

# Dietary and Plasmodium challenge effects on cuticular hydrocarbons in *Anopheles albimanus*

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## Research Article

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## Abstract

The cuticular hydrocarbon (CHC) profile reflects the insect's physiological states. These states include age, sex, reproductive stage, or gravidity. Environmental factors such as diet, relative humidity, or exposure to insecticides also affect the CHCs composition in mosquitoes. In this work, the CHC profile was analyzed in two *Anopheles albimanus* phenotypes with different degrees of susceptibility to *Plasmodium*: the susceptible-White and resistant-Brown phenotypes. The effects of the CHC profile were considered under a carbon-rich diet (sugar), a protein-rich diet (blood), and an infectious challenge (blood containing *Plasmodium berghei* ookinetes). The CHCs were analyzed by gas chromatography coupled with either mass spectrometry or flame ionization detection, identifying 19 CHCs with chain lengths ranging from 20 to 37 carbons. The qualitative and quantitative changes observed in the CHCs composition were dependent on the diet and parasite challenge, and independent of the phenotype. The exception was the challenged condition, where significant differences between the phenotypes were observed in Z-12 pentacosane, hexacosane 9-octyl, methyl-nonacosane, and methyl-hentriacontane. Since the lipid metabolism in *Anopheles* mosquitoes has been shown crucial for *Plasmodium* development, the changes in the CHC profiles associated with infection could have multiple effects on mosquito fitness and impacts on disease transmission.

## Introduction

The cuticle is the most extensive extracellular structure covering the insect's external surfaces, protecting them against the environment. It is mainly composed of chitin, proteins, lipids, waxes, and cement. The cuticle covers all the body parts exposed to the external environment, including cavities, the foregut and hindgut coatings, the luminal side of the trachea and tracheoles, as well as the wings. Therefore, the cuticle is the interface between the external milieu and the internal milieu of the insect; on one hand, it is the point of direct contact with the potentially hostile or challenging exterior, and on the other hand, the cuticle rests on a monolayer of epidermal cells that extends throughout the body of the mosquito, which in turn, is in close contact with the hemolymph through a thin basal lamina. The cuticle is formed as a product of: (i) the active secretion of the underlying epidermal cells (reviewed in, 1<sup>1</sup>), (ii) the prominent secretion of oenocytes located within or under the integument<sup>2</sup>, and (iii) the arrival of nutrients, principally derived from the fat body, through the hemolymph (reviewed in, 3, 4)<sup>3,4</sup>.

The cuticle composition is dynamic and changes depending on the age, developmental stage, and metabolic status of the insect (reviewed in, 1)<sup>1,2</sup>, exhibiting its essential function in the adaptation capacity and survival of the insect to changing environmental factors (reviewed in,4-6)<sup>4-6</sup>. Thus, the cuticle's composition is, in a way, the reflex of the insect physiological state. As a multifunctional structure, the cuticle participates in locomotion, desiccation resistance (reviewed in,3)<sup>3</sup>, defense against pathogens and toxins<sup>7,8</sup>, insecticide resistance (reviewed in, 4)<sup>4</sup>, and other mechanical and chemical insults.

The cuticle is comprised of multiple layers with different compositions and properties: (1) endocuticle, (2) exocuticle, (3) cuticuline, (4) cerosa, and (5) cement (reviewed in, 9)<sup>9</sup>. The endocuticle and exocuticle form in conjunction a region commonly named procuticle, which represents the major portion of the total cuticle and is composed mainly of chitin, proteins, lipids, salts, and pigments. The cuticuline, the cerosa, and the cement comprise the most external layer, rich in extractable lipids and known as epicuticle, which consists of a complex mixture of hydrocarbons, fatty acids, and waxes (reviewed in, 7, 10)<sup>7,10</sup>. In most insects, the cuticular hydrocarbons (CHCs) include n-alkanes, methyl-branched alkanes, and alkenes. The CHCs studied until recently have shown functions related to desiccation resistance, as mating signals, species and gender recognition cues, nestmate recognition, dominance and fertility cues, chemical mimicry, and for preventing infections (reviewed in, 3-5, 7, 10)<sup>3-5,7,10,11</sup>.

In *Anopheles* mosquitoes, the CHCs have been widely studied as a way for identifying species such as *An. gambiae*<sup>2,12-14</sup>, *An. culicifacies*<sup>15</sup>, *An. maculipennis*<sup>16</sup>, *An. maculatus*<sup>17</sup>, *An. darlingi*<sup>18</sup>, *An. stephensi*<sup>19</sup>, *An. quadrimaculatus*<sup>20</sup>, *An. claviger*, among others<sup>21</sup>. The analysis of the CHCs composition in anopheline mosquitoes has been made mainly from cuticular extracts with n-hexane and analyzed by GC-MS coupled with multivariate analysis (reviewed in, 10)<sup>10</sup>. This way, it has been confirmed that the principal CHCs in mosquitoes are n-alkanes with chain lengths ranging from 15 to 47 carbons (C15-C47), followed by monoinsaturated and saturated free fatty acids, and, to a lesser extent, alkenes<sup>14,15,17,20,22-24</sup>. The main reported function for the n-alkanes is the control of transcuticular water movement, while the unsaturated compounds and the methyl-branched CHCs, are more likely to be involved in communication (reviewed in, 3)<sup>3</sup>.

A notorious aspect of the characterization of CHCs in anopheline mosquitoes is that it is not only possible to distinguish between sibling species from different species complexes and karyotypes<sup>12,13,15,18,19,21,25,26</sup>, but also to determine age, sex, and reproductive status (reviewed in, 1, 5)<sup>1,5,14,22,23,27</sup>, the determination of vector or non-vector species<sup>17,21</sup>, and insecticide-resistant phenotypes (reviewed in, 4)<sup>4,24,28,29</sup>. However, despite being vectors of the parasite that causes malaria, little is known about the CHCs content or the cuticle's modifications during *Plasmodium* infection.

Female mosquitoes must feed on blood to initiate ovary development and lay eggs. However, this point is when the malaria parasites are transmitted from host to vector and vice versa. When a mosquito feeds on blood containing gametocytes, they mature into gametes and fertilization occurs. The resulting zygotes differentiate into ookinetes that invade the mosquito's midgut epithelium and transform into oocysts. With the support of nutrients from the mosquito, the oocysts grow in size from 5 to about 60 µm in diameter forming thousands of sporozoites that are freed to the hemolymph. The sporozoites then reach the salivary gland and invade it in order to be transmitted to new hosts upon subsequent feedings of the mosquito (reviewed in, 30)<sup>30</sup>.

*An. albimanus* is one of the principal human malaria vectors in Central America, the northern part of South America, and the Caribbean<sup>31</sup>. The Tapachula strain of *An. albimanus* was originally collected on the Coastal Plain of southern Chiapas-Mexico, and presents two phenotypic variants during its ontogeny<sup>32</sup>. These variants are distinguishable by the presence or absence of a morphological marker denominated *stripe*<sup>33</sup>, a white stripe visible in the dorsal side of the larvae and pupae. Mosquitoes lacking this marker present a homogenous brown coloration. The first observations related with these phenotypes showed that the *stripe* marker is dominantly inherited over the phenotype without the marker (*stripe*)<sup>34</sup>, where the genetic determinant is thought to reside in the

chromosome 3<sup>35</sup>. The first works regarding the association between the phenotypic variants with physiological characteristics in *An. albimanus* were developed around the phenomenon of insecticide and infection resistance<sup>33,36</sup>. The White (*stripe*<sup>+</sup>) phenotype mosquitoes are resistant to the insecticide dieldrine while significantly more susceptible to infections with *Plasmodium vivax* and *P. berghei* in comparison to the Brown (*stripe*<sup>-</sup>) phenotype<sup>37,38</sup>. Furthermore, the *stripe*'s presence has been associated with higher uric acid content in the larvae<sup>39</sup>. We have recently reported additional differences between the phenotypes at a transcriptional level and in the content of genomic 5-methylcytosine<sup>38</sup>. These differences in the susceptible-White phenotype in comparison to the resistant-Brown phenotype, consist in: (i) a lower genomic 5-methylcytosine content, (ii) lower basal transcriptional activity and (iii) lower transcriptional response to a *P. berghei* challenge. In this study, we show that the CHCs composition of *An. albimanus* mosquitoes is diet-dependent and that a *P. berghei* infection alters its composition.

## Results

To assess whether the CHCs composition in the susceptible-White and resistant-Brown phenotypes changed with diet and challenged status, the mosquitoes were separated into three different groups per phenotype: (i) sugar-fed, (ii) blood-fed and (iii) *P. berghei* (Pb)-challenged (Fig. 1A). The sugar-fed groups were maintained with 8% sugar solution for the entire experiment. Blood-fed and Pb-challenged groups were also maintained with 8% sugar solution but additionally fed with ookinete or mock cultures at five days post-emergence (dpe). The mosquitoes were left to lay eggs and at eight dpe mosquito samples were taken to analyze the CHCs and the infection parameters.

### Changes in the cuticular hydrocarbon profile are diet and infection dependent

The CHC profile of each phenotype submitted to different diets and exposure to the parasite was determined by gas chromatography coupled to electron ionization mass spectrometry for qualitative analysis, or coupled to a flame ionization detector for quantitative analysis. The qualitative analysis identified 19 different CHCs (Table 1) with the lack of phospholipids and acylglycerols, indicating that only cuticular and not epidermal compounds were extracted (reviewed in, 10)<sup>10</sup>. The CHC composition was different between the phenotypes and feeding treatments. The sugar-fed Brown (BS) phenotype presented all 19 CHCs detected and the sugar-fed White (WS) phenotype presented 18 out of 19 (Fig. 1B and Table 1). The blood feeding and parasite challenge caused the total number of CHCs to decrease. The blood-fed White (WB) phenotype presented 14 out of 19 and the blood-fed Brown (BB) phenotype, Pb-challenged Brown (BPb) and White (WPb) presented 15 out of 19 (Fig. 1B). These qualitative changes are resumed in Fig. 1C, where CHCs that could not be detected on a given feeding treatment are depicted (black boxes). Interestingly, the CHCs absent from the blood-fed and parasite-challenged mosquitoes were always short or long-chained (Fig. 1C), indicating a common metabolic response among the phenotypes and feeding/challenged status.

Although the BS mosquitoes presented all the detected CHCs, they had 1.25-fold less quantity than WS mosquitoes. This also happened with the BPb mosquitoes, with 1.23-fold less quantity than the WPb mosquitoes. In contrast, BB mosquitoes had 1.15-fold more than the WB mosquitoes (Table 1 and Fig. 1B). Despite these inter phenotype differences, the CHCs content of both White and Brown mosquitoes behaved similarly in response to the feeding/challenged status in terms of the quantity of each CHC. In general, all CHCs decreased after the blood-feeding but increased if the blood contained *Plasmodium* ookinetes. This is especially the case for pentadecane 8-hexyl, which increased almost 10-fold after the *Plasmodium* challenge, but not after the blood-feeding (Fig. 1B). An exception of this was the heptacosane, which decreased 2-fold after the *Plasmodium* challenge.

A principal component analysis (PCA) was carried out to determine the influence of the CHCs on the overall differences observed between the phenotypes and feeding treatments. It was found that nonacosane, methyl-hentriacontane, heptacosane, and to a lesser extent pentadecane 8-hexyl, Z-12 pentacosene, and hentriacontane, were responsible for explaining the greatest amount of the variation (Fig. 1D). That is, these CHCs account for the differences found between the phenotypes and feeding treatments. To further dissect these differences and establish to what kind of variation they explain for, a hierarchical cluster analysis was then performed on the PCA to find the dissimilarities between the phenotypes and feeding treatments. The resulting dendrogram clustered the groups according to their CHCs profiles, showing that the mosquitoes can be distinguished by their feeding, or challenged status (Fig. 1E). However, it was not possible to distinguish between the White and Brown phenotypes, which were grouped together in all cases. Thus, the CHCs profiles can be used to determine if the mosquitoes have fed on sugar, blood, or infected blood, but their susceptibility to *Plasmodium* can only be determined if the feeding status is known beforehand.

### The CHCs are modified differentially between the phenotypes after a *Plasmodium* challenge

Although the main differences in the CHCs were found between the feeding treatments, the White and Brown challenged mosquitoes also had differences that are worth exploring further. Figure 2A depicts the infection parameters of both phenotypes, showing that the Brown mosquitoes eliminated the parasites almost totally. On the contrary, 84% of the White mosquitoes could not resolve the infection, having on average four oocysts per mosquito. Interestingly, the Brown challenged mosquitoes weighted  $0.4 \pm 0.2$  mg less than the Brown blood-fed mosquitoes and  $0.5 \pm 0.09$  mg less than the White challenged mosquitoes (Fig. 2B). This is a reduction of 18% in the bodyweight, which suggests different nutrient management upon challenge, probably diverting nutrients to contend against the parasite without sacrificing egg development. This reduction can also explain why the Brown mosquitoes showed quantitative differences only in 6.25% of the CHCs relative to the blood-fed mosquitoes, whereas 68% of the CHCs changed significantly in the White phenotype after the *Plasmodium* challenge (Fig. 2C). A comparison between White and Brown mosquitoes after the *Plasmodium* challenge only showed changes in four CHCs (Fig. 2C); namely, Z-12 pentacosene, hexacosane 9-octyl, methyl-nonacosane, and methyl-hentriacontane, which are also included among the CHCs that changed significantly between the blood-fed and *Plasmodium*-challenged White mosquitoes (Fig. 2D). On the other hand, nonacosanol was the only CHC that changed in the Brown phenotype, a change that is not shared with the White phenotype.

## Discussion

It is known that many physiological aspects that impact on lifespan, general health, and reproductive potential are deeply affected by dietary composition (reviewed in, 1, 9)<sup>1,9,40</sup>. This especially holds for mosquitoes, which need a copious protein-rich blood meal to reproduce. Notwithstanding, this is also a source of infections for the mosquitoes and for the humans they feed on. Therefore, understanding the mosquito physiology concerning their feeding requirements and the eventual interaction with parasites, may give clues on how to block the transmission of vector-borne diseases. In this work, it was observed that the dietary composition affects the CHC profile in *An. albimanus*. This makes it possible to detect the mosquitoes' dietary/reproductive status and, most importantly, to detect mosquitoes that have entered in contact with the malaria parasite just by the determination of the CHC profile. The determination of the CHC profile brings several advantages in comparison to other techniques, like midgut or salivary gland dissection or PCR analysis. The most significant advantage is that the mosquitoes do not need preservation or a cold chain to be analyzed, as the mosquitoes can be damaged, dried, or even old (pinned museum specimens for example)<sup>17</sup>. Additionally, the CHC extraction with hexane is non-destructive, so mosquitoes can still be used afterwards.

The determination of the causes or effects of the observed changes in the CHC composition is a more challenging task and requires further experimentation. The results presented herein however, provide the basis for establishing new hypothesis that will deepen our understanding of the general mosquito metabolism after a blood feeding and parasite challenge. The sugar diet represents an abundant source of carbons that is reflected in the greater proportion of CHCs, as well as the presence of longer CHCs in the sugar-fed mosquitoes. The blood, whether infected or not, caused the total amount and composition of the CHCs to decrease, especially for the very long-chain CHCs, which may indicate a shortage of carbons and an inhibition/limitation of the fatty acid synthases or elongases activity<sup>2</sup>. Previous studies in *Drosophila melanogaster* reported similar results, where a diet based on yeast or sugar caused the CHC composition to change in opposite directions<sup>40</sup>. Fedina, *et al.* (2012) reported that a yeast-based diet reduced the relative abundance of the CHCs, while a sugar-based diet increased the relative abundance. In this regard, one of the mechanisms by which mosquitoes acquire resistance to insecticides is by limiting the penetration of chemicals through the thickening and alteration of the cuticle composition (reviewed in, 4)<sup>4,24,28</sup>. In the case of *An. albimanus* this is interesting in two ways: first, the White mosquitoes, in contrast to the Brown mosquitoes, have been shown to be resistant against insecticides, and although it is not known why, it is possible that the greater amount of CHCs may play a role in such phenomenon. Second, following the same argument, a blood meal may cause the mosquitoes to become more susceptible to insecticides due to the reduction in the CHC content and composition.

Consider that the CHC profile of the sugar-fed mosquitoes portrays a basal state. In that case, the 30% reduction in the CHC content in the White blood-fed mosquitoes and the 16% reduction in the Brown blood-fed mosquitoes represents a drastic change in the CHC content in a short amount of time (Fig. 1B; Table 1). The above is consistent with previous reports of the change in the CHC profile from one day to the next, allowing mosquitoes to be age-graded with a one-day-old difference<sup>14</sup>. This has been used to determine the probability for a female to be old enough to transmit the *Plasmodium* parasite<sup>23</sup>, for example. An interesting observation was that the Brown-resistant phenotype decreased its weight when exposed to *P. berghei*, which implies the possible activation of molecular mechanisms at the cost of energy consumption during the immune response (reviewed in, 41, 42)<sup>41,42</sup>. In *D. melanogaster*, it has long been recognized that the activation of immune cells leads to a metabolic switch associated with increased energy consumption (reviewed in, 43)<sup>43</sup>. Further studies should be carried out at later time points after the mosquito's feeding to determine the dynamics of the CHCs in time. In the case of the *Plasmodium*-challenged mosquitoes, subsequent studies should evaluate whether the changes in the CHC profile persist or change during the progression of the infection in the insect tissues.

Besides its importance in the adaptive responses to the environment, the CHCs also function as attractiveness signals for mating purposes. It has been hypothesized that the reduction in heneicosane and tricosane in *An. gambiae* females after mating reduce their attractiveness to courting males, reducing mating in already inseminated females<sup>22</sup>. In *An. albimanus*, all females at 5 dpe are considered already inseminated, and in agreement with previous reports the heneicosane is present in the sugar-fed females while undetectable in the blood-fed females. Remarkably, in the *Plasmodium*-challenged mosquitoes the heneicosane is present and quantitative indistinguishable to the sugar-fed mosquitoes, probably making the *Plasmodium*-challenged females still responsive to males. Albeit a tradeoff between reproduction and immunity have not been determined in *An. albimanus*, as has been shown for *An. stephensi*<sup>44</sup>, the presence of heneicosane in the challenged mosquitoes may indicate that such tradeoffs also occur in *An. albimanus*.

We identified four cuticular components with significant differences between the susceptible and resistant strains to *P. berghei* infection (Fig 2D): Z-12 pentacosane, hexacosane 9-octyl, methyl-nonacosane, and methyl-hentriacontane. Comparative analyzes in multiple species of insects have revealed that these CHCs have evolved to perform a number of functions in chemical communication with special emphasis on their reproductive biology (reviewed in, 3, 10)<sup>3,10</sup>. Studies on Z-12 pentacosane reveal that it is a potent oviposition pheromone<sup>45</sup>. Similarly, hexacosane, 9-octyl functions as a sex attractant pheromone<sup>46</sup>, and nestmate recognition<sup>47</sup>. Meanwhile, methyl-nonacosane has been identified to have a role in fertility signalling<sup>48</sup> and behavior<sup>49</sup>. Several studies exist for the role of methyl-hentriacontane, and has been proposed to be part of the phenotypic variability<sup>50</sup> and behavior<sup>49</sup>. However the specific contribution of these CHCs in mosquitoes is not clear, so these molecules should be analyzed to investigate their potential role in the behavior of infected mosquitoes.

Interestingly, pentadecane, 8-hexyl tends to increase in both strains only when an infection is involved (Fig. 1B; Table 1). This compound (along with octacosane and triacontane, also detected in *An. albimanus*'s cuticle) is present in extracts from green seaweed and has been implicated in antibacterial and fish pathogen functions<sup>51</sup>. It will be interesting to examine whether this molecule exerts an anti-*Plasmodium* activity.

The blood meal represents the principal source of proteins and lipids for the mosquito, which are firstly metabolized in the midgut. Here, cholesterol, phosphatidyl choline, phosphatidyl ethanolamine, cholesteryl ester, and diacylglyceride are the major lipoprotein-associated lipids<sup>52</sup>. The mosquito cell's common lipid uptake pathway relies on the lipophorin complex of proteins<sup>52</sup>. These proteins are present in the hemolymph and bind to lipids, carrying them as a reusable shuttle to or from the fat body and other organs for metabolism and storage. Lipophorins have been shown to be upregulated after a *Plasmodium* infection<sup>53</sup>, and the knockdown of lipophorins led to a strong restriction in oocysts numbers by 90% in *Ae. aegypti*<sup>53</sup> and of 4-fold in *An. gambiae*<sup>54</sup>. Several studies in mosquitoes have revealed that the steroid hormone 20-hydroxyecdysone (20-E) regulates vector competence, however the molecular mechanisms

by which the infection parameters are affected are poorly understood (reviewed in, 55)<sup>55</sup>. Werling, et al., (2019) showed that the 20E mediates a positive correlation between egg and oocyst numbers. When this hormone cascade is interrupted, the oocyst numbers are reduced, however the parasite development is accelerated, causing the mosquitoes to become infectious earlier. This effect was attributed to the accumulation of lipophorin due to a misregulation of egg development<sup>56</sup>. Since *Plasmodium* partially lacks the genes required for lipid synthesis, the parasite hijacks these lipids from the lipophorins for its growth, increasing its infectivity, virulence, and quantity of sporozoites (reviewed in, 57)<sup>57</sup>. Thus, the mosquito lipid metabolism is crucial for *Plasmodium* development, especially during sporogony, where the oocysts increase in size about 10-fold producing thousands of sporozoites, each with its own lipid membrane<sup>30</sup>.

The free aminoacids obtained through diet such as valine, isoleucine and methionine are substrate for the synthesis of propionyl-CoA by the mosquito, which in conjunction with malonyl-CoA, are incorporated to hydrocarbons to form methyl-branched hydrocarbons (reviewed in, 10)<sup>10</sup>. The CHCs are synthesized from acetyl-CoA in an elongation reaction to form a long-chained fatty acyl-CoA in the oenocytes<sup>2</sup>. Then P450 decarbonylase converts them to long-chained hydrocarbons that are transported to the cuticle. The first studies that addressed the effects of *Plasmodium* infection on the physiology of mosquitoes reported that the contents of valine, isoleucine, methionine, histidine and lysine in the hemolymph of infected mosquitoes markedly decreases<sup>58,59</sup> while their midguts use eight times as much glucose<sup>60</sup>. Other effects of *Plasmodium* infection on mosquitoes are changes in their behaviour. The infected mosquitoes have increased probing time, more persistent in biting, increases the frequency of multiple feeding, and greater feeding-associated mortality (Reviewed in, 42)<sup>42</sup>. In *An. albimanus* the infection with *P. berghei* lead to a differential expression of proteins in the infected midgut<sup>61</sup> and an increase in the concentration of brain proteins involved in the cellular metabolic pathway and neural function<sup>62</sup>.

Here, we address whether the composition of the CHCs of the malaria vector *An. albimanus* is altered by environmental factors such as diet and a parasitic infection. The changes observed in the CHCs composition were dependent on the diet and parasite challenge, which reflects its close relationship with the mosquito physiology and its importance in several adaptive responses against the environment. It is interesting that multiple CHCs that changed upon infection are associated with the reproductive biology of insects, indicating that a *Plasmodium* infection may be associated with changes in the behaviour of *An. albimanus*. This work opens new perspectives for the understanding of the molecular mechanism in this ecological triad between parasites, ambient and vectors.

## Methods

### *Mosquito maintenance*

*An. albimanus* White and Brown phenotypes were derived from the parental Tapachula strain and were reared at 28 °C under 70 - 80% humidity and at a 12/12 h day/night cycle in the insectary of the INSP, Cuernavaca Morelos.

*An. albimanus* phenotype selection was carried out in the pupal stage, discriminating against the presence of a white stripe in the dorsal side surrounded by the brownish to the grey coloration of the rest of the body (White) or by the absence of this stripe (Brown). Six hundred pupae were selected for each phenotype and allowed to emerge to adults.

Groups of 100 female mosquitoes were separated two days post-emergence (dpe) to form the following experimental treatment groups: White + sugar (WS), Brown + sugar (BS), White + blood (WB), Brown + blood (BB), White + *P. berghei* (WPb) and Brown + *P. berghei* (BPb). Female mosquitoes were fed ad-libitum every 24 h with sterile cotton balls dampened with maintenance solution (8 % sucrose (Molecular Probes), 0.05 % para-aminobenzoic acid (Sigma) and penicilin, streptomycin and neomicin (PSN, Sigma).

### *Plasmodium berghei* ookinete culture and mosquito feedings

Ookinete culture was carried out as described in 63<sup>63</sup>. Briefly, 6-8 weeks old male BALB/c mice were treated with 200 µl of phenylhydrazine (6 mg/ml in saline) intraperitoneally (IP) two days before IP inoculation of 2-4 x 10<sup>8</sup> GFP-expressing parasites. When the parasitemia reached 15 - 25%, and after gametocyte viability verification, the infected blood was extracted by cardiac puncture with a heparinized syringe from the CO<sub>2</sub> euthanized mice and incubated at 19 - 20°C for 20 - 24 h in RPMI-Ook medium (1:4, blood medium) to allow ookinete formation. The ookinetes were counted using a Neubauer chamber, and the culture was centrifuged at 2000 rpm for 5 min to resuspend the pellet in fetal calf serum (FCB, Hyclone) at a density of 900 ookinetes per microliter.

Mosquito feedings were carried out by the Standard Membrane Feeding Assay, at 5 dpe, using GFP-expressing ookinete cultures or uninfected-blood treated as for the ookinete cultures. Feeders were left in contact with the mosquitoes for 30 - 60 min at 37°C, using a water re-circulator. After feeding, partially or non-engorged females were excluded from the groups. Mosquitoes were kept at 19 - 20°C and fed on sterile cotton pads dampened with maintenance sugar solution *ad-libitum* until CHC extraction and infection quantification. The cottons pads were replaced every 24 h. This study was approved by the Biosafety and Ethics Committees of the Instituto Nacional de Salud Pública (INSP, Mexico). We confirm that all experiments were performed in accordance with the ARRIVE guidelines.

### *Prevalence, abundance, and intensity of infections.*

Three days post-infection (dpi), groups of 30 females were anesthetized at 4°C for 15 min to dissect the midguts. The midguts were mounted on microscopy slides with PBS and were observed by epifluorescence microscopy (Leica DM1000 microscope with a mercury lamp and the Leica H3 filter cube). *P. berghei*-GFP oocysts were counted, and the prevalence, abundance, and intensity of infections were determined in two independent experiments. The infection parameters were calculated as follows: prevalence = percentage of infected mosquitoes; intensity = average number of oocysts per infected mosquito;

abundance = average number of oocysts per total mosquitoes. Data was analyzed with the Mann-Whitney test. The values of the prevalence, intensity, abundance and the p value are indicated above the violin plots.

#### *Mosquito weight measurement and lipid extraction*

Grupos de 50 hembras de 8 dpe de cada tratamiento (sacarosa, sangre y P. berghei) were anesthetized at 4 °C for 15 min. Mosquitoes' weight was measured using an analytical scale (Gibco). Cuticular hydrocarbons extraction was performed by hexane immersion; 50 females were placed in 1.5 ml amber-crystal vials and incubated with 1 ml of hexane (Merck; HPLC grade) for 15 min at room temperature. The resulting liquid was transferred to a new vial and gently evaporated under an N<sub>2</sub> stream. The samples were analyzed by gas chromatography coupled to either mass spectrometry or flame ionization detectors.

For the qualitative identification of hydrocarbon, aliquots of 1 µl of extract samples were analysed using a 6890N gas chromatograph (GC, Agilent Technologies, Inc., Santa Clara, CA, USA), coupled with an EI-quadrupole 5973 mass spectrometer (MS, Agilent Technologies).

The GC was equipped with a ZB-5MS capillary column (Zebron Phenomenex, Inc., Torrance, CA, USA; 30 m × 0.25 mm, with a 0.25 µm phase coating thickness) and the carrier gas was ultra-high purity helium with a column head pressure of 10 psi. Oven was set at 60°C for 3 min, then the temperature was increased from 60°C to 110°C at 25°C/min rate and from 110°C to 310°C at 5°C/min rate. The final temperature was maintained for 20 minutes. The transfer line was at 290°C. Splitless injection (3 min. hold) was performed using an automated sampler with an injector temperature of 280°C. Mass spectral data were acquired in full scan mode over a range of 40 – 600 m/z. Integration of chromatograms and the analysis of hydrocarbon was made following procedures described by Witek et al., (2013)<sup>64</sup>. The quantitative analyses were performed by GC coupled to a Flame Ionization Detector (FID) by using a Shimadzu gas chromatograph hi (GC-2010 Plus) at the same conditions and using the same column as described above.

#### *Statistical analysis*

PCA and dendrogram analysis, as well as the evaluation of the mosquito infections were carried out in R version 4.0.2, Student's t-test comparisons were carried out in Prism 6.

## Declarations

### Ethical review

This study was approved by the Biosafety and Ethics Committees of the Instituto Nacional de Salud Pública (INSP, Mexico).

### Author's contribution

M.M. and H.L.M. conceived and designed the experiments. F.C.P, B.R.T. and M.M. performed all experiments, F.C.P, B.R.T., J.C.C., R.C and H.L.M analyzed the data. F.C.P, B.R.T., J.C.C., R.C and H.L.M wrote the paper. All authors read and approved the final version of the manuscript. Research direction H.L.M. and M.M.

### Competing interests

The authors declare no competing interests.

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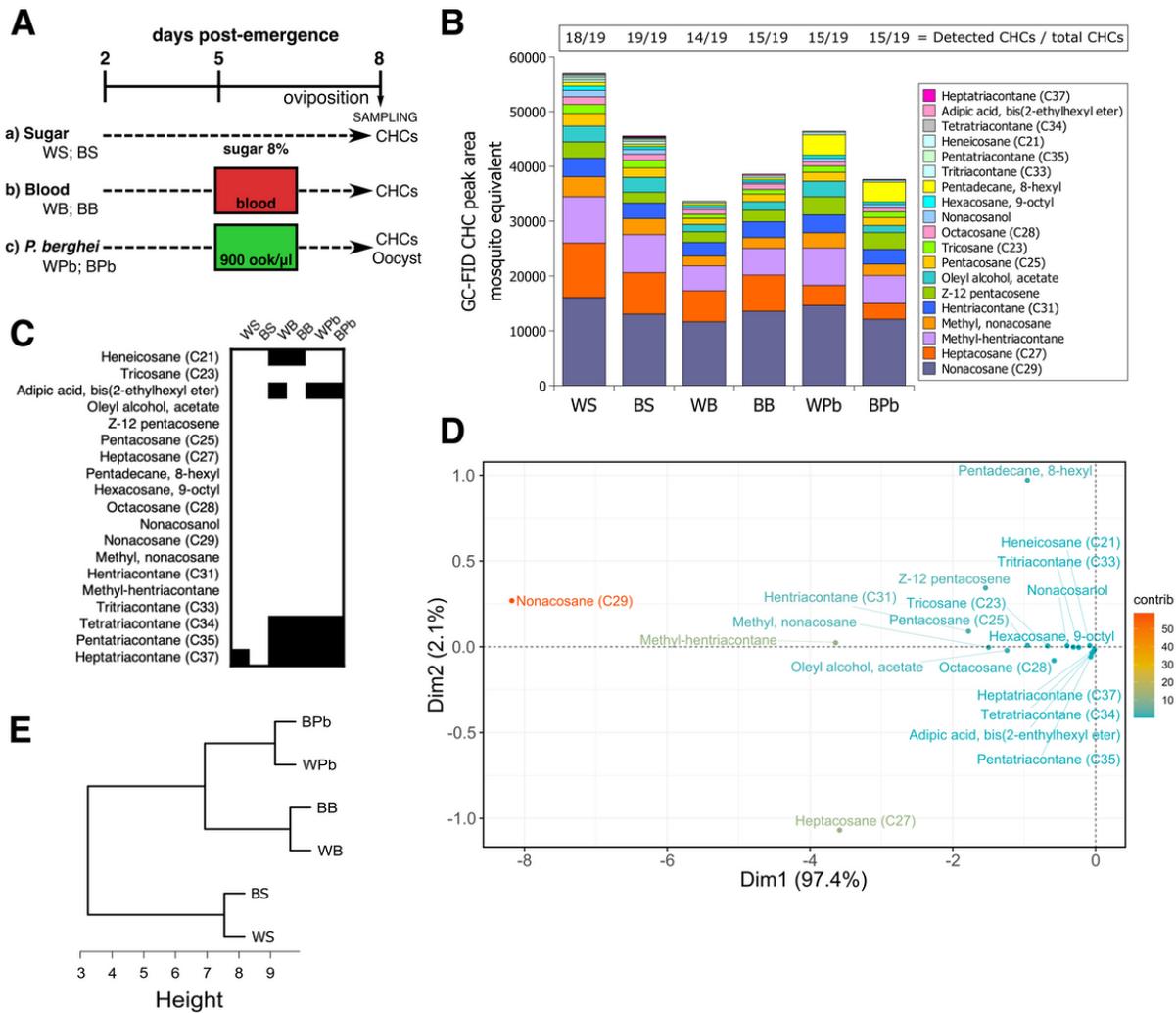
## Table

Table 1. Cuticular hydrocarbons and their abundance found in the White and Brown phenotypes fed with sugar, blood, or ookinete-containing blood (GC-FID peak area mosquito equivalent).

| Cuticular hydrocarbons               | White sugar |   |         | Brown sugar |   |        | White blood |   |       | Brown blood |   |        | White / |         |
|--------------------------------------|-------------|---|---------|-------------|---|--------|-------------|---|-------|-------------|---|--------|---------|---------|
|                                      | Mean        | ± | SE      | Mean        | ± | SE     | Mean        | ± | SE    | Mean        | ± | SE     | Mean    | ±       |
| Heneicosane (C21)                    | 304.0       |   | 103.8   | 238.1       |   | 238.1  | ND          |   | ND    | ND          |   | ND     |         | 230.6   |
| Tricosane (C23)                      | 1642.4      |   | 473.0   | 1385.0      |   | 1.9    | 738.2       |   | 3.7   | 845.7       |   | 167.3  |         | 1156.9  |
| Adipic acid, bis(2-ethylhexyl ether) | 107.7       |   | 107.6   | 141.4       |   | 141.4  | ND          |   | ND    | 281.8       |   | 71.5   |         | ND      |
| Oleyl alcohol, acetate               | 2903.3      |   | 389.6   | 2716.9      |   | 186.8  | 1333.3      |   | 169.1 | 1572.6      |   | 1239.4 |         | 2844.0  |
| Z-12 pentacosene                     | 2927.8      |   | 2043.6  | 1956.6      |   | 1157.9 | 1938.8      |   | 174.0 | 2091.3      |   | 1095.6 |         | 3292.3  |
| Pentacosane (C25)                    | 2316.1      |   | 670.3   | 1727.3      |   | 46.6   | 1110.2      |   | 39.1  | 1380.1      |   | 208.2  |         | 1618.0  |
| Heptacosane (C27)                    | 9922.9      |   | 2033.6  | 7568.1      |   | 1210.8 | 5648.9      |   | 286.4 | 6599.2      |   | 217.7  |         | 3657.8  |
| Pentadecane, 8-hexyl                 | 656.2       |   | 186.1   | 437.9       |   | 68.9   | 400.9       |   | 60.0  | 394.7       |   | 118.2  |         | 3729.7  |
| Hexacosane, 9-octyl                  | 784.3       |   | 222.3   | 528.6       |   | 45.9   | 407.1       |   | 14.7  | 427.4       |   | 37.1   |         | 542.0   |
| Octacosane (C28)                     | 1383.1      |   | 312.5   | 1124.8      |   | 101.5  | 856.5       |   | 0.9   | 1006.3      |   | 5.2    |         | 790.1   |
| Nonacosanol                          | 1185.3      |   | 566.0   | 864.3       |   | 133.4  | 392.4       |   | 168.9 | 312.8       |   | 26.5   |         | 654.5   |
| Nonacosane (C29)                     | 16101.1     |   | 3245.7  | 13055.1     |   | 1876.3 | 11667.5     |   | 345.1 | 13580.3     |   | 605.4  |         | 14648.6 |
| Methyl, nonacosane                   | 3628.8      |   | 792.4   | 2914.8      |   | 242.3  | 1792.9      |   | 66.6  | 1953.8      |   | 621.0  |         | 2784.3  |
| Hentriacontane (C31)                 | 3426.1      |   | 804.4   | 2851.5      |   | 290.4  | 2501.8      |   | 61.7  | 2902.7      |   | 223.3  |         | 3262.4  |
| Methyl-hentriacontane                | 8454.6      |   | 1864.5  | 6941.6      |   | 763.2  | 4528.1      |   | 260.2 | 4877.7      |   | 1531.3 |         | 6810.8  |
| Tritriacontane (C33)                 | 465.4       |   | 167.6   | 496.3       |   | 122.4  | 294.2       |   | 16.0  | 357.0       |   | 25.6   |         | 395.5   |
| Tetratriacontane (C34)               | 246.5       |   | 22.5    | 114.5       |   | 114.5  | ND          |   | ND    | ND          |   | ND     |         | ND      |
| Pentatriacontane (C35)               | 443.0       |   | 171.7   | 335.8       |   | 335.8  | ND          |   | ND    | ND          |   | ND     |         | ND      |
| Heptatriacontane (C37)               | ND          |   | ND      | 175.7       |   | 175.7  | ND          |   | ND    | ND          |   | ND     |         | ND      |
| Total                                | 56898.5     |   | 13833.9 | 45574.1     |   | 62.8   | 33610.7     |   | 56.0  | 38583.4     |   | 6193.3 |         | 46417.4 |

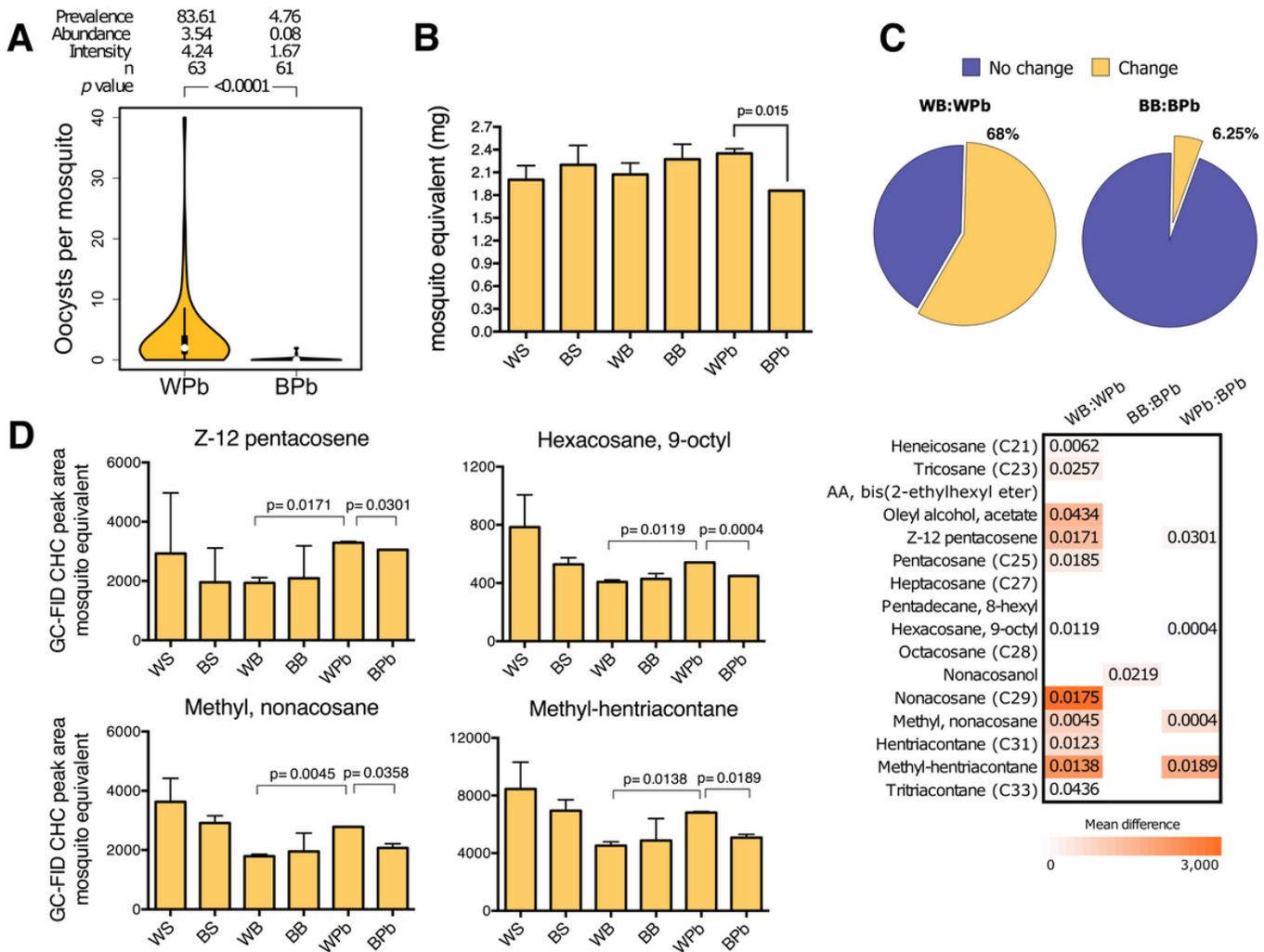
SE = standard error, ND = not detected

## Figures



**Figure 1**

The composition of cuticular hydrocarbons in *An. albimanus* mosquito is diet dependent. (A) Experimental setup for the determination of the CHC composition and abundance of the *An. albimanus* White-susceptible and Brown-resistant phenotypes. White (W) and Brown (B) 5 days post-emergence (dpe) females were fed on 8% sucrose solution (WS; BS), blood-fed (WB; BB), or fed with 900 *P. berghei* ookinetes per microlitre (WPb; BPb). CHC extraction was carried out three days post feeding from 50 mosquitoes per group for identification by GC. Oocyst counts per mosquito were also determined at eight dpe. (B) Total area of CHCs per mosquito-equivalent, composition, and abundance in the two phenotypes with different diets as determined in (A). The proportion of compounds identified is shown in the numbers above each column. Representation of the arithmetic mean of two independent experiments. (C) Qualitative CHC changes in the two phenotypes with different diets and challenged with *P. berghei*. The boxes show the presence (white) or absence (black) of the compound by GC-FID in the extracts of mosquitoes fed with 8% sucrose (WS; BS), blood (WB; BB), and infected blood (WPb; BPb). (D) Principal component analysis of the CHC influence in explaining the variation observed between the groups (WS, BS, WB, BB, WPb, BPb). The gradient of color (contrib) indicates the contribution of the compound on the distribution of the data. (E) Cluster dendrogram analysis. The distance matrix data of groups WS, BS, WB, BB, WPb, and BPb were grouped hierarchically on basis of the minimum distance between the principal components of the group, merging the two nearest groups until all groups are merged into a single group.



**Figure 2**

*P. berghei* infection alters the CHC composition (A) Infection parameters of the White-susceptible and Brown-resistant phenotypes. White and Brown females 5 (dpe) were challenged with 900 ookinetes per microliter of a *P. berghei* strain that constitutively expresses the GFP protein (WPb; BPb). Three days post-challenge, the midguts were dissected, and the abundance, intensity and prevalence of the infection were determined by fluorescence microscopy. Results of two independent experiments with approximately  $n = 30$  mosquitoes for each group. Data was analyzed with the Mann-Whitney test. The values of the prevalence, intensity, abundance and p-value are indicated above the violin plots. (B) Mosquito-equivalent mass of the White and Brown phenotypes with different diets based on 8% sucrose solution (WS; BS), blood (WB; BB), and infected blood (WPb; BPb). Three days post-feeding, the mass of 50 mosquitoes per group was measured to extract the cuticular hydrocarbons afterwards. Representation of the mean  $\pm$  standard error of the mean (SEM) of two independent experiments. t test, WPb vs. BPb;  $p = 0.0150$ . (C) Percentage changes in CHC composition in the White and Brown phenotypes after a *P. berghei* challenge and a Heat map of the identified CHC and those that showed significant changes in susceptible and resistant mosquitoes challenged with *P. berghei* (WPb:WB; BPb:BB). The color gradient represents the mean difference of the CHC GC-FID area of mosquito extracts between the different conditions (WPb:WB, BPb:BB, and WPb:BPb). Only compounds with significant differences were represented with color, while white boxes represent no significant change. The numbers in the boxes are the p-values calculated by a two-tailed t test of two independent experiments. (D) CHCs with significant changes between the susceptible and resistant phenotypes challenged with *P. berghei* (WPb:BPb). GC-FID lipid area of mosquito extracts of the two phenotypes with diets based on 8% sucrose (WS; BS), blood (WB; BB), and infected blood (WPb; BPb). Mean  $\pm$  SEM of two independent experiments, each with 50 mosquitoes per group. p-values of a two-tailed t test.