Reviewer 1 (Geoffrey Hill)

Basic reporting

This is a solid study. In my "comments to Authors" I give many small suggestions for improvement but I find no serious flaw in the study.

Experimental design

The experimental design seems solid. More justification of use of the specific diquat treatment would strengthen the paper, but I think the treatment can be justified.

AUTHORS: We have added expanded the justification for the diquat treatment in the Introduction (page 8) and Methods (page 12, first paragraph; see also page 36, L5-8).

Validity of the findings

The patterns revealed by this study are certainly valid and important. I do not agree with all interpretations, but the viewpoints of the authors are valid and important.

Comments for the Author

COMMENT 1. Carotenoid-based ornaments are honest signals of individual quality. This has been well established through many studies in the late twentieth and early 21st centuries. What remains uncertain is how this association between pigmentation and individual quality comes about.

The Alonso-Alvarez lab has been at the forefront of designing experiments to test alternative hypotheses for how carotenoid coloration and particularly the concentration of ketolated carotenoids serves as an honest signal of individual condition. The study submitted here is a valuable contribution to the effort to understand the signal content of carotenoid coloration.

I have some questions about protocols and methods and I have some concerns (and certainly differences of opinion) about how data are interpreted, but my overall assessment is that this is a solid contribution to the field.

AUTHORS: Thanks to Prof Hill for these kind words.

COMMENT 2. Abstract lines 20-23 – the logic of how the results suggest a trade-off is not clear. I would either more explicitly explain why the differences caused by supplementation indicate a trade-off or omit this speculation from the abstract. This study includes an array of measurements and manipulations that sets up an unprecedented and extremely valuable opportunity to evaluate interactions among physiological variables that may be related to color expression. I suggest reframing some aspects of the abstract—and perhaps discussion—to emphasize the study’s overall focus on complex interactions.

AUTHORS: We have the expanded the abstract to better explain our view with regard to the trade-off. We have added some other sentences in the text in this regard (page 35, lines 18-21, and page 40, lines 27-31). Moreover, we have briefly mentioned that our results are coherent with the recent and exciting discovery of an oxidase enzyme probably involved in converting yellow to red pigments in different avian species (i.e. Lopes et al. 2016 Curr Biol; Mundy et al. 2016 Curr Biol). Proff Hill was involved in one of these studies. This was now addressed in the abstract, Introduction (page 6, lines 25-26) and Discussion (page 35, L1-6 and page 38, L24-27).

COMMENT 3. Page 4 Lines 5-6 – With respect to Simons et al. 2012, I don’t think the evidence of carotenoids as immune stimulants is nearly conclusive; note that only response to PHA injection was significantly related to either carotenoids or trait redness, among 5 measurements of immune response (Fig. 2). While many studies find some correlative support for links between some aspects of immune response with some aspects of carotenoid prevalence or coloration, I think it is important to keep a broader and more objective perspective on the state of the debate of carotenoids as immune boosters (or not).

AUTHORS: We have rewritten the sentence to note that the support for this role is mostly base on inflammatory skin responses, explicitly citing the PHA (page 5, L5-6).

COMMENT 4. Page 7 Lines 13-18 – This seems like a core justification of your experimental design and interpretation. As such, I would devote more space to explaining how this works. How, specifically—mechanistically—might an increased production of RONS help ketolase activity? Also, might RONS directly modify carotenoids—and if so, how?

AUTHORS: We have added several sentences to explain these points at the end of Introduction. We mention that the way in which higher RONS levels could increase ketolase activity can only be speculated. In the case of RONS directly acting on carotenoids, we formulated a prediction: if diquat directly oxidizes hydroxycarotenoids, the resulting ketocarotenoids should be found in blood, where diquat should firstly be present. The results did not support this view as neither astaxanthin nor any other ketocarotenoid was found in other tissues that ornaments.

COMMENT 5. Page 8 Lines 12-14 – It’s important to further justify / describe the quantities of carotenoids the partridges ingested from the various diets and how the levels provided relate to what a wild partridge would ingest. Is the quantity of carotenoids similar to what we might expect to be “natural” for this species? The authors focus on ratios of pigment types, but if the overall availability of carotenoids is well above or below what a wild partridge would have access to, then that completely changes how the results will be interpreted. The poultry industry often gluts the diets of food or egg birds to adjust the coloration of products for consumption, and so the numbers from poultry research may not have biological relevance. The quantities of carotenoids consumed and therefore physiologically available is key to this study. The authors should cite the following in considering carotenoid dose:

Rebecca E. Koch, Alan E. Wilson, and Geoffrey E. Hill. 2016 The Importance of Carotenoid Dose in Supplementation Studies with Songbirds. Physiological and Biochemical Zoology 89:1, 61-71.

AUTHORS: We have added the cited reference. We have also explicitly commented that few is known about the natural diet of this species and nothing about the natural carotenoid content. The species lives currently in a strongly modified environment (cereal fields). We, however, know that captive birds show lower carotenoid levels even when pigments are supplied with the diet, and also they are paler. Commercial food tries to compensate this problem by adding carotenoids, but levels are usually low and inefficient as carotenoid supplements are extremely expensive. We anyway used commercial concentrations as an initial reference as we know that they do not produce health problems. Moreover, carotenoid supplements did not induce any negative effect in terms of survival, body mass, breeding output or oxidative stress was detected other that increased redness.

COMMENT 6. Page 8 Lines 27-29 – Can’t high temp and pressure also turn carotenoids into pro-oxidants? Do you have any verification that the nature of the carotenoids themselves wasn’t altered by the pelleting process?

AUTHORS: We have no verification, but we did not detect any toxicological effect in CAR treated birds (see the previous point).

COMMENT 7. Page 8 Line 30 – If tocopherol is going to be important to your results / discussion, I think it needs more introduction. What is it, and what role does it play in the interactions you are studying?

AUTHORS: we have added a sentence here (page 10, L23-24), but the question is also addressed in the Discussion (section 4.1, also page 40, first paragraph).

COMMENT 8. Page 10 Lines 10-12 – It is important to take the time to justify the diquat dose. What symptoms does it induce in the birds? Has it been shown to induce oxidative damage? In justifying the use of diquat and considering the importance of correct dose the authors should cite: Koch, R. E. and Hill, G. E. (2016), An assessment of techniques to manipulate oxidative stress in animals. Funct Ecol. Accepted Author Manuscript. doi:10.1111/1365-2435.12664

AUTHORS: This was better explained (page 12, L 8-10). In our previous pilot study in red-legged partridges, increased levels of oxidative damage in blood lipids were found, but not body mass change (Supplementary fig 1 in Alonso-Alvarez et al. 2010 PLoS One 6, e19403). In the subsequent experiment, we also found lower levels of the antioxidant glutathione (GSH) in erythrocytes, though birds were able to reduce oxidative damage (Galván & Alonso-Alvarez 2009), which suggests that our dose was relatively mild. We have also added the cited reference in different parts of the text (page 8, L8 and L26, page 39, L21). We also propose that our mild diquat treatment could in some way resemble other natural sources of free radicals such as exercise (see page 38, last sentence), by citing Costantini et al 2013 and Jenni-Eiermann et al. 2014 studies showing increased oxidative stress in birds due to flying effort.

COMMENT 9. Page 10 Line 18 – I question the quantification of the spectral data. The focus of this paper is on carotenoid ketolation. High versus low performance regarding carotenoid ketolation is typically manifest in the ratio of yellow unmodified pigments to red modified pigments in tissue, which affects the position of the reflectance curve, which is measured as hue. If the hue measure presented by the authors (and then confounded with brightness) is a measure of the position of the reflectance peak, then the authors should explain this more clearly. I’m not sure why the authors followed the techniques of Saks et al., which was used to measure yellow coloration in Greenfinches, when Montgomerie has detailed methods for red coloration in his 2006 book chapter:

Montgomerie R (2006) Analyzing colors. In: Hill & McGraw, editor. Bird Coloration: Volume 1, Mechanisms and Measurements. Cambridge, Massachusetts: Harvard University Press. pp. 90–147.

AUTHORS: The equation used to calculate hue was in fact obtained from the cited Montgomerie chapter (see Table 3.2). Montgomerie used the same formulae (based on Endler’s 1990) on page 122. The equation is general and not specific for yellow or red colors. Perhaps the confusion is due to the fact that we forgot to describe the red component in the equation, though we used it. We added total brightness as a covariate to control for its potential confounding effect because the original formulae introduce it in both the numerator and denominator of the equation, thus canceling out the supposed correcting effect.

COMMENT 10. Page 20: I interpreted, from the methods, that diquat was administered after “time 2”—day 48. Is that correct? If so, how are the coloration results shown here—from day 48—relevant to diquat effects? More importantly, it seems that the “control” coloration values are missing from the graphs in Fig. 5.

AUTHORS. Yes, diquat was administered from time 2 (equal to day 48). Results show in that page in the original version of the manuscript referred to color differences due to CAR treatment only. The reviewer’s confusion is because we erroneously maintained the (o) symbols (control vs diquat) at the top of figure 5. They are now removed. The legend indicates that these least squared means +- se are those obtained from the models after controlling for the diquat factor. This implies a larger sample size (a better statistical power) that allowed detecting subtle but relevant differences between LZ and ZL supplemented groups. Note that previous repeated measure analyses on blood or color directly removed data from diquat-treated birds (half of the sample) after time 2. This is highlighted in page 35, lines 12-16. The figure showing diquat x CAR is in the Supporting material Fig 2SM. We note that the interaction was not significant, but the figure was created for informative purposes.

COMMENT 11. Page 22, lines 2-7: Wouldn’t these results (effects of supplementation on pap. concentration) be more appropriately presented in section 3.2?

AUTHORS: In our opinion, this would be difficult to interpret. We have followed a temporal line, from repeated measures on blood and color variables only (excluding diquat) to models testing the entire body (sacrificed birds) at the last sample (including diquat treatment). The 3.3 subheading mentions "Variability after diquat exposure”. However, the cited lines are about the effect of the CAR treatment only, as it is strongly significant in the models AFTER diquat treatment but independently of diquat, and this merits to be addressed too. In fact, the use of models with larger sample sizes allowed detecting differences between LZ and ZL in color (see the previous comment).

COMMENT 12. Page 21-22: Why are the effects of diquat on lutein/zeaxanthin concentrations not described here? Were they not considered important—and if so, why not?

AUTHORS: We did not find measurable lutein or zeaxanthin levels in ornaments (now added in page 23, line 4).

COMMENT 13. Page 22, lines 8-14: By this point in the paper, I think the reader may have forgotten the purpose of including tocopherol. A one sentence statement of the relevance of tocohperol would be very useful.

AUTHORS: We have added a sentence about the purpose of testing and showing tocopherol and retinol variability (page 25, first sentence).

COMMENT 14. Page 22, lines 9-14: I’m guessing you report values only for lutein and zeaxanthin because the other pigments are not found in circulation. If so, that should be stated explicitly in this part of the paper.

AUTHORS: Prof Hill is right. We have added a sentence (page 26, last sentence).

COMMENT 15. Page 29, lines 12-14: I’m not sure the logic here is clear. What is the interaction between carotenoids and tocopherol?

AUTHORS: The sentence now says: “This suggests that a higher carotenoid availability among CAR-treated birds buffered tocopherol consumption for combating free radicals, which supports the idea of mutual recycling and protective roles between tocopherol and carotenoids”

COMMENT 16. Page 30, lines 19 to 27. To complete this discussion of constraints on absorption of ketolasted carotenoids, the authors should note that House Finches with red feather coloration readily take up canthaxanthin:

Hill 2002. Red bird in brown bag. Oxford Press.

Even more interesting, American Goldfinches, which never use ketolated carotenoids as feather pigments, absorbed canthaxanthin from diet and grew red feathers.

McGraw, K. J. and G. E. Hill. 2001. Carotenoid access and intraspecific variation in plumage pigmentation in male American Goldfinches (Carduelis tristis) and Northern Cardinals (Cardinalis cardinalis). Funct. Ecol. 15:732-739.

AUTHORS: We have added these two references and a related sentence (page 33, last sentence).

COMMENT 17. Page 31, lines 19-20: I just looked through Endler 1980 and he never speculates about foraging for specific carotenoids. He only talks about “good diets” and “poor diets”. So far as I know, the first time foraging for specific carotenoids was discussed is in:

Hill, G. E. 1994. Trait elaboration via adaptive mate choice: sexual conflict in the evolution of signals of male quality. Ethology, Ecology and Evolution 6: 351-370.

And discussed in more detail in

Hill, G. E. 2002. A red bird in a brown bag. Oxford Univ. Press, Oxford.

AUTHORS: We have added these references.

COMMENT 18. Page 32, lines 18-19: I think the documented effects of diquat need to directly stated somewhere near the beginning of the results. Much of the inference drawn from the paper depends on diquat increasing free radical production in tissues relevant to the research questions without unwanted targeted effects such as changing uptake of carotenoids in the gut. The more solidly the authors can justify their use of diquat, the stronger the paper becomes.

AUTHORS: we have addressed this at the end of Introduction (last paragraph), and again in Discussion (section 4.4). We have removed an incorrect reference here (Alonso-Alvarez et al. 2009). With regard to a possible negative effect of the gut in terms of reduced lipid and carotenoid absorption, we have tested the impact of diquat on triglycerides, total cholesterol, and LDL-cholesterol, which are key constituents of lipoprotein acting as carotenoid carriers in the blood (McGraw & Parker 2006, now added). They did not report any significant change related to the treatment, and the addition of these covariates to models testing diquat effects did not provide any effect. This is now described in point 2.7.

COMMENT 19. Page 36, lines 6-8: I don’t understand the logic that supports a trade-off. Trade-off between what, and what?

AUTHORS: I suppose this is for L10-13. We have rewritten this, also adding a sentence for clarifying the idea (page 40, lines 27-31 also page 35, lines 18-21).

COMMENT 20. METHODS/RESULTS: I suggest consistently using either “time 1/2/3” or “day 0/48/82” across methods and results. Shifting between designations left me confused about what was performed when.

AUTHORS: We have only used day 0/48/82 for consistency.

COMMENT 21. RESULTS: I would further clarify that that section 3.2 deals with the effects of supplementation only over time, while 3.3 looks at the effects of diquat treatment (on one time point only). Although this is mentioned in the statistical methods, it would be effective to briefly reiterate these distinctions—otherwise, discussions of the effects of supplementation on coloration seem repetitive.

AUTHORS: we have modified the 3.2 subheading and added a sentence at the start of 3.3. Results on color measures seem to be repetitive between 3.2 and 3.3 sections. However, the 3.3 section reports the analyses performed in other dataset, as birds allocated to diquat treatment are also used for testing differences among CAR groups. This was explained in Methods (page 16, second parag.) and again in Discussion (page 35, lines 12-16).

COMMENT 22. RESULTS: I recommend restructuring the results section. I think it maybe could benefit from a cleaner separation of the results into “Coloration,” “Carotenoid composition,” and “Oxidative stress” subheadings, discussing within each subsection which treatments had significant effects (or not). As currently written, the results section is more confusing than necessary because it often shifts among time points, treatments, ornaments, or other physiological parameters, and the reasons why some (but not all) measurements are mentioned in any subsection are not clear. I think that if you instead divide the section into three subsections that describe the effects of diquat and supplementation on each of the three main physiological systems, it will be much clearer.

AUTHORS: We acknowledge Prof Hill’s suggestion. We have largely discussed on how to better present so huge amount of results. We finally decided to try a method based on the type of statistics. Thus, we first describe results about coloration and plasma where three repeated measures were available (Table 2). Subsequently, we provide tests for final values of any variable, which mostly includes variables obtained by sacrificing birds (internal tissues; Table 3, and 4 for best fitted final models). Please, note that by dividing sections by the subject (i.e. “Coloration”, “carotenoid composition”, “oxidative stress” such as suggested by Prof Hill), the readers should simultaneously move across the three tables, which is also very difficult. We have not changed the structure but added sentences to clarify the content (above). We are anyway open to any editorial suggestion.

COMMENT 23. RESULTS 3.2: If you measured ornament carotenoid pigment composition at the end of the experiment, why don’t you present the effects of supplementation treatments on pigments (in non-diquat birds) here? I see the results on carotenoid pigments in section 3.3 (on diquat), but it seems like it would be applicable here in section 3.2 as well.

AUTHORS: please, see the previous response.

COMMENT 24. RESULTS and DISCUSSION: The interaction between diquat treatment and sex is a particularly interesting and unexpected finding of this study, and should be further highlighted. Why might we expect these differences? Could there be sex-based differences in strategy, and allocation to ornamentation, reproduction, and antioxidants?

AUTHORS: This is true, but we have previously debated this question (see now page 36, L11-20), where we mention that female partridges may incur in additional reproductive costs derived from egg production. In fact, we have previously described a higher oxidative damage in plasma lipids among female partridges producing eggs with better hatching success (which is related to antioxidant allocation to eggs). We, however, expanded this by noting that males allocated more carotenoid and tocopherol (antioxidants) to ornaments, which contradicts the other findings.

COMMENT 25. DISCUSSION: The concept of hormesis should be further emphasized in the discussion because it may be critical to understanding how diquat could increase coloration of ornaments.

AUTHORS: We have now addressed this question in Discussion (page 37, last sentence and page 38, L23) and at the end of Introduction (page 8, L20-21).

LIST OF REFERENCES

We have modified the reference list. Some references to respond Prof Hill arguments were added (i.e. Hill 1994; McGraw & Hill 2001; Hill 2002; McGraw et al. 2005; McGraw & Parker 2006; Hurd & Murphy 2009; Koch & Hill 2016; Koch et al. 2016) and also the references to the new avian ketolase (Lopes et al. 2016; Mundy et al. 2016). Choi et al. 2007; Makino et al. 2008 were substituted by Fraser et al. 1997; Schoefs et al. 2001 as the last ones are the original studies proposing that the ketolase uses oxygen, Fe+2 and NADPH. Sohal & Orr 2012 was also removed as Jones 2006 was enough to defend the involvement of redox signaling disruption in the oxidative stress concept.

Finally, we have modified the title to be more attractive, though we are open to any editor suggestion.