INTRODUCTION

Carotenoid-based displays have become textbook examples of honest signals of individual quality (Dugatkin, 2013). Although some studies have failed to find consistent evidence that carotenoid-based traits are related to physiological performance (Dale, 2000; Dowling & Mulder, 2006; Smith, Raberg, Ohlsson, Granbom, & Hasselquist, 2007), behavioural ecologists have generally embraced the idea that carotenoid-based coloration can serve as a condition-dependent signal of quality (Alonso-Alvarez et al., 2004; Hill, 1991; Kemp, Herberstein, & Grether, 2012; McGraw & Ardia, 2003; Peters, Denk, Delhey, & Kempenaers, 2004; Velando, Beamonte-Barrientos, & Torres, 2006). Accordingly, recent research on carotenoid-based ornamentation has focused on the mechanisms by which coloration can be a reliable signal of fundamental aspects of performance, such as immune system function or avoidance of oxidative stress.

Current discussions of the mechanisms that might create a link between carotenoid coloration and performance have been dominated by the resource trade-off hypothesis, which proposes that carotenoid coloration is an honest signal of individual quality because only high-quality individuals can allocate sufficient carotenoid resources away from critical physiological processes to achieve full-colour ornamentation (Figure 1). The resource trade-off hypothesis is founded on three key assumptions: (1) carotenoid pigments are limited resources such that most individuals do not possess sufficient carotenoids to maximize performance in all avenues simultaneously; (2) carotenoid pigments play important roles in physiological processes, particularly in immune response and/or antioxidant defence; and hence, (3) carotenoid-based ornaments reflect quality because only high-quality individuals can allocate to both sides of the trade-off associated with carotenoid pigments.

The main purpose of this synthesis was to provide a critical assessment of these three major assumptions of the resource trade-off hypothesis for explaining honesty in carotenoid-based coloration. We limit the scope of our discussion to studies of birds, which are the group of animals that have been most extensively studied with regard to carotenoid signalling.
Comparisons among classes of vertebrates or between vertebrates and invertebrates can provide important insights, but tackling physiological differences among major taxonomic groups further complicate an already complex topic. By focusing on birds in this review, we hope to highlight patterns and questions that will have important implications for other taxa, and we encourage application of the questions raised here to other systems.

In this review, we assess trade-off mechanisms proposed to explain honest signalling in carotenoid-coloured ornaments while also considering evidence for the direct physiological benefits of carotenoids to the individual. In the process of evaluating the current state of understanding of the main assumptions of the carotenoid resource trade-off hypothesis, we examined 179 empirical studies of carotenoid-based traits in birds published between 1992 and 2017; we provide a list of these studies for reference (Supporting Information Table S1, Appendix S1, Figures S1 and S2 for more detail). While debates regarding the physiological benefits of carotenoids or whether carotenoids are limited resources are not new, here, we focus on a specific, unifying framework and its main predictions. We highlight where assessment can be made based on currently available data and highlight where more information is needed to adequately test the carotenoid resource trade-off hypothesis.

2 ASSUMPTION 1: CAROTENOID LIMITATION

Without carotenoid limitation, carotenoid resource trade-offs cannot be the basis for honest signalling in carotenoid-based traits. Thus, the question of whether carotenoids are limiting resources for birds is a central question related to the resource trade-off hypothesis in avian species (Hadfield & Owens, 2006; Hill, 1994; Hudon, 1994; Olson & Owens, 1998; Simons, Maia, Leenknegt, & Verhulst, 2014). An important distinction is that carotenoid limitation can be interpreted from two distinct perspectives: individuals may be limited in the quantity of carotenoids they can acquire from the environment, or individuals may be limited in the size of their internal “pool” of carotenoid resources available for physiological use. While these two interpretations of carotenoid limitation are related—an individual cannot acquire internal carotenoid resources without consuming carotenoids from the environment—it is important to consider that internal carotenoid limitation can exist without environmental constraints (e.g., due to limitations in quantities that can be assimilated from the diet), and vice versa (e.g., finite quantities in the environment may still be more than sufficient for all processes).

Environmental carotenoid limitation was among the earliest explanations for variation in the expression of carotenoid-based coloration in both fish (Endler, 1980; Grether, Hudon, & Millie, 1999) and...
birds (Hill, 1990; Hill, Inouye, & Montgomerie, 2002). The necessity that animals acquire carotenoids through their diet is sometimes taken as clear evidence that carotenoid acquisition is limiting, but carotenoid resources can be finite without being limiting. It is possible that essentially all wild birds ingest sufficient carotenoids to meet their physiological needs (Hudson, 1994). Unfortunately, questions related to environmental carotenoid limitation are difficult to answer under natural conditions wherein a population of birds may consume a wide variety of food items, individuals may differ in which and how much of those various items they consume, and the internal processes of carotenoid assimilation (which may differ among individuals, species, season and even specific carotenoid pigments; McGraw, 2005; Tella et al., 2004) are largely unknown in any given study system. Studies testing for preferences for carotenoid-rich food in birds with carotenoid-based ornamentation have found only mixed support (Bascuñán, Tourville, Toomey, & McGraw, 2009; Behbahaninia, Butler, Toomey, & McGraw, 2012; Catoni, Metzger, Schaefer, & Bairlein, 2011; Senar et al., 2010), indicating that many colourful birds do not need to preferentially forage for carotenoid-rich food in order to produce colourful ornaments (but see Walker et al., 2014). Currently, environmental carotenoid limitation is not widely considered to be a major determinant of carotenoid-based colour signalling (Hadfield & Owens, 2006; Möller et al., 2000; Svensson & Wong, 2011).

While there is general acceptance that most populations of birds are not limited by the quantity of carotenoids they can acquire from the environment, experimental studies show that even wild populations of birds can respond to dietary carotenoid supplementation (Ewen, Thorogood, Karadas, & Cassey, 2008; Sternalski, Mougeot, Perez-Rodriguez, & Bretagnolle, 2012). If natural environments are not carotenoid-limited, then how can supplementation affect the coloration or physiology of wild birds? That supplementation has sometimes been found to increase coloration, immune response or other measurements in birds with access to their normal diets raises questions about our assumptions of environmental carotenoid limitation—and about supplementation itself.

It is difficult to validate the biological meaning of response to artificial carotenoid supplementation. Dietary supplementation inherently provides carotenoids in quantities and frequencies that differ from levels that would normally be consumed in the wild. Even if such supplementation does not lead to abnormally high plasma carotenoid levels, supplemented birds may be rapidly transporting excess carotenoids to other tissues. Carotenoids provided in supplementation can also enable birds to bypass metabolic pathways. For instance, when the red carotenoid canthaxanthin is provided in diet (Hill, 1992), birds can bypass the oxidation of yellow pigments to red (Lopes et al., 2016). More subtle changes in the ratio of carotenoids in supplemented diets—such as the ratio of lutein to zeaxanthin—can further alter the effects of supplementation (Fitze, Tschirren, Gasparini, & Richner, 2007; García-de Blas, Mateo, & Alonso-Alvarez, 2016). Experimental supplementation may therefore provide different quantities of various carotenoids at artificially high relative abundances and for different periods of time than what any bird would experience in the wild (Koch, Wilson, & Hill, 2015), which makes it difficult to assess the relevance of results of supplementation to variation observed in wild populations.

We can reconcile conflict between general acceptance that wild birds are not environmentally limited by carotenoids with findings that supplementation can modify bird phenotype if we accept that carotenoid supplementation provides birds with unnatural quantities or types of carotenoids with uncertain consequences for physiology and ornamental trait expression. Future research into honest carotenoid signalling may benefit from a shift away from using carotenoid supplementation in experiments. Given the number of variables already interacting to affect how supplemental carotenoid regimes alter avian physiology (dose, duration, type, life-history stage of subjects and season), it may be most productive for future studies of wild birds to avoid dietary manipulation and instead focus on physiological challenges or measurements of standing variation. In studies of birds held in captivity, it may be most useful to provide subjects with a seed, insect or fruit diet that more closely replicates natural carotenoid availability, without the potentially confounding effects of dosing with purified synthesized carotenoids. Over 40% of the studies we examined performed carotenoid supplementation (Supporting Information), and such experiments have made clear contributions to our understanding of avian physiology and colouration; however, we argue that future studies would be better served by focusing on internal carotenoid use rather than external carotenoid access.

Even if acquiring carotenoids from the environment poses no limitation on carotenoid use, the challenges of distributing carotenoids internally could still create trade-offs in how carotenoids are allocated. This concept of internal carotenoid limitation assumes that the quantities of carotenoids absorbed from the diet, transported through the body and stored for future use are finite and small enough that birds may not be able to allocate sufficient carotenoids to both external ornamentation and internal processes. Importantly, it is well known that internal carotenoid resources and their allocation may differ widely through seasons based on breeding (particularly for females depositing carotenoids in yolk) or moult (for species with plumage coloration), and studies must consider that limitation may be present in some seasons but absent in others—though changes in dietary consumption may also compensate for changing need (Hill, 1995; Isaksson, Von Post, & Andersson, 2007; McGraw, Nolan, & Crino, 2011; Sassani, Sevy, Strasser, Anderson, & Heath, 2016). Internal carotenoid limitation is most commonly tested by assessing correlations among different physiological processes that may require carotenoids, such as immune system function and ornamental colour production. We discuss these types of studies in detail in the following sections.

However, carotenoid limitation is, at its heart, a quantitative topic that depends on the amounts of carotenoids that are available to a given physiological process at the time they are needed. There are now sufficient data to estimate the quantities of carotenoids that an average individual in a well-studied species like the house finch (Haemorhous mexicanus) may require to properly colour its feathers.
(Supporting Information Appendix S2); we estimated that the average moult ing male house finch requires about 41 μg of carotenoids in total to colour his ornamental plumage, while he possesses about 58 μg in his liver, blood and fat at any given time during moult. That carotenoids may be limiting is conceivable if a male house finch possesses only 17 μg of excess carotenoids in his body during moult, stored throughout various tissues. However, our calculations do not estimate the quantities of carotenoids that are entering each bird’s body every day through its diet, offsetting the quantities of carotenoids in his body during moult, thereby necessitating internal storage. These quantities of carotenoids should be validated by tracking of carotenoids through an animal’s body. Ultimately, questions of internal carotenoid limitation should be answered by measuring the size of the internal resource “pool” in a bird’s tissues with the quantities of carotenoids required to fully colour an ornament and/or participate in an important physiological process.

3 | ASSUMPTION 2: PHYSIOLOGICAL BENEFITS OF CAROTENOIDS

It is widely stated that carotenoids play an important role in immune system function in birds, but their direct involvement as antioxidants in avian systems is uncertain and contentious (Costantini & Möller, 2008; Hartley & Kennedy, 2004; Perez-Rodriguez, 2009; Simons, Cohen, & Verhulst, 2012). Studies aimed at testing physiological functions of carotenoids have been conducted with such diverse approaches that a quantitative meta-analysis, while useful for assessing general patterns (Simons et al., 2012; Weaver, Santos, Tucker, Wilson, & Hill, 2018), misses important relationships between particular experimental designs, study systems and results. Thus, we assessed the evidence that carotenoids play a key role in immune defence and in the avoidance of oxidative stress using a qualitative assessment of published studies. Moreover, we provide a brief description of a wide variety of studies related to the carotenoid resource trade-off hypothesis in Supporting information Table S1, we aim to avoid overgeneralization of studies by “vote-counting” positive or negative relationships” (Koricheva, Gurevitch, & Mengersen, 2013), and instead encourage readers to assess results in-context within publications themselves. In the following sections, we highlight some important sources of variation and outlying questions regarding tests of the physiological benefits of carotenoids. The question of whether or not carotenoids play important physiological roles besides serving as pigments for coloration is fundamental to determining whether carotenoids are valuable resources that must be differentially allocated among functions at all.

3.1 | Carotenoids as immune boosters

Carotenoids are so widely accepted as enhancers of vertebrate immune function that some recent studies state the role of carotenoids as beneficial to immunocompetence as a well-established truth (Benito, Gonzalez-Solis, & Becker, 2011; Girauduche, Chavez, Toomey, & McGraw, 2015; Merrill, Naylor, & Grindstaff, 2016). Originally founded on a small number of empirical papers examining the potential benefits of a carotenoid-rich diet in humans (Krinsky, 1989; Stahl & Sies, 2005) and then expanded to behavioural ecology by foundational theoretical papers such as Lozano (1994), studies have since examined correlations between carotenoid-based coloration, circulating carotenoid levels, carotenoid consumption and measurements of immune response to test whether carotenoids enhance immunocompetence.

The specific means by which carotenoids may improve immune system performance are rarely articulated and remain largely unresolved. Studies investigating carotenoids and immune benefits often cite studies of mammalian species (Bendich, 1989; Jyonouchi, Zhang, Gross, & Tomita, 1994; Kim et al., 2000), or a review by Chew and Park (2004) that also discusses experiments performed almost entirely on mammalian subjects. Briefly, carotenoids have been implicated in lymphocyte proliferation and activity, though their biochemical participation in such processes remains uncertain (Chew & Park, 2004). It is possible that carotenoids may be localized to specific organelles, such as the mitochondria, where they may prevent oxidative damage and therefore facilitate proper cellular function in immune cells (Chew & Park, 2004; Hill, 2014; Koch, Josefson, & Hill, 2017)—but this hypothesis remains to be tested. Carotenoids have also been cited as important to preventing damage to self during the innate immune process of oxidative burst in which immune cells target pathogens for oxidative damage by rapidly releasing pro-oxidants (Chew & Park, 2004), though the process functions differently in mammalian vs. avian cells (Harmon, 1998), and thus far there is little evidence to suggest a role of carotenoids in oxidative burst in birds (Koch et al., 2018; Sild, Sepp, Manniste, & Horak, 2011). While research into the potential benefit of dietary carotenoid supplements in poultry has been fairly extensive, such studies are not focused on providing biologically relevant doses and are largely not useful tests of how carotenoid function may contribute to honest signalling (Koutsos, Lopez, & Klasing, 2007; Koutsos, López, & Klasing, 2006; Meriwether, Humphrey, Peterson, Klasing, & Koutsos, 2010; Shanmugasundaram & Selvaraj, 2011; Surai, 2002, 2012).

The first step towards understanding the role of carotenoids in immune response within an ecologically relevant context is to perform studies of how, where and when carotenoids are absorbed, transported throughout different tissues in the body and deposited in ornaments. Carotenoid trade-offs can be convincingly established only if the endpoints of carotenoid mobilization are determined. Using techniques to label carotenoid pigments and track their absorption, transport and conversion throughout the body (see Conclusions) will be imperative for elucidating carotenoid movement among and storage within tissues and how these patterns may differ among individuals, sexes and species. By combining such methods with various immune challenges, conclusive tests of allocation trade-offs are possible.

In conjunction with studies that track the allocation of carotenoids, we encourage the development and use of experimental...
manipulations that more closely simulate the kinds of pathogenic or parasitic challenges a bird may be expected to encounter in nature. The carotenoid trade-off literature is dominated by results based on quantification of parasites (such as coccidia) or assessment of immune function based on swelling response to injection of phytohemagglutinin (PHA; Supporting information Table S1). A primary reason that these measures are so widespread is that they have no apparent long-term effects on a bird and can be performed even in the field with no obvious side effects. While methods like PHA injection are convenient and pose relatively low risk of harm to experimental subjects, the results can be difficult to interpret (Adamo, 2004; Biard, Hardy, Motreuil, & Moreau, 2009). Challenging birds with a live pathogen or parasite is arguably the most biologically relevant means to test whether carotenoids function in immune response (Brawner, Hill, & Sundermann, 2000; Lindstrom & Lundstrom, 2000), particularly when we do not yet know the specific immune processes that may involve carotenoids. However, the experimental benefits of using live pathogens or parasites must be weighed against the ethics of inducing suffering in vertebrate animals.

Better methods for measuring immune response is a focus of the growing field of eco-immunology (Demas, Zysling, Beechler, Muehlenbein, & French, 2011; Graham et al., 2011; Martin, Well, & Nelson, 2006), and we urge ecologists, animal behaviourists and evolutionary biologists alike that study carotenoid coloration to adopt new and better techniques for assessing immune system function. We also suggest that information in the avian and poultry disease literature (Boseret, Losson, Mainil, Thiry, & Saegerman, 2013; Dorrestein, 2009; Joseph, 2003; Lister & Houghton-Wallace, 2012; Pattison, McMullin, Bradbury, & Alexander, 2008) is an important resource for finding pathogens that can be dosed to subjects in a controlled setting. Information from songbird veterinary studies may be particularly applicable to studies that keep captive populations of passerines commonly used in tests of carotenoid-based signalling, like zebra finches (Taeniopygia guttata), European greenfinches (Carduelis chloris) or house finches. A wide variety of viral, bacterial and fungal diseases have been described in passerine species (Dorrestein, 2009; Joseph, 2003), each of which may be a potentially useful challenge for a study of wild birds held in captivity. Using diseases known to veterinary medicine may help researchers reduce harm to animals by taking advantage of known treatments and also may provide a useful resource for how to detect and measure symptoms.

In sum, that carotenoids are essential to proper immune function is not yet justified by consistent empirical evidence. There is persistent variation in the results of studies testing for relationships between carotenoids and immunocompetence such that a straightforward, universally beneficial effect of carotenoids seems unlikely. We urge future research on these problems to: (1) perform in-depth analyses of where and in what quantities carotenoids move through the body to develop specific hypotheses for mechanisms by which carotenoids may interact with the immune system; then, (2) carry out targeted immune challenges and/or measurements that hone in one these specific hypothesized processes.

Before the former has been accomplished (developing specific hypothesis for where carotenoids participate in immune defence), studies interested in testing for a benefit of carotenoids should avoid using specific immune tests with questionable relevance to overall disease resistance (e.g., PHA). We instead suggest studies consider using challenges and measurements that best gauge biologically relevant immune defence, such as pathogen infection and measurement of clearance (Lindstrom & Lundstrom, 2000), tolerance (Adelman, Kirkpatrick, Grodlo, & Hawley, 2013), survival (Hanssen, Hasselquist, Folstad, & Erikstad, 2004) or a comprehensive suite of measures integrating among branches of the immune system (Adamo, 2004; Demas et al., 2011; Millet, Bennett, Lee, Hau, & Klaing, 2007; Salvante, 2006). Injecting birds with bacterial lipopolysaccharide (LPS) may be a useful option for inducing systemic immune response without a live pathogen, but similarly, we encourage use of this technique to be combined with overall measures of performance (such as body temperature, cytokine levels; Adamo, 2004; Demas et al., 2011; Millet et al., 2007). With any challenge, however, we emphasize that a decrease in coloration during immune response does not alone signify that carotenoid pigments are being used directly in the response. Isolating the movement of carotenoids through the animal body, particularly during states of challenge, is a key to advancing the field.

### 3.2 Carotenoids as antioxidants

Whether or not carotenoids function as important antioxidants in the bodies of birds is contentious (Costantini & Møller, 2008; Perez-Rodriguez, 2009; Svensson & Wong, 2011). Two meta-analyses have examined relationships between circulating carotenoids and/or carotenoid-based coloration and oxidative stress parameters and both reported small and generally non-significant effect sizes (Costantini & Møller, 2008; Simons et al., 2012). Others have suggested that carotenoids may indicate oxidative state without directly participating as antioxidants, such as by becoming "bleached" during oxidative stress (Hartley & Kennedy, 2004) or because only particular oxidative states (García-de Blas et al., 2016) or high levels of cellular functionality (Johnson & Hill, 2013) facilitate the conversion of dietary carotenoids into ornamental carotenoids. While carotenoids are, by their chemical nature, effective electron receptors under most conditions and hence are potential antioxidants (El-Agamey et al., 2004; Krinsky & Yeum, 2003; Stahl & Sies, 2003), it is uncertain whether the physiological systems of birds are adapted to deploy carotenoids as important antioxidants to maintain redox balance (Perez-Rodriguez, 2009). The key question is whether the right forms of carotenoids are present in sufficient quantities in the necessary organ-level, cellular or even subcellular locations to have a biologically significant effect on an individual’s ability to maintain healthy oxidative balance.

A challenge central to any studies examining oxidative stress parameters is that the interplay between pro-oxidants and antioxidants causes complex and variable fluctuation in levels of antioxidants, pro-oxidants and oxidative damage (Costantini, 2014; Hörak...
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& Cohen, 2010; Monaghan, Metcalfe, & Torres, 2009), and such levels may vary between different locations in an individual’s body. As in many tests of immune function, studies on live animals are restricted to assessing oxidative stress from blood samples to avoid terminal experimentation (Cohen, de Magalhães, & Gohil, 2010), although levels of oxidative damage markers or antioxidant capacity in the blood may have limited relevance to oxidative stress in other organs (Santos, 2017; but see Stier et al., 2017). Nonetheless, given that blood is a main location of carotenoid storage and transport, measuring blood-based oxidative stress parameters may be considered relevant to gauging carotenoid antioxidant activity. However, centralized and metabolically active organs like the liver are likely to play a more direct role in maintaining the oxidative state of birds, so estimates of carotenoids and oxidative stress in blood will likely be only an indirect measure of functions occurring elsewhere in the body.

It is important to note that experiments would ideally examine the relationships between antioxidants, pro-oxidants and damage in the specific tissues most pertinent to the research objectives. For example, measuring oxidative parameters in flight muscle may be a good target for testing activity-related oxidative stress, while sites of infection or immune cell congregation may be better suited for testing immune-related oxidative stress. In addition, the chemical paraquat is sometimes administered in oral doses as a generalized oxidative challenge, but it can have localized effects where it accumulates within the digestive tract (Koch & Hill, 2017). As a result, measuring oxidative damage in the blood may only indirectly correspond to any responses to oxidative stress occurring due to paraquat—and a paraquat challenge may not be expected to alter oxidative stress in tissues important to the production of coloured ornaments (Isaksson & Andersson, 2008). A whole-body challenge like low-dose ionizing radiation may be more effective than more localized challenges like diquat or paraquat (Koch & Hill, 2017) in inducing oxidative stress that can be meaningfully assessed in blood-based measures.

A recent study by Tomášek et al. (2016) has proposed that an important explanation for inconsistency in relationships between carotenoids and oxidative stress is that the current commonly used methods for measuring antioxidant defences fail to capture the effects of carotenoids because they focus on hydrophilic rather than lipophilic reactions (given that carotenoids themselves are lipophilic). García-de Blas et al. (2016) also found that hydrophilic antioxidant capacity in red-legged partridges was largely affected by hydrophilic antioxidants rather than lipophilic carotenoids. The implication that the most commonly used methods for estimating antioxidant defences in studies of carotenoid-based coloration in birds are likely to miss effects of carotenoid activity is intriguing and may provide some explanation for the inconsistency of previous studies, although measurement of antioxidant capacity is only one aspect of oxidative stress that has been tested.

Overall, studies of the relationships between carotenoids and oxidative stress have yielded complex results that are hard to interpret and do not clearly support the hypothesis that carotenoids serve as key antioxidants in birds. As with tests of the immune benefits of carotenoids, a critical next step is performing detailed experiments to try to isolate where and in what quantities carotenoids are present in the avian body and how, specifically, these carotenoids may or may not interact with pro-oxidants. Labelling carotenoid molecules (see Conclusions) will also allow for new tests of where carotenoids are present, and—in combination with new oxidative challenge techniques (Koch & Hill, 2017)—how and where they may be transformed in response to oxidative stress. A better understanding of the subcellular locations of carotenoids and the role that they play in antioxidant defences is essential to drawing conclusions about whether the antioxidant potential of carotenoids in vitro does in fact reflect an important and significant role of carotenoids as antioxidants in vivo—and how the deposition of carotenoids in coloured ornaments may or may not alter the ability of an individual to maintain a healthy oxidative balance (von Schantz, Bensch, Grathn, Hasselquist, & Wittzell, 1999). Given ongoing ambiguity in the antioxidant function of carotenoids in the avian body, we encourage researchers to avoid making general assumptions that carotenoid antioxidant activity may explain patterns in results.

### 4 | ASSUMPTION 3: HIGH-QUALITY INDIVIDUALS ARE LESS CHALLENGED BY TRADE-OFFS

The literature associated with the resource trade-off hypothesis is founded on the prediction that, in populations of animals with carotenoid-based colour displays, carotenoid coloration enables assessment of individual quality. The various ways in which quality can be defined and assessed with respect to condition-dependent signals have been considered in detail in several recent papers (Hill, 2011, 2014; Lailvaux & Kasumovic, 2011; Wilson & Nussey, 2010). Here, it is important to consider how concepts of quality can influence the interpretation of physiological data with regard to carotenoid resource trade-offs. There is a general lack of consistency within studies that test carotenoid resource trade-offs in how individual quality is defined, resulting in added confusion in the literature (Hill, 2011). Studies of the honesty of carotenoid-based ornaments tend to invoke individual quality from one of three broad perspectives:

- **1** oxidative or immune health state,
- **2** magnitude of internal carotenoid resource pools or
- **3** some aspect of inherent, underlying functionality (Table 1). These three aspects of quality are not mutually exclusive, but instead tend to represent the way that “high” or “low” quality is interpreted and tested within a given study.

In studies of carotenoid signalling that are focused on health state, it is commonly observed that birds presented with an immune challenge, such as a pathogen or parasite infection, develop paler carotenoid coloration than control individuals (Brawner et al., 2000; Faivre, Gregoire, Preault, Cezilly, & Sorci, 2003; Rosenthal, Murphy, Darling, & Tarvin, 2012). In these cases, carotenoid-based traits may be interpreted as honest signals of quality because increasing physiological challenge inhibits full colour expression (Table 1). In other
<table>
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<th>Axis of quality</th>
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<td>Experimental alterations of health state (e.g., administering an oxidative or pathogen challenge)</td>
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<tr>
<td>Internal carotenoid levels</td>
<td>Carotenoid coloration reflects internal carotenoid resource availability</td>
<td>Higher quality individuals have larger pools of internal carotenoid resources</td>
<td>Dietary carotenoid supplementation or deprivation</td>
<td>Individuals with larger carotenoid resource pools will have increased carotenoid coloration</td>
<td>The biological relevance of artificially increasing or decreasing dietary carotenoid levels is almost impossible to verify. We do not know how a given dietary programme may actually affect internal carotenoid levels, given variation in carotenoid absorption and transportation that is difficult to measure.</td>
</tr>
<tr>
<td>Interaction of health state and carotenoid levels</td>
<td>Carotenoid coloration reflects the combined effects of health state and internal carotenoid resource availability</td>
<td>Healthy, carotenoid-rich individuals may be considered high quality, but intermediate relationships are uncertain</td>
<td>Factorial-type manipulations of both health state and dietary carotenoid access</td>
<td>Uncertain</td>
<td>Without clear knowledge of the relative quantities of carotenoids “used up” (or not) by a health challenge, it is difficult to predict in any given system how dietary carotenoid supplementation or deprivation may affect coloration and/or response.</td>
</tr>
<tr>
<td>Underlying functionality</td>
<td>Carotenoid coloration reflects some aspect of intrinsic physiological performance</td>
<td>Higher quality individuals are those that perform best within one treatment group (e.g., the individuals with best coloration despite an immune challenge)</td>
<td>Comparisons between variables in resting individuals, or individuals within one treatment group</td>
<td>Under identical conditions, individuals will vary in response variables (immune response, oxidative stress measures, carotenoid coloration); some individuals will exhibit superior performance across multiple metrics compared with others</td>
<td>Without isolating the genetic and/or physiological for underlying variation in individual performance, it is difficult to disentangle underlying differences in performance from external differences in environment and other external factors (even within individuals held under the same conditions).</td>
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experiments, studies report positive relationships between supplementation of dietary carotenoids, amount of circulating carotenoid pigments (a proxy for the size of internal carotenoid resource pools) and the expression of colourful traits (Hörak, Sild, Soomets, Sepp, & Kilk, 2010; McGraw & Ardia, 2003). Such observations suggest that individuals with more pigments (larger resource pools) have higher quality ornaments (Table 1) and that coloration is affected by changing access to dietary pigments. When these two general patterns are considered in combination with the hypothesis that carotenoids may directly boost physiological function, predictions regarding the relationships between internal carotenoid pigments, physiological performance and external carotenoid coloration become extremely challenging to formulate (Table 1).

It is difficult to articulate a hypothesis that incorporates the negative effects of physiological challenges on carotenoids, positive effects of carotenoids on coloration and positive effects of carotenoids on physiological function. The main challenge is that we do not know the specifics of how or where carotenoids are used by immune or antioxidant systems, as described in the previous section. Thus, it is not possible to make realistic predictions in experiments that investigate the interactions among these variables. For example, one might propose that internal carotenoid pigments are “used up” while boosting physiological function, in which case the prediction would be a negative relationship between strength of physiological response and carotenoid levels. Alternatively, carotenoids might boost immune function without being consumed, in which case the prediction would be a positive relationship between physiological response and carotenoid levels. These two predictions both assume that carotenoids serve a direct beneficial role in individual performance, but they yield opposite predictions regarding the direction of the relationship between internal carotenoid levels and measures of that performance.

One method to attempt to test whether carotenoids are consumed in the process of boosting response (and whether they are involved in the response at all) may be to induce a systemic physiological challenge while manipulating dietary carotenoid access and measuring carotenoids or ornamental coloration. Hypothetically, if carotenoids are consumed during response and are present in physiologically limiting amounts, then only supplemented individuals should be able to maintain coloration during a challenge. However, results of studies testing for these interactions are often difficult to interpret. For example, both Sild et al. (2011) in greenfinches and Alonso-Alvarez et al. (2004) in zebra finches found no significant interaction between immune challenge (LPS injection) and carotenoid supplementation on plasma carotenoid levels or ornamental coloration, respectively. In contrast to predictions, they found that immune challenge decreased circulating carotenoids or coloration by a constant amount, regardless of dietary carotenoid manipulation—though supplemented individuals always had greater circulating carotenoids or coloration when compared to control individuals. The question then becomes, what caused the decrease in carotenoid circulation or ornamental expression during the challenge? Detailed measurement of the strength of individual response and the mobilization of internal carotenoid resources pools would be necessary to distinguish whether these challenged individuals paid a set “price” in carotenoid resources to boost response to the challenge, or whether they simply decreased carotenoid absorption by a set amount while mounting a response.

The study by Alonso-Alvarez et al. (2004) also demonstrates an interesting case of how examining the same results from a different perspective can alter interpretations. The authors found no direct, categorical effect of carotenoid supplementation on the ability of zebra finch red blood cells to resist oxidative attack; however, they did find that individuals with the greatest increase in plasma carotenoid content had the strongest performance on the oxidative attack test (Figure 2). This latter result could be seen as supportive of the resource trade-off hypothesis: perhaps individuals with the greatest quantity of carotenoids in internal tissues gained the largest benefit in resistance to oxidative damage. Without knowing how and where carotenoids may perform such antioxidant function, however, we cannot separate this resource trade-off interpretation from alternative hypotheses. It is also possible, for example, that some individuals (i.e., high-quality individuals) had fundamentally superior performance across multiple physiological arenas such that they had stronger defence against free radical attack as well as a greater capacity to absorb carotenoids from the diet (Hill, 2011). This latter hypothesis may be a better fit in that it predicts the same pattern of association between increase in plasma carotenoid levels and increase in resistance to oxidative stress, and it also explains a lack of direct effect of supplementation on oxidative damage resistance (Figure 2), as carotenoids were no longer required to directly participate in antioxidant reactions. These alternative interpretations emphasize that it is important to consider the complexity of the system under study and to be explicit regarding the assumptions that lead to a conclusion that carotenoid trade-offs are involved. The use of this additional analysis demonstrates how examining variation at the individual level rather than among discrete treatments may be particularly fruitful for interpreting results, given that variation within treatment groups or problems with the treatments themselves (e.g., supplementation dose) may obscure meaningful patterns.

To summarize, widespread uncertainty exists in the carotenoid literature about whether we define high-quality individuals as those that are (1) currently healthy, (2) currently possessing large quantities of carotenoids, some combination of both or (3) inherently superior in one or more physiological metrics. The result of this uncertainty is that a wide range of observations can be used to support resource trade-off hypothesis even if alternative explanations exist, such as index hypotheses that propose trait quality as an indicator of internal conditions rather than as a direct product of costly trade-offs (Biemanskie, Grafen, & Perry, 2014; Hill, 2011; Weaver, Koch & Hill, 2017). While it is not erroneous to discuss how results fit (or fail to fit) particular frameworks, the danger is in suggesting most every observation to be supportive of a favoured hypothesis. A lack of exclusive predictions hinders our ability to draw accurate conclusions about support for or against the resource trade-off hypothesis and discourages consideration of alternative hypotheses as stated...
evidence for the current trade-off paradigm continues to build. We encourage future studies to consider approaching experiments with the perspective of quality as the "underlying functionality" of individuals (Table 1), as it sidesteps many potential methodological issues (e.g., comparing supplemented to unsupplemented individuals when the supplemental dose has unknown biological relevance) and hones in on the physiological differences among individuals that allow some to express higher quality coloration than others.

5 | CONCLUSIONS

Several common threads emerge from our consideration of the carotenoid resource trade-off hypothesis. There is a critical need for better understanding of the specific biochemical activity of carotenoids in the animal body, for more detailed observations of the quantities and types of carotenoids present in various organs and cellular locations (and their movements between these locations), and for the articulation and testing of specific mechanisms that link production of carotenoid-based colour displays to individual quality. For no species have we yet quantified and characterized the full journey of carotenoids through an individual's body including the quantities of carotenoids absorbed, where they are stored, how and when stored carotenoids are used, the quantities and locations of carotenoids needed to achieve full coloration of an ornament, and the effects of experimental supplementation or physiological challenge on processes like carotenoid absorption and distribution. While these processes will vary among species and among individuals within a species, establishing the general patterns in these processes even in model avian species will significantly advance our ability to understand the role of carotenoids in physiological performance as well as coloration.

It is already possible to estimate the quantities of carotenoids present in the body or deposited in the feathers in species where detailed carotenoid analyses of many tissues have been reported, such as the house finch (Supporting Information Appendix S2). However, it will be more informative to experimentally label specific carotenoid molecules and follow them directly as they travel through the body and undergo transformations. Carotenoid radiolabelling has been used sparingly in animals, but radiolabelled canthaxanthin has been used in two studies that tracked carotenoid absorption and/or metabolism in chickens (Schedt, 1989) and trout (Hardy, Torrisen, & Scott, 1990); developing labelled dietary carotenoids for use in modern studies will open up a rich new resource for discovering patterns of carotenoid movement and use in the body (Jansen & Lugtenburg, 1996). Characterizing carotenoid absorption, transport, storage and conversion in both healthy and immune-challenged birds, for example, will provide clear answers to long-standing questions about how immune activation modifies carotenoid coloration, and whether the carotenoids appear to directly participate in response (e.g., if carotenoids are found to be transported to immune cells or tissues).

In addition, breakthroughs in deducing the enzymes that control carotenoid coloration in birds (Lopes et al., 2016; Mundy et al., 2016; Toews, Hofmeister, & Taylor, 2017; Toomey et al., 2017) provide exciting opportunities to measure gene expression and to conduct knock-down or knockout experiments to further parse how
and why individuals may differ in their internally sequestered and externally displayed levels of carotenoids. A knock-down in a main carotenoid absorption gene, SCARB1, has already been characterized in the domestic canary, a species with carotenoid-based plumage coloration both in domestic and in wild populations (Toomey et al., 2017). Studying SCARB1 knock-downs like that of the canary provides researchers the unique opportunity to test the effects of severe physiological carotenoid depletion without relying on dietary manipulations (Koch et al., 2018). As technology continues to advance and allow for manipulation of target genes within vertebrate systems, studying knock-outs in carotenoid absorption (Toomey et al., 2017) or transformation (Lopes et al., 2016; Mundy et al., 2016) genes will yield transformative information regarding the costs or benefits of specific types of carotenoids and the processes involving them. Simultaneously, examining the expression of these genes under different environmental, seasonal or developmental stages offers a new opportunity to advance our understanding of the mechanisms underlying colour variation and to make inferences about the role of such mechanisms in signal honesty. We note several of these gene products and their functions in the caption of Figure 1.

Until we have established a better fundamental understanding of carotenoid physiology, we urge researchers studying carotenoid coloration to maintain an open perspective with regard to whether or not carotenoids serve key physiological functions, such as immune enhancement or free radical scavenging, and whether resource trade-offs are the bases for honest carotenoid coloration. Correlations between carotenoid levels, coloration and physiological performance can only yield so much information without a better understanding of underlying mechanisms. The goal of this study was not to review and evaluate alternative hypotheses for the resource trade-off hypothesis (Hill, 2011; Weaver, Koch, & Hill, 2017), but it is important to keep alternatives in mind when interpreting results that are non-significant or do not match the predictions of carotenoid resource trade-offs. Tackling old questions with new approaches—be they new genetic techniques or new perspectives on the role of carotenoids in physiological function—will be key to substantiating or questioning the carotenoid resource trade-off hypothesis across systems.

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REFERENCES
Cohen, A. A., de Magalhães, J. P., & Gohil, K. (2010). Ecological, biomedical and epidemiological approaches to understanding oxidative balance


SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.