

Courtship behaviour in the genus *Nomada* – antennal grabbing and possible transfer of male secretions

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Abstract

Due to low population densities, copulation in the cuckoo bee genus *Nomada* has not previously been observed, although a seminal paper by Tengö and Bergström (1977) on the chemomimesis between these parasitic bees and their *Andrena* or *Melitta* hosts postulated that secretions from male glands might be sprayed onto females during copulation. Our observations on the initiation and insertion phase of copulation in three species of *Nomada* now indicate antennal grabbing as a mechanism by which chemicals are transferred between the sexes. Histological studies of the antennae of *N. fucata* and *N. latburiiana* reveal antennal modifications associated with cell aggregations that represent glandular cells, and SEM studies revealed numerous excretory canals.

Keywords

Antennal structure, copulation, chemomimesis, male secretions, cuckoo bees

Introduction

Bees (Apoidea, Apiformes) use visual, auditory and chemical signals to locate potential mates, and males may search for females at flowers, nest sites, or perch spots by non-aggressively patrolling along a strict route or by defending a territory in which females are likely to be encountered (Alcock et al. 1978, Eickwort and Ginsberg 1980). Observations on the actual copulation, which usually occurs on the wing, are scarce (Alcock et al. 1978, Michener 2007). Especially kleptoparasitic bees, which have low population densities, are rarely observed during mating, which comprises initiation, insertion, and separation. Here we report on the mating behavior of three species of *Nomada*, a genus of kleptoparasitic bees in the tribe Nomadini of the family Apidae (Hedtke et al. 2013) that comprises around 700 species occurring throughout the Holarctic, the Neotropics, sub-Saharan Africa, and the oriental region (Michener 2007; Ascher and Pickering 2017).

Nomada parasitizes predominantly species of *Andrena* (Tengö and Bergström 1977, Michener 2007), and the females use olfactory cues for detecting suitable host nests, but visual cues for finding nest entrance holes (Cane 1983, Schindler 2005). *Nomada* courtship includes “swarming” of males at shrubs of willow (*Salix* spec.) and gooseberry (*Ribes uva-crispa*; Friese 1923: 384) or males patrolling in groups with males of the host species (Tengö and Bergström 1977). Alcock (1978) saw males rubbing their abdomen and the lower surface of their head against blades of grass from which he inferred that they mark rendezvous places with sexual pheromones, an inference supported by an observation of a *N. fabriciana* male rubbing itself against a leaf (Smit 2005). Female dummies prepared with extracts of conspecific females elicit the initiation of copulation (Fleck 1995). The mandibular gland secretions of *N. bifida*, *N. flavoguttata*, *N. flavopicta*, *N. goodeniana*, *N. leucophthalma*, *N. lineola*, *N. marshamella*, and *N. panzeri* contain chemical compounds identical to those of the Dufour’s glands of their host species (Tengö and Bergström 1977), and Tengö and Bergström hypothesized that these secretions are sprayed on the females during mating to help them enter the host species’ nests. Figure 3 in Bergström (2008) shows a *Nomada lathburiana* female waiting to enter the nest of an *Andrena cineraria* female.

The present study of the courtship and mating behaviour of *Nomada* is based on field and lab observations of *N. fucata* and *N. lathburiana* in central Germany (Schindler 2005) and field studies on *N. flavoguttata* in southern Germany. *Nomada flavoguttata* is distributed across Eurasia, *N. fucata* from Portugal to Central Asia and from Sweden to Northern Africa, and *N. lathburiana* in Eurasia and Northern Africa (Scheuchl and Willner 2016). Observation of a previously unreported behavior during the initiation of copulation, which we term “antennal grabbing”, indicated that males transfer a chemical substance to the antennae of the females. This led us to investigate the morphology and histology of the antennae.

Methods

Behavioral observations

Courtship behavior in *Nomada fucata* F. and *N. lathburiana* (K.) was studied both in the field and in the lab. The courtship phase was observed at nesting sites of the host species *Andrena flavipes* Panz. and *A. vaga* Panz. and at foraging plants in an abandoned gravel pit near Bonn (50.773293°N, 7.147145°E, about 50 m a.s.l.) in 1998 and 1999. The initiation and insertion phase of copulation in *N. flavoguttata* (K.) was observed on 24 May 2017 in the conservation area “Allacher Heide” in Munich (48.206729°N, 11.474383°E, 517 m a.s.l.). It was the first sunny and warm day after a longer period of cold and moist weather, leading to a major hatching event of several *Nomada* species. Within 5 m², more than 50 *Nomada* bees could be observed foraging and mating on *Taraxacum officinale* s.l. (Fig. 1). Three bees were also identified via DNA barcoding (see Appendix 1 for a description of lab procedures).

Studies in the lab were carried out with virgin males and females of *N. fucata* and *N. lathburiana*. Bees were collected with emergence traps at the nests and released in flight cages (4 × 2 × 2 m) or transferred in petri-dishes (ø 15 cm) for observation. *Nomada* individuals were kept in refrigerators at 6 °C before being placed into the arena. Petri-dishes were illuminated with artificial light. Courtship behavior was documented with macro photographs and video records that were then analyzed frame by frame. Courtship was divided into the following phases, a) Courtship or pre-copula phase: males discovering mates, males approaching females, males mounting the abdomen of the females and attempting to copulate. b) Copula phase: male inserting his genital into the female’s genital aperture. We did not observe the separation phase of copulation.

Morphological studies

Flagella morphology of males of *Nomada fucata* and *N. lathburiana* was studied under a photo stereomicroscope, with photos taken at 10 to 40 x. Morphological terminology for the antennae follows Michener (1944), those for the cuticular structures Es-slen and Kaissling (1976). Elevated modifications on the flagella of *Nomada* males are called tyloids, following Schönitzer et al. (2000). Morphological structures of the outer and the inner surface of the flagella of *N. fucata* and *N. lathburiana* males were examined under scanning electron microscopy (SEM). Study objects were dried, mounted on aluminum stubs and gold coated or in the case of the flagella of *N. flavoguttata* platinum coated. Some antennae were cleaned by macerating in 5% KOH for 12 hours at 40 °C and dehydrated in graded ethanol series. To study the inner surface of the flagella, they were embedded in hot-melt glue, opened transversally with industrial blades, macerated and dehydrated as described above.

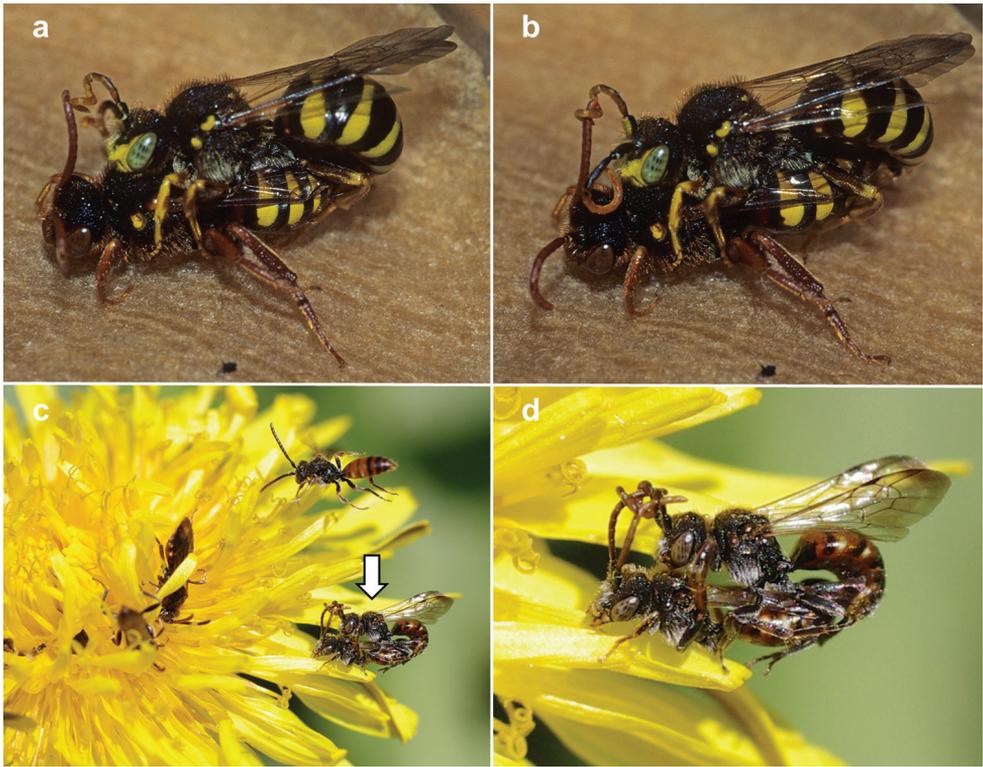


Figure 1. Mating of *Nomada lathburiana* (a, b) in the lab and of *N. flavoguttata* on *Taraxacum officinale* s.l. (c, d). a ♂ mounts the ♀ and fixes its wings with its mid and hind legs and tries to entangle the female's antennae spirally with its flagella. ♂ lifts the abdomen of the ♀ with its hind legs and tries to insert its genitalia in the female's genital opening b ♂ repeatedly spirally entangles the female's antennae with its left and right flagellum and pulls it off medially to apically. ♂ tries to insert its genitalia repeatedly c ♂♂ patrolling at a blossom of *T. officinale* d Insertion phase of copulation. Antennation of ♂ as described in (b).

Histological studies

Antennae of males of *N. fucata* and *N. lathburiana* were fixed in a Duboscq-Brasil solution (Romeis 1989) and then transferred in 70% ethanol. They were dissected (transversally and longitudinally), dehydrated in graded ethanol series and embedded in EMbed 812 (Electron Microscopy Sciences, Hatfield, PA). Semi-thin sections of flagella 4–7 (*N. fucata*) and flagella 9–11 (*N. lathburiana*) were made with glass knives on a rotation microtome. Sections were stained with toluidinblue and analysed under a photo-microscope.

Results

Courtship behavior of *Nomada flavoguttata*, *N. fucata*, and *N. lathburiana*

Field observations revealed that males of *N. fucata* patrol together with males of *Andrena flavipes*, the host species, at foraging plants and above the entrances of the

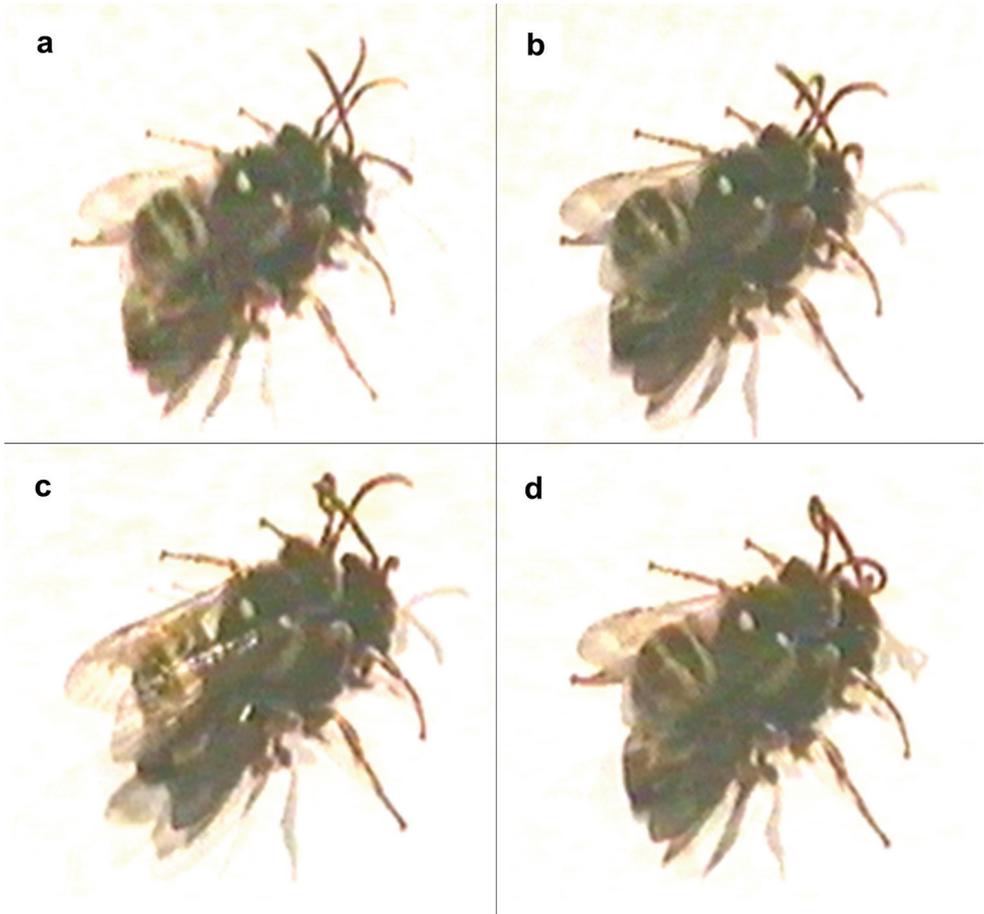


Figure 2. Mating of *Nomada fucata* (singular images of a video sequence). **a** ♂ mounts the ♀ and fixes its wings with its mid and hind legs. ♂ lifts the abdomen of the ♀ with its hind legs and tries to insert its genitalia in the female's genital opening **b** ♂ entangles the female's left antenna with its left flagellum **c** ♂ pulls its left flagellum from medial to apical along the female antenna **d** ♂ repeatedly entangles the female's antennae with its left and right flagellum and pulls it off medially to apically. ♂ tries to insert its genitalia.

fossorial host nests. During these flights, we observed neither inter- nor intraspecific aggression. *Nomada* males attempted to copulate with females that they discovered on flowers or on the ground, but females rejected the males in all observed attempts ($n = 11$). Males of *N. lathburiana* did not patrol potential rendezvous sites.

For both *N. fucata* and *N. lathburiana*, copulation was studied in the laboratory, with females chilled down for immobilization (since mobile females repelled the males in the lab experiment). For *N. fucata*, two copulations were observed, and for *N. lathburiana* four. Males mounted the abdomen of the females, fixed their forelegs on the side of the females' thorax, clinched the wings of the females with their middle legs and lifted their mates' abdomen with their hind legs to insert the genitalia. In this position the males' head was above the pronotum of the female. During the initiation of copulation males repeatedly wound their flagella (in case of *N. lathburiana* in a spiral) around

Table 1. Tyloid-like structures on the flagella of central-European *Nomada* males. The preselection of most species followed descriptions from Celary 1995 and Scheuchl 2000. Systematics after Alexander and Schwarz (1994); Abbreviations: gr. = Group, fig. = flagellomere.

Species	Species-group	Tyloid-like structures	
		position	morphology
<i>N. bifasciata</i> Olivier	<i>bifasciata</i> -gr.	figs 3–8	inconspicuous tubercles
<i>N. fucata</i> Panzer	<i>bifasciata</i> -gr.	figs 3–8	inconspicuous tubercles
<i>N. melanopyga</i> Schmied.	<i>bifasciata</i> -gr.	figs 4–9	inconspicuous tubercles
<i>N. succincta</i> Panzer ¹	<i>bifasciata</i> -gr.	figs 3–8	inconspicuous tubercles
<i>N. flavoguttata</i> (Kirby)	<i>flavoguttata</i> -gr.	figs. 3–7	inconspicuous tubercles
<i>N. bluethgeni</i> Stöckhert	<i>furva</i> -gr.	figs 2–4	spiky spots
<i>N. distinguenda</i> Morawitz	<i>furva</i> -gr.	figs 3–5	inconspicuous tubercles
<i>N. furvoides</i> Stöckhert	<i>furva</i> -gr.	figs 3–5	spiky to rounded elevation
<i>N. kohli</i> Schmied.	<i>furva</i> -gr.	figs 3–5	rounded spots
<i>N. posthuma</i> Blüthgen ²	c.f. <i>furva</i> -gr.	figs 3–6	spots
<i>N. argentata</i> Herr.-Schäf.	<i>integra</i> -gr.	figs 4–8	tubercles
<i>N. beaumonti</i> Schwarz	<i>integra</i> -gr.	figs 4–10	spiky spots
<i>N. facilis</i> Schwarz	<i>integra</i> -gr.	figs 3–9	spiky spots
<i>N. integra</i> Brullé	<i>integra</i> -gr.	figs 3–10	transverse ridges
<i>N. pleurosticta</i> Herr.-Schäf.	<i>integra</i> -gr.	figs 4–9	transverse ridges
<i>N. stigma</i> Fabricius	<i>integra</i> -gr.	figs 4–9	transverse ridges
<i>N. braunsiana</i> Schmied.	<i>ruficornis</i> -gr.	figs 2–9	spots
<i>N. castellana</i> Dusmet	<i>ruficornis</i> -gr.	figs 4–12	spots
<i>N. lathburiana</i> (Kirby)	<i>ruficornis</i> -gr.	figs 1–11	thorn-like spots
<i>N. striata</i> Fabricius	<i>ruficornis</i> -gr.	figs 3–7	inconspicuous tubercles

¹ the taxa *Nomada succincta* and *Nomada goodenia* were not separated,

² species for this study not available.

the female's antennae and then stroked the female's antennae (Fig. 1 and 2). For *N. flavoguttata*, the first stages of copulation were observed in the field (Fig. 1). We observed about 50 males and females foraging and resting on flowers of dandelion (*Taraxacum*). Males attempted to mount females from the back and grabbed the females' antennae with their flagella, similar to the behavior observed in the lab for *N. lathburiana*.

Morphology of the flagella

In all three species, the flagella of the males' antennae bear tyloid like-structures (Table 1, Fig. 3). In *N. fucata*, these tyloids are inconspicuous tubercles at the lateral side of flagella 3 to 8. The tubercles show numerous pores with diameters of 0.6 to 1 µm out of which paste-like substances were secreted. In *N. flavoguttata*, the tyloids are most prominent laterally on flagella 3 to 7 but can also be seen on flagella 8 to 10. There also are numerous pores (ca. 0.5 µm) and 3 to 5 µm-long setae. In *N. lathburiana*, thorn-like cuticular modifications can be seen at the ventral side of flagella 2 to 11, the surface of which appears to be coated with secretions; there were no pores.

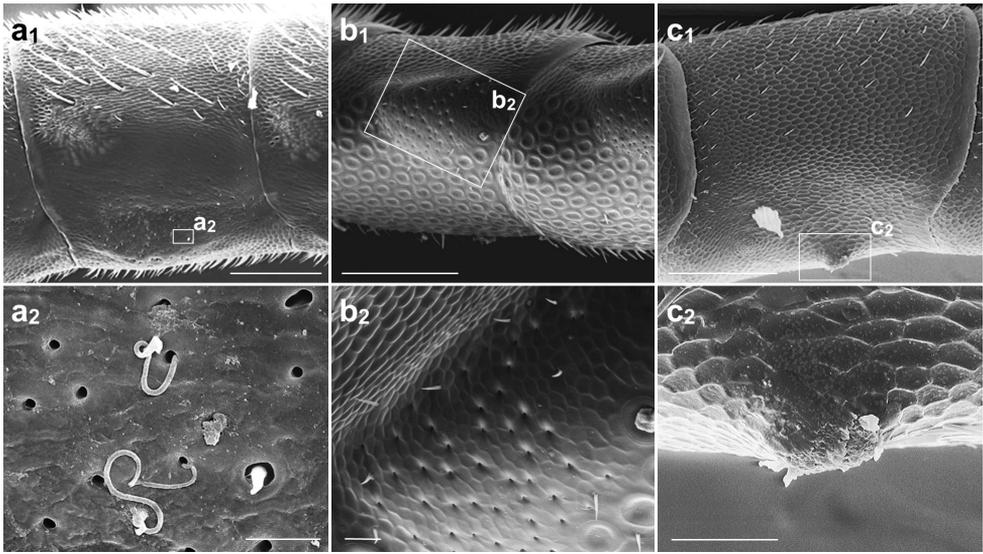


Figure 3. Modifications on the antennae of *Nomada fucata* ♂ (a) (left antenna, dorsal), *N. flavoguttata* ♂ (b) (right antenna, ventral) and *N. lathburiana* ♂ (c) (left antenna, lateral). SEM micrographs. **a₁** weak elevation on flagellum 6 laterally. Scale unit: 0.1 mm **b₁** weak elevations on flagellum 4 ventrally. Scale unit: 0.1 mm **c₁** nodule on flagellum 9. Scale unit: 0.1 mm **a₂** pores and secretions on the elevation. Scale unit: 0.01 mm **b₂** pores and setae on the elevations. Scale unit: 0.01 mm **c₂** nodule covered with secretions. Scale unit: 0.02 mm.

Antennal glands

Histological studies of *N. fucata* and *N. lathburiana* corroborated that these antennal modifications are associated with glands (Fig. 4). Longitudinal and cross semi-thin sections of several flagella revealed two cell aggregations that represent glandular cells of type 1 and type 3 (Noirot and Quennedy 1991). SEM studies of the inner surface of the modifications revealed numerous excretory canals (see Fig. 5)

Discussion

This is the first report of the mating behavior in *Nomada*. The males use their antennae to grab the females' antennae for a chemical signal transfer, while they use their feet to pin down the female. In Hymenoptera and other insects, transfer of sexual pheromones from the males to the females is widespread (e.g. Hymenoptera, Terebrantes: Dahms 1984, Bin and Vinson 1986, Isidoro et al. 1999; Hymenoptera, Aculeata: Isidoro et al. 1996, 2002, Romani et al. 2003; Coleoptera: de Marzo and Vit 1983, Bartlet et al. 1994; Trichoptera: Roemhild 1980), with the organs of transfer usually assumed to be the antennae on the basis of histological studies that revealed male antennal glands. Antennal movements during mating have been observed in Halictidae, Megachilidae,

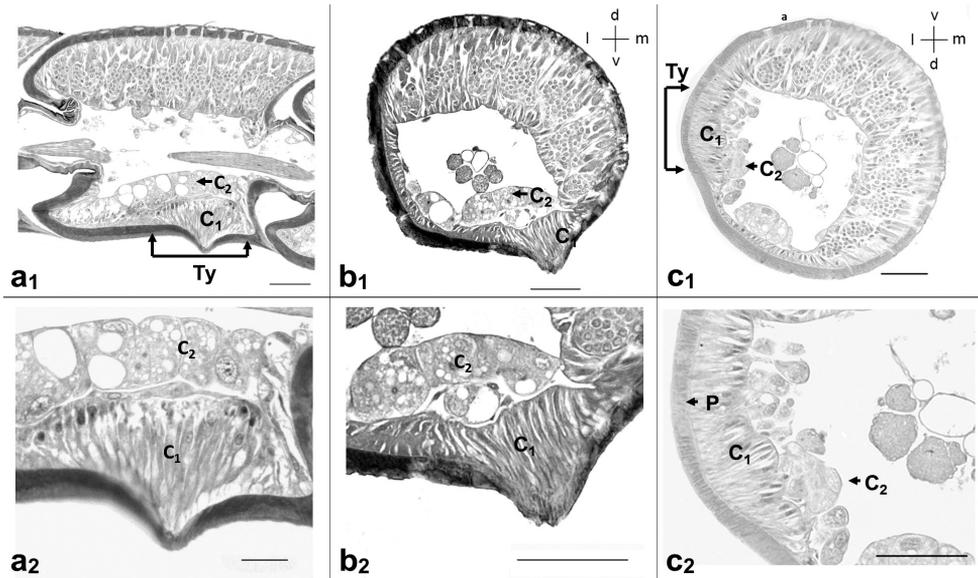


Figure 4. Semi-thin sections of antennomere 9 of *Nomada lathburiana* ♂ [a=longitudinal, b=cross. Ty=Thyloid, C₁=compact epidermal cells, C₂= gland cells, type III (Noirot & Quennedy 1991). Scale unit: 0.05 mm (a₁, b₁, b₂), 0.02 mm (a₂). Topography: l=lateral, m=medial, v=ventral, d=dorsal] and of antennomere 7 of *Nomada fucata* ♂ [c=cross. Ty=Tyloid, C₁= compact epidermal cells, C₂=gland cells, type III, P=porous cuticle (Noirot & Quennedy 1991). Scale unit: 0.05 mm (c₁, c₂) Topography: l=lateral, m=medial, v=ventral, d=dorsal].

Colletidae, and Anthophoridae (Barrows 1975, Batra 1978, Wcislo et al. 1992, Wcislo 1995, Wcislo and Buchmann 1995, Felicioli et al. 1998). A winding of the male's antennae around the females' antennae, which we here term “antennal grabbing,” has been observed in the widespread west Palearctic species *Melecta albifrons* (Jacobi 2005), and similar antennal rubbing behaviors have been documented for Dryinidae (Waloff 1974), Tiphiidae (Rivers et al. 1979), Sphecidae (Blösch 1999, 2000), and Vespidae (Romani et al. 2005).

Male antennation has been interpreted in terms of sensory exploration of visual and tactile stimuli as well as the transfer of pheromones (Isidoro et al. 1996, Bin et al. 1989, Schönitzer et al. 2000, Ayasse et al. 2001, Romani et al. 2003). Our observations suggest that *Nomada* males transfer liquid pheromones secreted from antennal glands to the females. Paste-like excretions as we saw on flagella pores of *N. fucata* may serve as contact pheromones that are spread directly onto the female antennae (Wittmann and Blochtein 1995, Isidoro et al. 1996). In *Osmia cornuta*, males in copula position rhythmically move their antennae, but without touching the females' antennae (Felicioli et al. 1998), suggesting that volatile, not liquid, pheromones are applied onto the females' antennae. In *Megachile* and *Xylocopa*, by contrast, males bring their modified fore or middle leg basitarsus in contact with the female flagella (Wittmann & Blochtein 1995, Wittmann et al. 2004), suggesting the

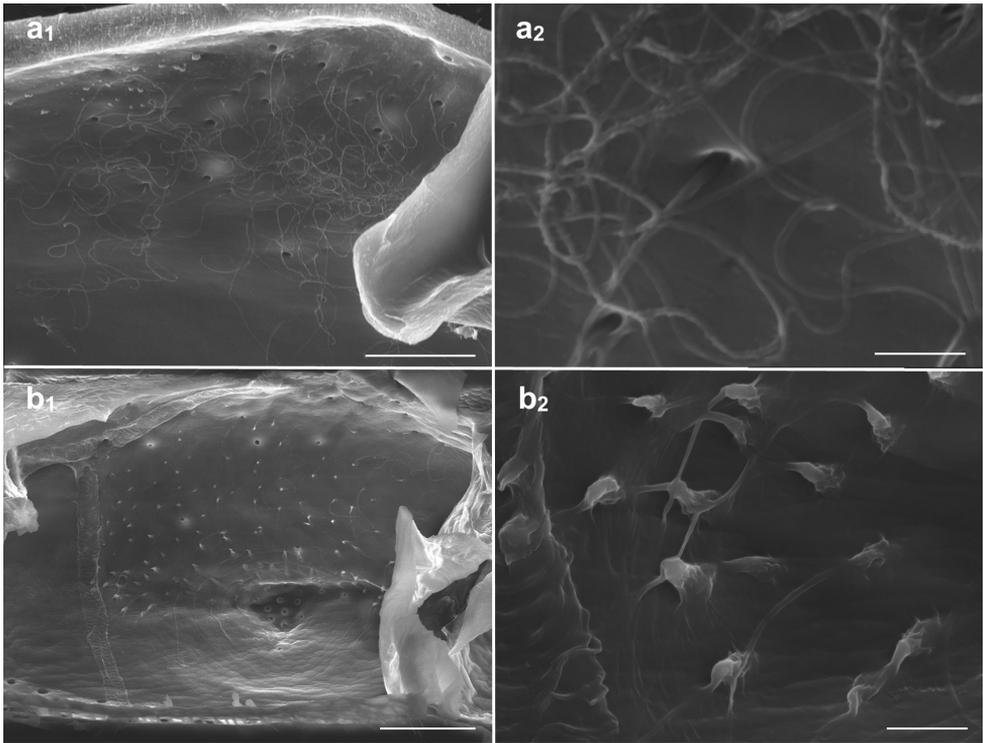


Figure 5. Excretory canals at the inner surface of the tyloid-like structures on the antennomere 6 of *Nomada fucata* ♂ (**a₁**, **a₂**) and antennomere 5 of *Nomada lathburiana* ♂ (**b₁**, **b₂**). SEM micrographs. Scale unit: 0.03mm (**a₁**), 0,004mm (**a₂**), 0.06 mm (**b₁**), 0.01 mm (**b₂**).

transfer of pheromones from tarsal glands. In males of *Anthophora plumipes*, finally, sexual pheromones produced in abdominal glands are transferred with the hind legs to specialized setae of the elongate middle legs that then brush them onto the females' antennae (Wittmann et al. 2004).

The biological function of the substance transferred by *Nomada* males to females during antennal grabbing may lie in making inseminated females unattractive to other males as suggested for *Centris adani* (Frankie et al. 1980) or in males perfuming females to facilitate odor mimetism of the host bees as suggested for five of eight chemically investigated *Nomada*/host pairs (Tengö and Bergström 1977). Identical compounds (isoprenoid and straight chain esters of short acids) in the nest-parasitic *Nomada* females and their *Andrena* or *Melitta* hosts presumably make it easier for the parasites to gain entrance into the nests of host-bee females without being attacked. Tengö and Bergström (1977) reported that the *Nomada* compounds they investigated are produced in the males' mandibular glands (in the head), but it appears that entire heads may have been used for chemical extraction. These authors also had no opportunity to observe any mating behavior. Interestingly, *Nomada flavoguttata*, the only species included in both studies, was not found to produce identical compounds as its host.

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Appendix I

DNA extraction, amplification and sequencing

DNA was isolated with the QIAGEN DNeasy-Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany), using one leg per bee and following the QIAGEN Quick-Start Protocol (January 2011) with two modifications: The legs were incubated in Lysis Buffer (ATL) and Proteinase K for at least 48 h at room temperature and 5 to 10 hours at 56 °C. To increase DNA concentration, we used 100 µL elution buffer (PE; 5 mM Tris/HCL pH 8.5). A fragment of the mitochondrial cytochrome c oxidase (COI) gene with a 658 bp target region near the 5' terminus of COI was amplified using primers described in Schmidt et al. (2015), namely COIfor (ATT CAA CCA ATC ATA AAG ATA TTG G) and COIrev (TAA ACT TCT GGA TGT CCA AAA AAT CA). Polymerase chain reactions (PCR) were performed using standard conditions. Amplified products were sequenced on an ABI 3100 Avant capillary sequencer (Applied Biosystems), and forward and reverse sequences were manually edited and assembled, using Sequencher 5.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA) and BLAST searching in GenBank. All species sequenced for this study have been previously barcoded for the GBOL-project, and reference sequences were therefore available in NCBI GenBank under DOIs: dx.doi.org/10.5883/DS-GBAPI and dx.doi.org/10.5883/DS-GBAPS. Three new sequences were generated for this study and are available under as GenBank accessions MG845937, MG845938, and MG845939.

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