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SHORT COMMUNICATION

Double cuticle barrier in two global pests, the whitefly *Trialeurodes vaporariorum* and the bedbug *Cimex lectularius*

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ABSTRACT

The integument protects the organism against penetration of xenobiotics and water that would potentially interfere with homeostasis. In insects that play key roles in a variety of agricultural and ecological habitats, this inward barrier has barely been investigated. In order to advance knowledge in this field, we studied integumental barrier (cuticle) permeability in the two global pests *Trialeurodes vaporariorum* (greenhouse whitefly) and *Cimex lectularius* (bedbug), applying a simple dye-penetration assay. In agreement with our recent findings in *Drosophila melanogaster*, we show that the surface of these insects is regionalised. We also show that, in contrast to the single barrier in *D. melanogaster*, two barriers with distinct temperature-sensitive and lipid-based physico-chemical material properties act in parallel to protect these insects against penetration of hydrophilic molecules. These findings imply the existence of unexplored mechanisms by which the cuticle acts as a protective coat against the penetration of water and xenobiotics, including pollutants and insecticides.

KEY WORDS: Cuticular hydrocarbon (CHC), Water, Xenobiotics

INTRODUCTION

Generally, in most habitats, insects are in close contact with their wet or solid environment, running the risk of undesired uptake of water, solutes and toxic molecules. They erect a composite barrier – the cuticle – at the apical side of their epidermis that protects them against these hazards. The first, physical barrier in penetration protection is constituted by cuticle nanostructures and macrostructures protruding at their surface (Watson et al., 2010; Sun et al., 2012; Gundersen et al., 2014; Darmanin and Guittard, 2015). The material properties of the second inward barrier are to a large extent defined by lipophilic compounds including waxes and free cuticular hydrocarbons (CHCs) at the cuticle surface (Ramsay, 1935; Noble-Nesbitt, 1970; Gibbs, 1998; Rourke and Gibbs, 1999; Gibbs, 2002; Moussian, 2010; Gibbs, 2011). According to the lipid-melting model of Ramsay (1935), water flow across the cuticle depends on the temperature at which cuticular waxes and CHCs exhibit a phase transition from solid to fluid, termed the critical temperature (T_c). The T_c values vary between species (Gibbs, 2002). These compounds are transported to the surface via a cuticular canal system comprising pore and wax canals that are continuous with the apical plasma membrane of epidermal cells (Wigglesworth, 1975). These canals are potential routes of uncontrolled penetration of water and solutes (Locke, 1965; Wigglesworth, 1986, 1990).

While cuticular microstructures and nanostructures are being analysed extensively (Darmanin and Guittard, 2015), the wax/CHC-based inward barrier of insects has not been studied in detail. A central problem in this field is to visualise penetration. Recently, we showed that the easily visualised harmless and water-soluble dye Eosin Y penetrates into the cuticle of several insect species dependent on lipid solvent-soluble CHCs and temperature (Wang et al., 2016). Interestingly, Eosin Y did not penetrate the surface uniformly, but at regionally different rates. We hypothesised that CHC composition is regionalised reflecting body region-specific physiological, and by consequence genetic, programmes. We reason that these differences may have an impact on the activity and efficiency of contact insecticides that are taken up by the cuticle. To study inward permeability patterns in the cuticle of insects with applied relevance, we used the Eosin Y penetration assay on various insect species. Here, we report on our analyses of the permeability barrier in two global human pests: the whitefly *Trialeurodes vaporariorum* Westwood 1856, a widespread greenhouse pest, and the human parasite bedbug *Cimex lectularius* Linnaeus 1758. Our data demonstrate that, as in *Drosophila melanogaster*, temperature-dependent permeability to Eosin Y is regionalised in these two species. In contrast to the situation in *D. melanogaster*, however, permeability in *T. vaporariorum* and *C. lectularius* is also regionalised after removal of surface lipids with chloroform. Similar results were obtained in penetration assays using other, chemically different dyes such as Bromophenol Blue and Methylene Blue. We infer that in contrast to *D. melanogaster*, two distinct barriers are present in the whitefly and bedbug cuticle.

MATERIALS AND METHODS

Animal husbandry and dye incubation

Whitefly (*T. vaporariorum*) adults were obtained from Nuetzlinge.de (Ammerbuch, Germany) and Katz Biotech AG (Baruth/Mark, Germany). We focused on their wings, which are rather robust in handling compared to the rest of the whitefly body. Bedbugs (*C. lectularius*) that were reared at 22°C in plastic vials furnished with a filter paper were obtained from the Reinhardt laboratory at the TU Dresden (Reinhardt et al., 2003). Eosin Y staining followed the recently described procedure (Wang et al., 2016). In brief, animals were incubated in plastic vials at different temperatures in 1 ml 0.5% (w/v) Eosin Y and 0.1% Triton X-100 (both Sigma Aldrich) using standard thermo-shakers. Bromophenol Blue (Sigma, 0.5% w/v, 0.1% Triton X-100) and Methylene Blue (Sigma, 0.1% w/v, 0.1% Triton X-100) were applied following the same protocol. All three dyes are generally used in histology as unspecific detectors of a variety of molecules (Selman, 1960; Marconi and Quintana, 1998; Fischer et al., 2008). For chloroform washes, insects were incubated in the solvent for 2 min in glass vials prior to staining with dyes at respective temperatures.

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Microscopy and imaging

A Leica MZ4 stereo-microscope with in-built camera was used for imaging whole insects. Images were taken and details were observed using a Nikon AZ100 zoom microscope equipped with a Digital Sight DS-Fi1 camera. Images were formatted and prepared for publication with Adobe Photoshop and Illustrator CS6 software without manipulation of the initial settings of the microscopes.

RESULTS AND DISCUSSION

Eosin Y penetration in whiteflies depends on temperature and chloroform-soluble components

Eosin Y was excluded from the *T. vaporariorum* wing cuticle at 25 and 60°C (Fig. 1). Penetration of the dye started at 70°C. A broad area at the basis, the margin and the single wing vein was stained at 70°C, and the entire wing surface was stained at 80°C. Prolonged incubation of whiteflies in Eosin Y at 60 or 70°C did not alter the dye penetration pattern (Fig. S1) suggesting that lateral diffusion does not play a role in this process. Cooling down whiteflies from 80°C to 0°C prior to staining at 25°C prevented Eosin Y uptake. This result underlines that our staining method does not inflict wounds that would entail uncontrolled dye penetration. Together, these findings indicate that permeability of the wing cuticle to Eosin Y is temperature dependent and restorable. Barrier restoration, i.e. reversibility of high-temperature permeabilisation, allows us to exclude the involvement of surface microstructures and nanostructures in Eosin Y repellence, assuming that cuticular structures are not temperature sensitive. Moreover, similar to the situation in the fruit fly *D. melanogaster* (Wang et al., 2016), whitefly wings are regionalised with respect to permeability to Eosin Y.

Next, we investigated the permeability properties of the wing cuticle surface with respect to Eosin Y penetration after incubation in chloroform, a widely used lipid solvent (Fig. 1). Eosin Y penetrated the wing margin and the wing vein after chloroform incubation, while the wing blade remained unstained. This finding suggests that permeability to Eosin Y is partially and regionally lipid dependent.

To further scrutinise the properties of the barrier against penetration in *T. vaporariorum*, we combined the effects of temperature with those of chloroform on permeability to Eosin Y (Fig. 1). After incubation in chloroform, Eosin Y stained the wing blade in addition to the wing margin at temperatures of 60°C and above. This observation indicates that the effects of temperature and chloroform on the penetration barrier are additive. In turn, this suggests that the penetration barrier consists of at least two different components, one of which is only sensitive to temperature, while the other is damageable by lipid solvents. The situation is even more complex, as staining of Eosin Y was uniform and did not intensify at 80°C regardless of chloroform treatment. Thus, the putative solvent-soluble component is also temperature sensitive but loses its barrier properties only at very

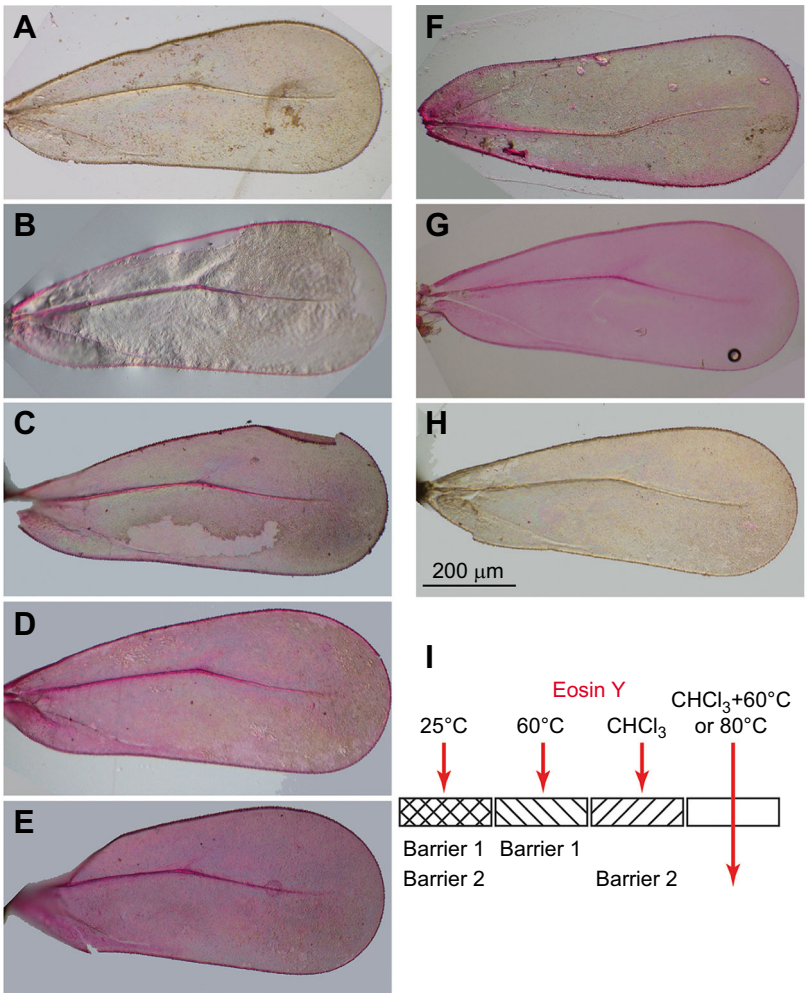

Fig. 1. Two cuticular barriers in *Trialeurodes vaporariorum*. (A) Wings do not take up Eosin Y at 60°C. (B) Eosin Y penetrates the wing margin and vein after chloroform wash. (C) The entire wing is stained by Eosin Y when incubated at 60°C after chloroform wash. (D) The entire wing is red when stained at 80°C. (E) Staining at 80°C is not enhanced after chloroform wash. (F) At 70°C, the wing margin, the vein and an area at the wing joint are stained by Eosin Y. (G) Eosin Y penetrates the entire tissue after incubation of specimens at 60°C following incubation at 80°C and annealing. (H) Impermeability to Eosin Y at 25°C is restored when animals are cooled down to 0°C after incubation at 80°C. (I) In summary, two barriers – one temperature dependent (barrier 1) and one sensitive to chloroform (barrier 2) – prevent Eosin Y penetration into the wing blade. Barrier 2 components denature at 80°C. $CHCl_3$, chloroform.

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high temperatures. Complete breakdown of both penetration barriers, in conclusion, occurs at 80°C. This effect cannot be reversed as Eosin Y is taken up by the entire surface after incubation of whiteflies at 60°C following chilling after 80°C incubation. In *D. melanogaster*, the situation seems to be simpler (Wang et al., 2016). Upon incubation in chloroform, Eosin Y stains the entire wing of *D. melanogaster*. Hence, lipid solvent application and temperature do not have an additive affect in this insect. A putative temperature-sensitive barrier in *D. melanogaster* does not play any role if the solvent-sensitive barrier is broken.

Chloroform and temperature have an additive effect on Eosin Y penetration in the bedbug cuticle

Eosin Y did not infiltrate the surface of *C. lectularius* first instar nymphs at temperatures below 65°C (Fig. 2). The first regions taking up Eosin Y at 65°C were the tips of the antennae. At 70°C, the entire antennae, the thorax, the posterior end of the abdomen and the tarsi of these animals became red. The whole animal was stained by Eosin Y at 75°C. However, at this temperature, redness of the leg joints was more intense compared with the other leg regions. Staining intensity of the legs was uniform at 80°C.

Washing first instar nymphs in chloroform before staining with Eosin Y led to dye uptake into the antennae, the thorax and the posterior region of the abdomen at 25°C, while the legs and a large abdominal area remained unstained (Fig. 2). Washing these animals with chloroform followed by their incubation at 60°C resulted in complete staining of the animal body, excluding the legs. Leg joints of chloroform-treated nymphs were stained at temperatures above 70°C. Uniform leg staining was observed after incubation at 75°C and did not intensify at 80°C. These observations indicate that for Eosin Y to penetrate into the bedbug cuticle, especially in the abdomen, two penetration barriers have to be trespassed. Re-incubation of bedbugs at 60°C after a cycle of incubation at 80°C and chilling resulted in complete Eosin Y uptake, while re-incubation at 25°C after the same initial incubation–chilling cycle resulted in no staining (Fig. 2). These findings support our assumption of a double inward barrier in bedbug nymphs.

Bromophenol Blue and Methylene Blue behave like Eosin Y in penetration assays

To test whether the results obtained with Eosin Y are specific to this dye, we performed the same set of experiments described above using Bromophenol Blue or Methylene Blue. In principle, Bromophenol Blue and Methylene Blue mimicked the findings with Eosin Y. These results are shown in Figs S2 and S3.

Conclusions and outlook

Together, our experiments allow the distinction of regions of inward permeability conferred by two sub-barriers in the cuticle of *T. vaporariorum* and *C. lectularius*. What is the nature of these barriers? Our experiments addressed physico-chemical, i.e. material, rather than physiological (cell-based) properties of the cuticle. These properties nevertheless correspond to biological properties of the extracellular cuticle. Insensitivity of the barrier to relatively high temperatures between 40 and 60°C, at which most if not all proteins denature, and restorability together argue against an important role of cuticle proteins in constituting these barriers. Conceptually, we postulate that two distinct classes of Eosin Y-repellent, i.e. lipophilic, compounds are involved in inward barrier constitution in *T. vaporariorum* and *C. lectularius* (Figs 1I and 2O). A fraction of free and solvent-soluble and high temperature-sensitive lipophilic compounds, probably CHCs, constitute one sub-barrier. The other sub-barrier is composed of a fraction of non-soluble lipophilic compounds that are sensitive to temperatures lower than those that modify the function of the first barrier. Lipophilic compounds, especially at the cuticle surface, have been repeatedly demonstrated to prevent dehydration in various insect species (Wigglesworth, 1971; Gibbs, 2002). Thus, the inward and outward barriers seem, at least concerning the CHCs, to rely on the same class of cuticular components. Thus,

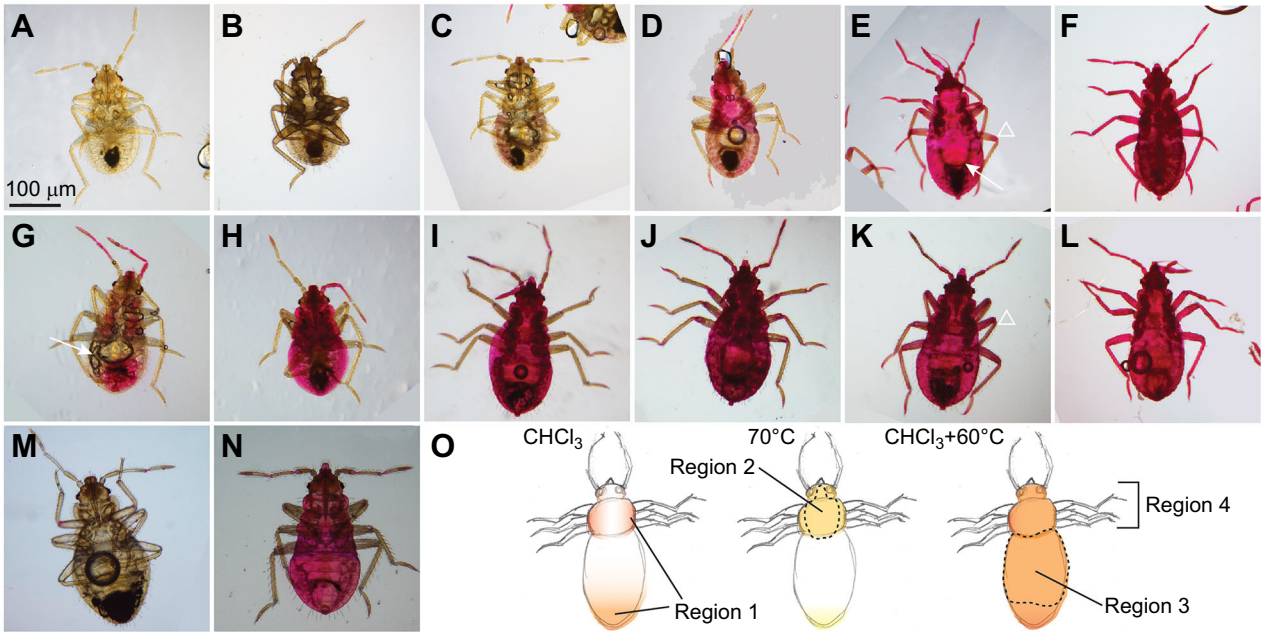

Fig. 2. Two cuticular barriers in *Cimex lectularius*. (A–L) Eosin Y staining of *C. lectularius* first instar nymphs at 25°C (A,G), 60°C (B,H), 65°C (C,I), 70°C (D,J), 75°C (E,K) and 80°C (F,L) alone (A–F) and after chloroform wash (G–L). (M,N) Cooling down nymphs to 0°C after incubation at 80°C restores impermeability to Eosin Y at 25°C (M), but not at 60°C (N). (O) Together, these experiments allow distinction of four regions of permeability.

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the lipid-melting model for phase transition of surface lipids formulated by Ramsey for the outward barrier may also apply to the inward barrier.

Structurally, the double barrier does not have to necessarily be attributed to the envelope only. The pore canals, as potential sites of uncontrolled water flow, may also be equipped with material (CHCs or any other hydrophobic substance) to plug this route, at the same time preventing dye uptake. In this scenario, the two experimentally separable barriers may also be spatially distinct structures. Regardless, barrier properties are simpler in the model insect *D. melanogaster*, where no additive effects of two permeability barriers were observed. Detailed molecular and histological analyses of the whitefly and bedbug cuticles are needed to understand the bipartite barrier against penetration of hydrophilic molecules like Eosin Y, Bromophenol Blue and Methylene Blue. In theory, work on the inward barrier will also enhance our knowledge on the mode of action of hydrophobic molecules during cuticle penetration. The probable regionalised distribution of lipids and waxes would determine the preferred site of entry of this type of molecule at ambient temperature. Together, exploration of insect cuticle barrier properties may enable better understanding of the route of penetration used by contact insecticides that have to overcome the cuticle barrier in order to eliminate the insect during pest control.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Y.W. and B.M. designed the experiments and they were performed by Y.W., R.G.C. and B.M. B.M. wrote the manuscript with contributions from Y.W. All authors approved the submitted manuscript.

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Data availability
Data are available from the Dryad Digital Repository (Wang et al., 2017): http://dx.doi.org/10.5061/dryad.60nc3.

Supplementary information
Supplementary information available online at
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