Regionalization of surface lipids in insects

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Cuticular hydrocarbons (CHCs) play a critical role in the establishment of the waterproof barrier that prevents dehydration and wetting in insects. While rich data are available on CHC composition in different species, we know little about their distribution and organization. Here, we report on our studies of the surface barrier of the fruit fly Drosophila melanogaster applying a newly developed Eosin Y staining method. The inert Eosin Y penetrates different regions of the adult body at distinct temperatures. By contrast, the larval body takes up the dye rather uniformly and gradually with increasing temperature. Cooling down specimens to 25°C after incubation at higher temperatures restores impermeability. Eosin Y penetration is also sensitive to lipid solvents such as chloroform indicating that permeability depends on CHCs. As in D. melanogaster adult flies, Eosin Y penetration is regionalized in Tenebrio molitor larvae, whereas it is not in Locusta migratoria nymphs. Regionalization of the fly surface implies tissue-specific variation of the genetic or biochemical programmes of CHC production and deposition. The Eosin Y-based map of CHC distribution may serve to identify the respective factors that are activated to accommodate ecological needs.

1. Introduction

An impermeable cuticle, which protects insects against dehydration and wetting, is probably one of the most important reasons for successful radiation of insects into almost every terrestrial niche. Free cuticular hydrocarbons (CHCs) covering the surface of the animal are believed to play a critical role in the establishment of this barrier [1]. An insect may harbour more than 100 different CHCs that are easily extracted with organic solvents like hexane or chloroform [2–4]. They range from simple n-alkanes to unsaturated and methyl-branched compounds [1,2,5]. The CHC composition, which is species-specific, is proposed to define the melting temperature (Tm) of the CHC layer that according to the lipid-melting model of Ramsey [6] correlates with the critical temperature (Tc) above which water is lost dramatically, leading to death [6–13]. Sudden increase in water loss above the Tc value is believed to rely on the conversion of CHCs from an impermeable solid state to a permeable fluid state [6,12,14,15]. Interestingly, CHC compositions are not uniform over the complete cuticle of an animal, the Tm can therefore differ from region to region [16].

Detailed analyses of whole body and body region Tc and Tm are particularly effective in large insects, such as locusts and cockroaches [8,16]. Fourier transform infrared (FTIR) spectrometers, for instance, allow studying lipid properties in cuticular lipids extracted from individuals or isolated organs in some insect species like Tenebrio molitor (mealworm), Melanoplus sanguinipes (grasshopper) and Allonemobius fasciatus (cricket) [12,16,17]. By contrast, the small body size of many insect species such as Drosophila melanogaster can hamper this type of work. Indeed, the available amount of CHCs from one individual small animal or parts of it may often be insufficient for analyses. Experiments using a mix of individuals cause loss of information on variations between individuals [12,18–20].
The ecological impact of CHCs has been well investigated in the *Drosophila* genus. A comparison between 18 different *Drosophila* species did not reveal any convincing correlation between CHC composition and temperature-dependent water loss [21]. Likewise, in the desert fruit fly *D. mojavensis*, changes in CHC amounts and composition in response to temperature increase did not improve desiccation resistance [22]. By contrast, desiccation-selected *D. melanogaster* had longer CHCs than control flies suggesting that higher *Tm* may contribute to reduced water loss [23]. Overall, however, at least in the genus *Drosophila*, the major adaptive mechanism to minimize water loss is to adjust gas exchange via the respiratory system [21]. Hence, besides their minor role in desiccation resistance, CHCs have other functions that are modulated during niche adaption and evolution. One of these functions is their well-perceived influence on mating behaviour as sex pheromones [24].

Together with cuticle surface nano- and microstructures [25,26], the CHC surface layer is also involved in avoiding wetting, a serious hazard that may compromise respiration and locomotion [3]. This aspect is, despite its importance, marginally studied. In particular, the impact of temperature on cuticle wettability has not been addressed.

Here, we present a new *in situ* method designed to analyse the barrier function of the CHC layer in dependence of the temperature. Instead of assessing water loss at different temperatures, a common method in CHC research, by light microscopy, we study the ability of the animal to take up the inert dye Eosin Y reflecting cuticle barrier function at the tissue level. In the strict sense, of course, we cannot exclude that other cuticle components than CHC are responsible for the effects that we observed in this project. This method is applicable to the fruit fly *D. melanogaster*, to the locust *Locusta migratoria* and to the mealworm *T. molitor*, suggesting that it probably can be applied to all insects.

2. Material and methods

(a) Insects and Eosin Y staining

In principle, insects were initially stained at the rearing temperature (i.e. 25°C for *D. melanogaster* and *T. molitor*, and 30°C for *L. migratoria*). We next chose temperatures at and around the *Tm* or *Tc* values published: 30°C (*Tm* [21]) for *D. melanogaster* (*Tc* unknown), 50–60°C (*Tc* [8]) for *T. molitor* and 48°C (*Tc* [9]) for *L. migratoria*. Generally, negative staining results prompted us to increase the dye incubation temperature, which, on the contrary, was decreased after positive staining results.

To stain adult flies, 2–3-day-old wasps flies were anaesthetized with CO₂ and transferred into a 2 ml microcentrifuge tube containing 1 ml dye solution (0.5% Eosin Y (W/V) and 0.1% Triton X-100) tempered at 4°C. After 10 min staining at room temperature (22°C), flies were washed twice with water (25°C) and analysed immediately by microscopy. All incubation experiments were performed at least twice, each with 10 animals.

To obtain larvae for staining, Oregon R flies (WT) were kept at 25°C in egg laying cages on an apple juice agar plate garnished with a spot of fresh baker’s yeast. Eggs were dechorionated in order to identify and collect late stage 17 embryos. These eggs were transferred to a new apple juice agar plate with yeast paste, and incubated at 18°C. Around twenty 24 h (L1) and 48 h (L2) old larvae were picked from the plate and transferred into 2 ml microcentrifuge tubes containing 200 μl water (25°C). One millilitre of 0.5% Eosin Y (W/V) preheated to 25°, 45°, 50° or 55°C was added to the tubes. Tubes were incubated at the indicated temperatures for further 30 min. Larvae were then washed three or four times with water (25°C) before microscopy. All incubation experiments were performed at least twice, each with more than 20 animals.

The mealworm larvae (Claus GmbH) with body lengths between 14 and 16 mm or pupae were transferred into a 2 ml microcentrifuge tube. One millilitre dye solution (0.5% Eosin Y (W/V) and 0.1% Triton X-100) preheated to 25°, 50°, 60°, 70°, 80° or 90°C was immediately added to the tubes. Tubes were incubated at the respective temperatures for 30 min. After incubation, the animals were washed three times with water (25°C). They were immediately analysed by microscopy. In total, five larvae or three pupae were tested at each temperature in two independent experiments.

To stain *L. migratoria* nymphs, eggs were incubated in a growth chamber (Yiheng, China) at 30°C under a relative humidity of 50%. First-instar nymphs without pigmentation were collected just after hatching. They were anaesthetized with CO₂ and transferred into a 2 ml microcentrifuge tube containing 1 ml dye solution (0.5% Eosin Y (W/V)) preheated to 30°, 45°, 48°, 50°, 53° or 55°C. Tubes were incubated at the respective temperature for further 30 min. Specimens were then washed three or four times with water (25°C) before microscopy. To remove surface lipids, we washed locusts with chloroform for 5 min. They were then incubated in Eosin Y solution for 10 min at room temperature (22°C).

Before microscopy, animals were washed three or four times with water until the wash water was clear. All experiments were performed at least four times, each with two or three animals.

(b) Microscopy

A Leica MZ4 stereo-microscope with in-built camera was used for imaging large insects. Images were taken and details were observed using a Nikon AZ100 zoom microscope equipped with a Digital Sight DS-F11 camera. Images were formatted and prepared for publication with Adobe Photoshop and Illustrator CS6 software.

3. Results

(a) Different parts of the fruit fly surface have different

*TPr* for Eosin Y

To illustrate cuticle impermeability, we first tested whether Eosin Y can penetrate the cuticle to stain the inside tissues of the animal at common temperatures of fruit fly breeding at 18°C and 25°C. At these two temperatures, most parts of the insects' surface were impermeable to Eosin Y (figure 1). Only two sensory organs on the head, the antennal and maxillary palps, were stained after 20 min immersion in the dye (figure 1; electronic supplementary material, figure S1). This shows that Eosin Y could penetrate the surface of these two organs. It is important to note that these results are generally independent from immersion time (data not show). Next, we stained animals at 4°C, and found that the antennal and maxillary palps did not stain. This result suggests that the permeability of the antennal and maxillary palps is temperature-dependent. Their critical temperature for Eosin Y penetration (*TPr*) lies between 4°C and 18°C.
We also studied the effects of temperatures higher than 25°C on cuticle permeability. Staining of the fly body with Eosin Y was unchanged at 30°C (which corresponds roughly to the Tm value of 29°C [21]), 37°C and 40°C (data not shown). In summary, as shown in figure 1 and electronic supplementary material figures S1 and S2, we found that different body regions became permeable to Eosin Y at distinct temperatures above 40°C. The dye started to penetrate the lower edge of the wing at 45°C, while it stained the wing’s upper edge at 58°C (figure 1). Similarly, staining of the legs was non-uniform. The joints and the claws were first stained at 50°C (electronic supplementary material, figure S2), the tarsal segment at 55°C. Finally, the whole legs became red at 58°C (figure 1). By this method, some tissue-specific TpE values could be determined exactly. For instance, the labellum remained impermeable until a temperature of 37°C, but became suddenly permeable to Eosin Y at 40°C.

To examine whether specimen treatment (particularly staining at higher temperatures) may injure the animals, thereby facilitating or enabling dye uptake, we cut wings or legs of wild-type flies before staining with Eosin Y (electronic supplementary material, figure S3). Eosin Y was taken up locally at the wound site, but did not spread into the entire organ or body. Other possible entry sites for Eosin Y are the tracheal openings (spiracles). To answer the question whether these structures might be responsible for uncontrolled Eosin Y penetration, we microscoped the spiracles of the thorax and the abdomen after incubation in Eosin Y at 25°C and 50°C (electronic supplementary material, figure S4). Eosin Y did not stain the spiracles at 25°C, but marked them at 50°C. The surrounding tissue, however, remained unstained. Thus, Eosin Y penetrates the fly body preferably through the cuticle.

In conclusion, Eosin Y incubation assays revealed a temperature-dependent regionalization of the fly surface for penetration of a hydrophilic molecule.

(b) Eosin Y penetration in the mealworm and locust cuticle is temperature-dependent

To evaluate the Eosin Y experiment with the fruit fly, we analysed Eosin Y penetration into the cuticle of T. molitor (mealworm) larvae and pupae, and freshly hatched
First-instar nymphs of the locust *L. migratoria* at different temperatures (figures 2 and 3). Mealworm larvae have a high $T_m$ value above 80°C [8,16]. Consistently, Eosin Y did not penetrate the cuticle of mealworm larvae at 25°C and 50°C (figure 2). At 60°C, the spiracle became red, and the dye passed a bit into the body through the respiration system. This is comparable with the published $T_c$ value of 50–60°C in larvae [8]. At 80°C, most soft parts of the cuticle became permeable to Eosin Y. At 90°C, Eosin Y stained also the sclerotized parts of the cuticle. Of note, the $T_m$ value for *T. molitor* larval exuviae has been measured to be over 80°C [16]. Interestingly, the anterior part of the arthrodial membrane (aa) remains unstained at this high temperature. These results are summarized in (f), showing a longitudinal section through the arthrodial membrane (aa and pa) connecting abdominal segments 1 and 2. In (a–e), a dorsal view on two abdominal segments (1 and 2) is shown. The segments were unfolded after a cut at the ventral side.

First-instar locust nymphs before pigmentation allowed easy scoring for staining with Eosin Y. Locusts were immersed in Eosin Y solution at 30°C, 45°C, 48°C, 50°C, 53°C or 55°C (figure 3). Eosin Y did not penetrate the cuticle at 30°C, which was the common temperature for locust husbandry. Likewise, at 45°C, most parts of the insects’ surface remained impermeable. By contrast, the insects’ surface is slightly stained at 48°C. This is very close to the $T_c$ value of 47°C determined for the adult locust [9]. Staining gradually intensified with an increase of the temperature to 55°C. Thus, together, in young locust nymphs surface seems not to be regionalized.

**Figure 2.** Eosin Y penetrates the cuticle of mealworm larva at high temperatures. At 25°C and 50°C, Eosin Y does not pass through the abdominal cuticle of mealworm larvae (a–b’). At 60°C, the spiracles (arrow) in the lateral cuticle of mealworm larvae take up Eosin Y, while other body parts remain unstained (c’). At 80°C, Eosin Y stains the posterior half of the arthrodial membrane (pa) that connects the tergites (d). At 90°C, also the tergites (t) are stained by Eosin Y (e). Interestingly, the anterior half of the arthrodial membrane (aa) remains unstained at this high temperature. These results are summarized in (f), showing a longitudinal section through the arthrodial membrane (aa and pa) connecting abdominal segments 1 and 2. In (a–e), a dorsal view on two abdominal segments (1 and 2) is shown. The segments were unfolded after a cut at the ventral side.

(c) The temperature-dependent permeability of fruit fly cuticle is reversible

To test whether the observed temperature-dependent permeability changes are reversible, we designed an annealing experiment. We heated the flies to 58°C for 20 min, cooled them slowly down on ice and immersed them for 20 min in Eosin Y at 25°C. These flies were not stained by Eosin Y, just like flies that were directly immersed in Eosin Y at 25°C without pre-heating (figure 4). This result indicates that temperature-dependent cuticle permeability is reversible.

We applied this method also to *D. melanogaster* L1 and L2 larvae. Both L1 and L2 larvae started to take up Eosin Y through their skin at 50°C (electronic supplementary material, figure S6). They were completely stained red at 55°C. In contrast to the situation in adult flies, cuticle impermeability of these larvae can only be partially restored.
In summary, the \textit{D. melanogaster} cuticle is a temperature-sensitive barrier for hydrophilic molecules that is better developed in adult flies than in larvae.

(d) Cuticle impermeability is overcome by washing with lipid solvents

We next investigated whether cuticle impermeability is due to the CHC layer at the surface of the animal. For this purpose, we stained flies with Eosin Y after washing them with chloroform, hexane and heptane, three lipid solvents that are traditionally used to extract insect surface lipids. We found that all solvents can systematically annihilate cuticle impermeability. Chloroform showed the strongest effect. Flies incubated with Eosin Y took in the dye acutely, becoming dark red, while hexane- or heptane-washed flies became light red. The difference was especially obvious in the wings, legs and halters (figure 5).

As in the fruit fly, chloroform application enabled Eosin Y penetration in locust nymphs. Thus, the surface barrier in locust nymphs seems to involve CHCs.

4. Discussion

The insect cuticle is a barrier against dehydration, at the same time preventing penetration of potentially harmful substances. Here, we report on the visualization of the temperature-dependent collapse of cuticle impermeability in locust nymphs. Thus, the surface barrier in locust nymphs seems to involve CHCs.

(a) First conclusion: Eosin Y penetration is blocked by cuticular hydrocarbons

At 58°C, Eosin Y, a red inert dye with molecular weight of 648 Da, penetrates the entire cuticle of the \textit{D. melanogaster} imago. Comparable results were obtained with \textit{L. migratoria}.
and T. molitor. After incubation in chloroform, a solvent often used to extract surface lipids in insects, Eosin Y penetrates the entire adult cuticle of the adult fly and the locust nymph already at 25°C. Consistently, flies mutant for ca that codes for a GTPase required for lipid transport have a weaker barrier against Eosin Y penetration. The wings of these flies become red already at 55°C upon Eosin Y staining. These observations hint at a lipid-, possibly CHC-dependent mechanism of Eosin Y penetration protection, naturally, without excluding that other cuticle components (e.g. proteins might be involved). In the literature, there are only a few comprehensive reports on the function of CHCs in blocking penetration of hydrophilic substances into the arthropod cuticle [25,29]. Commonly, the surface nano- and micro-morphology of the cuticle is also considered as an important entity that prevents wetting [26,30–32]. In this work, we have not studied the effects of temperature and lipid solvents on the surface nanostructures of D. melanogaster, L. migratoria or T. molitor in Eosin Y staining experiments. However, we think that the restoration of cuticle impermeability in flies cooled down after heat treatment implies a simple, CHC-involving annealing mechanism rather than the reconstitution of a complex cuticular nanostructure. To corroborate this assumption, along with ultra-structural analyses of the insect surface, CHC composition and abundance before and after annealing have to be determined by gas chromatography and mass spectrometry. Together, our data nevertheless suggest that Eosin Y penetration depends on surface lipids (i.e. CHCs that change their aggregate state at the utmost at 58°C in D. melanogaster).

(b) Second conclusion: Eosin Y application reveals body surface regionalization in Drosophila melanogaster and Tenebrio molitor

Different regions of the D. melanogaster adult fly surface become permeable to Eosin Y at different temperatures. Likewise, Eosin Y penetrates different parts of the cuticle of mealworm larvae at distinct temperatures. These findings indicate that different regions of the insect surface are composed of specific structural and chemical factors including CHCs. This conclusion is in agreement with recent mass spectrometric data on the distribution of neutral lipids on the surface of D. melanogaster [33]. Combining mass spectrometry with laser-assisted ionization imaging, among others, it was shown that different lipids cover the anterior and posterior halves of the wing. This mirrors our finding that the Eosin Y is soaked in by the anterior half of the wing at a higher temperature than by its posterior half. Moreover, the melting temperatures of CHCs of different body parts of the grasshopper Melanoplus sanguinipes, a large insect, have been demonstrated by FTIR spectroscopy to vary [16]. Conceivably, regionalization of the insect surface suggests region-specific genetic programmes deployed during cuticle development or reparation. In principle, the notion of surface regionalization is in line with the hypothesis of spatial separation of lipid phases on the cuticle formulated by Allen Gibbs in 2002 [13]. He postulated spatially different distribution of surface lipids (especially of high-melting alkanes) in order to explain why water loss rates do not correlate perfectly with the measured ‘bulk’ lipid-melting point. Thanks to the arsenal of molecular and genetic tools available, including tissue-specific gene silencing methods [34], D. melanogaster is the perfect model insect to uncover the respective underlying genetic and molecular factors.

In contrast to the situation in D. melanogaster adults and T. molitor larvae, the surface of locust first-instar nymphs and D. melanogaster larvae is not regionalized. How can these cases be explained in the light of the data discussed above? In the case of D. melanogaster, it may be that larvae as relatively simple life forms with limited behavioural repertoire and a uniform soft cuticle (note that the head skeleton is inverted) do not require differential CHC distribution for communication with the environment or survival. Comparably, body organization in alternating soft and hard cuticle types in T. molitor larvae is mirrored by differential distribution of CHCs. In the case of locust nymphs, it may simply be that freshly hatched and unpigmented nymphs have not yet developed an elaborate CHC coat. Although highly speculative at the moment, it may also be that a differential CHC distribution has not evolved in the more ancestral hemimetabolous insect species such as L. migratoria, but has in holometabolous
insects (T. molitor, D. melanogaster) [35,36]. Of course, it is equally possible that simplification of CHC distribution in locusts is a derived trait. These possibilities shall be tested in appropriate staining experiments using older animals and additional species representing different taxa and modes of life.

In any case, the Eosin Y penetration method established for D. melanogaster, T. molitor larvae and L. migratoria nymphs may potentially be used to plot maps of the surface of any insect of interest. These maps, in turn, may serve to study the mode of action of contact insecticides or any other lipophilic substance that interact with cuticular lipids in pest insects. Contact insecticides such as cypermethrin, sumithion, dieldrin and carbaryl [37–40] may associate with a preferred region-specific composition of CHCs. Indeed, in the case of dieldrin, distribution of the insecticide within the body of cockroaches (Periplaneta americana) was more effective after application on the pronotum than on the wing [37]. The pulvilli of tsetse flies (Glossina spp.) have been found to be very effective in DT and pyrethrum uptake [41]. Beyond that, diflubenzuron and flufenoxuron, two derivatives of the lipophlic benzoylurea-based chitin synthesis inhibitors, pass through the bumblebee (Bombus terrestris) cuticle with different efficiencies [42]. Taking these arguments together, knowledge about the preferred region of cuticle penetration and identification of the respective CHCs may help to optimize the impact of an insecticide by chemical modifications. Ultimately, uptake optimization might result in the development of species-specific insecticides.

(c) Hypothesis: Eosin Y penetration mirrors anti-wetting barrier constitution

Eosin Y penetrates the surface of different body regions at respective temperatures ranging from 18°C to 58°C. Except for the T_{PE} measured for parts of the head, the T_{PE} values of the other body parts are higher than the lipid-melting temperature T_m of around 29°C determined for D. melanogaster [21]. To what extent is it reasonable to compare T_{PE} with T_m? Or would we compare apples and oranges? Indeed, T_{PE} characterizes the outside-in (penetration) barrier, whereas T_m describes a property of the inside-out (transpiration) barrier that relies largely on CHCs. As reckoned in our first conclusion (see above), our results collectively suggest that CHCs may also be responsible for the penetration barrier. Considering hence CHCs as the central element of both types of barrier, we conjecture two alternative explanations for the discrepancy between T_m and most T_{PE} values in D. melanogaster. First, in a simple scenario, the size of the molecule that is able to pass through the CHC layer is directly proportional to the temperature that influences the physical properties of the barrier. Water, a small molecule, diffuses through the CHC barrier at moderate, physiological temperatures around the melting and critical temperatures [13,21]. Bigger molecules (Eosin Y, 648 Da) are retained at these temperatures, but pass through the CHC layer at higher temperatures. Application of other dyes with different sizes in similar experiments described here should serve to challenge this possibility.

Second, admittedly a less probable scenario, transpiration (water) and penetration (Eosin Y) may rely on distinct properties of the CHC layer. In other words, T_m and T_{PE} reflect distinct characteristics of the CHC layer. Wetting, a prerequisite for penetration, occurs at higher temperatures than transpiration and is independent of CHC melting in this scenario. Interestingly, T_{PE} values in some parts of the head, where many sensory organs are placed, are low, enabling communication with the environment. By contrast, they are not physiological in the other body regions suggesting that CHCs seal these regions perfectly against penetration at physiological conditions beyond necessity.

Previously, it was shown that T_m values of CHCs in species of the Drosophila genus living in mesic or xeric habitats are around 30°C irrespective of the habitat. This suggests a similar CHC composition in these species. Our aim is to scrutinize whether the T_{PE} values of these species are also similar.

(d) Concluding remarks

Regionalization of the insect penetration barrier is probably reflected by the differential distribution of CHCs. In order to identify the region-specific CHC species, we propose to combine our Eosin Y staining method with the method of solid-phase micro-extraction (SPME) prior to gas chromatography and mass spectrometry (GC-MS). Indeed, SPME-GC-MS has been applied successfully for the identification of CHCs on single insects [43–45].

Finally, we should consider that surface regionalization described and discussed here may be specific to Eosin Y. Using our approach, the behaviour of other inert dyes like bromophenol blue should be tested for a generalized statement.

Ethics. All experiments described here were performed according to local guidelines. Appropriate ethical approval and licences were obtained.

Data accessibility. All data from this work will be publicly available in the Dryad Digital Repository http://dx.doi.org/10.5061/dryad.kk52g.

Authors’ contributions. B.M. and Y.W. conceived and designed the experiments. Y.W. and Z.Y. performed the experiments. All authors analysed and discussed the data and wrote the manuscript and approved the final version.

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