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ORIGINAL PAPER

Correlated changes in breeding status and polyunsaturated cuticular hydrocarbons: the chemical basis of nestmate recognition in the burying beetle *Nicrophorus vespilloides*?

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Abstract Nestmate recognition in eusocial insects has received a lot of attention in the last decades. Recognition in subsocial species, in contrast, has been ignored almost completely and consequently, and little is known about proximate mechanisms of recognition in subsocial systems. We studied one subsocial species, the biparental brood caring burying beetle *Nicrophorus vespilloides*, an interesting model organism for studies of recognition because of its ability to discriminate between breeding partners and conspecific competitors. Recognition appears to be based on a chemical cue closely linked to the breeding status of individuals. Breeding and non-breeding beetles consistently differ in their relative proportions of polyunsaturated cuticular hydrocarbons. To investigate the function of these polyenes in the burying beetles' recognition system, we quantified their concentration on the cuticle during the early state of a breeding attempt and tested the response of breeding beetles in corresponding behavioural experiments. We observed a rapid increase in the proportion of polyunsaturated hydrocarbons of both males and females after they were provided with a carcass suitable for reproduction. Furthermore, we found that the relative amount of polyenes on an individual's surface was closely correlated with its chance of being accepted as breeding partner. Our results support the idea that polyunsaturated hydrocarbons are involved in breeding partner recognition in *N. vespilloides*, functioning as a signal that conveys information about the individual's breeding status. Breeding females have greater amount of polyenes than breeding males, and females ingest more carrion during the first days on the carcass, which supports our hypothesis that precursors for the respective polyenes are derived from ingested carrion.

Keywords Burying beetles · *Nicrophorus* · Nestmate recognition · Chemical cues · Polyunsaturated hydrocarbons

Introduction

The ability to discriminate between individuals that belong to the same species, sex, family, or social group, and ones that do not, is important in biological systems and essential for many behavioural functions, including mate choice, pair bonding and group cohesion. In insects, recognition of conspecifics is often mediated by chemical cues which may provide information about multiple facets of identity such as species, age, sex, reproductive state, or colony membership (e.g. Wyatt 2003). The particular chemical cues used vary widely between species, but cuticular hydrocarbons are known to play a paramount role in insect communication (see Singer 1998 and Howard and Blomquist 2005 for reviews). Primarily in eusocial insects, studies have conclusively demonstrated the involvement of cuticular hydrocarbons in recognition systems (e.g. Bagnéres et al. 1991; e.g. Lahav et al. 1999; Thomas et al. 1999; Wagner et al. 2000). To avoid any exploitation of group resources by intruders, social insects like termites, ants, bees, or wasps need to distinguish nestmates from non-nestmates, and the cuticular pattern is known to convey information about colony membership. Often, a complex chemical

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pattern consisting of a mixture of several compounds and hydrocarbon classes appears to be used (e.g. Kaib et al. 2004), but the specific substances involved remain unknown for most species. However, to fully understand recognition mechanisms, identifying the exact recognition cues would be helpful. One approach to obtain a better understanding about the evolution of nestmate recognition via cuticular hydrocarbons is to consider the ‘other insect societies’ (Costa 2006), the pre-, sub- and semisocial species (Wilson 1971; Eickwort 1981). Although in these species there is possibly no need for nestmate recognition in the sense that they need to distinguish between their own nestmates and individuals from other nesting or breeding sites, these species still have the necessity to discriminate between breeding partners or co-breeders (nestmates) and undesirable non-breeding intruders.

Burying beetles of the genus *Nicrophorus* are considered subsocial due to their elaborate biparental brood care (Choe and Crespi 1997). Usually, a pair of beetles cooperate in burying a small vertebrate carcass and in the subsequent feeding and (Pukowski 1933; Eggert and Müller 1997) defense of the brood (Trumbo 2006; Trumbo and Valletta 2007). Conspecific competitors may discover the buried carcass; if they do, they attempt to take over the resource for their own reproduction and kill the residents’ brood (Scott 1990; Trumbo 1990a, b, 2006; Trumbo and Valletta 2007). Breeding burying beetles distinguish between their breeding partner and intruders and attack intruders while tolerating their partner (Trumbo and Wilson 1993; Müller et al. 2003). No individual recognition is involved in the distinction between nestmates and intruders, but rather, all individuals that are providing care for larvae share a common chemical cue that distinguishes them from intruding non-breeding individuals (Müller et al. 2003; Steiger et al. 2007). However, in contrast to most eusocial insects, the similarity of the chemical substances is not due to relatedness, the exchange of chemicals among breeding beetles, or the acquisition of similar substances from a shared environment, but is due to parallel physiological changes that all beetles undergo during a breeding attempt on a carcass (Müller et al. 2003). Physiological changes in the course of a breeding attempt include a sudden increase in juvenile hormone titre in the initial stages of a breeding attempt (Trumbo et al. 1995; Trumbo 1997; Scott et al. 2001) as well as a behavioural switch from infanticide to parental care approximately 48 h after egg laying, the time the beetles’ own larvae hatch (Müller and Eggert 1990). These physiological changes appear to affect the composition of the beetles’ cuticular pattern as well. A recent study demonstrated differences in the composition of cuticular hydrocarbon mixtures between breeding and non-breeding beetles that are sufficient to allow for nestmate recognition (Steiger et al. 2007). Gas chromatographic and mass spectroscopic analysis revealed that breeding beetles were characterised by a high relative amount of some unusual long chained polyunsaturated hydrocarbons.

The aim of the current study was to investigate the role of such polyunsaturated hydrocarbons in the nestmate recognition system of the burying beetle *Nicrophorus vespilloides*. We documented the temporal change in the amount of polyunsaturated hydrocarbons when beetles were provided with a carcass, and we examined whether the amount of polyenes is correlated with behavioural interactions. We previously hypothesised that a carrion diet provides precursors for the polyenes observed in parental beetles (Steiger et al. 2007). If precursors are derived from the diet, sex differences in feeding rate could be an explanation for our finding that breeding females produced larger amounts of polyunsaturated hydrocarbons than males. We tested this hypothesis by weighing males and females before and during breeding.

Materials and methods

Collection and maintenance of experimental animals

Experimental animals were the first-generation offspring of *N. vespilloides* beetles collected in carrion-baited pitfall traps in the field in August and September, 2005. The field site was a deciduous forest near Freiburg, Germany (48°00′ N, 07°51′ E). The beetles were maintained in temperature-controlled chambers at 20°C on a 16:8-h light/dark cycle. Groups of up to six adults of the same sex were kept in small transparent plastic containers (10×10×6 cm) filled with moist peat and were fed freshly killed mealworms twice a week. Beetles used in the experiments were 30 to 40 days old and similar in size.

Sampling and quantitative analysis of polyenes

At different times during the course of reproduction, beetles were killed by freezing at −27°C for 15 min, then thawed for 30 min at room temperature and extracted. For extraction of cuticular compounds, beetles were placed individually in flasks with 3 ml *n*-pentane (>99%, Fluka, Switzerland) and shaken for 15 min on an orbital shaker. The extract was then transferred to a clean vial and reduced by evaporation using a stream of gaseous nitrogen until approximately 0.1 ml remained. Samples were quantified on an HP 6890 gas chromatograph with a split/splitless injector (300°C, automatic sampling, injection of 1 µl). We used a fused silica column (DB-1, 30 m×0.25 mm ID, 0.25 µm, J & W Scientific, Folsom, Canada) with a helium flow of 1 ml/min. The oven temperature was programmed as follows: 2 min at 35°C, to 100°C at a rate of 20°/min, to

300°C at 6°C/min, 25 min at 300°C. The flame ionisation detector was run with 40 ml H_2 per minute and 450 ml air per minute. *N. vespilloides* cuticular hydrocarbons are a complex blend of primarily *n*-alkanes, olefins and methyl-branched alkanes ranging from C_{18} to C_{31} in chain length. Nine of the 91 chemical compounds identified by mass spectrometry were polyunsaturated hydrocarbons (Table 1; see Steiger et al. 2007 for identification). The peaks representing the polyenes were integrated manually; their relative amounts were determined as their proportion relative to *n*-C_{24}. In *N. vespilloides*, the relative amount (in relation to all cuticular lipids found in *N. vespilloides*) of the alkane *n*-C_{24} exhibits little variation between individuals, including beetles of different sex and breeding condition (Steiger et al. 2007 and unpublished data), making it an appropriate standard for analysing changes in polyene amounts during a breeding attempt.

Breeding status and polyene production

To investigate the temporal course of polyene production during a breeding attempt, we provided pairs of beetles (n= 88) with a 10 g mouse carcass in a plastic box with moist filter paper. After the beetles had begun to inspect the carcass, beetles and carcass were carefully transferred to a new container with moist peat where the beetles began to bury the carcass. These containers were first placed inside an environmental chamber under the regular light regime and were transferred to a dark chamber at the same temperature 24 h later where they remained for the rest of the experiment. At the beginning of the experiment and every 16 h thereafter (0, 16, 32, 48 and 64 h after the pairs had been provided with a carcass), some of the male and female beetles were killed for chemical analyses ($n \geq 12$ for each point of time). In addition, some pairs (n=12) were killed after caring for larvae for 16 (±4) h, about 80–100 h after the first contact with the carcass. Such ‘parental’ beetles had been treated as described above, but had been transferred with their carcass to boxes with clean peat after 48 h. The old boxes were then checked regularly for newly hatched larvae. Once we observed larvae, we placed ten first-instar larvae on the carcass with the respective pair. The standardisation in larval number was done, as we cannot rule out that brood size has an effect on the physiological condition, and therefore, on the cuticular pattern of the breeding beetles. The standardisation required the occasional use of larvae that were not the pair’s own offspring. The use of unrelated larvae is of no consequence to the present study because parental beetles do not distinguish between their own and unrelated larvae, provided they appear on the carcass at the right time (Müller and Eggert 1990).

Table 1 Polyunsaturated hydrocarbons found in the surface extracts of male and female *N. vespilloides* (Steiger et al. 2007)

Compound	Retention index
6,9,12,15-C_{25}tetraene	2420
3,6,9,12,15-C_{25}pentaene	2425
C_{25}triene	2430
C_{27}hexaene	2605
6,9,12,15-C_{27}tetraene	2620
3,6,9,12,15-C_{27}pentaene	2625
C_{29}hexaene	2805
6,9,12,15-C_{29}tetraene	2820
3,6,9,12,15-C_{29}pentaene	2825

Behavioural response

We examined whether beetles with a higher relative amount of polyenes are more likely to be accepted as a breeding partner by conspecifics caring for larvae. We used the experimental specimens from the above experiment immediately after they had been frozen and thawed and presented them to ‘parental’ beetles of the opposite sex (=‘discriminating’ individuals). To avoid any sex differences in behavioural responses due to the described treatment, half of the discriminating beetles transferred to a new carcass were males and half were females. For observations of encounters, the dead ‘cue-bearing’ males and females were placed on the peat near the carcass or on the carcass itself in their natural (dorsal side up) position. Discriminating males were always presented with dead females and vice versa. We observed the beetles’ behaviour in the first five encounters and classified their reactions as aggressive or tolerant (cp. Müller et al. 2003). The behaviour was considered aggressive if the beetles grasped and bit the cue beetle in at least four of the five encounters and as tolerant if there was no aggressive behaviour in at least four of the five encounters. If beetles showed two or three aggressive responses and three or two tolerant ones, we considered their behaviour ambivalent and excluded these trials from further analyses.

Nutritional basis of polyene production

To test the hypothesis that differences in polyene production between males and females are due to a different feeding rate on the carcass, we provided pairs (n=20) of beetles with a 10 g carcass and measured the body mass of the beetles before the carcass was given and again once their first larvae hatched when females had completed oviposition (Müller 1987). The pairs with their carcass were gently transferred 48 h after they had been provided with a dead mouse. The old boxes were checked every 6 h for newly hatched larvae. Before contact with a carcass, the sexes did not differ in body mass (t test, n_1=n_2=20, df=38, t=0.94, p=0.35).

Statistical analyses

Statistical analyses were carried out using SPSS 14 and R 2.4.1. To test for an effect of time and sex on the polyene production, a two-way analysis of variance (ANOVA) with a post hoc Bonferroni analysis was performed. To analyse the effect of the amount of polyenes on behaviour, we used a logistic regression with polyene amount as the independent and behavioural response (aggressive or tolerant) as the dependent variable. To test for a difference in weight change between males and females, we performed a repeated measures ANOVA with body mass before and during breeding as dependent variables. Time was treated as a within-subjects factor and sex as a between-subjects factor.

Results

Polyene production in the course of a breeding attempt

The relative amount of polyenes produced by a beetle was significantly affected by the individual's sex and the amount of time it had spent on a carcass. This is true when the total relative amount of polyenes is taken into account (ANOVA, effect of time: $F_{5,164}$=33.09, p<0.001; effect of sex: $F_{1,164}$=71.34, p<0.001; Fig. 1b) as well as when looking at the relative amount of one particular polyene, Δ6,9,12,15-C$_{25}$tetraene (ANOVA, effect of time: $F_{5,164}$= 24.53, p<0.001; effect of sex: $F_{1,164}$=23.70, p<0.001; Fig. 1a). The longer a beetle had been on a carcass, the higher the relative amount of polyenes on its surface; a difference in comparison with the initial amount was present as soon as 32 h later (adjusted α-level after

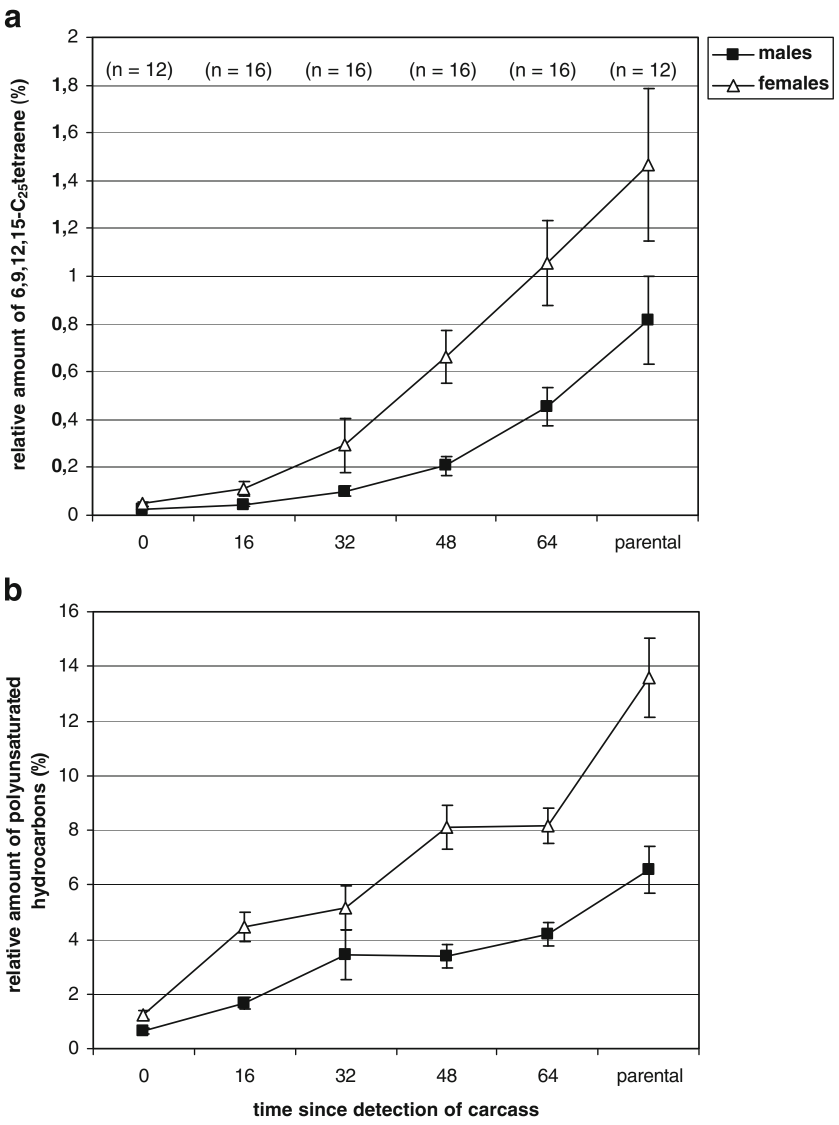

Fig. 1 Temporal change in the percentage of one exemplary polyene: 6,9,12,15-C25tetraene (**a**) and the sum of all nine polyenes found on the cuticle of male and female *N. vespilloides* (**b**). The proportion of these hydrocarbons was calculated relative to n-C$_{24}$. Mean±SE is given. Sample sizes shown at the *top of the figure* apply to males and females separately. The 6,9,12, 15-C25tetraene was chosen as an example because it is one of the polyenes which is only affected by breeding state and not by carrion diet (Steiger et al. 2007)

Bonferroni correction: $\alpha^1=0.003$, $p<0.001$). The increase of polyenes over time differed between the sexes: Females showed a greater increase than males (ANOVA, effect of time × sex: Δ6,9,12,15-C_{25}tetraene, $F_{5,164}=2.62$, $p=0.03$; total amount of polyenes, $F_{5,164}=4.34$, $p<0.001$).

Behavioural response and relative amount of polyenes

When putative 'cue-bearing' beetles were presented to parental males and females, most of the 'discriminating' individuals ($n=88$ males and 88 females) responded with obvious aggression or tolerance; only 11 males and nine females exhibited ambivalent behaviour (see "Materials and methods"). There was a clear correlation between the total relative amount of polyenes on a beetle's surface and how that individual was treated by a parental beetle of the opposite sex (Wald test, effect of polyenes: $n=156$, $df=1$ Wald=10.59, $p=0.001$; Fig. 2). Beetles with a high relative amount of polyenes were more likely to be accepted as nestmates by a parental beetle than beetles with a low amount. The correlation was significantly different between the sexes: female 'cue-bearing' beetles required a higher amount of polyenes to be tolerated by parental males than male 'cue-bearing' beetles did to be tolerated by parental females (Wald test, effect of sex × polyenes: $df=1$, Wald=6.06, $p=0.01$), a result visualised by the different slopes of the logistic regression curves (Fig. 2). Sex per se had no effect on the behavioural response of parental beetles (Wald test, effect of sex: $df=1$, Wald=0.01, $p=0.91$), meaning that the frequency of aggressive behaviour towards males (by females) was approximately the same as towards females (by males). This statement may appear confusing, as our earlier statement that parental males required greater amounts of polyenes than did females might have suggested that parental males were generally more aggressive towards 'cue-bearing' beetles of the opposite sex. However, the higher amount of polyenes that parental males required for tolerant behaviour was matched by the greater increase in the amount of polyenes exhibited by females (see Fig. 1b, previous section).

Nutritional basis of polyene production

There was no difference in the body mass between the sexes (repeated measures ANOVA, factor sex: $F_{1,38}=0.02$, $p=0.90$), but both males and females were heavier at the time larvae began to hatch than in the beginning of the breeding attempt (repeated measures ANOVA, factor time: $F_{1,38}=408.97$, $p<0.001$). Moreover, females actually gained more mass than did males in these first days of a breeding attempt (repeated measures ANOVA, factor sex × time: $F_{1,38}=10.74$, $p=0.002$; mean±SE of relative change in body mass: males, +19.88%±1.56%; females, +28.16% ±2.22%).

Discussion

The results presented here agree with our earlier finding (Steiger et al. 2007) that the cuticular pattern of breeding and non-breeding individuals differed in the relative amount of polyunsaturated long-chain hydrocarbons. In this paper, we demonstrated a rapid increase in the amount of polyenes during the initial stages of a reproductive attempt; the more time the beetles had spent on a carcass, the higher was their amount of unsaturated hydrocarbons. This result strongly suggests that breeding status and polyene production covary in burying beetles of either sex. Our results are also consistent with the hypothesis that polyunsaturated fatty acids derived from the ingestion of carrion on the carcass may play a role as precursors for the polyenes. Breeding females ingested more carrion than did males, and they also produced more polyenes than males. Vertebrate tissue contains polyunsaturated fatty acids

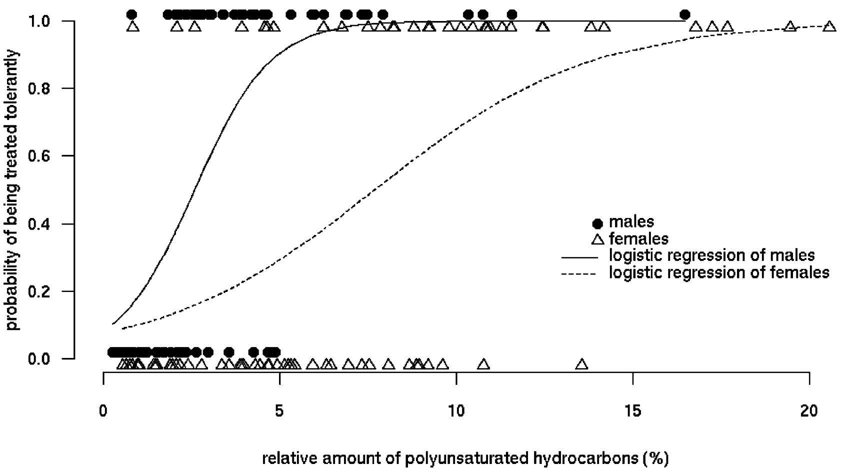

Fig. 2 Probability of being tolerated by breeding beetles as a function of the total amount of polyenes on a 'cue-bearing' beetle's cuticle. Breeding 'discriminating' beetles were of the opposite sex and had been reproducing with a partner. The *symbols* represent the original data (cue males: $n=77$; cue females $n=79$); the *curves* represent the resulting logistic regressions and show the probability for a beetle with a given amount of polyenes to be tolerated by an opposite-sex breeder (males: logit $f(x)=-2.5+0.32x$; females: logit $f(x)=-2.4+0.94x$)

related to linoleic or linolenic acid with double bonds at the same positions, as some of the polyenes found on burying beetles. For example, a pentaenoic acid isolated from beef liver has the same 3,6,9,12,15-pentaene structure (Montag et al. 1957) as the C_{25}-, C_{27}- and,C_{29}-pentaenes found on the surface of caring *N. vespilloides*.

The ingestion of carrion alone may provide the precursors, but cannot explain the difference in polyunsaturated hydrocarbons between breeding and non-breeding beetles. Breeding status has a much stronger effect on the quantity and quality of the polyunsaturated hydrocarbons than does a diet of carrion (Steiger et al. 2007), and non-breeding carrion-fed beetles are not accepted as nestmates (Müller et al. 2003). In our earlier paper (Steiger et al. 2007), we hypothesised that juvenile hormone governs the production of the recognition cue. This hypothesis was based on two pieces of evidence: first, JH titres in burying beetles change significantly during a breeding attempt, both in males and females (Trumbo et al. 1995; Trumbo 1997; Scott et al. 2001; Panaitof et al. 2004) and second, research in other insects has shown the involvement of JH in regulating the production of cuticular hydrocarbons and contact pheromones (e.g. Tillman et al. 1999; Sledge et al. 2004). In *Sarcophaga bullata* for example, JH has a direct effect on the activity of oenocytes, cells in which cuticular hydrocarbons are synthesised (Stoppie et al. 1981). By applying juvenile hormone or its analogue methoprene on non-breeding burying beetles fed with carrion and subjecting them to chemical analysis, our hypothesis could be tested.

Müller et al. (2003) found that the recognition cue of burying beetles appears to be present slightly before a beetle's readiness to exhibit parental care. The pattern of the temporal change of the polyenes shown in the present study can explain this phenomenon on a proximate level: It is not an abrupt rise, but rather a steady increase. It should be quite difficult to time the production of polyenes in such a manner that the amount which is necessary to be accepted as nestmate (acceptance threshold; Reeve 1989) is reached at the same time the behaviour switches from infanticide to parental care. To play it safe that the own breeding partner is not dispelled, it is better to accept a beetle with a polyene level being reached shortly before the behaviour changes. Thus, the acceptance threshold should be positioned in such a way that the rejection error becomes low (Reeve 1989). The question arises why the beetles do not circumvent the whole problem by a sudden development of the recognition cue which could be perfectly coupled with the transition in behaviour. The more or less slow production of the unsaturated hydrocarbons is likely to be a physiological constraint: Burying beetles could simply be not able to build up a high amount of polyenes at once. We think that a long time involved in the production of high amount of polyenes are necessary for the reliability of the recognition cue; a part of the costs of reliability would be time wasted. In general, it would be beneficial for an intruding conspecific to pretend to be a breeding individual because it would not be attacked by a resident beetle of the opposite sex. However, time is an important factor on a carcass, as the amount of carrion rapidly decreases due to consumption by the larvae and their parents and new larvae cannot be raised if there is not enough nutrition left (Eggert and Müller 1997). Thus, to wait 2 days or more for the full production of the chemical signal would involve high costs and low benefit for intruding conspecifics. Clearly, the suggested costs of wasted time are not enough to make the signal reliable. Beetles could just produce a high level of the polyenes throughout their lives. However, assuming that a high titre of JH is a precondition for the production of the chemical signal, it is likely that a permanent production would involve severe physiological costs (e.g. Rantala et al. 2003).

The close correlation between the amount of polyenes a beetle had on its surface and the frequency with which a beetle was tolerated suggests a signal function of polyunsaturated hydrocarbons. However, although the evidence appears persuasive, we have only documented a correlation and not a causal relationship. Information about an individual's breeding status could be conveyed through other cuticular substances that covary with the polyenes. We recently found that there is a shift in the ratio between relatively short-chain and long-chain hydrocarbons between breeding and non-breeding beetles, and this ratio could also act as a signal (Steiger, unpublished data). In addition, we do not know how many of the nine polyenes, or which particular ones, are involved in nestmate recognition. Ultimately, the behavioural activity of specific cuticular hydrocarbons can only be confirmed in experiments with synthetic chemicals. Unfortunately, the polyunsaturated components found in *N. vespilloides* are not commercially available nor are they easily synthesised. As a first step towards confirming the involvement of polyunsaturated hydrocarbons in the recognition of breeding status, surface extracts should be fractionated and the activity of the fractions tested in bioassays. More evidence could be gathered by examining the cuticular pattern during and after the period of parental care. There must be a time at which males and females lose the signal such that they are no more accepted as nestmates by breeding beetles. A corresponding decrease in the amount of polyenes would provide additional support for the 'polyene hypothesis'. Although burying beetles are the first insects in which a correlation between breeding status and the cuticular pattern has been documented, there are comparable phenomena in other insects. In some eusocial insects, female ovarian activity is correlated with the composition of the cuticle

(Ayasse et al. 1995; Peeters et al. 1999; Liebig et al. 2000; Cuvillier-Hot et al. 2001, 2004, 2005; De Biseau et al. 2004; Sledge et al. 2004; Dietemann et al. 2005; Hartmann et al. 2005). It is assumed that the chemical information plays a role in worker policing. Although polyunsaturated hydrocarbons do not appear to be involved in these examples, different kinds of hydrocarbons were attributed communicative function. However, none of the above studies were able to provide direct evidence of relevant hydrocarbons using bioassays with synthetic compounds.

If the polyenes are involved in the mate recognition system of *N. vespilloides*, the recognition mechanism appears to be slightly different in males and females. The cuticle of females was characterised by a higher amount of polyenes than males, and higher amounts were required for ‘cue-bearing’ females to be accepted as breeding partners by males. The results of the different strategies in sending and responding to the chemical signal support our earlier prediction (Steiger et al. 2007) that the gender-specific task specialisation of burying beetles, whose males appear to have a greater tendency to guard the brood than females (Trumbo 2006), may lead to different acceptance thresholds for males and females. However, considering our experiments, we cannot rule out that it is vice versa and males have a higher acceptance threshold because females have a higher amount of polyunsaturated hydrocarbons as a by-product of their feeding behaviour. In general, the current study and previous work highlight the fact that burying beetles are attractive model organisms for the study of recognition processes and the evolution of signals and may contribute to general concepts of signal theories.

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