J Mater Sci (2011) 46:6154–6157
DOI 10.1007/s10853-011-5624-3

LETTER

Evidence of biocomposite structure in *Colletes halophilus* nest material

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Received: 31 March 2011 / Accepted: 9 May 2011 / Published online: 19 May 2011

Introduction

Recently, materials scientists have taken interest in the nest cell linings of *Colletes* bees as a promising model for the synthesis of natural polymers, though they have been of long-standing interest to entomologists for taxonomic and evolutionary classification [1]. *Colletes* bees are capable of producing a high molecular weight, linear polyester [2] that is naturally derived, robust and strongly resistant to chemical degradation [2–4]. It has also been shown to be biodegradable [5] and may serve as a model for non-petroleum derived polymers in the future.

The source of the nest cell lining material has been identified as the Dufour's gland of *Colletes* bees, an enlarged gland containing a variety of macrocyclic lactones located in the bees' abdomens [2, 3, 6–8]. However, the process of nest cell lining construction is contested. Batra [8] suggests the Dufour's gland secretions are ingested and mixed with salivary secretions in the crop before being licked onto the nest cell walls. In contrast, Torchio et al. [9] suggests that the bee first applies salivary secretions to the nest cell walls before using its bilobed tongue to lick on the Dufour's gland secretions separately. In both cases the mechanism for polymerization is unknown, though it is assumed to be the result of the addition of salivary secretions since the Dufour's gland secretions do not self-polymerize [2, 10].

Investigations of the nest cell lining material have focused principally on material origin and, to a lesser extent, chemical composition. Though the nest cell lining material is composed of a polyester [2], combustion and amino acid analysis of nest cell linings show a significant presence of protein [3].

Little attention has been given to nest cell lining material microstructure. Observations of other species in the same family as *Colletes* have noted fibre-like striations in the nest cell lining material [11, 12]. These have been seen in *Hylaeus cressoni* [11] and attributed to texture as result of licking application in *Chilicola venticola* [12]. However, observations within *Colletes* are less clear; some investigations have shown the material to be quiet smooth [3] while others have noted fibre-like structures and attributed them to larval excrement or cocoon spinning [13].

This study addresses the discrepancies in the published literature on nest cell structure and construction by analysis of the microstructure of the nest cell lining of *Colletes halophilus*. The objective was to ascertain if one or both of the components of the lining material were natural polyester or if they were different in nature, thereby accounting for the protein detected. Use of advanced microscopy and analysis techniques provide new information on the morphology and chemistry of the nest cell lining material and give a clearer understanding of its potential as biopolymer.

Materials and methods

Nest cell collection

In this study the nest cell lining of *Colletes halophilus*, a solitary bee native to eastern England and much of continental Europe, was examined. Complete nest cell samples were excavated from sandy nesting areas in East Tilbury, UK and were roughly 5–17 mm in length and 7–9 mm in diameter with a wall thickness of roughly 30 μm. They were washed with water to remove pollen and debris before being

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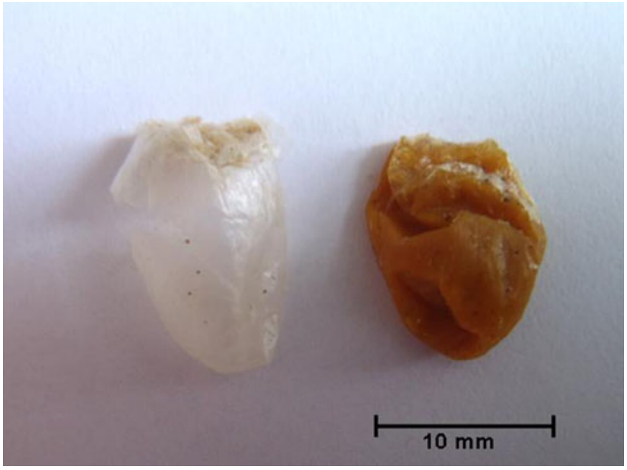

Fig. 1 *Colletes halophilus* nest cell linings, washed (*left*) and unwashed (*right*)

sealed in vials and stored at 4 °C until use. Typical nest cell linings, both before and after cleaning, can be seen in Fig. 1.

Microstructural analysis

Initial microstructure investigations were completed on nest cell linings using a JEOL 6480LV scanning electron microscope (SEM). Samples were gold sputter-coated to render them conductive then examined in the SEM using an accelerating voltage of 10 kV. The inner and outer surfaces of the nest cell lining were examined using this method as well as transverse sections. The latter were obtained using tensile fracture as well as by freeze fracturing samples using liquid nitrogen.

Further investigations, relating chemistry to microstructure, were carried out using a LSM 510META Zeiss confocal microscope. To prepare samples of the nest cell linings for confocal microscopy they were stained with Krypton™ Protein Stain, produced by Thermo Fisher Scientific Inc. This stain was selected for its affinity to protein, such that it would selectively stain the fibres if they were proteinaceous in nature, leaving the known polyester material unstained. The staining protocol used, provided by the manufacturers, was akin to that recommended for gel staining, replacing the gel with nest cell lining material and allowing samples to remain in staining solution overnight [14]. Once stained, the nest cell linings were immediately analyzed with a confocal microscope using a HeNe 543 nm laser for excitation.

Results and discussion

Morphology of nest cell lining

SEM was used to analyze the morphology of the nest cell lining. Figure 2 shows a transverse view of the nest cell lining obtained through tensile fracture. Inset in Fig. 2 is a higher magnification SEM image showing the laminated through thickness appearance of the nest lining which could relate to the way in which the lining is laid down in consecutive layers. Although previously the nest lining was considered to be a continuous film [3], Fig. 2 shows the presence of distinct fibres ranging in size from 2 to 7 μm in diameter. Not only is the presence of fibres confirmed, it can be seen that the fibres occur exclusively on the outer surface of the material. Figure 3 shows the external surface of the nest cell lining, highlighting the fibre location. External fibre placement indicates that the fibres must be laid down by the adults of the species and are not a larval product as previously suggested [13]. This confirms that the construction of the nest cell lining is more complicated than previously thought, with the *Colletes* bees producing a composite material with two distinct main components. In contrast, the internal surfaces of the nest lining were featureless and planar in aspect.

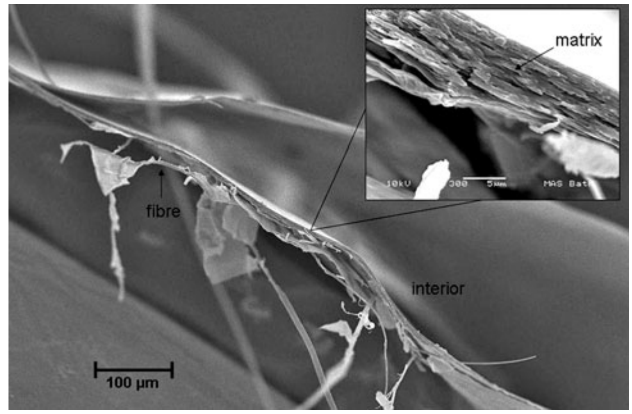

Fig. 2 SEM image of transverse section of nest cell lining

Composition of nest cell lining

The presence of distinct fibres raises the question as to whether or not the fibres are composed of the polyester

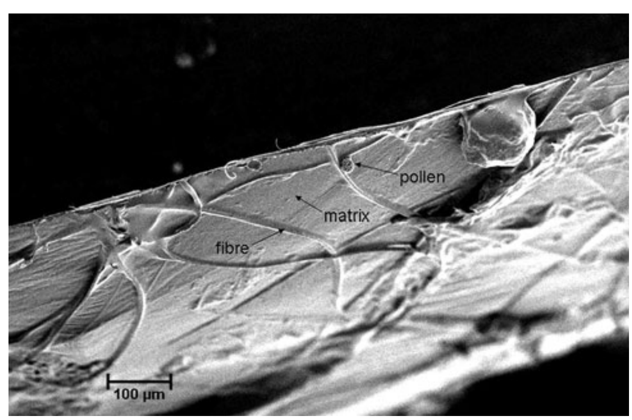

Fig. 3 SEM image of the exterior surface of nest cell lining

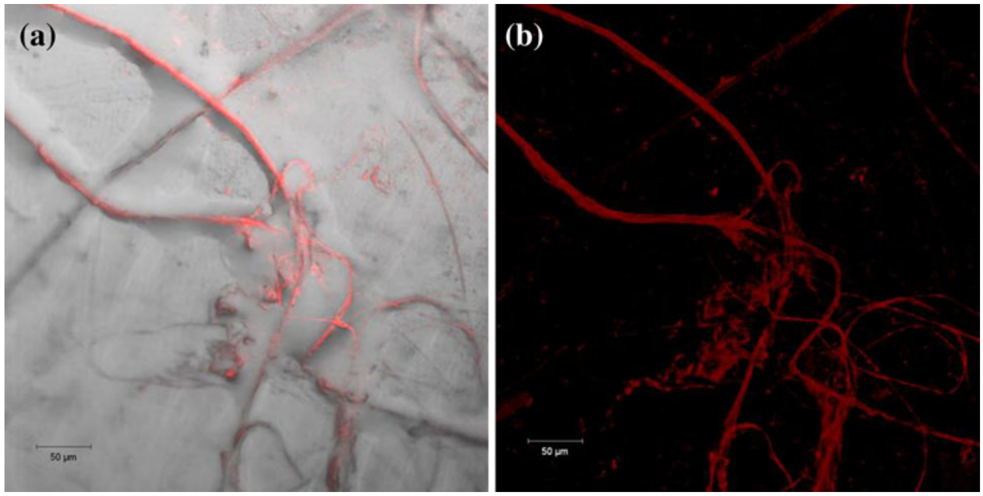

Fig. 4 Confocal image showing **a** stained fibres with unstained matrix included and **b** stained fibres isolated from the matrix

material previously found to constitute the nest cell lining. Using confocal microscopy it can be seen that these two components are indeed distinct with the fibres being positively stained for protein while the matrix remained unaffected by the Krypton^{TM} Protein Stain. This is illustrated in Fig. 4, showing two images of the stained nest cell lining (a) where the unstained matrix has been left in the image and (b) where the stained fibres have been isolated from the image. Initial amino acid analysis of *Colletes halophlius* nest cell linings has shown high quantities of alanine and glutamic acid, a composition akin to some insect silks [3, 15]. The data, coupled with the discovery of proteinaceous fibres, suggests that the nest cell lining is a composite material composed of silk fibres in a polyester matrix as supported by the literature [2–4, 8].

The nest cell linings produced by adult *Hylaeus* bees, a genus of the same family as *Colletes*, have been shown to contain both silk and polyester lipid [8, 16] and it is possible *Colletes* bees use a similar silk production mechanism. In this case, the salivary glands could be expected to be the source of silk production [8]. A two-step process of nest cell construction, where fibres are extruded separately from the matrix material, seems the most likely explanation for the formation of this type of composite material. This lends support to Torchio's [9] observations where *Colletes kincaidii* was seen applying salivary secretions before licking on Dufour's gland material. The silk could be extruded in this initial step when the *Colletes* bee applies the salivary secretions and could aid in the polymerization of the Dufour's gland material.

Conclusions

Colletes bees have been considered remarkable for their production of a linear polyester that is both naturally derived and robust. The results of this study show that *Colletes* females are the source of the silk fibres which are a fundamental component of the composite nest cell lining. Although the origin of silk production is not yet known, it is likely to be a secretion of the salivary glands. Since it is believed an additional salivary gland secretion is key to the formation of the polyester, it is possible that the production of the silk fibres aids in polymerization. Alternatively, the fibres may provide a scaffold for the application of the polyester material. Continued understanding of the role and production of the nest cell lining components may inform the development of more robust biopolymers in the future.

Acknowledgements The authors would like to acknowledge the Fulbright Commission for the award of a scholarship to one of the authors in support of this study. Thanks go to the MAS Centre at the University of Bath for help with the electron and confocal microscopy, and to Franklin W. Olin College of Engineering for their continued collaboration throughout this project.

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