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Tergal Gland Secretion of the Rove Beetle *Aleochara pseudochrysorrhoa* (Staphylinidae: Aleocharinae): Chemical Composition and Biological Roles

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Aleochara pseudochrysorroa has a glandular complex known as the tergal gland. Generally, the tergal gland secretion (TGS) has been described to have defensive function, but some reports point to a possible secondary function of this complex. For example, the TGS of the related species *A. curtuna* has been demonstrated to possess an important role in intraspecies communication. In this work we describe the chemical composition of the TGS of *A. pseudochrysorrhoa* males and females. Eleven compounds were identified based on GC-MS and GC-FTIR analyses, retention indexes and derivatization products. Furthermore, a brief study regarding the biological function of the TGS in mating behavior is provided, in which the stimulation of male grasping response reaction by female TGS proved to be dependent on concentration.

Keywords: Allomones • Defensive behavior • *Aleochara pseudochrysorrhoa* • pheromones • quinones

Introduction

The rove beetles of the family Staphylinidae comprise about 58,000 species[1, 2] and many of them possess abdominal defensive glands, which have arisen independently in the different subfamilies. [3] A possible reason for this evolutionary pattern is the fact that rove beetles usually have a soft unsclerotized abdomen, completely unprotected from attack. [4] Members of different subfamilies show variations in the position of the glandular system (e.g. in Aleocharinae, the tergal gland is located between sixth and seventh abdominal segments, while in Staphylininae, the pygidial gland is located in the apex of the abdomen), and also in the chemical composition of defensive secretion that is produced and released by these glands (tergal gland secretion, TGS). [5, 6]

Adults and larvae of *Aleochara* spp. are known as parasitoids of cyclorrhaphous dipteran pupae[7 – 10] and adults prey on eggs, larvae and pupae of dipterous species. Consequently, *Aleochara* spp. are usually found in fly-infested habitats such as decaying organic matter and animal droppings and are considered to be an important natural fly regulator, making this group important for ecological and forensic studies. [11 – 15]

Most of Aleocharinae species possess a glandular complex known as tergal gland, a bilobate reservoir with a bright yellow color and a characteristic pungent odor. This complex is formed by an invagination of the intersegmental membrane between the sixth and seventh abdominal tergites (Figure 1). [6, 16,17] It has been reported that when Aleocharinae beetles are disturbed, they bend their abdomen towards the aggressor and discharge the secretion from the tergal gland as defense. [18– 22, 16] Furthermore, it has also been described that the TGS of female *Aleochara curtula* acts as an aphrodisiac stimulating grasping reaction in males, which is the second step of mating sequence behavior of this species. [23]

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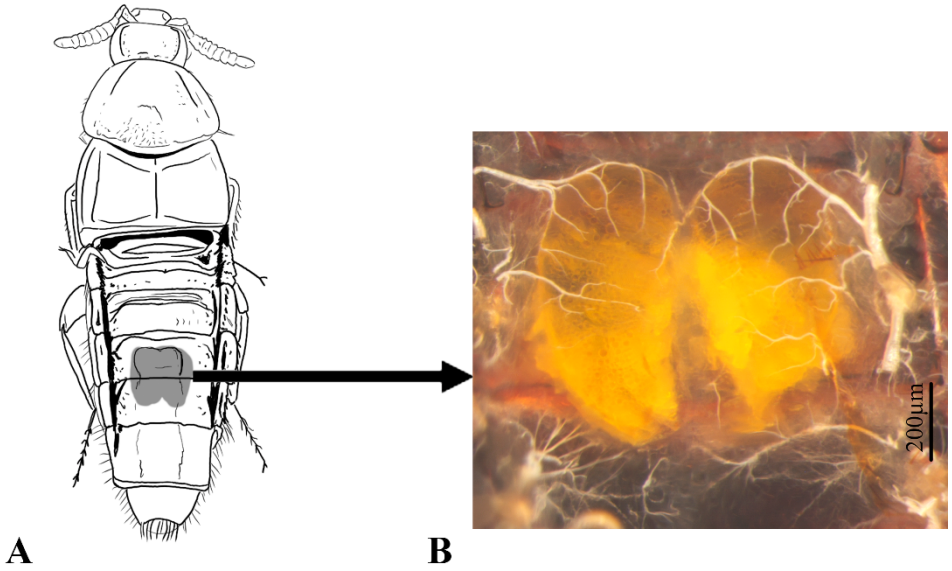


Figure 1. *a)* Schematic drawn of the *Aleochara pseudochrysorroa* specimen showing the tergal gland reservoir in dorsal view. *b)* tergal gland reservoir of *Aleochara pseudochrysorrhoa* in dorsal view in glycerin.

Aleochara pseudochrysorrhoa Caron, Mise & Klimaszewski (Staphylinidae: Aleocharinae) is an abundant species in southern Brazil. This species is an important fly regulator in this area[9, 10] and can be easily collected in animal carcasses. Even though the mating behavior has been described for many Staphylinidae spp., the function of volatile compounds mediating reproduction remains scarcely studied in the literature. *Aleochara curtula* (Goeze) is the only species of the subfamily Aleocharinae in which the volatiles that mediate its mating behavior were studied.[24] In the present work, the chemical composition and the biological role of the tergal gland secretions (TGS) of *A. pseudochrysorrhoa* were studied.

Results and Discussion

Chemical composition of the tergal gland secretion

GC analyses of hexane extracts of the tergal glands of male and female *A. pseudochrysorrhoa* (Figure 2) led to the identification of eleven compounds (1-11, Figure 3). The chemical structures were elucidated based on their mass and infrared spectra, retention indexes, and mass spectra of derivatization products (see Supporting Information).

Four compounds were identified as straight chain alkanes: decane (**1**), undecane (**4**), dodecane (**6**) and tridecane (**9**) based on their mass spectra, which presented characteristic fragmentation pattern composed of a homologous series of peaks separated by 14 mass units (43, 57, 71, 85, etc.), resulting from the loss of CH_2 groups (See Supporting Information). Compounds **3**, **7** and **8** showed spectral data indicative of straight chain alkenes, with fairly similar mass spectra characterized also by fragment ion peaks 14 mass units apart, but 2 units lower than in the alkanes (41, 55, 69, 83, etc.) (Figure 3). The mass spectrum of compound **3** (RI: 1096) showed a molecular ion at *m/z* 154Da, suggesting the compound to be an undecene. Similarly, compounds **7** (RI: 1291) and **8** (RI: 1296) with molecular ions at *m/z* 182Da were proposed to be tridecenes. The GC-FTIR spectra from these compounds exhibited typical bands of hydrocarbons: aliphatic methylene and methyl axial deformations (υ) at 2850-2957 cm^{-1} and angular deformation of aliphatic methine protons (υ) at 1452-1459 cm^{-1}. At 2997-3006 cm^{-1} a characteristic band of the C-H axial deformation of a Z-configured double bond[25] was clearly detectable in the spectra of all three compounds (see Supplementary Material). The C-C double bond position of compounds **3**, **7** and **8** was determined by micro-derivatization of the natural extract with dimethyldisulfide (DMDS) (Figure 4). GC-MS analysis of the resulting adducts showed relatively strong fragments that allowed assigning the location of the C-C double bond of the parental alkenes.

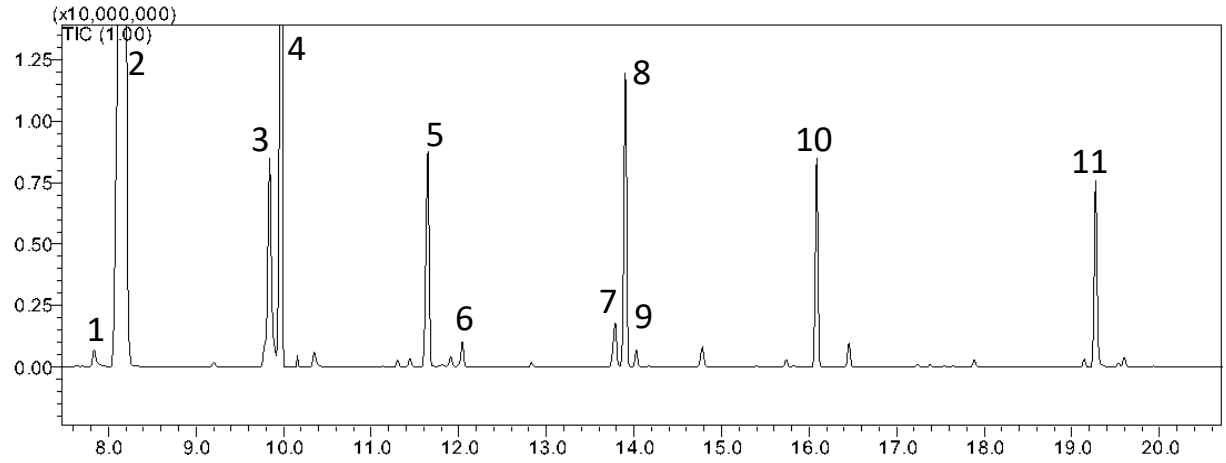


Figure 2. GC-MS analysesof the female tergal gland components (TGC) of *Aleochara pseudochrysorrhoa* showing the eleven identified compounds. Analysis conditions: Colunm DB-5, 50 °C to 270°C at 7 °C min^{-1}.

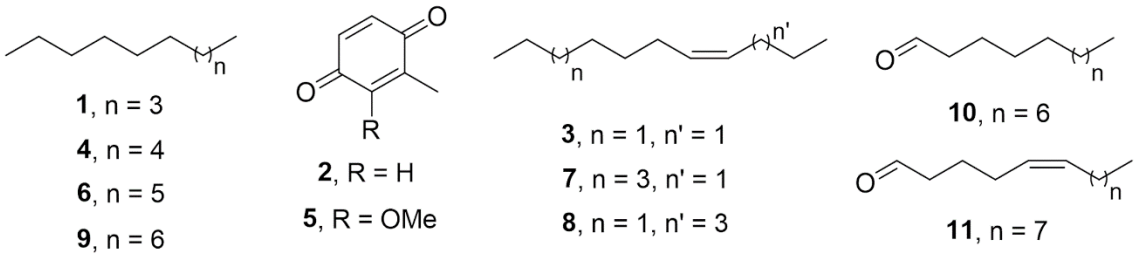


Figure 3. Substances identified as TGC of A. *pseudochrysorrhoa* males and females

The mass spectrum of the DMDS adduct from **3** presented fragments at *m/z* 103 and 145Da, corresponding to a cleavage between carbons at positions 4 and 5 bearing methylthio groups, resulting in the assignment of (*Z*)-undec-4-ene as the natural compound (**3**) (Figure 4). Following the same principle, (*Z*)-tridec-4-ene and (*Z*)-tridec-6-ene were suggested as the structures for compounds **7** and **8**, respectively.

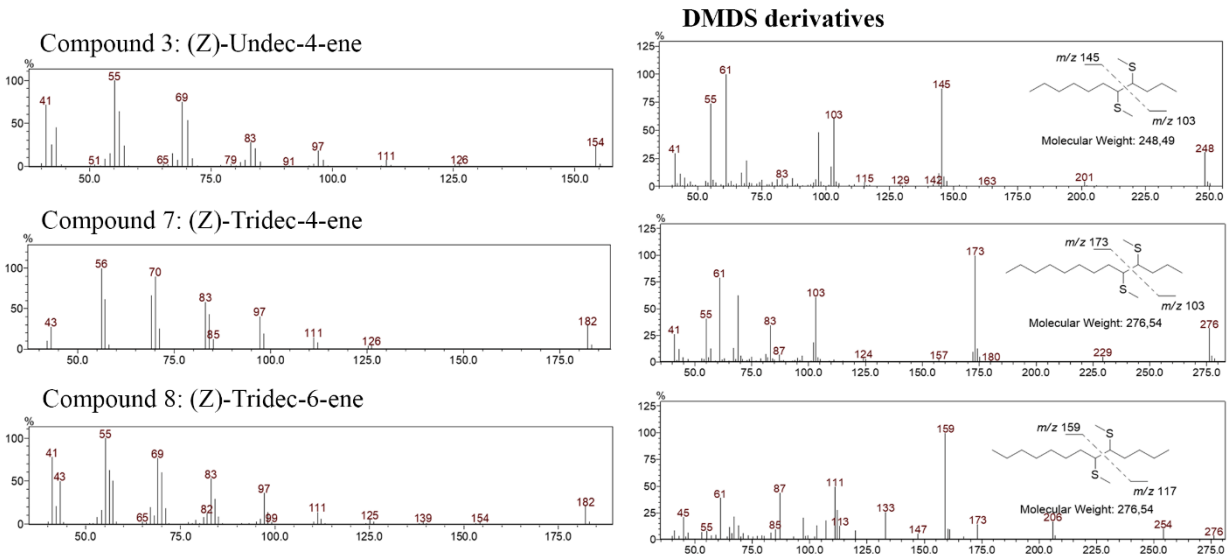
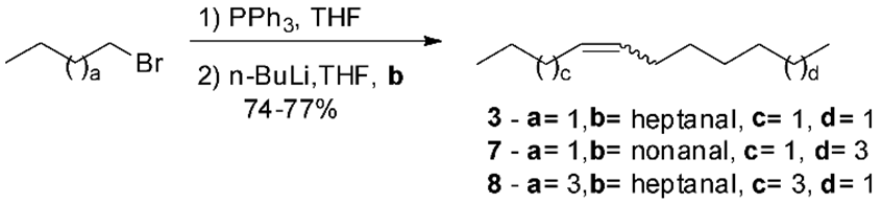


Figure 4. Comparative fragment patters of compounds **3**, **7** and **8** of the extract with their corresponding DMDS derivatives.

Aiming to confirm the suggesting chemical structures, (*Z*)-undec-4-ene, (*Z*)-tridec-4-ene, and (*Z*)-tridec-6-ene were synthesized in 76, 77, and 74% yield respectively, based on Wittig reactions, starting from the phosphonium salts from 1-bromobutane or 1-bromohexane and heptanal or nonanal, as illustrated on Scheme 1. As expected, the Wittig reactions resulted in a mixture of *E* and *Z* isomers (ratio close to 3:1), which were separated by column chromatography on silica gel impregnated with $AgNO_3$ (5%). Retention times and spectral data of the synthetic (*Z*)-alkenes were identical to those of the insect-produced compounds.



Scheme 1. Chemical synthesis of alkenes **3**, **7** and **8** identified in TGC extract of *Aleochara pseudochrysorrhoa*

The mass spectrum of compound **2** (RI: 1014), the major component found in extracts of the tergal glands, showed a highly intense molecular ion at *m/z* 122Da, a base peak at *m/z* 94Da, and relatively intense fragment ions at *m/z* 40, 54, 66 and 84Da. The FTIR spectrum from **2** presented an intense band at 1654 cm^{-1}, suggesting the presence of a conjugated carbonyl group. Furthermore, three characteristic bands of the C-H axial deformation of trisubstituted alkenes[26] or α,β-unsaturated ketones[27] were observed at 3039, 3058 and 3075 cm^{-1}. This spectral data indicated a quinone structure, and compound **2** was identified as 2-methylcyclohexa-2,5-diene-1,4-dione by comparison with a commercial standard.

The mass spectrum of compound **5** (RI: 1181) was very similar to that of **2**, with a molecular ion at *m/z* 152Da (+ 30Da) and relatively intense fragments at *m/z* 53, 66, 109 and 122Da, suggesting an additional methoxy group. Indeed, the GC-FTIR spectrum showed C-O stretching bands from an ether at 1214 and 1089 cm^{-1}, two intense C=O stretching bands at 1653 and 1671 cm^{-1}, and two bands related to the C-H axial deformation of alkenes at 3013 and 3066 cm^{-1}. Based on the comparison of the spectral data with literature values, compound **5** was tentatively identified as 2-methoxy-3-methyl-*p*-benzoquinone, a compound usually found in the TGS of Aleocharinae species. [6, 28]

Finally, the remaining compounds **10** and **11** showed spectral data characteristic of straight chain aldehydes (See Supporting Information). Compound **10** (RI: 1410) was readily identified as dodecanal, while compound **11** (RI: 1594) showed spectral data indicative of an unsaturated aldehyde. The retention index and the mass spectrum of **11** suggested a tetradecenal structure, with M^+-18 at *m/z* 192Da (after loss of water), while the band of C-H axial deformation at 2999 cm^{-1} in the GC-FTIR spectrum pointed to a double bond with *Z* configuration.[27] The mass spectrum of the DMDS adduct revealed the double bond to be located between positions 5-6, leading to the proposal of (*Z*)-tetradec-5-enal as the structure of compound **11**, which was confirmed by comparison with a synthetic standard.

The major compound found in the TGC of *A.pseudochrysorrhoa* is 2-methylcyclohexa-2,5-diene-1,4-dione (**2**), which has been previously reported as the most abundant compound in the TGS of other 19 out of 26 Aleocharinae species in which chemical composition of the TGS was investigated (personal data base, unpublished). This compound seems to be of very primitive origin since it occurs in many other Arthropod groups, for example as a component produced by scent glands of Opiliones. [29, 30] In Staphylinidae, 2-methylcyclohexa-2,5-diene-1,4-dione (**2**) is also found in species of the subfamily Oxytelinae, but not as the major compound of gland secretions.[31 – 33] Due to its strong repellent effect, a defensive function has been proposed for this compound [6, 16, 23], but there are also reports of it acting as a potent bactericide and fungicide.[34].

The second most abundant compound found in *Aleochara pseudochrysorrhoa* TGS is undecane (**4**). This compound was found in 12 out of 26 Aleocharinae species for which the TGS composition has been analyzed (personal data base, unpublished). This class of compounds is usually found blended with quinones in defensive secretions, mainly acting as a solvent to enhance the impregnation of defensive compounds.[6, 35 – 37].

The aldehydes found in *A. pseudochrysorrhoa* TGC are very similar to those found in the TGS of *A. curtula*, *Atheta crassicornis* Fabricius, *Drusilla canaliculata* Fabricius, *Oxypoda alternans* Gravenhorst, *Pella funestus* Gravenhorst, *Pella humeralis* Gravenhorst, *Zyras lugens* Gravenhorst, and *Zyras funestus* Gravenhorst. [6, 16, 21, 38]. This class of compounds is related to self-defense and protection against bacteria and fungi, but it is also related to the stimulation of male mating behavior sequence.[23, 34] The TGS of Aleocharinae species has been attributed a defensive function since 1913,[18] when it was observed that ants instantly died after being confined in a tube containing the TGS of *Lomechusa strumosa* Gravenhorst. The minor compounds found in the TGS of Aleochrianae species have been interpreted as by-products of biochemical pathways,[20, 39] but Kremmer[40] was the first to propose a secondary function of the TGS, based on observations that Aleocharinae individuals emitted a pungent odor when confined and male mating behavior sequence was

also observed. In 1983, Peschke[23] was the first to demonstrate conclusively a secondary function for the TGS of *Aleochara curtula*. The TGS of female *A. curtula* also acts as an aphrodisiac, stimulating male copulation activity.

Quantification of tergal gland components

The amount of natural compounds present in extracts of tergal glands of both males and females is reported in Table 1. As can be seen, female extracts presented higher concentration of all substances when compared to male extracts, but this difference was not statistically significant.

Table 1. Quantification of TGC from *A. pseudochrysorrhoa* males and females in nanogram per insect

Peak Number	Linear retention index	Compound	Male Medium in ng[a]	Female Medium in ng[a]
1	1000	Decane	31.6 ± 4.5	65.9 ± 19.8
2	1014	2-Methylcyclohexa-2,5-diene-1,4-dione	4151.0 ± 399.6	4834.5 ± 367.9
3	1096	(*Z*)-Undec-4-ene	182.2 ± 35.7	306.0 ± 62.2
4	1103	Undecane	2538.5 ± 404.7	2906.0 ± 437.4
5	1181	2-Methoxy-3-methyl-1,4-benzoquinone	584.2 ± 84.9	1079.3 ± 205.0
6	1200	Dodecane	37.9 ± 9.9	48.9 ± 11.6
7	1291	(*Z*)-Tridec-4-ene	80.3 ± 15.8	85.7 ± 17.5
8	1296	(*Z*)-Tridec-6-ene	325.7 ± 51.1	562.3 ± 117.3
9	1302	Tridecane	19.7 ± 4.0	32.1 ± 9.2
10	1410	Dodecanal	353.5 ± 55.3	560.6 ± 122.9
11	1591	(*Z*)-Tetradec-5-enal	98.06 ± 42.0	132.8 ± 46.6

[a] Expressed as the mean ± SD determined from ten different extracts.

Male grasping response to different doses of TGS

The male grasping response of *A. pseudochrysorrhoa* (i.e. the exposition of the genital and attempt of copulation with the female) was investigated exposing the male to different doses of female TGS (Figure 5). The bioassays showed that fresh freeze-killed females with intact tergal glands had the highest copulation response of males (89% of positive response) (N=58).

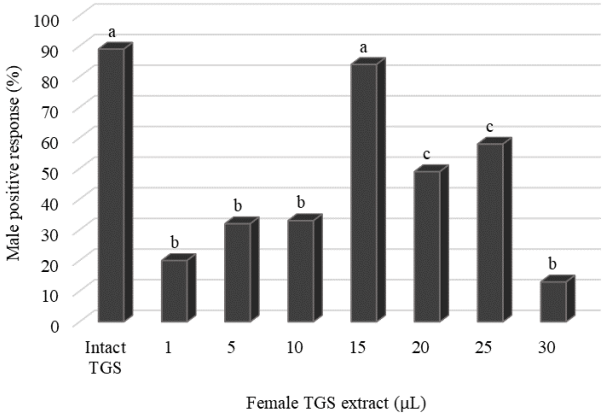


Figure 5. Graph showing the differences between the stimuli of fresh freeze killed female (intact tergal gland) and the volumes of female TGS (µL) used to stimulate male copulation. Different letters indicate statistical differences at $p < 0.05$ in the Chi-square test.

When 0.01 female equivalents (FE) of the natural extract of female TGS was tested, the stimulated copulation response was only 20% of males assayed (N=13). No significant difference was observed in the copulation response when the dose was increased to 0.05 FE (22%) (N=13). Application of 0.15 FE of female TGS extract resulted in 84% (N=55) of copulation behavior response. However, when males were exposed to 0.2 FE of female TGS extract, a

significant decrease in male copulation activity was observed (49% of positive response, N=32). When males were presented to rinsed female cadavers treated with 0.25 FE of female TGS extract, 58% of males attempted copulation (N=38). A significant decrease in male copulation attempts was observed with 0.3 FE of the female TGS extract (30% of positive response, N=20). The global Chi-square test with all the amounts used showed a statistical difference between the stimulus and female TGS extract doses (χ^2 = 120.979 d.f.= 7 p=<0.0001). Pairwise test showed a statistical difference between the fresh freeze-killed female and the doses of 0.01 female equivalents (FE) (χ^2 = 62.843 d.f.= 1 p= <0.0001), 0.05 FE (χ^2 = 42.538 d.f.= 1 p=<0.0001), 0.1 FE (χ^2 = 39.526 d.f.= 1 p=<0.0001), 0.2 FE (χ^2 = 22.226 d.f.= 1 p=<0.0001), 0.25 FE (χ^2 = 14.114 d.f.= 1 p=0.0002), and 0.3 FE (χ^2 = 43.609 d.f.= 1 p=<0.0001).

The male TGS also stimulated male copulation behavior response, but the number of positive responses was smaller in comparison to female TGS (Figure 6).

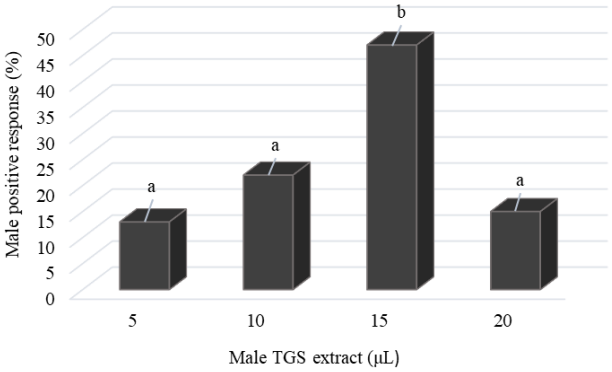


Figure 6. Graph showing the differences of male copulation behavior response to different volumes of the male tergal gland components extract. Different letters indicate statistical differences at p < 0.05 in the Chi-square test.

The dose of 0.5 male equivalents (ME) of male TGS extract elicited only 13% of copulation attempts (N=6). Copulation response was observed in 28% of males that were assayed with 0.1 ME of male TGS extract (N=10). The highest percentage of copulation response was obtained when males were assayed with 0.15 ME of male TGS (46% of positive responses) (N=21). Copulation response of 15% was observed when males were assayed with 0.2 ME of male TGS (N=7). The global Chi-square test with all the bioassays showed that there is a statistical difference between the stimulus and male TGS extract doses used χ^2 = 17.086 d.f.= 3 p=<0.0007. Pairwise test showed a statistical difference between the doses of 0.15 male equivalents (ME) and 0.05 ME (χ2 = 11.905 d.f.= 1 p=<0.0007), 0.15 ME and 0.1 ME (χ^2 = 5.954 d.f.= 1 p=<0.01) and 0.15 ME and 0.20 ME (χ^2 = 10.161 d.f.= 1 p=<0.001). Individual synthetic compounds identified in female TGS or a blend of these compounds did not stimulate any male copulation behavior response in bioassays.

Our results show that the chemical compounds present in the TGS of *A. pseudochrysorrhoa* play an important role in the chemical communication of this species. Based on the experiments performed here, the female tergal gland components possess a dual function; it can be used to defend against natural enemies, or to mediate the copulatory behavior. The female can use higher amounts of the tergal gland components to avoid copula with conspecific males, or can use lower amounts to initiate male copulatory responses. The male copulation response to compounds present in the female TGS of *A .pseudochrysorrhoa* is higher than in other species (38% of male copulation response in *A. curtula*, while 84% of male copulation response in *A. pseudochrysorrhoa*). In these two species, the stimulation of male grasping response reaction by female TGS is dependent on concentration. However, no male copulation response to synthetic compounds was observed in male *A. pseudochrysorrhoa* , while the synthetic compound dodecanal, identified from *A. curtula* female TGS, stimulates 38% of copulation response in *A. curtula* males.[23]

The occurrence of homosexual behavior in *A. pseudochrysorrhoa* is observed when males are kept in groups. Our experiments demonstrated that male TGS stimulates male grasping reaction response. It might be related to intraspecific competition for a food source, once Peschke [41] have demonstrated that old mature males of *A. curtula* do not show homosexual behavior when fed, but young males can accept homosexual copula to have access to food sources.

Conclusions

Eleven compounds were identified from the extract of TGS of male and female *A. pseudochrysorrhoa*. Ten of the identifications were confirmed through co-injection with synthetic standards. The chemical composition of the TGS of *A. pseudochrysorrhoa* proved to be very similar to other Aleocharinae species, comprising saturated and unsaturated linear hydrocarbons, aldehydes and quinones.

Both sexes possess a tergal gland, and no sex-specific compound could be identified. However, the bioassay of the stimulation of male grasping response reaction by female TGS proved to be dose-dependent, with 0.15 FE of TGS extract leading to 84% of male mating response. The bioassays also demonstrated that male TGS stimulated male grasping reaction response. The occurrence of homosexual behavior in *A. pseudochrysorrhoa* was observed when males are kept in groups.

The bioassays showed that the TGS of *A. pseudochrysorrhoa*, in addition to a defensive function, has a secondary function in the mate identification and copulation system, by stimulating the mating sequence behavior of males. Overall, the role of the TGS of *A. pseudochrysorrhoa* is not yet fully understood.

Experimental Section

Laboratory cultures

A. pseudochrysorrhoa adults were collected by the first author in animal carcasses at Federal University of Paraná (UFPR), located in Curitiba-PR, Brazil (S25.45, W49.23) between May 2015 to December 2016 under the zoological material permanent collect license number 48904, provided by ICMBIO (Instituto Chico Mendes de Conservação da Biodiversidade Ministério do Meio Ambiente). The identification and sex separation were also performed by the first author (based on Caron, Mise & Klimaszewski[9]). Rearing was established in laboratory by placing adults in plastic boxes (20 x 20 cm), containing smaller plastic boxes with moistened soil for oviposition. Adults were fed with first and second larval instar of *Sarconesia chlorogaster* (Wiedemann) (Diptera: Calliphoridae). The first instar larvae of *A. pseudochrysorroa* were reared in plastic boxes (10 x 10 cm) filled with moistened soil and *Sarchonesia chloragaster* pupae were offered as hosts. Immediately after the emergence of adults, beetles were separated by sex and kept in groups of ten individuals in plastic boxes (20 x 20 cm) with moistened filter paper. Beetles were kept in a controlled environmental chamber at 25 ºC, with photoperiod of 12 h. [10, 43]

Tergal gland components extraction

The tergal glands of males and females (n=10) were extracted individually from freeze killed virgin beetles at least 21 days old (30 min at -17°C). [23] The extractions were performed under a Zeiss Discovery V8 stereoscopic microscope. Lateral incisions were done on both sides of beetles' abdomen; the tergal gland located in the sixth and seventh abdominal tergites was removed and immediately immersed in 100 µl of hexanes during 45 minutes. The extracts were stored at -17 ºC.

Chemical analyses

GC-MS analyses were performed using a Shimadzu GC2010 gas chromatograph coupled to a Shimadzu QP2010 Plus spectrometer with electron impact ionization detector. The injector was operated in the splitless mode, at 250 °C with an injection volume of 1 µL. A DB-5 capillary column (Agilent Technologies; 30 m × 0.25 m × 0.25 µm) was employed, with gradient temperature programming starting at 50 °C held for 1 min and an increase rate of 7 °C min^{-1} to 270 °C. Quantitative determination for all extract components (n=10) was performed employing nonadecane (10 ng μl^{-1}) as internal standard. Retention indexes (RI) were calculated on a DB-5 column using commercial standards of alkanes (C10 to C26 10 ng μl^{-1}) as reference (equipment parameters as described above). GC coupled with Fourier transform infrared spectroscopy (GC-FTIR) analyses were performed in a Shimadzu GC-2010 equipped with a RTX-5 column (30 m x 0.25 mm x 0,25 µm, Restek Chromatography Products, USA) (same conditions as described above), coupled to a DiscovIR-GC spectrometer (DANI Instruments, Marlborough, Massachusetts, USA), with a scan rage of 4000-750 cm^{-1} and resolution of 8 cm^{-1}. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra of the synthetic compounds (See supporting information) were acquired on an ARX-200 spectrometer (Bruker, Billerica, USA) at 200 and 50 MHz respectively, as $CDCl_3$ solutions. Chemical shifts are expressed in ppm relative to tetramethylsilane (0 ppm).

Micro-derivatization (methylthiolation)

A stock solution of iodine was prepared in carbon disulfide (2 mL, 5% w/w). In an ampoule, 10 µL of dimethyldisulfide (Sigma-Aldrich, USA), 10 µL of the previously prepared iodine solution, and 10 µL of the crude natural extract were mixed. The ampoule was sealed and the mixture was stirred at room temperature during 12 h. The resulting solution was washed with a 10% solution of $Na_2S_2O_3$ and filtered over anhydrous Na_2SO_4 prior to GC-MS analysis. [25]

Chemicals

Chemical structures were confirmed through co-injection with synthetic standards. Decane (**1**), 2-methylcyclohexa-2,5-diene-1,4-dione (**2**), undecane (**4**), dodecane (**6**), tridecane (**9**) and dodecanal (**10**) were purchased from Sigma-Aldrich Chemical Company (Milwaukee, Wisconsin, USA). The structure of 2-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione (**5**) was proposed by comparison of the mass spectra and retention index previously described. [6, 16] The

unsaturated hydrocarbons (*Z*)-undec-4-ene (**3**), (*Z*)-tridec-4-ene (**7**) and (*Z*)-tridec-6-ene (**8**) were synthesized by Wittig reactions, starting from different aldehydes and bromides as described below. The unsaturated aldehyde (*Z*)-tetradec-5-enal (**11**) was prepared through pyridinium chlorochromate oxidation of the corresponding alcohol as described below.

General synthetic procedures

Alkenes **3**, **7** and **8** were synthesized by adding the corresponding bromide (2.1 mmol) and triphenylphosphine (551 mg, 2.1 mmol) to an ampoule that was sealed and stirred for 24 h at 100 ºC. The corresponding phosphonium salt was added without further purification to a reaction flask and suspended in THF (25 mL). The resulting mixture was then stirred for 20 min and cooled to -78 ºC, followed by the addition of *n*-buthyllithium solution (2.2 mmol in hexane). The solution was slowly warmed to room temperature and stirred for additional 30 min. Subsequently, the reaction was cooled to -78 ºC and a solution of the corresponding aldehyde (2.0 mmol in 5 mL of THF) was added dropwise. The reaction mixture was slowly warmed to room temperature overnight. The reaction was quenched with saturated NH_4Cl solution. The aqueous layer was extracted with pentane; the organic layers were combined, washed with brine, dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by flash chromatography using pentane as eluent, yielding a mixture of the corresponding (*Z*)- and (*E*)-alkenes, which were separated by column chromatography on silica gel impregnated with $AgNO_3$ (5%).

(*Z*)-undec-4-ene (**3**): Prepared according to procedure described above, starting from 1-bromobutane (287 mg, 2.1 mmol) and heptanal (228 mg, 2.0 mmol). Yield: 76%. ^{1}H NMR (200 MHz, $CDCl_3$): 0.82 – 0.98 (m, 6H), 1.19 – 1.45 (m, 10H), 1.88 – 2.16 (m, 4H), 5.28 – 5.45 (m, 2H). ^{13}C NMR (50 MHz, $CDCl_3$): 13.7, 14.0, 22.6, 22.8, 27.2, 29.0, 29.2, 29.7, 31.7, 129.5, 130.0.

(*Z*)-tridec-4-ene (**7**): Prepared according to procedure described above, starting from 1-bromobutane (287 mg, 2.1 mmol) and nonanal (285 mg, 2.0 mmol). Yield: 77%. ^{1}H NMR (200 MHz, $CDCl_3$): 0.83 – 0.95 (m, 6H), 1.21 – 1.43 (m, 14H), 1.90 – 2.10 (m, 4H), 5.25 – 5.46 (m, 2H). ^{13}C NMR (50 MHz, $CDCl_3$): 13.8, 14.1, 22.7, 22.9, 27.2, 29.3, 29.5, 29.8, 31.6, 31.9, 32.6, 129.6, 130.1.

(*Z*)-tridec-6-ene (**8**): Prepared according to procedure described above, starting from 1-bromohexane (346 mg, 2.1 mmol) and heptanal (228 mg, 2.0 mmol). Yield: 74%. ^{1}H NMR (200 MHz, $CDCl_3$): 0.82 – 0.96 (m, 6H), 1.21 – 1.43 (m, 14H), 1.91 – 2.08 (m, 4H), 5.31 – 5.43 (m, 2H). ^{13}C NMR (50 MHz, $CDCl_3$): 14.1, 22.6, 22.6, 27.1, 29.0, 29.4, 29.7, 31.4, 31.5, 31.8, 32.6, 129.9, 130.3.

(*Z*)-tetradec-5-enal (**11**): To a suspension of pyridinium chlorochromate (PCC, 10 mg), CeliteTM (10 mg), and sodium acetate (1 mg) in dichloromethane (300 µL) was added a solution of (*Z*)-tetradec-5-en-1-ol in hexane (1 mL, 1000 ppm). The reaction was stirred at room temperature for 4 h. The resulting mixture was filtered over a silica gel/celite (1:1) column, washed exhaustively with dichloromethane. The solution was concentrated to 100 µL under a gentle argon flow and promptly analyzed by GC-MS and GC-FTIR.

Bioassays

Assays were performed with virgin insects at least 21 days old, which had been separated according to sex immediately after adult emergence. [23] The influence of chemical odorants present in female/male TGS on male mating behavior was initially assayed by placing freshly killed females/males (n=5) glued by the pronotum in a bamboo stick with 13 live males (one at a time) in plastic boxes (10 x 10 cm) for 1 minute. Then, the same procedure was repeated with another four female cadavers, totalizing five female cadavers. Grasping behavior (when the male exposes its genital and tries to copulate with the female) was used as the criterion for the beginning of male copulation response sequence. In the next experiment, female cadavers were rinsed sequentially in two aliquots of hexane for 45 minutes (the volume of solvent used was the necessary to cover the whole body of the insect) for complete removal of TGS compounds, then the female cadavers were presented to males. Rinsed female cadavers that did not stimulate male grasping were used in the next treatments. Rinsed female cadaver was reconstituted with different volumes of the female TGS extracts (1 µL, 5 µL, 10 µL, 15 µL, 20 µL, 25 µL and 30 µL). In each experiment, five female cadavers were reconstituted and introduced to 13 live males sequentially in the arena. One male at a time was introduced and given 1 min to respond. Each volume was prepared from an extract of one female tergal gland in 100 µL of hexane. The influence of male TGS on male behavior was assayed as described above. Five rinsed male cadavers were reconstituted with different volumes of male TGS extracts (5 µL, 10 µL, 15 µL and 20 µL) and sequentially introduced to nine live males.

Bioassays were also performed with synthetic compounds for all identified compounds (except 2-methoxy-3-methyl-1,4-benzoquinone) and a mixture of these synthetic compounds was used as follows: hydrocarbons; aldehydes; hydrocarbons + quinone; aldehydes + hydrocarbons and aldehydes +

quinones using the same methodology as described above. The dose of synthetic compounds used was based on the amount of each compound in 15 µL of the volume solutions (one female tergal gland in 100 µL), since this volume had the best score in initiate male copulatory responses.

Statistical analysis

Statistical analyses were performed with the software BioEstat 5.3. The Chi-square was used to test significant differences between all bioassays. Differences in mean quantities between males and females of the identified compounds in ten extracts were tested by Shapiro-Wilk normality test, followed by T-test or Mann-Whitney (Wilcoxon rank-sum test). An experiment-wise error response of 0.05 was considered for all statistical analyses.

Supplementary Material

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/MS-number.

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Author Contribution Statement

M. R. S., D. M. V. and L. F. performed the experiments, data analysis and interpretation, and wrote the manuscript; P. T. B. contributed to the writing, review and editing; J. B. aided to the analyses of results and to writing of the manuscript; P. H. G. Z. designed and supervised the research.

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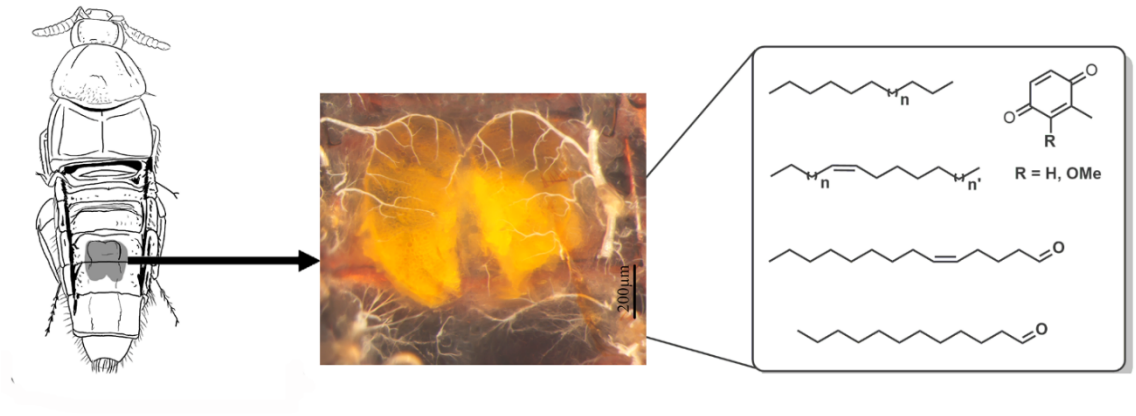

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Eleven compounds were identified from the natural extract of tergal gland components (TGC) of *Aleochara pseudochrysorrhoa*, which comprise saturated and unsaturated linear hydrocarbons, aldehydes and quinones.