

## Larval exocrine glands in the galerucine *Agelastica alni* L. (Coleoptera: Chrysomelidae): their morphology and possible functions

Martina Bünnige and Monika Hilker

Institut für Zoologie, Haderslebener Strasse 9, D-12163 Berlin, Germany, e-mail: hilker@zedat.fu-berlin.de

**Summary.** Larvae of the alder leaf beetle *Agelastica alni* (Coleoptera: Chrysomelidae) discharge a fluid from openings located dorsolaterally on paired tubercles of the first to eighth abdominal segments, when disturbed. These paired openings have been described as apertures of defensive glands, whereas the discharged fluid (= tubercle fluid) is often mentioned as reflex blood. Up to now, clear evidence for the presence of glandular cells below the precisely described apertures was lacking, and detailed knowledge of the composition and defensive efficiency of the discharged fluid was not available. In this study, the internal structures around the paired abdominal openings in *A. alni* larvae were investigated by scanning electron microscopy and histological methods. Additionally, the composition of tubercle fluid and larval haemolymph was compared by microscopy, tlc, and electrophoresis. The effects of tubercle fluid and haemolymph on ants and conspecific larvae were studied by olfactory and feeding bioassays.

The morphological studies revealed that *A. alni* larvae possess sac-like cuticular invaginations which open dorsolaterally from the spiracle on tubercles of the abdominal segments 1–8. A single voluminous gland cell (type III) opens into each invaginated cuticular sac. Each sac may be turned from the inside to the outside and retracted by muscles. The discharged tubercle fluid conforms with haemolymph in its pattern of haemocytes and proteins (SDS-PAGE). Tlc patterns of the tubercle fluid and haemolymph differ by a single spot when using a high polarity eluent. Both tubercle fluid and haemolymph significantly deter the ant *Myrmica rubra* from feeding. Heating, treating by proteinase K, or exclusion of molecules > 3 kD did not inactivate the feeding deterrent quality of tubercle fluid and haemolymph, thus indicating that the feeding deterrents are small, non-volatile, non-proteinous molecules present both in the haemolymph and the tubercle fluid. However, the bioactivity of volatiles from tubercle fluid and haemolymph differed, since the volatiles from tubercle fluid act as alarm pheromone for conspecific larvae, whereas volatiles from the haemolymph do not. The function of the glandular cells associated with the segmental cuticular invaginations of *A. alni* larvae is discussed.

**Key words.** reflex bleeding – larval exocrine glands – alarm pheromone – *Agelastica alni* – Galerucinae – Chrysomelidae

### Introduction

Larvae of Chrysomelidae are able to protect themselves against predators by a wide variety of devices, ranging from inconspicuous colour and shape, hairs and spines, elaborated faecal shields, reflex bleeding to defensive exocrine secretions (Blum 1994; Crowson 1981; Dettner & Schwinger 1987; Erber 1968; Pasteels *et al.* 1988; Wallace & Blum 1971). The presence of larval defensive glands in the Chrysomelidae has been used as a taxonomic character and also serves as an argument in some phylogenetic studies (Cox 1982; Lee 1993; Steinhäuser 1994; Takizawa 1972, 1994, 1997).

Defensive glands of larvae in the subfamily Chrysomelinae have intensively been studied by histological, different microscopic and chemical methods. These larval glands are well-known to consist of glandular cells (type III), which enter a cuticular reservoir that opens either intersegmentally or dorsolaterally on a tubercle of a thoracic and abdominal segment, respectively (Garb 1915; Hinton 1951; Renner 1970). The main components of the defensive secretions are of impressive diversity between species and even between populations (Blum *et al.* 1972; Dettner & Schwinger 1987; Gross & Hilker 1996; Matsuda & Sugawara 1980; Pasteels *et al.* 1984; Sugawara *et al.* 1979).

In the chrysomelid taxon Galerucinae, species with so-called glanduliferous larvae (*i.e.*, larvae which are said to possess paired exocrine glands on thoracic and/or abdominal segments) are known as well as species with non-glanduliferous larvae (no paired larval exocrine glands) (Takizawa 1972). Takizawa (1972) divides larvae of the subfamily Galerucinae into five groups based on the number and localisation of their glands (Table 1). For the glanduliferous galerucine larvae, precise and excellent descriptions of the localisation and external structures of the gland openings are available, but no study showed the presence of glandular cells yet (Böving 1929; Paterson 1931; Takizawa 1972). This lack of evidence for the presence of glandular cells might be the cause for a puzzling interpretation

of the defensive responses in larvae of the alder leaf beetle, *Agelastica alni*. On one hand, larvae of this galerucine species are said to have defensive glands aggregated in the wall of sacs which can be turned to the outside at tubercles located pairwise dorsolaterally on the abdominal segments 1–8 (Böving 1929). On the other hand, the yellow fluid discharged by *A. alni* larvae from dorsolateral tubercles after molestation is considered as reflex blood (Baur & Rank 1996; Hollande 1911; Tischler 1977). Larvae of *A. alni* belong to the tribe Sermylini within the subfamily Galerucinae (compare Table 1, Seeno & Wilcox 1982). They mainly feed upon alder (*Alnus* spp.) and live in aggregations of about 20–60 individuals during their first and second instar.

In the present study, we investigated the internal structures of the dorsolateral tubercles in *A. alni* larvae to elucidate whether or not glandular cells are present. The fluid discharged from the tubercles (=tubercle fluid) was compared to haemolymph by microscopy and by chemical methods in order to examine whether the discharged fluid contains haemolymph or not. To study the defensive mode and the efficiency of the tubercle fluid against a generalist predator, feeding assays were carried out with the ant *Myrmica rubra*. Since observations of *A. alni* larvae during our experiments indicated that the release of tubercle fluid of a single larva disturbs the cohesion of a larval aggregation, we additionally studied the effects of tubercle fluid on conspecific larvae.

## Materials and methods

### Insects

Gravid females of *A. alni* were collected in Berlin from *Alnus glutinosa* and kept in small cages on alder twigs. Egg masses were transferred into Petri dishes which were lined with filterpaper. As soon as larvae hatched they were provided with alder leaves ad libitum. These larvae were used for morphological, chemical and ethological studies. All stages of *A. alni* were kept at 20°C (15°C), 70% RH, and 16 h/8 h light/dark cycles.

### Scanning electron microscopic studies

Individuals of all three larval instars of *A. alni* were prepared for scanning electron microscopy (SEM). For comparison, 3rd instar larvae of two chrysomeline species, *Gastrophysa viridula* DeGeer and *Lineaidea aenea* L., were prepared in the same way. After sagittal dissection of previously frozen larvae into halves, they were treated with 5% KOH for 30 h at room temperature. Samples were dehy-

drated with acetone (10–100%; at 10% intervals, 15 min for each concentration), critical-point-dried (CPD 030, Balzers) and mounted on aluminium stubs. They were sputter coated with gold (SCD 040, Balzers) and examined with a scanning electron microscope (Philips SEM 515).

### Light microscopic studies of tubercle fluid, haemolymph and histological sections of larvae

Samples were studied by using a Leitz Dialux 22 microscope and photographs were taken with a Wild MPS 11 camera. For microscopic investigation of tubercle fluid, a larva was slightly squeezed with forceps and the emerging tubercle fluid was dipped directly from the larva into a droplet of citrate-EDTA-buffer (pH 4.6) on a glass slide (buffer composition: KCl 69 mM, NaCl 27 mM, NaHCO<sub>3</sub> 2 mM, D(+)-glucose 100 mM, tripotassiumcitrate 30 mM, citric acid 26 mM, Na<sub>2</sub>-EDTA 10 mM). For studying larval haemolymph microscopically, a larval foreleg was cut and the emerging haemolymph was again dipped directly into a droplet of buffer solution.

For the preparation of larval histological sections, 2nd and 3rd instar larvae of *A. alni* were fixed in Bouin's solution for 40 h, dehydrated with ethanol (30%, 50%, 70%, 80%, 90% and 100%, 30 min for each concentration), and embedded in paraffin wax with isobutanol as an intermedium. Sagittal and horizontal sections were made at 7–10 µm intervals and stained with haematoxylin and counterstained with eosin (0.5% in water).

### Bioassays

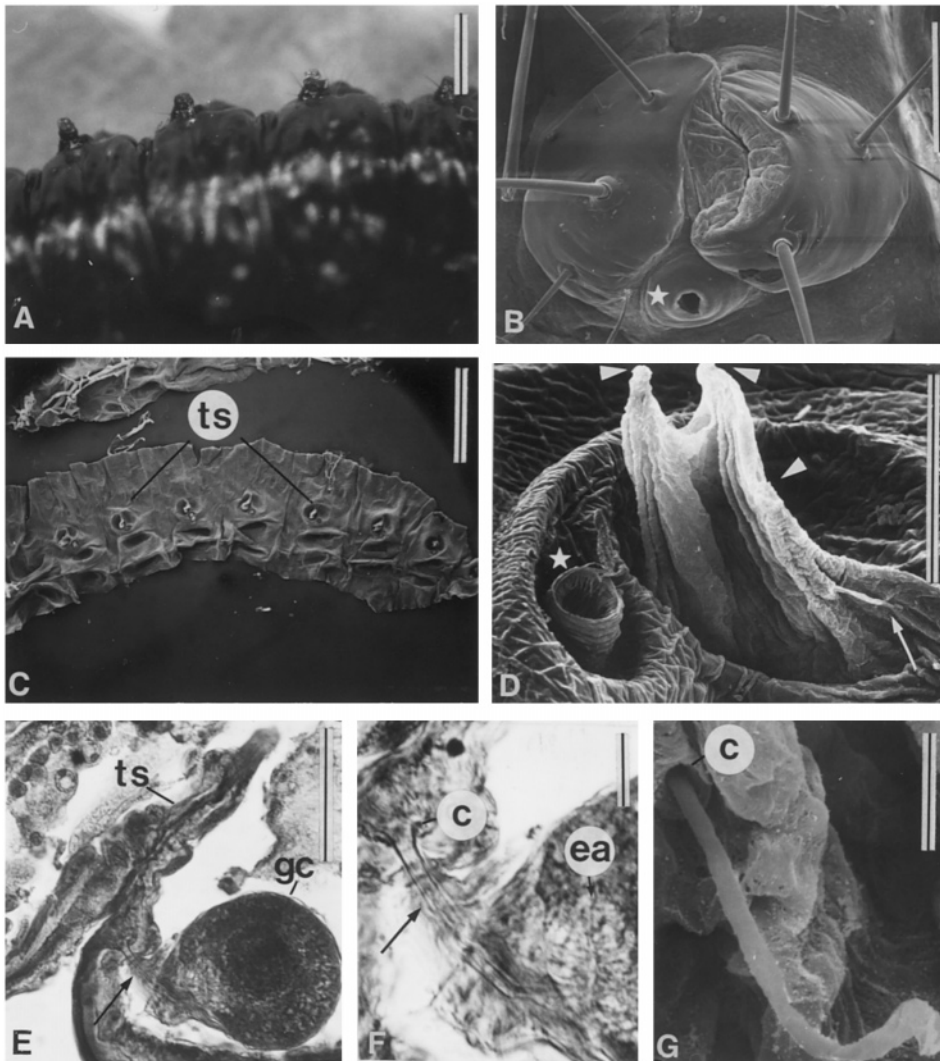
#### Feeding bioassay with ants

Tubercle fluid was collected onto small pieces of filter paper by slightly squeezing L3 larvae and collecting the fluid emerging from the abdominal tubercles. Haemolymph was collected on a piece of filter paper by cutting forelegs of the same larvae. In order to examine the effect of tubercle fluid and haemolymph on the feeding behaviour of a generalist predator, choice tests were carried out with twenty ants from a colony of *M. rubra* L. (Hymenoptera: Formicidae). The ants were placed into a Petri dish (Ø 9 cm), deprived of food for three days, but provided with water. A choice test was started by offering simultaneously 10 µl of a test and a control sample, respectively, on a glass slide. The test samples were freshly collected tubercle fluid (8 mg fresh weight in 240 µl 0.1 M aqueous sucrose solution) and freshly collected haemolymph from a dissected leg (7 mg in 220 µl 0.1 M aqueous sucrose solution), respectively. Aqueous sucrose solution (0.1 M) served as control. After five minutes, ants feeding on every sugar droplet were counted. The assay was replicated twenty times with different groups of ants. Wilcoxon signed-rank test for paired observations was used for statistical analysis.

To get information on the heat resistance, the molecular weight, and the volatility of the feeding deterrent component(s) of tubercle fluid and haemolymph, the freshly collected test samples were treated by three different methods: (1) They were heated for 10 min in a water bath at 90°C. After heating, samples were centrifuged (5 min, 8832 g) and supernatants mixed with 0.2 M sucrose solution (1:1 ratio) were tested. (2) Heated test samples were additionally treated by a proteinase (50 µl proteinase K bound to latex beads, Merck) to exclude the possibility that heat resistant proteins cause a feeding

**Table 1** Division of Galerucinae in groups according to the number and localisation of defensive glands in galerucine larvae (after Takizawa 1972). Further arguments for grouping are presence and arrangement of larval sclerites. Metacyclini were not investigated and are thus not mentioned. Classification is according to tribes based on adult characters (Seeno & Wilcox 1982). *Agelastica alni* belongs to group III

(Tribe) Tagmata	Group I (Sermylini)	Group II (Sermylini)	Group III (Sermylini)	Group IV (Luperini)	Group V (Galerucini, Oidini)
Prothorax	1 pair	none	none	none	none
Meso-/Metathorax	2 pairs	4 pairs	none	none	none
Abdomen	8–9 pairs	8–9 pairs	8 pairs	none	none



**Fig. 1** Segmental abdominal tubercles of *Agelastica alni* L. larvae (3rd instar). (A) Larva everting cuticular sacs through openings located at tubercle tips; droplets of fluid are visible at two tubercle tips. External view from ventrolateral side, bar: 1 mm. (B) Dorsolaterally located tubercle on the abdomen, above the spiracle. The tubercular sac is invaginated in the body cavity. External view by SEM, bar: 100  $\mu$ m. (C) Larval integument, internal view. Each half of an abdominal segment contains a sac-like cuticular structure. SEM, bar: 1 mm. (D) Larval tubercular sac. SEM, bar: 100  $\mu$ m. (E) Cuticular sac and connected gland. Microscopic view, haematoxylin/eosin stain, bar: 10  $\mu$ m. (F) Glandular cell with cuticular canal that is connected to the sac; cuticular end apparatus within the glandular cell. Microscopic view, haematoxylin/eosin stain, bar: 10  $\mu$ m. (G) Cuticular canal leading to the sac. SEM, bar 10  $\mu$ m. Abbreviations: **ts**: tubercle sac; **gc**: gland cell; **ea**: end apparatus; **c**: connection between canal and cuticular sac; thin and elongated arrows mark conducting canals, thick and white arrows mark localisation of muscle insertion, asterisks indicate spiracle and tracheal trunk, respectively

deterrent effect. Samples were incubated with proteinase for 3 h at room temperature. After twofold centrifugation (10 min, 8832 g), supernatants mixed with 0.2 M sucrose solution (1:1 ratio) were tested for their effects on *M. rubra*. (3) Heat treated samples were ultrafiltered (Millipor, Centricon 3000) excluding molecules > 3 kD; ultrafiltrates mixed with 0.2 M sucrose solution (1:1 ratio) were tested. (4) Freshly collected tubercle fluid (8 mg) was evaporated to dryness by a vacuum pump (Univapo 100 H Concentrator, Uniequip GmbH Berlin). Residues were resuspended in 240  $\mu$ l of a 0.1 M aqueous sucrose solution.

#### Bioassay to examine intraspecific effects

For testing the effects of tubercle fluid and haemolymph on conspecific larvae, three to ten 1st instar larvae were placed on alder leaf discs (diameter: 17 mm) in Petri dishes lined with moistened filter paper. After a few minutes, larvae approached each other and were feeding in small groups of three to ten individuals. When larvae had been aggregated, a piece of filter paper (2  $\times$  2 mm) was placed close to one of the larvae for one minute (control). The number of larvae starting to walk was registered. Then, a piece of filter paper soaked either with larval haemolymph taken from a dissected leg or with tubercle fluid was placed in the same position as the control paper for one minute. Again, the number of larvae starting to walk around was registered. The Wilcoxon signed-rank test for paired differences was used for statistical analysis.

#### (Bio)chemical comparison of tubercle fluid and haemolymph

##### Electrophoresis

Samples of freshly collected tubercle fluid and haemolymph, respectively, were taken from four larvae of each instar as described above for the bioassays. For analysing these samples by electrophoresis, filter papers soaked with sample fluids were transferred to a SDS sample buffer (0.15 M TrisHCl, pH 6.8; 1% SDS; 1% glycerine, traces of bromophenol blue). These untreated samples, and additionally, samples treated by the methods 1 and 2 described above for feeding bioassay samples (heat, proteinase K) were investigated electrophoretically. Prior to application to the electrophoresis gel, all samples were centrifuged (5 min, 8832 g) for the removal of haemocytes. Twenty-five microliters (containing 0.1–0.5 mg larval fluid) of the supernatants were used for SDS-PAGE carried out for 2 h at 200 mV and 10°C in a gradient gel (content of polyacrylic acid 5–17.5%) using the mighty small system (Hoefer). A low molecular weight calibration kit (LMW 17-0446-01, Pharmacia, Germany) was used as electrophoresis standard. Separated proteins were stained by 0.5 g/l Coomassie Brilliant Blue R 250 in 10% acetic acid and 50% methanol.

##### Thin layer chromatography

Filter papers soaked with either tubercle fluid or haemolymph from L3 larvae were transferred into ice-cold methanol (7 mg/100  $\mu$ l

methanol) and treated by ultrasound for 5 min. Thirty  $\mu\text{l}$  of each sample were applied on silica gel 60 F<sub>254</sub> (0.25 mm). Three eluents of different polarity were used:

- hexane, 2-propanol, water (80:9.5:0.5, v:v:v);
- ethyl acetate, methanol, water (85:10:5, v:v:v);
- 2-propanol, ammonia (25%), saturated boric acid, water (60:20:10:10, v:v:v:v).

Two different samples of tubercle fluid and haemolymph, respectively, were separated. After fractionation the plates were sprayed with

- \* molybdato-phosphoric acid for detection of reducing compounds, steroids, sterols, lipids, fatty acids, and their methyl esters,
- \* p-anisaldehyde-sulfuric acid for detection of sugars, terpenes, steroids, phenols,
- \* thymol-sulfuric acid for detection of sugars,
- \* dragendorff's reagent for detection of alkaloids.

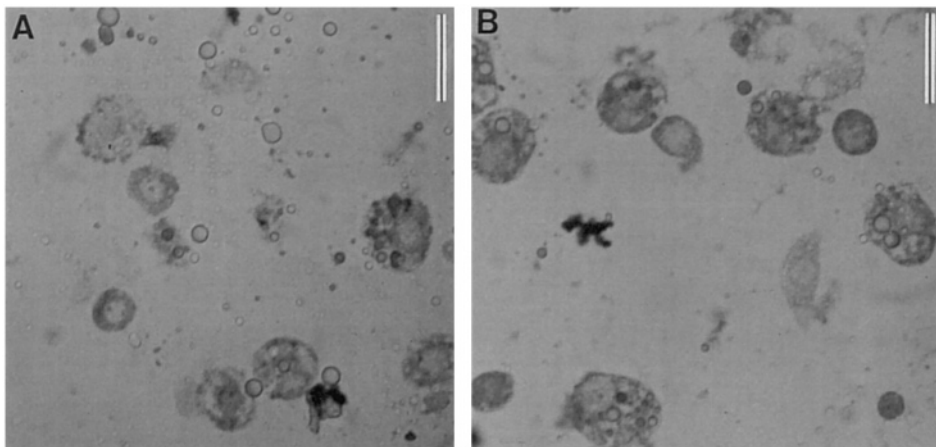
## Results

### Morphological studies

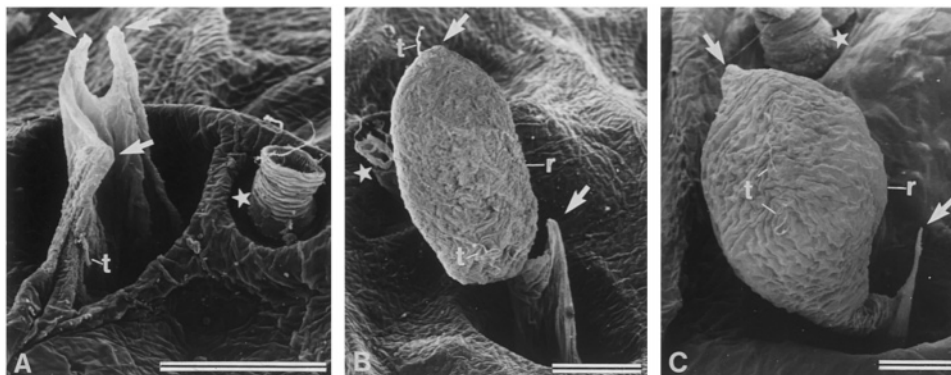
*A. alni* larvae of each instar release yellow, sticky droplets from tubercles located dorsolaterally on the abdominal segments 1 to 8, when briefly molested with the tips of forceps (Fig. 1A). These droplets appear at the tip of inverted cuticular, sac-like structures which are normally invaginated in the body (Fig. 1B). The

cuticle is inverted at each tubercle near the spiracle by forming a two-tailed sac which is attached to three muscles (Fig. 1C, D). A gland enters each sac at its base (Fig. 1D–G). The gland (type III after Noiro & Quennedy, 1974) consists of a single voluminous glandular cell (Fig. 1E), which is connected to the sac by a cuticular canal (Fig. 1F). This cuticular canal is also visible after KOH-maceration by SEM observations (Fig. 1G). The tubercle fluid which is discharged by everting the cuticular sacs to the outside contains the same haemocytes of 10–25  $\mu\text{m}$  diameter as the haemolymph. The high abundance of lipid droplets in all samples of tubercle fluid was striking when compared to the few lipid droplets observed in samples of haemolymph taken from a foreleg (Fig. 2A, B).

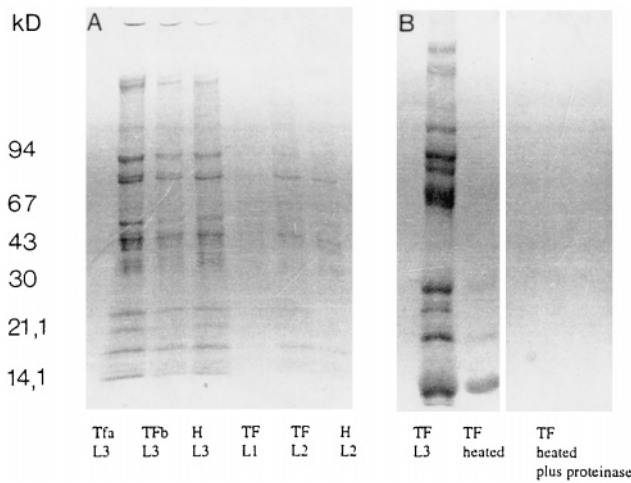
A comparison of the segmental cuticular sacs of *A. alni* larvae with the larval defensive glands of two chrysomeline species, *G. viridula* and *L. aenea*, revealed that the well-known glandular reservoir of these chrysomeline larvae is at its base connected to a structure that matches well with the shape, size, and localisation of the eversible cuticular sacs of *A. alni* larvae (Fig. 3A, B, C). Similar to the cuticular sac of *A. alni* larvae, also the base structures entered by the chrysomeline glandular reservoir are inserted by muscles.



**Fig. 2** Light microscopic comparison of tubercle fluid (A) and haemolymph (B) of *Agelastica alni* L. larvae (3rd instar). Stained by Giemsa, bar: 20  $\mu\text{m}$ . Different haemocytes and droplets of lipid are visible



**Fig. 3** SEM views of abdominal larval eversible sacs. (A) *Agelastica alni* L., L3, tubercle sac. (B) *Gastrophysa viridula* DeGeer, L3, reservoir of abdominal defensive gland connected at its base to a cuticular sac (C) *Linaeidea aenea* L., L3, reservoir of abdominal defensive gland connected at its base to a cuticular sac. Abbreviations: r: reservoir; t: tubulus of gland leading into the reservoir. White arrows indicate locations of muscle insertion. Asterisks indicate spiracles or end of trachea. Bars: 100  $\mu\text{m}$



**Fig. 4** SDS-PAGE electrophoresis of tubercle fluid (=TF) and haemolymph (H) from (A) different instars (L1, L2, L3) of larvae of *Agelastica alni* L. TFa = tubercle fluid which poured out after strong molestation; TFb = tubercle fluid which was discharged after brief molestation, and (B) tubercle fluid after heating and after heating plus incubation with a proteinase. Molecular weights of separated proteins range from 10 kD to >200 kD

#### (Bio)chemical comparison of tubercle fluid and haemolymph

Electrophoretic investigation of the tubercle fluid and the haemolymph revealed no significant differences in the protein patterns. When comparing protein patterns of samples from L1, L2, and L3 larvae, most proteins were detected in samples of the eldest larvae (Fig. 4A). Larger proteins of tubercle fluid were destroyed after heat treatment for 10 min. When heat treatment of the tubercle fluid was followed by a proteinase incubation, no longer were any proteins detected (Fig. 4B).

Tlc patterns of tubercle fluid and haemolymph (Table 2) showed no differences when using eluents of low (eluent 1) and medium polarity (eluent 2). When separating the samples by using a high polarity eluent (eluent 3), one fraction only present in tubercle fluid samples but not in haemolymph samples was detected after spraying with thymol-sulfuric acid and p-anisaldehyde-sulfuric acid ( $R_f$ -value: 0.44–0.46).

#### Bioassays

The feeding bioassays revealed that both tubercle fluid and haemolymph significantly deter the ant *M. rubra* from feeding (Table 3). Neither heating nor incubation of heated samples with a proteinase did influence the feeding deterring activity of tubercle fluid and haemolymph. After ultrafiltration of tubercle fluid, the fraction with molecules < 3 kD retained its significant feeding deterring activity on the ants, whereas the fraction with molecules > 3 kD showed no significant deterring effect after five minutes of observation. Drying of tubercle fluid did not diminish the feeding deterring effect against ants.

Volatiles from tubercle fluid exhibited a distinct intraspecific effect on a group of *A. alni* larvae. As soon as larvae were exposed to volatiles from tubercle fluid, they started moving around so that the cohesion of their aggregation was lost (Table 4). Odour from the haemolymph had no significant effect on the aggregation behaviour of the larvae (Table 4).

#### Discussion

This study clearly showed that larvae of *A. alni* possess exocrine glandular cells (type III) which are connected to cuticular sacs located close to the spiracle at the abdominal segments 1–8. Each sac may be everted at molestation through the opening at the tip of a tubercle close to each spiracle at the respective segments. The discharged tubercle fluid shows a strong feeding deterring effect on ants and an up to now unknown effect in the Chrysomelidae as alarm pheromone among conspecific larvae. The fluid discharged from these tubercle openings contains haemolymph and most probably also glandular secretion. The presence of haemolymph within the tubercle fluid is shown by the results obtained from tlc and SDS-PAGE. The presence of glandular secretion within the discharged tubercle fluid is indicated by (1) the location of the glandular duct and (2) the alarm effect of tubercle fluid but the ineffectiveness of haemolymph towards conspecific larvae. Furthermore, also the tlc spot detected in the tubercle fluid in addition to the haemolymph fractions may suggest that glandular secretion is added to haemolymph when released. Addition of glandular secretion to discharged haemolymph is also known in other insects. The tiger moth *Arctia caja* L. and the grasshopper *Poeciloceris bufonius* (Klug) are well-known to emit mixtures of haemolymph and secretions of exocrine glands for defensive purposes. They blow up their discharged fluids by air; these foamy defensive froths are interpreted as carrier masses to effectively spread a small amount of defensive secretion over the body (Euw *et al.* 1967; Rothschild & Haskell 1966).

The alarm effect of discharged tubercle fluid of *A. alni* larvae might be caused by the glandular secretion. Another function of the glandular secretions could be to fasten coagulation of discharged haemolymph, thereby enhancing the glutinous properties of the tubercle fluid. Ants attacking *A. alni* larvae were immediately entangled with mandibles, antennae and legs in the sticky fluid, while larger predators like the carabid beetle *Poecilus versicolor* (Sturm) and the pentatomid *Arma custos* (Fabricius) were not (pers. obs. by M. Bünnige). Not only does the discharged tubercle fluid physically disturb feeding ants, but it also contains a feeding deterrent that is still active after heating which destroys sticky large proteins. The effect of tubercle fluid of *A. alni* on ants seems to be caused by the same components as the feeding deterring activity of the haemolymph. Our results suggest that the feeding deterring component is not proteinous and non-volatile.

	R <sub>F</sub> -values
<b>ELUENT 1</b>	
Molybdatophosphoric acid	
TF	0; 0.05; 0.12; 0.18; 0.22; 0.24; 0.31; <b>0.33</b> ; 0.71; <b>0.77</b>
H	0; 0.05; 0.12; 0.18; 0.23; 0.25; 0.33; <b>0.35</b> ; 0.69; <b>0.77</b>
Anisaldehyde – sulfuric acid	
TF	0; 0.17; 0.21; 0.23; 0.30; <b>0.32</b> ; 0.71; <b>0.77</b>
H	0; 0.17; 0.21; 0.23; 0.30; <b>0.32</b> ; 0.71; <b>0.77</b>
Thymol – sulfuric acid	
TF	0
H	0
<b>ELUENT 2</b>	
Molybdatophosphoric acid	
TF	0; 0.03; 0.09; <u>0.77</u> ; <b>0.83</b>
H	0; 0.03; 0.08; <u>0.77</u> ; <b>0.83</b>
Thymol – sulfuric acid	
TF	0; 0.02
H	0; 0.02
<b>ELUENT 3</b>	
Molybdatophosphoric acid	
TF	0.24; 0.50; <u>0.67</u> ; 0.77; <b>0.90</b>
H	0.24; 0.50; <u>0.67</u> ; 0.76; <b>0.90</b>
Anisaldehyde – sulfuric acid	
TF	0.25; 0.44; 0.50; <u>0.67</u> ; 0.78; <b>0.90</b>
H	0.25; 0.50; <u>0.66</u> ; 0.78; <b>0.90</b>
Thymol – sulfuric acid	
TF	0.19; 0.22; 0.27; <u>0.46</u> ; 0.51; <u>0.67</u> ; 0.75; <b>0.90</b>
H	0.19; 0.22; 0.27; 0.51; <u>0.67</u> ; 0.75; <b>0.90</b>
Dragendorff's reagent	
TF	no reaction
H	no reaction

**Table 2** Tlc patterns of tubercle fluid and haemolymph samples of *A. alni* larvae, respectively, after spraying with different reagents, using three different solvent systems (eluent 1: hexane, 2-propanol, water (80:9.5:0.5, v:v:v), eluent 2: ethyl acetate, methanol, water (85:10:5, v:v:v), and eluent 3: 2-propanol, ammonia, boric acid, water (60:20:10:10, v:v:v:v). Spots which reacted with the spraying reagent, but are visible at daylight before spraying, are indicated by bold types. Spots which reacted with the spraying reagent, but are visible at UV<sub>254</sub>-light before spraying, are indicated by underlining. R<sub>F</sub>-values of fractions only detectable in tubercle fluid samples are written in italics. TF: tubercle fluid samples, H: haemolymph samples

The defense of *A. alni* larvae seems to be based on both release of haemolymph and exocrine secretion. Such a combination of haemolymph and secretion for defense is unknown in other Galerucinae and Chrysomelinae. While numerous chrysomeline larvae are known to rely on defensive volatiles discharged by exocrine glands (Pasteels *et al.* 1988), several galerucine larvae on the other hand defend themselves by adverse physical properties of haemolymph (Wallace & Blum 1971) or by toxins and feeding deterrents from the haemolymph (Ferguson & Metcalf 1985; Hilker *et al.* 1992; Howard *et al.* 1982).

The morphology of the larval exocrine glands described here for the galerucine *A. alni* shows similarities

to the structures of the defensive glands of chrysomeline larvae located at the meso- and metathorax, and the abdominal segments 1–7. Especially the cuticular structures that form the base of the huge glandular reservoirs of the chrysomeline larvae *G. viridula* and *L. aenea* are very similar to the cuticular sacs of *A. alni* larvae. These similarities raise the question whether or not exocrine defensive glands of galerucine and chrysomeline larvae are homologous. Lee (1993) uses the presence of defensive glands in the larvae of the subfamilies Galerucinae and Chrysomelinae as a synapomorphic character, which requires homology. On the other hand, Reid (1995) considers the homology of larval defensive glands of Galerucinae and Chrysomeli-

Test solutions	Ants (mean ± SD) feeding upon		N	p-level
	Test	Control		
Tubercle fluid (TF)	0.9 ± 1.2	5.2 ± 2.8	20	<0.001
Haemolymph (H)	1.3 ± 1.8	5.1 ± 2.8	18	<0.01
TF, heated to 90°C for 10 min	1.3 ± 1.4	3.8 ± 1.9	18	<0.01
H, heated to 90°C for 10 min	2.4 ± 1.7	5.0 ± 2.4	20	<0.01
TF, treated with proteinase K	1.8 ± 1.4	5.0 ± 1.7	20	<0.001
TF, heated to 90°C fraction < 3 kD	0.8 ± 0.9	6.1 ± 1.4	19	<0.001
TF, heated to 90°C fraction > 3 kD	3.4 ± 2.8	3.6 ± 1.8	20	0.57
TF, dried and resuspended	1.2 ± 1.3	4.2 ± 1.5	20	<0.001

**Table 3** Bioassay with the ant *Myrmica rubra* L. feeding upon tubercle fluid and haemolymph of *Agelastica alni* L. larvae (3rd instar). Recording of the number of feeding ants after five minutes. *Test*: ants feeding upon test solution mixed with aqueous sucrose solution, *control*: ants feeding upon an identically treated 0.1 M aqueous sucrose solution. *N*: number of replications. Wilcoxon signed-rank test for paired observations

**Table 4** Intraspecific effects of tubercle fluid of *Agelastica alni* L. larvae (L3) on aggregations of conspecific larvae (L1). *Tubercle fluid*, *haemolymph*: larvae walking away from the aggregation when responding to odour of a filter paper soaked with tubercle fluid and haemolymph, respectively. *Control*: larvae walking away from the aggregation when responding to odour of filter paper. *N*: number of replications. Wilcoxon signed-rank test for paired observations

Test	Percentage (mean $\pm$ SD) of moving larvae	N	p-level
	Control		
<i>Tubercle fluid</i>			
88.3 $\pm$ 27.8	7.2 $\pm$ 12.4	28	<0.001
<i>Haemolymph</i>			
15.0 $\pm$ 32.7	4.7 $\pm$ 10.0	25	0.09

nae as uncertain. Crowson & Crowson (1996) suggest that numerous similar characters of chrysomeline and galerucine larvae evolved convergently. Structures of defensive devices and chemistry of defensive components released by galerucine larvae at molestation need intensive further studies to elucidate the evolution of the Galerucinae and their relationship to Chrysomelinae.

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