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Investigating chemical communication in oribatid and astigmatid mites in bioassays - Pitfalls and suggestions

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Abstract

A pair of exocrine opisthonotal oil glands from which more than one hundred chemical compounds have been described characterizes the glandulate Oribatida and the Astigmata. While allomonal and pheromonal properties were demonstrated for some of these compounds in some species, the biological function has remained unknown in most cases. The few existing studies on chemical communication used different kinds of experimental designs with bioassays and impregnated filter paper as source for scent dispersal. Like this, the existence of alarm-, aggregation- and sex-pheromones has been demonstrated. Here, we show that most of these studies may have suffered from some shortcomings regarding two parts of the experimental design: i) proper replication and ii) source for scent dispersal.

Hence, this contribution has two principle parts: in the first part we focus on bioassay design and the occurrence of pseudoreplication by analyzing published studies with astigmatid and oribatid mites in a literature survey. The second part concerns the source for scent dispersal used in bioassays: we investigated the evaporation dynamics of multi-component-secretions from paper and paper/clay combinations and show that these represent two different principal kinds of sources (instantaneous vs. continuous release of scents). Paper alone is an improper source for long-standing bioassays (i.e. several minutes) because different compounds evaporate with different rates leading to a dramatic change in relative composition. This is much less pronounced in a paper/clay combination.

Keywords: chemical ecology, pseudoreplication, bioassay, emitting source, diffusion, Oribatida, Astigmata

1. Introduction

Most oribatid and astigmatid mites bear an exocrine opisthonotal oil gland system that has been suggested to be a synapomorphic character for a clade in which the Astigmata originated within 'Oribatida' (Sakata & Norton 2001). To date more than one hundred compounds – chemically belonging to the classes of hydrocarbons, aromatics, terpenes and alkaloids – have been described for oribatid and astigmatid mites (reviewed in Kuwahara 2004, Raspotnig et al. 2011). Many of the compounds probably are important allomones for predator defense in oribatid mites (Raspotnig 2006, Heethoff et al. 2011, Heethoff & Raspotnig 2012), and some have been demonstrated to act as alarm-pheromones as well (Shimano et al. 2002, Raspotnig

2006). While the two latter reports are the only papers dealing with the pheromonal activity of oil gland secretions of oribatid mites, functional aspects have been studied to a much broader extent in Astigmata: more than 30 cases of alarm-, aggregation- or sex-pheromones have been published (Kuwahara 2004). Hence, the pheromonal activity of oil gland secretions in oribatid mites (with about 10.000 described species; Schatz 2002) certainly deserves research intensification.

Oil gland derived pheromones in astigmatid and oribatid mites probably all act as releasers, resulting in an immediate response of receivers (see Law & Regnier 1971). The expected initial responses include two easily discriminable behaviors of the receivers: moving towards the emitter (attraction: in case of sex- or aggregation-pheromones; e.g. Shimizu et al. 2001) or moving away from the emitter (repellence: in cases of alarm-pheromones; e.g. Raspotnig 2006). Hence, it seems that a first functional classification of oil gland compounds (neutral, attractive, repellent) could be easily investigated in experimental bioassays. Such bioassays include a test arena (or olfactometer), a source emitting semiochemicals to be tested and test specimens from which the reaction upon the candidate semiochemicals is recorded. Bioassays should be properly replicated to enable the application of inferential statistics. Improper replication (=pseudoreplication) occurs when treatments are spatially or temporally segregated, replicates of a treatment are somehow interconnected, or replicates are only samples from a single experimental unit (e.g. a group of specimens; Hurlbert 1984). Pseudoreplication has been a big issue in field ecological studies and occurred in 27% (48% if inferential statistics were used) of publications between 1960 and 1984 (Hurlbert 1984), but is not only an elapsed topic of ecological studies: Lazic (2010) analyzed a single issue of *Nature Neuroscience* (one of the leading journals in this field with a 2010 ISI impact factor of 14.2) and found that 12–48% of the statistical analyses were based on pseudoreplication. Concerning our contribution here, Ramirez et al. (2000) analyzed the occurrence of pseudoreplication in experimental designs of olfactometric laboratory studies and demonstrated that almost half (46.7%) of the evaluated studies suffered from some dependency of replicates. Pseudoreplication cannot always be circumvented with an acceptable effort, but if included in the experimental design, inferential statistics have to be avoided (Hurlbert 1984). On the contrary, Oksanen (2001) stated that 'whether the experiment is replicated or not, inferential statistics should always be used to enable the reader to judge how well the apparent patterns in samples reflect real patterns in statistical populations'. He further argues that, as long as the signal in the data is high compared to the noise of the background, pseudoreplication may also lead to clear-cut results. Whether conclusions made from such experiments reflect the 'truth' cannot generally be decided by just identifying dependency among the replicates. However, we think it is important to clearly state in a publication what the 'experimental unit' (Hurlbert 1984) was. We reviewed available literature on oil gland secretions that used bioassays with oribatid and astigmatid mites with respect to experimental design and found that most of the experiments were either not replicated at all or were based on pseudoreplication (without mentioning the lack of independency in the data).

Another important part of the experimental design of olfactometric bioassays is the source for scent dispersal. Bossert & Wilson (1963) considered four general cases of diffusion in chemical communication: i) instantaneous release in still air, ii) continuous release in still air, iii) continuous release from a moving source, and iv) continuous release in wind. Cases i and ii are probably the most common among oribatid and astigmatid mites. All available studies on the chemical communication in astigmatid and oribatid mites used impregnated paper as source material and implicitly assumed continuous releases (case ii) of the applied semiochemicals,

but this has never been tested. Here, we investigated the evaporation dynamics of multi-component-secretions from paper and show that this material instead widely resembles the 'instantaneous' (case i) release. Furthermore, different compounds evaporated from the paper with different rates leading to a dramatic change in relative composition. We modified the source by adding clay particles to the paper and show that this combination provides another kind of diffusion (i.e. case ii). Not only are the compounds released much slower from the paper/clay combination than from the paper alone, the relative composition remained quite stable for the time frame investigated.

While we assume that most conclusions on pheromonal functions made from the existing studies with oribatid and astigmatid mites are based on so strong evidence that the experimental problems regarding the source and statistical error arising from designs with pseudoreplications do probably not affect the general findings (see Oksanen 2001), we also think that it would be helpful to discuss where existing experiments may have had general shortcomings.

2. Materials and Methods

Literature-survey: Studies were excluded if they used identical (or nearly identical) bioassays from earlier studies that were already included in the survey to avoid inclusion of 'inherited' errors. This, however, also means that the true number of publications with experimental shortcomings is higher than presented here. The following remaining eleven papers were included (in alphabetical order of the first author): Hiraoka et al. (2003), Kuwahara et al. (1975, 1980), Mizoguchi et al. (2003), Mori et al. (1996), Nishimura et al. (2002), Raspotnig (2006), Shimano et al. (2002), Shimizu et al. (2001), Tatami et al. (2001), Tomita et al. (2003). Publications were screened for i) type of arena and experimental procedure including the occurrence of pseudoreplication (e.g. arena was used more than once, animals were used more than once, groups of animals were used and treated as replicates), and ii) type of source used for presenting semiochemicals.

Animals: Adult specimens were taken from the laboratory strain *Archegozetes longisetosus* ran (Acari, Oribatida; Heethoff et al. 2007). Oil gland secretions of *A. longisetosus* consist of eleven compounds: 2,6-HMBD (= 2-hydroxy-6-methyl-benzaldehyde), neral, geranial, neryl formate, γ-acaridial (= 3-hydroxybenzene-1,2-dicarbaldehyde), tridecane, pentadecene, pentadecane, heptadecadiene, heptadecene and heptadecane (Heethoff & Raspotnig 2011). An approximation of absolute amounts of oil gland secretions (~320 ng) for individuals of this strain is given in Raspotnig & Föttinger (2008) and is also used here for quantification purposes.

Large-scale extraction (LSE) of oil gland secretions: Three hundred adult specimens were carefully collected from the culture with a fine brush and extracted in 600 µl hexane for 5 minutes. All collected specimens had fed (visible food boli) and were actively moving.

Source-materials: White paper (80 g/m^2, cut in 4*4 mm pieces) and clay granula (0.5–1 mm particle size, SERAMIS) were impregnated with the equivalent of secretions from one oil gland (1 µl from the LSE described above corresponding to ~160 ng of secretion). Clay granulate has previously been fractionated by a sieving-array and was washed and stored in hexane. New pieces of paper and clay particles were used for every measurement.

Experimental procedure: Impregnated paper/clay were exposed to still air in petri-dishes with 55 mm diameter and 14 mm height at 23°C (new dishes were used for every application), covered by a lid immediately after application of secretions to the paper/clay. As a control, 1 µl of LSE was diluted in 50 µl hexane and quantified as described below. Impregnated pieces

of paper were extracted for 5 min in 50 µl hexane at 0, 30, 60, 90, 120, 300 and 600 sec after application of LSE. Combinations of paper and each one piece of clay particles were extracted at 0, 120 and 300 sec after application of LSE. Each treatment (control, paper alone at different times, paper/clay at different times) was replicated three times.

GC/MS and quantification*:* A trace gas chromatograph (GC) coupled to a DSQ I mass spectrometer (MS; both from Thermo, Vienna, Austria) and equipped with a ZB-5MS fused silica capillary column (30 m length, 0.25 mm diameter, 0.25 µm film thickness, Phenomenex, Germany) was used for the analyses. Amounts of secretions were calculated based on the integration of peak areas in the chromatograms relative to a constant amount of an internal standard (5 ng of 6-methyl-5-hepten-2-one), and thus expressed in % peak area of this standard or in % peak area of the secretion (Heethoff & Raspotnig 2011). Absolute quantification was based on the data given in Raspotnig & Föttinger (2008).

Statistics: Data (amounts of secretions) were tested for normal distribution by a Kolmogorov-Smirnov-test and subsequently tested for significant differences by analyses of variance (ANOVA); homogeneous groups were identified by a post-hoc Tukey-HSD test. All statistical analyses were calculated in SPSS 20 (IBM 2011).

3. Results and discussion

Part I: Literature survey: In the following section we will describe the arenas and sources used in olfactometric bioassays on oribatid and astigmatid mites and evaluate experimental designs regarding pseudoreplication. To our knowledge, the eleven publications included here cover all of the published principal experimental setups investigating chemical communication in oribatid and astigmatid mites. Remaining studies not mentioned here used similar procedures.

Circular arenas such as glass plates or Petri dishes with diameters ranging from 7 mm to 13 cm were used in nine of the studies (Kuwahara et al. 1975, Mori et al. 1996, Tatami et al. 2001, Shimizu et al. 2001, Shimano et al. 2002, Hiraoka et al. 2003, Tomita et al. 2003, Mizoguchi et al. 2003, Raspotnig 2006). Kuwahara et al. (1980) used glass cover slips (23*36 mm) and Nishimura et al. (2002) used Pasteur pipets with a gradated paper inside on which specimens were placed. These Pasteur pipets represent more or less one-dimensional arenas in which tested specimens could move either to or away from the scent-emitting source. All other studies used two-dimensional schemes where mites either moved out of a circle away from the source or towards the scent source in a two-dimensional space. All studies used impregnated (filter) paper that was placed inside the arenas as source for scent dispersal except for Nishimura et al. (2002) who placed the paper in an eyedropper, mounted it to a pipet and expressed it once to send the scent through the pipet. None of the studies explicitly used new (or at least cleaned) arenas for each measurement.

Single specimens were used only by Tomita et al. (2003) and to some extent by Raspotnig (2006). Other bioassays were performed with groups ranging from five (Tatami et al. 2001) to 100,000 (Kuwahara et al. 1975) specimens.

Seven (64%) of the studies did not allow the application of inferential statistics: three studies (Kuwahara et al. 1975, 1980; Shimano et al. 2002) did not use any kind of replication and four studies (Nishimura et al. 2002, Hiraoka et al. 2003, Tomita et al. 2003, Raspotnig 2006) included pseudoreplicated data without mentioning the dependency. Of these, only Raspotnig (2006) did not apply inferential statistics. The remaining four studies (36%) did not provide sufficient information to attest proper replication on all experimental levels and thus are at least suspected to contain pseudoreplication. These studies (Mori et al. 1996, Tatami

et al. 2001, Shimizu et al. 2001, Hiraoka et al. 2003) 'repeated' instead of 'replicated' the measurements. It cannot be decided with certainty what 'repeated' means in this context - 'repeat' in the context of statistical terminology is different from 'replicate' and would mean that the same arena with the same specimens was used multiple times und thus needs to be treated as pseudoreplication.

To conclude, 36–72% of all bioassays included pseudoreplication and the remaining studies did not replicate at all. This alarming result brings us to the point where we like to make some suggestions for future experimental designs that will allow proper application of inferential statistics.

First of all, we strongly suggest using single specimens in bioassays with sufficient replications. For each replicate, a new (or at least cleaned) arena, a fresh specimen and a new source should be used - all of these should be discarded after the measurement. If only a limited number of arenas or specimens is available, we suggest using a randomized application of treatments to those units that are used in more than one trial. Using fresh materials 'ensures' that replications are independent and thus can be used for inferential statistics (e.g. Hurlbert 1984, Ramirez et al. 2000). If more specimens are used in each trial, these have to be treated as one unit (=one point in the data matrix). Another strong argument for using single specimens instead of groups is that any influence (e.g. positive or negative behavioral feedback) individuals may have on each other is excluded. Whatever experimental design is used, it should be clearly stated in the Materials & Methods, so that it becomes clear to the reader what exactly has been done in the study. Following Lazic (2010) we also strongly suggest to explicitly report the sample size, degrees of freedom, used test statistic, and p-values so that the reader can judge on his own about the strength of the results (see also Oksanen 2001).

Most studies used circular arenas to discriminate mainly between attraction or repellency of oil gland secretions or their compounds (there are some exceptions regarding sex-pheromones and their influence on the behavior, but these are subsequent mounting experiments not covered here). We think that this initial one-dimensional response (moving towards or away from the source) could be better investigated in more or less one-dimensional arenas. The Pasteur pipet used by Nishimura et al. (2002) presents a pretty good concept, although the mechanism of scent disposal (by a single 'puff') represents another type of dispersal compared to all other sources used (something like 'instantaneous release in wind', not covered by Bossert & Wilson 1963). Hence, we suggest another possible design for an arena that is less intricate and cheaper (each complete bioassay costs only about 0.15 €), but quite similar in its general setup: plastic boxes with lids (80*7*7 mm, produced by e.g. Licefa, www.licefa.com) could be used as disposable arenas. The bottom can be covered by a piece of paper with a gradation (mm-scale) and a point printed in the middle marks the starting position for the test specimen (these can be kept at the starting position by some food supply or by a small ring). The source is applied at one end of the arena, (the ring is removed), the lid is closed and the position of the test specimen is recorded (mm moved towards or away from the source) after a certain time. The whole arena is discarded and a new one is used for the next measurement. Our suggestions avoid pseudoreplication by using single specimens and fresh (or at least randomized) bioassays for every measurement and thus allow data to be analyzed with inferential statistics.

Part II: Sources for scent dispersal in bioassays: All studies reported in the literature survey above used paper as source of sent dispersal in the bioassays. The paper was impregnated with variable amounts of single fractions or compounds or with raw extracts of whole secretions from one or several specimens (e.g. Mori et al. 1996, Nishimura at al. 2002, Tomita et al. 2003). In some cases, several minutes were interposed between impregnation of the paper and usage in

the bioassay (Nishimura et al. 2002, Tomita et al. 2003). This implies that probably the source changed with respect to the number of applied molecules in the time between application and presentation in the bioassay. When using complex multi-component secretions, differences in volatility of components probably lead to a shift in relative composition of the secretion after some time (some components evaporate fast, others slow). Although such dynamics can lead to unpredictable experimental situations (e.g. some compounds may act as aggregation- or alarm-pheromones, depending on the dose, Nishimura et al. 2002), this topic has never been studied to our knowledge. Hence, as an example, we analyzed the temporal dynamics of the multi-compound-secretions from *A. longisetosus* applied to pieces of paper and clay granula.

Quantification of the control (1 µl of LSE) resulted in an average of 402% of the standard MHO (= 160 ng, Raspotnig & Föttinger 2008). The pieces of paper immediately absorbed about the half (204% / 81 ng, which we use here as the 0 sec-reference for this time course). About half of the absorbed secretions had evaporated already after 30 sec (52% / 43 ng remaining, see Tab. 1, Fig. 1). After 1 min, 29% of the 0 sec-amount (23 ng) remained in the paper, this further dropped down to 19% of 0 sec (15 ng) after 2 min and to 12% (10 ng) after 4 min. After 10 min, 96% of secretions had evaporated (only 4% / 3 ng remained in the impregnated paper). Data were normally distributed (K-S-test, $p = 0.063$) and ANOVA resulted in significant differences ($F_{7,17} = 49$, $p < 0.001$, Fig. 1).

When adding a piece of clay granule to the piece of paper, the initial absorbing capacity was increased to 93% (373% of standard, 148 ng, 0 sec-reference for this experiment, Tab. 1, Fig. 2). After 2 min, 63% of 0 sec (93 ng) remained in the paper/clay combination and after 5 min still 49% (72 ng) of the initially absorbed secretion could be re-extracted. Data were normally distributed (K-S-test, $p = 0.964$) and ANOVA resulted in significant differences ($F_{3,9} = 16$, $p = 0.001$). The evaporation rate between paper alone and the paper/clay combination strongly differed: After 2 min already 81% of the absorbed secretion were emitted from the paper, but only 27% from the paper/clay combination. Additionally, in contrast to the paper alone, no significant differences were found between the control and the 0 sec-reference-point. This indicates that the initial amount of secretions presented as source in a bioassay would closely resemble the amount of secretions used for impregnation of the source only when a combination of paper and clay is used.

Tab. 1 Amounts (3 replicates) of oil gland secretions (dissolved in hexane) remaining in paper and paper/clay combinations after exposition to still air for certain times (sec).

	time	amount of secretion (% of standard)
Paper	control	350, 474, 383
	0	271, 192, 148
	30	135, 99, 87
	60	53, 60, 63
	90	47, 43, 44
	120	41, 40, 34
	300	23, 27, 24
	600	7, 9, 7
Paper / clay	0	382, 326, 410
	120	269, 247, 187
	300	191, 149, 206

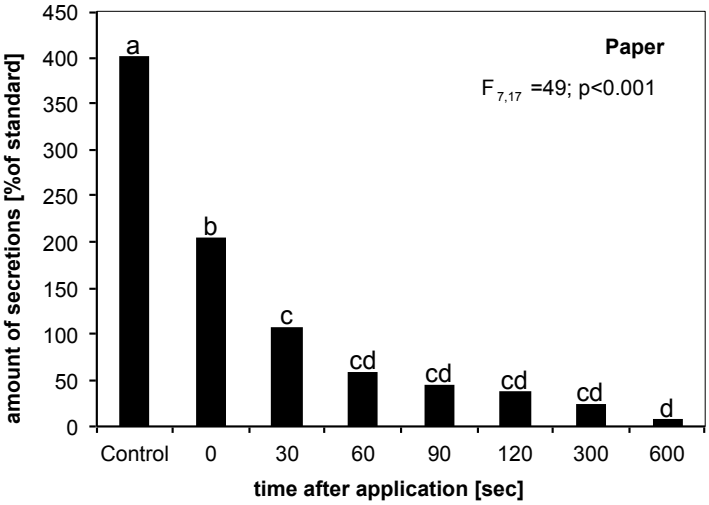

Fig. 1 Comparison of absolute amounts of secretions (% of standard, average of three replicates, see Tab. 1) at certain times (sec) after application on paper. Letters indicate homogeneous groups (Tukey-HSD).

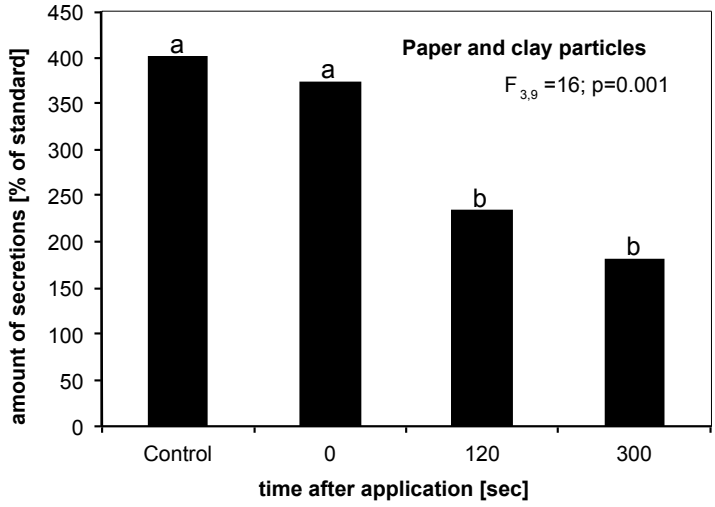

Fig. 2 Comparison of absolute amounts of secretions (% of standard, average of three replicates, see Tab. 1), certain times (sec) after application on a combination of paper and a clay particle. Letters indicate homogeneous groups (Tukey-HSD).

Compounds were analyzed regarding their evaporation-behavior in detail if they contributed with at least 5% to the whole secretion in any measurement. Like this, 7 of the 11 compounds were included in the following analyses (Tab. 2, Figs 3, 4). Some compounds evaporated faster than others from the paper: about half of the γ-acaridial, neryl formate and neral were already gone after 30 sec; 2,6-HMBD even dropped to less than 20% of the initial amount and was completely absent after 60 sec. The hydrocarbons, on the other hand, evaporated

comparably slow and after 5 min, almost no heptadecene had evaporated, about 60% of pentadecane remained in the paper and also 50% of pentadecene (Fig. 3). These differences in volatility of the compounds led to a dramatic change in the dynamics of the relative composition (Fig. 3). While γ-acaridial remained the main compound for the first 2 min, neryl formate was replaced by pentadecane as the second-most compound already after 60 sec and then by heptadecene after 2 min. After 10 min, the secretion in the filter paper consisted solely of pentadecane and heptadecene.

Tab. 2 Absolute and relative amounts of single compounds remaining in paper and paper/clay combinations after exposition to still air for certain times (sec). Three replicates, given in % of standard (abs) and % of secretion (rel). **A**: γ-acaridial; **B**: neryl formate; **C**: 2,6-HMBD; **D**: neral; **E**: pentadecane; **F**: pentadecene; **G**: heptadecene.

		A		**B**		**C**		**D**		**E**		**F**		**G**	
	time	abs	rel	abs	rel	abs	rel	abs	rel	abs	rel	abs	rel	abs	rel
paper	0	106	39	77	28	28	10	15	5	17	6	5	2	9	3
		77	40	53	28	18	10	10	5	13	7	4	2	6	3
		59	40	40	27	14	10	7	5	11	7	3	2	6	4
	30	52	39	27	20	5	4	6	5	14	11	8	6	11	8
		38	39	22	22	3	3	5	5	13	13	5	5	7	7
		34	39	19	22	3	3	4	5	12	13	3	3	6	7
	60	18	34	9	17	0	0	3	5	11	21	2	4	6	11
		22	37	11	18	0	0	3	5	11	19	2	4	7	11
		23	37	11	17	0	0	3	5	12	19	3	4	7	12
	90	15	32	7	16	0	0	2	4	11	24	3	6	6	13
		15	35	8	18	0	0	2	4	9	21	2	4	5	12
		16	35	6	13	0	0	2	4	11	24	2	4	7	15
	120	15	37	5	12	0	0	0	0	11	27	2	5	8	19
		14	36	5	12	0	0	0	0	11	28	2	6	7	19
		10	28	3	9	0	0	0	0	11	32	3	9	8	22
	300	6	25	1	5	0	0	0	0	8	35	2	8	6	28
		8	30	2	6	0	0	0	0	9	33	2	6	7	25
		7	20	1	6	0	0	0	0	8	32	2	9	6	25
	600	0	0	0	0	0	0	0	0	4	50	0	0	4	50
		0	0	0	0	0	0	0	0	4	50	0	0	4	50
		0	0	0	0	0	0	0	0	3	43	0	0	4	57
paper / clay	0	132	35	83	22	42	11	30	8	28	7	9	3	21	5
		115	35	70	22	34	10	25	8	26	8	9	3	19	6
		139	34	68	17	45	11	33	8	31	8	10	3	24	6
	120	94	35	56	21	26	10	20	8	25	9	8	3	17	6
		84	34	56	23	24	10	20	8	21	8	7	3	15	6
		71	38	33	18	16	9	11	6	19	10	6	3	13	7
	300	75	39	27	14	16	9	9	5	24	12	7	4	17	9
		59	39	21	14	11	7	7	5	19	13	6	4	13	9
		75	36	40	19	18	9	14	7	20	10	6	3	15	7

This changed when a clay particle was added to the piece of paper (Tab. 2, Fig. 4) and both were impregnated together. The absolute amount of remaining secretion was also reduced over time by evaporation, but much slower when compared to the paper alone. After 2 min, 64% of γ-acaridial remained in the source, which is four times more than with the paper alone. Regarding neryl formate, the remaining amount was more than nine times higher after 2 min. 2,6-HMBD, which had evaporated completely from the paper already after 60 sec, was reduced in the paper/clay combination after 2 min by less than 50%. Even more important than the general slower evaporation processes were the differences in relative composition dynamics when a paper/clay combination was used (Fig. 4). After 2 min, γ-acaridial still represented the main component, followed by neryl formate and 2,6-HMBD reflecting the

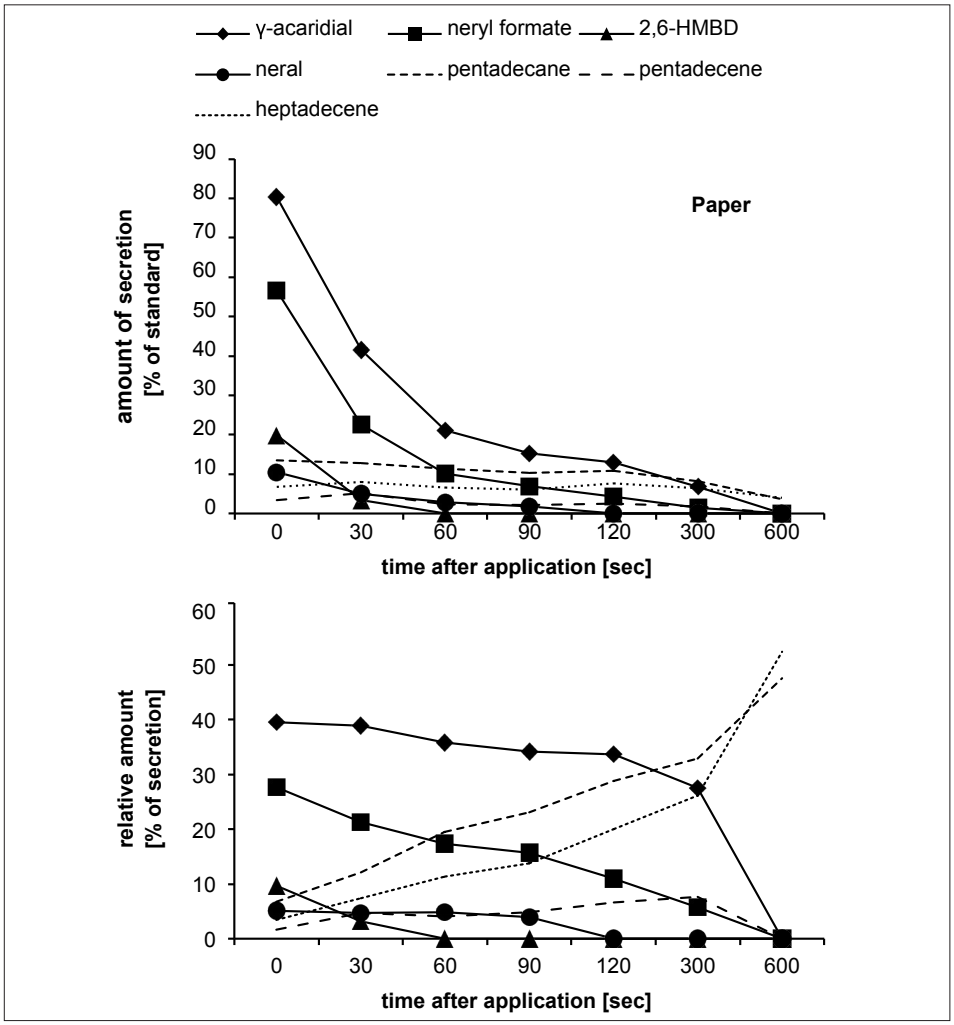

Fig. 3 Temporal dynamics of compounds in a multi-component-secretion after application on paper (averages from each three replicates, values are given in Tab. 2).

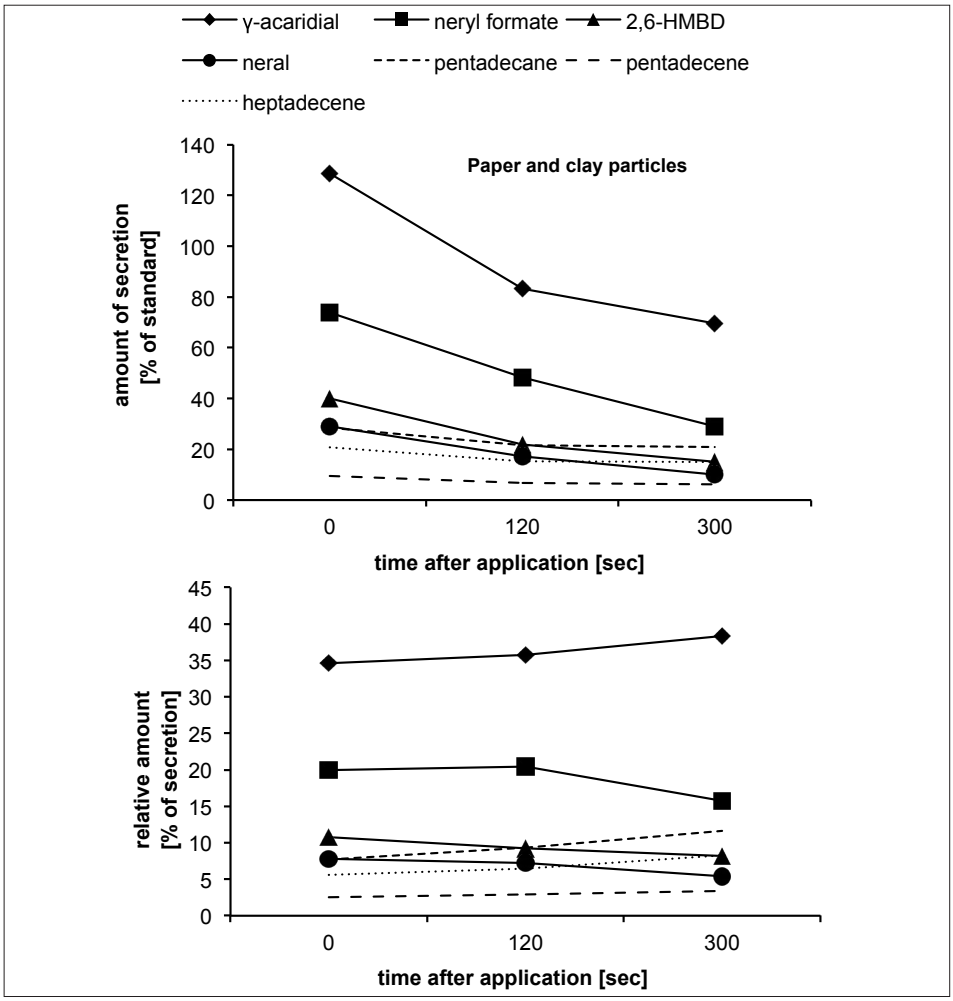

Fig. 4 Temporal dynamics of compounds in a multi-component-secretion after application on a combination of paper and a clay particle (averages from each three replicates, values are given in Tab. 2).

starting conditions. The hydrocarbons (pentadecane and heptadecene) slightly shifted upwards in relative amounts during the time course, but this was much less pronounced when compared to the paper alone (Figs 3, 4). Regarding the relative amounts of neryl formate, 2,6-HMBD and neral, which have been shown to be pheromones in some oribatid and astigmatid mites (Kuwahara 2004, Raspotnig 2006), their relative contribution to the secretion remained relatively stable for at least 5 min.

What does this mean for the interpretation of results from the above-mentioned former studies using filter paper alone as source in bioassays? None of the studies using raw extracts with multiple components made any quantification purposes but used the raw extract only to i) demonstrate its general pheromonal activity (e.g. Tomita et al. 2003) and/or ii) determine

the active fraction separated with SiO_2 column in varying ether/hexane concentrations (e.g. Mizoguchi et al. 2003). However, Tomita et al. (2003) waited 'a few minutes' before the impregnated filter paper with the raw extracts of three females (containing 0.5 ng of neryl formate) was presented in the bioassay and they determined neryl formate as the alarm pheromone. The amount of neryl formate in our study had dropped by 93% (from 23 ng to 1.6 ng) after 2 min and by 98% (to 0.4 ng) after 5 min. Hence, we assume that after waiting 'a few minutes' probably almost no neryl formate had been left in the filter paper in the bioassay of Tomita et al. (2003). Tatami et al. (2001) identified 2,6-HMBD as a female sex pheromone and studied amounts of 5–50 ng of the substance (provided on filter paper) for 10 minutes. We have shown that 2,6-HMBD had completely evaporated from the paper in less than a minute (the starting amount of 8 ng was reduced to 1.5 ng after 30 sec and was completely gone at 60 sec). Hence, at least the dose-dependent quantitative conclusions of Tatami et al. (2001) probably need some reconsideration. Neral has been considered pheromonally active (dose-dependent as alarm- and aggregation-pheromone, Kuwahara et al. 2001, Nishimura et al. 2002): alarm function was demonstrated at doses between 10 and 100 ng (Kuwahara et al. 2001) and aggregation at doses up to 3 ng. In our experiment, more than 50% of neral evaporated from the paper in the first 30 sec (from 4 ng to 2 ng) and all neral had evaporated after 2 min. Hence, at least the exact doses in which neral is active as alarm- or aggregation-pheromone need to be reconsidered (Kuwahara et al. 2001, Nishimura et al. 2002).

In terms of the general cases of diffusion in chemical communication (Bossert & Wilson 1963) we think that the two sources (filter paper alone and filter paper/clay combination) represent two different principles. The paper alone probably closely resembles the 'instantaneous release' (case i), where all of the pheromone is released at once (or during a short time) while the filter paper/clay combination can be seen more as a 'continuous release' (case ii) of pheromones. This implies that bioassays using filter paper alone should focus on immediate changes in behavior of the receiver while bioassays with combined (paper/clay) sources should be used to observe behavior over a time of several minutes.

The results shown here are based on white copy-paper (80 g/m^2) because this paper can be used to print the arena gradation and mark points directly on it with a laser printer. We also made some measurements with impregnated filter paper (data not shown) because this paper has probably a more fibrous structure than copy-paper (and it was used in other bioassays), but the results were similar and evaporation of compounds was only slightly slower when compared to the printer paper.

4. Conclusions

The principles of chemical communication in oribatid and astigmatid mites are far from being unraveled and, as we demonstrated here, many of the available data have to be treated with some caution with respect to bioassay setup, statistical significance and given thresholds inducing first behavioral responses to certain chemical stimuli. Our suggestions given for the bioassay and experimental design are to be seen as a first step and a thought-provoking impulse for further improvement. A tremendous intensification of research will be necessary to promote our understanding of the biological role of complex multi-component oil gland secretions in chemical communication of oribatid and astigmatid mites.

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