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Maternal food provisioning in relation to condition-dependent offspring odours in burrower bugs (*Sehirus cinctus*)

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The sensory modalities used for communication among family members have at least partly evolved within an organism's pre-existing sensory context. Given the well-known general importance of chemical communication in insects, we hypothesized in sub-social insects with parental care that chemical signals emitted by larvae to influence parental care (i.e. solicitation pheromones) would have evolved. To test this hypothesis, we performed an experiment in the burrower bug *Sehirus cinctus* (Hemiptera: Cydnidae) where nymphs were hand-reared under high- or low-food conditions. These hand-reared clutches were used as a source of volatiles. The volatiles were collected for chemical analysis and delivered to caring mothers to quantify their behavioural response. As predicted, mothers exposed to volatiles from nymphs in poor condition provisioned significantly more food than those exposed to air (controls) or volatiles from high-condition nymphs. Chemical analysis revealed that nymphs emitted a blend of eight compounds of which α-pinene and camphene showed the strongest relationship with food treatment. Exposure to pure synthetic α-pinene and camphene did not affect maternal provisioning, however, suggesting that the functional significance of α-pinene and/or camphene may occur in a blend with other compounds. This study shows a clear effect of condition-dependent offspring odours on maternal food provisioning and identifies, for the first time, candidate compounds for a potential chemical offspring begging signal.

Keywords: parental care; begging; family conflicts; chemical communication; *Sehirus cinctus*

1. INTRODUCTION

The evolution of family interactions and the sensory modalities used for communication among family members is at least partly shaped by the pre-existing sensory context of an organism. Parent–offspring conflict, sibling rivalry (Trivers 1974; Godfray 1995; Mock & Parker 1997) and the coadaptation of parental provisioning and offspring demand (Kölliker *et al.* 2005*a*) are expected to drive the evolution of signals within sensory modalities, but across potential modalities pre-existing sensory capacities of parents (i.e. adults) provide the environment for the initial evolution of offspring signalling traits.

For instance, birds are well known for their visual and vocal capacities in general (Bradbury & Vehrencamp 1998) and, correspondingly, the begging behaviour of nestling birds is a highly visual and acoustic display (Kilner & Johnstone 1997; Mock & Parker 1997; Budden & Wright 2001; Wright & Leonard 2002). On the other hand, insects are legendary for the importance of chemical communication in most, if not all, aspects of their life history (Baker & Longhurst 1981; Eisner & Meinwald 1995; Blomqvist & Vogt 2003; Wyatt 2003). This chemosensory pre-disposition of adult insects is, in species with post-zygotic parental care (Tallamy 1984), a social selective environment (West-Eberhard 1983; Wolf *et al.* 1999) for the evolution of solicitation traits in offspring. As a consequence, the evolution of chemical signals displaying the need (Godfray 1991) or demand (Parker *et al.* 2002*a*) for parental care may be expected in such insect species. Depending on the importance of other sensory channels for gathering information about their environments, tactile, visual and acoustic display components may also play a role.

In a recent paper, we postulated the existence of a 'solicitation pheromone' in the burrower bug *Sehirus cinctus* (Kölliker *et al.* 2005*b*), a hemipteran with maternal care including the provisioning of food (i.e. *Lamium purpureum* nutlets) to hatched nymphs through second instar (Sites & McPherson 1982; Kight 1997; Agrawal *et al.* 2001). The hypothesis was tested by exposing burrower bug mothers to crude cuticular extracts from nymphs (obtained from hexane washes) hand-reared under low- or high-food conditions. Consistent with the hypothesis, assay mothers exposed to extracts from low-food nymphs provisioned more food than assay mothers exposed to extracts from high-food nymphs. Contrary to the hypothesis, however, nymph extracts had an overall inhibitory effect on maternal food provisioning, suggesting

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that the chemical communication between *S. cinctus* nymphs and their mothers was more complex than a simple linear provisioning stimulating solicitation pheromone (Kölliker *et al.* 2005*b*).

To further explore the effects of chemical signals on maternal behaviour, and to allow for the identification and quantification of specific chemical compounds, we examined volatiles emitted by nymphs. To test whether chemical solicitation influenced parent–offspring interactions, we used a combination of experimental manipulation of nymph condition, bioassay of the effects of volatiles on maternal behaviour, collection and chemical analysis of compounds, and exposure to synthetic compounds. We built an experimental apparatus that allowed us to simultaneously collect the volatiles emitted by the nymphs and expose assay mothers to these volatiles. Under our hypothesis of a volatile 'solicitation pheromone', we predicted that (i) assay mothers should provision more nutlets when exposed to odours of low-condition nymphs than when exposed to odours of high-condition nymphs, (ii) the quantity of particular chemical compounds would be related to the condition of nymphs and (iii) exposure to synthetics of the condition-dependent compounds would affect maternal provisioning.

2. MATERIAL AND METHODS

(a) *General methods*

Female burrower bugs were collected from agricultural fields containing the mint *L. purpureum* in Bloomington, IN, between 15 and 21 April, 2004. We transferred the bugs to the laboratory where we set them up individually in petri dishes (polystyrene, 100×15 mm) containing moist sand. We used the published protocols for maintenance and handling of burrower bugs in the lab (Agrawal *et al.* 2001; Kölliker *et al.* 2005*b*). Experimental families were set-up one day after initiation of hatching. Two groups of experimental families were set-up: 'biosource clutches' that would serve as the odour source, and 'assay clutches' that would be exposed to the odours produced by the biosource clutches.

Newly hatched clutches were randomly assigned as biosource or assay clutches. Biosource clutches were divided into two sets of 30 nymphs; each set was placed in a new dish (polystyrene, 100×15 mm) with sand and a small PVC shelter. Because of the possibility that nymphs may need to behaviourally interact with a tending female to release the hypothesized solicitation pheromone, one set of nymphs was set-up with the mother, while the other set of nymphs from the same family was reared without a mother. Pairs of biosource clutches (i.e. with mother and without mother) were hand-provisioned with *L. purpureum* nutlets according to either a high-food or low-food protocol. The quantity of mint nutlets provided daily in the high-food treatment was taken as the seventy-fifth percentile of the per-day distribution of the provisioning rate observed from bug mothers (Kölliker *et al.* 2005*b*). The low-food treatment corresponded to the tenth percentile. We placed the nutlets in vicinity of the clutch. To compensate for the presence of mothers for the biosource clutches containing the mother (i.e. mothers may feed on the nutlets, too), we added one extra seed in the centre of the dish. Nutlets that became mouldy were removed daily. We used the biosource clutches for the odour collection/exposure experiments (see below) on the day after initiation of moult to second instar (at age 5–7 days). Nymphs from the same clutch usually moult simultaneously (within 12–24 h of each other).

Assay families consisting of 50 nymphs and their mother were set-up at hatching in larger petri dishes (polystyrene, 150×15 mm), serving as the arena in which food provisioning would be quantified. The arena consisted of a small PVC shelter and three bottle caps (each containing six mint nutlets) at the three, six and nine o'clock direction as food sources. The basic set-up corresponded to that used in previous studies on maternal food provisioning in this species (Agrawal *et al.* 2001, 2005; Kölliker *et al.* 2005*b*). To keep the condition of the assay nymphs as standardized as possible, we limited food supply available to the mother for provisioning by giving her three bottle caps containing six nutlets daily (as opposed to the nine nutlets or more per cap used in earlier studies; Agrawal *et al.* 2001), and removed all except three of the previously provisioned nutlets daily. On the second day after nymphs began moult to second instar (age 6–8 days), the assay clutches were used in the odour exposure experiments (see below).

(b) *Collection of and exposure to nymph odours*

Because maternal provisioning in *S. cinctus* occurs at a relatively low and highly variable rate (mean (± s.d.) over 24 h, 12.1 (±11.8) nutlets; Kölliker *et al.* 2005*b*), and due to the expected low quantities of odours produced by nymphs per unit time, we designed an apparatus that allowed us to simultaneously collect odours from a biosource clutch and expose assay mothers to these odours over long time-intervals (see electronic supplementary material). The apparatus and its properties are also described in detail elsewhere (Chuckalovcak *et al.* submitted). Air blown at a flow rate of 1.25 l min^{-1} over nymphs from the low- or high-food treatment, respectively, was partly (i.e. approximately. 0.60 l min^{-1}) pulled by a vacuum pump through a volatile collection trap (VTC) packed with 30 mg of the SuperQ polymer. The remaining 0.65 l min^{-1} of the air was passively blown into the assay arena where mothers were exposed to the air.

On each test day, an assay mother experienced two trials, one in which she was exposed to odours from a biosource clutch reared with a tending mother and one in which she was exposed to the related biosource clutch reared without a tending mother. Biosource clutches were chilled on ice for approximately 1 h to immobilize the nymphs and allow us to transfer them to the biosource chamber. Only the nymphs from biosource clutches (i.e. not the mothers) were placed in the biosource chamber. The order of exposure was randomized between the two experimental sessions. Each assay mother was tested on only a single day.

Before each session, we held the assay clutches for approximately 30 min at 4 °C. The assay mothers and clutches were then placed back under the shelter, if required. The apparatus blew air directly under the shelter through a hole in the sidewall of the petri dish. Each exposure experiment lasted 3 h and the provisioning rate of each assay mother was quantified over the two daily sessions (i.e. biosource clutch reared with and without mother) and a total of 6 h.

The presence of mothers during rearing of a biosource clutch did not significantly affect the provisioning rate of assay mothers (Wilcoxon signed-ranks test, $n=38$, $S=39.5$, $p=0.351$), so we pooled the data for each assay-mother to obtain more robust provisioning data over the full exposure duration of 6 h.

(c) *Chemical analysis*

After the experiments, we eluted the VCTs with 200 µl methylene chloride (CH_2Cl_2; EMD Chemicals, Inc., Norwood, OH, USA), captured the elution in autosampler vials (12×35 mm, Fisherbrand), and stored the samples at −80 °C until chemical analysis using gas chromatography–mass spectrometry (GC–MS; see electronic supplementary material for more detail). The peaks on the GC-traces that corresponded to nymphal compounds (figure 2) were identified by comparing the peaks from nymph collections to those from control collections. Peaks that were absent in control collections were assumed to be produced by nymphs. Identifications focused on monoterpenes, because they were the principal constituents that distinguished treatment and control collections. The chemical identity of each peak was then inferred by matching the obtained mass spectra to a reference library (NIST98), and retention times with purchased standards. An initial randomly selected 50% of the collected samples were run on a 30 m DB-WAX column, and a second half of the samples on a 60 m DB-WAX column. One of the compounds (α-pinene) was missed on the 30 m column since it eluted from the column before termination of the 5 min solvent delay. Thus, data on α-pinene are available for the second random half of the samples only. The peak of another compound (terpenolene) overlapped with the peak of a contaminant on the 60 m column and could not be accurately quantified. Therefore, only the first random half of the collected samples could be used for quantification of terpenolene.

We calculated the quantity (in nanograms) of the chemical compounds as the ratio of the compound's area under the GC-peak over peak area from an added internal standard (Undecane; $C_{11}H_{24}$; see electronic supplementary material). Multiplying this ratio by 5 ng (i.e. the quantity of internal standard) yields the absolute quantity in nanograms.

(d) *Synthetic compound exposure experiments*

Candidate compounds were identified as those that covaried with the food treatment in the odour collection experiments. The conditions used in the synthetic exposure experiment were identical to the ones in the above experiments, except that the experiments were run for 6 h without interruption. The described apparatus (electronic supplementary material) was modified to allow insertion of synthetic compound probes (purchased from Sigma-Aldrich, Milwaukee, WI, USA). Air was blown from a compressed air tank (highly purified air) over the probes at a flow of 0.65 l min^{-1} into the assay arena where provisioning of assay mothers was quantified. The synthetics (diluted in the solvent hexane, 99% pure; Sigma-Aldrich, St Louis, MO, USA) were applied on pieces of filterpaper and quantities were chosen to mimic as closely as possible the quantities collected in the above experiments. The control treatment consisted of the solvent hexane applied to the filter paper. See the electronic supplementary material for more details.

(e) *Statistical analysis*

The distribution of provisioning data in *S. cinctus* is typically skewed positively (Kölliker *et al.* 2005*b*), and we used Poisson-regression (log-linear) models (proc GENMOD; SAS 1999) incorporating planned contrasts to test for both an overall effect of exposure treatment on maternal provisioning and differences in provisioning rate among individual treatments (Kölliker *et al.* 2005*b*). Means and standard errors for provisioning were calculated on the logarithmic scale and transformed back to the linear scale for illustrative purposes. Similarly, compound quantities were square root transformed before analysis to achieve approximate normality. Means and measures of variability were calculated on the square root scale and transformed back to the linear scale for representation in figures.

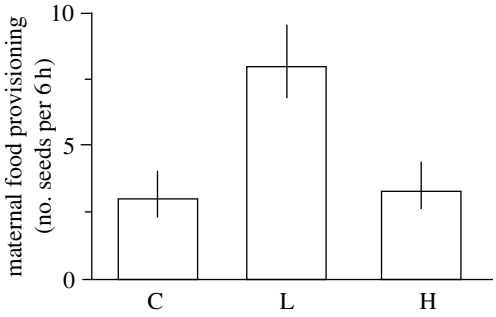

Figure 1. Odour mediated effect of nymph condition on maternal provisioning. Shown is the mean (+s.e.m) number of mint nutlets provisioned over the sum of two 3 h experimental sessions on the same day. C, Control; L, assay mothers exposed to odours from low-food biosource clutches; H, assay mothers exposed to odours from high-food biosource clutches.

We tested for differences in compound quantities among clutches of the low-food versus high-food experimental treatments using a multivariate analysis of variance (MANOVA) approach (proc mixed; SAS 1999). The collected quantities of the chemical components were defined as repeated measure, and the food treatment of the biosource clutches as the between-subject factor. Individual ANOVAs were used to test each compound for its relationship with food-treatment.

One of the compounds (D-limonene) was collected from control collections, indicating that it was a contaminant in the system, and the chemical identity of another compound could not be determined. These two compounds were, therefore, not included in the statistical tests, but we report descriptive statistics since both the compounds were clearly produced by the nymphs.

Significance tests are two-tailed and symmetrical throughout, except for the contrast in provisioning among assay mothers exposed to odours of low-food versus high-food biosource clutches. For this case, we had the *a priori* directional prediction of higher provisioning when exposed to low-food biosource clutches (Kölliker *et al.* 2005*b*), and we used directional significance test (Rice & Gaines 1994). The corresponding *p*-value is denoted as p_{OHT}.

3. RESULTS

(a) *Effect of nymph odours on maternal provisioning*

As predicted from our solicitation pheromone hypothesis, the exposure treatment had a significant effect on the provisioning of assay mothers (figure 1; Poisson-regression, $F_{2,57}=6.62$, $p<0.003$). Mothers exposed to odours from low-condition nymphs provisioned at a significantly higher rate than either control mothers (figure 1; $F_{1,57}=10.12$, $p<0.003$) or mothers exposed to odours from high-condition nymphs (figure 1; $F_{1,57}=8.95$, $p_{OHT}<0.004$). The provisioning rate did not differ significantly between control mothers and mothers

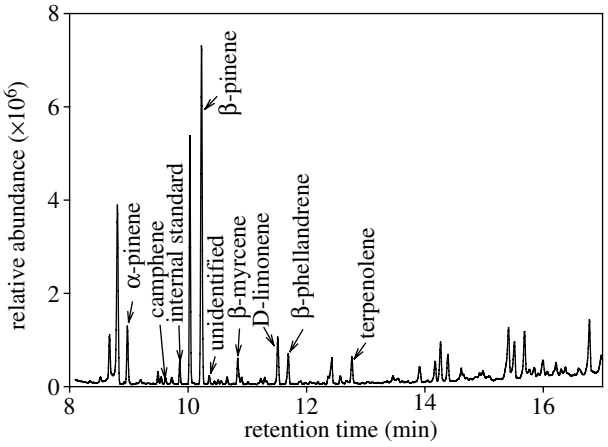

Figure 2. GC-trace of a volatile collection from burrower bug nymphs. Nymphal compounds were distinguished from contaminants by comparing the peaks to the traces from control volatile collections.

exposed to odours of high-condition nymphs (figure 1; $F_{1,57}=0.06$, $p=0.811$).

(b) *Effect of nutritional condition on nymph odour production*

GC–MS analysis revealed that the nymphs emit a blend of eight compounds, all belonging to the chemical category of monoterpenes (figures 2 and 3): α-pinene, camphene, β-pinene, β-myrcene, D-limonene, β-phellandrene, terpenolene and one compound that remained unidentified. The quantitative contribution of each of these compounds to the blend varied considerably (figure 3; MANOVA, $F_{5,31}=132.15$, $p<0.0001$), with β-pinene being the clear dominating component. Overall, nymphs from the high-food treatment tended to release a higher total amount of monoterpenes (figure 3; MANOVA, $F_{1,31}=4.08$, $p=0.052$), and the significant interaction between the food treatment and compound quantities shows that the quantitative composition of the blend differed between the high- and low-food treatment (figure 3; MANOVA, $F_{5,31}=2.65$, $p=0.042$). Among the six compounds tested in the model, α-pinene ($r^2=0.164$; $F_{1,31}=6.09$, $p=0.019$) and camphene ($r^2=0.111$; $F_{1,31}=3.86$, $p=0.059$) showed the strongest relationship with treatment, both in terms of effect size (r^2) and statistical significance, with higher collected quantities in the high-food than the low-food treatment (figure 3).

(c) *Exposure to synthetic compounds*

Based on these results, α-pinene and camphene were considered as prime candidates for the causal agents mediating the observed condition-dependent effect of nymph odours on maternal provisioning (figure 1). Exposure to two quantities each of synthetic α-pinene and camphene, respectively, did not significantly affect maternal food provisioning (Poisson-regression; α-pinene, $F_{2,56}=0.37$, $p=0.69$; camphene, $F_{2,52}=0.10$, $p=0.91$).

4. DISCUSSION

Experimental research on parental provisioning and offspring demand has focussed on parental responses to auditory and visual offspring solicitation displays (Kilner & Johnstone 1997; Budden & Wright 2001; Wright & Leonard 2002). Here we report results from experiments in the burrower bug *S. cinctus* that test predictions from the hypothesis that sub-social insects may have evolved solicitation pheromones emitted by nymphs to partly regulate maternal provisioning. We show that condition-dependent odours produced by nymphs influence maternal provisioning in this species, hence convey information about nutritional requirements to which mothers are sensitive, and provide for the first time a description of volatile compounds collected from offspring that might play the role of such a solicitation pheromone.

The effect of nymph odours on maternal provisioning is consistent with the hypothesis of a solicitation pheromone, and the predictions of honest signalling and scramble models for the resolution of the parent–offspring conflict (Godfray 1991; Parker *et al.* 2002*b*). As predicted, mothers exposed to odours from nymphs in low-condition provisioned more nutlets than the ones exposed to odours from nymphs in high condition. No overall inhibitory effect of nymph cues occurred, contrary to the result of Kölliker *et al.* (2005*b*) where a different exposure design was used based on crude cuticular nymph extracts. These results suggest that the inhibitory effect found by Kölliker *et al.* (2005*b*) may be due to non-volatile compounds on the cuticule of the nymphs which were part of the extracts in that experiment but not in the volatiles examined in the present study. Alternatively, the physical manipulation necessary for cuticular extraction may have caused nymphs to release an alarm pheromone (becoming part of the extracts), resulting in reduced maternal food provisioning in the earlier study (Kölliker *et al.* 2005*b*).

We describe eight compounds emitted by nymphs that are candidate compounds mediating the odour effect on provisioning. Nymphs in high-nutritional condition tended to release more volatiles overall, and the relative contribution of individual compounds to the blend differed significantly from the one released by nymphs in poor nutritional condition. α-Pinene was mostly responsible for this interaction between compound quantities and the food treatment, followed in importance by camphene. The direction of the relationships observed was opposite to our *a priori* prediction for a solicitation pheromone actively synthesized by the nymphs. Rather than finding a higher quantity (i.e. more begging) in the low-food treatment, we found a lower quantity. This relationship initially appears more consistent with a chemical cue that is passively produced by nymphs as a by-product of physiological processes related to food ingestion and digestion (Kölliker *et al.* 2005*b*). Indeed, we have detected α-pinene, camphene, β-pinene, β-myrcene and D-limonene in volatile collections from crushed, but not intact, nutlets (our unpublished preliminary results).

However, the significant interaction between compound quantities and food treatment shows that the blend ratios emitted by the nymphs were affected by the nutritional condition of nymphs. This effect suggests active release modification of some of the compounds by nymphs. For instance, if the functional significance of, for example, α-pinene occurs in a blend with other compounds, and the dependence of α-pinene on the quantity of ingested food would really reflect a passive by-product, then the independence of other compounds in the blend potentially reflects the active maintenance of quantity levels by the nymphs. If so, α-pinene would be a critical

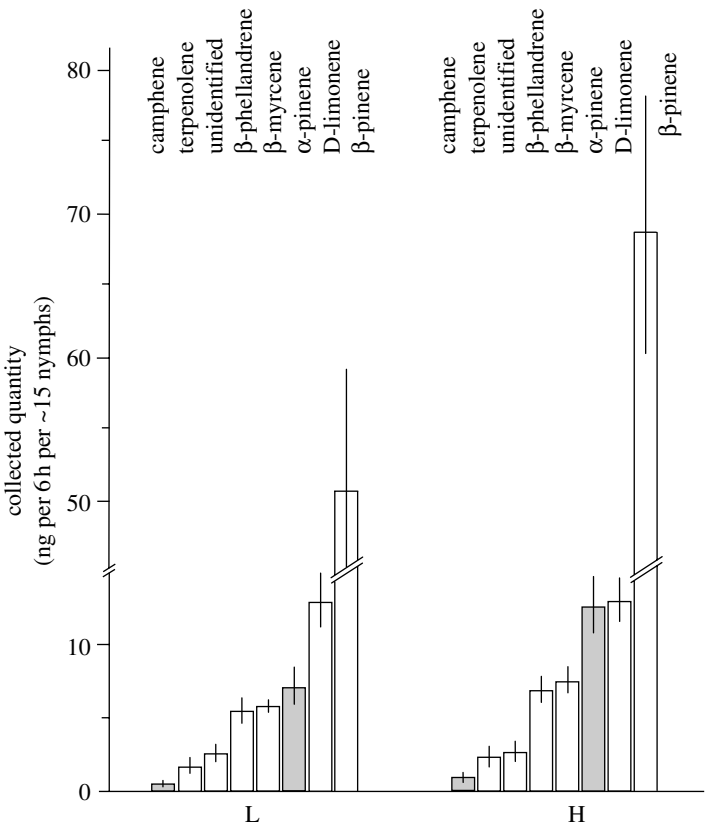

Figure 3. Mean (+s.e.m) collected quantities of volatile compounds in the low-food (L) and high-food (H) experimental treatment. The blend compositions are shown ordered with respect to the relative quantities of individual compounds. The shaded bars depict our prime candidates, α-pinene and camphene, which showed the strongest relationship with the food treatment (α-pinene, $r^2=0.164$, $F_{1,31}=6.09$, $p=0.019$; camphene, $r^2=0.111$, $F_{1,31}=3.86$, $p=0.059$; β-pinene, $r^2=0.069$, $F_{1,31}=2.29$, $p=0.140$; β-myrcene, $r^2=0.082$, $F_{1,31}=2.77$, $p=0.106$; β-phellandrene, $r^2=0.059$, $F_{1,31}=1.95$, $p=0.172$; terpenolene, $r^2=0.016$, $F_{1,30}=0.48$, $p=0.495$).

component of a blend solicitation pheromone where components of the blend would be actively modulated by nymphs while the α-pinene passively follows food intake, i.e. is the food-limited signal component.

The question of whether the observed effect of nutritional condition on nymph odour production reflects passive cues or actively released signal components has important implications for understanding the evolutionary significance of the observed condition dependence (Bradbury & Vehrencamp 1998). In the case of passive cues, the maternal sensitivity would provide mothers with the ability to directly gauge their own recent investment, allowing her full behavioural control over her level of investment. This scenario suggests that selection from family conflicts has not (yet) shaped nymphal odour (despite maternal sensitivity potentially allowing selection from parent–offspring conflict to favour manipulation of odour production by nymphs). Production of passive cues is further expected to be cost-free to the nymphs. Only if nymphs actively modify their odour production can we consider these compounds a solicitation pheromone (i.e. a signal). In such cases, selection from family conflicts might have lead to active modification of the odour components to which mothers are responsive (much like bird and mammal vocal and behavioural begging—and following models of conflict resolution). Contrary to the case of a passive cue, a solicitation pheromone would be expected to involve production costs to the offspring (Godfray 1991; Parker *et al.* 2002*a*).

Several of the described compounds (α-pinene, β-pinene, β-myrcene, D-limonene, terpenolene) were previously identified as components of the defensive secretions released by adult *S. cinctus* when attacked or agitated (Krall *et al.* 1997). This finding further suggests that a physiological machinery to actively release the compounds may be in place (at least in adults), although the quantities collected from the nymphs are much lower than in the alarm secretion (Krall *et al.* 1997; our unpublished results). The qualitative similarity of chemical compounds potentially involved in the regulation of food provisioning and involved in predator defence raises the possibility that provisioning cues secondarily evolved from cues that originally functioned to regulate maternal attendance of the clutch for the protection of nymphs against natural enemies and/or triggering nymph dispersal and independent foraging (Smiseth *et al.* 2003; Agrawal *et al.* 2004; Kölliker *et al.* 2005*b*). Maternal attendance for protection of the clutch is likely one of the early evolved forms of parental care (Tallamy 1984; Clutton-Brock 1991; Tallamy & Schaefer 1997). Quantity and context-dependent shifts in pheromone functionality are not unusual and have been described for other aspects of social behaviour in insects (Blum & Brand 1972; Nault & Phelan 1984; the 'secondary function hypothesis', see review in Haynes & Potter 1995). Chemical compounds originally involved in the maintenance of family groups may thus have secondarily evolved more specific functions, such as the solicitation of maternal food provisioning.

Our study shows that condition-dependent offspring odours from nymphs affect maternal food provisioning, but the relationship between provisioning and the identified compounds (individual compounds or blends) is correlational at present. A first attempt to identify the causally involved potential signal components by exposing mothers to pure synthetic candidates (α-pinene or camphene) showed no effect, suggesting that the functional significance of the compounds arises in blends. Experimental manipulations of quantities of synthetics of the chemical compounds in blends may, therefore, be required to causally identify the critical blend ratios characterizing the signal components.

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