

# What Does Carotenoid-Dependent Coloration Tell? Plasma Carotenoid Level Signals Immunocompetence and Oxidative Stress State in Birds—A Meta-Analysis

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## Abstract

Mechanisms maintaining honesty of sexual signals are far from resolved, limiting our understanding of sexual selection and potential important parts of physiology. Carotenoid pigmented visual signals are among the most extensively studied sexual displays, but evidence regarding hypotheses on how carotenoids ensure signal honesty is mixed. Using a phylogenetically controlled meta-analysis of 357 effect sizes across 88 different species of birds, we tested two prominent hypotheses in the field: that carotenoid-dependent coloration signals i) immunocompetence and/or ii) oxidative stress state. Separate meta-analyses were performed for the relationships of trait coloration and circulating carotenoid level with different measures of immunocompetence and oxidative stress state. For immunocompetence we find that carotenoid levels ( $r=0.20$ ) and trait color intensity ( $r=0.17$ ) are significantly positively related to PHA response. Additionally we find that carotenoids are significantly positively related to antioxidant capacity ( $r=0.10$ ), but not significantly related to oxidative damage ( $r=-0.02$ ). Thus our analyses provide support for both hypotheses, in that at least for some aspects of immunity and oxidative stress state the predicted correlations were found. Furthermore, we tested for differences in effect size between experimental and observational studies; a larger effect in observational studies would indicate that co-variation might not be causal. However, we detected no significant difference, suggesting that the relationships we found are causal. The overall effect sizes we report are modest and we discuss potential factors contributing to this, including differences between species. We suggest complementary mechanisms maintaining honesty rather than the involvement of carotenoids in immune function and oxidative stress and suggest experiments on how to test these.

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## Introduction

Mate choice for highly ornamented partners is common in the animal kingdom [1]. These ornaments are usually considered to have evolved through Fisherian runaway selection processes [2] or sensory drive [3], and can evolve into honest signals of phenotypic quality [4,5]. Small preferences at the population level can rapidly select for increased chooser preference and for increased ornamentation of the chosen sex, given that attractive offspring result from sex with attractive mates. Associated costs of ornaments can limit their further elaboration [2]. For instance, if resources required for the development of the ornament are limited this prevents further elaboration. Variation in the ornament can now honestly signal genetic and phenotypic variation in the ability to acquire and/or maintain these resources [4–6]. Therefore choosing mates with these costly signals yields indirect genetic benefits, siring offspring that will be attractive and of high quality. It can also yield direct benefits if the costs of the ornament reflect or are directly related to resources that underlie variation in reproductive performance [7]. Theoretically, sexual ornaments are thus predicted to reliably signal phenotypic quality. However, empirical evidence of costs is scarce [6,8]. The handicap

principle [4,5] states that strategic investment into sexual signals, at the expense of some cost, maintains signal honesty. Not all honest signals require handicapping, but can also be maintained via a diversity of other mechanisms, (reviewed in [8], e.g. social punishment of cheaters). The operating honesty maintenance mechanism and its evolution can only be understood by identifying the fitness costs of sexual signals. Additionally, honest handicap signals which feature in mate choice are predicted to be closely linked to important physiological processes within the animal [9], given that this provides most signaling value and makes it difficult to avoid costs (i.e. cheat). The study of sexual signaling will thus likely yield both insights into its evolution and also into important physiological trade-offs.

Carotenoid dependent sexual traits have received considerable attention with respect to the mechanisms that could maintain their honesty [10–15]. Mate choice for more elaborate carotenoid dependent traits has been described in multiple species (e.g. [16–21]). Carotenoids cannot be synthesized *de novo* by vertebrates making them a scarce commodity [12]. Indeed supplementation with carotenoids increases redness of sexual traits (e.g. [22–25]).

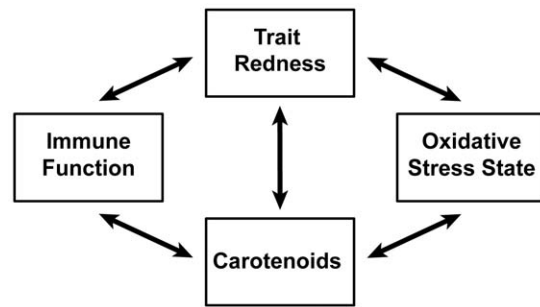
Carotenoids have multiple functions, including the chemical potential to act as antioxidants [26]. However the significance of

their role as antioxidant *in vivo* has been questioned [26,27]. This was corroborated by an earlier meta-analysis in birds which reported no association between carotenoid level and oxidative stress state [28]. Antioxidants prevent damage by free radicals, e.g. reactive oxygen species, to crucial parts of the cell, such as DNA [29]. When antioxidant systems do not adequately quench free radicals, oxidative damage to cell components is increased, which is termed oxidative stress [29,30]. In life history theory, oxidative stress has been hypothesized to shape lifespan and reproductive investment [31]. Possibly ignoring the debatable *in vivo* antioxidant potential of carotenoids [26,27], redness of carotenoid dependent ornaments has been hypothesized to signal oxidative stress state of individuals [14,15,32]. The supplementation of non-carotenoid antioxidants generally increases carotenoid dependent sexual coloration [33–35], but see [25], suggesting that oxidative stress is involved in the determination of carotenoid-dependent coloration.

If redness of sexual traits reliably signals oxidative stress state, mate choice for these traits should yield direct and/or indirect fitness benefits. The precise mechanism through which carotenoid availability is honestly signaling oxidative stress state might be more complicated than carotenoids serving a substantial antioxidant role *in vivo*, but this does not mean that it is not doing just that. Carotenoid levels may function as indicators of oxidative damage, without contributing substantially to the antioxidant barrier, but indicating damage that is not adequately quenched by other antioxidants [27,36]. The reason why these other antioxidants are not used in pigmentation of sexual signals, signaling antioxidant capacity more directly, may simply be because these antioxidants do not absorb light in the way that carotenoids do [27].

Another mechanism by which carotenoid levels may honestly signal aspects of condition is their role in supporting immune function. The immune system is one of the main contributors to total free radical production in vertebrates, and measures of oxidative stress state increase when birds are faced with an immune challenge [37]. The immune system itself is however also sensitive to oxidative stress compromising the integrity of immune cells, especially so because their plasma membranes contain large amounts of polyunsaturated fatty acids [38]. Immunosenescence is also attributed to increased oxidative stress, and has been shown to be reversed by antioxidant treatment [38,39]. Carotenoids may therefore improve immune system functioning via their (debated) antioxidant function [40–42]. Alternatively carotenoids may also improve immune functioning via retinoids, which are derived from carotenoids [43,44]. Retinoids also serve a wide range of other physiological roles involved in tissue repair and gene regulation [27].

The aim of this study was to examine whether the honesty of carotenoid-dependent signals is maintained via the antioxidant and/or immune function action of carotenoids. To this end we carried out meta-analyses. In meta-analysis standardized metrics of multiple study outcomes, effect sizes (ESs) [45,46], are combined to test hypotheses across studies [47,48]. We focused on one class in the animal kingdom, birds, in which carotenoid-dependent signaling is both prevalent [49–51] and mechanistically studied [49]. We summarized five phenotypic relationships: circulating carotenoid levels with trait redness, immune function and oxidative stress state; and trait redness with immune function and oxidative stress state (figure 1). The relationships with trait redness represent signaling value, i.e. the information that can be obtained by a choosing individual regarding the physiological state of the signaler. The relationships with carotenoid levels represent the hypothesized mechanisms maintaining signal honesty.



**Figure 1. The five connections we investigated between, trait redness, carotenoids, immune function and oxidative stress state.**

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## Methods and Approach

### Literature Search

Literature searches were conducted using Google Scholar, with our last search dating to January 2012. We used wide search terms (PRISMA S1, S2) which resulted in the screening of the maximum of 1000 hits Google Scholar provides. Additionally we screened the references of the articles we viewed full text. The details on the number of studies screened for eligibility is therefore a minimal estimate, given that cross-referencing was also used. Our search is described according to the PRISMA [52] flowchart (PRISMA S1). In our field of research no standard reviewing protocols exist that we could use.

Our exclusion criteria were as follows: i) Animals other than birds were studied ii) Necessary information to calculate effect size was not reported and authors did not respond to requests for this information. Authors were always contacted when information necessary to calculate relevant effect sizes was not reported. iii) An immune challenge or oxidative stress challenge was given after which carotenoid levels or sexual coloration were assessed. Our focus here is whether carotenoid levels or carotenoid-dependent coloration predict oxidative stress parameters or immune response. The question of whether challenges reduce carotenoid levels or redness of sexual coloration is relevant [53,54], and this mechanism may in part or fully underlie between individual variation in sexual coloration. However, the effects of experimentally induced immune or oxidative stress cannot be directly scaled to natural variation or direct manipulation of carotenoid levels and may involve different trade-offs and hence we excluded such studies. iv) When carotenoid supplementation was applied experimentally, but data on natural variation in circulating carotenoid levels or coloration were also available we used the latter because this is the variation that a choosing potential mate is confronted with.

### Meta-analytic Technique

Effect sizes were expressed as Pearson's  $r$  and were either directly extracted, calculated from statistics reported using the appropriate conversion formula [45] or measured from graphs (using ImageJ [55]). Pearson's  $r$ 's were transformed to Fisher's  $Z$ 's before analysis [46]. These effect sizes were weighted using the total sample size ( $N$ )  $-3$  [46]. When effect sizes were calculated from statistics where only the degrees of freedom (DF) were reported we used  $N = DF + 2$ . To correct for statistical non-independence of brood-mates, the number of broods rather than the number of nestlings measured was used as  $N$ .

**Table 1.** Overview of the parameters included in the meta-analyses, marked with x, per association investigated.

Analysis		Parameters									
		Phylogeny	Study	Species	Sex	Juvenile	Supplemen- tation	Exp. variation	Assay	Carotenoid color	Site of coloration
trait redness	antibody response	x	x	x				x			x
	H:L ratio	x	x	x							
	parasite load	x	x	x	x						x
	PHA response	x	x	x		x		x			x
	white blood cells	x	x	x							
	antioxidant capacity	x	x	x	x			x	x		x
	oxidative damage	x	x	x				x	x		
carotenoid level	trait redness	x	x	x	x	x	x	x			x
	antibody response	x	x	x	x		x				
	H:L ratio		x	x							
	parasite load	x	x	x							
	PHA response	x	x	x	x	x	x	x		x	
	white blood cells	x	x	x		x					
	antioxidant capacity	x	x	x	x	x	x	x	x	x	
oxidative damage	x	x	x	x	x	x	x	x	x		

See also data S1 and text for inclusion criteria of the moderators.  
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Meta-analyses were performed using Bayesian mixed models implemented in MCMCglmm [56,57] in R [58]. This approach is highly flexible and allows for the inclusion of study, species and phylogeny as random effects [47,56]. Note that phylogeny was only included when the analysis contained more than three species (in practice only one analysis was run without phylogeny, H:L ratio against carotenoid level, table 1). Inverse Wishart priors were used ( $V = 1$ ,  $\nu = 0.002$ ) and models were run three times each, with 10,000,000 iterations, burnin interval of 2,500,000 and thinning interval of 250. Convergence of the models was assessed using Gelman-Rubin statistics, which all were lower (except for the H:L ratio against carotenoid level model which probably included too few data per level to converge readily) than 1.05, which is lower than the recommended criterion of potential scale reduction of 1.1 among chains [59,60].

In several cases multiple effect sizes were extracted from one study and/or from the same species, and controlling for the non-independence of these effect sizes yields a more precise estimate of the effects and their confidence limits. This approach can be considered conservative when compared to treating each effect size estimate as an independent data point. The phylogeny included was a pruned supertree of birds [61]. This is a maximum parsimony tree and therefore without estimates of branch lengths. To date no comparable supertree of birds is available with estimates of branch lengths, therefore we assumed equal branch lengths and scaled these to obtain an ultrametric tree. Branch

lengths between nodes thus equaled one divided by the number of nodes from root to tip.

Publication bias can be a potential caveat in meta-analysis given that the tendency not to publish non-significant relationships can inflate average effect sizes [62]. These biases become apparent in asymmetry of a funnel plot, in which effects sizes are plotted against the corresponding sample sizes (funnel plots S1). Additionally, publication bias is less likely to be present when data is obtained from the authors directly when not all statistics of interest to a meta-analysis were reported. This was the case in 25% of the effect sizes included in the present study (data S1). The rank correlation test for funnel plot asymmetry [48] did not reach significance in any of the analyses (all  $p > 0.05$ ).

### Moderators

Effect sizes can differ between studies for many reasons, including stochastic variation, but also potential moderating variables. Therefore, we included several moderating variables to test whether they explain variation in effect sizes. In each separate meta-analysis we only included moderators for which we had at least three effect size estimates per level (data S1, table 1). These moderators were added simultaneously to the corresponding models. For moderators that showed at least a trend ( $p < 0.1$ ), separate meta-analyses were run within the levels of moderators to investigate overall effects sizes in these subcategories. For example,

when sex showed a trend in the overall model, we ran separate models for each sex. We chose to investigate trends in addition to significant effects for two reasons: i) to be conservative in controlling for potential confounding variables and ii) because multi-level moderators with a low number of effect sizes per level may be hard to detect, but may provide new testable hypotheses.

We included the following moderators: i) Sex, for which we coded unknown sex as 0, females as  $-1$ , and males as  $1$  ii) Whether adults or juveniles were studied. iii) Whether carotenoids were supplemented or not. iv) Whether the effect size was subject to experimental variation, caused by treatments other than carotenoid supplementation, which potentially increased variation in the traits of interest. To avoid such effects we selected pre-experimental (including carotenoid supplementation studies) values or results of analyses of the control group only, when possible. v) The oxidative stress state assay used. For antioxidant capacity the following levels were distinguished: the OXY test [63], the TEAC test [64] and the KRL test [65], which differ in components of the plasma antioxidant barrier that are measured [63]. For oxidative damage analyses the following moderator levels were distinguished: MDA, TBARS, [66] and d-ROM [67]. vi) In the analyses of the associations with carotenoid levels we included a moderator indicating whether the study species exhibited carotenoid-dependent coloration or not. Because the precise usage of carotenoids in pigmentation is unknown for many species, this was judged by the presence of yellow, orange or red traits characteristic for carotenoid-based pigmentation [51,68]. Images were searched via Google Images with a search query that included genus and species name. The first nine pictures of the search results were viewed to be positive of the species identification and to control for variability in picture quality and coloration. Species for which these colors are known to be based on other pigments were coded as exhibiting no carotenoid pigmentation (e.g. *Gallus gallus* [69,70]) or in a special case, the blue-footed booby, blue coloration was considered carotenoid based as this was previously demonstrated [71]. When carotenoids are used to pigment sexual traits one may expect higher effect sizes, given that sexual selection may increase variance in carotenoids due to investment into sexual traits. Additionally carotenoid-based signals are perhaps more likely to evolve in species in which carotenoids play a major physiological role. vii) Within the sexual signal analyses, we included a moderator indicating whether the carotenoid-dependent coloration was expressed in plumage or other tissue (e.g. the bill), using the same pictures as described above. Plumage pigmentation reflects physiological state at molt and may therefore signal current physiological state less reliably than carotenoid-dependent coloration in other tissues, such as the bill, that can change more rapidly.

## Color

Aspects of light reflectance together composing the perception of variation in colors are described in many ways, for example brightness, hue and chroma [72] or principal components [73]. These aspects can also be captured in various ways, for example by comparing color charts [74], digital photography [75,76] or spectrophotometry [73]. Not surprisingly, studies from which we extracted effect sizes related to coloration used different descriptions of color. Effect sizes of these studies were interpreted as follows: i) Measures which corresponded to a shifted weighted spectrum towards red were interpreted as representing increasing carotenoid content in a trait. This includes a shift in the shape of the curve towards the red part of the spectrum (corresponding to hue) and an increase in the relative reflectance in the red part of

the spectrum (corresponding to chroma). Papers with color measures that corresponded to total reflectance (brightness) could not be interpreted as either increasing or decreasing carotenoid content and were not included, except when the authors presented evidence of it reflecting carotenoid content of the trait considered. ii) When multiple relationships of the considered (see above) color metrics were reported we took an average of effect sizes across these metrics. The sign of the effect size was expressed as positive when the relationship showed a positive relationship with carotenoid-dependent color intensity (i.e. trait redness).

## Immune System Components

The immune system is complex, and several components of the immune system have been studied in relation to carotenoid levels and carotenoid-dependent coloration. In our analyses we considered the measures of the immune system of which we found four or more independent studies. These measures were as follows: PHA response, antibody production against experimentally induced antigens, parasite load and white blood cell counts.

Swelling induced by the subcutaneous injection of phytohaemagglutinin (PHA, a lectin found in plants used as mitogen) is a widely used test in birds and other vertebrates [77]. Larger swellings are interpreted as a stronger immune response, a view supported by the finding that larger swellings are usually found in individuals or experimental groups that can be considered to be in a better state [77–80]. However, the specific immunology behind PHA responses in birds is still debatable possibly limiting such straightforward interpretation [81]. Especially the common interpretation that PHA responses represent a T-cell mediated immune response alone may be incomplete [78,79].

Antibody responses are commonly assumed to be more effective with increasing amount of antibodies produced [82–84]. We did not discriminate between the different antigens used to induce an immune response, because several antigens were only used in one or a few studies (see data S1).

White blood cell counts are more difficult to interpret, because they can both indicate current infections or high immunocompetence. Separate populations of white blood cells may be abundant because of a current infection or higher levels may indicate the ability to launch a more potent immune response [82]. We analyzed studies reporting on separate types of white blood cells together and averaged correlations reported with our variables of interest across separate types of white blood cells when they were reported within a single study to make the most of the available data. Higher ratios of heterophils over lymphocytes are considered a reliable indicator of higher stress [85], which we therefore analyzed in a separate analysis.

Parasite infection may either reflect inability to clear parasites or the ability to tolerate parasites [86]. In the case of carotenoid-dependent coloration, resource allocation of carotenoids towards signal intensity is either predicted to increase parasite load in bright males, or, when resource availability differs between individuals, parasite load is predicted to co-vary negatively with signal intensity [87]. A positive relationship between parasite load and signal intensity can also become apparent by selective disappearance of highly parasitized low-quality individuals harboring signals of low intensity [88].

## Oxidative Stress State

The imbalance between the production of free radicals and antioxidant defenses that quench them is termed oxidative stress [29]. Free radical damage to “crucial” cell components can impair physiological function and it is this impairment that is viewed as a major agent of senescence [29]. To capture aspects of oxidative

stress, measures of antioxidant defense and oxidative damage are employed [30]. To interpret differences in oxidative stress state between individuals both these measures are required [30]. When for example antioxidant capacity increases in response to increased exposure to free radicals, conclusions based solely on either antioxidant capacity or oxidative damage will lead to opposite conclusions (e.g. antioxidant capacity is higher, indicating lower oxidative stress; oxidative damage is higher indicating higher oxidative stress). Therefore we performed separate meta-analyses for effect sizes of antioxidant capacity and oxidative damage.

Assuming that carotenoids do not directly increase free radical production an association of carotenoid level with higher antioxidant capacity is likely to reflect a positive effect of carotenoids on resistance against free radicals. This is expected to also result in lowered oxidative damage measured, possibly depending on the composition of antioxidant defenses and the proxy of oxidative damage measured. We therefore speculate that associations of carotenoids with decreased measured oxidative damage or increased antioxidant capacity can be interpreted as a reduction in oxidative stress, improving oxidative stress state. Associations with trait redness are more elusive, because it cannot be excluded that free radical production is associated with trait expression.

## Carotenoids

There are many subtypes of carotenoids and species differ in the combinations of carotenoids they incorporate into sexual traits (reviewed in [49]) as well as in the relative levels in plasma [89]. Because of the diversity of ways that carotenoid levels are reported discrimination between different types of carotenoids was not feasible in our meta-analyses, and we pooled separate correlations between levels of carotenoid subtypes and the variables of interest when they were reported separately. In many studies carotenoid levels in plasma are assessed colorimetrically which only yields a total plasma concentration of carotenoids (e.g. [33]). Additionally, information on the precise mechanisms of specific carotenoid incorporation in pigmentation is lacking for many species [49] and carotenoids are likely metabolized into different subtypes [49,90,91]. Hence a more detailed treatment of carotenoid levels was not feasible, even though we recognize that this could provide more informative estimates.

## Differences between Species

Relationships between different antioxidants, including carotenoids, and antioxidant capacity vary substantially across species [89]. Sexual selection for carotenoid-dependent coloration may also differ between species. In some species carotenoid-dependent coloration may not have evolved into a costly signal that advertises condition, may be a remnant of past selection, or may serve other roles such as sex and species recognition [1,92]. To assess the importance of interspecific variation in our meta-analyses we first compared whether adding species and phylogeny to a model which included only study improved the model, as judged by the deviance information criterion (DIC) (table 2). Lower DIC values indicate a better fit and can be considered the Bayesian counterpart of the Akaike information criterion (AIC) [60]. Additionally we calculated the proportion of heterogeneity explained by species and phylogeny in the model [47,60]. To visualize some of these differences between species and directly test within-species effects we performed within-species meta-analyses within the datasets for which we found at least three separate studies per single species. These analyses were performed using the metafor package [48] in R [58] using random-effects meta-analysis estimated using REML, given that the complex data structure for

which we employed MCMCglmm is not present within species. Multiple effect sizes per study were pooled by using a weighted average for sample size.

## Residual Heterogeneity

Heterogeneity between effect sizes due to factors other than the moderators described above can suggest potential for additional moderators to explain variation among effect sizes. We calculated the residual heterogeneity according to Nakagawa and Santos [47]. The variance component of study and the residual variance were summed per sample along the chain and divided by the sum of all variance components and the typical sampling error variance [47] to calculate the proportion of residual heterogeneity. Low, moderate and high levels of heterogeneity are considered to be 25%, 50% and 75% respectively (equations 22–25 in [47]).

## Results

Our literature search identified 148 studies [22,23,25,35,54,64,67,71,89,93–229] on 88 species with information on 357 estimates of effect sizes falling into 15 categories of pairwise associations among relevant variables (data S1).

### Carotenoids, Trait Redness

As expected we found that carotenoid availability was positively related to redness of sexual traits (figure 2,  $p < 0.0001$ ). Experiments that supplemented carotenoids rather than correlations with concentrations of carotenoids in blood resulted in significantly larger effect sizes ( $p = 0.006$ ), but an analysis without the supplementation studies still yielded a highly significant overall effect size (table 2,  $p < 0.0001$ ). Males tended to show higher effect sizes than females (sex,  $p = 0.094$ ), though both sexes showed significant associations in stratified analyses (table 2,  $p < 0.01$ ).

### Immune Function

Of the five categories of immunological measurements only the PHA response was significantly and positively associated with higher carotenoid levels ( $p = 0.04$ ) and trait redness ( $p = 0.03$ ). We detected a tendency for higher carotenoid levels to be associated with lower parasite load (figure 2,  $p = 0.06$ ). This was however not reflected in the relationship between trait redness and parasite load ( $p = 0.93$ ). We did not detect any effects of the moderating variables included (table 1).

### Oxidative Stress State

Carotenoid levels increased with antioxidant capacity (figure 2,  $p = 0.027$ ), but were not associated with oxidative damage (table 2,  $p = 0.70$ ). The relationships of trait redness with antioxidant capacity ( $p = 0.86$ ) and oxidative damage ( $p = 0.30$ ) were not significant (figure 2). Only for the relationship between carotenoid levels and oxidative damage did we detect any effect of the moderators included (table 1, table 2). Males tended to have a lower effect size ( $p = 0.097$ ), and within males we did not detect a significant overall effect size ( $p = 0.20$ ).

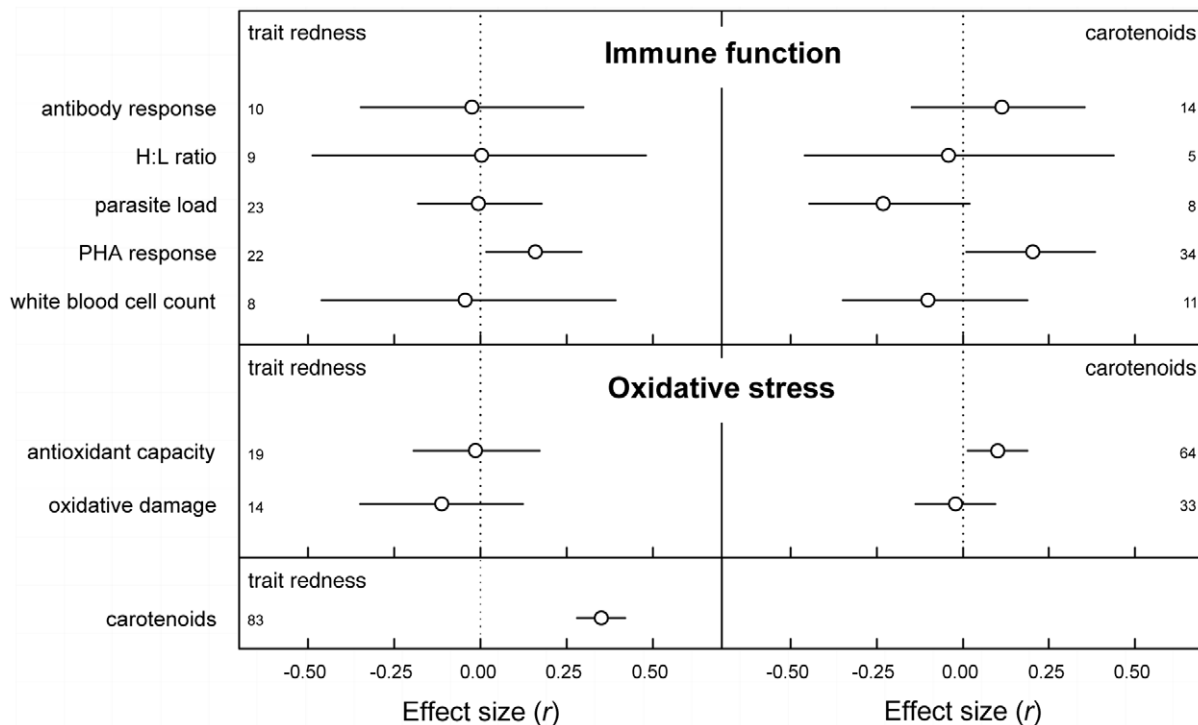
### Differences between Species

Of the 17 models, the within-moderator level analyses, 12 models improved when species and phylogeny were added to the model (table 2, as judged by reduction in DIC). This demonstrates variation between species over and above variation attributable to differences between studies or typical sampling error variance. Separate analyses of species for which three or more effect sizes were available also showed considerable variation (figure 3), but note that the analyses presented in table 2 are with all species

**Table 2.** Overview of the separate meta-analyses performed.

Analysis	Results (* marks significance)				DIC (* marks improved model)		Heterogeneity (%)	
	r (95% CI)	ESs	species	study only	full model	species and phylogeny	residual	
trait redness	immune system	antibody response	10	6	-15.77	-14.19	5	47
	Hi:L ratio	Hi:L ratio	9	6	1.28	-5.02*	2	7
	parasite load	parasite load	23	15	-11.39	-32.26*	74	14
	PHA response	PHA response	22	15	-37.29	-37.69*	8	28
oxidative stress	white blood cell count	white blood cell count	8	6	-1.72	-7.03*	4	5
	antioxidant capacity	antioxidant capacity	19	9	-43.35	-42.17	10	17
	oxidative damage	oxidative damage	14	8	-25.25	-28.92*	12	7
	color	oxidative damage	83	28	15.10	16.59	4	64
carotenoid level	trait redness (all)	trait redness (all)	45	21	-53.46	-51.96	6	61
	trait redness (males)	trait redness (males)	17	15	-19.40	-26.35*	7	9
	trait redness (females)	trait redness (females)	55	19	0.45	-1.34*	8	50
	trait redness (all without supplementation)	trait redness (all without supplementation)	14	7	-27.59	-28.28*	10	15
immune system	antibody response	antibody response	5	3	-12.08	-12.13*	1	10
	Hi:L ratio	Hi:L ratio	8	6	-20.53	-20.13	11	10
	parasite load	parasite load	34	19	-31.62	-35.97*	8	57
	PHA response	PHA response	11	8	-22.10	-25.17*	20	5
oxidative stress	white blood cell count	white blood cell count	64	37	-156.13	-176.54*	13	7
	antioxidant capacity	antioxidant capacity	33	15	-79.92	-77.5	7	13
	oxidative damage	oxidative damage	17	10	-40.41	-39.37	10	10
	oxidative damage (males)	oxidative damage (males)						

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**Figure 2. Effect sizes ( $\pm 95\%$  confidence interval) of the main 15 meta-analyses.** Note that when the confidence interval does not intersect the dotted line (which reflects  $r=0$ ), the effect size is statistically significant. Numbers along the y-axis depict the number of effect sizes included in each analysis. The variables along the y-axis depict the relationships analyzed with the variables depicted in the upper corners of each panel. doi:10.1371/journal.pone.0043088.g002

included. The separate analyses (figure 3) also show that in some species significant overall effects can be detected which are in accordance with the overall effects (figure 2), whereas in other species they are not, further illustrating the heterogeneity between species.

### Causality

If carotenoid levels covary with the physiological variables we summarized here without being causally involved, inclusion of studies that supplement carotenoids would lower effect sizes. For the relationship of carotenoid levels with both antioxidant capacity and PHA response we found positive non-significant associations with the supplementation moderator (estimate = 0.14 ( $-0.06:0.34$  95% CI),  $p=0.15$  and estimate = 0.13 ( $-0.23:0.51$ , 95% CI),  $p=0.48$  respectively). That these associations are non-significant implies that the effects of natural and experimental variation cannot be distinguished, indicating that carotenoids are likely to be mechanistically involved. Carotenoid supplementation was associated with higher effect sizes of the increase in trait redness (see above). This suggests that either the dosages used induce carotenoid levels outside of the normal range or that it decreases variance between individuals, both can increase effect size.

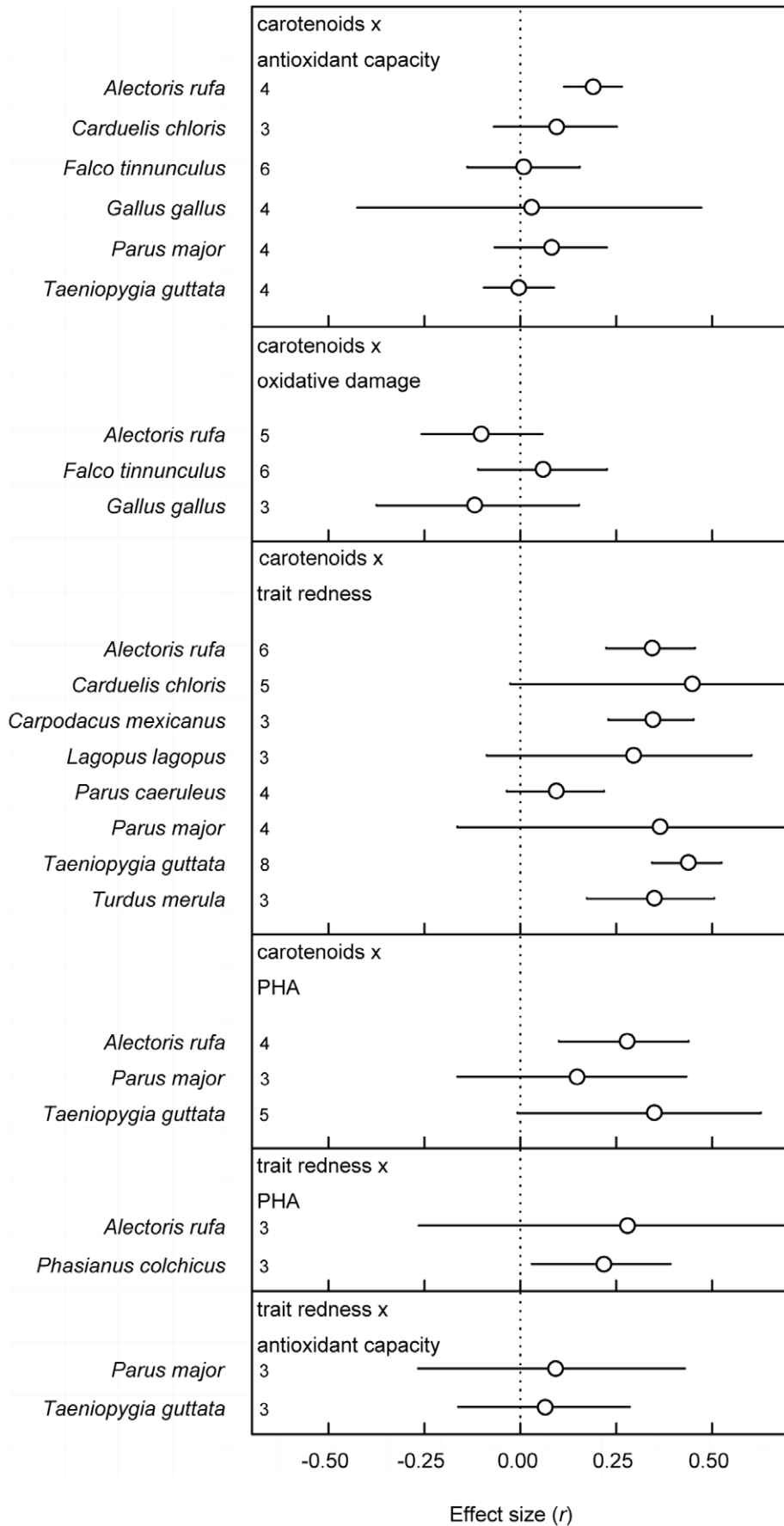
### Discussion

Pooling a large number of studies through meta-analysis, we found evidence for the hypothesized honesty maintenance mechanisms of the associations of carotenoid levels with immune functioning and oxidative stress state (figures 2 and 4). Honest signaling via carotenoids was only apparent in PHA response, given that carotenoids and trait redness were both associated with

a greater swelling in response to PHA injection. Carotenoids tended to be associated with lower parasite abundance suggesting that carotenoids may signal multiple components of the immune system; however, this effect was not mimicked in the association with trait redness. Future studies may reveal whether carotenoids directly increase the efficacy of components of the immune system involved in the PHA response. Alternatively, carotenoids may alter oxidative stress state which may in turn affect immune responses [37,40–42].

For oxidative stress state, we show that carotenoids signal higher antioxidant capacity, increased resistance against free radicals. However no association between trait redness and oxidative stress state was found (figure 2), and hence we find no evidence that antioxidant capacity is signaled via carotenoid-dependent traits. The size of the overall effect is modest suggesting that carotenoids are indeed only minor contributors to antioxidant capacity *in vivo* as suggested previously [27,28,89,230]. However the significance of the overall effect size does show that carotenoids can signal oxidative stress state in birds, and our finding that the effect size was independent of whether carotenoids were experimentally supplemented or not suggests this relationship to be causal. Moreover, carotenoids may be more important in regulating oxidative balance in other parts of the body than in the blood circulation, such as cell membranes, information not necessarily captured by the plasma carotenoid levels we used in this study.

An alternative explanation for the modest overall effect sizes we find is that carotenoid levels are required for retinoid production or indicate the amount of free radicals that are not quenched by other antioxidant machinery (i.e. enzymatic and non-enzymatic) [27]. In this scenario carotenoids are not contributing substantially to the quenching of free radicals but are damaged (i.e. bleached)





**Figure 3. Plot of separate overall effect sizes ( $\pm 95\%$  confidence interval) per species.** An overall effect size per species was only calculated when three or more studies were available per relationship of interest. Numbers along the y-axis depict the number of effect sizes included in each overall effect size.

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by them. A correlation between carotenoids and oxidative stress state is then still expected, but may be weak. Under this scenario, the total carotenoid store in the body could be viewed as dynamic indicator of past levels of free radicals that were not quenched by other antioxidants. Measures of antioxidant capacity and oxidative damage are relatively flexible within an individual as demonstrated by their modest within individual repeatability. These repeatabilities in birds range from 0.12 (Beamonte-Barrientos & Verhulst submitted), 0.14 [231], 0.3 [127] to 0.49 [232] for antioxidant capacity and 0.18 [231], 0.42 (Beamonte-Barrientos & Verhulst submitted) to 0.60 [127] for oxidative damage. Correlations with these flexible parameters are therefore predicted to be weak when the carotenoid store integrates past damage and is therefore both lagging and less flexible. The same reasoning holds for the redness of carotenoid-dependent sexual traits, which are considered relatively consistent and have also been experimentally shown to lag in response to immune challenges [53,106]. In addition, variation between studies in the assays used for antioxidant capacity [63] and oxidative damage assays [233] used may weaken the relationship between carotenoids and oxidative stress state. However we did not detect a moderating effect of assay method.

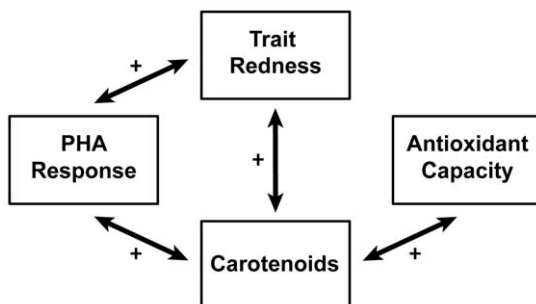
The interspecific variation that was apparent in most analyses (table 2) can also result in lower overall effect sizes. When in some species carotenoid levels and trait redness signal different aspects of physiology or are physiologically less important, effect sizes across species are expected to be lower. Also in some species carotenoid-dependent coloration may not currently be under sexual selection or choosiness for these traits may differ between species. Ideally, we would want to relate the preference for carotenoid-dependent coloration per species to the differences in effect sizes we report. However for carotenoid-dependent traits relatively few species (reviewed in [234], 15 species with little overlap with the species in this study) have been studied in mate-choice experiments and there is only one quantitative meta-analysis within a species, the zebra finch [16]. Given that mate-choice experiments are difficult to conduct or variable in general, judging from its repeatability [235], this may prove difficult.

Interestingly, there is no single species in which all effect sizes that were overall significant were also significant within that species (figure 3). The study of carotenoid-dependent signaling

may thus profit from both in-depth study of a single species, and by examining different species or explaining between-species variation. Which carotenoids are used in pigmenting their traits or how they metabolize or sequester carotenoids may be key. Residual heterogeneity was low in general (table 2) except for the correlations of carotenoid levels with PHA response and trait redness in which residual heterogeneity was moderate. The latter may be due to the differences between species in carotenoid usage. This may also be a reason why some of the between species moderators we examined in this study failed to reach significance.

Whether or not a species evolved carotenoid-dependent coloration as a signal may depend, among other things, on the importance of carotenoids in its physiology, or on the scarcity of carotenoids in the environment, and such effects could be reflected in the relationships between carotenoids on the one hand, and aspects of immune function and oxidative stress state on the other hand. However, whether or not a species exhibited carotenoid-dependent coloration did not affect effect size estimates in our analyses. This may suggest that carotenoids serve the same physiological functions in species exhibiting carotenoid-dependent coloration and those lacking them. Additionally it suggests that the increase in variance caused by sexual selection for carotenoid incorporation into sexual coloration is too small or variable to detect. This may also be the reason why we only detected two trends of lowered effect sizes in females, the generally choosier sex. Within the analyses on trait redness we also did not detect a moderating effect of whether the trait was plumage related and thus subject to molting patterns. A reduction in effect size was expected given that plumage can only signal carotenoid content at molt and is therefore less flexible than for instance skin coloration [236]. The lack of such an association might actually be a reflection of how long past oxidative stress is encoded into carotenoid stores and sexual traits, but this awaits future study.

The two aspects of physiology associated with carotenoids we summarized here, immune function and oxidative stress state, may help maintain honesty of carotenoid-based signals. The use of carotenoids in sexual traits diverts carotenoids away from its benefit for immune function and antioxidant capacity creating a handicap. These aspects are not mutually exclusive with the hypothesis that carotenoid levels integrate information on past exposure to free radicals not quenched by other more potent antioxidants or antioxidant machinery. All three mechanisms have in common that a larger supply of carotenoids into the body will yield a more colorful sexual ornament, increasing mating success. The sequestering of carotenoids, in terms of foraging, assimilation, transport abilities or the like, is under positive selective pressure. This may still be the major mechanism maintaining honesty, together with the beneficial physiological functions of carotenoids, which may be indicative of shorter-term history. Examining how large the contributions of carotenoid sequestering on the one hand and carotenoid use within the body on the other hand are in determining honesty of carotenoid-dependent coloration will require the direct measurement of sequestration and carotenoid turnover, preferably also under both immunological and oxidative stress. This will be exciting yet experimentally challenging. It seems the more complete our understanding of carotenoid-dependent signaling becomes the more areas of biology become involved. The integrated involvement of carotenoids in major physiological areas in combination with their light absorbing



**Figure 4. Overview of the significant overall effect sizes framed in the layout of figure 1.** A full circle argument can only be made for the relationship of carotenoids and trait redness with PHA response. Carotenoids do however signal lowered antioxidant capacity, but there was no significant relationship with trait redness. Pluses and minuses indicate positive or negative relationships.

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properties may be why carotenoid-based sexual signals are common.

## Supporting Information

**Funnel Plots S1** Plotted in the order of table 1 and data S1 are the separate datasets of effect sizes plotted against their corresponding sample sizes. The title depicts the relationship plotted.

(PDF)

**Data S1** Excel file of the datasets collected for the separate meta-analyses.

(XLSX)

**PRISMA S1** PRISMA flow diagram showing the literature search procedure.

(PDF)

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**PRISMA S2** PRISMA checklist for meta-analyses.  
(PDF)

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## Author Contributions

Conceived and designed the experiments: MJPS AAC SV. Analyzed the data: MJPS. Wrote the paper: MJPS AAC SV.

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