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Original article



New insights into the unusual nesting biology of the bee *Trichocolletes orientalis* (Hymenoptera: Colletidae, Neopasiphaeinae), particularly its larval 'oil bath'

Terry F. HOUSTON¹, Ken DODS², Lynne A. MILNE^{2,3}, Richard STEPHENS², and Daniela SCACCABAROZZI^{4,5}

¹ Collections and Research, Western Australian Museum, 49 Kew Street, Welshpool, Western Australia 6106, Australia ² ChemCentre, Resources and Chemistry Precinct, Research Service, Curtin University, Bentley, WA 6102, Australia

³ School of Earth and Planetary Sciences, Curtin University, Bentley, WA 6102, Australia

⁴ School of Molecular and Life Sciences, Curtin University, Bentley, WA 6102, Australia

⁵ School of Pharmaceutical Science and Technology, Tianjin University, Nankai District, Tianjin 300072, China

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Abstract – *Trichocolletes orientalis* is an Australian solitary, ground-nesting bee, reported to have some unusual aspects to its nesting biology. Prime among these, and a focus for the present study, is the production of copious amounts of oil by post-feeding pre-defaecating larvae. To better understand the mechanism of oil production, we examined the bee's floral resources and larval provisions and compared the fatty acid profiles of the provision and larval oil exudate using gas chromatography. The study population was monolectic on the legume *Hardenbergia comptoniana*. Unusually for Neopasiphaeinae, larval provisions were liquid but contained no obvious free oil. Differences in the fatty acid composition of the provision and larval oil lead us to conclude that larvae must secrete the oil. Fully fed larvae prevented from curling produced a yellowish, non-oily liquid from the anus. The Malpighian tubules are implicated in the production of this liquid and perhaps the oil (which has not been reported for any other bee species). While likely preventing water loss from resting larvae, the oil contains some fatty acids with known antimicrobial and antifungal properties and might protect the larvae from pathogens. Additionally, we provide a complete life cycle calendar for the bee.

life cycle / monolecty / provision / larval exocrine secretions

1. INTRODUCTION

The bee family Colletidae has a worldwide distribution but is most diverse in the southern hemisphere and, in Australia, contains over 50% of the estimated 2000 bee species (Michener 1965, 1979; Almeida et al. 2012). Knowledge of the behaviour, nesting biology, floral preferences and chemical

Corresponding author: T. F. Houston, Terry.Houston@museum.wa.gov.au Manuscript Editor: Klaus Hartfelder ecology of southern hemisphere colletids is far from complete, and detailed studies are few. This is not unexpected when the majority of bee species are ground nesters (Cane 2003; Antoine and Forrest 2021) and are much more difficult to study than cavity nesters (Winfree 2010). Consequently, generalisations about their nesting biology might be unreliable, and novel facts might yet emerge.

Recently, for example, Houston (2020) reported that cell plugs of the Australian colletid bee *Trichocolletes orientalis* Batley and Houston were coated on the inside with a dry, waxy foam, evidently secreted and applied by the mother

bee. Such a material was previously unknown among Colletidae, indeed among bees. Houston also observed that fully fed larvae of *T. orientalis*, prior to entering summer dormancy, exuded copious amounts of a yellowish oil that pooled in the bottoms of their cells.

Bees possess a wide array of exocrine glands secreting many diverse substances, and their bodies can be thought of as miniature chemical factories. Best known among bee secretions are those of the domesticated honeybee (Apis mellifera Linnaeus) such as wax, venom and royal jelly. Solitary and primitively social bees also secrete diverse materials ranging from solids used to line, waterproof and disinfect their brood cells to volatile substances that function as pheromones, markers or repellants (Cane and Tengo 1981; Cane et al. 1983; Mitra 2013; Danforth et al. 2019). In relation to brood cell linings, the glandular sources and chemical nature of the cellophane-like linings unique to the Colletidae have been the subject of several investigations (for a summary, see Almeida 2008).

In contrast to adults, bee larvae seldom produce exocrine secretions other than silk for the formation of cocoons (and only in certain groups of bees). Fully fed larvae (prepupae) commonly enter periods of dormancy lasting many months or even years and, in a few species, possess thick, waxy cuticles that are believed to reduce water loss (Torchio 1975; Houston and Thorp 1984; Rozen 2016). The source of the wax coatings, however, was not determined. The present study, among other things, elucidates the origin of the oil produced by larvae of *T. orientalis*.

Trichocolletes Cockerell is a genus of mediumsized, hairy bees found chiefly in the Bassian regions of south-eastern and south-western Australia and is comprised of 40 named species, most of which forage at pea flowers (Fabaceae) (Batley and Houston 2012). Some species pollinate *Diuris* orchids that mimic their co-occurring forage plants (Beardsell et al. 1986; Indsto et al. 2006; Scaccabarozzi et al. 2018; Scaccabarozzi et al. 2020a, b).

To date, the nesting biology of only one species, tentatively identified as *T. orientalis* Batley and Houston, has been studied in some detail. Houston (2020) observed a nesting aggregation in south-western Australia, excavating brood cells after the end of the flight season and following the development of fully fed larvae through to the imago. His study revealed some very unusual aspects of the bee's life history that we considered worthy of further investigation. Foremost was that, after consuming its provision, the larva exuded copious amounts of a yellowish oil which formed a pool in the bottom of its cell and in which it lay dormant during summer. Houston deduced that the oil was pollenkitt (a sticky, oily coating on pollen grains) regurgitated from the pollen-filled midgut. We came to question this deduction after observing females of the same population collecting pollen bearing no visible pollenkitt.

Secondly, while Houston's (2020) study revealed that periods of dormancy occurred not only in the post-fed, pre-defaecating larvae but also in pharate adults and newly eclosed adults, a complete picture of the bee's life cycle was wanting.

In the present study, our aims were to (1) identify the floral resources utilised by females to provision their brood cells, (2) determine the composition of the larval provision and compare its fatty acid profile with that of the oil exuded by fully fed larvae (this we hoped would clarify whether the larvae simply regurgitate lipids they have consumed or secrete the oil) and (3) provide a complete life cycle calendar for the bee.

To simplify the discussion in the text below, we refer to the oily exudate of fully fed larvae as 'larval oil'.

2. METHODS AND MATERIALS

2.1. Study area: flora and observations of adult behaviour

The field components of this study were undertaken in and around Boomerang Gorge, Yanchep National Park, c. 48 km north of Perth, Western Australia, the same site where Houston (2020) made his observations. Yanchep National Park is located in the south-western Australian biodiversity hotspot and contains diverse habitats and vegetation communities. Field observations and collection of material underpinning this study were made by DS and TFH (separately or together) on several occasions between August 2019 and November 2021. Voucher specimens of the bee *T. orientalis* are lodged in the entomology collection of the Western Australian Museum, Perth.

Available floral resources were checked during the bee's adult flight and foraging season. Two transects of 200 m length were made, one along the path through the gorge, the other along the path on the rim of the gorge, both in Tuart (*Eucalyptus gomphocephala* DC) woodland community. These were expected to cover the bee's foraging territory around the nesting area. Searches for the bee were also undertaken in *Banksia* woodland community 0.5–1.5 km to the east and 4.0 km north of Boomerang Gorge.

2.2. Collection and handling of brood cell samples

Brood cells were extracted from excavated soil by putting the soil through a 5-mm sieve. Relatively intact cells were stabilised in soil-filled plastic vials and transported to the laboratory in a chilled cooler box. Loose larvae from badly broken cells were placed in small glass tubes, except for several that were preserved in 70% ethanol.

Following the methodology of Houston (2020), we maintained live larvae at ambient room temperatures in a large insulated cooler box with an open container of saturated salt solution and a sealed 1-L container of water as heat ballast. The live larvae were individually numbered, photographed, and examined at least once a week to check their condition and stage of development.

Three cells containing fresh larval provision and two cells containing dormant larvae resting in oil were frozen at -20 °C for subsequent lipid analysis.

2.3. Miscellaneous tests

To test whether fully fed larvae excrete oil from the mouth or the anus, ten such larvae

excavated on 17 November were placed in small plastic containers specially constructed to prevent them curling into a C-shape (Figure 1). Paper discs were placed in the bottoms of the containers to absorb any liquid exudates.

To check if larvae perforate the cellophane-like linings in the bottoms of their cells prior to defaecation (as in some New World diphaglossine bees; vide Rozen 1984), the faecal masses in several cells were softened with warm water and detergent before being carefully peeled away. The membranes were then examined under a stereomicroscope.

To compare the ability of cell bottoms to hold oil before and after pupation, olive oil was pipetted into eight cells in which dormant larvae had died and two that had contained live pharate adults. Dead larvae were left in place, but live pharate adults were carefully removed prior to the addition of oil.

2.4. Identification of pollen

Samples of three fresh larval provisions, a dissected, fully fed larva and larval faeces from two old cells were palynologically processed to determine their pollen content. Samples were acetolysed by heating to boiling point in 9:1 acetic anhydride and sulphuric acid for 8-10 min, then rehydrated in several rinses of acetic acid (Erdtmann 1960), followed by rinses in distilled H₂O; all centrifuged at 3000 rpm for 3 min. Residues were sieved with a 120-µm nylon mesh. Strew slides were permanently mounted in the plastic medium 'Eukitt' and examined with an OLYMPUS-BX51 microscope. Photomicrographs were taken with an OLYMPUS-P71 digital camera. Pollen was identified from the pollen database of the third author (L.A.M.), and voucher slides are deposited in her collection.

Additional samples from the midguts of preserved, fully fed larvae and from the faecal masses (meconia) of old brood cells were taken by TH. The faecal samples likely represented the waste material of numerous bee generations. The samples were observed microscopically in saline solution or in glycerine and compared with the acetolysed pollen.



Figure 1. Larval 'constrainment chamber': **a** section of a plastic vial glued to glass microscope slide with short section of plastic tubing glued acentrically within it to prevent larva from curling into a C-shape; **b** top view of chamber showing fully fed larva and its excretion (arrowed).

2.5. Lipid analysis of larval provision and larval oil

To confirm and localise the presence of lipids in the larval provision and in pollen, samples of both were stained with Sudan IV and examined on slides under compound microscope magnification of $400 \times$ after sample solubilisation in 50% ethanol following Southworth (1973). Several drops of a saturated solution of Sudan IV in 70% ethanol were added. After 10 min, more 50% ethanol was added. Sudan IV stains fats, oils and waxes pink to orange.

Three samples of larval provision and two brood cells containing live, fully fed, pre-defaecating larvae partly immersed in oil were prepared on 15 February 2021 for chemical analysis.

Oil in two brood cells with dormant larvae was withdrawn after three rinses with 0.5 mL hexane. After removing the larvae using dressing forceps, the cells were rinsed again as before to ensure that the total oil content was withdrawn.

Fatty acid profile preparation was carried out by gas chromatography using flame ionisation detection (GCFID) on the esterified methyl esters of lipids extracted from the samples, according to Methods 41.1.28 (969.33) and 41.1.29 (963.22)

applicable to common plant and animal fats (AOAC 2000). The range of fatty acids used for reference is from C10 to C30. Prior to extracting fats, samples were subjected to esterification by adding the hexane solution into a 50-mL round-bottom flask. After boiling off the hexane, 4 mL of caustic methanol solution was added and refluxed on a boiling water bath until all of the fats were saponified. Then, 5 mL of boron trifluoride in methanol was added through the top of the condenser, keeping the reaction mixture under reflux for a further 3 min. Furthermore, 5 mL of hexane was added to the top condenser and refluxed for a further 2 min. The flasks were cooled, remaining attached to the condenser for 1-2 min. Extraction was done by disconnecting the condenser from the flasks and adding saturated sodium chloride solution to float the solvent layer up into the neck of the flask.

The fatty acid methyl ester solution was transferred into a 25-mL screw-capped vial containing anhydrous sodium sulphate and, after being shaken well, was transferred to a 2-mL vial suitable for the GC autosampler.

3. RESULTS

3.1. Observations of bee activity at nests and flowers

We observed females of *T. orientalis* flying into or departing from burrows at the nesting site on 21 August 2019 and 24 July, 28 August and 3 September 2020. Activity peaked in September. Females commenced foraging at flowers of *Hardenbergia comptoniana* (Andrews) Benth. in July, and during August and September, females were observed returning to burrows carrying loads of white pollen in their scopae.

During the bee's flight season, we recorded the following potential sources of pollen and nectar for *Trichocolletes* species in and around Boomerang Gorge (all are Fabaceae): *Bossiaea eriocarpa* Benth., *Daviesia divaricata* Benth., *H. comptoniana*, *Hovea trisperma* Benth. and *Jacksonia sternbergiana* Huegel. While flowers of some of these species (particularly *D*. *divaricata* and *J. sternbergiana*) attracted numerous individuals and several species of *Trichocolletes*, adults of *T. orientalis* were found only at flowers of *H. comptoniana* (Figure 2). This last pea species was dominant near the nesting area and throughout Boomerang Gorge where it was noted to be in early flower on 24 July 2020 and at the peak of flowering on 28 August 2020.

Two females of *T. orientalis* collected while flying over the nesting ground were observed to have clumps of pollen grains adhering to the undersides of the midsection of their proboscises. This section, composed of the stipites and prementum, is conspicuously bristly (Figure 3c). The pollen was identified as that of *H. comptoniana* (see more in Section 3.3). Sizes of the female bee proboscis and the flower androecium (Figure 3a, b) mean that, when the proboscis is inserted to withdraw nectar, the anthers contact the bristles, likely depositing pollen among them. Males of *Trichocolletes*, by contrast, have a relatively bare proboscis.

3.2. Brood cells and larval provision

The plugs of several freshly provisioned and sealed cells excavated on 3 September 2020 possessed a bright white waxy lining on their inner surfaces (Figure 4) as described for older cells by Houston (2020).

Cells containing fresh larval provision were excavated on 3 September 2020 and 22 October 2021. On the first occasion, we opened a recently completed brood cell that was at least half-filled with a yellowish, liquid provision with an egg floating on its upper surface (Figure 4). The provision consisted of a uniform paste of pollen suspended in a clear liquid (presumably nectar) and resembled the provisions of such colletids as Colletes and Hylaeus. On both occasions, similar provision was found in numerous cells that were broken. In these cases, the liquid portion of the provision rapidly soaked into the earthen walls of the cells, leaving the pollen component as a semisolid mass. In one cell collected on 3



Figure 2. Flowers of Hardenbergia comptoniana.

September, dry, white pollen lay in the bottom. In no case was any free oil observed in the provision.

Microscopic examination of samples of provision from cells of different ages revealed the presence of yeast cells in a few of them. The yeast was no more abundant in older cells than newer ones, and none of the samples had a detectable odour.

Excavation of further brood cells on 22 October 2021 revealed that many still contained some liquid provision on which larvae were feeding. The consistency of the provision was much the same as described for newly completed cells, and microscopic examination revealed no yeasts in these older provisions.

3.3. Identification and characteristics of collected pollen

Only one kind of pollen, identified as that of *H. comptoniana*, was observed in over 40 samples taken from adult females, brood cell provisions, larval midguts and larval faeces.

H. comptoniana pollen sampled from stamens was colourless under high magnification (Figure 5) and en masse appeared white. It dispersed readily in water, suggesting that it carried little oily pollenkitt. Staining with Sudan IV, however, did reveal some amorphous material on the surfaces of grains.



Figure 3. a Flower of *Hardenbergia comptoniana* in lateral view with petals of near side removed to show androecium; **b** anterior end of female of *Trichocolletes orientalis* (left lateral view) to same scale (arrow indicates partly extended proboscis; **c** proboscis (left lateral view) enlarged to show its bristly underside (lower right).

Microscopic examination of some clumps of *H. comptoniana* pollen grains found adhering to the bristly underside of the proboscis of two females collected at the nesting site revealed some clear amorphous material between the grains that probably acted as an adhesive (Figure 6).

While pollen in the provision readily dispersed in water, a sample first stained with Sudan IV, revealed globules of an oily substance among the grains (Figure 7).

Pollen sampled from the midgut of dormant larvae had no or much reduced cytoplasmic contents, but the exines retained their shape. Pollen grains in meconia, by contrast, were mostly collapsed, although enough retained their shape to confirm their identity as *H. comptoniana*.

3.4. Immatures and their development

Observations of the early (feeding and growth) stages of larval development were made as follows:

1. A fully provisioned and sealed cell opened on 3 September 2020 (Figure 4) contained



Figure 4. Freshly sealed cell of *Trichocolletes orientalis* opened to show white wax-lined cell cap (left) and yellowish liquid provision.

an egg floating on the surface of the liquid provision; 2 days later, the egg had hatched to a first instar larva.

- Nine brood cells excavated on 22 October 2021 contained early stage to near fully grown larvae feeding on provision.
- 3. All but one of 11 larvae excavated on 17 November 2021 were fully grown and had completed their provision.

Larvae from this last sample were held initially in small glass vials with wads of tissue paper in the bases. Within 3 days, six of them had soiled the tissue paper on which they rested with a yellowish liquid. Seven of the larvae were transferred to constrainment containers (Figure 1) where they continued to produce a yellowish liquid from the anus over 2 days (Figure 1b). However, the flow ceased with the exudate congealing about the anus and three larvae began to discolour. All larvae were immediately frozen for later dissection (see Section 3.7).

Observations of post-feeding development were based on specimens excavated on 3 December 2020. Nineteen relatively intact cells containing dormant larvae were stabilised in soil-filled





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Figure 6. Pollen grains of *Hardenbergia comptoniana*: from underside of proboscis of a female of *Trichocolletes orientalis* (note colourless, amorphous material between grains).

plastic vials and transported to the laboratory in a chilled cooler box. Fourteen further larvae that fell from badly broken cells were placed in small glass tubes and were maintained in the same cooler box as the larvae in cells.

Larvae in glass tubes survived for varying lengths of time. Some produced sufficient oil to coat themselves and the bottoms of their vials, while others remained relatively dry. Those with the greatest amount of oil around them survived longest, but all had died by April 2021 with none proceeding to the defaecation stage. Three larvae died by 22 December 2020 and quickly became mouldy, while the remaining, more oily larvae stayed mould-free. Mites (probably *Tyrophagus putrescentiae*)



Figure 7. Pollen grains of *Hardenbergia comptoniana*: from fresh provision stained with Sudan IV (note lumps of amorphous material stained red-brown on and between grains).



had infested two dead larvae by 28 January 2021, and the infestation gradually spread to all dead larvae. By 8 December 2021, several larvae had been eaten out, leaving just the exoskeletons.

The 19 fully fed, pre-defaecating larvae that were collected in brood cells (or parts of them) fared better, but still suffered a high rate of mortality with only four reaching defaecation stage and two becoming pharate adults.

On 9 February 2021, four larvae curled in the bases of their cells exhibited movements, and by the 15th, all had assumed upright positions (Figure 8).

At this stage, no oil could be observed in the bottoms of the cells. All four larvae commenced defaecating between 15 and 19 February. Two produced liquid faeces in which their posterior ends were submerged (Figure 9). This liquid was quite miscible in water but immiscible in castor oil, and microscopic examination showed that it was composed of a suspension of partly digested pollen grains in clear liquid. By 21 February, the faecal matter had become semisolid, and it continued to dry and shrink over the following days. The other two larvae began producing semisolid faeces but died after some days. The walls of their cells were found to have holes through which most of the larval oil had escaped.

The two surviving larvae pupated on 1–2 March. These pupae developed pink eyes by 9 March, dark brown eyes by 20 March, a darkened head and mesosoma with setae clearly visible beneath the cuticle by 10 April and were fully pigmented pharate adults by 18 April. On 4 May, when the pharate adults were removed from their cells to test the cells' permeability to oil, one individual was freshly deceased; the other made vigorous movements of its mandibles and legs but was dead on 22 May.

3.5. Analysis of lipids in larval provision and larval oil

Analysis by gas chromatography identified 17 fatty acids (FAs) in the larval provision and 27 in



Figure 8. Fully fed larva of *Trichocolletes orientalis* sitting erect in brood cell shortly after stirring from its summer dormancy.

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Figure 9. Post-defaecating larva of Trichocolletes orientalis sitting erect in brood cell in pool of liquid faecal material.

the larval oil (Table I). As well, some unknown FAs were found in both samples (4.64 and 3.72%, respectively). Thirteen FAs were common to both samples but constituted different relative percentages. In the larval provision, the relatively most abundant fatty acids were linolenic isomer 3 C18:3 t9, c12, t15 (31.48%), followed by palmitic C16 (17.79%), linoleic isomer 2 C18:2 t9, c12, (15.4%) and lauric C12 (9.99%). In the larval oil sample, the highest relative lipid concentrations were represented by lauric C12 (37.26%), linolenic isomer 8 C18:3 c6,9,12 or c9,12,15 (15.88%) and stearic C18. An array of linoleic isomers (six structure types of polyunsaturated FAs) occurred in the larval oil sample, with three isomers in the larval provision (Table II).

The relative percentages of FAs in the larval oil differed from those in the larval provision to varying degrees: saturated and unsaturated FAs— $2\times$, polyunsaturated omega 3 and 6— $0.5\times$ and trans FAs (hydrogenated trans fats)— $0.2\times$.

Oil produced by larvae had no noticeable odour. Where produced in small glass vials, the oil persisted after the death of the larvae for more than a year, so it was clearly not highly volatile.

3.6. Fate of the larval oil

When, on 15 February, four fully fed, predefaecating larvae sat erect after their summer torpor, the oil in which they had earlier been lying was no longer visible. Examination under a stereomicroscope revealed no evidence that the larvae had perforated the cellophane-like membranes lining their cells, the membranes appearing to be wholly intact. Furthermore, two 'pupal' cells retained olive oil pipetted into them for many months, four cells that had contained dormant larvae retained the oil over several days, and some other cells lost the oil immediately or over several days. In these last cases, fractures were found in the cell walls.

3.7. Characteristics and origin of larval anal excretion

The anal excretion from fully fed larvae (excavated on 17 November 2021) congealed to a clear, gummy, solid state. This gummy material revealed no uric acid granules when examined microscopically and dissolved readily in water.

	Larval provision			Larval oil			
	Fatty acid	% Relative	g/100	Fatty acid	% Relative	g/100	
Same	Lauric C12	9.99	0.97510	Lauric C12	37.26	0.11326	
	Palmitic C16	17.79	1.73709	Palmitic C16	2.43	0.00737	
	Palmitelaidic C16:1 t9	4.19	0.40915	Palmitelaidic C16:1 t9	5.30	0.01612	
	Palmitoleic C16:1 c9	0.66	0.06437	Palmitoleic C16:1 c9	0.32	0.00096	
	Heptadecenoic C17:1 c10	1.10	0.10763	Heptadecenoic C17:1 c10	0.03	0.00010	
	Stearic C18	1.59	0.15520	Stearic C18	13.61	0.04139	
	Petroselinic C18:1 c6	6.73	0.65685	Petroselinic C18:1 c6	8.31	0.02527	
	Linolenic isomer 3 C18:3 t9, c12, t15	31.48	3.07346	Linoleic isomer 3 C18:2 c9, t12	1.28	0.00389	
	Linolenic isomer 8 C18:3 c6,9,12 or c9,12,15	2.83	0.27597	Linolenic isomer 8 C18:3 c6,9,12 or c9,12,15	15.88	0.04827	
	Docosapentaenoic C22:5 n3	0.23	0.02216	Docosapentaenoic C22:5 n3	0.61	0.00184	
	Cerotic C26	0.58	0.05692	Cerotic C26	2.70	0.00820	
	Octacosanoic C28	0.60	0.05885	Octacosanoic C28	0.36	0.00109	
	Triacontanoic C30	0.43	0.04217	Triacontanoic C30	0.52	0.00158	
Distinct	Linoleic isomer 2 C18:2 t9, c12	15.40	1.50363	Myristic C14	0.17	0.00051	
	Eicosadienoic C20:2 n6	0.43	0.04243	Heptadecenoic C17:1 t10	1.16	0.00010	
	Eicosapentaenoic C20:5 n3	0.97	0.09520	Oleic C18:1 c9	3.28	0.00996	
	Tricosanoic C23/docosa- tetraenoic C22:4	0.31	0.04217	Linoleic isomer 1 C18:2 t9,12	0.24	0.00072	
				Linoleic isomer 4 C18:2 c9,12	0.12	0.00036	
				Linolenic isomer 4 C18:3 c9, t12, t15	0.60	0.00183	
				Linolenic isomer 7 C18:3 t9, c12, c15	0.05	0.00015	
				Arachidonic C20:4 n3	0.84	0.00256	
				Heneicosanoic C21	0.02	0.00007	
				Behenic C22	0.02	0.00007	
				Brassidic C22:1 t13	0.02	0.00007	
				Docosahexaenoic C22:6 n3	0.31	0.00094	
				Lignoceric C24	0.43	0.00132	
				Heptacosanoic C27	0.42	0.00128	
	Unknown	4.64	0.45260	Unknown	3.72	0.011317	

Table I Fatty acid profiles of the larval provision and the oil exuded by fully fed larvae

Dissection of larvae that had begun producing the yellowish exudate revealed that the hindgut was expanded into a thin-walled sac at its midsection (Figure 10), and in one case, this sac was observed to be full of yellowish liquid. No glands or glandular cells were detected about the hindgut except for the Malpighian tubules of which there were four. The tubules were very long and thin compared with those of the honeybee (illustrated by Snodgrass 1956 and Nelson 1924), reaching forward to the anterior end of the midgut and doubling back to the hind end.

Larval provision			Larval oil			
Lipid	g/100 g	% Relative	Lipid	g/100 g	% Relative	
Saturated	3.06	31.29	Saturated	0.18	57.94	
Unsaturated	1.72	17.59	Unsaturated	0.10	33.41	
Mono-unsaturated	0.83	8.49	Mono-unsaturated	0.04	11.94	
Polyunsaturated	0.44	4.46	Polyunsaturated	0.05	17.75	
Unknown unsaturated	0.45	4.64	Unknown unsaturated	0.01	3.72	
Trans	4.99	51.06	Trans	0.03	8.64	
Omega-3	3.39	34.76	Omega-3	0.05	17.67	
Omega-6	1.90	19.43	Omega-6	0.06	18.13	
Total lipid	9.76		Total lipid	0.30		

Table II Comparison of the classes of fatty acids identified in the larval provision and the larval oil

3.8. Associated organisms

No parasitic organisms were encountered in the bee's brood cells. However, several female mutillid wasps were observed crawling around among leaf litter over nest entrances on 3 September (two specimens are lodged in the Western Australian Museum). Based on images of them, they were identified as *Ephutomorpha macracantha* Turner by Christopher Taylor (pers. comm. 2020). Two empty cocoons found in old brood cells conformed to those of Mutillidae.

4. DISCUSSION

4.1. Brood cells

The brood cells collected for this study conformed to the description given by Houston (2020). Our observation that cell caps of newly completed cells have the same white, waxy, dried



Figure 10. Dissected ileum (hindgut) of fully fed, pre-defaecating larva of *Trichocolletes orientalis* showing sac-like midsection (arrowed). Position of adjoining ventriculus (midgut) represented by broken line. Only basal ends of Malpighian tubules are represented.

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foam lining on their inner surfaces as described by Houston for older cells provides strong evidence that it is the mother bee that makes the lining.

4.2. Floral resources and larval provision

Despite the flowering of several genera and species of Fabaceae within or adjacent to Boomerang Gorge during August and September, pollen of *H. comptoniana* was the only kind identified from larval provisions, midguts and faeces. Pollen samples taken from old brood cells would have represented multiple past generations. Evidently, the Boomerang Gorge population is monolectic, something newly recorded in the genus *Trichocolletes* and a generally uncommon trait among bees (Danforth et al. 2019; Cane 2021).

Our observations suggest that the very bristly proboscis of females of *T. orientalis* is an adaptation for collecting pollen from relatively small pea flowers. The proboscis of females of many other *Trichocolletes* species is similarly bristly. While bristles on the mouthparts of bees serving to aid pollen collection have been reported for various bees (summarised by Danforth et al. 2019), none of those bees was a pea flower specialist.

As is common among bees, females of *T. orientalis* initially deposited loads of dry pollen in the bases of their brood cells, later adding nectar and mixing the ingredients to form a liquid paste. When freshly completed cells were broken, the liquid component of the provision soaked into the earthen cell wall suggesting that females thicken the nectar only marginally or not at all before adding it to a cell.

Almeida (2008) expressed the view that a larval provision consisting of a semisolid ball was typical of the 'Paracolletinae' (subsequently to become known as the Neopasiphaeinae with the removal of *Paracolletes* Smith; Almeida et al. 2012). The observations herein and those of Houston (2018) for '*Paracolletes* (*Anthoglossa*) *nigrocinctus* Cockerell' suggest that unformed, liquid provision might be more common in the Neopasiphaeinae than once thought. Pollen of *H. comptoniana* is white en masse, but the completed provision was yellow. The addition of nectar alone does not explain this colour change, so perhaps, some enzyme secreted by the mother bee and added to the provision is responsible.

An amorphous material found among pollen grains collected by the bees and which stained red with Sudan IV was presumed to be pollenkitt. Its quantity, though, seemed insufficient for it to be the source of all the oil exuded by larvae. Some colletid bees have been observed to add material to their provision from the apex of the abdomen (Torchio 1984), and oily droplets were found on the surface of provisions of *Ptiloglossa arizonensis* Timberlake (Rozen 1984). We observed nothing similar.

4.3. Life cycle

Our observations of the life cycle of *T. orientalis* corroborate and extend those of Houston (2020). We determined that the dormancy of the post-feeding, pre-defaecated larval stage ended in early February and was followed closely by defaecation. A summary of the life cycle is presented graphically in Figure 11.

Dormancy in fully fed, pre-defaecating larvae is most unusual among bees but has been recorded for a *Sphecodes* species (Halictidae) by Torchio (1975).

4.4. Origin, function and fate of the larval oil

Our lipid analyses revealed that the larval oil exhibits a greater diversity of FAs than the larval provision and contains different relative amounts of the shared FAs. So, the larval oil is not simply regurgitated from consumed provision as was suggested by Houston (2020). Furthermore, it does not consist of breakdown products derived from the digestion of pollen lipids (these would consist of short-chain FAs). We conclude that the larval oil is secreted by the larvae themselves.



Figure 11. Graphic representation of life cycle of Trichocolletes orientalis. Individuals vary somewhat in the timing of transition from one stage to the next (indicated by oblique dividers). Eggs and feeding larvae are present over a substantial part of the early nesting season (indicated by the colour gradient yellow to orange). 'Pharate' refers to the pharate adult stage (purple) when the newly formed adult is still enclosed in the pupal skin. Houston (2020) found that fully fed, predefaecating larvae of *T. orientalis* confined in gelatine capsules accumulated a yellowish oil about their anal region. However, because the larvae curl 'nose to tail', he considered it possible that the oil was exuded from the mouth onto the anal region. Our use of 'constrainment containers' revealed that larvae that had recently finished feeding produced a yellowish liquid from the anus, but nothing from the mouth. Perplexingly, the liquid was small in amount and watersoluble. Perhaps, by preventing larvae from curling up in their usual manner, we interfered with their physiological processes.

Although the sac in the middle of the hindgut seemed to serve as a reservoir for the yellow liquid, there were no obvious glandular cells closely associated with the sac. We consider the Malpighian tubules, of which there are four, to be the most likely source of both the yellow, water-soluble secretion and the yellow oil. The tubules, while functioning chiefly as excretory organs and producing uric acid, have been reported to secrete various other substances in a variety of insects including bees. For example, a silk-like substance linking the larval faeces of some halictids was reported by Hirashima (1960), and a clear gelatinous substance contributing to the cocoon has been reported by Torchio (1965) for Colletes ciliatoides Stephen and by Mello & Kerr (1984) for some meliponines. Moreover, prepupae of various bees have been reported to have integumental coverings of waxy or chemically unknown materials: Hesperapis rhodocerata (Cockerell), Melittidae (Rozen 2016); Sphecodes sp. Halictidae (Torchio 1975); Stenotritus greavesi (Rayment), Stenotritidae (Houston and Thorp 1984). In none of these cases was the source of the material identified. The production of oil, as far as we can determine, is unique to T. orientalis.

There are few descriptions of the Malpighian tubules in bee larvae. In *A. mellifera*, there are four relatively thick tubules that lie along the sides of the ventriculus and end near its anterior end (Nelson 1924; Dade 1962). Larvae of *T. orientalis* likewise have four tubules, but they are

comparatively much thinner and longer. Barbosa-Costa et al. (2012) reported that the number of tubules in larvae of various meliponines varies from four to eight. Torchio (1965) noted that larvae of C. ciliatoides, in addition to the usual four Malpighian tubules, possessed a pair of tubules that joined the posterior end of the hindgut and supposed that these produced the gelatinous substance that formed part of the cocoon. Clearly, the number, form and function of larval Malpighian tubules vary among bee taxa, and further research could prove rewarding. Nelson (1924) noted that the Malpighian tubules of A. mellifera larvae discharge their contents only at the time of defaecation when the ventriculus develops a connection with the hindgut. However, the presence of urate granules in the oil produced by pre-defaecating larvae of T. orientalis (Houston 2020) suggests that the Malpighian tubules of this species must discharge at an earlier stage.

The oil produced by larvae of T. orientalis is not highly volatile and persisted in glass vials beyond the time of pupation. Circumstantial evidence suggests that the oil bath is necessary for complete development. Two larvae that did not have their full complement of oil produced semisolid faeces instead of liquid faeces and died before pupating. Furthermore, we observed that, when larvae stirred from their summer dormancy and sat erect, oil was no longer visible in their cells. Our experiments showed that olive oil pipetted into cells with holes or slight cracks in the lower portion leaked out quickly, but two cells in which pupae had developed retained the olive oil for many months. We conclude, therefore, that the bee oil would not have leaked out, and we suspect that larvae imbibe it prior to sitting erect and defaecating.

Two likely functions of the oil baths of postfeeding larvae of *T. orientalis* are the prevention of dehydration through dry summer months and the suppression of the growth of microbial pathogens. Some of the FAs composing the larval oil (namely lauric, linoleic, myristic and oleic acids) are known to have antimicrobial properties (Carballeira 2008; Hornitsky 2003; Kabara 1984; Matsue et al. 2019). Lauric acid is a particularly effective antibacterial agent (Bergsson et al. 2011; Drake et al. 2008; Fischer et al. 2014) and was the most abundant FA in the larval oil.

4.5. Larval defaecation

Faeces produced by bee larvae most commonly take the form of solid pellets that lie loosely in the cell, semisolid ribbons that are plastered onto the cell walls or, less commonly, a semi-liquid that congeals into a cake in the lower part of the cell (Stephen et al. 1969; Danforth et al. 2019). Members of the colletid subfamily Diphaglossinae are unusual in producing very liquid faeces (Rozen 1984), and in some species, larvae perforate the cellophane-like lining in the lower part of their brood cells to permit drainage of faecal liquid into the soil. Larvae of T. orientalis produce similarly liquid faeces but, as far as we could determine, do not perforate the cell lining. Still, the faecal material dries to a solid cake around the pupa, and we surmise that water vapour escapes via the pore in the cell cap.

For an insect living in dry soil during hot summer months, it would seem maladaptive that larvae of *T. orientalis* should produce such liquid faeces, and the question arises 'where does the water in the faeces come from?' It is well known that many animals including insects that live in dry environments metabolise dietary fats to water (Downer and Matthews 1976; Fraenkel and Blewett 1944; Mellanby 1942), so perhaps, *T. orientalis* larvae re-ingest the oil in which they lie dormant over summer and metabolise it to water.

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AUTHOR CONTRIBUTION

Conception and design of this study, field observations and sample collection were undertaken by TH and DS. Analysis of the fatty acid contents of samples was directed by KD and performed by RS. LM guided and participated in the palynological aspects of this study. The manuscript was drafted by TH and DS with input from the other authors. All authors read and approved the final manuscript.

DATA AVAILABILITY

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

CODE AVAILABILITY

Not applicable.

DECLARATIONS

Ethics approval Not applicable.

Consent to participate Not applicable.

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Competing interests The authors declare no competing interests.

REFERENCES

- Almeida EAB (2008) Colletidae nesting biology (Hymenoptera: Apoidea). Apidologie 39:16–29. https://doi.org/10.1051/apido:2007049
- Almeida EAB, Pie OMR, Brady SG, Danforth BN (2012) Biogeography and diversification of colletid bees (Hymenoptera: Colletidae): emerging patterns from the southern end of the world. J Biogeogr 39:526–544. https://doi.org/10.1111/j. 1365-2699.2011.02624.x
- Antoine CM, Forrest JRK (2021) Nesting habitat of ground-nesting bees: a review. Ecol Entomol 46:143–159. https://doi.org/10.1111/een.12986
- AOAC (2000) Official methods of analysis. 17th Edition: The association of official analytical chemists. Gaithersburg MD, USA
- Barbosa-Costa K, Kerr WE, Carvalho-Zilse GA (2012) Number of Malpighian tubules in larvae and adults of stingless bees (Hymenoptera: Apidae) from Amazonia. Neotropical Entomol 41:42–45. https:// doi.org/10.1007/s13744-011-0017-5
- Batley M, Houston TF (2012) Revision of the Australian bee genus *Trichocolletes* Cockerell (Hymenoptera: Colletidae: Paracolletini). Rec Australian Mus 64:1– 50. https://doi.org/10.3853/j.0067-1975.64.2012.1589

- Beardsell DV, Clements MA, Hutchinson JF, Williams EG (1986) Pollination of *Diuris maculata* R. Br. (Orchidaceae) by floral mimicry of the native legumes *Daviesia* spp. and *Pultenaea scabra* R. Br Australian J Botany 34:165–173. https://doi.org/ 10.1071/BT9860165
- Bergsson G, Hilmarsson H, Thormar H (2011) Chapter 3: Antibacterial, antiviral and antifungal activities of lipids. In: Thormar H (ed) Lipids and essential oils as antimicrobial agents. John Wiley & Sons, Ltd; Chichester, UK, pp 47–80. https:// doi.org/10.1002/9780470976623
- Cane JH (2003). Exotic non-social bees (Hymenoptera: Apoidea) in North America: ecological implications. In: Strickler KV, Cane JH (eds). For non-native crops, whence pollinators of the future? Thomas Say Publications in Entomology, Entomological Society of America, Lanham, MD, pp. 113–126
- Cane JH (2021) A brief review of monolecty in bees and benefits of a broadened definition. Apidologie 52:17–22. https://doi.org/10.1007/s13592-020-00785-y
- Cane JH, Gerdin S, Wife G (1983) Mandibular gland secretions of solitary bees (Hymenoptera: Apoidea): potential for nest cell disinfection. J Kans Ent Soc 56:199–204
- Cane JH, Tengo JO (1981) Pheromonal cues direct mate-seeking behavior of male *Colletes cunicularius* (Hymenoptera: Colletidae). J Chem Ecology 7:427–436. https://doi.org/10.1007/BF00995765
- Carballeira NM (2008) New advances in fatty acids as antimalarial, antimycobacterial and antifungal agents. Prog Lipid Res 47:50–61. https://doi.org/ 10.1016/j.plipres.2007.10.002
- Dade HA (1962) Anatomy and dissection of the honeybee. Bee Research Association, pp. 158
- Danforth BN, Minckley RL, Neff JL (2019) The solitary bees: biology, evolution conservation. Princeton University Press, pp. 472
- Downer RGH, Matthews JR (1976) Patterns of lipid distribution and utilization in insects. Am Zool 16:733–745. https://doi.org/10.1093/icb/16.4.733
- Drake DR, Brogden KA, Dawson DV, Wertz PW (2008) Antimicrobial lipids at the skin surface. J Lipid Res 49:4–11. https://doi.org/10.1194/jlr.R700016-JLR200
- Erdtman G (1960) The acetolysis method. A Revised Description Svensk Botanisk Tidskrift 54:561–564
- Fischer CL, Blanchette DR, Brogden KA, Dawson DV, Drake DR, Hill JR, Wertz PW (2014) The roles of cutaneous lipids in host defense. Biochem Biophys Acta 1841:319–322. https://doi.org/10. 1016/j.bbalip.2013.08.012
- Fraenkel G, Blewett M (1944) The utilisation of metabolic water in insects. Bull Entomol Res 35:127– 139. https://doi.org/10.1017/S0007485300017351
- Hirashima Y (1960) An interesting habit of the fullgrown larvae of *Halictus affinis* Smith, apparently correlated with the function of the Malpighian tubes (Hymenoptera, Halictidae). Mushi 33:85–88

- Hornitsky M (2003) Fatty acids an alternative control strategy for honeybee diseases. A report for the Rural Industries Research and Development Corporation. RIRDC Publication No 03/028, vi + 1–13
- Houston TF (2018) A guide to native bees of Australia. CSIRO Publications, Clayton South, Victoria, vii + 272 pp
- Houston TF (2020) On the remarkable nesting biology of an Australian bee in the genus *Trichocolletes* Cockerell (Hymenoptera: Colletidae). Austral Entomology 59:593–601. https://doi.org/10.1111/ aen.12462
- Houston TF, Thorp RW (1984) Bionomics of the bee Stenotritus greavesi and ethological characteristics of Stenotritidae (Hymenoptera). Records of the Western Australian Museum 11:375–385
- Indsto JO, Weston PH, Clements MA, Dyer AG, Batley M, Whelan RJ (2006) Pollination of *Diuris maculata* (Orchidaceae) by male *Trichocolletes venustus* bees. Aust J Bot 54:669–679. https://doi.org/10. 1071/BT05146
- Kabara JJ (1984) Antimicrobial agents derived from fatty acids. Journal of the American Oil Chemists' Society 61:397–403. https://doi.org/10.1007/ BF02678802
- Matsue M, Mori Y, Nagase S, Sugiyama Y, Hirano R, Ogai K, Ogura K, Kurihara S, Okamoto S (2019) Measuring the antimicrobial activity of lauric acid against various bacteria in human gut microbiota using a new method. Cell Transplant 28:1528– 1541. https://doi.org/10.1177/0963689719881366
- Mellanby K (1942) Metabolic water and desiccation. Nature 150(3792):21. https://doi.org/10.1038/150021a0
- Mello ML, Kerr WE (1984) Histochemistry of salivary gland and Malpighian tubule secretions contributing to the cocoon in *Plebeia droryana* and *Scaptotrigona postica* (Hym., Apoidea). Zoologischer Anzeiger Jena 213:177–189
- Michener CD (1965) A classification of the bees of the Australian and South Pacific regions. Bull Am Mus Nat Hist 130:1–362
- Michener CD (1979) Biogeography of the bees. Ann Mo Bot Gard 66:277–347. https://doi.org/10.2307/ 2398833
- Mitra A (2013) Function of the Dufour's gland in solitary and social Hymenoptera. J Hymenopt Res 35:33–58. https://doi.org/10.3897/JHR.35.4783
- Nelson JA (1924) Morphology of the honeybee larva. J Agric Res 28:1167–1213
- Rozen JG jr (1984) Nesting biology of diphaglossine bees (Hymenoptera, Colletidae). Am Mus Novit 2786:1–33
- Rozen JG jr (2016) Hesperapis rhodocerata: behavioral biology, egg, and larval instars, including behavioral and larval comparisons with H. larreae (Hymenoptera: Melittidae: Dasypodainae). Am Mus Novit 3856:1–19
- Scaccabarozzi D, Cozzolino S, Guzzetti L, Galimberti A, Milne M, Dixon KW, Phillips RD (2018)

Masquerading as pea plants: behavioural and morphological evidence for mimicry of multiple models in an Australian orchid. Ann Bot 122:1061–1073. https://doi.org/10.1093/aob/mcy166

- Scaccabarozzi D, Guzzetti L, Phillips RD, Milne L, Tommasi N, Cozzolino S, Dixon KW (2020a) Ecological factors driving pollination success in an orchid that mimics a range of Fabaceae. Bot J Linn Soc 20:1–17. https://doi. org/10.1093/botlinnean/boaa039
- Scaccabarozzi D, Dixon KW, Tomlinson S, Milne L, Bohman B, Phillips RD, Cozzolino S (2020b) Pronounced differences in visitation by potential pollinators to co-occurring species of Fabaceae in the southwest Australian biodiversity hotspot. Bot J Linn Soc 194:308–325. https://doi.org/10.1093/ botlinnean/boaa053
- Snodgrass RE (1956) Anatomy of the Honey Bee. Cornell University Press, Ithaca.Southworth D (1973) Cytochemical reactivity of pollen walls. J Histochem Cytochem 21:73–80. https://doi.org/10.1177/21.1.73
- Southworth D (1973) Cytochemical reactivity of pollen walls. J Histochem Cytochem 21:73–80
- Stephen WP, Bohart GE, Torchio PF (1969) The biology and external morphology of bees. Agricultural Experiment Station, Oregon State University, Corvallis; ii + 140 pp
- Torchio PF (1965) Observations on the biology of *Colletes ciliatoides* (Hymenoptera: Apoidea, Colletidae). J Kansas Entomol Soc 38:182–187

- Torchio PF (1975) The biology of *Perdita nuda* and descriptions of its immature forms and those of its *Sphecodes* parasite (Hymenoptera: Apoidea). J Kansas Entomol Soc 48:257–279
- Torchio PF (1984) The nesting biology of Hylaeus bisinuatus Forster and development of its immature forms (Hymenoptera: Colletidae). J Kansas Entomol Soc 57:276–297
- Winfree R (2010) The conservation and restoration of wild bees. Ann N Y Acad Sci 1195:169–197. https://doi.org/10.1111/j.1749-6632.2010.05449.x

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