



Microcrystals coating the wing membranes of a living insect (Psocoptera: Psyllipsocidae) from a Brazilian cave

Charles Lienhard¹, Rodrigo L. Ferreira², Edwin Gnos¹, John Hollier¹, Urs Eggenberger³ & André Piuze¹

¹Natural History Museum of the City of Geneva, CP 6434, CH-1211 Geneva 6, Switzerland, ²Federal University of Lavras, Biology Department (Zoology), CP 3037, CEP 37200-000 Lavras (MG), Brazil, ³Institute for Geological Sciences, University of Bern, Baltzerstrasse 1-3, CH-3012 Bern, Switzerland.

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Correspondence and requests for materials should be addressed to C.L. (charleslienhard@bluewin.ch)

Two specimens of *Psyllipsocus yucatan* with black wings were found with normal individuals of this species on guano piles produced by the common vampire bat *Desmodus rotundus*. These specimens have both pairs of wings dorsally and ventrally covered by a black crystalline layer. They did not exhibit any signs of reduced vitality in the field and their morphology is completely normal. This ultrathin (1.5 μm) crystalline layer, naturally deposited on a biological membrane, is documented by photographs, SEM micrographs, energy dispersive spectroscopy (EDS) and X-ray diffractometry (XRD). The crystalline deposit contains iron, carbon and oxygen, but the mineral species could not be identified. Guano probably played a role in its formation; the presence of iron may be a consequence of the excretion of iron by the common vampire bat. This enigmatic phenomenon lacks obvious biological significance but may inspire bionic applications. Nothing similar has ever been observed in terrestrial arthropods.

The observation reported here is an accessory result of the studies of the Brazilian cave fauna initiated by the Federal University of Lavras (Minas Gerais state, Brazil). Of the arthropods collected, members of the insect order Psocoptera have proved to be particularly interesting. Some remarkable new species of the family Prionoglarididae have recently been described¹ and many new species of the family Psyllipsocidae, mostly belonging to the genus *Psyllipsocus* Selys-Longchamps, have been recognized and will be described in the near future. Among the hundreds of individuals of the latter genus sent to the Geneva Natural History Museum for identification there are numerous specimens of *Psyllipsocus yucatan* Gurney originating from 11 different caves situated in 6 Brazilian states (Alagoas, Bahia, Ceará, Espírito Santo, Minas Gerais, São Paulo). *P. yucatan* appears to be one of the most common cave-dwelling psocids in Brazil. This is the only *Psyllipsocus* species previously recorded from Brazil (Pará state, Belém, on walls of an artificial grotto in the Parque Municipal)^{2,3}. *P. yucatan* is also known from Mexico⁴, from where it was originally described⁵. The species is easy to identify using the original description⁵ and the augmented description and key published recently by Mockford³.

P. yucatan shows the typical habitus of a fully winged *Psyllipsocus*, a tiny, weakly sclerotized, light brown insect (body length 1.2–1.6 mm) with two pairs of wings folded roof-like over the abdomen in resting position (Figure 1). The almost hyaline transparent wing membranes are very thin and completely smooth. The Brazilian population of *P. yucatan* that interests us here is from the cave known as "Toca da Tiquara", a dolomitic cave located in the municipality of Campo Formoso, north eastern Bahia state (hereafter called Tiquara cave). The cave entrance is in the bottom of a subsidence sinkhole (Figure 2). The cave is 1010 meters long and comprises three main conduits which are predominantly dry. The only water is in two small pools in the final portion of one of the conduits. Numerous individuals (males, females and nymphs) of *P. yucatan* were collected in Tiquara cave during three biospeleological expeditions between 2008 and 2010. These insects were found living on guano piles, mainly produced by the common vampire bat *Desmodus rotundus* (Geoffroy). The psocids were especially abundant in a chamber located about 100 metres from the cave entrance (Figure 3a, b), and exhibit the normal habitus of *P. yucatan*, as shown in Figure 1.

However, in January 2008, two aberrant individuals of *Psyllipsocus*, with black and completely opaque wings, were captured in this chamber of Tiquara cave (called "black *Psyllipsocus*" or "black specimens" in the following). The purpose of this paper is to document the characteristics of these aberrant specimens by habitus photographs, SEM micrographs and the results of analyses by energy dispersive spectroscopy (EDS) on the SEM and by X-ray diffractometry (XRD). In the final discussion the phenomenon is compared with the present knowledge on solid



Figure 1 | *Psyllipsocus yucatan*, habitus in dorsal view, male from cave "Lapa do Salobo", Arinos municipality, Minas Gerais state, Brazil. Body length 1.5 mm (from head to apex of abdomen).

adherents to cuticular surfaces in terrestrial arthropods, and a hypothesis concerning the origin of this aberrant habitus is presented.

Results

General observations on the specimens examined. Collecting data of the two black specimens of *Psyllipsocus*: 1 male, 1 female, Brazil, Bahia state, Campo Formoso municipality, Tiquara cave (10°27'03"S and 40°32'15"W), 8th January 2008, leg. R. L. Ferreira, on guano piles located in a chamber some 100 metres from the cave entrance (Figure 3). The specimens were captured by hand and immediately transferred into 70–80% ethanol; they were alive when discovered on the guano deposit and did not show any sign of reduced vitality when collected. The male was kept intact in alcohol, except for the slide-mounted terminalia. The wings of the female were consumed during the SEM, EDS and XRD treatment, the remaining parts (head, legs and terminalia) are slide-mounted. The material is deposited in the following institutions: Universidade Federal de Lavras, Departamento de Biologia (Coleção de Invertebrados Subterrâneos), Lavras, Brazil (ISLA); Muséum d'histoire naturelle de la Ville de Genève, Geneva, Switzerland (MHNG).



Figure 2 | Entrance of Tiquara cave, Campo Formoso municipality, Bahia state, Brazil (10°27'03"S and 40°32'15"W).



Figure 3 | Tiquara cave, Campo Formoso municipality, Bahia state, Brazil. (a) Chamber with guano piles wherein the black specimens of *Psyllipsocus yucatan* were collected. (b) Some normal specimens of *P. yucatan* in bat guano at the same place (white circle = nymph; yellow circle = adult specimen).

Light Microscopy. When first examining the black specimens under a stereomicroscope it became immediately evident that the black colour of the wings was not due to pigmentation of the wing membrane but to a uniform black deposit on their surfaces giving them a velvety aspect (Figures 4, 5). This black layer is generally absent on rugose parts of the wings (margin and veins). However, it homogeneously covers the dorsal and ventral surface of the membrane of fore wings and hind wings, except for their basal-most parts and of some limited places where it is slightly damaged (probably due to manipulation of the specimen during collecting and/or sorting in the laboratory, see Figure 4). The remaining body of the specimens is completely clean, except for a few small spots of black substance on dorsal side of the abdomen in the male (Figure 5a). General and genital morphology of the black male and the black female exactly correspond to *Psyllipsocus yucatan*, therefore the black specimens could be assigned to this species, which is common in Tiquara cave (see Introduction). They apparently differ from the normal form of *P. yucatan* only by the presence of the enigmatic black layer on wing membranes.

Scanning Electron Microscopy. Figure 6a shows the general view of the right fore wing (ventral surface) of the female, air-dried but untreated, before using this wing for crystallographic analysis. The deposit layer on the membrane is almost intact but the cuticular microstructures of the margin and veins are well visible due to their incomplete covering by this layer (for details see Figures 6b and 7a). Some SEM micrographs of the gold coated left wings of the same specimen are presented in Figures 6 and 7. They show



Figure 4 | *Psyllipsocus yucatan*, black female, body length 1.3 mm. (a) Habitus in latero-dorsal view, black layer slightly damaged near apex of left fore wing, revealing transparent wing membrane and light brown veins. (b) Same in ventral view.

that the layer covering wing membranes consists of a compact deposit of numerous slightly elongate, suboval microcrystals (width somewhat less than 1 μm , length about 2 μm). The layer of parallel oriented crystals tightly adheres to the dorsal and ventral surface of the wing membrane (Figure 7c). Due to mechanical

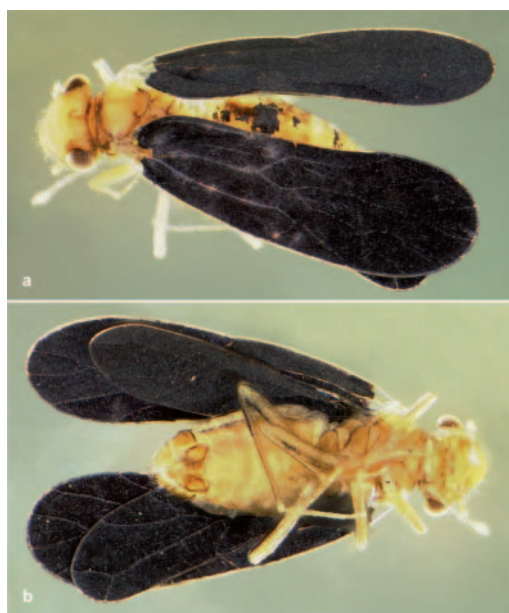


Figure 5 | *Psyllipsocus yucatan*, black male, body length 1.2 mm. (a) Habitus in dorso-lateral view. (b) Same in ventral view.

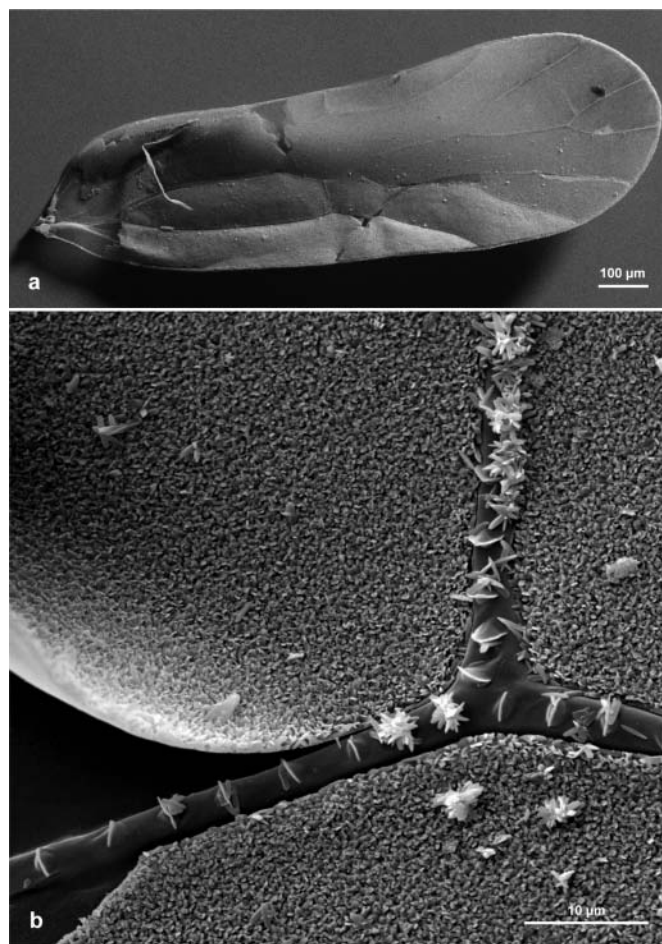


Figure 6 | *Psyllipsocus yucatan*, black female, SEM micrographs. (a) Right fore wing (untreated), general view of ventral surface (micrograph A. Wetzel, Bern). (b) Left fore wing (gold coated), ventral surface, completely smooth membrane visible at left due to local detachment of crystalline layer.

bending of the wings for SEM preparation this crystalline layer is locally detached from the wing membrane (Figures 6b, 7b). The wing membrane is smooth and appears completely unstructured in SEM (Figure 6b, at left along vein), it is about 0.25 μm thick (Figure 7c). The crystalline layer on the dorsal surface of the wing is about 1.5 μm thick, that on the ventral surface slightly thinner (Figure 7b, c). The parts of the wings bearing cuticular sculpture, as the veins (Figure 6b) and the margin (Figure 7a) are bare or only incompletely covered by microcrystals.

EDS analyses on the SEM were performed on the untreated right hind wing of the black female. They showed that the crystals on its surface contain iron, carbon and oxygen (Figure 8). All other element signals are from the carbon adhesive tape (Figure 8a, b) or the aluminium stub (Figure 8c). Iron was not detected during the control analysis of the wings of a normal female of *Psyllipsocus yucatan* collected in Tiquara cave on the same date as the black specimens (Figure 8b).

Crystallography. An initial test by Raman spectroscopy showed that the black mineral layer is not amorphous but crystalline. A first run on the XRD using the complete wing yielded only one weak peak confirming the strong orientation of the crystals. For obtaining a diffractogram of randomly oriented crystals, the wing was then crushed, the material placed onto a single crystal Si disc for underground noise reduction and analysed at maximum spectral

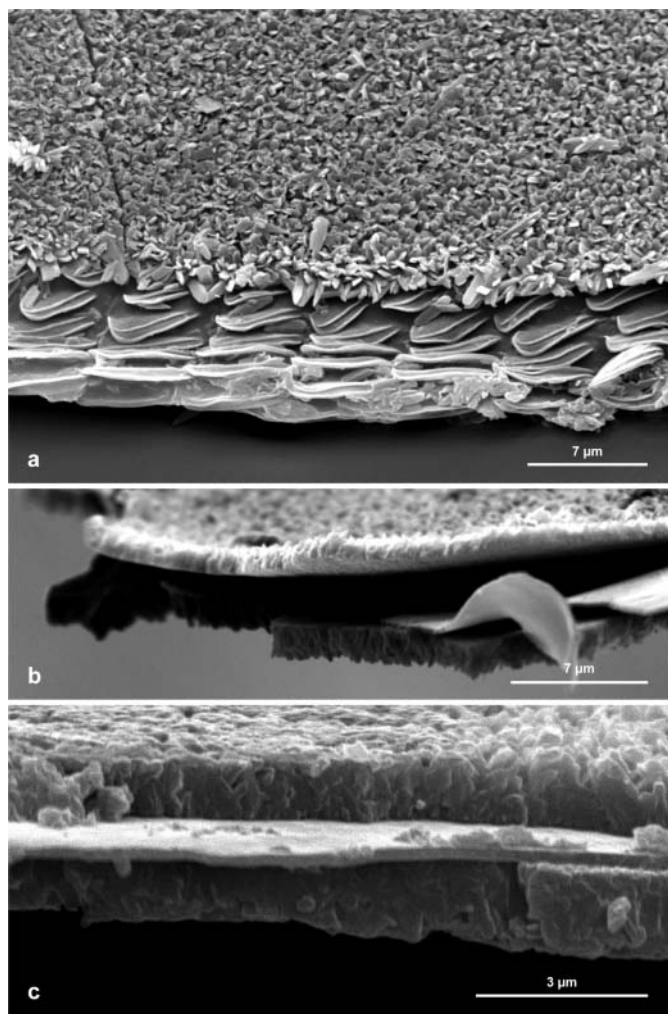


Figure 7 | *Psyllipsocus yucatan*, black female, SEM micrographs (gold coated). (a) Right fore wing, near apex, ventral surface and strongly sculptured wing margin. (b) Right fore wing, broken about in middle, cross-section showing damaged wing membrane and crystalline layers (dorsal side of wing downwards in the figure). (c) Right hind wing, broken about in middle, cross-section showing wing membrane and crystalline layers (dorsal side of wing downwards in the figure), smooth wing membrane with some debris of detached crystalline layer.

resolution of $0.01^\circ/\text{step}$. Due to the small grain size of the crystals, the diffractogram yielded only three very weak peaks (Table 1).

Discussion

The mineral coating of the black specimens bears a superficial resemblance to several phenomena widespread amongst terrestrial arthropods. Many insects, and other arthropods, camouflage themselves with material garnered from the environment. Specialised, sometimes long, often glandular hairs trap environmental particles such as dust, sand, green algae and fragments of wood or prey^{6,7}. This is well known in the Psocoptera, and algae covering bark-dwelling nymphs of *Loensia fasciata* (Fabricius) are illustrated by Lienhard⁸. Such camouflage layers are normally heterogeneous, amorphous and not crystalline. A case of passive covering with crystalline material has been reported for a cave-dwelling harvestman⁹, but examination of the specimens showed that the covering was uneven and of varying thickness, and was restricted to the highest part of the back. This appears to be due to mechanical contact with the calcareous substrate of the cave, and thus unlike the even coating seen on the black specimens of *Psyllipsocus yucatan*.

Insects in many orders produce visible wax secretions from hypodermal glands¹⁰. In some groups, notably the Coniopterygidae (Neuroptera), the wax which is secreted principally on the abdomen is spread using the legs to coat the body and the wings in a fine powdery layer¹¹. In certain Cicadellidae and Membracidae (Auchenorrhyncha), secretions containing brochosomes (characteristically shaped structures produced by the Mapighian tubules) are spread onto the cuticle, usually after moulting¹². In both cases the material spread on the cuticle is produced by the insect itself and therefore fundamentally unlike the mineral crystals on the black specimens encountered in Tiquara cave. The thin, even, coating of crystals seen on the black specimens of *Psyllipsocus yucatan* seems never to have been observed in nature before.

As explained above, the two black specimens of *Psyllipsocus yucatan* were (as were all other specimens of *Psyllipsocus* and most of the other arthropods collected during this study of Brazilian caves) captured manually rather than trapped, and directly transferred into ethanol with no special fixative. No other arthropod from Tiquara cave (apart from a third black psocid unfortunately lost but assumed to be of the same species), or from any other Brazilian cave, was observed to have such a crystalline deposit on the cuticle or wing membrane. Guano deposits in Tiquara cave were exploited in the past as a source of saltpetre for gun powder production. The extraction changed the topography of the cave floor, and a considerable amount of waste was left behind. These works were illuminated using inefficient, very smoky, oil lamps. The cave is not intensively used by humans at present, and few visitors (essentially only speleologists) visit it. It is unlikely that such visitors would release anything, such as smoke or chemical waste, that could have caused the observed phenomenon. Therefore technical artefact and human impact on the cave environment can virtually be excluded as the direct cause of the formation of the observed black crystalline layers on *Psyllipsocus* wings.

The crystallographic analysis of this black layer showed that even with maximum XRD sensitivity it was not possible to obtain more than a very weak diffractogram of the small crystallites, comprising only three clear peaks (Table 1). This was not sufficient to obtain a clear mineral identification. Moreover, it is possible that organic crystals with low electron scattering, producing only very weak X-ray diffraction are present. They would not be recorded with this XRD technique. However, the diffractogram clearly confirms that the deposit consists of crystals.

These data suggest the following hypotheses. Unknown microclimatic phenomena, probably specific to the particular cave, could possibly be at the origin of occasional formation of crystalline precipitates from the locally supersaturated atmosphere, leading to accidental deposits of microcrystals on cave organisms. In the case of the *Psyllipsocus* observed the wings had to be in a spread-out position during the phase of condensation, because the precipitate was uniformly deposited on dorsal and ventral surface of fore and hind wings. Perhaps the condensation process was triggered by high frequency vibration of the wings (at rest or during flight). The insects concerned survived this "accident" due to the fact that only wing membranes and no other parts of the body were affected. The crystalline precipitate appears only to adhere to the completely smooth surface of the wing membranes and not the hairy or slightly rugose parts of the rest of the exoskeleton. Probably even the reproductive capacity of these insects would not be greatly altered, because they have completely normal genitalia. A positive consequence could be their possibly reduced palatability for predators. Guano probably played a role in the formation of the crystal deposit on the wings. The presence of iron could be a consequence of the important excretion of iron by the common vampire bat, due to the severely limited percentage of iron absorbed from its blood meal¹³. However, even in Tiquara cave, this kind of accident is clearly very rare, because the two black specimens of *Psyllipsocus yucatan* examined for this study

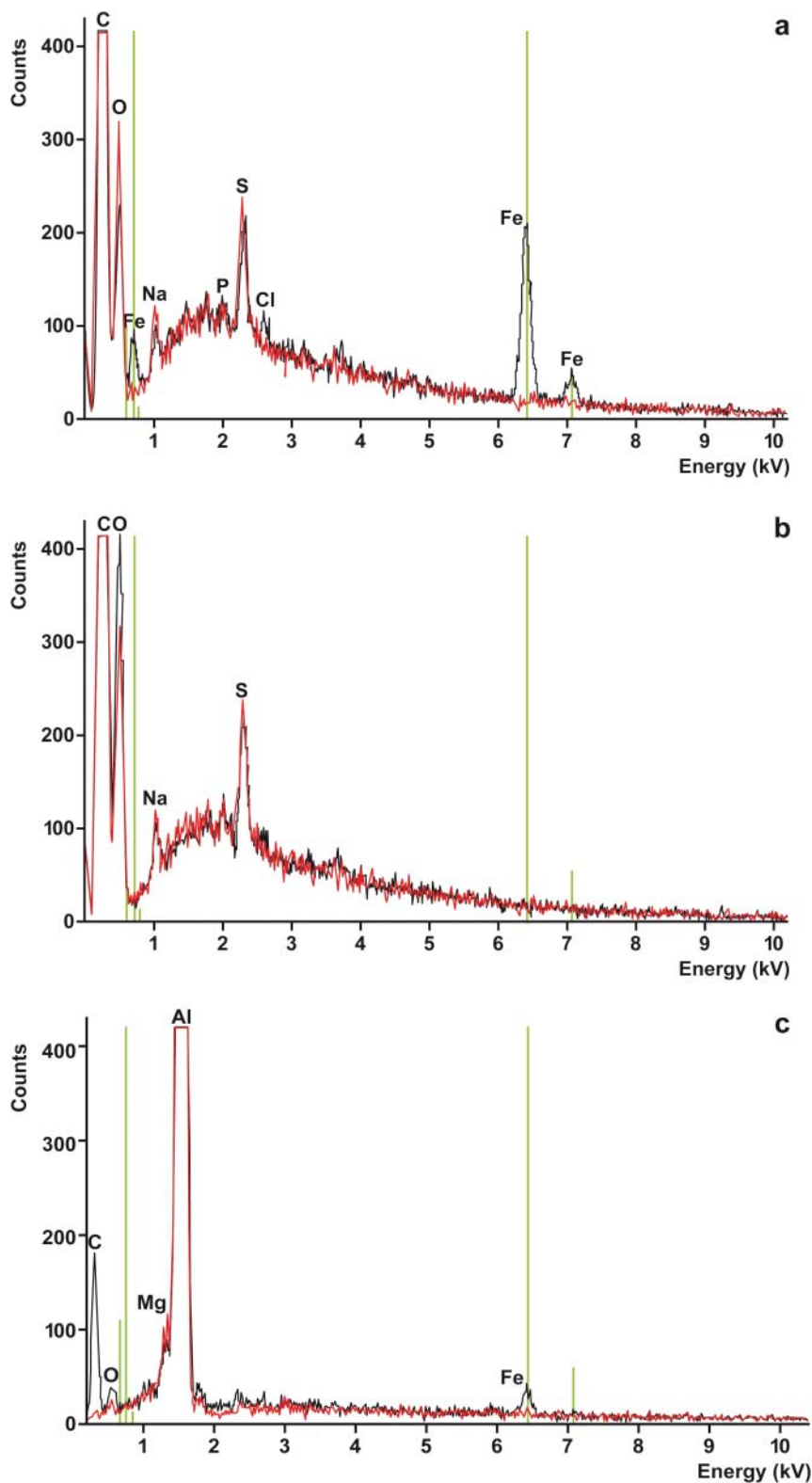


Figure 8 | EDS analysis of *Psyllipocus yucatan*, female. The green lines indicate positions of characteristic X-ray lines of Fe. (a) Spectrum of wing of black specimen analysed on carbon adhesive tape (black spectrum), and adhesive tape only (red spectrum). Note the presence of Fe from the crystal deposit. (b) Spectrum of wing of normal specimen on carbon adhesive tape (black spectrum), and adhesive tape only (red spectrum). (c) Spectrum of wing crystal deposit of black specimen analysed on aluminium stub (black spectrum), and stub only (red spectrum). Note the presence of C and O in addition to Fe in the crystals.

were the only invertebrates collected with such crystalline deposits on their bodies (apart from a third black psocid unfortunately lost but assumed to be of the same species). Thus, based on the limited

data available at present, we have to consider this surprising phenomenon as exceptional and purely accidental, lacking any evident biological significance. Nevertheless, if not really biologically



Table 1 | 2-theta (Th) values, counts (cts), calculated lattice d-spacing and relative intensity of peaks of crystal grown on the wing. The data were obtained using CuK α radiation

Pos. [$^{\circ}$ 2Th.]	Height [cts]	d-spacing [Å]	Rel. Int. [%]
28.3510	66.58	3.14806	88.98
29.4514	56.23	3.03289	75.15
31.6856	74.82	2.82395	100.00

relevant, this natural application of an ultrathin layer of microcrystals to an absolutely smooth biological membrane may be interesting from a point of view of bionics, if the latter is defined as the use of a design found in nature as a model for designing artificial systems¹⁴.

Methods

Dissection and slide-mounting of terminalia and some other parts of the specimens followed the method described by Lienhard⁸. Habitus photographs (in 70–80% ethanol) were made with the AutoMontage[®] system using a JVC[®] video camera mounted on a Leica[®] MZ APO stereomicroscope and slightly reworked with ADOBE Photoshop[®].

SEM analysis (EDS and micrographs): Left wings of the black female were used for micrographs. Both wings were broken about in middle to allow examination in cross-section. After gold coating (Cressington[®] sputter coater 108 auto), necessary for high-resolution SEM imaging, the SEM studies were completed on a Zeiss[®] DSM940a. EDS (Noran[®] System Six) was performed separately, with dried but otherwise untreated wings (i. e. not coated by gold or carbon). The right hind wing of the black female was used for EDS. Two analyses were performed for this wing, the first one with the wing fixed with adhesive carbon tape (Bal-Tec[®] carbon adhesive Leit-Tabs), the second one by simply placing some debris of the crystal deposit on a Balzers Union[®] aluminium stub. Control data were obtained using wings of a normal female collected in Tiquara cave on the same date as the black specimens.

Crystallographic analysis: After an initial test by Raman spectroscopy, using a Renishaw[®] inVia Raman microscope, the right fore wing of the female was used for crystallographic analysis. Before being destroyed for this purpose a SEM micrograph of the dried but untreated and uncoated wing was made on a low vacuum Zeiss[®] EVO 50 electron microscope at the University of Bern (Figure 6a). The wing was then crushed in an agate mortar under ethanol in order to remove the crystalline deposit, and the alcohol-powder mix transferred onto a polished single crystal Si disc, dried and analyzed on a Philips[®] PW3060 X-ray diffractometer (XRD). A CuK α radiation and sample spinner were used. Analysis covered a 2-theta range of 5–60 $^{\circ}$.

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Author contributions

RLF carried out the fieldwork and made the biotope photographs, CL identified the specimens and made the habitus photographs, AP carried out the EDS analysis and made the SEM micrographs, UE carried out the XRD analysis, UE and EG analysed the XRD data, AP and EG analysed the EDS data, CL, JH, EG, RLF and AP wrote the manuscript and all authors reviewed the manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests

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