MS #61026  
  
Dear Author:  
  
The Editorial Board of the American Naturalist has not yet made a decision on your article, "Population-specific patterns of toxin sequestration in monarch butterflies from around the world," in its current form. Let me apologize for the unusual delay in coming to a determination on your MS. Circumstances sometimes conspire to slow down the evaluation process beyond what we would wish, and regrettably that has happened in this case. One of our associate editors (Dr. Marcelo A. Aizen) has evaluated your manuscript and obtained reviewer comments. Having read your submission and the reviews, I agree with the Associate Editor’s suggestions, and am therefore requesting revisions to your article before we make a final decision about its disposition.   
  
The three reviewers, Associate Editor, and I were all quite positive about this submission. Indeed, it is rare to have three reviewers all so effusively complimentary. It is clear your paper is well-written and of substantial interest particularly given the charismatic species in question. The reviewers did all provide some additional comments pointing out areas where your presentation could be improved or clarified. These are excellent constructive comments meant to improve the impact this work will have. I’ve read through the paper and find the comments generally on point, and I think you will be reasonably able to address the stated concerns. One, that I want to highlight, is a comment raised by the Associate Editor. Although the monarch system is rightly famous and charismatic, I’d like you to work on more effectively conveying in the introduction, abstract, and discussion, the broader implications, what this teaches us about evolutionary ecology or plant-insect  
interactions generally, lessons transcending the monarch system in particular. This requires some modest rewriting. Likewise the reviewer comments generally represent revisions to text, or some modest analytical or graphical changes.  
  
The final thing that I want to raise is one you cannot address. It is a concern and might normally be something that stops a manuscript at this stage: you have a single replicate of the low bird predation setting (Guam). That's no fault of your own, it is the biological reality of this study species. Yet, it limits your inferential ability a bit. The most interesting comparison of your paper is effectively unreplicated, and not replicable. I wrestled for some time with whether this troubles me to the point that I'd reject the paper, and I have rejected other papers for similar limitations in the past. Yet, in this case the merits of the work largely outweigh this significant limitation. So, I'd like at a minimum to ask that you acknowledge this limitation with suitable disclaimers. of course if you can find replicated Guams, great, but I doubt that's an option.

We have updated the discussion of the paper to more clearly acknowledge this limitation and to suggest alternative approaches that could be used for measuring impacts of variation in predation on sequestration. The added text reads:

“That we only have a single bird-free island certainly limits the power of our inferences regarding predation intensity and natural selection on sequestration. Future studies of sequestration ability in relation to predators could instead focus on quantitative variation in predation intensity (e.g. Camara 1997) or leverage experimental evolution approaches in a short-lived species with experimentally tractable predators (e.g. the western corn rootworm and its nematode predators [Robert et al. 2017]).”

Please be careful in your revision to add as little as possible to the length of the text, and any ability to condense a bit here and there would be appreciated. Our journal is under extreme competition for space among many excellent articles, so we are forced to consider the value of a paper relative to the number of pages it requires. Therefore an idea needs to be presented concisely to maximize its probability of success in publication.

To the extent possible, we cut paragraphs from the introduction and discussion of the paper that were not critical for understanding the main points of the manuscript. The main text of the paper (not including the abstract or references) is currently 4825 words, which is slightly longer than the original submission (4605 words) but includes many additions requested by reviewers.  
  
We provide a list of “Best Practices” for our authors and reviewers, which we hope you find useful in checking over your paper during revisions. You can find this list at:  
<https://www.amnat.org/announcements/MS-Checklist.html>. In particular, both simulations and code-based statistical analyses are vulnerable to author error. Even a tiny coding error can flip a paper’s results, so an excess of caution is a good idea here. I wish we were in a position to provide thorough code review, but that’s not a standard policy yet. I would strongly encourage you to proofread your code as carefully as you proofread your prose. Or, better yet, have a colleague examine your code for errors.   
  
As a requirement for publication, authors of all accepted manuscripts should upload their raw data and analysis code to Dryad/zenodo ([www.datadryad.org](http://www.datadryad.org)), including adequately annotated materia and a README file that makes the archive usable. You can find The American Naturalist’s guidelines for data and code archiving here: <https://comments.amnat.org/2021/12/guidelines-for-archiving-code-with-data.html>. Dryad also publishes a list of best practices (<https://datadryad.org/stash/best_practices>) you may wish to consult. You will not be charged for a Dryad repository when publishing with The American Naturalist.   
  
Authors may use other repositories if there is good reason to do so (government or industry requirements, genomic data and gene sequences, methods video, etc) but Dryad/zenodo is always preferred because it is curated, permanent, and free to authors of published papers in this journal. Users of GitHub can obtain a zenodo DOI for their code deposits, though our recommendation is to move code and related files over to zenodo. If authors require an exception to any archiving policy, they should contact the Editor in advance.   
  
To ensure that your work satisfies The American Naturalist’s commitment to transparency and Open Data, your manuscript and data repository will be evaluated by one of our Data Editors. If your article involves data or code, one of our Data Editors will generate a report identifying deficiencies and making suggestions to make your data/code repository complete and useful. Our goal is to adopt a simple and painless process to improve compliance with data archiving, to avoid post-publication problems that may arise from incomplete data archives or poorly documented metadata.  We feel that review of data repositories is crucial to ensure that the work is reproducible. Recent evidence also suggests that a useful data repository will increase the impact of your work. If your data and code is accessible and usable, readers may be more likely to embrace and build upon your exciting findings!   
  
Please submit your revised manuscript to the AmNat web peer review website within 60 days. Please upload a detailed explanation of your responses to all of the comments below. Your responses will be available to any subsequent reviewers, so to maintain double blind review (unless you opt out), please do not include identifying information.  
  
Thank you for submitting your work to The American Naturalist. I look forward to reading your revision.  
  
Sincerely,  
  
Daniel I. Bolnick  
Editor-in-Chief  
The American Naturalist  
Professor, Department of Ecology and Evolutionary Biology, University of Connecticut  
  
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Associate Editor Dr. Marcelo A. Aizen's Recommendation  
  
Dear Dan,  
  
In "Population-specific patterns of toxin sequestration in monarch butterflies from around the world" the authors focus on different (micro)evolutionary aspects associated with the use of chemical defenses that animals sequester from plants against predators. In particular, using the monarch’s butterfly as a model system the authors assess adaptation in cardenolide sequestration from different milkweed hostplants and whether cardenolide sequestration relaxes under diminishing avian predation. Using a cross-factorial experiment involving individuals of six monarch populations and their associated hostplants, they found substantial variation in sequestration ability but limited support for increased sequestration on sympatric hosts that will support the hypothesis of local adaptation in toxin uptake.  However, they report evidence for diminishing sequestration under reduced predation in distinctive populations, suggesting that this relaxation in toxin uptake is a  
genetically-based characteristic that can evolve in a few generations.  Based on these findings, they conclude that there is genetic variation in defense sequestration ability and that this ability can respond rapidly and adaptatively to changing species interactions.  
  
The reviewers and I enjoyed reading this paper, which blends successfully the description of some fascinating natural history with the assessment of intriguing and quite unexplored questions about the evolution of defense sequestration at the intraspecific level.  Reviewer 1 feels fascinated by the findings and praises the authors for their balanced conclusions. However, she/he requests clarification about the provenance of the collected seeds and the extent of spatial overlap between the sites of seed and monarch collection. Also, she/he questions whether a particular host plant that has been widely introduced in Eastern and Western North America can be considered allopatric. Reviewer 2 identifies several merits of this study, including the novel questions it poses and its experimental approach.  However, she/he requests the inclusion of a paragraph for the non-specialist, describing how the cardenolides were separated and identified.  Reviewer 3 also finds this to be a  
strong contribution that has important implications for our understanding of the evolution of defense sequestration.  However, she/he points out to several missing pieces of methodological information. She/he also requests clarification on data sharing between publications and further explanation of several important methodological aspects like the authors’ working definition of sequestration efficiency.  I find all these and the other several comments made by the reviewers highly pertinent, and suggest the authors to consider each of them in detail.  
  
Even though I share the reviewers’ enthusiasm about this article, I have two comments of my own.  The first is about data analysis.  In addition, to the comment on the random structure of the model made by Reviewer 1, I wonder why the authors did not consider “plant species” and “monarch population” as random factors.  After all, the selected milkweed species and monarch populations are samples of all existing milkweed species and monarch populations, respectively.  Therefore, estimations of variance components that can be attributed to these two main factors and the interaction between both can lead to more general conclusions about variation in toxin sequestration.  This could be complemented with partial-pooling estimations of means and SEs to depict reaction norms.  My second comment is that, despite being well-written and well-structured, I find the whole contribution too focused on monarchs and cardenolides with very limited cross-comparisons with other systems  
involving sequestration of secondary compounds or defense recycling, or even with the results of studies looking at specialization in plant-herbivore interactions. Also, I missed seeing some general implications of this research beyond the study system, particularly in the last paragraph of the Discussion. In the same line, in several parts of the manuscript (e.g., last sentence of the Abstract) cardenolide sequestration could be replaced by toxin sequestration, defense sequestration or sequestration of plant secondary compounds to widen the scope of this presentation. More generally and while avoiding entering into vague speculation, I would suggest the authors make an effort to allure non-specialists into the reading of this exciting contribution.

Data analysis:

While we agree with the general suggestion of assigning random intercepts for milkweed species and monarch populations, this approach leads to two issues within our dataset. The first issue is methodological: because of the relatively small number of milkweed species and monarch populations included in this study (n = 6 for both), a model that includes milkweed species, monarch population, and their interaction as random effects has issues with model convergence and singularity (this effect is even more pronounced because the model of sequestration only includes 4 of the 6 milkweed species). There is relatively little change in the estimated means of this fully random model compared to the model that treats them as fixed effects, although there is some evidence of shrinkage, whereby within-species differences in sequestration ability appear less pronounced between populations in a random effects-only model (see tables at the bottom of this response). The package that we used for our statistical inference (lme4) generates artificially small standard error estimates for each milkweed species x monarch population combination (hence the warning about singularity). This is fundamentally an issue of experimental design: we have too few factor levels to confidently estimate variation in the overall “populations” of milkweed species and monarch populations. We could potentially use a Bayesian framework in JAGS or STAN with informative priors to bypass some of these concerns, although intuitively it seems as though this may be an instance (balanced experimental design with a small number of factor levels but decent replication within levels) where the mode of inference is unlikely to affect our point estimates. To the extent that we do implement partial pooling, it is to deal with unequal sampling of milkweed populations within species (see Table S1 and Figure S7b).

The second issue, somewhat related to the first, is more philosophical. While it is true that the chosen milkweed species and monarch populations are samples from the “population” of all existing milkweed species and monarch populations, they are not random, *per se*. The definition of fixed versus random effects is of course somewhat fraught, but in our case, we deliberately chose milkweed species because of their unique prevalence and relevance as host plants throughout the monarch’s global range. In this way, they are not random selections from the broader pool of all milkweed species, but rather are biased selections because of their special importance as host plants. A related complication that we are unable to address due to a shortage of published data is that our model assumes milkweed species and monarch populations are independent, when in fact there is underlying phylogenetic signal (i.e. some milkweed species are more closely related to each other than others, and likewise for monarch populations).

In summary, a study design that included more milkweed species and more monarch populations would have been better-suited for making conclusions about the generality of local adaptation in systems involving toxin sequestration. In particular, it would have been useful to sample milkweed taxa with a greater emphasis on phylogenetic diversity. However, for logistical reasons, this would have been difficult (particularly in the case of increasing the number of monarch populations). Instead, we are left with treating each monarch population, milkweed species, and their interaction as their own discrete and idiosyncratic instances of an evolutionary process. We hope that this does not take away from the overall tenor of our findings, and we are willing to revisit the analysis again if you feel that it is warranted.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Model with random effects** | | | | |
|  | *G. physocarpus* | *A. curassavica* | *A. syriaca* | *A. speciosa* |
| Australia | 5.45 | 14.61 | 6.78 | 3.38 |
| California | 5.49 | 12.21 | 6.46 | 3.95 |
| Eastern N. America | 5.66 | 13.30 | 6.17 | 3.54 |
| Guam | 5.20 | 9.28 | 6.83 | 4.93 |
| Hawaii | 5.05 | 11.01 | 7.34 | 4.61 |
| Puerto Rico | 7.73 | 15.18 | 1.70 | 1.43 |

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Model with fixed effects** | | | | |
|  | *G. physocarpus* | *A. curassavica* | *A. syriaca* | *A. speciosa* |
| Australia | 5.24 | 14.61 | 7.16 | 3.02 |
| California | 4.82 | 12.57 | 5.25 | 4.66 |
| Eastern N. America | 5.35 | 13.08 | 6.20 | 1.91 |
| Guam | 4.72 | 8.89 | 6.62 | 3.62 |
| Hawaii | 4.99 | 11.01 | 7.37 | 4.95 |
| Puerto Rico | 7.69 | 14.96 | 1.06 | 0.90 |

Generality:

Thank you for raising this comment. We have updated the abstract, introduction, and discussion of the paper in an attempt to make our findings less organism-specific. In particular, the two opening paragraphs of the introduction now include numerous examples of toxin sequestration in non-monarch systems, and we attempt to make the point that our study is mainly novel in that it explores intraspecific genetic variation in the ability to sequester toxins. We also added a paragraph to the discussion that highlights a poison-dart frog system as an example of a species where, for various reasons, one might expect to find a stronger signature of local adaptation for toxin sequestration.

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Reviewer #1: Review of Manuscript #61026: Population-specific patterns of toxic sequestration in monarch butterflies from around the world   
  
This manuscript explores cardenolide sequestration in monarchs and environmental interactions that may influence sequestration. The authors use the worldwide distribution of monarchs as natural experiments, collecting monarchs from six different populations. Monarch individuals from these different populations were raised from eggs at the host institution under standardized conditions on the various hostplants in a factorial design. Cardenolide concentration as well as chemical profile was measured for the resulting adults. Additionally, the authors explored the potential for monarchs to adapt to reduce predation pressure by reducing cardenolide sequestration, by comparing monarchs from Guam to a nearby island. They find a significant GxE interaction, largely due to Puerto Rican monarchs. They do not find a significant effect in cross-host rearing. There is a difference in cardenolide sequestration between the two islands.    
  
Overall, this is very interesting and novel set of experiments that explores cardenolide sequestration in a new way and finds some very interesting results. I was fascinated by the findings and was also impressed with the conclusions the authors made with the data. It is often the case, in my opinion, that conclusions from natural populations that are influenced by a multitude of factors are vastly overstated. This was not at all the case here.    
  
I have two major comments/questions that need to be addressed to fully understand the implications of the study and the way in which the analysis was done.    
  
It is not clear where the plants came from and their genetic relatedness. The appendix states seeds were collected "from around the world" and that seeds were collected by fruit so that they were all from the same family. So, were all of one plant species collected in the same place from the same family, or was there genetic variation? Where exactly were seeds collected? In the same place as the monarchs? Line 163 indicates the authors collected ACUR from both Guam and Rota. Perhaps related, two of the models (Lines 226 and 241) specify "plant population" as a factor in the model only explained as "plant population of origin." Perhaps I am missing something, but when cardenolide concentration can vary in plants, I think this matters and should be better explained.

Thank for you raising this point. We agree that the provenance of the milkweed species used in this study is important to describe, and we have updated the methods section to direct readers to a newly added Table S1, which includes a full description of where all of the milkweed genotypes used in this study originated. We include a brief description here:

1. *Gomphocarpus physocarpus* was collected from both Hawaii and Australia in the same locations that monarch populations were collected. Hawaiian *G. physocarpus* consisted of seed collected from eight plants on Maui. Australian *G. physocarpus* consisted of seed collected from six plants, four of which came from Queensland and two of which came from New South Wales.
2. *Asclepias curassavica* was collected from the Mariana Islands and consisted of two pools of bulked seed (multiple plants) from Guam and from Rota. We also included seed from a single self-fertilized *A. curassavica* plant from Guam that set seed in the greenhouse in Davis in 2017. We did not include Puerto Rican genotypes of *A. curassavica*, though we still consider the species to be sympatric for Puerto Rican monarchs. Subsequent sampling (not presented here) has shown that *A. curassavica* genotypes from Guam and from Puerto Rico have similar cardenolide profiles, both in concentration and relative abundance of cardenolides; thus, the genotypes from Guam should be decent chemical approximations of the *A. curassavica* plants that Puerto Rican monarchs would encounter in the wild.
3. *Asclepias incarnata* was collected from a number of locations in Washtenaw County, Michigan and consisted of seed sourced from five plants. These locations are within the range of the eastern North American monarch population.
4. *Asclepias fascicularis* was sourced from a bulk seed collection from Hedgerow Farms in Yolo County, California. This bulk seed collection includes plants sourced from the Central Valley of California and overlaps the range of the western North American monarch population.
5. *Asclepias speciosa* was sourced from two locations: a commercial seed vendor (Seed Needs LLC) whose provenance information is unavailable and seed from plants in Yolo County, California. While provenance information is unavailable for commercially-sourced A. speciosa, we note that the majority of *A. speciosa*’s range is in western North America.
6. *Asclepias syriaca* was sourced from two locations: Washtenaw County, Michigan and Ithaca, New York. Seed from Michigan came from six plants; seed from New York came from a bulk collection made in 2012 and a single plant sampled in 2017. All *w* plants were sampled from within the range of the eastern monarch population.

Throughout the manuscript and in statistical models, each seed source is referred to as a “population” and treated as a proxy for plant provenance.

As you suggested, cardenolide concentration did vary between plant populations within species (though was much less pronounced than variation between species). We now report means and sample sizes for each plant population in Table S1. However, as shown in Figure S9 and the newly added Figure S10, we also note that cardenolide concentrations in butterfly wings were surprisingly independent of cardenolide concentrations in host plants.

In this cross-host study of sympatric and allopatric relationships, it is incredibly important to acknowledge that Ascelpias curassavica has been widely introduced in the Eastern North American and California populations over the last few decades. Therefore, I do not think these can be considered allopatric in the model, as I see they are in Figure 4a. I understand this study is focused on understanding evolutionary time scales, but there is still the potential for hundreds of generations that have been exposed to ACUR.

You are definitely correct that *Asclepias curassavica* has become widely cultivated and is encountered fairly regularly by monarchs from both eastern and western North America. Similarly, it is present in both Hawaii and Australia and could conceivably be considered sympatric for those populations as well. We chose to treat it as allopatric for two reasons:

1. Despite *A. curassavica*’s presence in these locations, its availability as a host is probably limited to particular areas (Gulf Coast, Florida, southeastern seaboard, coastal California) and particular times (primarily winter-breeding periods). These relatively limited spatial and temporal use patterns probably prevent it from being a major source of selection for sequestration behavior in the wild. Formally determining the prevalence of *A. curassavica* as a host would require cardenolide fingerprinting of wild-caught butterflies from these locations (e.g., Satterfield et al. 2018, Ecology Letters). In eastern North America, some estimates suggest that *A. syriaca* serves as the natal host plant for 85-90% of all overwintering butterflies (Seiber et al. 1986), suggesting it is the primary host used there in late summer.
2. As you mention, it is important to consider evolutionary time scales when considering monarch interactions with *A. curassavica*. This consideration is two-fold: *A. curassavica* has probably only been introduced to locations in eastern and western North America (with perhaps the exception of south Florida) in the past few decades, and so not that many generations of migratory monarchs have been exposed to it (perhaps hundreds, although maybe less when considering the limited spatial and temporal patterns of overlap). Secondly, there is an argument to be made that *A. curassavica* is perhaps the ancestral host for ALL monarch populations included in this study. Prior to the onset of widespread continent-scale migration within North America ~20,000 years ago, monarchs in North and Central America may have relied heavily on *A. curassavica* as a host. This point is somewhat speculative, but it has some support from studies showing that *A. curassavica* is always the favored host plant in oviposition preference experiments, and also supports among the highest growth rates of any milkweed species tested in cross-species comparisons. Thus, one could contend that *A. curassavica* could be considered sympatric (in a broader evolutionary sense, based on its likely historical prominence as a host) for all populations included in this study. For ease of interpretation, we chose to only consider populations whose present-day ranges support year-round reliance on *A. curassavica* as a host as sympatric.

Having said this, we did still test a statistical model that treated *A. curassavica* as sympatric with eastern and western North American monarch populations. This update did not meaningfully impact any of our inferences, and the magnitude of the sympatric/allopatric contrast did not change appreciably (original p value of sympatric/allopatric contrast = 0.687, updated p value of sympatric/allopatric contrast = 0.900).  
  
Here are some additional notes and suggestions on places where more information or clarification is needed for readers to understand and a few questions about experimental design:   
  
The abstract would benefit from a major revision to make it understandable to a broader audience. It is full of technical jargon and language. This is especially important given the intense interest in monarchs from scientists on all levels.

We have rewritten the abstract in an attempt to make it more understandable to a broader audience. Here is the text of the updated abstract:

“Animals frequently defend themselves against predators and parasites using toxins obtained from their diets. Monarch butterflies are a preeminent example of toxin sequestration and gain protection from cardenolides in their milkweed host plants. Although sequestration behavior is well-studied in monarchs, relatively little research has studied genetic variation in sequestration ability. In this study, we use the monarch’s global range expansion to test hypotheses about how cardenolide sequestration has evolved over recent evolutionary history. First, using a reciprocal rearing experiment involving six monarch populations and six associated milkweed host species, we test for whether natural selection has increased cardenolide sequestration in monarch populations reared on their sympatric hosts. Second, we test for whether contemporary species interactions affect sequestration by measuring cardenolides in monarchs from Guam, an oceanic island where bird predators have been functionally absent for approximately 40 years. We find evidence for substantial genetic variation in sequestration ability, although no consistent pattern of enhanced sequestration in sympatric monarch/milkweed combinations. One monarch population (from Puerto Rico) shows strong support for cross-hosts tradeoffs in sequestration ability, with elevated sequestration from two tropical milkweed species (*Asclepias curassavica*, *Gomphocarpus physocarpus*) but greatly reduced sequestration from two temperate species (*A. syriaca*, *A. speciosa*). Monarchs from Guam show some evidence for reduced cardenolide sequestration in both a cross-island comparison of wild-caught butterflies as well as a population-level comparisons of greenhouse-reared butterflies. Our results suggest that processes involved in toxin sequestration are subject to natural selection and may evolve in response to contemporary changes in species interactions.”

While we acknowledge that there may still be some technical jargon (e.g. the words “sequestration” and “sympatric“), we feel that these concepts are central to the economy of the manuscript and cannot be avoided or thoroughly explained in a 250 word abstract.

Sentence starting Line 35: I would recommend offering a bit more detail here. I realize the focus is on cardenolide sequestration, not host plant specification, but it is an underlying concept for the whole paper. Either that, or I would remove it.

We ended up removing this sentence altogether, as we agree that it would require additional explanation and space to make the connection between host plant specialization and sequestration more clear.   
  
Paragraph starting Line 151: The authors should include a line and citations explaining the major predators of monarchs.

As suggested, we have added the following text. We added it to the first section of the methods, now entitled “Study system and natural history.”

“Monarch butterflies are subject to predation throughout their lifetime: major larval predators/parasites include Tachinid flies (Oberhauser et al. 2017), Polistine wasps (Baker and Potter 2020), ants (Calvert 2004), and various opportunistic generalists including earwigs (Hermann et al. 2019). Adults are thought to be primarily attacked by birds (Calvert et al. 1979, Brower 1988, Groen and Whiteman 2021), although mice are likely also a major source of predation, especially at overwintering locations (Glendinning and Brower 1990, Weinstein and Dearing 2021).”  
  
Model 2, Line 241: I believe the factor (1|maternal.family) would be nested within mon.pop since a family could not be from multiple populations.

Thank you for bringing this point up. You are definitely correct that the term for maternal family should be treated as a nested term within monarch population, since each family belongs to only one population. The model that we used in lme4, as written, detects the nested structure of the underlying variance/covariance matrix and treats a model of the form A + (1 | B) as equivalent to A + (1 | A/B). This can be seen in the fact that the random intercept estimates for maternal family are not only centered around an overall mean of 0, but also are centered around 0 within each monarch population. We have updated Figure S7a to show this pattern more clearly (with random intercepts shown separately for each monarch population). We note that the same pattern applies for milkweed populations: they are nested within milkweed species and are modeled similarly (see Figure S7b)  
  
Lines 289 to 297: This is a really interesting result!

We agree! Subsequent research involving similar comparisons but with slightly modified rearing and chromatographic techniques found slightly less pronounced differences between Puerto Rican and North American monarchs reared on *A. syriaca* (approximately a 4-fold difference in total concentrations, compared to the 5.5-fold difference reported here), and we are currently following up on this result to try to pinpoint the genetic basis of this difference.  
  
Section starting Line 299: Did the authors consider looking at larval mass on day 8 (which they collected) as a potential factor in cardenolide sequestration? There are factors other than cardenolides between milkweed species that influence larval feeding pattern (both positively and negatively) that may not be reflected in days to eclosion. The mass could reflect the amount of plant consumed, which would reflect the amount of cardenolides they were exposed to.

Yes, we did consider including larval mass on day 8 in analyses of sequestration. However, this approach is complicated by the fact that we did not keep track of the identity of individual larvae. Thus, although we can match each individual butterfly to the individual host plant on which it was reared, we cannot relate its levels of sequestered cardenolides to its specific mass on day 8. This is why we instead chose to use the number of days that elapsed between hatching and adult eclosion, which provides a measure of development time that is highly correlated with larval mass. We have updated our methods section to reflect this. It would have definitely been preferable to be able to match larval mass on day 8 to levels of sequestered cardenolides (as done in Agrawal et al. 2021, PNAS) to see whether processes involved in sequestration impose a physiological burden that slows development time.  
  
Clause starting line 311 to 314: This higher concentration on Guam is very interesting! Is this amount of variation normal between populations? Did the authors see this type of variation between other plant populations (see Major question #1 above)?

Yes, we agree that this result is interesting. Research by Agrawal et al. (2021) suggests that *A. curassavica* genotypes collected around the world are highly variable in their production of cardenolides, with 8-fold variation in total cardenolide concentrations between individual plants. So, it is perhaps not surprising to find differences of this magnitude (~30%) between *A. curassavica* plants from Guam and Rota, and we do not necessarily feel that the differences in *A. curassavica* chemistry between Guam and Rota are attributable to natural selection by monarchs. We also note that these differences were measured in field-collected plants that grew under different environmental conditions; when we only include greenhouse-grown *A. curassavica* from Guam and Rota, there is no statistical difference between them in cardenolide production (see population-level means presented Table S1).  
  
Line 405: The authors state (in revision) evidence for monarchs between Guam and Rota being genetically distinct. Do they happen to know if the plants are genetically distinct? I assume if monarchs are not moving between islands that milkweeds are not either, but if there is any information out there, it would be helpful.

Unfortunately we do not have any data about genetic differentiation between *A. curassavica* plants (more broadly, we are not aware of any population genetic studies involving *A. curassavica*). We agree with your general contention that there is likely some degree of divergence between these plant populations, although as noted before, the differences in cardenolide concentrations measured in field plants were no longer evident when each population was grown under common garden conditions.  
  
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Reviewer #2:   
  
The authors compare widely separated disjunct populations of monarch butterflies with different evolutionary histories for their ability to sequester cardiac glycosides from different species of milkweed.  The classic monarch-milkweed system has received a lot of study and parallel adaptations to cardenolides in other species has recently added a new dimension to the topic.  The new dimension added by this manuscript is intra-specific evolution in sequestration ability by the monarchs, using common-garden experiments of a sort as well as exploiting geographic variation in milkweed host availability and predation pressure.  This new dimension merits publication in the American Naturalist.  
  
The experimental design is powerful, the chemical and statistical analysis is appropriate, the writing is exceptionally clear, and the conclusions are justified by the data presented.  I can find very little to criticize except for the following point:  
  
In the Methods section, the authors should add a short paragraph describing how the cardenolides are separated and identified, that is accessible by evolutionary biologists with no analytical experience.  Citing the name of the column gives no insight into the physical principles that the authors, as experts, take for granted.  "A library of cardenolide peaks" has no meaning to the non-specialist.  The authors should find two or three colleagues with no analytical experience and no knowledge of the cardenolide system, ask them to read the paragraph, and then ask them to explain how cardenolides are separated and identified without referring to the paragraph.  Repeat the process until convinced that the message is getting through.

We have followed your suggestion and updated the methods section to the following text. Please let us know if you feel that this section would still be unclear to a non-specialist:

“We used ultra-performance liquid chromatography (UPLC) to separate cardenolides based on their polarity; compounds with early retention times are more polar than those that elute later (see Figure 2A for an example of a chromatogram generated from wing tissue). Peaks with absorbance spectra between 216-222 nm were considered to be cardenolides, in accordance with previously published methods (Malcolm and Zalucki 1996, Zehnder and Hunter 2007). Across all samples, we identified 70 distinct peaks, each of which should correspond to a unique cardenolide compound. We note that some of these peaks are likely constituent fragments of larger, more intact cardenolides; for example, the widespread cardenolides calotropin, calotoxin, calactin, and uscharin share a common aglycone precursor (calotropogenin). In order to verify the identity of some major sequestered compounds, we tested authentic standards for the compounds calactin, calotropin, and frugoside—reported to be the three major compounds sequestered from *A. curassavica* (Agrawal et al. 2021)—as well as aspecioside, reported to be a major sequestered compound from *A. syriaca* (Seiber et al. 1986; Malcolm et al. 1989) (Table S3). Authentic standards were provided by A. Agrawal and are the same as those used in Agrawal et al. (2021).

Cardenolide peak areas were integrated using Chromeleon™ software (Thermo-Fisher). Each sample was prepared with an internal standard (digitoxin) added at a known concentration, which allowed for quantification of total cardenolide concentrations. Total cardenolide concentrations (expressed in mg of cardenolide per g of dry tissue) were calculated by summing across all peak areas, dividing by the peak area for digitoxin (0.15 mg/mL), dividing by 0.8 to account for the fraction of cardenolide extract saved after centrifugation, and dividing by the corresponding dry tissue mass in grams. We note that we did not record voruscharin from leaf tissue of either *A. curassavica* or *G. physocarpus* (see Agrawal et al. 2021) because of its very late retention time and potential degradation under our storage conditions, although we did record its sequestered derivatives calactin and calotropin in monarch wings.”  
  
line 442, of course the links to Dryad need to appear in the published version.

All of our data and scripts have been uploaded to Dryad and are available at the following link: https://datadryad.org/stash/dataset/doi:10.25338/B8TD1F.  
  
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Reviewer #3:   
  
This paper was a delight to read; it is well-written and addresses several very interesting questions.  While this manuscript is focused on monarch butterflies and their host plants, it also provides some potential insight into the evolution of sequestration in other taxa.  
  
While this is a strong manuscript, I was frustrated by the large amount of data that were in appendices and that a lot of pertinent information was in another paper.  While I understand that there are many reasons for this, it did make reading the paper more difficult.  In addition, there are some methodological issues that need to be dealt with (see below).

We apologize for all of the information included in appendices and in a previously published paper. We have updated our supplemental materials to include relevant details that were reported in Freedman et al. (2020 – Evolution), and if you feel that it is warranted, we can also update the main figures and tables of the manuscript to include the information that you feel is most necessary for understanding the paper.  
  
Strengths of the manuscript:  
  
1)  Well and clearly written.  
2)  Addresses several very interesting questions with innovative methodology (e.g., monarchs on Guam where there are no bird predators compared to island where there are birds).  
3)  Adds to our understanding of monarch biology, but also has important implications for other sequestering species.  
  
Questions and concerns:  
  
General:  it looks like the data in this manuscript are the same as those in the Freedman et al. (2020) Evolution paper.  Clearly the questions being addressed are different, but if the same data set is used, that should be explicitly stated.

Yes, the data in this manuscript are the result of the same experiment described in Freedman et al. (2020), although that paper did not include information on cardenolide sequestration. We have updated the methods section of this paper to more clearly reflect that the underlying experiment is the same as this previous paper.  
  
p. 6, Lines 103 and following:  Given the large amount of data in this manuscript an the fact that there are several different questions being addressed, it would be useful to number the specific questions and then structure the methods and results around these different questions.  From this paragraph, there are 3 basic questions and organizing this paragraph and the methods and results specifically around these 3 questions would be helpful.

As suggested, we have updated the methods section so that it is split into two major subsections: Approach 1 (Testing for GxE interactions and local adaptation in sequestration behavior) and Approach 2 (How does loss of bird predation affect cardenolide sequestration?). We also added these two headings to the results section as well as a subsection entitled “Overall patterns of variation in milkweed and monarch cardenolides.”  
  
p. 9, L 179:  explain why you chose all peaks with absorbance spectra between 216 and 222 nm.

This corresponds to the region of maximum absorbance for cardenolides, where interference from non-cardenolide compounds should be lowest. This seems to be the standard used across multiple previously published papers (e.g. Zalucki and Malcolm 1996, Zehnder and Hunter 2007). We have updated our method to reflect this.  
  
p. 9, L. 188 and 190:  where were authentic standards obtained?  Please state.

All authentic standards were the same as those described in Agrawal et al. (2021, PNAS) and were provided by the lead author of that paper. Those compounds were isolated from pooled samples of caterpillars reared on either *A. curassavica* or *A. syriaca* and then isolated using column chromatography. Digitoxin was obtained from Sigma-Aldrich. We have updated our methods to reflect this.  
  
p. 10, L. 206-208:  I was unclear about how the sequestration ratio was determined; were individual sequestration ratios calculated for monarchs reared on specific host plants, thus there was a specific host plant paired with a specific butterfly?  That does not appear to be the case. Given that individual host plants can vary substantially in their cardenolide content, it seems that these sequestration ratios would have to be calculated based on specific host plant-butterfly pairs.  It was unclear to me how these were calculated.  Please more clearly explain.

Apologies for the confusion. We have updated the text to hopefully make this point more clear.

Ideally calculating sequestration ratios would involve paired measurements of cardenolide concentration from individual butterflies and the individual host plant on which they developed. However, because we only collected tissue samples from a relatively small subset of milkweed plants, we were unable to generate a sequestration ratio using this 1:1 butterfly:plant correspondence. Instead, the ratios simply reflect overall averages (i.e. global mean butterfly concentration divided by global mean plant concentration for each milkweed species). As you note, there is substantial variation among plant genotypes in their cardenolide production, and this approach ignores that variation. However, because we assigned caterpillars to plants randomly and used a nearly completely balanced rearing design, these sequestration ratios should still provide a good approximation of what would be observed using the mean of many individual butterfly/plant pairings. Reviewer 1 also noted the potential for variation in cardenolide concentration within milkweed species to affect sequestration. In response, we added Supplemental Table S1, which shows variation in plant cardenolide concentration as a function of population of origin. We also note Figure S9 and the newly added Figure S10, which shows that there is a surprisingly weak correspondence within milkweed species between plant and butterfly cardenolide concentrations.  
  
p. 12, L. 249-253:  The definition of sequestration efficiency given in this manuscript is different from that in Tao and Hunter (2015) to which the manuscript refers.  Tao and Hunter define this as "the proportion of ingested defense chemical that is retained by the herbivore", not as cardenolide concentration divided by developmental rate.  Please elaborate on your method for calculating sequestration efficiency and explain how your methods relates to that of Tao and Hunter.  It's not clear that the method used here is really an appropriate measure of sequestration efficiency.

Thank for you bringing up this point. We agree that our original phrasing was unclear, and we have removed all mentions of the term “sequestration efficiency” from our methods. In the narrow sense (as first used by Roeske et al. 1976), measuring sequestration efficiency requires measuring the cardenolide content of inputs (leaves), the quantity of leaf tissue consumed, the cardenolide content of outputs (frass) and the quantity of frass produced, and the amount of cardenolide stored in larval tissue. Instead, we only measured adult butterfly cardenolide content, which integrates over the entire larval and pupal developmental window and does not directly account for variation in cardenolide input or output. We have updated our methods section with the following paragraph to reflect this fact:

“We note that all of our analyses use total cardenolide concentrations in adult hindwings as our response variable. This approach involves a number of important assumptions. First, when testing for local adaptation, the implicit assumption is that natural selection favors higher cardenolide concentrations in sympatric combinations. Second, by using total cardenolide concentration in adults, we are integrating over the entire larval and pupal developmental process and not explicitly considering factors such as the time required to complete development or the quantity of milkweed tissue consumed during development. Thus, our measure does not necessarily capture sequestration efficiency *per se*, which typically is expressed in units of sequestered cardenolide per unit of plant material ingested (e.g. Roeske et al. 1976, Tao and Hunter 2015). However, we did conduct an analysis that accounted for monarch development time, by dividing butterfly cardenolide concentrations by the number of days from egg hatching to adult eclosion and using this measure as our response variable; this analysis accounts for the fact that a longer larval development window affords more time to process and sequester milkweed cardenolides.”

p. 12, L. 254:  better to rephrase:  "For monarch and plant samples collected in the field…"

We agree and have updated this phrasing.  
  
p. 15, L. 311 and following:  I am a little concerned that the use of the mean of 31% higher concentrations of cardenolides on Guam to modify cardenolide concentrations and then adjust sequestration is not appropriate, especially given the large standard errors associated with the mean plant cardenolide concentrations.

Yes, we agree that this is an important caveat in interpretation and that using “adjusted” cardenolide concentrations that take into account plant concentrations on Rota vs. Guam is not an ideal comparison. For this reason, we tried to be circumspect in our discussion: we refer to the evidence for reduced sequestration on Guam as “modest” and also reiterate that sequestration differences in wild-caught monarchs are only statistically significant after accounting for differences in *A. curassavica* cardenolide concentrations across islands. As per the suggestion of the head editor, we have also modified this paragraph of the discussion to further highlight the limits to inference that are inherent in using a single bird-free island. In general, we feel that the reduced cardenolide concentrations seen in common garden-reared monarchs from Guam on *A. curassavica* provides much stronger evidence for genetically-based reductions in cardenolide sequestration from this island. We hope that our discussion of these limitations is sufficiently circumspect as written.   
  
Figure 1:  should probably be A and B instead of top and bottom (for consistency).

This has been updated.

Figure 4B:  I didn't see any faint lines in the background, which are referred to in the figure legend.

Apologies, this seems to have arisen when the manuscript was converted to PDF form in the submission portal. We have updated this figure so that the faint lines should now be visible.  
  
Figure S4:  need to say that PR is orange, since say Australia is cyan.  Also, need to italicize A. syriaca.

This has been updated.  
  
  
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General Instructions  
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