Biol 17:156–164.

Viechtbauer W. 2010. Conducting meta-analyses in R with the metafor package. J Stat Softw 36:1–48.

von Schantz T., S. Bensch, M. Grahn, D. Hasselquist, and H. Wittzell. 1999. Good genes, oxidative stress and condition-dependent sexual signals. Proc R Soc B 266:1–12.