Female choice in the red mason bee, *Osmia rufa* (L.) (Megachilidae)

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SUMMARY

Females are often thought to use several cues and more than one modality in selection of a mate, possibly because they offer complementary information on a mate's suitability. In the red mason bee, *Osmia rufa*, we investigated the criteria a female uses to choose a mating partner. We hypothesized that the female uses male thorax vibrations and size as signs of male viability and male odor for kin discrimination and assessment of genetic relatedness. We therefore compared males that had been accepted by a female for copulation with those rejected, in terms of their size, their immediate precopulatory vibrations (using laser vibrometry), the genetic relatedness of unmated and mated pairs (using microsatellite markers) and emitted volatiles (using chemical analyses). Females showed a preference for intermediate-sized males that were slightly larger than the modal male size. Furthermore, male precopulatory vibration burst duration was significantly longer in males accepted for copulation compared with rejected males. Vibrations may indicate vigor and assure that males selected by females are metabolically active and healthy. Females preferentially copulated with males that were genetically more closely related, possibly to avoid outbreeding depression. Volatiles of the cuticular surface differed significantly between accepted and rejected males in the relative amounts of certain hydrocarbons, although the relationship between male odor and female preference was complex. Females may therefore also use differences in odor bouquet to select among males. Our investigations show that *O. rufa* females appear to use multiple cues in selecting a male. Future investigations are needed to demonstrate whether odor plays a role in kin recognition and how the multiple cues are integrated in mate choice by females.

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Key words: *Osmia rufa*, mating behavior, optimal outbreeding, thorax vibrations.

INTRODUCTION

Female choice of a mating partner is widespread in the animal kingdom (Andersson, 1994; Bateson, 1983; Darwin, 1871; Thornhill and Alcock, 1983). Theory suggests that if one sex invests significantly more heavily in offspring, this sex will try to maximize its reproductive fitness by choosing the best possible mate (Roitberg and Isman, 1992). Females typically invest more heavily than males in offspring, and they use a variety of male traits such as color (Bezzel and Prinzinger, 1990), size (Partridge et al., 1987), mating song (Ryan et al., 1982) or scent (Smith, 1983) to evaluate the quality of a male before accepting him for mating.

These selected traits either provide information about the male's viability, e.g. the biggest male is the most viable (Partridge et al., 1987), or about other components of his quality such as his suitability, e.g. the relatedness of a male to a female. Female choice on the basis of relatedness may lead to 'optimal outbreeding' (Bateson, 1983), an idea which is based on the observation that both inbreeding and outbreeding have costs. Optimal outbreeding therefore predicts that mate choice helps to minimize both of these costs (Bateson, 1983).

Given the multiple facets of quality that a female may select in a mate, it may be anticipated that females use multiple cues encompassing two or more modalities in selecting a male. Multiple cues may provide additional information on a mate's suitability beyond that provided by a single cue, and it is possible that a combination of different traits is necessary for a male to be successful in mating (Candolin, 2003). In that case, it might not necessarily be the biggest male but a male with the best combination of traits that a female selects (Candolin, 2003). To understand the targets of sexual selection and the information a female uses in mate selection, it is therefore important to examine multiple male traits rather than focusing on a single one.

Pheromones play an important role in mating in a wide variety of insects (Ayasse et al., 2001; Wyatt, 2003). Prominent examples are seen in various Lepidoptera, in which the female emits small amounts of sex pheromone to attract males. Males, in return, have developed specialized antennae to recognize minimal amounts of female sex pheromone (Roitberg and Isman, 1992). In bees, female sex pheromones are used to attract males, to identify receptive females and to elicit territorial behavior and courtship behavior in males (Ayasse et al., 1999; Ayasse et al., 2001; Fraberger and Ayasse, 2007; Krieger et al., 2006). In the red mason bee, *Osmia rufa* (Linnaeus) (Hymenoptera: Megachilidae), virgin females attract males by a sex pheromone that elicits an intensive courtship behavior, during which males even try to mount each other in an attempt to mate (Rosner, 1994).

It has been repeatedly shown that odor also plays a predominant role in kin recognition in bees (Smith, 1983; Smith and Ayasse, 1987; Smith and Wenzel, 1988). In the sweat bee, *Lasioglossum zephyrum*, males can distinguish females according to their degree

of relatedness; they are able to recognize kin using a chemical signal from the Dufour's gland in which the relative proportions of macrocyclic lactones vary less among close than among distant relatives (Smith and Ayasse, 1987; Smith and Wenzel, 1988). These studies show that kinship correlates strongly with similarities in odor, which may be highly important in selecting a mate (Alcock et al., 1976; Ayasse et al., 2001; Smith, 1983). In addition, male (and female) bees release a range of other odors that may not be involved in kin discrimination (Ayasse et al., 2001; Eickwort and Ginsberg, 1980).

In diverse groups of eusocial bees (bumblebees, stingless bees and honeybees), thoracic vibrations are used in recruitment communication (Esch, 1961; Hrncir et al., 2006a; Michelsen et al., 1986; Nieh and Tautz, 2000), pollen collection (Buchmann, 1983; King, 1993; Michener, 1962; Wille, 1963) and defense (Schneider, 1975; Sen-Sorma et al., 2002). Little research has been done on the function of vibration signals in bee mating behavior, although several species are known to emit these signals while mating (Eickwort and Ginsberg, 1980). There are also several arthropod species from orders other than the Hymenoptera that show a broad spectrum of vibrational displays during mating (Cocroft and Rodríguez, 2005). These include fiddler crabs (Crustacea), stoneflies (Plecoptera), alderflies (Megaloptera), crickets and grasshoppers (Orthoptera), beetles (Coleoptera), flies (Diptera), spiders (Arachnida) and many more (Hill, 2008).

Although information exists on vibrations being used as signals for species recognition and sexual selection during courtship (Cocroft and Rodríguez, 2005), little research has been undertaken to date on the relevance of vibrations as fitness signals in mating rituals of insects, particularly for those species that do not possess specialized organs to produce sound (Hedrick, 1986; Klappert and Reinhold, 2003). In bees, vibrations are generated by rhythmic thoracic oscillations using a motor pattern in which the wings hardly move. Increasing muscle contraction leads to an increased stiffening of the thorax and, thus, to higher oscillating frequencies (Hrncir et al., 2006b).

The vibrations that bees produce during mating may indicate male vigor. Stronger or healthier males are likely to be able to produce a longer or more intense vibration owing to the fact that they have more energy for continuous thorax oscillations. In that case, a female may select a male with the 'best' (e.g. longest or with greatest amplitude) vibrational display because that male also is the most viable (Hedrick, 1986; Hill, 2008; Klappert and Reinhold, 2003; Ryan et al., 1982).

It is well known that size is correlated with fitness in some bees (Paxton, 2005). The biggest male is usually the strongest and most vigorous, and females are therefore thought to be under selection to choose the biggest (Eickwort and Ginsberg, 1980). However, there are several examples in hymenopteran insects in which females select smaller males over larger males (Alcock, 1996b; Larsson, 1991). There is also evidence that size does not play such an important role in mate selection in *O. rufa* (Seidelmann, 1995).

O. rufa is a widespread solitary bee of Central and Northern Europe. Owing to its ready acceptance of artificial nesting boxes, the species is amenable to experimental investigation and aspects of the basic reproductive biology of *O. rufa* are well known (Seidelmann, 1995). During precopulatory courtship, the male embraces the female by standing on her back and engaging in a series of behaviors in order to persuade the female to mate. The male may vibrate his thorax, rub himself against the female and pass his antennae repeatedly over those of the female and his forelegs over the female's compound eyes (Seidelmann, 1995). During

precopulatory courtship, the female may reject the male by physically pushing him off her back, yet which of the male cues a female uses in mate selection is still not known.

Here we analyzed four potential male cues – male size, male vibrations, relatedness between a pre-copulatory pair and odor – as possible cues by which a female *O. rufa* may select a male that has engaged in a pre-copulatory embrace with her. These cues would allow females to gather information on kinship or signals of vigor, or a combination of both (Candolin, 2003). The following specific questions were addressed: does the size of a male play a role in female choice in *O. rufa*; are the precopulatory thorax vibrations produced by a male used by the female to evaluate the male's suitability; does the female choose a male based on the pair's relatedness; and is the female able to use male odor in mate selection?

MATERIALS AND METHODS
Animals

We used *O. rufa* reared at the University of Ulm in trap nests consisting of a wooden box (50 cm×20 cm×23 cm), with 7 rows of 22 holes (diameter 6 mm), on the roof of the University building. The population of *O. rufa* was mixed, consisting of individuals from Ulm, Vienna and Gattersleben, locations with different climatic conditions. *O. rufa* cocoons containing teneral adults were overwintered in a cold room at 5°C. Both sexes normally emerge in late March to early April, although *O. rufa* is a protandrous species in which males eclose, on average, approximately two weeks before females (Seidelmann, 1995). We held bees in a refrigerator until they were required for experiments, and then moved them into a room-temperature environment, where they emerged. In addition to the population reared in the lab, we collected pairs engaged in pre-copulation from the field. The numerical sex ratio is generally slightly male biased and females only mate once whereas males mate repeatedly (Seidelmann, 1995), hence there is considerable potential for female choice based on male traits (Paxton, 2005).

Mating in *O. rufa* consists of three phases: precopulatory courtship, copulation and postcopulatory embrace (Seidelmann, 1995). After a female first emerges in spring, males in the vicinity approach the female. Once a male has established a position on the dorsum of a female, other males quickly retreat and leave the pair alone (Seidelmann, 1995). During this precopulatory phase, the male sits on the back of the female and embraces the female's mesothorax with his first and second pairs of legs. The male's antennae point anteriorly whereas the female's antennae point laterally (Seidelmann, 1995). The male then tries to motivate the female to copulate by moving his antennae in a stroking motion over the female's antennae. At the same time, the male moves his front legs over the female's eyes. To the human ear, every stroking motion is accompanied by a high-pitched humming sound, which develops into a buzzing sound when the male tries to copulate (see supplementary material Movie 1, Audio 1).

Thereafter, the male moves backwards on the female and tries to insert his genitalia into the female's genital chamber while using his antennae to produce a tremolo (a continuous drumming) on the female's face (Seidelmann, 1995). If the female accepts the male, copulation begins. However, the female can also reject the male by bending her abdomen downwards and trying to shake the male off. In that case, the male stops the attempt at insertion and either departs or begins the precopulatory ritual again. If the male is successful, copulation lasts for several minutes and is then usually followed by a postcopulatory phase of up to 13 min in duration, during which time the male continuously strokes his abdomen over the female in

the posterior to anterior direction in order to apply an antiaphrodisiac (A.M., G. Dutzler, F. Schiestl, F. Ibarra and W.F., unpublished data) (Seidelmann, 1995).

For each male trait we measured, different sets of individuals (males and females) were used in experiments and then sacrificed. We were therefore unable to examine interactions between the four cues (size, vibrations, relatedness and odor) in mate selection. Each individual male or female was only used and measured once to avoid pseudoreplication.

Size measurements

The minimum interocular distance (the smallest distance between the eyes) was measured in male bees either from our laboratory experiments or collected in the field, in front of nesting boxes, using a Leica photomicroscope ($\times$40 magnification; Wetzlar, Germany) and the Leica Application Suite software. Measures of head width and interocular distance are linearly and isometrically related to body size (wet and dry weight) in a number of other bee species in which, like *O. rufa*, males do not exhibit extreme size variation linked to alternative mating strategies (Danforth and Desjardins, 1999; Marlovits, 1994; Paxton and Tengö, 1996; Rust, 1991).

Laser vibrometry

Vibrations were recorded under daylight between 10 May and 14 June 2007. Male and female bees used for the recordings had emerged in separate flight cages at room temperature. They were provided *ad libitum* with a 50% (w/w) sugar solution of APIInvert (72.7%; Südzucker AG, Rain, Germany; 1 g citric acid and 3 g potassium sorbate were added per liter API-Invert solution). To obtain mating pairs, one female was introduced at a time into a flight cage containing approximately 40 males that originated from numerous trap nests. New males were added to the flight cage periodically (every 4–5 days), which led to a medley of older and younger males. We only used unmated males and, for each male, we only allowed one copulation attempt. Once a mating pair had become established in pre-copulatory embrace (one male sitting on a female and the other males retreating), the pair was taken out of the flight cage and put in a plastic box (40 cm$\times$20 cm$\times$10 cm).

The thoracic vibrations produced by males during the precopulatory phase were recorded with a laser vibrometer (Polytec PDV-100; Waldbronn, Germany) connected to a laptop computer using a 32-bit sound card and Soundforge 8.0 software (SonicFoundry, Madison, WI, USA) at a sampling rate of 44.1 kHz. The files were later analyzed using Avisoft SasLab Pro (Avisoft Bioacoustics, Berlin, Germany). All males were marked with a white spot on their thorax to enhance the reflection of the laser beam. The following parameters were measured: the duration of one burst and the time between bursts during which no vibrations were recorded. We recorded and compared the vibrations of males that were either accepted (permitted by the female to copulate) or rejected by the female. Females occasionally moved during the recordings and the angle between the laser and the bees changed. Hence, we were unable to compare the signal amplitudes of the different males.

Sampling of mating pairs

The kinship experiments were conducted under artificial light between 18 June and 20 July 2007. We used bees from cocoons of single nesting tubes. Because all the bees from each nesting tube are normally produced by a single female (Seidelmann, 1991), we expected all the bees from one nesting tube to be brothers and sisters. Male and female cocoons were separated by sex and tube. The bees emerged at room temperature in individual round plastic boxes (13 cm diameter), which were placed next to a window as a source of daylight.

After emergence, male bees were marked on the thorax with different colors according to their nest of origin. Approximately 40 males were kept together in a flight cage at all times (see Laser vibrometry). A single female was then introduced and, once a mating pair had established, she was taken out and observed until the male was either accepted for copulation or rejected. In addition to the pairs from the lab experiments, pairs from the field were also collected. Each pair was then frozen in liquid nitrogen. Owing to the fact that many males rubbed off their thoracic marks and some males escaped, it was not possible to establish exactly which males were present at all times in mating cages. Also, while a nest owner forages for food, her nest may be taken over by another female (M.A., unpublished observations), leading to nests containing a mix of full sibs and unrelated individuals. We therefore cannot determine with certainty what brother/unrelated male ratio existed in the cages. However, we estimate that the percentage of brothers in the flight cage, based on affiliation to single tubes, was approximately 10% for each female (*N*=45 nests).

After observations and freezing, both males and females were stored in ethanol (99%, Merck KgaA, Darmstadt, Germany) at 4°C. Because there was no statistical difference in size between bees from the field and the lab (*t*-test, *P*<0.05), data were pooled for further analysis. The number of copulating pairs studied was 97 and the number of non-copulating or unmated pairs was 35.

Genetic analysis

DNA was extracted from 264 individuals, both males and females, using a high-salt extraction protocol (Paxton et al., 1996) and analyzed at six microsatellite loci developed for the species (Neumann and Seidelmann, 2006). Polymerase chain reaction (PCR) was used to amplify the alleles, which were labeled radioactively. PCRs were performed using 1$\times$ PCR Buffer (Promega), 75 μM each of dGTP, dCTP and dTTP, 6 μmol l^{-1} dATP, 4 pmol l^{-1} of each primer (forward and reverse), 0.4 U *Taq* polymerase, 0.125 μCi ^{33}Pα dATP and 1.00 μl DNA template (*ca.* 10 ng), made to a total volume of 10.0 μl with H$_2$O. Multiplexing was possible for three pairs of loci, namely: OruE5/OruA8, OruS8/OruC4 and Oru10/OruS4. PCRs were run for 40 cycles at 94°C for 30 s, 50°C for 30 s and 72°C for 44 s, with an initial 94°C for 3 min and an additional 10 min at 72°C afterwards.

Polyacrylamide gel electrophoresis was used to resolve alleles (6% polyacrylamide, 8 mol l^{-1} urea). Gels were run at 70 W, 55 mA, 2000 V for 2.5 h for OruE5/A8, and 2 h for OruS8/OruC4 and Oru10/OruS4. Gels were then dried in a vacuum dryer at 80°C for 1 h, and an autoradiography film was placed on them for 4 days. Films were then developed using an autodeveloper and alleles were scored by eye. Two individuals of known genotype (PCR product length) were examined with every 20 individuals to allow absolute lengths of alleles to be scored across gels.

Chemical analysis

In order to obtain volatiles of the male cuticular surface, a bee's abdomen was put into a vial with 1 ml pentane (99%, Sigma Aldrich Chemie GmbH), shaken for 1 min and then removed. The pentane was evaporated under a nitrogen stream to a volume of 30 μl, and an internal standard of 1 μg octadecane added.

Subsequently, the sample was analyzed using a gas chromatograph (GC) (HP 5890, Series II; Hewlett Packard, Palo Alto, CA, USA) equipped with a flame ionization detector with a

non-polar DB-5 column (30 m×0.25 mm i.d.×50 μm film; J&W, Levittown, PA, USA) and hydrogen (2 ml min^{-1}) as carrier gas. One μl of an odor sample was injected splitless at an initial oven temperature of 50°C. After 1 min, the splitting valve was opened and the temperature then increased by 10°C min^{-1} to 310°C, at which it was kept constant for 50 min. To ensure consistency of the analyses, a GC run with a synthetic alkane standard mixture was regularly performed. Structure elucidation of individual compounds was performed with an HP 6890 gas chromatograph (Hewlett Packard) connected to a mass selective detector (GCMS; Quadrupol 5972, Agilent, Santa Clara, CA, USA). The temperature program was the same as described above. Helium was used as the carrier gas (1.5 ml min^{-1} constant flow). Based on our previous work (Ibarra, 2002; Rosner, 1994), structure assignments were carried out by comparison of mass spectra and retention times of natural products with corresponding data from synthetic reference samples, using the NIST database and a database of the Institute of Experimental Ecology at the University of Ulm. Peak identities between different runs were confirmed by GCMS.

Statistics

For statistical analysis of the data, we used the software SigmaStat 3.1 (Systat Software, Chicago, IL, USA), Sigma Plot 9 (Systat Software), R (R Development Core Team, 2009) and SPSS 13.0 (SPSS Inc., München, Germany). All data were checked for deviation from a normal distribution using a Kolmogorov–Smirnov test. Because the laser vibrometry data were not normally distributed, those data were compared using Mann–Whitney U-tests. Relatedness was calculated using the program 'Relatedness' (Queller and Goodnight, 1989). A t-test was then used to compare the relatedness of accepted males to the female (mated pairs) *versus* rejected males to the female (unmated pairs), and a Mann–Whitney U-test was performed to compare the size of successful (accepted) *versus* unsuccessful (rejected) males. Because there was no difference between the size of males from the laboratory and those from the field, the two groups were pooled for further analysis.

Results of chemical analyses were compared using a principal component analysis (PCA) on the relative amounts of all 27 compounds detected. The resulting five principal components (PCs) with an eigenvalue above one were used in a stepwise statistical test for differences in odor bouquets by means of a discriminant function analysis. The standardized discriminant function coefficients and the factor loadings after varimax rotation were used to assess the importance of individual compounds. A compound was considered to have a high factor loading if the loading was above 0.5. Finally, relative proportions of individual compounds were compared between accepted and rejected males using Mann–Whitney U-tests.

For all male traits measured, we explored the relationship between male trait and female preference with cubic splines (non-parametric regression), using the method developed by Schluter (Schluter, 1988) (http://www.zoology.ubc.ca/~schluter/software.html). We used R to generate cubic splines using the mgcv library with bionomial errors of the (binary) response variable 'preference' (accept *versus* reject) and a logit link function. We also used logistic regression to test for the significance of the relationship between female preference and male trait, incorporating both linear and quadratic terms into the regression function to examine linear and non-linear relationships.

As described above, different individuals were used for each series of tests of size, vibration, relatedness and odor; therefore, relationships among criteria that females use to select an embracing male for copulation could not be explored.

RESULTS
Size

There was no significant difference in size between the accepted and the rejected males (Mann–Whitney U-test, $P>0.05$, $N=53$ for rejected and 34 for accepted) (Fig. 1). However, the shape of the female preference function for male size (Fig. 2) clearly indicates weak directional selection by females for males that are slightly larger than the mean male size. Both linear and quadratic terms of the logistic regression of female preference upon male size were significant ($P<0.001$, $N=87$).

Vibrations

The sound analyses showed that male vibrations occur in 1 to 9 trains (periods of vibrations), with each train consisting of approximately 10 to 220 bursts (Fig. 3). Because male vibrations could only be recorded after a precopulatory mating pair had already embraced and vibrations commenced, it was not possible to compare differences in the total numbers of trains or bursts among accepted (for copulation) *versus* rejected (for copulation) males. Accepted males showed significantly longer burst duration than males rejected before copulation (Mann–Whitney U-test, $P<0.05$, $N=23$). The shape of the female preference function (Fig. 4) clearly shows that females prefer males with a longer burst duration; all three males with a burst duration >600 ms were accepted. However, the linear and quadratic terms of the logistic regression of female preference upon male vibration duration were not significant ($P>0.05$). The time between bursts appeared to be shorter in accepted males (Fig. 3); however, the difference was not significant (Mann–Whitney U-tests, $P>0.05$).

Genetic relatedness

The six microsatellites exhibited, on average, six alleles per locus, with the most polyallelic locus, OruC4, having seven alleles. Reciprocal relatedness among full-sib nestmates was calculated using Relatedness as 0.443 [95% confidence intervals (CI)=0.378 to 0.502], a value not significantly different from 0.5, as expected among haplodiploid full sibs (a mix of brothers and sisters). The Relatedness software estimated individuals from different nests to be unrelated (0.019, 95% CI=–0.011 to 0.049). Therefore, the genetic markers provided good resolving power with which to determine relatedness (Goodnight and Queller, 1999).

There was a significant difference in genetic relatedness (r-value) of mated pairs (accepted male to female) compared to unmated pairs
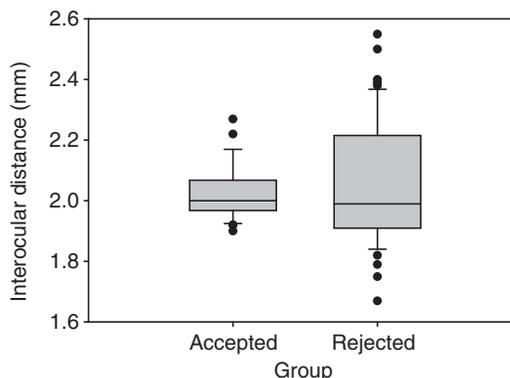

Fig. 1. Interocular distance of accepted and rejected males in laboratory experiments and collected from the field (pooled). The median, quartiles and outliers (circles) are shown. There is no significant difference between the two groups (Mann–Whitney U-test, $P>0.05$, $N=53$ rejected, 34 accepted).
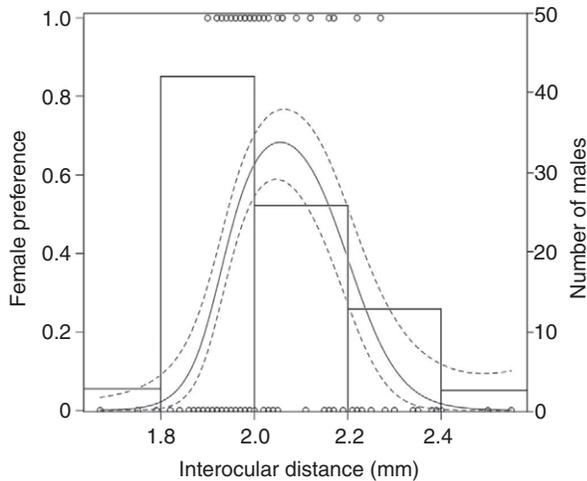
Fig. 2. The effect of male size (interocular distance) on the probability of male acceptance by a female when in a precopulatory embrace. A cubic spline (±1 s.e.) is fitted to data points using R. The histogram shows the observed size distribution of males, which have been binned into size classes for ease of visualization.

(rejected male to female) (*t*-test, $P<0.05$, $N=35$ for unmated and 97 for mated, mean mated $r=0.125\pm0.0361$, unmated $r=-0.047\pm0.0481$). As *r*-values derived from the Relatedness software are regression values and are therefore relative to the complete number of individuals measured, they can lie between -1 and 1, where a value of 0 indicates unrelated or random (with respect to relatedness) individuals, 1 indicates for clones and -1, in our case, indicates individuals from different populations (Queller and Goodnight, 1989). Female preference for males on the basis of relatedness (Fig. 5) showed a monotonic increase, suggesting that females rejected males from different populations. The linear term

of the logistic regression of female preference upon relatedness was significant ($P<0.05$, $N=132$) but the quadratic term was not.

Cuticular volatiles

We identified 27 substances on the cuticular surface of males, amongst them mainly alkanes and alkenes plus one ester (Fig. 6). In a PCA performed with the relative amounts of all of the compounds, five PCs explained 83.9% of the total variance. In a stepwise discriminant function analysis, only the first PC led to a significant difference in the odor bouquets of accepted and rejected males (Wilks' lambda=0.908, $\chi^2=4.782$, d.f.=5, $P=0.029$). According to the values of the standardized discriminant function coefficients and the factor loadings, 19 compounds contributed most to the discrimination between chosen and rejected males and explained 40% of the total variance. Alkenes with the double-bond positions 7 and 9 and also some alkanes contributed the most to the difference between male groups. In univariate comparisons of these components, two alkenes and two alkanes differed significantly between rejected and accepted males (Mann–Whitney *U*-test, $P<0.05$): accepted males had lower relative amounts of Z9-C23, Z7-C26 and C28 than the rejected males and higher amounts of C25 (Fig. 7). Females exhibited a clear preference for males with high quantities of C25 (Fig. 8A) and low quantities of C28 (Fig. 8B), yet their preferences for Z9-C23 and Z7-C26 were complex and not monotonic (Fig. 8C,D). Logistic regression terms of female preference upon male traits were non-significant for all odor components, possibly owing to small sample size.

DISCUSSION
The importance of size

Our finding that females had a preference for intermediate-sized males is in accordance with the results of Seidelmann (Seidelmann, 1999). There are many cases of females choosing larger males (Alcock, 1996a; O'Neill and Evans, 1983; Thornhill and Alcock, 1983), and it is generally understood that a larger male represents a more vigorous one (Eickwort and Ginsberg, 1980). However, large body size might
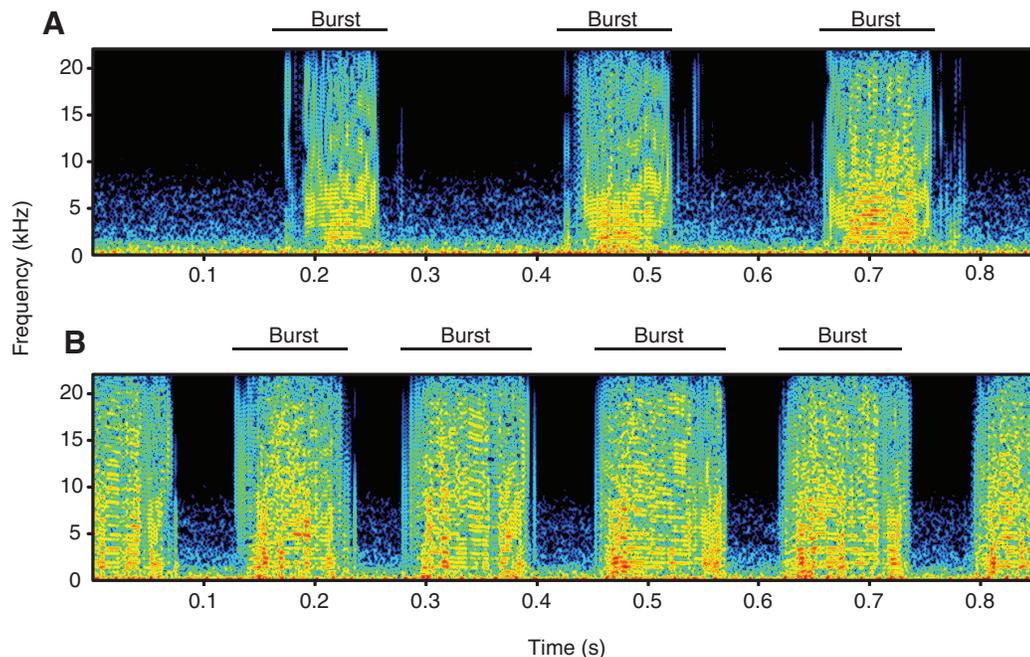

Fig. 3. Vibrogram (frequency over time) of thorax vibrations of a series of 'bursts' from a finally rejected male (A) and a finally accepted male (B). In the accepted male (B), the time between the bursts is shorter (not significant) and the bursts are longer ($P<0.05$, $N=23$) than in the rejected male (A).
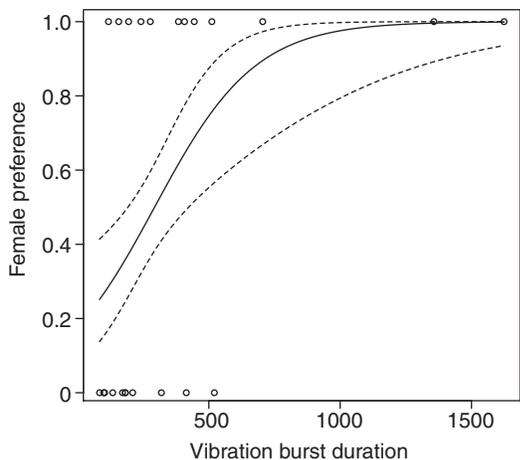
Fig. 4. The effect of male burst duration (number of bursts) on the probability of male acceptance by a female when in a precopulatory embrace. A cubic spline (±1 s.e.) is fitted to data points using R.

become a disadvantage after a certain threshold (Larsson, 1989). For example, in the burrowing bee *Amegilla dawsoni*, larger males face a much higher risk of predation and wing damage owing to male-to-male combat, which leads to a shorter lifespan than that of smaller males (Alcock, 1996b). Larsson also found a correlation between small-male mating success and increasing temperature in the sphecid wasp *Bembix rostrata* (Larsson, 1991). Lack of or weak directional selection on male size – selecting for males of intermediate size – seems typical of bees in which, like *O. rufa*, males practice scramble competition for females (Paxton, 2005). That the male size distribution of *O. rufa* is only subtly different from the shape of the female preference function is also consistent with the hypothesis of signal-preference coevolution (Ritchie, 1996).

The importance of male vibrations

Male *O. rufa* exhibit a pattern of vibrations that is characterized by a series of trains that are made up of several bursts. Bursts lasted significantly longer in successfully copulating males than in rejected
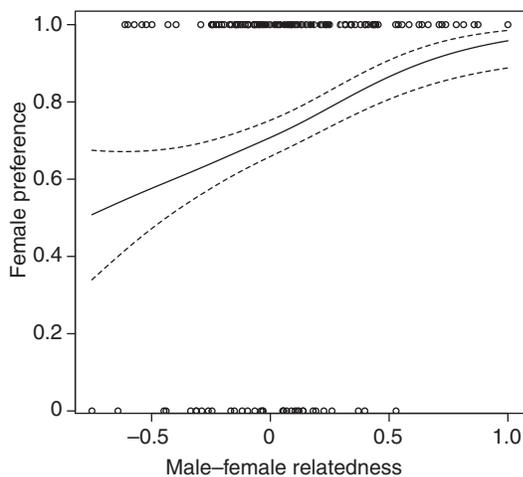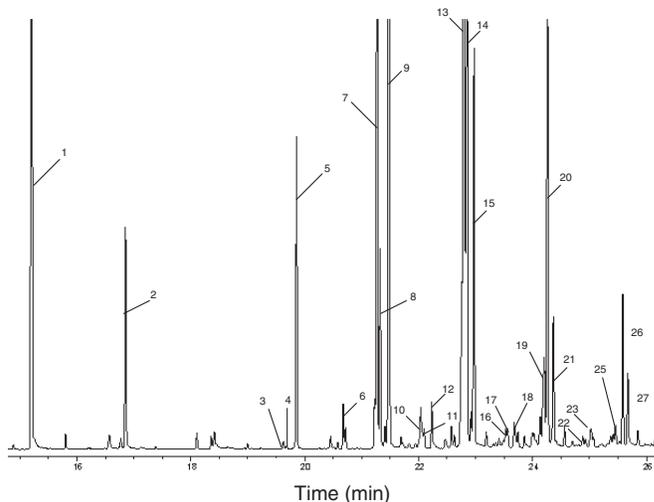

Fig. 6. Gas chromotography trace of a male cuticular extract. Chemical separations were performed on a non-polar DB-5 mass spectrometry (MS) column and numbered peaks were identified using GCMS.

1 Z7-Ethylhexadecenoate	15 Heptacosane
2 Henicosane	16 Z9-Octocosene
3 Z9-Tricosene	17 Z7-Octacosene
4 Z7-Tricosene	18 Octacosane
5 Tricosane	19 Z9-Nonacosene
6 Tetracosane	20 Z7-Nonacosene
7 Z9-Pentacosene	21 Nonacosane
8 Z7-Pentacosene	22 Z9-Triacontene
9 Pentacosane	23 Z7-Triacontene
10 Z9-Hexacosene	24 Triacontane
11 Z7-Hexacosene	25 Z9-Hentriacontene
12 Hexacosane	26 Z7-Hentriacontene
13 Z9-Heptacosene	27 Hentriacontane
14 Z7-Heptacosene	

ones. The time between bursts showed a trend towards being shorter in the males that were finally accepted, although this was not significant. Clearly, females prefer to copulate with males that exhibit longer vibrational bursts. Given that the production of vibrations requires thorax contractions that are presumably energetically costly (Gewecke, 1995), females might use vibrations
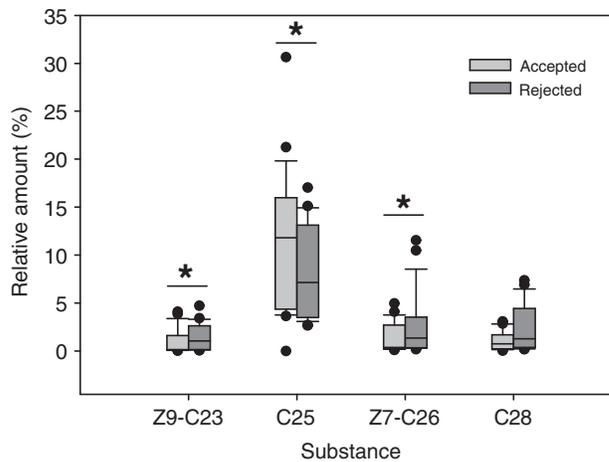

Fig. 5. The effect of male–female relatedness (genetic relatedness as derived from microsatellites) on the probability of male acceptance by a female when in a precopulatory embrace. A cubic spline (±1 s.e.) is fitted to data points using R.

Fig. 7. Relative amounts of cuticular volatiles of accepted (N=28) and rejected (N=24) males. The medians, quartiles and outliers (circles) are shown. Significant differences are marked by asterisks (Mann–Whitney U-test, P<0.05).
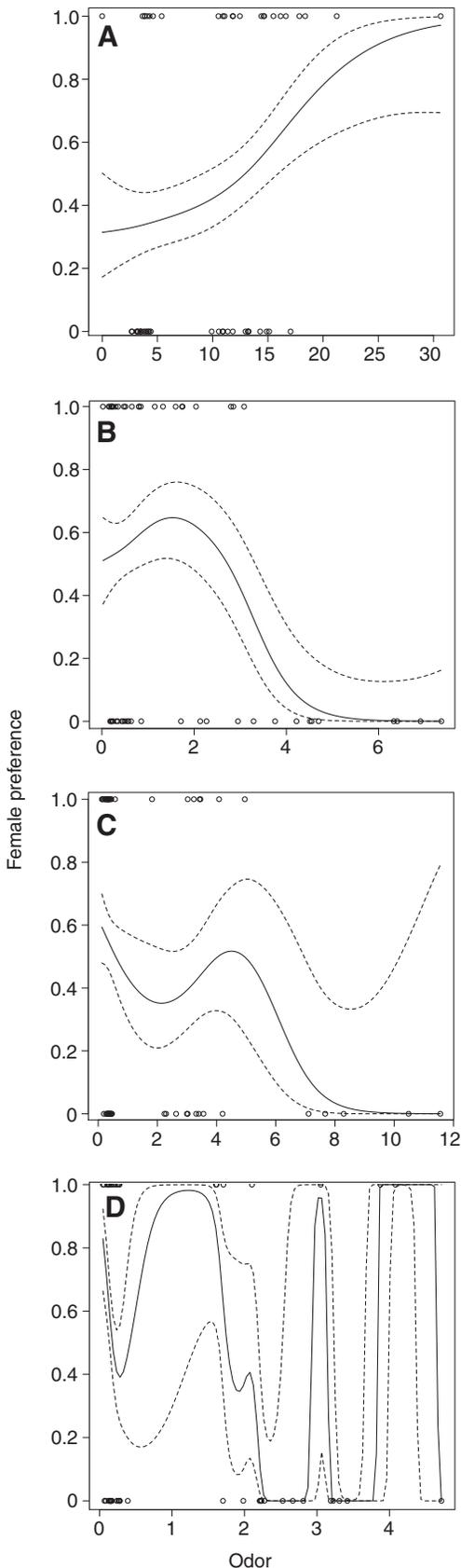
Fig. 8. The effect of male odor component (relative amount of an odor component) on the probability of male acceptance by a female when in a precopulatory embrace. A cubic spline (±1 s.e.) is fitted to data points using R. (A) C25, (B) C28, (C) Z7-C26 and (D) Z9-C23.

as a signal of vigor and health. This might also lead to discrimination against older males because they lack the energy to produce long bursts. Of relevance are Seidelmann's observations that older males even 'lose interest' in females (Seidelmann, 1995), suggesting that mating pairs including older males probably did not establish in our experimental setup.

Although there is little known about the importance of male vibrations in female choice, there is evidence that, in many cases, vibrations might be used by females to discriminate amongst males (Hill, 2008; Mappes et al., 1996; Rodríguez et al., 2006; Singer et al., 2000). Because the vibrational signals in *O. rufa* are based on muscle contractions and are, therefore, energetically costly, they are likely to represent an honest signal. Therefore, it is plausible that longer bursts and shorter times between bursts of *O. rufa* male vibrations are favored by females as honest signals of vigor. Experiments in which male vigor, health and age are manipulated while their precopulatory vibrations are recorded are needed to support these ideas.

Kin discrimination

Relatedness as a criterion in mate choice has been discussed in general and also with regard to bees in particular (Bateson, 1983; Partridge et al., 1987; Shields, 1982; Smith, 1979; Smith and Ayasse, 1987). According to our genetic analyses, a female accepts a male that is more related to her over males that are genetically more distant.

Inbreeding in Hymenoptera with complementary sex determination (CSD) increases the production of inviable or sterile diploid males (Zayed, 2009). Therefore, at first glance, our results are counterintuitive because one might anticipate females to preferentially reject close kin as mating partners. However, there is little evidence that females of other Hymenoptera selectively accept or reject males on the basis of signaling of sex alleles at the CSD locus (see Paxton et al., 2000).

On closer inspection, our results may rather be interpreted in terms of optimal outbreeding (Bateson, 1983). Our investigation was performed with a mixed artificial population consisting of individuals from Ulm, Vienna and Gatersleben. Therefore, females, while choosing a mate, may have had to select between males that were either closely related, distantly related from the same population, or from a different population. Selected males were more related to their mates than rejected males; this difference in relatedness may be interpreted under our experimental paradigm as females selecting males from their own population and not from a distant population. Selecting a male from the same population may be a reasonable choice for the female considering that the individuals in a population that the female belongs to probably have valuable adaptations to the local microhabitat, which a male from a different population might lack (Bateson, 1983). Alternatively, or in addition, females may be selected to reject heterospecific males, which might be interpreted as a lower relatedness of rejected *versus* accepted males in our experiment. Whether *O. rufa* females also selectively reject close kin as mates, as we originally expected, will require explicit testing of their mate selection using related *versus* unrelated males from the same population. It will also require a different experimental paradigm in which a female can select between two or a few males, rather than our current design in which a female was offered *ca*. 40 males (*ca*. 10% brothers) in a large arena and in which we analyzed mating pairs that had already engaged in pre-copulatory embrace.

The role of odor

It has been repeatedly demonstrated that odor bouquets can be used to identify relatedness in a range of animals, including bees (Ayasse et al., 2001; Busquet and Baudin, 2005; Gerlach and Lysiak, 2005; Olsén, 1989; Smith, 1983; Sun and Müller-Schwarze, 1997). We found that there were differences between the scents of accepted and rejected males. The relative amounts of C28 were significantly lower in accepted versus rejected males whereas C25 was present in higher relative amounts in accepted males. The relative amounts of Z9-C23 and Z7-C26 exhibited a complex relationship with female acceptance. The females used in our mate-choice experiments were a random collection of all the genotypes from our mixed population that may represent different odor phenotypes (Vienna, Ulm or Gattersleben). Therefore, the differences in the odor bouquets we found between accepted and rejected males is unlikely to be due to some form of kin discrimination or preference. If they were indeed kin signals, then, under the hypothesis of a self-referent template, some females might be expected to choose males with higher relative amounts and some with lower amounts, depending on their own odor bouquet and kinship to the potential mate. In this respect, Z9-C23 and Z7-C26 are candidate kin discriminators.

An explanation of our results could be that odor is used as another signal of vigor. For example, young and old males may produce different odor bouquets and a female may select for younger, more vigorous males, as already shown in *Drosophila melanogaster* (Howse et al., 1998). It is also possible that the higher or lower relative amounts of odor components of selected males signal vigor independent of male age but correlated with male size, burst duration or health. In that case, we hypothesize that smaller males are only capable of producing lesser amounts of the alkane C28 and greater amounts of C25. However, because we did not use the same individuals for size measurements, vibration experiments and chemical analyses, a statistical test of these associations is not possible. Further studies will have to explicitly test this hypothesis, and gas chromatography with electroantennographic detection (GC-EAD) might be a useful technique to determine those compounds perceived by the female.

A female probably detects a male's odor during precopulatory courtship, when the male strokes the female's antennae with his own or when the female contacts the male body surface with the chemoreceptors of her own antennae. Future investigations will have to clarify the role of olfactory recognition cues in kin recognition for mate selection by *O. rufa*.

In summary, *O. rufa* females seem to use size, vibration and odor as signals in mate selection, and relatedness using currently unknown mechanisms. An important question is how these diverse signals are integrated by a female to select the most suitable mate (Candolin, 2003). *O. rufa* offers a model to experimentally investigate the extent to which these cues provide complementary information to a female in mate selection.

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