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Bornean caterpillar (Lepidoptera) constructs cocoon from *Vatica rassak* (Dipterocarpaceae) resin containing multiple deterrent compounds

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Many Lepidoptera larvae use pieces of vegetation bound with silk to construct or disguise their cocoons. Here we report the first known case of a caterpillar building its cocoon entirely out of fragments of resin, broken away from sheets of dried resin on the trunk of a tree and held together with silk. The behaviour of the larva (possibly *Negritothripa* sp. in the Nolidae), from the Kinabatangan Wildlife Sanctuary in Sabah, Borneo, is described. The cocoon was constructed on the trunk of *Vatica rassak* (Dipterocarpaceae). Analysis of resin from the cocoon, using gas chromatography-mass spectrometry, revealed a complex mixture of 260 components, dominated by sesquiterpenes and triterpenes. Many of these compounds have defensive properties, protecting the tree from herbivores and fungi. The larva appears to have evolved an elaborate and possibly unique behaviour, allowing it to harness the defensive properties of the resin to protect its pupa from predators and/or entomopathogenic fungi.

Keywords: defensive chemicals; Dipterocarpaceae; eligmine Nolidae; sesquiterpenes; terpenes; *Vatica rassak*

Introduction

Many Lepidoptera construct their cocoons by using their silk to bind together materials (e.g. sticks, leaves, bark) collected from the surrounding environment. Some of the best known examples are the bagworms (Psychidae), whose larvae collect together loose pieces of vegetation bound with silk to make a well-camouflaged mobile refuge, later fixed to a substrate and used as a site for pupation (Rhainds et al. 2009). Species in at least 10 other families of Lepidoptera do something similar (Rhainds et al. 2009). Some species are more selective than others but the case is built out of materials scavenged from the immediate area. As far as we are aware there are no previous reports of Lepidoptera larvae exclusively using potentially toxic resins collected from tree trunks for this purpose.

In 2011 an unidentified caterpillar was found on the trunk of a *Vatica rassak* tree (Dipterocarpaceae) near the Danau Girang Field Centre on the Kinabatangan River in Eastern Sabah. *Vatica rassak* is found in Malaysia, Indonesia, the Philippines, Papua New Guinea and elsewhere (Uphof 1968; Soerianegara and Lemmens 1994). This tree, locally known as rasak, produces abundant resin from wounds, which dries

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to produce hard sheets on the trunk and branches. This resin, known as rose dammar or damar rasak (Uphof 1968; Soerianegara and Lemmens 1994), is used for caulking boats and lighting, and is exported for use in a range of products including incense and as a 'herbal' remedy. Extracts from the bark have been studied extensively for bioactive compounds with potential use in medicine, oligostilbenoids in particular (Tanaka, Ito, Nakaya, Iinuma and Riswan 2000; Tanaka, Ito, Nakaya, Iinuma, Takahashi et al. 2000). Many resveratrol (3,5,40-trihydroxystilbene) derivatives have been shown to be highly cytotoxic and one dominant compound in particular, Vaticol C, was found to be effective against cancer cells, inducing apoptosis (Ito et al. 2002, 2003). No analyses were previously performed on the resin itself although this may contain some of the same compounds as the bark. Cytotoxic compounds have evolved in numerous plant species as a defence against attack by natural enemies, such as herbivores and fungi.

Our aim was to record the unique behaviour of this unidentified lepidopteran as it built its cocoon out of pieces of dried resin, bound together with silk. In addition we analysed the resin to determine whether it contained compounds likely to provide an effective defence for the pupa within such a cocoon.

Methods

Photographs of the larva building its cocoon were taken during July at 5°24′49.93″ N, 118°02′18.58″ E, on a tree bordering the pathway between the Kinabatangan river and the Danau Girang Field Centre buildings. The cocoon was located ~1 m above the ground on the north-east side of the trunk of a *V. rassak* tree. The camera used to record cocoon construction was a Panasonic Lumix™ DMC-FZ18 with Leica™ optics (Panasonic, Bracknell, Berkshire, UK).

Emergence of the moth was not observed and subsequently the empty cocoon was sent to Cardiff University. No remains of the larval skin were available. Images of the resin pieces from which the cocoon was constructed were first taken using a photomontage system at the National Museum of Wales (Syncroscopy Automontage v5.04 (Cambridge, UK) was used to stack the images from a JVC™ KYF70U camera (London, UK) mounted on a Leica™ MZ8 microscope with a Planapo™ lens (Milton Keynes, UK)). The pieces of resin were then examined in more detail using a scanning electron microscope located within the School of Earth and Ocean Sciences at Cardiff University (an FEI™ XL30 environmental SEM with field emission gun, Eindhoven, the Netherlands). The aim was to determine the structure of the blocks broken away from the sheets of resin on the tree trunk and to gain insight into the methods used by the caterpillar to obtain them.

Pieces of the resin were then analysed using mass spectrometry. Resin (1.5 mg) was weighed into 250 µl GC sample vials (Chromacol™, Welwyn Garden City, UK) and dissolved in 100 µl dichloromethane (HPLC grade, Fisher™, Loughborough, UK). A 1 µl sample of the solution was directly injected into the inlet of a gas chromatograph-mass spectrometer (GC: 5973, MS: 6890, Agilent™, Wokingham, UK) at 220°C in splitless mode and separated on a 30 m, 0.25 mm ID capillary column coated with 0.25 µm HP-5MS (Agilent) at a constant pressure of 8.5 psi Helium carrier gas. The initial temperature of the GC oven was 35°C for 2 min followed by a linear temperature increase of 5°C min^{-1} to 310°C and 10 min hold.

Mass spectra were acquired in electron impact mode after 3.5 min solvent delay from m/z 35 to 450.

Two n-alkane standards ranging from octane to eicosane (C_8–C_{20}, 13 components, 4 mg l^{-1} each, Supelco™, Gillingham, UK) and nonane to hexatriacontane (C_9–C_{36}, 16 components, Supelco) were analysed under the same conditions (0.1 µl and 1 µl injection volume respectively) to allow calculation of retention indices (RI) and provide a quantitative reference for abundance and limit of detection (LoD at a signal to noise ratio of 5 for C_8–C_{20} standard only).

Data were analysed and integrated using ChemStation™ (Build 75, Agilent). Amounts of components were determined as area of total ion count intensities under the corresponding signals in the chromatogram. Components were putatively identified by comparison of mass spectra and retention indices against NIST library data (National Institute of Standards and Technology 2005, Gaithersburg, MD, USA).

Results and discussion

The larva itself was orange, except for the posterior four segments of the body, which were white. It was covered with thick black hairs (four per segment), enlarged towards the tips, plus thinner white hairs of uniform thickness ventrally. The orange body suggests aposematic coloration which in turn would indicate sequestration of toxins from its *V. rassak* host (Ômura et al. 2006; Opitz and Müller 2009). This may be prepupal coloration (providing some defence during this vulnerable stage) and not that of the actively feeding larval instars. Construction of the pupal cocoon was observed over 6 h although it had already started when first observed. The larva constructed two parallel walls on either side of it, reaching out to obtain fresh resin from within the space between these walls (Figure 1A). Flakes appeared to be broken off by exerting pressure on the resin sheets with the head and mandibles. As the translucent walls were built up, each block was secured in position (Figure 1B). Once the walls were complete, threads of silk were strung between the tops of the two walls and manipulated by the caterpillar, which pulled on the threads. The walls 'pulsed' in and out until finally they were drawn together along the top and secured (Figure 1C). The caterpillar was then fully enclosed. The finished cocoon (Figure 1D) appeared white from light reflected from the semi-transparent flakes of resin. No plant or other material, apart from resin and silk, was incorporated in the construction.

The photomontage images show pieces of resin ~2–3 mm in size of random shapes (Figure 2A). The shapes would have resulted from the shearing planes within the sheets of resin, clearly shown in the EM images (Figure 2B–D). Shearing patterns appeared to be fractal and reflected both the way in which the material was laid down, in layers, and the physico-chemical properties of the material itself. In one image (Figure 2D) there is evidence of a groove which, it is thought, resulted from the pressure of the insect's mandibles as it endeavoured to break off a piece of resin. The relatively uniform size of the pieces indicates some 'trimming' or selection by the larva, although this was not directly observed. This relative uniformity permits regularity, and hence probably strength, in the construction of the 'walls'. The internal face of the walls was relatively smooth, while angular pieces of resin were allowed to project from the outer surface.

The resin dissolved completely in dichloromethane, and the resulting chromatogram showed a total of 260 components with abundances in total ion count above the

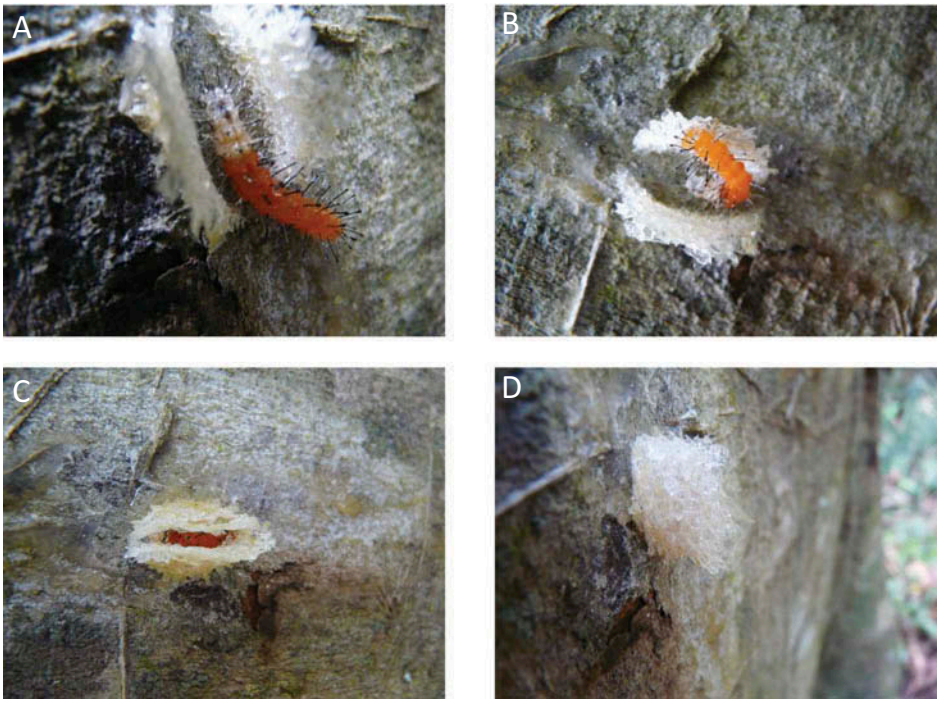

Figure 1. Stages in cocoon construction showing (A) larva reaching out to detach new pieces of resin from dried sheets on the tree trunk; (B) building pieces of resin into one of the two walls; (C) pulling together the two walls using threads of silk; and (D) the completed cocoon.

limit of detection of 4–24 pg μl^{-1}. Of these 170 (65%) fell into two distinct areas of the chromatogram between 20 and 36 min (RI: 1350–2038, 137 compounds) and from 54 and 60 min (RI: 3190–3700, 33 compounds), together accounting for 95% of the total sample amount.

A total of 112 (43%) components could be characterized as terpenes and their oxygen-containing derivatives, representing the most abundant compounds in the resin (83% of the total sample in terms of area count under the chromatogram), and clearly dominating the two distinct areas of the chromatogram. Between 20 and 36 min, 71% of all compounds (97 of 137) were classifiable as sesquiterpenes and their oxygen derivatives, representing 74.3% of the total amount of the sample and 89% of the total amount in that range. Another 15 compounds of a total of 33 (46%) eluting from 54 to 60 min were identified as triterpenes and their oxygenated derivatives, accounting for 8.7% of the total sample amount and 78% of the amount of compounds in that range.

Structurally, 14 sesquiterpenes could be identified putatively by mass spectra and retention index (Table 1). Whilst identification was not possible for any of the triterpenes, the data suggested structures related to the five carbon ring moiety found in lupeol and ursenol.

Terpenes and their derivatives exhibit antifungal, antimicrobial and antifeedant properties and are involved in a multitude of ecological interactions (Gershenzon and

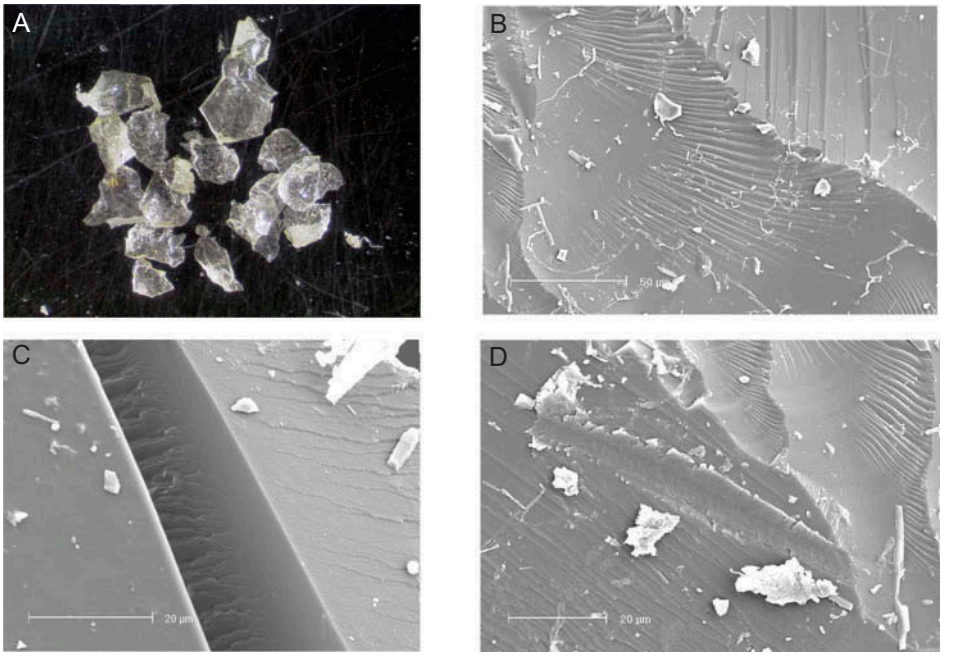

Figure 2. Pieces of resin taken from the cocoon and imaged (A) using photomontage; and (B–D) environmental electron microscopy. Images (B–D) show the elaborate shearing patterns within the resin. The centre of image (D) shows what may be a score mark in the surface of the resin made by the caterpillar.

Dudareva 2007). The compounds are likely to offer protection against infection and to actively repel predators and/or parasites. The resin would have chemically camouflaged the pupa, located as it was within large patches of the same dried resin, while the sharp angles of many of the resin flakes may have offered further defence. We do not know whether the lepidopteran was a specialist herbivore of *V. rassak* but given this unusual adaptation to the resin of that tree it is likely. It is however possible that it is a grazer of lichen and/or fungi, or even predatory. Species within the noctuoid family Erebidae, subfamily Boletobiinae, such as *Corgatha*, *Enispa*, *Laspeyria* and *Zurobata* spp., are possible candidates, often including bark, lichen and other substrate in their cocoon construction. Some of the larvae in these genera also have club-like primary setae (c.f. Figure 1).

However, the two-walled method of cocoon construction is a definitive feature of the family Nolidae (Holloway 2003), also Noctuoidea, and the larva itself and the general structure of the cocoon bear a striking resemblance to those illustrated by Sugi et al. (1987, Plate 104, images 7 and 8) for a Japanese species of *Negritothripa*. This genus belongs to the subfamily Eligminae (Holloway 2011) as redefined by Zahiri et al. (2013), but treated under Collomeninae by Holloway (2003), who listed three species in Borneo. Members of the subfamily generally have long primary setae, often black, but only *Negritothripa* has them apically clubbed; it also has shorter white setae ventrally. Sugi et al. (1987, p. 294) noted that these black setae and those

Table 1. Sesquiterpenes identified putatively in the resin sample. RI (exp)/(lit): experimental/NIST library retention index, Forward fit: goodness of fit of recorded to library mass spectrum, Reversed fit: goodness of fit of library to recorded spectrum, both max. 1000. Example papers under 'Repellency' implicate these sesquiterpenes as insect repellents, either alone or in combination with other compounds.

RI (EXP)	Name	Forward fit	Reversed fit	RI (LIT)	Repellency
1371	Ylangene	872	898	1371	Peterson et al. (2002)
1375	Copaene	938	938	1375	Kaoneka et al. (2007)
1384	β-Bourbonene	935	935	1382	Birkett et al. (2008)
1393	β-Elemene	939	939	1400	Sakasegawa et al. (2003)
1418	Caryophyllene	913	913	>1420	Kafle and Shih (2013)
1477	τ-Muurolene	938	938	1475	García et al. (2007)
1482	Germacrene D	910	932	1482	Ghirardo et al. (2012)
1486	Eudesma-4[14],11-diene	895	915	1482	
1500	α-Muurolene	941	944	1499	
1514	τ-Cadinene	896	898	1509	
1524	δ-Cadinene	862	876	1527	Yatagai et al. (2002)
1572	Iso-aromadendrene epoxide	842	851	1579	Pontes et al. (2010)
1580	(−)-Spathulenol	941	941	1576	Cantrell et al. (2005)
1584	Caryophyllene oxide	885	891	<1584	

of one other eligmine genus were incorporated into the cocoon to form stridulatory ridges on the interior surface.

The Japanese species feeds on oak trees (Fagaceae: Quercus). Host switching from Fagaceae to Dipterocarpaceae is not unprecedented, as reported for example in the highly diverse lycaenid genus *Arhopala* (Lycaenidae: Theclinae) (Megens et al. 2005). Many *Arhopala* species feed on Euphorbiaceae, and this is a plant family also utilized widely in the Eligminae, and several other resiniferous and lactiferous plant families, such as Anacardiaceae, Clusiaceae, Moraceae and, for the type genus *Eligma*, Simaroubaceae, have also been recorded (Holloway 2003, 2011).

The cocoon of the Japanese species is also similar but, instead of being constructed from resin, appears to be made of fragments of epidermis scraped from the stem on which it is constructed. It would appear to be a relatively small evolutionary step from scraping epidermis rich in tannins to collecting resins rich in sesquiterpenes and triterpenes, at least behaviourally. However, constructing a cocoon exclusively from pieces of potentially toxic resin, accompanied by the ability to detach the resin from hard sheets of this material, strongly suggests evolved behaviour, rather than fortuitous exploitation of available material.

Given the number and complexity of the compounds found within the resin, and the likely repellent/toxic properties of a large proportion of these, it is extraordinary that the larvae should have evolved to seek out this material. Having done so they have acquired an elegant and (as far as we know) unique defence system for

protecting their pupa from attack by natural enemies. We can find no record of similar larval behaviour. Although most of the macro-moths of Borneo (and many of the larger micro-moths) have been recorded and named (Holloway 1986–2011), few have had their life cycles analysed and therefore few can be linked to described larval morphologies. However, now that a possible identity has been established, at least to genus level, further work on this unusual phenomenon may be possible and is encouraged. A search for larvae and cocoons in the same area during July over the last two years has failed to find more specimens. A further search will take place in 2014.

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