Dear Dr. Sperling,

We thank you and the reviewers for your time and feedback and for the opportunity to revise our manuscript. We have carefully considered all comments and recommendations.

Based on your guidance, we have:

• published the dataset containing the raw extracted compounds on Zenodo (https://doi.org/10.5281/zenodo.6497483)

• explained the method used for hydrocarbon extraction and analyses in more detail (lines 131-146)

• revised the Figure 2 as a boxplot

• revised the figure labels and tables

• added a paragraph about cuticular hydrocarbon biosynthesis (lines 222-228)

• added explanations of how environment, diet, micro-habitat and predation could affect the cuticular hydrocarbon signature in our system (lines 284-332)

Below please find the detailed responses to the comments of the reviewers.

We feel that these revisions have improved the paper and hope that you will agree and now deem it worthy of publication.

Best wishes,

Marlène Dupraz

On behalf of all authors

Within and among population differences in cuticular hydrocarbons in the seabird tick Ixodes uriae

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https://doi.org/10.1101/2022.01.21.477272
Submitted by Marlene Dupraz 08 Feb 2022 13:00

Abstract

The hydrophobic layer of the arthropod cuticle acts to maintain water balance, but can also serve to transmit chemical signals via cuticular hydrocarbons (CHC), essential mediators of insect behavior. CHC signatures typically vary qualitatively among species, but also quantitatively among populations within a species, and have been used as taxonomic tools to differentiate species or populations in a variety of taxa. Most work in this area to date has focused on insects, with little known for other arthropod classes such as ticks. The worldwide distribution and extensive host-range of the seabird tick *Ixodes uriae* make it a good model to study the factors influencing CHC composition. Genetically differentiated host-races of *I. uriae* have evolved across the distribution of this species, but the factors promoting sympatric population divergence are still unknown. To test for a potential role of host-associated CHC in population isolation, we collected *I. uriae* specimens from two of its seabird hosts, the Atlantic puffin (*Fratercula arctica*) and the common guillemot (*Uria aalge*) in different colonies in Iceland. Using gas-chromatography and mass-spectrometry, we detected a complex cuticular mixture of 22 hydrocarbons, including n-alkanes, methyl-alkanes and alkenes ranging from 17 to 33 carbons in length. We found that each population had a distinct CHC profile, with long-chain hydrocarbons tending to be more abundant in puffin tick populations. As profiles also varied between host-associated groups, future work will now be required to tests whether the different CHC signals may reinforce assortative mating patterns, and thus *I. uriae* population divergence.

Keywords: Host race formation; GC-MS; colonial seabirds; Ixodidae; environmental variation

Round #1

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by Felix Sperling, 27 Mar 2022 21:26
Manuscript: **https://doi.org/10.1101/2022.01.21.477272**

Dear Marlène Dupraz and coauthors,

Two reviews of your manuscript have now been received, and I agree with both of them that this is an interesting and generally well written study. However, they raise some key issues and edits that should be addressed before publication can be considered. In particular, Reviewer 1 recommends the addition of further information on the methods as well as clarification of several items and explicit consideration of factors such as mating status and microclimate. Reviewer 2 suggests that the authors tie in hydrocarbon synthesis pathways, and include more explicit discussion of how environmental factors may alter the tick hydrocarbon profile. All points raised by the reviewers should be addressed, whether in a revised version of the ms or in rebuttal.

In addition, from my own review of the ms: 1) although this manuscript does a good job of considering the role CHCs may play in reproduction, the discussion seems a bit biased toward the hypothesized involvement of these hydrocarbons in host race formation. Other functions should be considered more fully. For example, Yoder and Domingus (2003) demonstrated that long chain hydrocarbons secreted by Dermacentor variabilis ticks act as a defence against ant predation.

We agree with this remark. A paragraph explaining the role of CHCs in the protection against predators was added (lines 325-332): "Fourth, CHCs can be altered by infection with different micro-organisms. For example, both the corn borer (*Ostrinia nubilalis*) and the common cockchafer (*Melolontha melolontha*) show altered CHC profiles when infected by fungi of the genus Beauveria (Lecuona et al, 1990). Moreover, Yoder and Domingus (2003) demonstrated that production of the CHCs n-C20 and n-C24 by *Dermacentor variabilis* ticks could protect against predatory behavior by ants. The abundances of n-C20, n-C21, n-C23 and n-C24 in the puffin tick pools of the present study may represent different infection statuses and/or that puffin burrows provide a less protective environment against predators, such as ants or spiders, than stones in common guillemot colonies."

Also, 2) this statement needs to be clarified: "For example, aging favors the production of longer hydrocarbon chains and decreased attractiveness in Drosophila melanogaster (Kuo et al., 2012)." It is not fully clear what kind of attractiveness is intended here.

Precision about the kind of attractiveness was added (lines 275-277): "For example, aging favors the production of longer hydrocarbon chains and decreased sexual attractiveness in *Drosophila melanogaster* (Kuo et al., 2012)."

Consequently, this paper still requires revision before it could be recommended. However when the methods and other items listed in the reviews have been clarified, and the discussion has been expanded to include the factors outlined above, this paper should be a valuable addition to the literature on the role of tick cuticular hydrocarbons.

- Felix Sperling

Reviews

Reviewed by anonymous reviewer, 16 Mar 2022 18:13

The manuscript by Dupraz et al. outlines and interesting study comparing CHC profiles from among different host and geopgraphic populations of Ixodes uriae ticks. Generally speaking this is a well written study, has been conducted using established techniques and has interesting results. That being said, I have outlined a number of key issues and edits in the manuscript which should be addressed before consideration for publication. Some of the more important of these include:

1. There are key pieces of information in the methods which have been left out of the text and need to be added. Its also unclear why the authors have not quantified their materials using a standard curve or internal standard, and are instead relying on ratios of abnundance?

The use of standard curve or internal standard is necessary when one aims at quantifying the amount of compound (e.g. in ng) extracted from the cuticle. We were not particularly interested

in quantifying the absolute amount of CHCs, we were interested in comparing the relative proportion of CHCs among samples. We have clarified this (line 127-129): ". We did not use an internal standard because the aim of our analysis was not to quantify the absolute amount of CHCs, but to compare the relative proportions of CHCs among samples."

2. I am concerned about the evaporation and reconstitution method used on the samples. This method will introduce a significant amount of variation in the recovery of solutes in re-adding the solvent. The lack of an internal standard in the samples unfortunately makes it impossible to know what lost in this process.

We added a precise amount of solvent after evaporation to help reduce the variation introduced during processing. A sentence has been added to better explain the evaporation process (lines 131-133): "Cuticular hydrocarbons are not very volatile, but to reduce variation during the evaporation process, all samples were treated in the same way, placed under the same fume hood in a temperature controlled room."

3. Several of the figure descriptions use acronyms and abbreviations which have not been explained.

Descriptions of acronyms and abbreviations have been added on figures n°1, 2, 4 and 5 and in the text (lines 92-94).

4. The amount of variation in the data is discussed, but Figure 2 does not provide a good look at the degree of variation present. I suggest that this figure should be reconfigured as a boxplot, including the individual data points, as well as outliers. I am concerned that the limited replicates used for these samples may be masking other trends that may be present.

As requested, Figure 2 was reconfigured as a boxplot and a sentence was added to the text (lines 141-142): "The abundance of each detected cuticular hydrocarbon was used to build a box plot using the ggplot 2 package with R software (version 4.1.1)."

5. In the discussion, there are a number of 'suggestions' made by the authors regarding pheromone-based function of these CHC's largely based upon insect literature which has documented such function in other species. In particular, the point is made that the data suggests that these CHCs are important for reproductive function, as these were collected from ticks during the reproductive season. However, the authors collected wild ticks, with no knowledge of mating status. Therefore its difficult to make any strong assumption regarding the behavioral role of these CHCs.

We agree with the reviewer and have modified the sentence about the role of CHC in tick reproduction (lines 244-248): "However, as mating in this tick species frequently takes place prior to feeding on the host, and in particular when nymphal ticks emerge as flat females (McCoy and Tirard, 2002), we cannot conclude on the mating status of collected females. Indeed, some of the profile variation in our data may be due to the inclusion of females in different reproductive states. Experimental studies will now be necessary to determine the potential role of the observed CHC compounds in mating behavior."

6. While the data support population based differences in CHC profiles, and the authors discuss the possible impact of different habitat and microclimatic differences, theres very little

description regarding the discrete differences in environmental variables between these sites (which at least appear to be very similar in geographic distribution).

Details about the discrete differences in micro-habitats and how they could explain cuticular hydrocarbons profiles were added (lines 295-304): “By positioning temperature and humidity captors during one year in the off-host environment in a heterospecific seabird breeding colony in northern Norway (70°22’ N, 31°10’ E), we observed that the average temperature and relative humidity (HR) ranged respectively from -7.5 to 18°C and from 0 to 110% HR in breeding sites of Common guillemots, and from -7.5 to 10°C and from 80 to 105% HR in those occupied by puffins (data not shown). The micro-habitat used by CG ticks may therefore be more exposed to temperature and humidity variation than the more stable deep burrows used by PF ticks. However, the presence and abundance of saturated CHCs, such as 9meC24 and 2meC24, were not more frequent in CG samples compared to PF samples. More detailed environmental data from each location and more sampled locations are therefore necessary to more fully evaluate this hypothesis.

This manuscript requires revisions addressing the points above and those outlined in the manuscript file before being considered for publication.

Download the review

Reviewed by anonymous reviewer, 18 Mar 2022 00:09

This manuscript is a valuable addition to the literature on tick cuticular hydrocarbons. The experiments attempt to determine the effects of host species and geography on the cuticular hydrocarbons of *Ixodes uriae.* There is great potential in this tick-seabird system to learn about factors that enhance population divergence.

The discussion would benefit from a brief account of what is known about hydrocarbon synthesis in insects (e.g. fig. 1 in Howard & Blomquist, 2005).

A description of cuticular hydrocarbon biosynthesis was added at the beginning of the discussion section (lines 222-228): “Cuticular hydrocarbons, including linear and methyl-branched alkanes and alkenes, are biosynthesized from an acetyl-CoA molecule in specialized secretory cells, the oenocytes which are mainly located in the epidermis of insects (Howard and Blomquist, 2005). They are shuttled through the hemolymph to the epicuticular surface via specialized pore canals penetrating the cuticular layers (Holze et al, 2021). Once fixed on the arthropod cuticle within a complex mixture of alcohols, esters, aldehydes, fatty acids, etc, hydrocarbons help prevent desiccation and serve in chemical communication, constituting essential mediators of insect behavior (Blomquist and Bagnères, 2010).”

Avian erythrocyte membrane lipids might be quickly mobilized by the tick for conversion to hydrocarbons, and the very long chain hydrocarbons are probably synthesized from pre-existing shorter-chain fatty acids (the *I. scapularis* genome has genes coding for proteins with acyl chain elongase-like sequences). Thus, the host erythrocyte lipid composition might provide a direct pathway for the host to influence the hydrocarbon composition of the tick cuticle. Future studies could benefit from collecting ticks and simultaneously obtaining blood samples of birds likely to have been hosts. It would be interesting to look for correlations between bird erythrocyte membrane lipid acyl chain composition and tick cuticular hydrocarbon composition.

The future analysis could also be expanded to include polar cuticular lipids, such as fatty acids and steroids, which have been reported as pheromones in metastriate ticks (J Chem Ecol 11, 1669-1694, 1985; Parasitology 129 Suppl, S405-425, 2004).

We thank the reviewer for this interesting insight. A paragraph explaining the potential role of the host in the acquisition of tick cuticular hydrocarbon has been added (lines 315-324) : "In our system, avian erythrocyte membrane lipids might provide a direct pathway for the tick to synthesize cuticular hydrocarbons from pre-existing shorter-chain fatty acids and could result in the acquisition of specific hydrocarbon mixtures. This type of acquisition could explain both the variation among ticks from different host species, and variation among colony locations, if seabird diets shift among locations. Futures studies could therefore be expanded to look for correlations between avian erythrocyte membrane lipid composition and tick cuticular signatures by comparing ticks and host blood samples. In addition, looking at patterns in tick cuticular lipids, such as fatty acids and steroids, which have been reported as pheromones in metastriate ticks (Sonenshine, 2004; Sonenshine et al, 1985), would also be of particular interest."

The manuscript does not present a clear argument for how environmental acquisition would alter the tick cuticular hydrocarbon profile.

We removed this paragraph and add a sentence about the environmental acquisition of hydrocarbons in relation to aggregation and recognition signals (lines 253-256): "Studies have also highlighted the importance of the environment in the acquisition of new hydrocarbons (d'Ettorre et al., 2006; d'Ettorre et al., 2002), acting for example as a recognition signal of their own nest for the social wasp Polistes metricus Say (1981) (Singer and Espelie, 1996; Espelie et al., 1990)."

I do not have expertise in the data analysis methods used by the authors, and I suggest that they post the raw GC-MS areas on the Zenodo site.

A dataset containing the raw extracted peak areas for each compound was published on Zenodo (https://doi.org/10.5281/zenodo.6497483) and this information has been added to the manuscript (line 138-141): "Compounds were then identified (raw data available on Zenodo at https://doi.org/10.5281/zenodo.6497483) and sorted on the basis of their mass spectra and retention time by comparison with standards (alkane standard solutions, Sigma-Aldrich, Saint Louis, MO, USA) and published spectra (NIST Library)."

There is a minor typo on line 365: the authors' names in the reference are given twice.

This repetition was removed from the reference. Thank you.