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An insect trap as habitat: cohesion-failure mechanism prevents adhesion of *Pameridea roridulae* bugs to the sticky surface of the plant *Roridula gorgonias*

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SUMMARY

The glandular trichomes of the plant *Roridula gorgonias* release an extremely adhesive, visco-elastic, resinous secretion that traps a variety of insects, including those having a considerable body size. However, the specialized mutualistic mirid bug *Pameridea roridulae* lives and walks on this sticky plant surface without being trapped. We have sought to reveal the mechanism underlying the apparent non-sticky nature of the cuticle of this bug. In this study, we have visualized intact plant and insect surfaces using cryo-scanning electron microscopy and measured the adhesive properties of the plant secretion on different surfaces. We present a combination of structural and experimental results that suggest that a thick and cohesively weak film of an outermost, epicuticular greasy secretion acts as a 'sloughing-off' layer, preventing the formation of contacts between the sticky plant secretion and the solid insect cuticle. In a comparative study of fresh cuticle fractures of flies representing a typical prey of *R. gorgonias*, a thin, fragmentary layer of epicuticular grease was revealed. These results indicate that, when trapping prey, the plant adhesive might form proper contact with solid islands of the insect cuticle that are free of epicuticular grease.

Key words: adhesion, biomechanics, cuticle, insect–plant interaction, plant resin.

INTRODUCTION

Glandular trichomes that release sticky fluids are common in a variety of plant species. They can have antixenotic effects on insects and mites, preventing them from settling and damaging plant surfaces (e.g. Levin, 1973). Several carnivorous and protocarnivorous plants use 'fly-paper' traps with tentacles and/or capitate trichomes to capture their prey (Lloyd, 1942; Juniper, 1986). The released glandular fluids make the surface shiny, and, by means of these reflective surface properties, a large number of insects are attracted and can be suddenly entangled when coming in contact with the sticky surface of the plant.

Viscid secretions at trichome tips can be, in general, of three kinds: oily (often aromatic), mucilaginous and resinous (Lloyd, 1942). In the genera *Byblis*, *Drosera*, *Drosophyllum* and *Pinguicula*, a hydrous mucilage is found, whereas, in the South African perennial shrubs *Roridula gorgonias* Planch. and *R. dentata* L. (Roridulaceae), the transparent exudates of their glands are very different from those of *Drosera* (Lloyd, 1934). The water-insoluble pellucid droplets of *Roridula* plants have been observed to persist indefinitely on dead and dried-up leaves (Bruce, 1907) or leaves preserved in formalin (Lloyd, 1934). Marloth (Marloth, 1925) washed dried leaves with chloroform and found a very viscid resinous residue containing ~10% of caoutchouc. These results were confirmed by Lloyd (Lloyd, 1934), who extracted fresh leaves with acetone, yielding a resin or a mixture of resins. Following this by extraction with petroleum ether yielded an acetone-insoluble material having properties of caoutchouc. However, no distinct chemical analyses on the composition of the *Roridula* secretion have been performed.

The presumably resinous nature led to the conclusion that proteolytic enzymes can be neither transported nor dissolved in the adhesive secretion (Marloth, 1925). That is why the 'carnivorous syndrome' of *Roridula* plants is considered controversial. Primarily, these plants were supposed to be carnivorous because of the similarity of the tentacle-shaped trichomes to those in representatives of the genera *Drosera* and *Drosophyllum* (Darwin, 1875; Marloth, 1903; Fenner, 1904; Bruce, 1907). As no digestive organs in the trichomes and leaves were found, *Roridula* was placed in the group of protocarnivorous plants – those that trap insects without the ability to digest them (Marloth, 1910; Marloth, 1925; Lloyd, 1934; Lloyd, 1942; Juniper et al., 1989). However, Midgley and Stock (Midgley and Stock, 1998) found higher levels of nitrogen and stronger ultraviolet reflectivity in the mature leaves of plants that captured insects, compared with control plants. These data confirm the carnivorous nature of *R. gorgonias*, despite the apparent lack of proteolytic enzymes. However, Płachno and colleagues (Płachno et al., 2006) observed phosphatase activity in the leaf epidermis using enzyme-labeled fluorescence microscopy.

The leaf surface of *Roridula* is covered with numerous tentacle-shaped and capitate trichomes of different size but similar structure, concentrated along leaf margins and the main vein (Fenner, 1904; Bruce, 1907; Uphof, 1962). The adhesive secretion of the trichomes is extremely effective, as shown by the conspicuous number of trapped insects of considerable size and mass, particularly flying ones (Marloth, 1910; Barthlott et al., 2004). Hartmeyer (Hartmeyer, 1998) suggested that the secretion of *Roridula* is the strongest glue of all insect-trapping plants. Stuck insect parts and entire corpses of occasionally considerable size and body mass have been found on the plant surface (Marloth, 1903; Marloth, 1910). Mean prey length averaged 3.55±0.57 mm (±s.d.; N=109) (Ellis and Midgley, 1996). However, no representative systematic study of the insect species trapped by *Roridula* plants has been performed to date. From

a small sample of *R. dentata*, representatives of Hymenoptera (25 individuals from subfamilies Sphecinae and Apinae), Diptera (20 individuals from the family Muscidae), Coleoptera (a few individuals from the families Coccinellidae and Scarabaeidae), Hemiptera (single individuals from the families Lygaeidae, Reduviidae and Membracidae) and Lepidoptera have been reported previously, whereas bees, wasps and flies were the most abundant specimens found (Marloth, 1903; Marloth, 1910). Samplings of individual leaf rosettes of 15 *R. gorgonias* plants after an eight-week period resulted in a total of 109 trapped individuals belonging to 32 macro-invertebrate species (>2 mm in length) consisting mainly of dipterans and coleopterans (Ellis and Midgley, 1996). Additionally, 122 micro-invertebrates (<2 mm in length) mainly from the orders Thysanoptera and Diptera have been counted. Illustrating their potency, South African farmers call the plant 'Vliegebos' and suspend the plants in their houses as flytraps (Marloth, 1925).

However, mirid bugs of the genus *Pameridea* (Heteroptera, Miridae, Bryocorinae, Dicyphini) are obligately associated with *Roridula* plants (Reuter, 1907; Dolling and Palmer, 1991; Picker et al., 2004) in a form of digestive and pollinating mutualism (Ellis and Midgley, 1996; Reiner, 2003; Anderson, 2005; Anderson, 2006; Anderson and Midgley, 2002; Anderson and Midgley, 2003; Anderson and Midgley, 2007; Anderson et al., 2003). They live omnivorously and walk confidently and quickly on the sticky plant surface without becoming entangled and without hindrance. The bugs feed on the glued insects and defecate on the leaves (Marloth, 1903; Lloyd, 1934). The nitrogen in the faeces of the bugs is absorbed through the thin leaf cuticle of the plant, thus providing up to 70% of the total plant nitrogen uptake (Ellis and Midgley, 1996; Anderson and Midgley, 2002; Anderson and Midgley, 2003). Similar relationships between mirid bugs and carnivorous plants are known from representatives of the genera *Byblis* and *Drosera* in Australia (Schuh, 1995). Moreover, mirid bugs from the subfamilies Orthotylinae and Bryocorinae seem to be specialized for living on glandular hairy plants (Reuter, 1913; Dolling and Palmer, 1991; Falkingham, 1995; Schuh, 1995; Wheeler, 2001; Sugiura and Yamazaki, 2006). For example, the mirid species *Dicyphus errans* Wolff (Bryocorinae, Dicyphini) avoids contact with sticky glandular secretions by means of morphological (slim body, long and slender legs, elongated curved claws) and behavioral (mode of locomotion, grooming) adaptations to hairy plant substrates (Southwood, 1986; Voigt et al., 2006a; Voigt et al., 2007). *D. errans* is often observed grooming various body parts (Voigt, 2005). By contrast, *Pameridea* bugs frequently touch the released viscid plant secretion, which is spread evenly over ovoid glands and often over the trichome multiseriate stalks and the plant stem (Marloth, 1910).

Previous authors (Lloyd, 1934; Hartmeyer, 1996; Hartmeyer, 1998) have suggested that bugs must have some kind of defense and resistance mechanisms to adhesive substances such as a sophisticated arrangement of short bristles capable of protecting the body against adhesives, specialized body cleaning adaptations and/or a locomotion mode in which the body is kept elevated above sticky trichomes. Intensive body grooming has been reported previously to be an essential adaptation of mirid bugs to living permanently, and walking, on hairy and glandular hairy plant substrates (Kullenberg, 1946; Voigt et al., 2006b). Our preliminary observations showed that *Pameridea roridulae* Reuter, after being wrapped in a sticky leaf of *R. gorgonias*, continued normal walking without body grooming.

Why does *P. roridulae* not stick to the adhesive secretion, whereas numerous other insect species do? Is such an anti-adhesive property of the mirid bug surface related to some specialized microstructure (Fig. 1C), similar to that previously described on unwettable surfaces of aquatic bugs (Anderson, 1976; Anderson, 1977; Anderson, 1982; Perez Goodwyn, in press; Perez Goodwyn et al., 2008)? Does the solid epicuticle have a strong repelling ability to nonpolar fluids (Fig. 1B)? Are there some fluids or easy-to-break solid layers on the epicuticle that function to prevent direct contact between plant secretions and the insect surface (Fig. 1D,E), as suggested by Lloyd (Lloyd, 1934)?

This study was performed to analyze the surface structure and adhesive characteristics of insect cuticle in contact with glandular droplets of trichomes of *R. gorgonias*. Using light and cryo-scanning electron microscopy (cryo-SEM), adhesive droplets of the plant, the surface of the insect cuticle and its prints on a glass surface were visualized in the intact state and after treatment with different solvents. The adhesion forces of adhesive droplets of *R. gorgonias* on the mutualistic mirid bug *P. roridulae*, the representative prey insect *Calliphora vicina* Rob.-Des. (Diptera, Calliphoridae) and a glass surface (control) were estimated through experimentation.

MATERIALS AND METHODS

Plants and insects

Seeded *R. gorgonias* plants, of age 1–3 years, occupied by *P. roridulae*, were obtained from a private greenhouse culture (Klaus Keller, Augsburg, Germany), kept under laboratory conditions during experiments (23.7±1.7°C, 47.3±10.0% relative humidity, 16 h photoperiod) and fed with wingless, adult *Drosophila melanogaster* Meigen fruit flies (Diptera, Drosophilidae; Zoo-Schöniger, Stuttgart, Germany).

Adult *C. vicina* blow-flies were reared from commercially offered larvae (Angelmarkt Stephan, Stuttgart, Germany).

Light microscopy

A stereomicroscope Olympus SZX 12 with a DF PLAPO 1×PF objective (Olympus Corporation, Tokyo, Japan) was used to observe *P. roridulae* mirid bugs and captured insects on *R. gorgonias*. Images were taken using a Nikon Coolpix E995 digital camera adapted to the stereomicroscope with a C-Mount adapter and a MDC 2 relay lens MXA 29005 (Nikon Corporation, Tokyo, Japan).

Cuticle prints of living and dead *P. roridulae* and *C. vicina*, pressed against a glass slide, were visualized using an upright AXIOPLAN microscope with an AxioCam MRc digital camera (Carl Zeiss MicroImaging GmbH, Jena, Germany) and AxioVision 3.1 software (AxioVision GmbH, München-Hallbergmoos, Germany).

Cryo-SEM

To visualize the details of interactions between plant and insect surfaces in the freshly frozen condition, microscopy studies were carried out using a Hitachi S-4800 cryo-SEM (Hitachi High-Technologies Corp., Tokyo, Japan) equipped with a Gatan ALTO 2500 cryo-preparation system (Gatan, Inc., Abingdon, UK). Samples were mounted on metal holders, frozen in the preparation chamber at –140°C, sputter-coated with gold–palladium (3 nm) and examined in a frozen state in the cryo-SEM at 3 kV accelerating voltage while at –120°C.

Plant trichomes, droplets and insect surface

Fresh samples of *R. gorgonias*, *P. roridulae* and *C. vicina* were prepared and studied as described above.

Plant adhesive secretion on different surfaces

Cryo-SEM has been reported previously to be a successful method for visualizing droplets of glycerine, water, lipids and

other biological fluids and their mixtures (Gorb et al., 2007). Using tweezers, droplets of single tentacle-shaped and capitate trichomes were manually brought into contact with surfaces of freshly killed insects (*C. vicina*, *P. roridulae*), and small hemispherical pieces of smooth Au–Pa-metallized epoxy resin Spurr (Spurr, 1969; Gorb, 2006), and examined according to the method described above. The solid metallized Spurr surface is known to increase the material contrast between the substrate and fluid droplets as well as, owing to the substrate profile, allowing the observation of droplets at an appreciable angle (Gorb 2006, Gorb et al., 2007).

Plant adhesive drops treated with different solvents

Using tweezers, single tentacle-shaped trichomes with glandular droplets were removed from the leaf margin, washed for 5 min in ethanol (Rotipuran® ≥99.8%, p.a., Carl Roth GmbH & Co. KG, Karlsruhe, Germany), acetone (Rotipuran® ≥99.8%, p.a., ACS, ISO, Carl Roth) or cold chloroform (≥99.8%, p.a., Merck KgaA, Darmstadt, Germany), placed on sample holders and observed as described above. The results were compared with data on untreated trichomes and those washed with aqua millipore.

Insect cuticle

Samples of *P. roridulae* and *C. vicina* – living, dead, and dead washed with cold chloroform (5 min) – were mounted on holders. Using a cold scalpel, fractures of frozen legs were performed in the preparation chamber of the cryo-SEM at –140°C. Next, samples were sputter-coated in the frozen condition (3 nm thickness of Au–Pa) and examined in the cryo-SEM at –120°C and an accelerating voltage of 3 kV.

From digital images of 10 randomly selected points, the thickness of the epicuticular grease layer was estimated, using Sigma Scan Pro 5 (SPSS, Inc., Chicago, IL, USA) software. The data obtained were statistically processed using Kruskal–Wallis one-way ANOVA on ranks and an all pairwise multiple comparison Tukey test (SigmaStat 3.1.1® software, Systat Software, Inc., Richmond, CA, USA).

Measurements of adhesion forces

The measurements were carried out using a force transducer (10 g capacity, Biopac Systems, Santa Barbara, CA, USA), attached to a motorized DC3314R micromanipulator with MS314 controller (World Precision Instruments, Sarasota, FL, USA). A piece of double-sided carbon tape (5×5 mm) was firmly attached to the force transducer. Using tweezers, a tentacle-like glandular trichome was removed from the leaf margin and attached by its base to the tape, perpendicular to the force transducer (Fig. 2). The trichome could be moved up and down with a velocity of 100 μm s^{-1}. The adhesive droplet on the trichome tip was brought into contact with the insect cuticle of the ventral side of the abdomen (living and dead bugs and flies, as well as chloroform-washed bugs) or with the glass surface. The droplet was preloaded to a maximum of 50 μN force and then withdrawn. Force–time curves were used to estimate the maximum pull-off (adhesion) force. For each test surface, 20 measurements on different sites (N=5 insects, n=4 measurements per insect), and 120 single measurements in total were carried out (N=6 surfaces, n=20 measurements per surface). Kruskal–Wallis one-way ANOVA on ranks followed by an all pairwise multiple comparison procedure (Tukey test) was used to evaluate differences in the adhesion force values between the substrates (SigmaStat 3.1.1® software, Systat Software, Inc.). Laboratory conditions were similar to those mentioned previously.

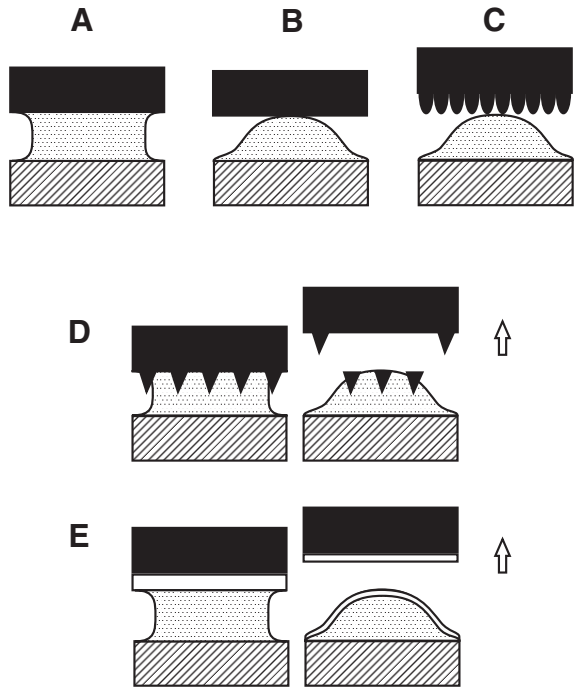

Fig. 1. Diagram of hypothetical interactions between the plant adhesive and the insect cuticle. (A) Cuticle with no anti-adhesive properties. (B) Epicuticle, non-wettable by the *Roridula* adhesive owing to the specific chemistry of the surface. (C) Microstructure preventing adhesion. (D) Easy-to-break solid layer preventing strong bonding of adhesive to the epicuticle. (E) Fluid layer providing cohesion failure. Black area, solid insect surface; dotted area, plant adhesive fluid; hatched area, plant surface; white area, fluid layer. Arrows indicate the direction of movement of the insect surface.

Using a few representative force–time curves, measured on both living *P. roridulae* (N=3) and *C. vicina* (N=3), force–distance curves were calculated to estimate the work necessary to retract the adhering plant trichome from an insect cuticle at a distance of 1.5 mm.

RESULTS

Insects on the surface of *R. gorgonias*

Leaves of *R. gorgonias* are densely covered with capitate trichomes of varying sizes, mainly situated on leaf margins as well as on adaxial and abaxial mid veins. All instars of *P. roridulae* bugs moved easily on the adhesive glandular plant surface (Fig. 3A–D). To attach to the plant during locomotion, mirid bugs either hooked themselves to single trichomes using claws or attached to the flat leaf surface between the trichomes apparently by using their adhesive pads, called pseudopulvilli. The body and especially the legs were held close to adhesive secretion droplets and contacted them frequently without being trapped. However, representatives of Homoptera, Lepidoptera, Coleoptera and Diptera were observed to be caught immediately after touching the plant surface, always with either the ventral or lateral body surface. An individual of *D. melanogaster*, for example, can be trapped by a single capitate trichome (Fig. 3E). Prey insects that struggled intensively to free themselves after capture were held by sticky secretion droplets of two or numerous trichomes. Insect activity apparently effected an increase in the number of droplets in contact with the body of the insect (Fig. 3F,G).

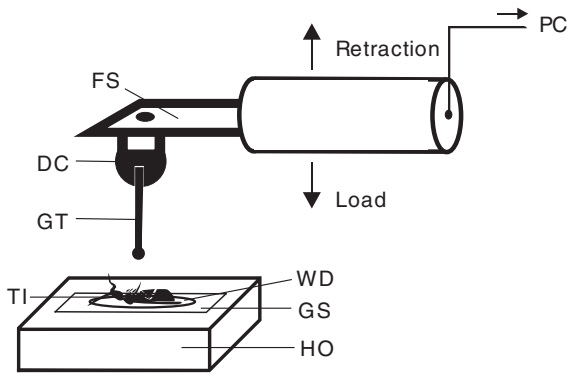

Fig. 2. Experimental design for adhesion force measurements of single droplets of the adhesive secretion in tentacle-shaped trichomes. The living or dead test insect (TI), with its dorsal side attached to a glass slide (GS) with a droplet of beeswax (WD), is mounted on a horizontal holder (HO). A tentacle-shaped glandular trichome (GT) with a distinct terminal droplet of adhesive secretion is attached to a piece of double-sided carbon tape (DC) adhering firmly to a force sensor (FS). The sensor with the trichome was moved down using a motorized micromanipulator until contact between the droplet and the test surface (insect, glass) occurred at a load of approximately 50 μN, and then the sensor with the trichome was pulled up. The time–force sensor signal was recorded and processed further in a computer (PC).

Properties of the plant adhesive secretion

Shapes of trichome tips

The capitate and tentacle-shaped trichomes of *R. gorgonias* consist of multicellular stalks and multicellular glandular heads releasing the adhesive secretion (Fig. 4). The width in the middle of secretion droplets ranged from 129 to 405 μm (226.4±54.8 μm, mean ± s.d., *N*=120). The consistency of the secretion seems to vary slightly between the very long, tentacle-shaped and shorter capitate trichomes. The latter possess round, apparently more viscous, stable droplets (Fig. 4B,C) that appear rough in the cryo-SEM preparations (Fig. 4D). The tips of the longer tentacle-shaped trichomes are equipped with ovoid, apparently less viscous, secretion droplets, having a smooth surface at high magnification (×1000) in the cryo-SEM (Fig. 4E). The adhesive fluid of different trichomes can spread over the trichome stalk (Fig. 4E,F) and even the leaf lamina (Fig. 4B). Furthermore, we observed fluid filaments of up to 5 cm in length into which the secretion, in particular those produced by the tentacle-shaped trichomes, can be pulled after contacting a surface (Fig. 3F,H, Fig. 4A).

Solubility of the adhesive secretion in different solvents

Treatment of the secretion droplets of the tentacle-shaped trichomes with different fluids demonstrated differences in droplet solubility. The droplets kept their volume and ovoid shape after treatment with aqua millipore (Fig. 5A). After trichome treatment with absolute ethanol, they were deformed, and the secretion was partially removed such that some intact, convex glandular cells could be seen (Fig. 5B,C).

After treatment with chloroform, fibrous residues of the glandular secretion covering the trichome tips were found (Fig. 5E). The stalk and gland of the trichome collapsed (Fig. 5D). Treatment of the trichome with acetone resulted in total removal of the glandular secretion from the trichome tip. The terminal gland with its opening was then clearly visible.

Contact behavior of the adhesive secretion on different substrates

Intact droplets were manually attached to insect cuticles or metallized epoxy-resin surfaces. Contact formation between the glandular tip of tentacle-shaped trichomes and *P. roridulae* was hardly detectable. The secretion spreads on the surface of the mirid bug (Fig. 6A,B). However, on single bug setae, residual round droplets of the secretion were observed (Fig. 6C). In contrast to the mirid bug, the surface of the fly *C. vicina* induced formation of round, droplet-shaped patches with a distinct boundary (Fig. 6D–F). Similar droplets formed on the surface of epoxy resin coated with Au–Pa (Fig. 6G,H). Furthermore, numerous flat micro-droplets were observed when the tip of a tentacle-shaped trichome briefly contacted the metallized surface (Fig. 6I).

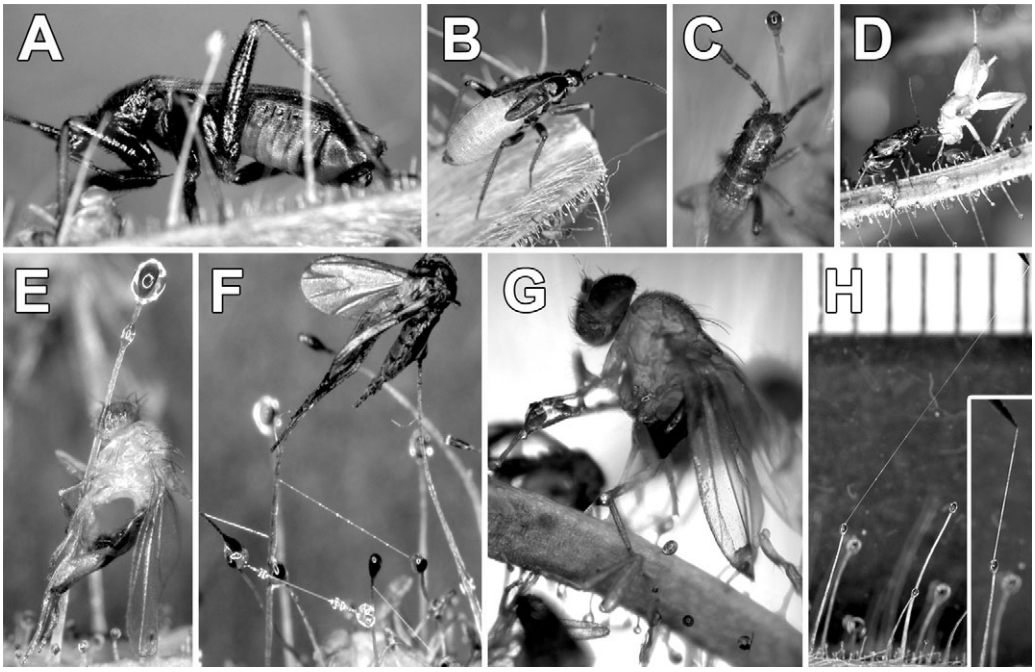

Fig. 3. (A–D) Digital images of *Pameridea roridulae* walking on leaves of *Roridula gorgonias* covered with glandular trichomes: (A) adult *P. roridulae*; (B) fifth-instar nymph; (C) first-instar larva; (D) adult sucking on a stuck cricket. (E–G) Prey insects adhering to the secretion: (E,G) ventrally trapped *Drosophila* sp.; (F) laterally trapped fly of the family Sciaridae. (H) Two images of adhesive secretion filaments pulled away with a needle from the tip of tentacle-shaped trichomes; scale units: millimetres.

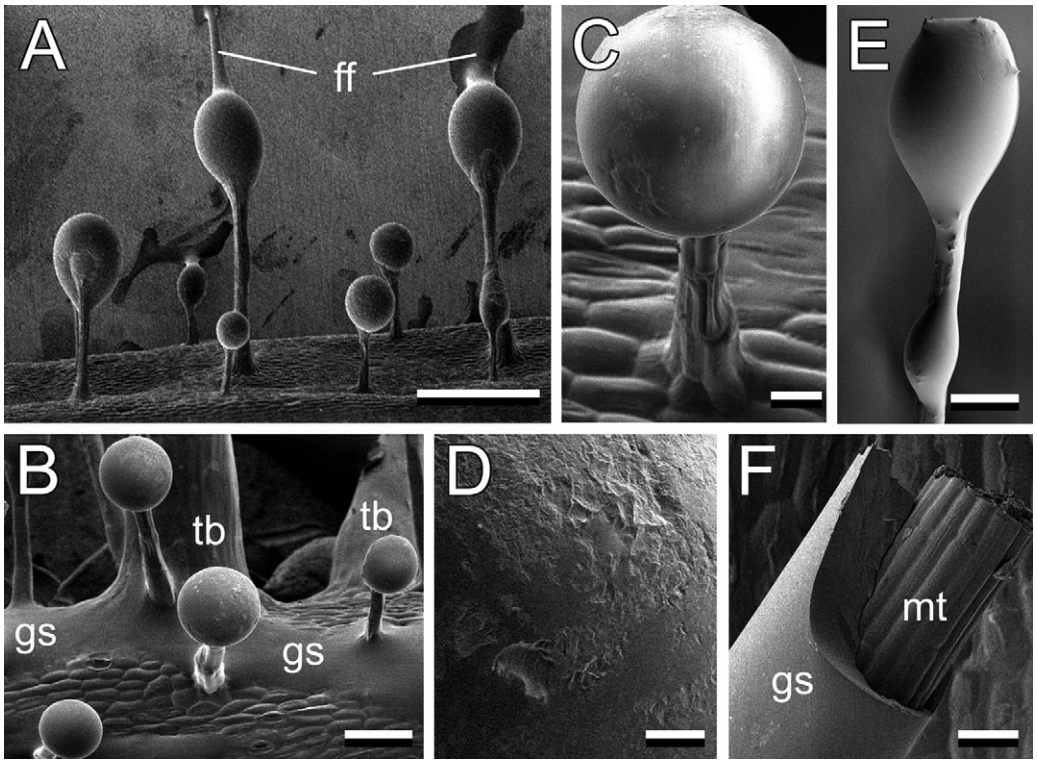

Fig. 4. Cryo-SEM micrographs of glandular trichomes and their adhesive secretion in *Roridula gorgonias*. (A) Leaf margin bearing capitate trichomes of varying lengths with glandular secretion droplets, partly adhering to the metal surface of the sample holder and partly transformed into fluid filaments. (B) Short capitate trichomes against a background of multicellular bases of tentacle-shaped trichomes, and a layer of glandular secretion on the leaf epidermis. (C) Short capitate trichome on the abaxial leaf lamina consisting of a large spherical, glandular head, covered by adhesive secretion, and a multicellular stalk. (D) Detail of the surface of the glandular secretion released by short capitate trichomes. Note the uneven profile of the fluid surface. (E) The tip of a long, tentacle-shaped trichome releasing an ovoid secretion droplet with a smooth surface. Note that the secretion runs down along the stalk of the trichome. (F) Detail of the fractured multicellular stalk of a tentacle-shaped trichome covered with a thick layer of glandular secretion. ff, fluid filaments; gs, glandular secretion; tb, base of the tentacle-shaped trichome; mt, multicellular trichome. Scale bars, 500 μm (A); 100 μm (B,E); 20 μm (C,D,F).

The adhesive force of the plant secretion on different surfaces

The measured adhesive force depended on the surface to which the tentacle-shaped trichomes were brought into contact (Fig. 7). The lowest values were obtained on living mirid bug cuticle, and the highest ones on the cuticle of living and dry flies, as well as on the cuticle of chloroform-washed mirid bugs. These differences were statistically highly significant. During the experiment, secretion frequently formed filaments with lengths ranging from 2 to 51 mm

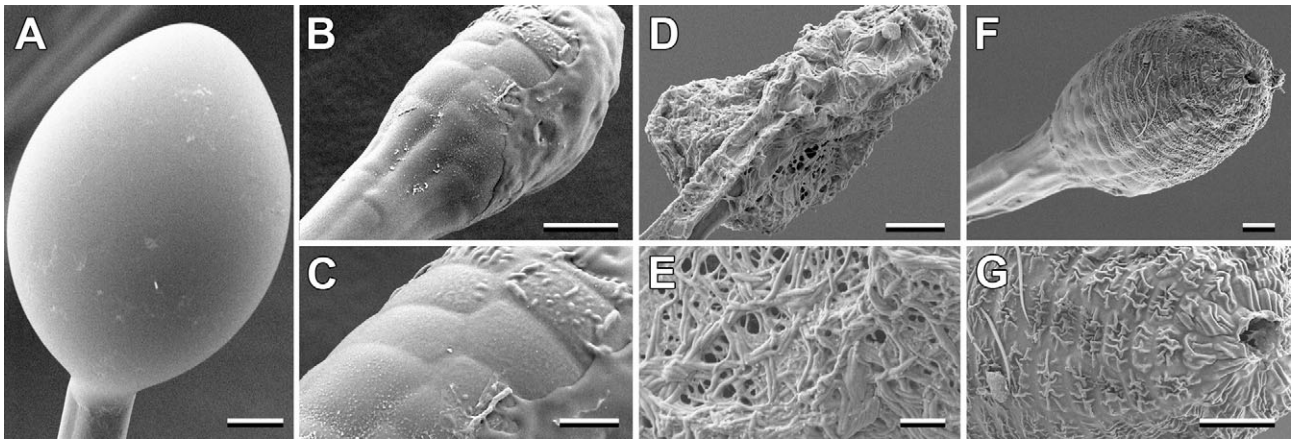

Fig. 5. Cryo-SEM micrographs of the glandular tips of tentacle-shaped trichomes in *Roridula gorgonias* after different treatments. (A) Intact voluminous, ovoid droplet of adhesive secretion after washing with aqua millipore. (B,C) Terminal gland covered with a fragmented layer of adhesive secretion after rinsing with ethanol. (D,E) Collapsed terminal gland with adhering fibrous formations of adhesive secretion residues after washing with chloroform. (F,G) Terminal gland after washing with acetone: the adhesive secretion is totally removed, and the glandular opening is clearly visible on the tip. Scale bars, 25 μm (A,B,D,F,G); 10 μm (C,E).

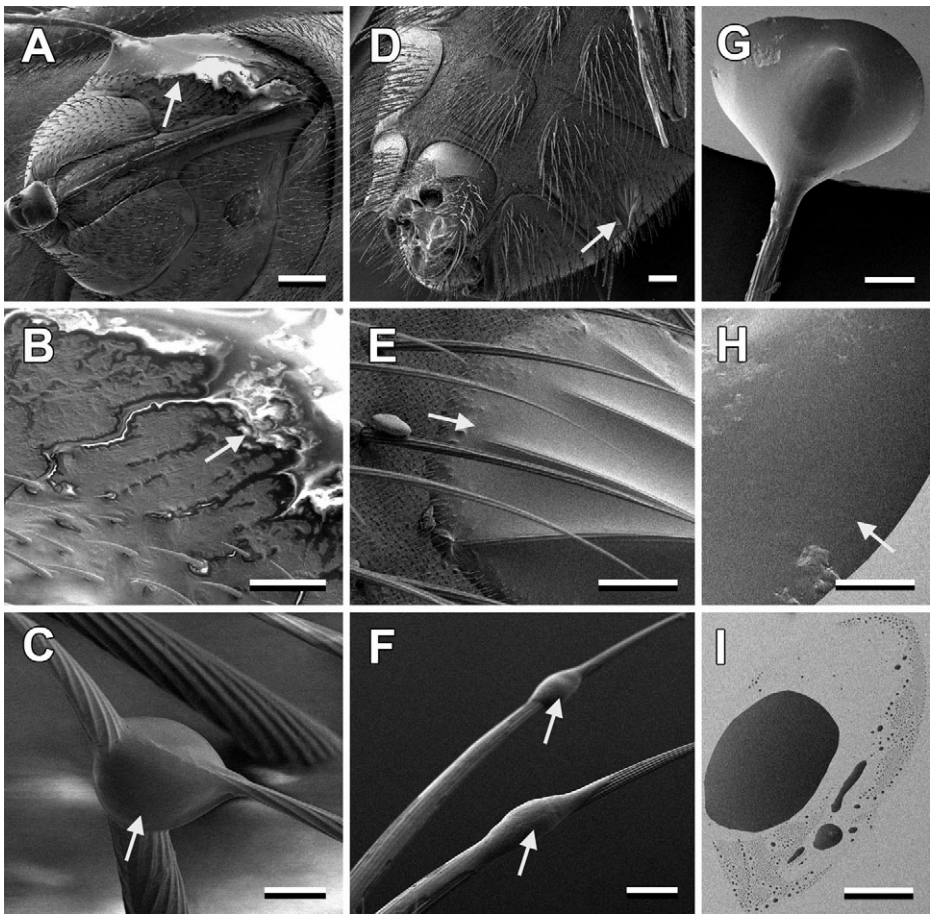

Fig. 6. Cryo-SEM micrographs showing the adhesive secretion of *Roridula gorgonias* in contact with (A–C) the surface of the mirid bug *Pameridea roridulae*, (D–F) the fly *Calliphora vicina* and (G–I) epoxy resin Spurr coated with gold–palladium. (A,B) Secretion droplet on the ventral abdomen of *P. roridulae*. (C) A spherical secretion droplet adhering to bristles in *P. roridulae*. (D,E) Secretion droplet on the ventral surface of the abdomen of *C. vicina*. (F) Adhesive secretion on setae of *C. vicina*. (G,H). Adhesive secretion in contact with a metallized Spurr surface. (I) Numerous micro-droplets left after the removal of a secretion droplet (G,H) from the metallized Spurr surface. Arrows point to secretion droplets on the insect surface. Scale bars, 200 μm (A,D,G); 50 μm (B,E,F,H,I); 5 μm (C).

in 48% of the individual tests (for details, see Materials and methods). The length of filaments (*l*) did not influence the measured pull-off force (F_A) (F_A=158.20–0.68*l*, r^2=0.01, $F_{1,58}$=0.41, P=0.52, linear-regression analysis). The work required to retract the trichome from the insect cuticle was estimated from force–distance curves (Fig. 8). It was much lower (0.07±0.026 J) in living *P. roridulae* than in living *C. vicina* (0.18±0.093 J) (for each species, N=3).

Insect cuticle

Both insect species studied have an integument that bears large setae (Fig. 6A,D) and a layer of much smaller, procumbent microtrichia. When light pressure was applied by a clean glass slide to the insect cuticle, prints of epicuticle grease became visible in the phase-contrast mode of a light microscope (Fig. 9). The cuticle of living and dried *P. roridulae* mirid bugs left a distinct amount of fluid

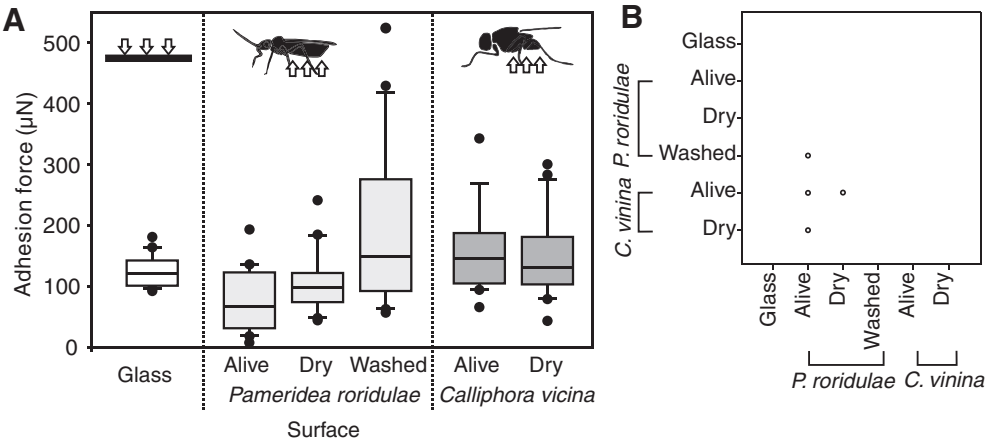

Fig. 7. (A) Box-and-whisker diagram of the adhesion force of single adhesive droplets in the tentacle-shaped trichomes of *Roridula gorgonias*, measured on different surfaces. The ends of the boxes define the 25th and 75th percentiles, with a line at the median and error bars defining the 10th and 19th percentiles. Arrows point to areas of substrates where force was measured. (B) Statistical differences between surfaces (Kruskal–Wallis one-way ANOVA on ranks, $H_{5,119}$=25.317, P≤0.001 and Tukey test, P<0.05).

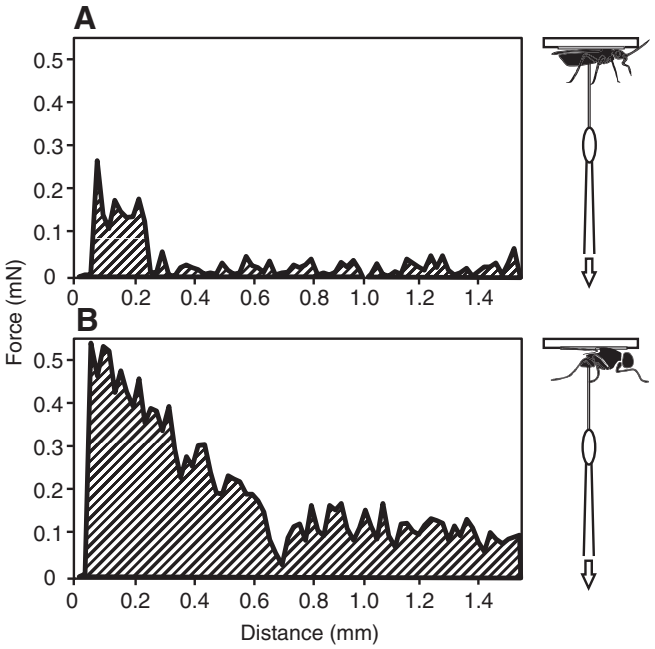

Fig. 8. Examples of force–distance curves obtained by adhering trichomes of *Roridula gorgonias* to the ventral surface of the abdominal cuticle of living insects. The curves were used to calculate the work (shaded areas) that had to be applied to retract the adhering trichomes to a distance of 1.5 mm. (A) Data obtained on *Pameridea roridulae*. (B) Data obtained on *Calliphora vicina*.

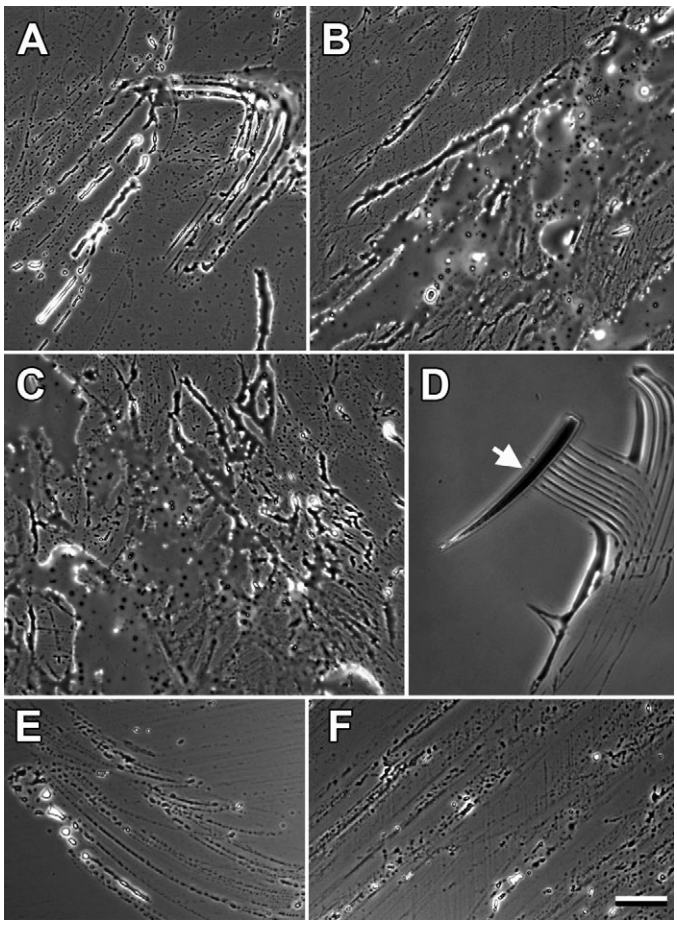

Fig. 9. Phase-contrast light-microscopy images of prints left after pressing an insect cuticle against a glass slide. (A–D) Prints of adult *Pameridea roridulae* showing considerable fluid residues from (A) the head of a living insect, (B) the abdomen of a living insect, (C) the wing of a living insect and (D) the wing of a dry insect. (E,F) Prints of living (E) adult *Calliphora vicina* wing and (F) abdomen, showing only scattered micropatterns of grease residues. The arrow points to a broken bug seta leaving traces of parallel oriented lines corresponding to its helical surface texture. Scale bar, 50 μm.

residues (Fig. 9A–D), in most cases forming a continuous layer on the glass surface. In contrast to the mirid bug cuticle, cuticle prints of living *C. vicina* flies were seen as discrete micro-droplets on the glass slide (Fig. 9E,F).

Cryo-SEM fractures clearly revealed an amorphous epicuticular grease layer on the surface of the cuticle in living and dry *P. roridulae*. This layer covers the entire insect surface, including procumbent microtrichia (Fig. 10A,B,D,E). In living and dry *C. vicina*, such a clear grease layer was not observed. The thickness values of the layer were significantly higher (~30-fold) in living and dry mirid bugs compared with those of living and dry flies (Table 1). In both insects treated with chloroform, the grease layers seemed to be nearly removed (significantly lower thickness values were measured; see Table 1). Furthermore, in chloroform-rinsed mirid bugs, a clean cuticle with clear uncovered microtrichia was found (Fig. 10F, compare with Fig. 10D,E).

DISCUSSION

It has been reported previously that the surface of *R. gorgonias* is very sticky (Marloth, 1910; Barthlott et al., 2004). We showed here that not only the tips of tentacle-shaped trichomes, but also their stalks and the leaf lamina, are mostly coated with an adhesive secretion. This coverage is responsible for the adhesive property of almost the entire plant. The adhesive secretion is released in the form of shiny droplets on the ovoid glandular heads of numerous capitate trichomes of varying lengths. Very long tentacle-shaped trichomes, shorter trichomes and very short capitate trichomes together comprise a three-dimensional trap. Once stuck to a fluid glandular droplet of a long, flexible, tentacle-shaped trichome, an insect will struggle and move, pulling the droplet into a long fluid filament. A moving insect usually remains trapped and contacts further droplets from the long tentacle-shaped trichomes. The arrangement of trichomes in combination with the insect behavior results in an entanglement of the insect prey between trichomes and pulled filaments. Finally, the insects, caught by the presumably more viscous secretion of the stiffer short capitate trichomes, adhere firmly to the plant surface. Our results show that the work required to break contacts between the fluid filaments of the plant and the insect surface is higher in flies than in mirid bugs. This result suggests that struggling flies, compared with *P. roridulae* mirid bugs, must apply considerably more energy to free themselves from the plant.

Captured insects were usually observed sticking ventrally or laterally to the plant surface. Insects initially contact the plant surface with their feet but finally become trapped at their ventral and lateral surfaces, which seem to be more sensitive to the sticky traps.

The presence of long, thin, extensible fluid filaments, caused by the pulling of adhesive droplets, and their ability to recover their shape afterwards, supports previous assumptions about the existence of a caoutchouc-like fluid in this plant (Marloth, 1925; Lloyd, 1934). This behavior indicates that the secretion possesses a viscoelastic property. The trichomes seem to release substances of varying viscosity. The fibrous residues we found on trichome tips after

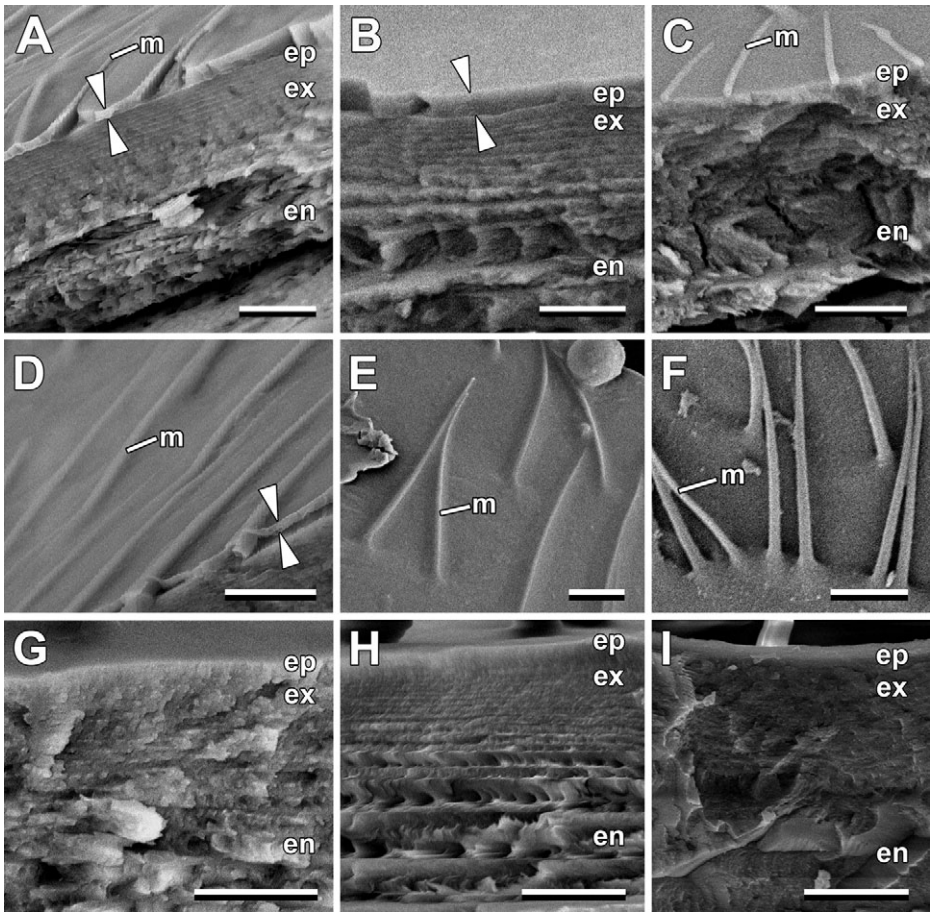

Fig. 10. Cryo-SEM micrographs of the leg cuticle of different insects. (A–F) Adult *Pameridea roridulae* bug. Cross-fractures of (A) a freshly killed and (B) a dead, dry mirid bug show a distinct layer of epicuticular grease. (C) The layer disappears after washing bugs in cold chloroform. Top views of cuticle in (D) a freshly killed, (E) a dead, dry and (F) a dry, chloroform-washed mirid bug. (G–I) Adult *Calliphora vicina* fly. Cross-fractures of (G) a freshly killed, (H) dead, dry and (I) a dry, chloroform-washed *C. vicina*. All preparations are missing a distinct epicuticular grease layer. The arrowheads indicate epicuticular grease layer; m, microtrichia; ep, epicuticle; ex, exocuticle; en, endocuticle. Scale bars, 2 μm.

washing them with cold chloroform might indicate a composite nature of the secretion consisting of a fibrous network embedded in a fluid matrix. Such a composition of the secretion could well explain its behavior as being similar to that of an elastomere with fluid-like behavior. Our efforts in visualizing solvent-treated droplets indicate the presence of a multicomponent substance, partly soluble in ethanol and chloroform and entirely soluble in acetone. These solubility characteristics suggest that the adhesive fluid might comprise various lipids and terpenes (polar resins). Thus, our results support previous observations that the adhesive secretion has a resinous character (Marloth, 1925; Lloyd, 1934). However, we did not observe an acetone-insoluble material, as reported by Marloth (Marloth, 1925). Terpenes in plant secretions have been reported to be released in great diversity and, normally, as a mixture of several terpenes (Michie and Reid, 1968; Schnepf, 1969; Dell and McComb, 1978). Fatty or oily substances have been found as concomitant fluids of essential oils. In the sticky sage *Salvia glutinosa* L. (Lamiaceae), for example, sticky secretions of glandular trichomes contain a large amount of lipids (Schnepf, 1969). In addition to the solvent-treatment experiment, the probable presence of lipid-like components was supported by the particular pattern of droplet prints spreading into numerous micro-droplets on a metallized epoxy-resin surface. The formation of such extremely small fluid droplets is hardly possible for a highly viscous material. That is why we assume that the margins of the large droplets contain oily substances. Also, the affinity of the adhesive secretion to the grease-covered, hydrophobic insect cuticle is an additional indicator of the possible presence of oily substances in the plant adhesive fluid.

The adhesive secretion of *R. gorgonias* has been reported previously to be very viscous and to be the strongest glue of all plant flypapers (Hartmeyer, 1998). In our adhesion measurements, we obtained pull-off forces of the secretion in tentacle-shaped trichomes at the micro-Newton scale. In the protocarnivorous tar flower *Befaria racemosa* Venten (Ericaceae), the stickiness of the

Table 1. Thickness (nm) of the epicuticular grease layer in fresh, dry and dry treated with chloroform *Pameridea roridulae* and *Calliphora vicina* measured from freeze-fractured pieces of the leg cuticle by cryo-SEM

	Freshly killed		Dry		Dry, treated with chloroform	
	Median	Min–max	Median	Min–max	Median	Min–max
P. roridulae	$622.0^{A,a}$	516–713	$557.0^{A,a}$	507–623	$8.3^{B,a}$	8.3–11.7
C. vicina	$20.1^{A,b}$	19.8–29.7	$19.8^{A,b}$	19.8–39.7	$6.6^{B,b}$	6.3–6.6

Different capital letters indicate statistical differences between values in the line (Kruskal–Wallis one-way ANOVA on ranks and all pairwise multiple comparison procedure on ranks, Tukey test, $P<0.05$; *P. roridulae*: $H_{2,29}=22.12$, $P\leq0.001$; *C. vicina*: $H_{2,29}=20.39$, $P\leq0.001$). Different lower-case letters show statistical differences between values in the column (Mann–Whitney rank sum test, for each: $T_{1,19}=155.0$, $P\leq0.001$).

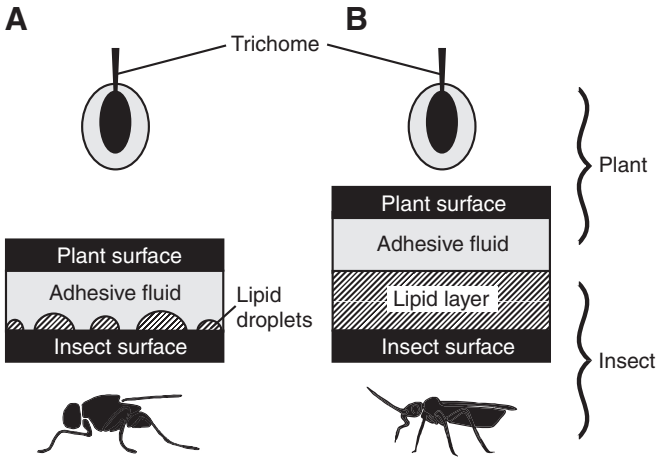

Fig. 11. Diagram demonstrating hypothetical interactions between the adhesive fluid of the plant *Roridula gorgonias* and the insect cuticle. (A) A thin greasy film, consisting of many single patches of tiny droplets, as demonstrated for the fly *Calliphora vicina*. Such a surface offers islands of solid cuticle as contact sites for the plant adhesive fluid. (B) A thick grease layer in the cuticle of the mirid bug *Pameridea roridulae* preventing the adhesion of the plant secretion by means of cohesion failure.

viscid secretion corresponded to that of commercial flypapers and ranged between 40 and 50 kPa (Eisner and Aneshansley, 1983). If we assume the width of secretion droplets (~100 μm; see Fig. 6I) to be the contact area, we can estimate the adhesive strength in *R. gorgonias* as being ~13 kPa. Such a discrepancy between our results and those in the literature might be explained by the fact that our experimental design differed drastically from that used in experiments on *B. racemosa*. Eisner and Aneshansley (Eisner and Aneshansley, 1983) measured the pressure-dependent adhesive strength on the fluid trapped between two flat surfaces, whereas we estimated the adhesion strength of a single ovoid droplet slightly brought into contact with the insect or glass surface and then retracted from it. Direct comparison of these two approaches is not possible because of the strong differences in the fluid thickness (a thinner adhesive layer attaches two bodies much more strongly), which was not measured in both studies. Our experiment was designed to simulate the natural situation in which insects contact the surface of *R. gorgonias* (usually through long tentacle-shaped trichomes) and then pull the adhesive secretion into the form of filaments. This is also the reason why we used long tentacle-shaped trichomes in our experiments.

P. roridulae bugs move easily and quickly on *R. gorgonias*, adhering neither to trichomes nor to the epidermis. Related mirid species avoid contact with sticky plant fluids by means of a combination of morphological and behavioral adaptations (Southwood, 1986; Voigt et al., 2006a; Voigt et al., 2007), whereas *P. roridulae* bugs seem not to avoid particular plant parts, even regions between the trichomes, and frequently touch the glandular fluid. We observed that they also seldom groom their body, contrary to previous suggestions (Lloyd, 1934; Hartmeyer, 1996; Hartmeyer, 1998). These authors discussed the arrangement of short setae as being the mechanism protecting the mirid bug against adhesives. Our comparative SEM study has revealed that the surfaces of both *P. roridulae* bugs and *C. vicina* flies are covered with cuticle outgrowths of different origins. Adhesive droplets adhere to cuticle protuberances of both mirid bugs and flies. However, flies are caught by *R. gorgonias*, whereas mirid bugs are not caught. Thus, we presume that the hairy covering of the surface is not the main reason for the resistance of the bug to the sticky secretion. The present study shows that *P. roridulae* bugs employ a different mechanism to resist plant adhesion.

Our experiments show that the adhesion force was significantly lower on surfaces of live and dead *P. roridulae* bugs compared with that of live and dead *C. vicina* as well as compared with that of chloroform-washed mirid bugs (the latter still bear a hairy covering). That is why our results support the assumption of Lloyd (Lloyd, 1934) that mirid bugs release some substances repelling the adhesive secretion of the plant. Even if the greasy layer does not really repel the adhesive secretion but rather seems to have a strong affinity for it (the adhesive wets the surface of the bug), this layer sloughs off easily (cohesion failure), preventing trapping of the bug. According to both light-microscopic data on cuticle prints of *P. roridulae* and to the cryo-SEM results showing the distinct amorphous layer on the surface of the epicuticle of *P. roridulae*, we hypothesize that an epicuticular, greasy substance, removable with chloroform, is responsible for the anti-adhesive property of the mirid bug surface. Lipid-containing epicuticular grease has been reported previously from other arthropods: *Calliphora erythrocephala* Meigen (Diptera, Calliphoridae) (Wolfe, 1954), *Rhodnius prolixus* Stål (Wigglesworth, 1933) (Heteroptera, Reduviidae), *Periplaneta* sp. (Blattaria, Blattidae) (Beament, 1945; Beament, 1958; Gilby and Cox, 1963), *Acheta domesticus* L. (Orthoptera, Gryllidae) (Hendricks and Hadley, 1983), *Locusta migratoria* L. (Orthoptera, Acrididae) (Vötsch et al., 2002), *Leptinotarsa decemlineata* Say (Coleoptera, Chrysomelidae) (Tower, 1906), Cicindelinae (Carabidae, Coleoptera) (Stegemann, 1929), *Boophilus microplus* Canestrini (Acarina, Ixodidae) (Gilby, 1957) and *Cupiennius salei* Keys (Araneae, Ctenidae) (McConney et al., 2007). Such a covering has been extracted from the fresh cuticle of the cockroach either as hard wax, mobile grease (Beament, 1945; Beament, 1958) or as a relatively fluid lipid layer hardening through chemical reactions (Gilby, 1962; Gilby and Cox, 1963). In *Periplaneta* sp., the polar, reducing-agent material spreads over the entire surface (Lees and Beament, 1948). Epicuticular grease has been considered to play a fundamental role in limiting water loss (e.g. Ramsay, 1935; Neville, 1975) and serving as a behavioral cue for insects (Dubis et al., 1987; Espelie et al., 1991). Its thickness previously has been suggested to be at a molecular scale (Beament, 1955), varying from less than 1 nm to several microns (Hendricks and Hadley, 1983), or ranging from 0.1 μm to 1.0 μm (Locke, 1964). In the hunting spider *Cupiennius salei*, the thickness of the surface viscous layer has been quantitatively estimated to be 20–40 nm (McConney et al., 2007). Atomic force microscopy of elytra of the Colorado potato beetle *L. decemlineata* revealed a grease layer with a thickness of ~8 nm (Gorb et al., 2008; Voigt et al., in press). Our measurements of grease thickness on leg cuticle in flies and mirid bugs confirm the previously suggested nanoscale dimensions. In *C. vicina*, a thin layer ranging from 19.8 to 29.7 nm was found. Remarkably, the grease thickness in *P. roridulae* is much more prominent, ranging from 516 nm to 713 nm. This layer covers the surface of the mirid bugs, embedding also their microtrichia.

Assuming that the epicuticular grease appears lipid like, we suggest that it functions as a layer causing cohesion failure when the plant adhesive contacts the surface of the mirid bug (Fig. 11). This means that plant adhesive secretions can adhere to the layer of grease, but, under pulling force, the grease layer breaks apart, preventing adherence to the underlying solid cuticle. On the surface of the fly, the very thin, greasy coverage presumably consists of fragmentary patches where the sticky plant secretion can get into

contact with the solid epicuticle by filling the gaps of the discontinuous greasy layer (Fig. 11A). The 30-fold thicker epicuticular layer of the surface of the mirid bug prevents contact formation between the plant adhesive and the bug cuticle (Fig. 11B). The microtrichia, submersed in the grease layer, might be interpreted as a microstructure maintaining the thickness of the layer. The sticky droplets cannot penetrate the epicuticular grease of the mirid bug. As a result, these mutualistic mirid bugs cannot be trapped by the plant.

Conclusion and outlook

An adhesive secretion enables the *R. gorgonias* plant to capture effectively a large number of prey insects. The secretion exhibits adhesive properties, presumably differing depending on the type of trichome. Further mechanical characterization of the adhesive secretion combined with the analysis of the chemical composition of secretion from different trichomes will aid in further understanding the trapping strategy of protocarnivorous *R. gorgonias*.

The mutualistic mirid bug *P. roridulae* bears a thick, anti-adhesive greasy layer, which is a 'smart' surface adaptation for living on the strongly adhesive surfaces of the plant. The anti-adhesion mechanism of the mirid bug is based on the cohesion failure caused by grease. The non-continuous, patch-like pattern of grease found in potential dipteran prey does not provide sufficient protection against the plant adhesive. Additionally, the presence of specific substances in the bug grease targeted to prevent adhesion of *Roridula* secretion cannot be entirely excluded. In future studies, this mechanism should be compared with that of the cuticle structures of various Heteroptera. Possibly, similar adaptations might be found in other insect–plant associations. Finally, the cohesion failure anti-adhesive mechanism might be potentially interesting for technical developments of novel biologically inspired surfaces.

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