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Crystalline wax coverage of the imaginal cuticle in *Calopteryx splendens* (Odonata: Calopterygidae)

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ABSTRACT

In this study we use high resolution SEM to describe the diversity of wax crystals and their distribution on different morphological structures in male individuals of *Calopteryx splendens*. The entire cuticle surface of this damselfly, with the exception of ommatidia and ocelli, is covered with crystalline wax in dimensions from sub-micron to micron range. It is shown that shape – rod-like, plate like, filamentous, etc. –, size, and density of crystals vary on different surfaces and in individuals of different ages. Additionally, we demonstrate different types of damage to the crystalline wax layer: scratches, compressions, wear, and contamination. The primary function of the wax crystalline coverage in odonates is, presumably, reduction of surface wettability by water (superhydrophobicity). However, other functions are also discussed, especially in such specialized body areas as postero-ventral parts of male abdomen, the so called ‘lantern’.

INTRODUCTION

It has been previously shown that pruinosity in Odonata is based on the microsculpture related to tiny wax crystals covering some areas of the cuticle (Gorb 1995). Such coverage can consist of platelets on the abdomen in libellulids, or filaments on axillary sclerites and on antero-dorsal surface of pterothorax in the calopterygid *Mnais pruinosa* Selys and the euphaeid *Bayadera indica* (Selys). It is generally accepted that pruinosity is a type of structural coloration (Parker 2000) that is likely to be involved in species and mate recognition in Odonata. The mechanism of such structural coloration is based on a light scattering effect.

More recently, pruinosity has been described as a dust-like wax crystalline coverage on both sides of the wing in the coenagrionids *Coenagrion puella* (Linnaeus) and *Pyrrosoma nymphula* (Sulzer) (Gorb et al. 2000). At the ultrastructural level, this wing vein coverage resembles the filamentous pruinosity found in various areas of the body of other damselflies. The wing membrane coverage consists of shorter crystals ranging in width from 30 to 100 nm. Because of the small dimension of these crystals – far below visible light wavelengths –, the wing membrane looks transparent to our eye. Having this in mind, we hypothesized that the entire odonate body might be covered with these crystals, which would explain the extreme unwettability of adult dragonflies and damselflies.

This study was undertaken to test the above mentioned hypothesis. We used a scanning electron microscope (SEM) at high resolution to describe the observed diversity of wax crystal structures and their spatial distribution on different morphological structures in male individuals of the calopterygid *Calopteryx splendens* (Harris). In addition, to determine the effect of male age on wax crystal structure and spatial distribution, we investigated males of three age groups: just emerged immature males, young mature males, and old mature males. Based on the wax coverage microstructure, possible functions, such as reduction of surface wettability, prevention of adhesion to sticky traps, modification of surface reflectivity, and involvement in color pattern formation are discussed.

MATERIAL AND METHODS

Study species

Male *Calopteryx splendens* were collected from central Finland (River Niemenjoki; 62°15 N, 26°19 E), during their flying season in 2007 and 2008. We captured males of three age groups for our study: (1) a recently emerged (teneral) immature male whose epicuticle was not yet hardened and thus had presumably emerged during the day of collection ($n = 1$); (2) young mature males which appeared at the river after a few days of teneral phase ($n = 3$); and (3) old mature males which had been at the river for at least 10 days after their teneral phase ($n = 2$). Age of oldest males was determined by marking and releasing young mature males with a unique three letter code on their hind wings. When samples were collected, individuals were captured with an entomological net and killed by freezing at -20°C for 0.5 h (young mature and old mature males, 2007) or by decapitating (one immature male, 2008). We did not use the vapour of strong solvents such as Chloroform and Ether in order to prevent damage to cuticle wax crystals. Insects were subsequently air dried at room temperature ($+21^{\circ}\text{C}$) and used for scanning electron microscopy (SEM).

SEM

Desired pieces of the cuticle were removed using forceps and fine scissors, then mounted on aluminum holders, sputter-coated with gold-palladium (10 nm), and examined under a SEM Hitachi S-4800 at 1-3 kV. Low accelerating voltage was used while operating at high resolution in order to prevent damage of fragile crystals by the focused electron beam. To obtain a side view of cuticle crystals, the cuticle was broken in some preparations and the fractured surface was exposed to view.

Terminology

Crystals of comparable thickness and different height had a different 'aspect ratio', which is the relationship between the crystal height and crystal thickness. Crystals with different aspect ratio are referred to by specific names in the remainder of the paper. If the aspect ratio was below 5-7 (Figs 1f, g), we called the crystals 'rod-like'. Crystals having a greater aspect ratio were called 'filamentous' (Fig. 1h). Terminology for cuticle protuberances follows Richards & Richards (1979).

RESULTS

Almost the entire cuticle surface of the male of *Calopteryx splendens* was covered with crystalline wax of a micron and submicron range dimension. Descriptions of the wax coverage for different body parts are given below.

Head

Head was completely covered with crystalline wax except for ommatidia of compound eyes, ocelli, and contacting zones of mouthparts. Head was also covered by cuticular knobs or papillae, short cuticle outgrowths sparsely distributed in a hexagonal pattern (Figs 1a, b). Their size ranged from 3 to 10 μm and distance between them was 6-15 μm . Wax crystals were rod-like (for a description see next paragraph). In the case of hair sensilla (setae), only their sockets, i.e. round structures at hair bases responsible for hair mobility, were partly covered by wax crystals; hair shafts usually remained smooth (Fig. 1b).

Thorax

Cuticle of dorsal surface of thorax was also covered with cuticular knobs/papillae (Figs 1c, h). Different parts of the dorsal thoracic cuticle had wax crystals of different aspect ratios, both rod-like (Figs 1f, g) and filamentous (Fig. 1h). Rod-like crystals usually did not build clusters, whereas filaments were often bundled into groups and even fused together, the latter building compact layers of crystals (see below). At the top of the knobs some traces of wear were visible: some islands of the cuticle lacked wax crystals (Fig. 1h).

Shafts of thoracic hair sensilla were not absolutely smooth, but rather covered with short, sparsely distributed wax platelets (Figs 1d, e). Plate width and height were in the range of 100-500 nm at a thickness of 30-100 nm.

Ventral side of thorax contained either flat cuticle covered with filamentous crystals (Figs 2a, b, f-h), or folded cuticle covered with short thick wax crystals (Figs 2c-e). Filaments were often clustered (Figs 2a, b), but very long filaments built an apparently gapless coverage (Fig. 2h). Long filaments were often fused together especially in older individuals (Fig. 2g). In areas of folded cuticle wax crystals were considerably thicker (200-300 nm) (Figs 2d, e) than single filaments (30-100 nm) (Fig. 2h). It seems that such thicker crystals were built by a few single crystals fused together. In folded areas of cuticle, platelet-like crystals (up to 1 μm wide) were also found (Fig. 2c).

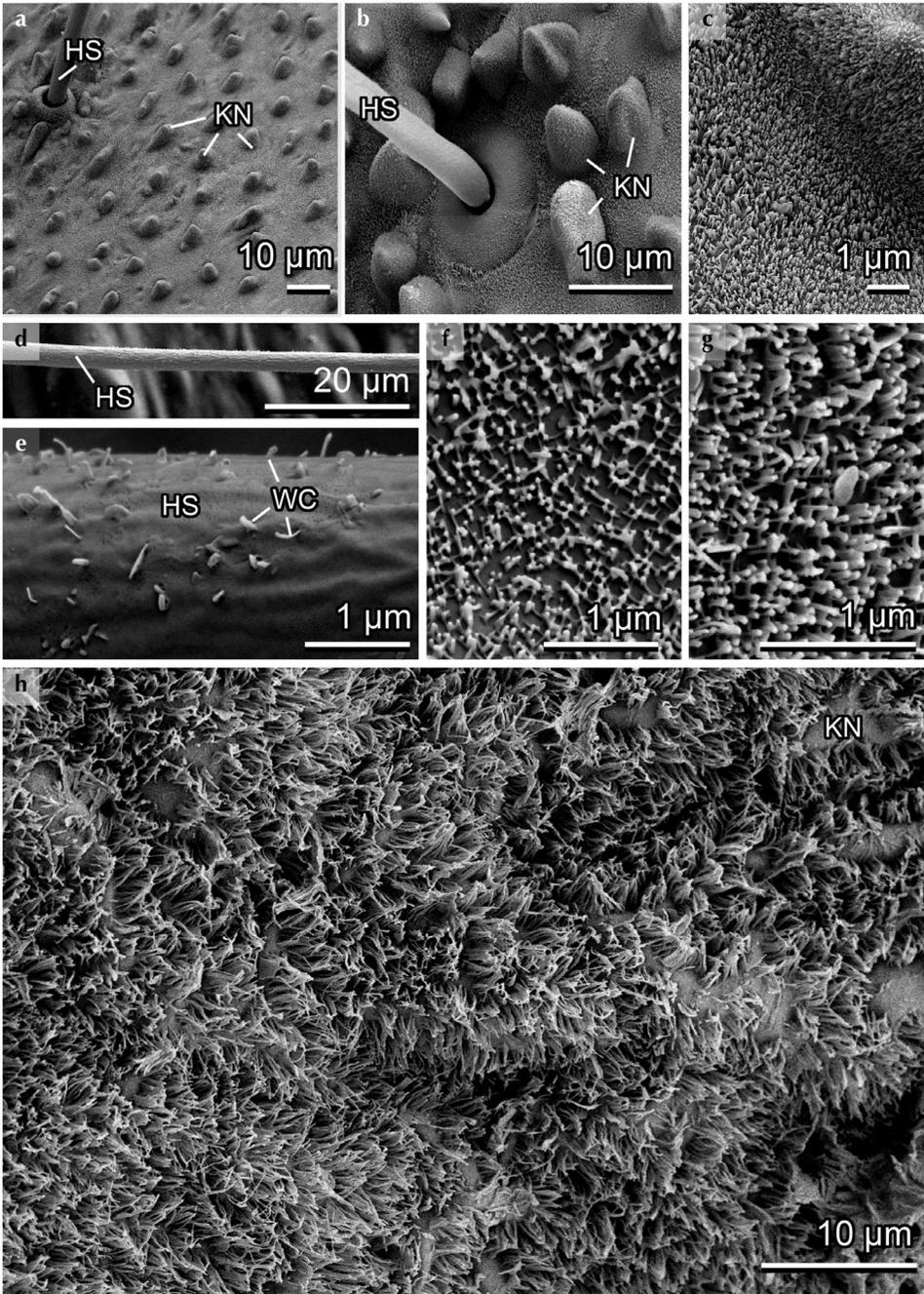


Figure 1: Cuticle surface of *Calopteryx splendens*, young mature male — (a, b) head; (c-h) thorax dorsally; — (a, b, d, e) hair sensilla; (c, f-h) cuticle surface. HS: hair sensilla; KN: cuticle knobs or papillae; WC: wax crystals.

Wings

Wings were covered with densely packed, rod-like crystals, oriented more or less perpendicular to cuticle surface (Figs 3a, b, h). Some crystals built platelets, which were often curled (Figs 3b, d). Some of them were remarkably elongated (Fig. 3e). Wing veins, the largest of which often contained spines, were also covered with rod-like or filamentous crystals (Fig. 3f), so that the entire wing was dorsally and ventrally completely covered with wax crystals of which only the dorsal side is shown here. The wing membrane in side view (Fig. 3h) showed single crystals of different lengths and approximately the same thickness. Single crystals often built 'duets' or groups, consisting of multiple fused crystals.

In older males, there were some traces of damage in the wax crystal coverage of the wing. Tips of vein spines were abraded from the coverage or contained coverage with crystals smeared into a homogenous wax layer (Fig. 3f). Similar kinds of damage were also found on wing membrane (Figs 3b, g). When wax was completely removed, tiny pore channels in wing membrane were seen using a lower SE-detector of the microscope (Figs 3b, c). These pore channels were more regular and much smaller, of a diameter of ca 20 nm, than the thickness of single wax rods/filaments.

Legs

Legs contained remarkable wax structure combinations, some of which were not found on other body parts. Basal leg segments from coxa to femur were mostly covered by wax rods, which sometimes were fused into narrow platelets (Fig. 4h). Tibia was mostly covered by wax platelets of different dimensions: small platelets ca 300 nm wide and high, and large platelets from 500 nm to 2.5 μm in width and height (Figs 4b-g). Some regions were covered by a combination of small and large platelets, which constituted two hierarchical levels of coverage (Figs 4b-d). Other regions were completely covered by large platelets, which were often slightly curled (Figs 4e, g). Together with cuticular structures such as acanthae (Figs 4b, c), knobs/papillae (Fig. 4i), and ridges (Figs 4j, k), three levels of microstructure on damselfly legs can be described. Acanthae and knobs are structures originating from one single epidermal cell and do not have a socket at their base. Wax platelets were oriented perpendicularly to cuticle, but due to their dimension and orientation they touched their neighbors and together built a foam-like network on the surface.

Sockets and shafts of hair sensilla (Figs 4a, d) as well as large thorns (Figs 4j, k) were covered with platelet wax crystals. Especially in older males, regions which regularly came in contact with other surfaces bore some traces of wear. Such an example was seen in tibial thorns, which are likely to be used in prey capture and grasping of females (Figs 4j, k). Exposed parts had lost their crystalline wax coverage due to abrasion and compression of wax crystals.

Abdomen

Dorsal surface of abdomen was corrugated, with cuticular folds running parallel or slightly sloped to a plane transverse to the longitudinal body axis (Fig. 5c). Cuticle was covered with short wax rods similar to those found on wing membrane surface (Fig. 5d). However, especially in immature individuals, there was an additional hierarchical level of wax coverage organization. This level consisted of large (0.5-7 μm wide, 0.5-2 μm high), sometimes perforated wax platelets, randomly orientated

on an axis perpendicular to the surface (Figs 5a, b). These crystals did not seem to originate from fusion of single wax rods and filaments, because (1) they embraced wax rods of lower wax layer; (2) they were perforated with rounded holes; and (3) they were corrugated at their margins, but dimensions of these corrugations did not correspond to dimensions of single (or several fused) rod-like crystals (Fig. 5b). In older individuals, large platelets were less evident (Fig. 5d), which might be the result of wear during grooming, contact with other surfaces or with water. Scratches in wax coverage (Fig. 5d) resembled those found on wing membrane.

Ventral surface of abdominal middle region contained cuticular knobs, covered with rod-like wax crystals (Figs 5e-g). Sockets and shafts of hair sensilla were covered with wax crystals. Damage to wax layer was due to abrasion of the crystals (Fig. 5g) or to contamination with some unknown amorphous material (Figs 5h, i).

Ventro-caudal regions of abdomen were covered with some more specialized wax structures. This was the case in the area between S8 and S10, which we call the

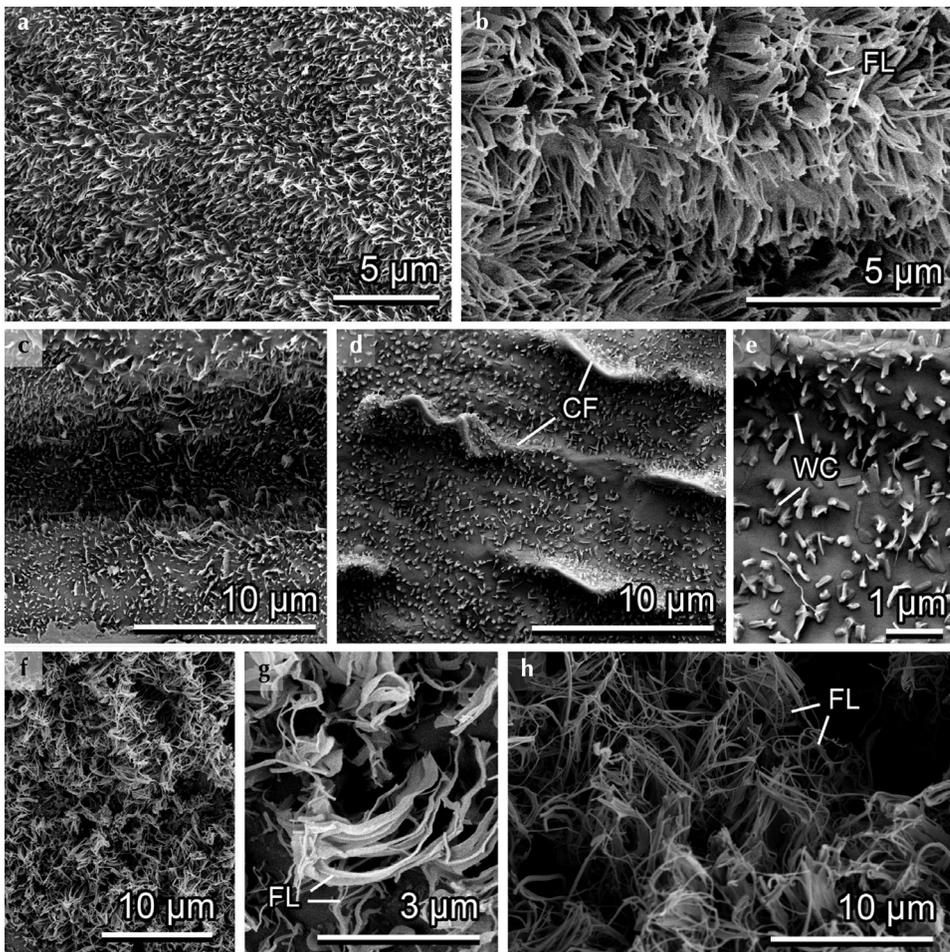


Figure 2: Cuticle surface of *Calopteryx splendens*, male, ventral part of thorax — (a-e, h) young mature males; (f, g) old mature males. CF: cuticle folds; FL: filamentous wax; WC: wax crystals.

lantern area. In *C. splendens* this area was white or pale yellowish, differing from the predominantly blue and dark color of other body parts. S8 in males was covered with cuticular acanthes (Figs 6b, i, j). Wax coverage on S10 was represented by extremely long filaments (3-15 μm ; Figs 6c-f), and this area was white and appeared pruinose to the human eye. Taking into account thickness of single filaments (at an average of 50 nm), their aspect ratio ranged between 60 and 300. However, in some regions of the lantern, single filaments were clustered (Fig. 6d) or fused together (Fig. 6a). Damage to wax layer, as on ventral region, was due to abrasion of the crystals (Fig. 6b) or to contamination of some unknown amorphous material (Figs 6g-h).

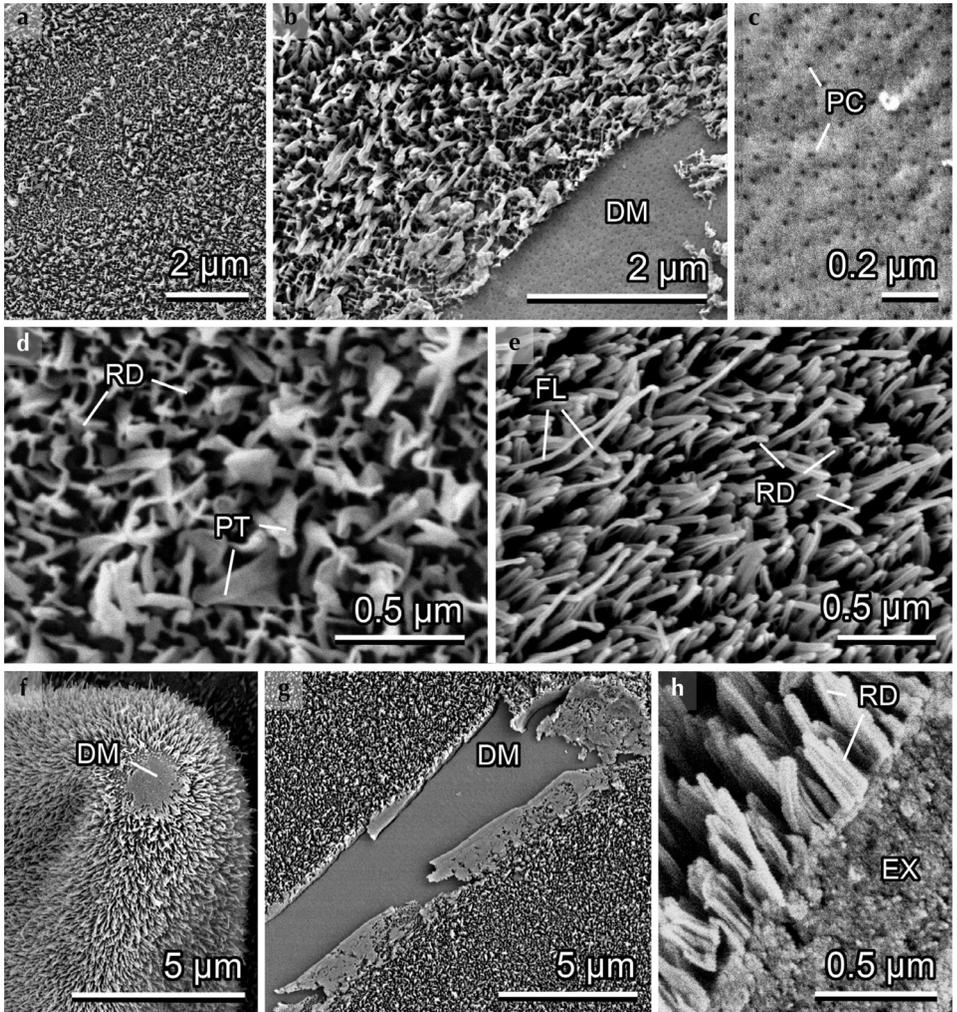


Figure 3: Dorsal wing surface of *Calopteryx splendens*, young mature male — (a, d, e) surface of wing membrane covered with crystalline wax; (b, c, g) wing membrane surface with damage (scratches) in crystalline layer; (f) spine on wing vein; (h) fractured wing membrane, side aspect. DM: damage/scratch; EX: exocuticle; FL: wax filaments; PC: porous channels of cuticle; PT: wax platelets; RD: wax rods; WC: wax crystals.

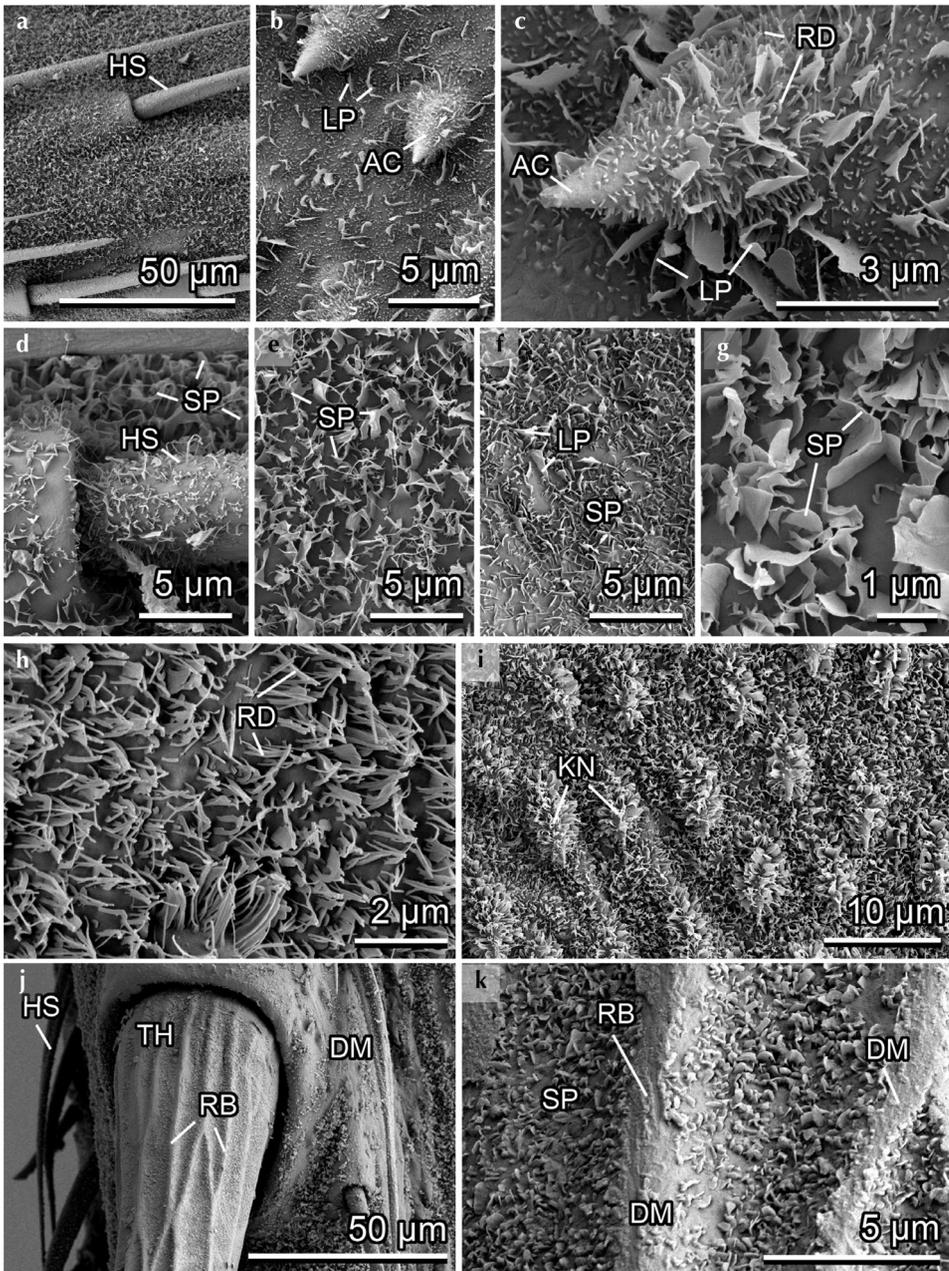


Figure 4: Leg surface of *Calopteryx splendens*, young mature male — (a, d, e, g, i-k) tibia; (b, c, h) femur; (f) tarsus; — (a, d) hair sensilla; (b, c) acanths; (j, k) tibial thorn. AC: acanths; DM: damage/wear; HS: hair sensilla; KN: cuticle knobs (papillae); LP: large plate-like crystals; RB: cuticular ribs; RD: wax rods; SP: small plate-like crystals; TH: thorn.

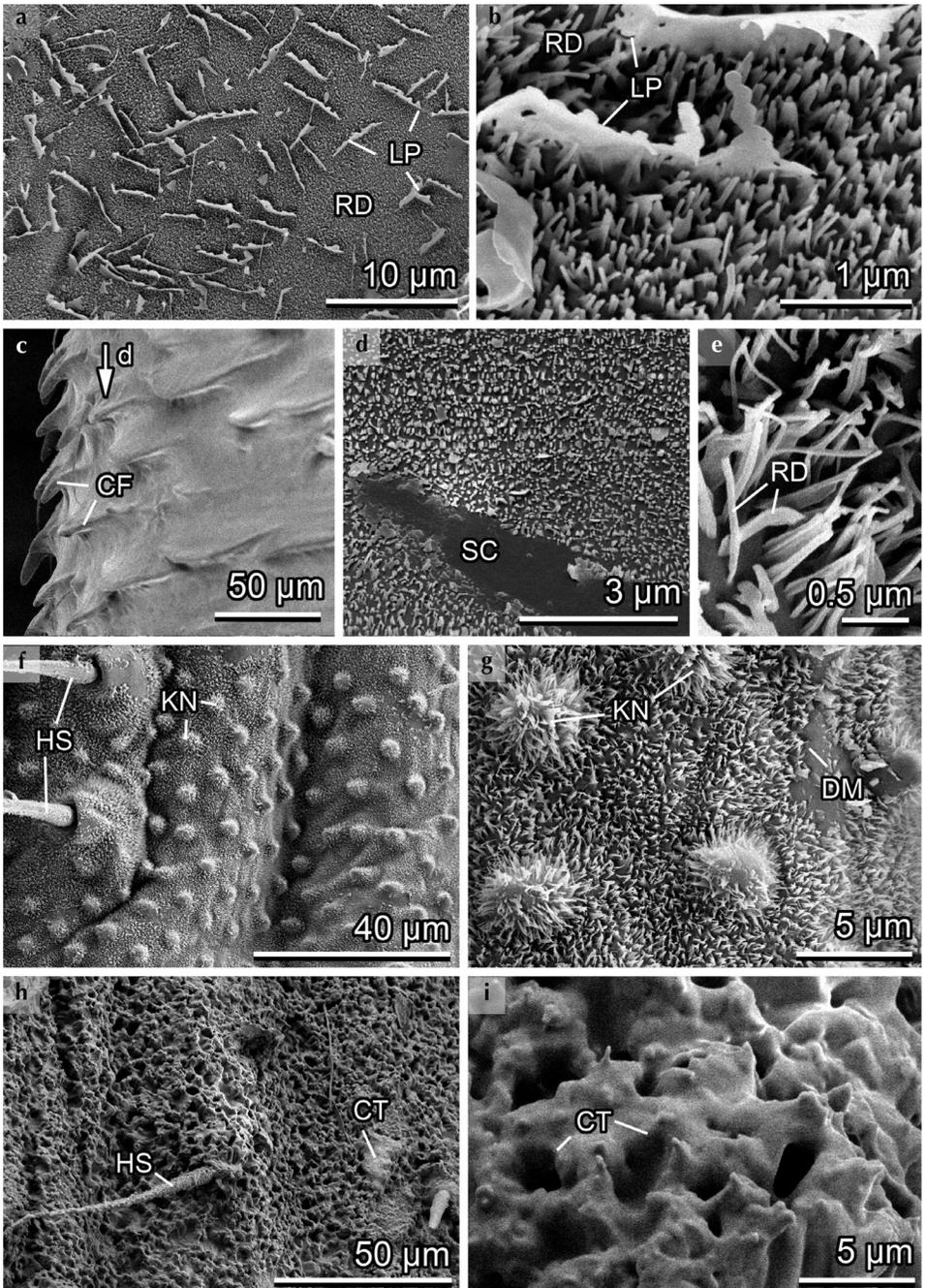


Figure 5: Abdomen surface of *Calopteryx splendens*, male — (a, b) immature male, dorsal; (c, d) young mature male, dorsal; (e-g) young mature male, ventral; (h, i) old mature male, ventral. CF: cuticular folds; d: distal direction (arrow); CT: contamination; DM: damage/wear; HS: hair sensilla; KN: cuticle knobs (papillae); LP: large plate-like crystals; RD: wax rods; SC: damage/scratch.

Surface of male anal appendages contained a regular pattern of cuticle knobs (Figs 7a-c) covered with rod-like (Fig. 7g) or filamentous wax crystals (Figs 7c-e). Cerci were partly covered with wax filaments that were ca 20 μm long (Figs 7d, e). Such slender structures were often clustered together and built a whitish coverage on cuticular surface (pruinosity). Due to continuous use of anal appendages for contact formation with corresponding structures of female, wax coverage was strongly abraded on some surfaces (Figs 7b, e). Some parts of anal appendages were also contaminated with an amorphous material (Figs 7f, h).

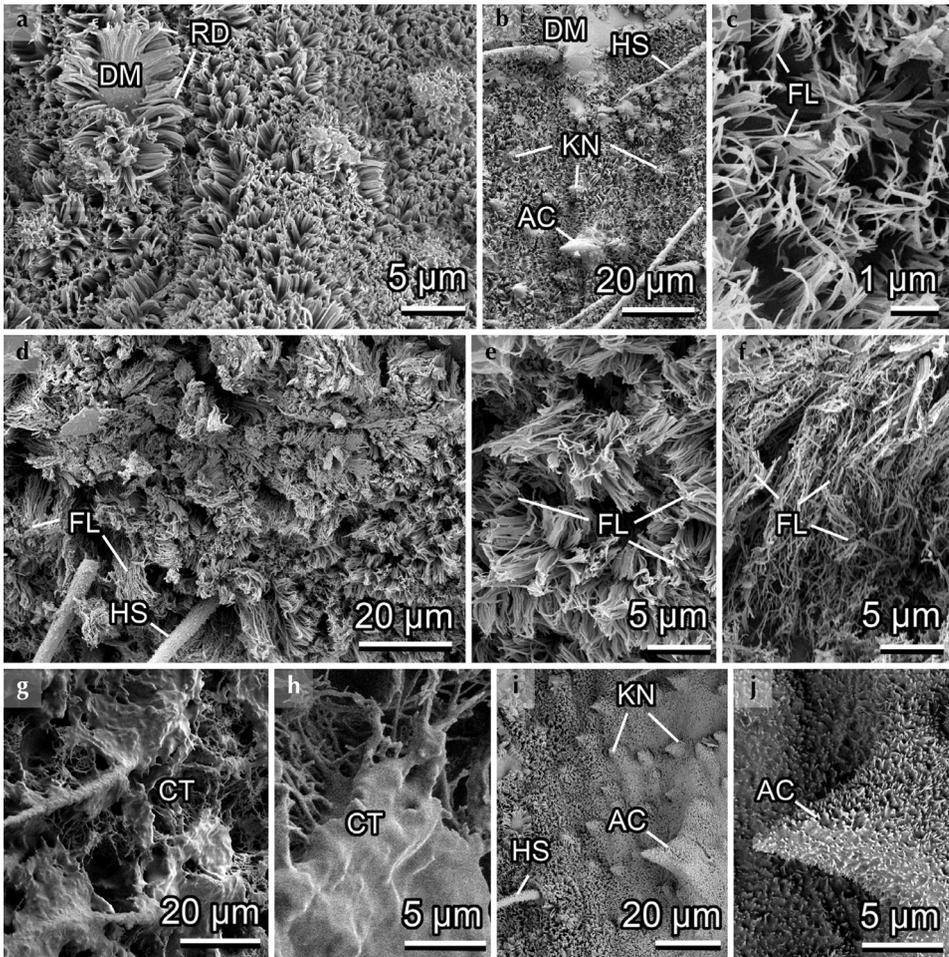


Figure 6: Lantern surface of *Calopteryx splendens*, abdomen ventral — (a-f, i, j) young mature male; (g, h) old mature male. AC: acanthae; CT: contamination; DM: damage/wear; HS: hair sensilla; FL: wax filaments; KN: cuticle knobs (papillae); RD: wax rods.

DISCUSSION

Homology of different shapes of odonate wax crystals

Presence of wax crystals on cuticular surface has been previously described in several different insect taxa, such as representatives of Odonata (Gorb 1995; Gorb et al. 2000), Ephemeroptera (Wagner et al. 1996), Auchenorrhyncha, Sternorrhyncha (Weber 1930), Lepidoptera (Locke 1959, 1960), and Planipennia (Wagner et al. 1996). Wax crystals are very diverse in size and shape. In Odonata, they are extremely small, but they may form quite complex, even hierarchical, structures. Using SEM analysis we can hypothesize homology of different wax crystal structures in Odonata (Fig. 8). We assume a rod-like pattern to be the initial structure, because this pattern (1) was found on almost all studied surfaces of *Calopteryx splendens* (Fig. 8a) and (2) corresponds to the density and distribution of the porous channels of the cuticle. In an earlier study, such pores were found in cross sections of odonate wing cuticle membrane but not on cuticular surface (Gorb et al. 2000). It has been assumed that the material composing the layer of wax crystals is presumably secreted within epidermal cells and transported to the cuticle surface by the system of tiny pore channels (Gorb 1997).

The elongated version of these crystals, here called filamentous (Fig. 8b), may build clusters (Fig. 8c) or fuse into platelets (Figs 8d, h). Similar platelet fusions were also described for rod-like crystals (Figs 8e, f). When filaments become extremely long (aspect ratio over 300), they can build networks (Fig. 8g). Sometimes waxes can build hierarchical structures containing crystals at two levels. In some cases, the larger crystals of the upper level are formed by fusion of single rod-like crystals, but sometimes, especially in immature individuals, they are formed by a substance different than the rod-like crystal material. This substance embeds rod-like crystals of a lower level (Fig. 8i). Different size, shape, and density of crystals, their combinations, and also their combinations with cuticle outgrowths are presumably responsible for different functions.

Some interesting patterns concerning distribution of different types of wax crystals are revealed in this study. First, crystalline waxes were not present on surfaces connected to the visual system (ommatidia, ocelli). This is in contrast to the ommatidia grating of other insects (Parker et al. 1998). Presence of wax crystals might reduce transparency of the surface, despite their small dimensions. This may be of importance in these visually hunting predators.

The second interesting observation is that legs, especially their distal parts, were mostly covered with platelets and not with rods or filaments. These platelets were randomly oriented and interconnected along their sides so that they form a foam-like network, which is presumably more mechanically stable compared to single crystals. Such a mechanical stability might be important on structures continuously coming into contact with other solid bodies, such as plant substrate, prey, or mates.

Damage of crystalline waxes

We studied animals of different ages, and detected traces of damage in older individuals. Damage was of three different types. In the first type, due to wiping off or abrasion which happens when force is applied (Fig. 8k), the underlying cuticle becomes fully exposed. Secondly, when force is applied perpendicularly to the surface

(compression), wax material may be deformed or smeared onto the surface (Fig. 8m). In this case, the cuticle is still covered with wax, but micro- and nanostructure of the surface disappears. The last type of damage is surface contamination, which was mainly observed on abdominal surface and partially on the head (Fig. 8m). The observed amorphous contaminating layer is of unknown nature. An alternative explanation could be that some sort of chemical substances resulted in the fusion of wax platelets and rods.

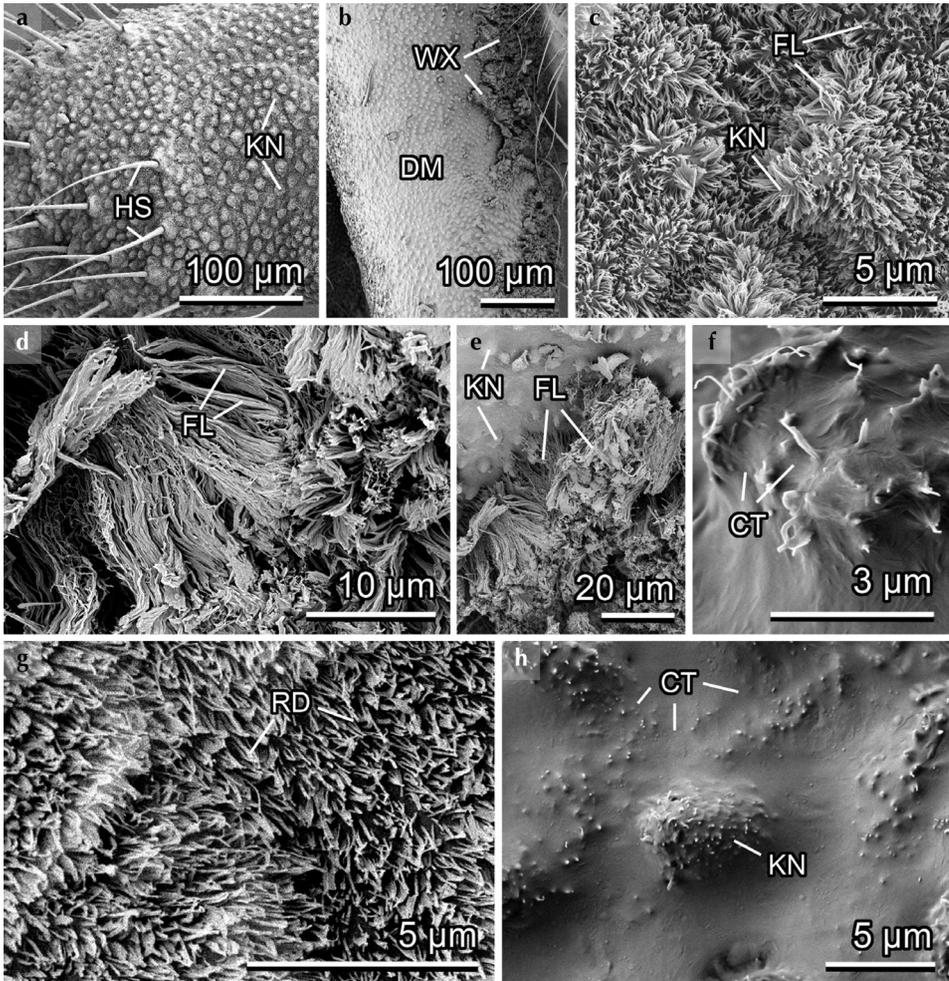


Figure 7: Cuticle surface of anal appendages of *Calopteryx splendens*, abdomen ventral — (a-f) cerci; (g, h) paraprocts; — (a, c, g) immature male; (b, d, e) young mature male; (f, h) old mature male. CT: contamination; DM: damage/wear; HS: hair sensilla; FL: wax filaments; KN: cuticle knobs (papillae); RD: wax rods. Distal part is on the left (a) and bottom (b).

Since damaged surface areas always lacked a next generation of crystals, one may assume that wax crystals are produced once in the damselfly's life and will not be renewed after damage. This means that damaged surfaces will not be able to perform their initial function compared to the surface of freshly emerged individuals. Because such damage accumulates with age, the performance of functions discussed below will be definitely less reliable in older, compared to younger individuals. For example, presence of contamination and removal of the crystalline wax will cause changes in surface chemistry, surface topography, and surface energy and these will influence proper functioning of the surface, for instance, by increasing surface wettability.

Effects caused by crystalline waxes

One possible function of wax coverage is to decrease wettability of the body, which can be essential for insects closely connected to bodies of water during their life-history. Males often defend their territories over water and could be trapped by the water meniscus in the event of contact caused by a wind pulse, rain or a crash during an attack by another male. Males also commonly land on the water surface (floating display) when courting females (Gibbons & Pain 1992).

Although we reported only on males, our preliminary observations show that females also have a wax coverage. Females are likely to benefit from unwettability because many zygopteran species lay their eggs in submerged plant substrate, and in some species, e.g. in our study species *C. splendens*, females can submerge under water to lay their eggs (review in Corbet 1999: 31-32). Thanks to the wax covering, the entire body remains dry, holding a reasonable amount of air that is sufficient to remain submerged for 70 min (*Enallagma hageni* Walsh, Fincke 1986) to 250 min (*Calopteryx cornelia* Selys, Tsubaki et al. 2006). Similar effects have been previously described for a number of plant surfaces (Barthlott & Neinhuis 1997).

The crystalline wax-like wing covering can also prevent wing contamination, as shown by Wagner et al. (1996). Due to surface roughness one may assume that adhesion of sticky material to the odonate cuticle, covered with crystalline waxes, will be reduced, similar to adhesion reduction of insect adhesive feet on plants that are covered with crystalline waxes (Gorb 2001; Gorb & Gorb 2002). Also waxy surfaces might generally reduce adhesion of some sticky substances. A reduced wettability of wing membrane and veins may aid in maintaining rigidity and structural integrity of wings so that the insect can fly after contact with water. Also, since *C. splendens* and many other zygopteran species fold their wings when at rest, the wax might also insure that wing pairs do not become stuck together by surface tension of a water layer between the wings. Previously it has been demonstrated that this effect can be caused by the reduction of the real contact area due to surface microstructure (Peressadko & Gorb 2004) and by contamination of adhesive surfaces by wax crystals removed from the counterpart surface (Gorb & Gorb 2002). Taking all this into account, one may hypothesize that odonate wax coverage may cause adhesion reduction to the sticky capture threads of predatory spiders.

Pruinose surfaces can scatter light, which is polarized under obliquely incident light (Parker 2000). Intensity of the resultant blue is increased when it is viewed against a dark background, such as melanin. Relative sizes of wax crystals determine the shade of blue. If the particles responsible for the scattering coalesce to form particles with a diameter greater than ca 1 μ m, then white light is observed (Mason

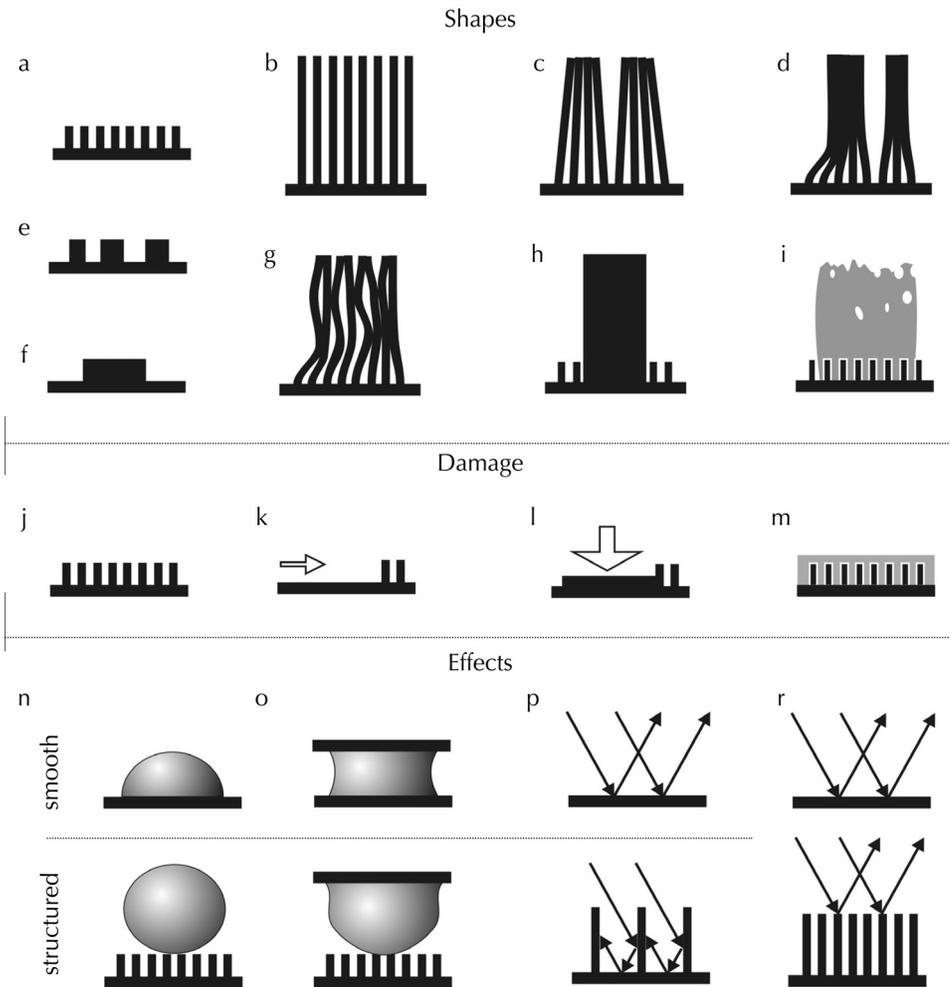


Figure 8: Diagram schematically summarizing shapes of wax crystals (shapes), types of wax coverage damage (damage), and hypothetical functional effects (effects).

Shapes — (a) rod-like wax crystals (low aspect ratio); (b) filamentous wax crystals (high aspect ratio); (c) clustering of filamentous wax crystals; (d-f) fusion of rod-like or filament-like wax crystals and formation of platelets; (g) formation of filamentous networks by crystals with very high aspect ratio; (h) two hierarchical levels of crystals, when large platelets are formed by fused filamentous wax crystals; (i) two hierarchical levels of crystals, when large platelets are formed by a substance differing from the material of rod-like crystals.

Damage — (j) intact wax crystals; (k) abraded crystals caused by action of shear force; (l) plastic deformation of wax crystals caused by compression force; (m) contamination by amorphous substances.

Effects — (n) reduction of wettability; (o) prevention of contamination and adhesion reduction; (p) anti-reflection; (r) changing of surface color (pruinosity). Structured surface is compared with a smooth one.

1926; Parker 2000). In any case, wax coverage by crystals with low aspect ratio and larger distances between them can decrease reflectivity of the surface with only minimal changes of its color or light transmission property (Fig. 8p). If the height of crystals increases and crystals fuse together, surface coloration can also become bluish and whitish (Fig. 8r).

Since wax covering located on thorax or abdomen of odonates can reflect ultraviolet light (Robertson 1984; Hilton 1986) and since dragonflies can recognize a UV coloration pattern (Frantsevich & Mokrushov 1984), we suggest that wax coverage may play a role in intra- and inter-specific interactions. Indeed, it has been previously shown that *Lestes* and *Coenagrion* males, which usually can easily recognize dead females with partly removed body parts, do not recognize females with removed wings (Mokrushov 1992; Gorb 1998). Pruinosity reflecting UV in odonate males probably aids in thermoregulation by reflecting radiation (Paulson 1983; Ubukata 1985). However, no reflectance peaks in wavelengths of UV area were observed in *C. splendens*, at least when reflectance of dried individuals was measured (KT, JSK, SNG, unpubl.).

In *C. splendens* males, there seems to be an especially thick wax filamentous coverage on the ventral side of S10, or in the lantern area, causing pruinosity. This area is interesting because *Calopteryx* species use the lantern in intra- and inter-sexual signaling (Pajunen 1966; Waage 1974; Gibbons & Pain 1992; Corbet 1999: 476-479). The lantern color might also play a role in species recognition since coloration of this area varies greatly between closely related species (see Dijkstra 2006). For example, males of the congener *C. virgo* (Linnaeus) have no pruinosity in the lantern area (KT pers. obs.).

CONCLUSIONS AND OUTLOOK

Odonata are one of the few insect groups having crystalline wax coverage on their body. Because different body parts have different functions, we assumed that the wax coverage must be different on different body parts. Our study documents the strong diversity of wax crystalline coverage within one *Calopteryx* species. We suggest that the structure and density of the coverage correlates with, or at least is adapted to, the functions of different body parts. Further experimental studies are in progress to quantify optical and physico-chemical properties of the wax coverage. These experiments will provide data for assessing the validity of our hypotheses on the functional load of different types of wax coverage.

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