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RESEARCH ARTICLE

Age-dependent release of and response to alarm pheromone in a ponerine ant

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ABSTRACT

Social insect societies are characterized by division of labour and communication within the colony. The most frequent mode of communication is by chemical signals. In general, pheromones elicit specific responses in the receiver, although reactions may vary depending on the receiving individual's physiological or motivational state. For example, it has been shown that pheromones can elicit different responses in morphological worker castes. However, comparably little is known about such effects in worker castes of monomorphic species. Here, we comprehensively studied a monomorphic species showing age polyethism, the thelytokous ant *Platythyrea punctata*. Our analyses revealed that the species' alarm pheromone consists of (*S*)-(−)-citronellal and (*S*)-(−)-actinidine, and is produced in the mandibular glands. Ants responded with increased movement activity and increasing ant density towards the pheromone source in whole-colony bioassays, confirming the alarming effect of these compounds. We found age classes to differ in their absolute pheromone content, in the propensity to release alarm pheromone upon disturbance and in their reaction towards the pheromone. Absolute amounts of pheromone content may differ simply because the biosynthesis of the pheromone begins only after adult eclosion. Nonetheless, our results indicate that this clonal species exhibits age-related polyethism in the emission of as well as in the response to its alarm pheromone.

KEY WORDS: Age polyethism, *Platythyrea punctata*, (*S*)-(−)-Citronellal, (*S*)-(−)-Actinidine

INTRODUCTION

Communication is essential to social insect societies, and chemical signalling is ubiquitous in governing individuals' behaviours and the overall organization within the colony (Leonhardt et al., 2016; Richard and Hunt, 2013). Such signals contribute to the maintenance of the social structure in many ways, including the suppression of worker reproduction by queen pheromones (Holman et al., 2010; Le Conte and Hefetz, 2008; Oi et al., 2016) and policing among workers based on recognition of worker-laid eggs (Endler et al., 2004; Oi et al., 2015; Smith et al., 2009). Chemical communication can also be vital for the survival and coherence of the colony. Trail pheromones enable recruitment to valuable resources (Hölldobler and Wilson, 1990; Jarau et al., 2010; Morgan, 2009), cuticular lipid profiles play a role in nestmate recognition (Pradella et al., 2015; Sturgis and Gordon, 2012; van Zweden and d'Ettorre, 2010), and alarm pheromones alert workers to danger (Hölldobler and Wilson, 1990). Alarm pheromones are common among social insects (Blum, 1969; Leonhardt et al., 2016) and workers' responses to such signals vary depending on species, caste and context (Hölldobler and Wilson, 2009; Schorkopf, 2016; Sasaki et al., 2014). In general, when alarmed individuals react by showing escape behaviours or relocating the brood, the reaction is termed panic alarm. If an alarm pheromone elicits aggressive alarm, individuals will move to confront the disturbance, prepared to attack (Wilson and Regnier, 1971).

Among the social Hymenoptera, ants are known to employ a remarkable diversity of chemical compounds for communication (Jackson and Morgan, 1993; Richard and Hunt, 2013). Ponerine ants, despite being thought to generally exhibit a more simple social structure (Peeters, 1997), are no exception and have been found to employ an array of chemical signals for essential social functions such as recruitment (Attygalle et al., 1991; Duncan and Crewe, 1994; Hölldobler et al., 1994; Leal and Oliveira, 1995; Witte and Maschwitz, 2000) and policing (Gobin et al., 1999; Kikuta and Tsuji, 1999), and as alarm pheromones (Longhurst et al., 1978, 1980; Wheeler and Blum, 1973; and see Schmidt and Shattuck, 2014).

The genus *Platythyrea* (Ponerinae, Platythyrini) appears to be exceptionally poor at producing and utilizing exocrine secretions. Of the 38 described species, there is only one report of characterized chemical compounds in an exocrine secretion, present in Dufour's glands of *Platythyrea cribinodis*, while no volatile compounds were detected in the venom gland of that species (Morgan et al., 2003). A related species, *Platythyrea punctata* (F. Smith 1858), possesses neither subepithelial glands (Gobin et al., 2003) nor a Dufour's gland, and samples taken from the venom gland and sternal glands were devoid of volatile material (Morgan et al., 2003). Nonetheless, we found this species to occasionally emit a lemon-like odour upon disturbance, indicating the presence of a volatile alarm pheromone. Apart from being a species for which no volatile signals have yet been reported, *P. punctata* is interesting to study because it is monomorphic and reproduces most often via thelytokous parthenogenesis (Hartmann et al., 2005; Heinze and Hölldobler, 1995). Thus, *P. punctata* is an ideal model organism for studies on age polyethism, as genetic effects within a colony can be expected to be minimal (Bernadou et al., 2015). The term age polyethism describes the phenomenon that age influences task allocation within the colony (Wilson, 1971; Robinson, 1992; Camargo et al., 2007). Younger individuals perform predominantly safe tasks such as nursing within the nest, and switch to more risky tasks outside of the nest, like foraging or guarding the nest, with increasing age (Wilson, 1971; Oster and Wilson, 1978; Seeley, 1982; Moroń et al., 2008; shown for *P. punctata* by Bernadou et al., 2015). Age polyethism, however, can not only govern the division of tasks but may also include differences in behavioural reactions to social signals (Cammaerts, 2014), as has been shown for morphological castes (Hölldobler and Wilson, 1990; Hughes and Goulson, 2001; Simola et al., 2016).

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In this study, we aimed to comprehensively elucidate the putative alarm pheromone of *P. punctata* by characterizing the compound(s) underlying the lemon-like odour, identifying the tissue of origin and determining the quantity of the compound(s) therein. We then investigated the behavioural effect of the compound(s), and tested the hypotheses that there is an age effect on both the production and emission of the compound(s), and that age influences the behavioural reaction towards the putative pheromone.

MATERIALS AND METHODS

All experiments were conducted with 10 colonies of *P. punctata* that were originally collected in Puerto Rico in 2012 (El Verde Field Station, El Yunque National Forest). They were kept in plastic boxes (20 cm×20 cm, 8.5 cm height, bottom covered with a layer of plaster of Paris) at 22–26°C and 75% humidity under a 12 h light:12 h dark cycle. The nest site in each box consisted of a chamber in the plaster floor that was covered with glass slides and kept dark by a sheet of black plastic on the glass. Water was provided *ad libitum* by means of a cotton wool-plugged plastic tube positioned in the box. Small cockroaches, fruit flies and diluted honey were provided as food 3 times a week after moistening the plaster floor to maintain high humidity in the boxes.

Colonies typically contained one, occasionally several, dominant reproductive individuals, with all other individuals acting as workers. Task allocation is influenced by an individual's age: young intranidals of *P. punctata* (YI, individuals with tan to brown coloration, aged <3 weeks) typically remain in the nest where they care for the brood, while foragers (FG, distinguishable from YI by the fully melanized black cuticula) are predominantly found outside the nest (Bernadou et al., 2015). All 10 colonies were tested in each of the experiments described below, providing n=10 replicates for every assay.

Chemical analyses

Pheromone source and composition

The results of headspace analyses (see 'Pheromone emission', below) supported the notion that the lemon-like odour is due to the putative alarm pheromone of *P. punctata*. In several other ponerine species, the mandibular glands are the source of alarm pheromone (Duffield and Blum, 1975; Longhurst et al., 1978), and we therefore considered the mandibular glands to be a likely source for the production and storage of this pheromone in *P. punctata*. In a preliminary analysis, we dissected a total of eight mandibular glands from FG that had been freeze-killed at −21°C. The glands were extracted singly in 20 µl of dichloromethane (DCM) containing 10 ng µl^{-1} methyl undecanoate as an internal standard (IS) for a minimum of 1 h, and the extracts were then subjected to chemical analysis by gas chromatography and coupled mass spectrometry (GC/MS) as described below. The mandibular gland extracts were found to contain citronellal (a monoterpene aldehyde, the same compound that was detected in the headspace analyses) and, additionally, actinidine (a monoterpene alkaloid). For the quantification of mandibular gland contents, we used ants from each of the 10 colonies. Single ants cannot easily be retrieved from their colony without disturbance (potentially causing them to emit alarm pheromone). Therefore, a minimum of four *P. punctata* workers were carefully isolated from their colony, and kept together in a Petri dish for 17–24 h to allow for some replenishment of pheromone content. The dish contained a water source (moistened tissue paper) and a shelter (folded piece of red plastic). Ventilation was ensured by a gauze-covered opening in the lid of the dish. The Petri dishes were kept in the same climate chamber as the colony boxes. FG and YI from each colony were kept together in their respective Petri dish. After the isolation period, the ants were flash-frozen by immersion in liquid nitrogen and subsequently kept at −21°C until dissection of mandibular glands. For all 10 colonies, we analysed one mandibular gland each from one FG and one YI. A sample consisted of one intact mandibular gland still attached to the mandible (Fig. 1C) extracted in 20 µl of DCM containing 10 ng µl^{-1} methyl undecanoate as IS for at least 3 h before chemical analysis (see below).

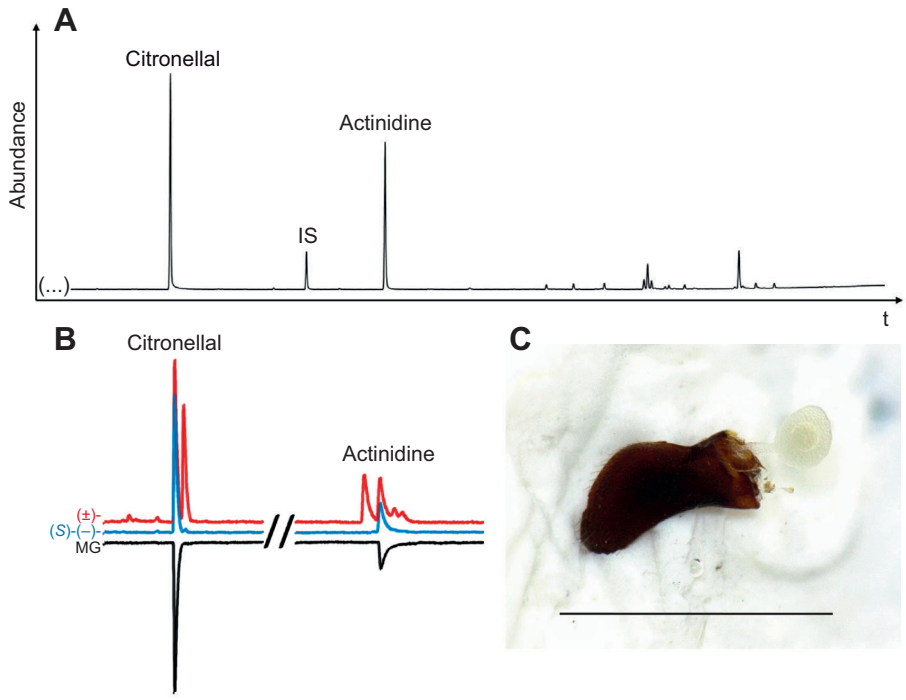

Fig. 1. Characterization of mandibular gland compounds. (A) Chromatogram of a mandibular gland extract with the two largest peaks representing the pheromone, and including methyl undecanoate as the internal standard (IS, 10 ng µl^{-1}). The smaller and later eluting peaks are components of the cuticular lipid profile. (B) Overlaid chromatograms (chiral column: Beta Dex 225) of the racemic mixture consisting of both enantiomers of citronellal and actinidine [(±)-, red], the mixture of (*S*)-(−)-citronellal and (*S*)-(−)-actinidine [(*S*)-(−)-, blue] and an extract of six pooled mandibular glands (MG, black). (C) Dissected mandibular gland still attached to the right mandible of a *Platythyrea punctata* forager. Scale bar: 1 mm.

Pheromone identification and quantification by GC/MS

Extracts from the mandibular glands and the headspace analyses (see 'Pheromone emission', below) were analysed on a Shimadzu QP2010 SE GC/MS equipped with a polar BP20 column (30 m length, 0.25 mm inner diameter, 0.25 µm thickness, SGE Analytical Science/Trajan Scientific, Melbourne, VIC, Australia) with an injection volume of 1 µl in splitless mode using helium as carrier gas (linear velocity 40 cm s^{-1}). The injection temperature was 240°C, and the oven was programmed to increase in temperature from 60°C to 240°C at 5°C min^{-1}. The final temperature was held constant for 10 min. Chromatograms were analysed using the software package GCMS solution version 4.11 (LabSolutions, Shimadzu, Kyoto, Japan). Compounds were identified by comparison of mass spectra and retention indices with those of synthetic standards. Quantification was accomplished by relating the peak areas of pheromone components to the peak area of the IS (10 ng $µl^{-1}$).

For the determination of the absolute configuration of the pheromone compounds, the GC was equipped with a Beta-Dex 225 column (30 m length, 0.25 mm inner diameter, 0.25 µm thickness, Supelco/Sigma-Aldrich, St Louis, MO, USA). Samples were injected splitless at a temperature of 220°C. The temperature program started at 60°C (held for 3 min) and was raised at 1°C min^{-1} to 100°C, which was held constant for 50 min. Subsequently, the temperature was raised to 220°C at 5°C min^{-1} and held at the final temperature for 10 min. Injection volume, carrier gas and velocity were the same as described above. The retention times of the peaks detected in an extract of six pooled mandibular glands were compared with those of a racemic mixture of both citronellal and actinidine, as well as with those of a mixture of (*S*)-(−)-citronellal (SC) and (*S*)-(−)-actinidine (SA). The authentic standard of SC was purchased (Merck, Darmstadt, Germany) and that of SA was prepared as described previously (Beckett et al., 2010).

Pheromone emission

Seventeen to 24 h before being tested, *P. punctata* workers were carefully isolated from their colony to allow for potential replenishment of pheromone content. FG and YI from each colony were again kept together in their respective Petri dish, and tested on the same day.

As volatile emission had previously been observed upon disturbance, we tested the effect of physical distress on volatile release using dynamic headspace analyses (purge-and-adsorbent trapping; Asfaw et al., 2018). Experiments were conducted at room temperature (around 21°C) with a custom-made sampling apparatus consisting of a 50 ml Erlenmeyer flask that contained a clean magnetic stir bar (8 mm×40 mm, Carl Roth, Karlsruhe, Germany), fitted with a wash bottle insert. The apparatus consisted exclusively of glass, stainless steel and Teflon parts in order to minimize contamination. Prior to every headspace sampling, all parts were cleaned with ethanol and deionized water and then heated for a minimum of 60 min at 100°C in a convection oven (VWR VENTI-Line VL 115). Incoming air was filtered through a glass tube containing 150 mg of activated charcoal (Supelco Orbo 32, small, Sigma-Aldrich, Taufkirchen, Germany). Volatile compounds from the headspace were purged for 15 min with an airflow of 60± 10 ml min^{-1} and trapped on commercially available adsorption tubes (65 mm length×5 mm inner diameter, 5 mg activated charcoal, Gränicher and Quartero, Daumazan, France). Trapped volatiles were eluted with 40 µl DCM containing 10 ng $µl^{-1}$ methyl decanoate as IS and subjected to analysis by GC/MS as described above.

For each replicate, three FG or three YI were carefully transferred to the Erlenmeyer flask. To keep the ants in the flask, the glass tube reaching into the Erlenmeyer flask as well as the top of the flask were blocked off using cleaned stainless steel mesh and Teflon tape. As a control, headspace was sampled for the three ants with the enclosed stir bar remaining motionless. After 15 min, the adsorption tube was replaced by a new one and the same three ants were sampled again. Now, using a magnet on the outside of the flask to move the enclosed stir bar, physical disturbance was elicited by deliberately bumping the stir bar into the ants for the duration of volatile collection (15 min).

We investigated the detection efficacy of the headspace setup after finding that the headspace analyses yielded SC only, while two compounds, SC and SA, could be detected in the mandibular gland extracts. Thus, 750 ng synthetic SC and 385 ng synthetic SA (amounts based on the preliminary quantification of one gland equivalent), each dissolved in DCM, were applied onto the same piece of clean filter paper (disc of 6 mm diameter), which was then tested in the headspace sampling setup for 15 min using the same experimental parameters as described for the tests with ants. Replicates (n=10) were always conducted with freshly prepared chemical-treated filter paper discs.

Behavioural experiments

Colony level

We evaluated the reaction of *P. punctata* towards the pheromone, using five different treatments: (1) mandibular gland extract (amount equivalent to one mandibular gland), (2) a synthetic pheromone mixture consisting of a combination of 480 ng synthetic SC and 350 ng synthetic SA (amounts based on the equivalent of one mandibular gland as estimated from a pooled extract of five mandibular glands), (3) 480 ng synthetic SC alone, or (4) 350 ng synthetic SA alone; we additionally tested a control treatment (5), using pure solvent (DCM). Each assay was conducted with all 10 colonies before starting the next. Before testing, a freely accessible test arena was set up for each of the 10 colonies. Every arena was placed in the box of the respective colony for a minimum of 17 h prior to testing in order to allow the ants to familiarize themselves with the arena. The arena consisted of a glass plate (49 mm×49 mm) with a roughened surface, covering an equally sized piece of paper. The paper was printed with concentric rings (hereafter referred to as 'lines'), dividing the area into different zones bordered by the respective lines. The lines and zones were labelled in the order: outer line (diameter d=48.5 mm), outer zone, middle line (d=34.5 mm), middle zone, centre line (d=20 mm) and centre zone. An additional ring (d=6 mm) printed around the centre point served to guide the placement of test compounds (see Fig. 3). Extracts and chemicals were applied to a clean filter paper disc (d=6 mm). After evaporation of the solvent (minimum of 5 s, until the paper appeared dry), the respective filter paper was mounted on the centre of the glass plate using a small piece of double-sided adhesive tape (about 3 mm×3 mm, Tesa SE, Hamburg, Germany) to prevent the ants from moving it from the original position.

For each replicate, the respective colony box was carefully placed under a video camera (Canon EOS M equipped with a Tamron 90 mm F/2.8 macro lens, recording from the top) in the climate chamber, and illuminated indirectly. Large pieces of white cardboard screen surrounded the setup to minimize distraction of the ants by external optical stimuli. Recording began 1 min after the box was set up. Introduction of the first filter paper (colony 'baseline' control, always the pure solvent: DCM) took place 3 min after the start of the recording, and the recording continued for another 3 min. After the recording was stopped, the first filter paper was removed, and the ants were given 3 min to settle. Subsequently,

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a filter paper impregnated with the respective test compound was introduced, and the second recording was started. The control treatment using pure DCM as test compound served to verify that the second placement of a filter paper per se would not influence the behaviour of the ants. Videos were analysed with The Observer XT 11 software (Noldus Information Technology, Wageningen, The Netherlands) in offline mode. Because of the volatility of the alarm pheromone, video analyses of colony assays covered the first minute after placement of a filter paper in the arena. The first 10 s were not included for analyses to avoid recording behaviour caused by the manipulation (introducing the filter paper), and to allow an odour gradient to develop. Two parameters were assessed: the number of times that ants crossed the printed lines (as a proxy for movement activity) and the number of ants present in each zone (a measure of ant density). An ant was defined to be present in a zone when more than half of its body was in the respective zone. Likewise, the crossing of a line was defined to have taken place when an ant moved from one zone to another in the designated time frame. The number of crossings over each of the three lines was documented for two consecutive seconds (to be able to assess the ants' movements) every 10 s, i.e. from 10th to 12th second, from 20th to 22nd second, from 30th to 32nd second, from 40th to 42nd second and lastly from 50th to 52nd second, resulting in a total observation time of 10 s. Density counts were taken by freezing the video every 5 s and counting the number of ants present in each zone, starting at the 10th second and ending with the last count at the 60th second, resulting in a total of 11 count events. Counts were taken without regard to ant identity, i.e. an ant present in the same zone during two consecutive density counts was counted once for each time point. The same principle was applied for the line crossings, with all crossings of every ant during the measuring time span being counted. The results were normalized by dividing the number of crossings over a line by its respective length, and by dividing the total number of ants for one zone by the respective zone's area.

Age groups

YI are rarely seen outside the nest under normal circumstances, and never ventured out of the nest cavity during bioassays on the colony level. Thus, the setup had to be modified to be able to test whether *P. punctata* shows age polyethism in the reaction to the pheromone.

The same arena as described before was placed in the colonies for a minimum of 17 h prior to testing, though now it was additionally positioned on top of a larger glass plate (12 cm×12 cm). As for the colony assays, this was done to let ants familiarize themselves with the arena. As YI do not usually leave the nest and the arena would therefore be novel to them, this allowed the arena to acquire at least some of the colony-typical odour left by colony members. The whole construction was taken out of the colony box directly before testing, carefully removing ants currently present on it, and positioned under the video camera in the same fashion as for the colony assays. An outer barrier (PE, 4.1 cm high with rounded corners, 6.6 cm×6.6 cm) coated with Fluon (PTFE dispersion, Whitford GmbH, Diez, Germany) was positioned around the arena to prevent ants from escaping. The barrier was fixed in place from the outside using glue pads (patafix, UHU GmbH & Co KG, Bühl, Germany). Three ants of the same age class (either FG or YI) were carefully taken from the colony using flexible forceps and placed within the barrier directly prior to the experiment. Video recording was conducted as described for the colony-level assays, with the difference that the time between the end of the first video (baseline control) and the onset of the test video was reduced to 1 min, as the removal of the filter paper did not cause any disturbance. Only the synthetic pheromone (mixture of synthetic SC and synthetic SA) was tested in this setup.

Video analyses for age group bioassays were conducted in a similar fashion to that described for the colony-level assays, but were adapted for the lower number of ants. The observation period lasted 15 s after the introduction of the filter paper, omitting the first 1.5 s. Line crossings were documented continuously from 2 s onwards, resulting in a total of 13 s. Density counts were taken every 1.5 s with the final count at the 15th second, resulting in a total of 10 count events. The results were normalized as described for the colony assays.

Ant housing, treatment, and all experiments conformed to the relevant regulatory standards.

Statistical analyses

Statistical analyses were conducted with R version 3.6.1 (https://www.R-project.org). Wilcoxon paired tests were employed to compare the paired data of the headspace assays between the control situation and during physical distress for the peak areas of SC for FG and YI. Analyses of age polyethism were conducted with Wilcoxon paired tests for pheromone quantification (amounts of the single compounds as well as their ratio to each other), and using the values resulting from the test situation for FG and YI in the headspace analyses of pheromone emission under physical distress. Data for FG and YI were considered paired by colony origin.

The behavioural experiments testing the ants' reaction to the pheromone (for both colony and the age groups) were analysed using linear mixed effect models (LMM, R packages 'lme4': Bates et al., 2015; 'car': Fox and Weisberg, 2019). Movement activity and ant density were analysed separately, for both the colony assay and the age group assay. To test for the effect of treatment (colony assay, test compounds: mandibular gland extract, synthetic pheromone, SC, SA and DCM control) or age (age group assay: FG and YI), and the three lines/zones (outer, middle and centre) on the response of the ants, we first calculated the response variables by subtracting each baseline control value from the respective movement/density value. We always started with a full model, including lines/zones and treatment or age as fixed effect factors, as well as the interactions between these factors. Colony identity was included as random factor. We obtained minimal models by a stepwise backward elimination procedure (successively removing non-significant terms from the models), and comparing the nested models with a maximum likelihood method. The minimal models were then estimated using a restricted maximum likelihood method (Pinheiro and Bates, 2000). To comply with the assumption of normality and homoscedasticity as much as possible, data were transformed where necessary: first a fixed value was added to the response variables (raising the lowest value to 1) and the data were subsequently $\log_{10}$ transformed. *Post hoc* multiple comparison procedures were conducted using Tukey all-pair comparisons with Bonferroni–Holm correction (R package 'multcomp': Hothorn et al., 2008).

RESULTS

Chemical analyses

The mandibular gland extracts contained two volatile compounds, SC and SA (Fig. 1A). The absolute configuration was determined by comparison with synthetic compounds (Fig. 1B). A single mandibular gland of FG contained on average 840±390 ng of SC and 510±220 ng of SA (Fig. 2A). Mandibular glands of YI contained significantly less of both: 340±120 ng SC and 140±70 ng

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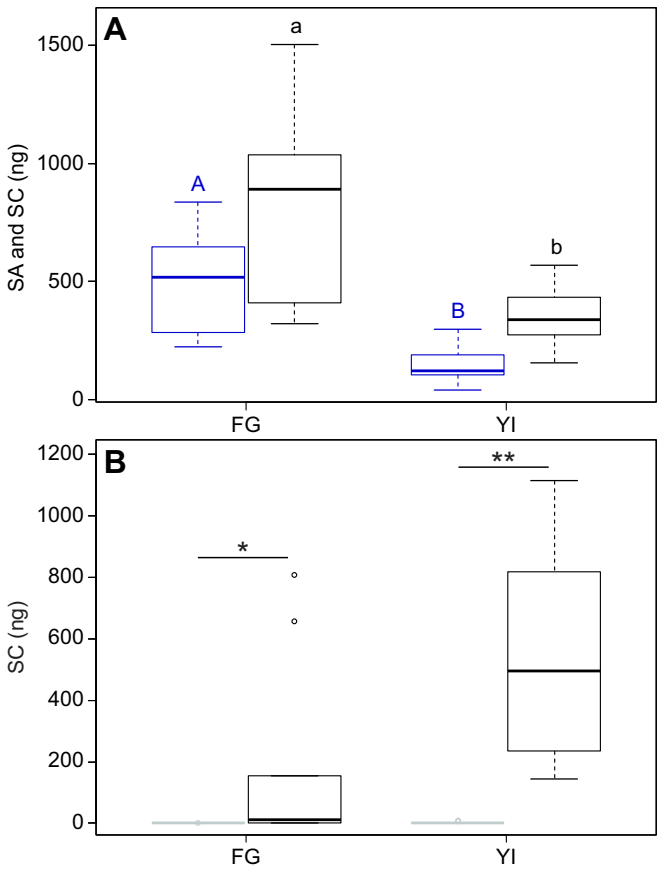

Fig. 2. Mandibular gland contents of and pheromone emission by *P. punctata*. (A) Amounts of (*S*)-(−)-actinidine (SA, blue) and (*S*)-(−)-citronellal (SC, black) found in extracts of one mandibular gland of foragers (FG) and young intranidals (YI). (B) Amount of SC recovered from the headspace over three undisturbed ants (light grey) and three ants under physical distress (black) for FG and YI. Boxplots show median and quartiles (the second and third data quartile lie within the box, whiskers encompass the first and fourth quartile). Outliers (more than 1.5 times the interquartile range, i.e. box height) are indicated by small circles. Significant differences are shown by letters or asterisks: A,B: P=0.006; a,b: P=0.014; *P=0.022, **P=0.002 (Wilcoxon paired tests, n=10 for each sample type).

SA per gland (Wilcoxon paired tests: v=51, P=0.014 for SC; v=0, P=0.006 for SA; Fig. 2A). Additionally, the ratio of the two compounds differed between the age classes (Wilcoxon paired test: v=0, P=0.006), with YI having relatively more SC (ratio of SC to SA: FG: 1.6±0.2, YI: 2.6±0.8; Fig. 2A).

When testing for pheromone emission with dynamic headspace analyses, SC could be detected in only one case (7 ng) in the control situation for FG. Upon physical distress, however, they released this compound in significantly larger amounts (170±300 ng in 7/10 replicates; Wilcoxon paired test: v=0, P=0.022; Fig. 2B). YI emitted SC in two out of 10 cases during the control setup (6 ng and 10 ng). In the physical distress situation, the pheromone was released in all 10 replicates, and in significantly higher amounts than during the control (540±370 ng, v=0, P=0.002; Fig. 2B). In comparison to the tested FG, YI tended to emit more SC (v=9, P=0.064; Fig. 2B). Headspace analyses testing the recovery rate for both compounds found in the mandibular glands could not recover any of the 385 ng of synthetic SA in any of the 10 replicates. Of the 750 ng of synthetic SC used in each replicate, 15 min of headspace sampling yielded on average 270±30 ng (recovery rate: 36%).

Behavioural experiments

In the colony-level bioassays, the response values of the DCM control treatment did not differ from zero (for both movement activity and ant density), i.e. there was no change of behaviour in response to pure solvent (movement–0: z=0.367, P=0.999, density–0: z=0.355, P=0.999; Figs 3 and 4A, Table 1). All other treatments differed significantly from zero (Table 1), indicating that mandibular gland extract, as well as the synthetic pheromone mixture and synthetic SC or SA alone led to an increase of ant activity (Fig. 3). For further LMM analyses, the control treatment was removed from the dataset. The minimal model for movement activity included both fixed factors as significant (treatment: χ^2=29.204, d.f.=3, P<0.001; line: χ^2=9.328, d.f.=2, P=0.009). Pairwise comparisons revealed that the behavioural effect of SA alone was less pronounced than in the other treatments (mandibular gland extract–SA: z=4.885, P<0.001; synthetic pheromone–SA: z=3.640, P=0.001; SC–SA: z=3.861, P<0.001; Figs 3 and 4A, Table 1). Additionally, the reactions differed for lines between outer and centre (z=−2.716, P=0.019; Figs 3 and 4A, Table 1), suggesting a decreasing response with increasing distance from the odour source. For ant density in colony-level bioassays, only treatments had a significant effect, with reactions to the SA treatment being less distinct than those to mandibular gland extracts (z=−3.520, P=0.003) and synthetic SC (z=−2.809, P=0.025; Figs 3 and 4B, Table 1). In all but the control assay, the average behavioural change, as measured by the difference of movement activity and density between the baseline control and test situation was pronounced (Movie 1), and tended to be higher the closer the respective zone/line was to the source of the test stimulus (Figs 3 and 4).

In the age group bioassays confronting three ants with the mixture of synthetic SC and SA, behavioural responses did not differ from zero for ant density (FG–0: z=−1.303, P=0.592; YI–0: z=0.0, P=1). For movement activity, however, behavioural reactions of FG were significantly different from zero (z=3.765, P<0.001), while YI showed only a trend to move more over the lines (z=2.391, P=0.056; Table 1). The minimal model included both factors age class (χ^2=3.844, d.f.=1, P=0.049; Fig. S1) and line (χ^2=6.317, d.f.=2, P=0.042) as significant, with the reaction differing between outer and centre line (z=−2.716, P=0.019; Fig. S1; Table 1). An ant crossing the centre line in the test situation was observed in five of the replicates for YI while FG did so in all 10 cases. In general, YI were less active than FG and tended to remain huddled together beyond the outer line, showing hardly any movement. Nonetheless, a reaction (rapid antennal movements and sometimes single ants beginning to move around) could be observed in most cases upon exposure to the pheromone.

DISCUSSION

Platythyrea punctata workers released their volatile pheromone only upon infliction of physical distress, and the source of the pheromone was found to be the mandibular gland. Additional tests indicated that *P. punctata* does not emit the pheromone upon contact with prey items (not to familiar, unfamiliar or chemically defended prey, as might be expected if the pheromone had a general alerting purpose; Table S1). Our bioassays in ant colonies showed that SC and SA elicit an increase in movement activity and lead to a higher density of ants near the point of pheromone release. We thus conclude that SC and SA function as an alarm pheromone in *P. punctata*.

The mandibular gland is the source of alarm pheromones for several other ant species in different subfamilies (e.g. Ponerinae:

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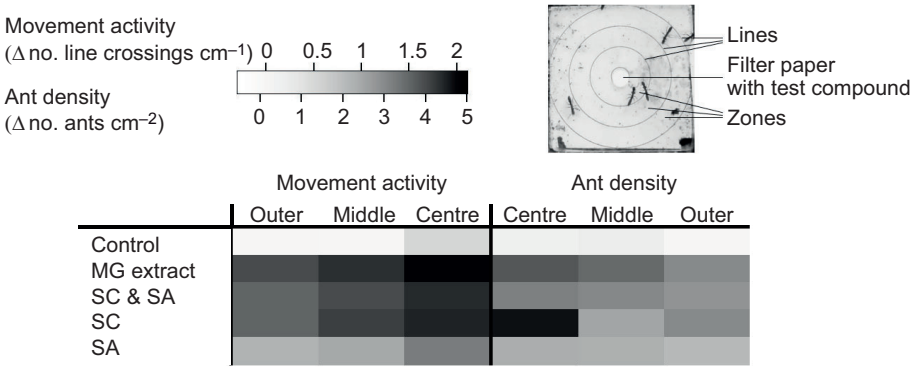

Fig. 3. Heatmap depicting the change in behavioural reaction to different treatments in colony-level bioassays of *P. punctata*. The test arena is shown top right with the heat map below. The darker the area of the heatmap, the larger the difference in movement activity or density between test and baseline control (dichloromethane, DCM). To better visualize the increase of movement activity and ant density towards the centre of the test arena, the right side of the heatmap (ant density) is arranged from centre to outer zone. Test treatments are given in the first column (n=10 for each treatment). In the control treatment, pure DCM was used in both the baseline control and test run. MG, mandibular gland; SC, (*S*)-(−)-citronellal; SA, (*S*)-(−)-actinidine.

Duffield and Blum, 1975; Longhurst et al., 1978; Formicinae: Bradshaw et al., 1979; Witte et al., 2007; Myrmicinae: Norman et al., 2017; Sasaki et al., 2014; Vander Meer et al., 2010; and see Hölldobler and Wilson, 1990). Both citronellal and actinidine have previously been reported as alarm/alerting pheromone components in ants. In *Acanthomyops claviger*, citronellal was found to be present in the mandibular glands and, in combination with the contents of Dufour's gland, to function as an alarm pheromone (Regnier and Wilson, 1968; Wilson and Regnier, 1971). Actinidine has been reported to be present in the pygidial gland contents of *Megaponera foetens*. It was suggested to have an alerting function, as more ants left a nest entrance treated with this compound than one treated with a solvent control (Janssen et al., 1995). In both cases, however, the absolute configuration of the compounds was not determined. The absolute configuration may be of importance as the behavioural reactions towards pheromone enantiomers and/or a racemic mixture can differ (see Riley et al., 1974; Wojtasek et al., 1998; Yu et al., 2014). In the present study, we demonstrated that *P. punctata* produces the (*S*)-enantiomers of both pheromone compounds. The dynamic headspace analyses yielded only SC, and the same was found in the recovery experiments with synthetic chemicals, clearly demonstrating that SA in natural doses was not detectable with this method. This was probably due to the compound's polar properties, which could lead to adsorption on the glass surfaces in the experimental setup.

The results of the colony-level bioassays showed that *P. punctata* reacts to both compounds of its mandibular pheromone (singly and in a mixture) in the same way. The ants showed increased movement and aggregated near the point of pheromone release. The reaction to synthetic SA alone, however, was less pronounced than the reaction to synthetic SC or a mixture of the two compounds. This could be due to the lower concentration of SA used in the assays (350 ng versus 480 ng of synthetic SC or the total of 830 ng in the pheromone mixture). Further studies would be needed to assess the potential effects of pheromone concentration on the behavioural reaction. The fact that the behavioural reaction of *P. punctata* to synthetic SC did not differ from that to the mixture of SC and SA indicates that there is no additive effect of the two single compounds. This is in contrast to reports on *Formica rufa* (Löfqvist, 1976), *Atta colombica* and two *Acromyrmex* species, where an extract containing all pheromone compounds elicited a stronger reaction than the most active single compound on its own (Norman et al., 2017). Multi-component alarm pheromones are common in ants (see Keeling et al., 2004) and other Hymenoptera (Brushini et al., 2010; Keeling et al., 2004; Slessor et al., 2005). In some cases, the various compounds have different functions that are related to their volatility, and thus come into play at different distances from the source (Bradshaw et al., 1975) or at different times after emission (Fujiwara-Tsujii et al., 2006). Although the vapour pressures of SC (0.2866 hPa at 25°C) and SA (0.1933 hPa at 25°C; both values from The Good Scents Company Information System: http://www.thegoodscentscompany.com/index.html) differ, the observed behavioural reactions were similar for the two compounds in the present study. Further studies could investigate whether the two alarm pheromone components differ in their effect over larger distances or with respect to time after emission. Furthermore, the potential of enantiospecificity in the alarm pheromone response of *P. punctata* deserves further investigation.

Species specificity is generally considered to be important for many types of pheromones, and can be achieved through unique compounds or specific mixtures of more common compounds (Wyatt, 2014). Alarm pheromones, however, can lack that characteristic. This is assumed to be caused by a comparatively low selection on specificity of alarm signals, as species can benefit from responding to alarm pheromones of sympatric species (Vander Meer and Alonso, 1998). It has also been found that congeneric species may produce the same main alarm compounds (e.g. eight species of *Atta*; see Norman et al., 2017). Although nothing is known about the alarm pheromones of other species of *Platythyrea*, two facts suggest that the alarm pheromone of *P. punctata* is not species specific. First, the two compounds have been reported for other ant species (although stereochemistry was not resolved: Regnier and Wilson, 1968; Wilson and Regnier, 1971; Janssen et al., 1995; see http://www.pherobase.com), and thus are probably not unique to *P. punctata*. Second, the reaction of the ants was comparable between SC alone and the mixture of SC and SA, i.e. the second compound does not seem to be required to elicit the full response. The effect of the alarm pheromone in bioassays with whole colonies was clearly visible (Movie 1). Ants were additionally observed to open their mandibles while approaching the pheromone-treated filter paper (but not the solvent control), making rapid sideways head movements with raised antennae as if searching for something to attack. In contrast, reactions of groups of only three FG were much less pronounced. This difference may not only be due to the higher number of ants in the colony, and thus a clearer increase in the reaction when exposed to the pheromone, but

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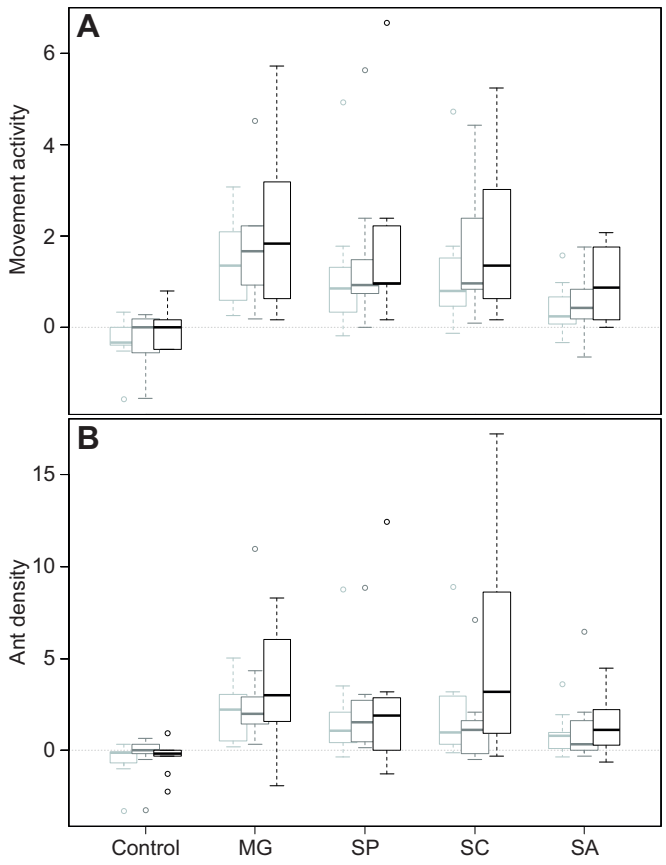

Fig. 4. Behavioural responses of *P. punctata* to five treatments in whole-colony bioassays. Values shown were obtained after subtraction of the baseline response (to DCM alone) from the response to the treatment. Control: DCM introduced a second time; MG: mandibular gland extract equivalent to one mandibular gland; SP: synthetic pheromone mixture of SC and SA; SC: (*S*)-(−)-citronellal alone; SA: (*S*)-(−)-actinidine alone (*n*=10 each). Observed behavioural responses were (A) movement activity [number of line crossings per cm for the outer (light grey), middle (dark grey) and centre (black) lines of the test arena; see Fig. 3] and (B) ant density [number of ants per cm² for the outer (light grey), middle (dark grey) and centre (black) zone of the test arena; see Fig. 3]. Boxplots show median and quartiles (the second and third data quartile lie within the box, whiskers encompass the first and fourth quartile). Outliers (more than 1.5 times the interquartile range, i.e. box height) are indicated by small circles. See Results and Table 1 for statistics.

Table 1. Reactions of *Platythyrea punctata* to alarm pheromone and single compounds (results of pairwise comparisons)

	z	*P*
Colony assay		
Movement reaction to treatment differing from zero		
Control–0	0.367	0.999
MG–0	6.798	<0.001*
SP–0	5.834	<0.001*
SC–0	6.005	<0.001*
SA–0	3.014	0.015*
Colony assay with control treatment removed from dataset		
Movement reaction differing between treatments		
MG–SP	1.245	0.639
MG–SC	1.024	0.639
MG–SA	4.885	<0.001*
SP–SC	−0.221	0.825
SP–SA	3.640	0.001*
SC–SA	3.861	<0.001*
Movement reaction differing between lines		
Outer–middle	−1.046	0.295
Outer–centre	−2.716	0.020*
Middle–centre	−1.670	0.190
Colony assay		
Density reaction to treatment differing from zero		
Control–0	0.355	0.999
MG–0	5.370	<0.001*
SP–0	4.292	<0.001*
SC–0	5.329	<0.001*
SA–0	2.712	0.037*
Colony assay with control treatment removed from dataset, transformed		
Density reaction differing between treatments		
MG–SP	1.672	0.284
MG–SC	0.712	0.674
MG–SA	3.520	0.003*
SP–SC	−0.960	0.674
SP–SA	1.848	0.258
SC–SA	2.809	0.025*
Age group assay		
Movement reaction to treatment differing from zero		
FG–0	3.765	<0.001*
YI–0	2.391	0.056
Movement reaction differing between lines		
Outer–middle	−0.096	0.436
Outer–centre	−2.394	0.050*
Middle–centre	−1.615	0.213

Analyses were conducted using linear mixed models (see Materials and Methods). The table lists only the results for comparisons against zero and pairwise tests where required for significant factors (see Results for model statistics). *Post hoc* Tukey pairwise comparisons were Bonferroni–Holm corrected, with accordingly adjusted *P*-values reported here. Significant results are marked with an asterisk.
MG, mandibular gland; SP, synthetic pheromone mixture of (*S*)-(−)-citronellal and (*S*)-(−)-actinidine; SC, (*S*)-(−)-citronellal; SA, (*S*)-(−)-actinidine; FG, forager; YI, young intranidal.

also be a consequence of context. Ants may react less strongly to alarm pheromones when fewer nestmates are around (Sakata and Katayama, 2001) or when they are not in the vicinity of the nest (Parmentier et al., 2015; Velásquez et al., 2006). It is also possible that within a colony the chemical alarm signal is reinforced by some of the newly approaching individuals (perhaps after contact with already alarmed individuals). The resulting increase in pheromone concentration could then lead to more pronounced or more prolonged reactions. Indeed, in the colony-level bioassays, the increased overall activity after pheromone exposure could be observed for a longer period of time than in assays with only three ants. A further explanation for a lower reaction in small groups compared with that at the colony level might be the subset of tested individuals itself. A study with *Rhytidoponera confusa* revealed that aggression was shown by only a small number of ants from a colony (Crosland, 1990). Similarly, a study on aggression in *P. puncata* found that individual ants varied in their level of aggression. While a minority of workers was involved in most aggressive interactions, several individuals avoided fighting altogether (Barth et al., 2010). The subsets of ants in the group bioassays may thus have contained relatively more individuals showing a less pronounced reaction/higher response threshold to the pheromone, while in the complete colony, many of the most responsive ants would have been attracted to the compounds presented in the arena and thus included in the counts.

We showed that *P. punctata* exhibits age polyethism not only in relation to performed tasks (Bernadou et al., 2015) but also in the production of and reaction to its alarm pheromone. YI had significantly less alarm pheromone in their mandibular glands than FG, and the ratio of the two compounds differed between the age classes. It seems highly unlikely that the compound ratios differ

as a result of selective emission of one of the compounds. Rather, this effect may be due to a difference in onset times or rates of biosynthesis for the two compounds, as has been shown for Dufour's gland components in *Formica sanguinea* (Fadl Ali et al., 1988). Our results suggest that young workers begin their adult lives with little or no alarm pheromone, as has been found for the compounds of the poison gland in *Harpegnathos saltator* (Haight, 2012). Despite having less pheromone in their mandibular glands, YI tended to release both a higher total amount and a higher proportion of the available pheromone upon physical distress than FG. When taking into account the average amount of SC in one mandibular gland and the recovery rate of this compound with the dynamic headspace analysis, YI emitted on average about 74% of their total SC when under physical distress, while the tested FG emitted only 9% of their available pheromone. Such an age-related difference in the propensity for release of alarm pheromone could be adaptive, as YI, under natural circumstances, would only come into contact with an aggressive alien intruder if that ant had already reached the nest chamber, probably posing a severe threat to the colony. Whether a higher pheromone concentration actually elicits a stronger response, remains to be shown. Additionally, experience might influence how much alarm pheromone is emitted in a certain context and could also explain the lower amounts of pheromone released by FG.

Reaction to the alarm pheromone in colony-level bioassays was shown exclusively by FG. Nest defence is often performed by older ants, a typical consequence of age polyethism in which more risky tasks are taken up only later in life (Wilson, 1971; Oster and Wilson, 1978; Seeley, 1982; Moroń et al., 2008). Our results follow the prediction that it is the older workers that show a more pronounced reaction to the alarm pheromone (see Norman et al., 2017; Cammaerts, 2014; but see Parmentier et al., 2015). Nonetheless, there were some YI that reacted to the alarm pheromone with increased movement. It would be interesting to study whether such a higher propensity to react to alarm pheromone could be a permanent individual trait (e.g. a lower than average response threshold) that remains enhanced in relation to others from the same age cohort. However, the reaction of YI might also be more likely in ants from certain colonies (i.e. a genetic effect) or dependent on colony size.

To our knowledge, this is the first study to demonstrate the composition and effect of an alarm pheromone in the genus *Platythyrea*, and the first report of a volatile pheromone in *P. punctata*. The ants exhibited age polyethism not only in regard to pheromone emission but also in their behavioural reaction. A valuable extension of this work would be an investigation of the influence of pheromone concentration on behaviour, and determination of whether differing response thresholds or other physiological factors explain the observed behavioural age polyethism.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: T.P., A.B., J.R.; Methodology: T.P., A.B., J.R.; Formal analysis: T.P., L.S.; Investigation: T.P., L.S., J.R.; Resources: J.E.H., A.B., J.R.; Writing - original draft: T.P.; Writing - review & editing: T.P., J.E.H., A.B., J.R.; Visualization: T.P.

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Supplementary information

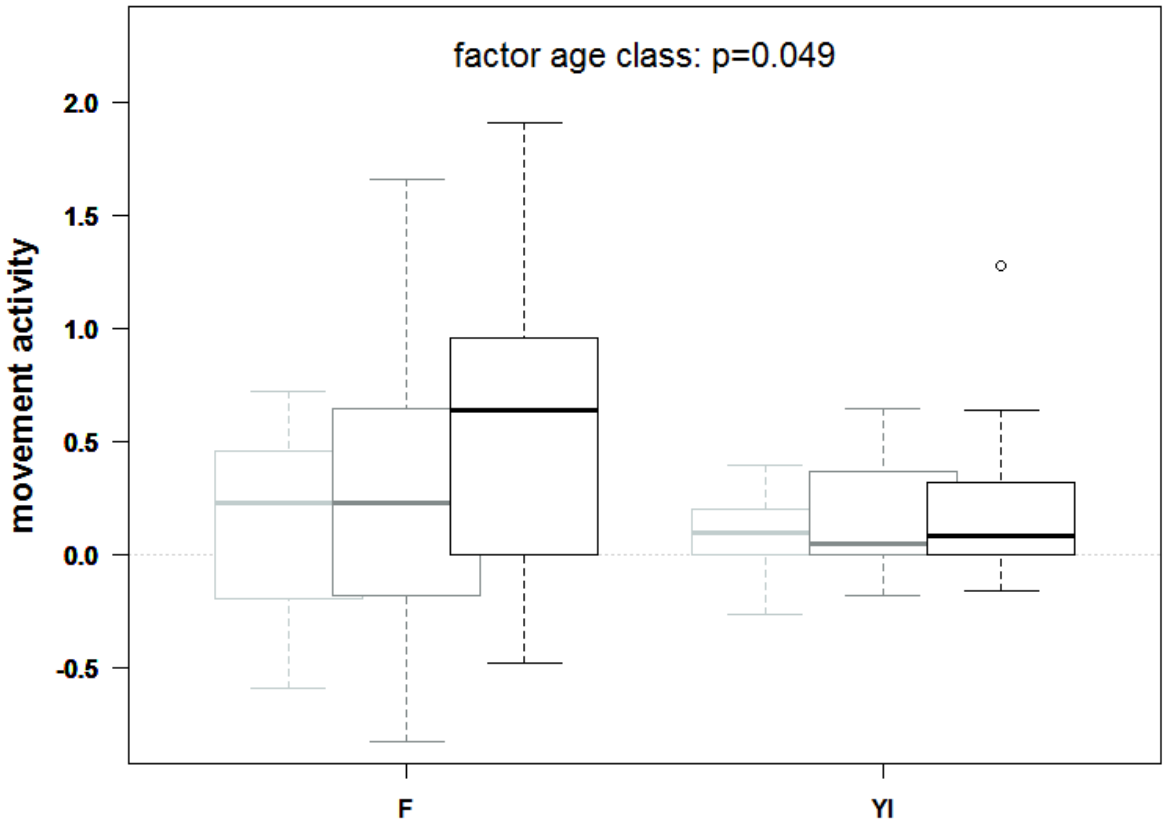
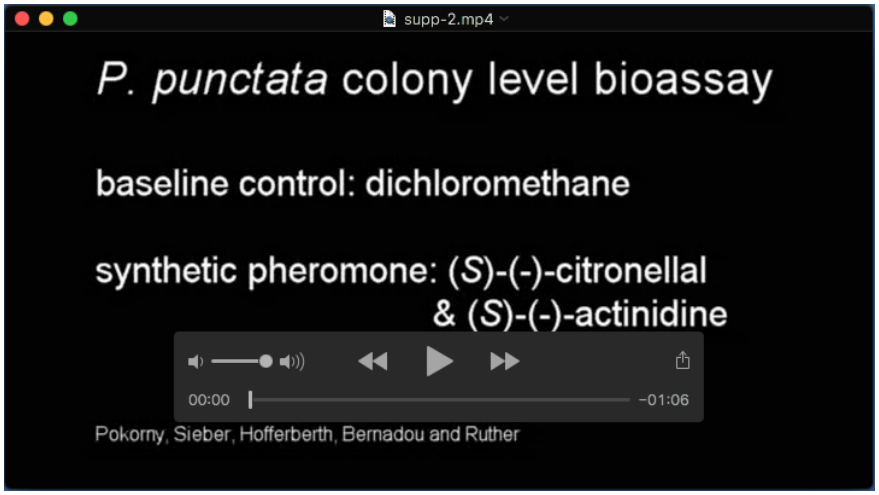


Figure S1: Age polyethism in the response to alarm pheromone. Changes in ant movement activity (number of line crossings divided by line length over each of the three lines surrounding the zones of the arena, see Fig. 3) in response to synthetic alarm pheromone (mixture of synthetic (*S*)-(-)-citronellal and synthetic (*S*)-(-)-actinidine, values of the baseline control subtracted from the values during the test situation) for groups of three foragers (F) and three young intranidals (YI), n=10 each. Boxplots show median and quartiles (the second and third data quartile lie within the box, whiskers encompass the first and fourth quartile). Outliers (more than 1.5 times the interquartile range, i.e. box height, above the third quartile) are indicated by small circles. See text for statistics.



Movie 1: Exemplary video recording of a colony level bioassay. Both sides show the same colony during exposition of the baseline control (left side) and the synthetic pheromone (480 ng (*S*)-(-)-citronellal with 350 ng (*S*)-(-)-actinidine, right side).

Table S1: The effect of prey encounter on pheromone emission by foragers of *P. punctata* using dynamic headspace analyses.

Stimulus	(*S*)-(-)-citronellal detected in control	(*S*)-(-)-citronellal detected in test
a) cockroach head (usual prey item)	10x 0.0 ng	9x 0.0 ng, 1x 5.5 ng
b) *L. caesar* larva (3rd instar) (novel prey item)	10x 0.0 ng	8x 0.0 ng, 1x 17.2 ng, 1x 3.8 ng
c) *P. cochleariae* larva (2nd instar) (novel but chemically defended prey item)	9x 0.0 ng, 1x 11.1 ng	10x 0.0 ng

Experimental setup followed the same protocol as for pheromone emission under physical distress (see text). Prior to testing, the colonies were starved for three days. The control situation consisted of the three ants to be tested in the otherwise empty Erlenmeyer flask. For the test situation, one of three stimuli was carefully added into the chamber before sample collection. There was no effect of (potential) prey encounter on pheromone emission, be it familiar or novel, chemically defended or not (Wilcoxon paired tests between control and test situation, for cockroach and *P. cochleariae* larva: p=1, for *L. caesar* larva p=0.371).