nature research

Peer Review Information

Journal: Nature Ecology & Evolution
Manuscript Title: Ecology shapes epistasis in a genotype-phenotype-fitness map for stick-insect colour
Corresponding author name(s): Patrik Nosil

Editorial Notes:

Reviewer Comments & Decisions:

Decision Letter, initial version:
17th June 2020

*Please ensure you delete the link to your author homepage in this e-mail if you wish to forward it to your co-authors.

Dear Patrik,

Your manuscript entitled "Ecology shapes epistasis in a genotype-phenotype-fitness map" has now been seen by three reviewers, whose comments are attached. The reviewers have raised a number of concerns which will need to be addressed before we can offer publication in Nature Ecology & Evolution. We will therefore need to see your responses to the criticisms raised and to some editorial concerns, along with a revised manuscript, before we can reach a final decision regarding publication.

We are particularly concerned about the questions raised by Reviewers 1 and 3 regarding the possibility that the results are dependent on the specific way you measured colour.

We therefore invite you to revise your manuscript taking into account all reviewer and editor comments. Please highlight all changes in the manuscript text file in Microsoft Word format.

We are committed to providing a fair and constructive peer-review process. Do not hesitate to contact us if there are specific requests from the reviewers that you believe are technically impossible or unlikely to yield a meaningful outcome.

When revising your manuscript:

* Include a "Response to reviewers" document detailing, point-by-point, how you addressed each

reviewer comment. If no action was taken to address a point, you must provide a compelling argument. This response will be sent back to the reviewers along with the revised manuscript.

* If you have not done so already please begin to revise your manuscript so that it conforms to our Article format instructions at http://www.nature.com/natecolevol/info/final-submission. Refer also to any guidelines provided in this letter.

* Include a revised version of any required reporting checklist. It will be available to referees (and, potentially, statisticians) to aid in their evaluation if the manuscript goes back for peer review. A revised checklist is essential for re-review of the paper.

Please use the link below to submit your revised manuscript and related files:

[REDACTED]

Note: This URL links to your confidential home page and associated information about manuscripts you may have submitted, or that you are reviewing for us. If you wish to forward this email to co-authors, please delete the link to your homepage.

We hope to receive your revised manuscript within four to eight weeks. If you cannot send it within this time, please let us know. We will be happy to consider your revision so long as nothing similar has been accepted for publication at Nature Ecology & Evolution or published elsewhere.

Nature Ecology & Evolution is committed to improving transparency in authorship. As part of our efforts in this direction, we are now requesting that all authors identified as 'corresponding author' on published papers create and link their Open Researcher and Contributor Identifier (ORCID) with their account on the Manuscript Tracking System (MTS), prior to acceptance. ORCID helps the scientific community achieve unambiguous attribution of all scholarly contributions. You can create and link your ORCID from the home page of the MTS by clicking on 'Modify my Springer Nature account'. For more information please visit please visit www.springernature.com/orcid.

Please do not hesitate to contact me if you have any questions or would like to discuss these revisions further.

We look forward to seeing the revised manuscript and thank you for the opportunity to review your work.

[REDACTED]

Reviewer expertise:

Reviewer #1: genetics of adaptation in wild populations

Reviewer #2: genetics of adaptation in wild populations

Reviewer #3: genetics of adaptation, fitness landscapes theory including epistasis

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Nosil et al. present a study in which they quantified the contribution of multidimensional epistasis to fitness variation in a natural population of stick insects. They decompose the problem of fitness epistasis by first addressing the contribution of non-additive gene interactions to phenotypic variation (color; the main work on deciphering the genetic basis of coloration seems to have been published in a paper that is currently in press and has been appended to this submission). They then assess the degree of epistasis on fitness variation (survival) in two different selection regimes. Finally, by reference to previous work in related stick insect species, they discuss the possible evolutionary feedback of fitness epistasis on genetic trait architecture. In summary, they find a slight contribution of non-additive allelic interactions on phenotypic variation (10% additional variance explained). Selection differential was small in the MM selection regime (preferred habitat), but strong in the (disruptive) AC selection regime that showed evidence for epistasis inducing a more rugged fitness landscape favoring certain allelic combinations over others.

I liked reading this well-written paper that addresses a long-standing question on the role of epistasis in adaptation which dates back to work by Bateson, Wright and Fisher at the onset of the last century. Nosil et al. are certainly not the first to do so. We have learned a lot from experimental evolution where genotypic combinations are often generated to assess the effects of epistasis (synergistic vs. antagonistic, magnitudinal vs. sign epistasis, etc.) and resulting fitness landscapes on evolutionary dynamics, often in a systems biology framework of metabolic pathways. It is also trivial that the fitness landscape is influenced by the environment, as found here. Yet, few, quantitative studies from natural systems exist that offer insight into the full circle from genotype to phenotype to selection. Therefore, in my view, the paper which supports its conclusions with thorough Bayesian statistics warrants publication. Find below a number of reservations, comments and suggestions.

1) Introduction
I think the introduction could be made more accessible to readers in places. I would recommend reducing 'vague generality claims' (e.g. p. 2 end of first paragraph) about 'understanding complex systems' (whatever that means) and rather stick to concrete evolutionary terminology. Non-linear selection is also not a very revealing term. I'd rather frame it as deviations from multiplicative or additive fitness and refer to the (vast) theoretical literature.

2) Definition phenotype
Trait definition influences the inferred genetic architecture. The closer to a molecule a trait is defined the less polygenic and arguably less epistatic its genetic architecture. Here the authors chose to focus on color, which has been shown to be subject to selection in previous work (and tends have a rather simple genetic architecture). They quantified color in the (human) visible spectrum (RGB channels) and then defined color as two composite traits: RG and GB. I would like to see more justification on this choice than the Endler citation. What happens if all channels are analyzed separately? Or if all three channels are projected on a single axis of major variation? What is the G-matrix of these components? Would a change in how the phenotype is conceptualized alter the conclusions on epistasis at any of the levels (genotyp-phenotype; phenotype-fitness)?

3) Phenotype free
Fitness epistasis is here assessed as mediated by a (largely) additive phenotype. This is common practice and informative on the underlying processes. I would be curious, however, what a GWAS directly relating genotype to fitness would infer. Will the same loci show up? Is the degree of epistasis similar? Eventually, this test should also help identify whether the selection regime is as exclusively related to selection on color, or whether additional forces are at play. The latter question is relevant, since pleiotropy is a major factor inducing epistasis, an aspect that also warrants more discussion.

4) Genetic variation
The vast majority of 'color loci' seem to reside in the Mel-Stripe genetic region. This makes me wonder how strongly the outcome of the GWAS is governed by the selective regime of the population under investigation. Would you expect the same loci to show up in controlled crosses in the lab? Color variation available to the selection experiment was already bimodal, possibly at least partly because of (the AC?) disruptive selection in the study population. The influence of the allele frequencies and patterns of LD (though seemingly controlled for) in the study population deserve some more attention, as they may bias the results.

5) Correlational selection
Does selection introduce the correlation, as suggested, or is selection only targeted at one of the two traits and trait architecture is correlated (G-matrix); see 'Definition phenotype' above.

6) Genome evolution
The aspect of genome evolution arising from fitness epistasis is highly interesting. We see strong LD (most likely because of selection) also in other systems (e.g. Hench et al. 2019 Nat Ecol Evol). How strongly does epistasis favor physical linkage of such loci? I would love to learn more about homology of the colour loci (and adjacent regions or genes) between the Timema cristinae and T. chumash system. It seems that investigation of this aspect would be possible and add to the value of this study.

Minor comments:

7) Figure 1: I found figure 1b somewhat confusing. It may be more informative for the reader to visualize epistasis in form of an adaptive landscape – maybe even for two different selection regimes.

8) P. 24 (phenotyp to fitness): Please provide more information on the actual model formulation, analogous to the explicit from in the previous section (p. 23 bottom).

9) One of the main merit of this study is certainly to investigate the genotype-phentoype-selection relationship in natural studies. It may be worth devoting some space of the discussion to the relative merit of experimental systems (e.g. Sackman& Tokyta 2018 Genetics) and empirical systems addressing this question (e.g. Hench et al. 2019 Nat Ecol Evol, Knief et al. 2019 Nat Ecol Evol). What is the advantage of studying this in natural populations over experimental systems? What are the challenges and shortcomings?

Reviewer #2 (Remarks to the Author):

This is a really exciting study. The paper addresses a fundamental question in biology: to what extent does epistasis underlie to relationship between genotype, phenotype and fitness. This question has been addressed in theory and to some extent in lab studies of model organisms, but to address it in a natural system, like the present study, is rare. The present work also serves as a case study of how to tease apart different aspects of epistasis in the field, which has become possible only after methodological developments (both computational and the ability to query large number of individual genomes). Importantly, the study clearly lays out two possible sources of epistasis and queries to what extent each of these affects genotype-phenotype-fitness relationships in a natural population. The present study will be of interest to a wide range of fields, ranging from evolutionary biology and ecology to molecular biology and genetics in model and non-model organisms. The manuscript is clearly written and results are placed in context of current open questions. Judged with my expertise, the manuscript does not have fatal flaws that would prohibit its publication. I recommend this manuscript to be invited for revisions.

Major comment

You show that coloration phenotype is mostly determined by additive effects of multiple loci with small but significant contribution of epistatically interacting variants. Additivity explains 80% of the variation and epistasis between five SNPs explains additional 10% of the variation. This result supports the role of epistasis in "cellular processes that convert genotype to phenotype".

In the next part you investigate if "epistasis arises via selective processes that connect phenotype to fitness". You find evidence for epistasis that arises due to ecological selection. On page 7, L3-5 you say "--the number of effects of epistasis on survival on AC was ~6". For someone not an expert on the specific methods used, it is difficult to evaluate what does this actually mean. How large is the effect of epistasis? Something comparable to the percentage of variation explained (as in the first experiment) would help to place your results into context.

Minor comments

P1, L34: Please provide a definition for epistasis in the beginning. Genetic interactions are not a strict synonym for epistasis, as genetic interactions can be additive as well. If you use interaction as a synonym for epistasis, please state this clearly. Clear definition at the onset would help reader to follow your reasoning. Figure 1 is very helpful in explaining the two ways in which epistasis can arise.

P4, L13-14: How many SNPs were associated with color within and outside the Mel-Stripe region? This will help to evaluate to what extent epistasis could occur outside this region. Also, it would be good to add statistical evidence of the associations in the text shortly even if the data is presented in the figure.

P4, L20-21: How significant were these 5 SNPs? It would help the reader to state here that you focused on SNPs on Mel-Stripe locus. Thus, there could be epistasis between Mel-Stripe and other regions of the genome, you just did not test for it?

P5, L25-26: How many survivors were captured? What percentage of transplanted individuals survived? Is this consistent with selection intensity in natural conditions? Again, this helps to place weight to your conclusions.

P6, L7-19: I wonder why are the analyses for color associated SNPs repeated? Is this data set the same or different to the one which was used earlier in the ms to map color associated SNPs?

P6, L33-34: Are these the same SNPs that were identified in the first experiment (i.e. P4)?

Reviewer #3 (Remarks to the Author):

Nosil et al. studied the genotype-phenotype-fitness relationships in stick insects. Here, the genotype space contains the variations at a few SNPs, the phenotype is the body color, and fitness is measured by the survival rate of the insects on two sets of plant hosts. They did a large amount of work on hundreds of insects (genotyping, GWAS, measuring body color, and estimating fitness). The question they study is interesting, but the results appear expected (at least for the phenotype-fitness relation) and are suspected to depend on a particular definition of phenotype. There are also important conceptual issues that are unclear. My detailed comments follow.

1. The authors measured the insect body color by RGB color codes. My understanding is that a color is represented by three parameters (red, green, and blue, respectively) in the RGB space. Why did the authors use only two parameters—RG and GB? Does RG mean R/G ratio and GB mean G/B ratio? I believe that these two ratios are insufficient to describe a color. That is, multiple colors can have the same pair of R/G and G/B ratios.
2. The authors showed that the mapping between SNPs and the two color parameters are largely additive. Is this result robust to different descriptions of color? For example, color can also be measured by wavelength. Will the mapping between SNPs and wavelength additive? More generally, every trait can be transformed. For example, one could consider either body mass or ln(body mass) as a measure of body size. If the relationship between SNPs and one of these measures is additive, the relationship between SNPs and the other measure must be non-additive. Because it is equally valid to use the two measures, emphasizing that the mapping is additive is not meaningful. The only exception may be when one can tell from the biological basis of body size (i.e., how body size is regulated) that one of the measures is more reasonable than the other. Is there biology of body color in these insects (e.g., based on how pigments are synthesized) that makes the authors think that the two parameters they use are more reasonable than a myriad of other possible measures of color? Furthermore, there are numerous papers showing epistatic effects of mutations on non-fitness phenotypes such as enzyme activities. So, their finding of additivity here is certainly not general.
3. As the authors explained, the colors of the A and C plants dictate that green and melanistic colorations of insects will be selected for on A and C plants, respectively. Hence, the non-additive mapping from body color phenotypes to fitness is expected. I don't see this to be generally true for other traits or other environments (e.g., industrial melanism).
4. My biggest concern is about the overarching goal of genotype-phenotype-fitness mapping. Given that pleiotropy is widespread, a SNP likely impacts multiple traits, and many of these traits may affect fitness. So, the SNPs identified to affect body color likely also affect other traits and influence fitness through these other traits. The negligence of these other traits in the study makes the mapping of genotype-phenotype-fitness incomplete and probably wrong. This is why I think genotype-phenotype-fitness mapping is almost impossible.
5. Related to the above point, the phenotype-fitness mapping in this study is based on a regression between body color and survival rate without controlling for other traits. Thus, in theory, it is possible that an unknown trait that is correlated with body color actually causes the fitness variation. If this were true, the phenotype-fitness mapping in this study would be meaningless. I am not saying that

this is definitely the case here, but it seems hard to rule out this possibility.

**********END**********

Author Rebuttal to Initial comments

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

Nosil et al. present a study in which they quantified the contribution of multidimensional epistasis to fitness variation in a natural population of stick insects. They decompose the problem of fitness epistasis by first addressing the contribution of non-additive gene interactions to phenotypic variation (color; the main work on deciphering the genetic basis of coloration seems to have been published in a paper that is currently in press and has been appended to this submission). They then assess the degree of epistasis on fitness variation (survival) in two different selection regimes. Finally, by reference to previous work in related stick insect species, they discuss the possible evolutionary feedback of fitness epistasis on genetic trait architecture.

In summary, they find a slight contribution of non-additive allelic interactions on phenotypic variation (10% additional variance explained). Selection differential was small in the MM selection regime (preferred habitat), but strong in the (disruptive) AC selection regime that showed evidence for epistasis inducing a more rugged fitness landscape favoring certain allelic combinations over others.

I liked reading this well-written paper that addresses a long-standing question on the role of epistasis in adaptation which dates back to work by Bateson, Wright and Fisher at the onset of the last century. Nosil et al. are certainly not the first to do so. We have learned a lot from experimental evolution where genotypic combinations are often generated to assess the effects of epistasis (synergistic vs. antagonistic, magnitudinal vs. sign epistasis, etc.) and resulting fitness landscapes on evolutionary dynamics, often in a systems biology framework of metabolic pathways. It is also trivial that the fitness landscape is influenced by the environment, as found here. Yet, few, quantitative studies from natural systems exist that offer insight into the full circle from genotype to phenotype to selection. Therefore, in my view, the paper which supports its conclusions with thorough Bayesian statistics warrants publication. Find below a number of reservations, comments and suggestions.

Response: We are pleased that the reviewer enjoyed reading the paper, considered it to address a major topic, and found our conclusions supported. We have addressed the issues raised below in revision and thank the reviewer for their efforts to improve the manuscript.

1) Introduction
I think the introduction could be made more accessible to readers in places. I would recommend reducing 'vague generality claims' (e.g. p. 2 end of first paragraph) about 'understanding complex systems'

(whatever that means) and rather stick to concrete evolutionary terminology. Non-linear selection is also not a very revealing term. I'd rather frame it as deviations from multiplicative or additive fitness and refer to the (vast) theoretical literature.

Response: Agreed. We have removed vague generality terms from the Introduction as suggested. We agree that 'non-linear' selection is not a useful term in the population genetics sense when referring to selection on alleles. However, it is a standard and well-used term in the literature on phenotypic selection, stemming back to Lande and Arnold's seminal paper in 1983. We have clarified in the revised manuscript that directional selection is linear and that other forms (stabilizing, disruptive, correlational) are non-linear, and that we use these terms in the context of phenotypic selection.

2) Definition phenotype
Trait definition influences the inferred genetic architecture. The closer to a molecule a trait is defined the less polygenic and arguably less epistatic its genetic architecture. Here the authors chose to focus on color, which has been shown to be subject to selection in previous work (and tends have a rather simple genetic architecture). They quantified color in the (human) visible spectrum (RGB channels) and then defined color as two composite traits: RG and GB. I would like to see more justification on this choice than the Endler citation. What happens if all channels are analyzed separately? Or if all three channels are projected on a single axis of major variation? What is the G-matrix of these components? Would a change in how the phenotype is conceptualized alter the conclusions on epistasis at any of the levels (genotype-phenotype; phenotype-fitness)?

Response: We agree that these points are important and that our description of the quantification of colour was not clear enough in the original submission. We have elaborated on this topic in the main text, and included a new explanatory figure (Figure 2 in the revised manuscript). Our approach is biologically rooted as it is based on the physiological processing of colour stimuli, as crystallized by Endler and cited over a thousand times (based on Web of Science searches on his 1990 and 2012 papers in the Biological Journal of the Linnaean Society). Using reflectance data, we show in the manuscript that *T. chumash* does not reflect ultraviolet light. Thus, we focused in the human visible spectrum (RGB, 400-700nm), which involves the following photoreceptors: cones that capture long wavelengths (L, red), medium wavelengths (M, green) and short wavelengths (S, blue). Differences in L-M and M-S activities are the most common responses used in colour sensation and discrimination (*i.e.* the chromatic response functions). The approach used in our work is an approximation to this physiological response, based on the *relative distances* between R and G, and G and B pixel values extracted from digital photographs. This approach yields values that are more biologically meaningful and more readily comparable across colour patterns and studies than using raw RGB values (and as such we do not consider R + G + B, which is best conceptualized as brightness not hue). RG and GB chromatic contrasts collectively represent the main variation in *T. chumash* colour, and form the focus of our work. These points have been clarified in the revised manuscript.

3) Phenotype free
Fitness epistasis is here assessed as mediated by a (largely) additive phenotype. This is common practice and informative on the underlying processes. I would be curious, however, what a GWAS directly

relating genotype to fitness would infer. Will the same loci show up? Is the degree of epistasis similar? Eventually, this test should also help identify whether the selection regime is as exclusively related to selection on color, or whether additional forces are at play. The latter question is relevant, since pleiotropy is a major factor inducing epistasis, an aspect that also warrants more discussion.

Response: The points raised by the reviewer are highly relevant to the theme of our study. We have thus conducted additional analyses that address the curiosity of the reviewer concerning a 'phenotype-free' approach, and that directly tackle the interesting and important issue of pleiotropy (note that these also address some of the key concerns of Reviewer 3, as well). These additional analyses comprise three main approaches, which we applied to the AC treatment where fitness epistasis was detected.

First, we have conducted additional Bayesian model averaging analyses that no longer factor colour into the response variable. Here, we use survival directly as our response variable, rather than using expected fitness based on phenotypic selection and colour scores. Our independent variables are the same five SNPs used in the main analyses based on expected fitness. We consider models with only main effects of these SNPs, and those which also include interactions between and among SNPs. These represent a pure additive model versus a model with both additive and epistatic effects, respectively. The results show substantial predictive power when considering only genotype and survival, including a marked increase in predictive power when epistasis is considered (Table 3).

Second, we have conducted a truly 'phenotype-free' analysis in MAPIT. Here, we quantify the epistatic effect of every SNP in the *Mel-Stripe* region on survival (response variable), rather than only considering the most strongly colour-associated SNPs. This revealed that the five specific SNPs exhibiting epistasis for colour show collective evidence of epistasis for survival (individual *P*-values are 0.11. 0.18, 0.15. 0.09 0.10, Fisher's combined probability test, $X^2 = 21.05$, d.f. = 10, $P = 0.007$). A similar trend, albeit weaker, was detected for the five colour-associated SNPs used in our Bayesian model-averaging survival analyses (individual *P*-values are 0.25, 0.52. 0.11. 0.15, 0.52, Fisher's combined probability test, $X^2 = 13.47$, d.f. = 10, $P = 0.051$). Our finding of fitness epistasis thus appears robust to different analytical approaches, but stronger results are obtained when considering selection acting through colour phenotype than when considering genotype alone.

Third, we have employed an approach for testing pleiotropy analogous to that introduced by Rennison and colleagues (from Dolph Schluter's group, Am Nat 2015), and applied to stickleback. The idea is to include both genotype (in our case, for the five colour-associated SNPs) and measured phenotype (in our case, RG and GB colour scores) as independent covariates in a model that predicts survival (response variable). If effects of genotype on survival are observed even when controlling for phenotype, this implies pleiotropy, i.e., effects of genotype on survival independent from colour, via unmeasured traits. Our results reveal a modest increase in cross-validation predictive power in the model with both covariates, relative to a model including only colour. Thus, the results maintain clear evidence for selection on colour phenotype, but also suggest some pleiotropic effects of colour loci on other (unmeasured) traits influencing fitness.

These findings increase the breadth and clarity of the manuscript, and have been added to the revised manuscript as a separate sub-section at the end of the results, with detailed results collated in a new Table (i.e., Table 3 in the revised manuscript).

4) Genetic variation
The vast majority of 'color loci' seem to reside in the Mel-Stripe genetic region. This makes me wonder how strongly the outcome of the GWAS is governed by the selective regime of the population under investigation. Would you expect the same loci to show up in controlled crosses in the lab? Color variation available to the selection experiment was already bimodal, possibly at least partly because of (the AC?) disruptive selection in the study population. The influence of the allele frequencies and patterns of LD (though seemingly controlled for) in the study population deserve some more attention, as they may bias the results.

Response: The reviewer is correct that the majority of color loci reside in the *Mel-Stripe* region. We note this explicitly in the revised manuscript, and refer readers to a paper focused on the genetics of colour for further details (Villoutreix et al. *Science*, in press). Our genetic mapping approach indeed controls for LD, as noted in our revised manuscript. Nonetheless, patterns of LD for the five most strongly associated loci are illustrated in the online materials for interested readers (Supplemental Figure 4).

5) Correlational selection
Does selection introduce the correlation, as suggested, or is selection only targeted at one of the two traits and trait architecture is correlated (G-matrix); see 'Definition phenotype' above.

Response: Correlational selection refers to selection for combinations of traits, which can then generate epistasis for fitness at loci underlying the traits. We have clarified this definition and point in the revised manuscript.

6) Genome evolution
The aspect of genome evolution arising from fitness epistasis is highly interesting. We see strong LD (most likely because of selection) also in other systems (e.g. Hench et al. 2019 Nat Ecol Evol). How strongly does epistasis favor physical linkage of such loci? I would love to learn more about homology of the colour loci (and adjacent regions or genes) between the Timema cristinae and T. chumash system. It seems that investigation of this aspect would be possible and add to the value of this study.

Response: We fully agree that the aspect of genome evolution arising from fitness epistasis is highly interesting. We have now cited the Hench et al. paper as an additional example. Moreover, we have fully addressed the point raised of homology (or more precisely, synteny) by generating a new *de novo* chromosome-level genome assembly for *T. chumash*, and compared it to the previously existing *T. cristinae* genome. This revealed three new findings, which are reported in the revised manuscript: (1) the SNPs we focus our analyses on are in synteny between the two species (same genomic location), (2) the SNPs exist as a single copy in the *T. chumash* genome, and (3) a chromosomal inversion on LG8 distinguishes these species, explaining reduced recombination. We agree that this adds value to the study and thank the reviewer for urging us in this direction.

Minor comments:

7) Figure 1: I found figure 1b somewhat confusing. It may be more informative for the reader to visualize epistasis in form of an adaptive landscape – maybe even for two different selection regimes.

Response: We agree that figure 1b was somewhat confusing; colleagues have independently noted so. We have thus removed it and ensured that the verbal description of fitness epistasis in our revised Introduction is totally clear.

8) P. 24 (phenotyp to fitness): Please provide more information on the actual model formulation, analogous to the explicit from in the previous section (p. 23 bottom).

Response: Done as requested.

9) One of the main merits of this study is certainly to investigate the genotype-phentoype-selection relationship in natural studies. It may be worth devoting some space of the discussion to the relative merit of experimental systems (e.g. Sackman & Tokyta 2018 Genetics) and empirical systems addressing this question (e.g. Hench et al. 2019 Nat Ecol Evol, Knief et al. 2019 Nat Ecol Evol). What is the advantage of studying this in natural populations over experimental systems? What are the challenges and shortcomings?

Response: Great idea. Done as requested, at least in brief due to space constraints, with reference to the citations noted (p. 10 and 11 of the revised manuscript).

Reviewer #2 (Remarks to the Author):

This is a really exciting study. The paper addresses a fundamental question in biology: to what extent does epistasis underlie to relationship between genotype, phenotype and fitness. This question has been addressed in theory and to some extent in lab studies of model organisms, but to address it in a natural system, like the present study, is rare. The present work also serves as a case study of how to tease apart different aspects of epistasis in the field, which has become possible only after methodological developments (both computational and the ability to query large number of individual genomes). Importantly, the study clearly lays out two possible sources of epistasis and queries to what extent each of these affects genotype-phenotype-fitness relationships in a natural population. The present study will be of interest to a wide range of fields, ranging from evolutionary biology and ecology to molecular biology and genetics in model and non-model organisms.

The manuscript is clearly written and results are placed in context of current open questions. Judged with my expertise, the manuscript does not have fatal flaws that would prohibit its publication. I recommend this manuscript to be invited for revisions.

Response: We are pleased to hear that the reviewer saw the merit of our study, and have revised it according to their useful suggestions.

Major comment

You show that coloration phenotype is mostly determined by additive effects of multiple loci with small but significant contribution of epistatically interacting variants. Additivity explains 80% of the variation and epistasis between five SNPs explains additional 10% of the variation. This result supports the role of epistasis in "cellular processes that convert genotype to phenotype".

In the next part you investigate if "epistasis arises via selective processes that connect phenotype to fitness". You find evidence for epistasis that arises due to ecological selection. On page 7, L3-5 you say "--the number of effects of epistasis on survival on AC was ~6". For someone not an expert on the specific methods used, it is difficult to evaluate what does this actually mean. How large is the effect of epistasis? Something comparable to the percentage of variation explained (as in the first experiment) would help to place your results into context.

Response: Great point. To address this we now report results of new analyses that explicitly compare the cross-validation power of models with versus without epistasis (our original submission reported only the former). This demonstrates that a model with additive and epistatic effects is 64% better (in terms of predictive power) than a model with only additive effects.

Minor comments

P1, L34: Please provide a definition for epistasis in the beginning. Genetic interactions are not a strict synonym for epistasis, as genetic interactions can be additive as well. If you use interaction as a synonym for epistasis, please state this clearly. Clear definition at the onset would help reader to follow your reasoning. Figure 1 is very helpful in explaining the two ways in which epistasis can arise.

Response: As requested, we have offered a clear definition of epistasis at the onset of the manuscript, based on the common definition of effects of an allele at one locus being dependent on another gene or genes in the genome.

P4, L13-14: How many SNPs were associated with color within and outside the Mel-Stripe region? This will help to evaluate to what extent epistasis could occur outside this region. Also, it would be good to add statistical evidence of the associations in the text shortly even if the data is presented in the figure.

Response: The majority of color loci reside in the *Mel-Stripe* region. We note this explicitly in the revised manuscript, and refer readers to a paper focused on the genetics of color for further details (Villoutreix et al. Science, in press). Along the lines of the reviewer suggestion, we have added a new table to the revised manuscript that explicitly reports the extent of associations, effect sizes, and their significance levels (thus addressing the point directly below, see Table 2 in the revised manuscript).

P4, L20-21: How significant were these 5 SNPs? It would help the reader to state here that you focused on SNPs on Mel-Stripe locus. Thus, there could be epistasis between Mel-Stripe and other regions of the genome, you just did not test for it?

Response: In the aforementioned new table we report the significance of these SNPs, which is extremely high (hundreds of times more likely than the lack of an association). It is true that there could be additional epistasis with other regions. Because this was beyond our focus on colour genes, we leave this for further work (and, the reviewer is correct that we did not test for it, which we now clarify in the revised manuscript by noting our exclusive focus on the *Mel-Stripe* locus).

P5, L25-26: How many survivors were captured? What percentage of transplanted individuals survived? Is this consistent with selection intensity in natural conditions? Again, this helps to place weight to your conclusions.

Response: Good questions. We have added a small table to the revised manuscript that provides details of our experimental bushes, blocks, recaptures, etc.

P6, L7-19: I wonder why are the analyses for color associated SNPs repeated? Is this data set the same or different to the one which was used earlier in the ms to map color associated SNPs?

Response: This is a good question concerning an aspect of our study, which we were not clear enough about in the original submission. The analyses are not repeated; they stem from the same core mapping of colour using GWAS in GEMMA. The reason the results are reported in different sections is that the mapping section focused on percent variance explained by additive versus epistatic models, not on individual SNPs. In contrast, the fitness analysis connects genetic variation at individual color-associated SNPs to survival. This distinction has been clarified in the revised manuscript.

P6, L33-34: Are these the same SNPs that were identified in the first experiment (i.e. P4)?

Response: Yes, they are, see point directly above.

Reviewer #3 (Remarks to the Author):

Nosil et al. studied the genotype-phenotype-fitness relationships in stick insects. Here, the genotype space contains the variations at a few SNPs, the phenotype is the body color, and fitness is measured by the survival rate of the insects on two sets of plant hosts. They did a large amount of work on hundreds of insects (genotyping, GWAS, measuring body color, and estimating fitness). The question they study is interesting, but the results appear expected (at least for the phenotype-fitness relation) and are suspected to depend on a particular definition of phenotype. There are also important conceptual issues that are unclear. My detailed comments follow.

Response: We are pleased that the reviewer recognized the work done and the interest of the topic. We have explicitly addressed the conceptual issues raised, which has improved the manuscript.

1. The authors measured the insect body color by RGB color codes. My understanding is that a color is represented by three parameters (red, green, and blue, respectively) in the RGB space. Why did the authors use only two parameters—RG and GB? Does RG mean R/G ratio and GB mean G/B ratio? I

believe that these two ratios are insufficient to describe a color. That is, multiple colors can have the same pair of R/G and G/B ratios.

Response: Please see detailed response to the first point of reviewer 1. Good points, which have been addressed. Most critically relevant to this specific comment, we have clarified that our colour variables are chromatic contrasts, as developed by Endler, rather than strict ratios per se.

2. The authors showed that the mapping between SNPs and the two color parameters are largely additive. Is this result robust to different descriptions of color? For example, color can also be measured by wavelength. Will the mapping between SNPs and wavelength additive? More generally, every trait can be transformed. For example, one could consider either body mass or ln(body mass) as a measure of body size. If the relationship between SNPs and one of these measures is additive, the relationship between SNPs and the other measure must be non-additive. Because it is equally valid to use the two measures, emphasizing that the mapping is additive is not meaningful. The only exception may be when one can tell from the biological basis of body size (i.e., how body size is regulated) that one of the measures is more reasonable than the other.

Is there biology of body color in these insects (e.g., based on how pigments are synthesized) that makes the authors think that the two parameters they use are more reasonable than a myriad of other possible measures of color? Furthermore, there are numerous papers showing epistatic effects of mutations on non-fitness phenotypes such as enzyme activities. So, their finding of additivity here is certainly not general.

Response: We have expanded our treatment of colour in a number of ways to address these issues. First, we emphasize that we provide a full treatment and analysis of spectral data and wavelengths specifically. This shows that *Timema* largely reflect in the visible spectrum and do not give off appreciable levels of ultraviolet. Moreover, modeling to the avian visual system shows that the visible spectrum is what is perceived by birds, a major predator of *Timema*. Moreover, the plants these insects rest upon are coloured in shades of green and red/brown, matching the colours of the insects. Thus, our focus on RG and GB is biologically defensible and relevant. We have clarified these points in the revised manuscript. Moreover, our results concerning pleiotropic effects of SNPs (details below) demonstrate that fitness epistasis occurs independent from our colour scores.

3. As the authors explained, the colors of the A and C plants dictate that green and melanistic colorations of insects will be selected for on A and C plants, respectively. Hence, the non-additive mapping from body color phenotypes to fitness is expected. I don't see this to be generally true for other traits or other environments (e.g., industrial melanism).

Response: The reviewer is correct that fitness epistasis is not expected in all cases; for example, directional selection on an additive trait will not generate fitness epistasis. However, our results are likely to apply for other traits subject to non-linear selection, and the influential review by Kingsolver et al. in the American Naturalist (2001) shows that non-linear selection is common in nature (as cited in the revised manuscript). We have clarified the context-dependent nature of our results, but also when they should apply generally, in our revised manuscript. We thank the reviewer for urging such balance.

4. My biggest concern is about the overarching goal of genotype-phenotype-fitness mapping. Given that pleiotropy is widespread, a SNP likely impacts multiple traits, and many of these traits may affect fitness. So, the SNPs identified to affect body color likely also affect other traits and influence fitness through these other traits. The negligence of these other traits in the study makes the mapping of genotype-phenotype-fitness incomplete and probably wrong. This is why I think genotype-phenotype-fitness mapping is almost impossible.

Response: The reviewer notes excellent points about pleiotropy (which we note apply to all studies attempting genotype-phenotype-fitness mapping, and are not specific to our study). Because this point is important, we have tackled it directly. As noted in response to point 3 from reviewer 1, we have employed an approach for testing pleiotropy analogous to that introduced by Rennison and colleagues (from Dolph Schluter's group, Am Nat 2015). The idea is to include both genotype (in our case, for the five color-associated SNPs) and measured phenotype (in our case, colour trait scores) as independent covariates in a model that predicts survival (dependent variable). If effects of genotype on survival are observed even when controlling for (measured) phenotype, this implies pleiotropy, i.e., effects of genotype on survival independent from colour, via unmeasured traits. Such an analysis maintains the clear evidence for selection on colour phenotype, but also revealed evidence for modest pleiotropic effects of colour loci on other (unmeasured) traits influencing fitness. These findings increase the breadth and clarity of the manuscript, and have been added to the revised manuscript (page 9, Table 3). Thus, although we agree with the reviewer that the genotype-phenotype-fitness map may always be incomplete, we have worked to increased how complete it is via this new analysis. Finally, we have clarified in the revised manuscript that a substantial body of work points to colour as a major axis of adaptation and target of selection in these insects (e.g., Sandoval 1994, Evolution and Biological Journal of the Linnean Society, Nosil and Crespi 2006 PNAS, Comeault et al. 2015 Current Biology).

5. Related to the above point, the phenotype-fitness mapping in this study is based on a regression between body color and survival rate without controlling for other traits. Thus, in theory, it is possible that an unknown trait that is correlated with body color actually causes the fitness variation. If this were true, the phenotype-fitness mapping in this study would be meaningless. I am not saying that this is definitely the case here, but it seems hard to rule out this possibility.

Response: Please see response to point 4 above, which does not entirely rule out the reviewer's possibility, but does begin to actively parse the contributions of colour versus other traits to survival. Our results reveal a modest increase in cross-validation predictive power in the model with both covariates, relative to a model including only colour.

Decision Letter, first revision:

11th August 2020

*Please ensure you delete the link to your author homepage in this e-mail if you wish to forward it to

your co-authors.

Dear Patrik,

Your manuscript entitled "Ecology shapes epistasis in a genotype-phenotype-fitness map" has now been seen by two of our reviewers, and in the light of their advice I am delighted to say that we can in principle offer to publish it. First, however, we would like you to revise your paper to ensure that it is as brief as possible and complies with our Guide to Authors at http://www.nature.com/natecolevol/info/final-submission.

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1. A two-sentence editorial summary of the paper will appear on the journal homepage with the link to the paper. This is our proposed summary: 'The causes of epistasis in nature are poorly understood. Measuring the genetic basis of cryptic colouration and survival in a field experiment with stick insects, the authors show that epistasis results from ecological variation in natural selection". Please let us know of any factual inaccuracies.

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4. Please include the subheading 'Results and Discussion'

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Reviewer Comments:

Reviewer #1 (Remarks to the Author):

The revised version covers all of my concerns in a satisfactory way. The introduction is more accessible, central terms are well defined, additional analyses broaden the explanatory power of the study and additional figures guide the reader through the complex subject matter. I recommend

publication.

Reviewer #3 (Remarks to the Author):

Authors have adequately addressed my comments. I look forward to seeing it published.

**********END**********

Final Decision Letter:

19th August 2020

Dear Patrik,

We are pleased to inform you that your Article entitled "Ecology shapes epistasis in a genotype-phenotype-fitness map for stick-insect colour", has now been accepted for publication in Nature Ecology & Evolution.

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