



Age-related changes in cuticular hydrocarbon bouquet and same-sex sexual behavior of mason bee males (*Osmia cornuta* and *O. bicornis*)

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Abstract – Finding a mate is the central task in the lives of many male insects. In many solitary bees, sexual competition between males is high due to monandry and a pronounced male-biased sex ratio. Males searching for females will inspect and mount any object of suitable size and habitus. Species, sex, and reproductive status are encoded in the bee's bouquet of cuticular hydrocarbons (CHCs) and can only be identified at a short distance from the object encountered. In two species of mason bees, this CHC-based system for recognizing newly emerged, virgin females is not error-free. Newly emerged individuals of both sexes carry similar CHC bouquets, facilitating the confusion of newly emerged males with newly emerged, virgin females. Misidentification leads to same-sex sexual behavior by males seeking a mate. However, individuals of both sexes modify their CHC bouquet sex-specifically within a few days. The changes in the bouquet prevent further sexual harassment. In addition, within 3 days of emergence, males begin to release species-specific carboxylic acid esters, which may act as courtship-inhibiting pheromones to extend the range of their sex-specific scent tag. Due to the pronounced protandry of both species, SSB is restricted to the early flight season, when females are not yet present, and imposes no apparent cost on the two males involved.

cuticular hydrocarbons / sex-specific bouquets / mistaken identity / courtship inhibiting pheromone / isopropyl-9-hexadecenoate / ethyl-9-hexadecenoate

1. INTRODUCTION

Same-sex sexual behavior (SSB) is a widespread phenomenon in animals (Bailey and Zuk 2009). Even males of solitary mason bees, such as the horn-faced mason bee *Osmia cornuta* Latreille and the red mason bee *Osmia bicornis* Linnaeus (syn. *Osmia rufa* L.), occasionally engage in same-sex sexual behavior (SSB) early in the flight season (Figure 1).

Courting males regularly display the generic courtship behavior (Seidelmann 2014) and attempt to copulate with mounted males. SSB has generally been associated with several adaptive benefits as well as mistakes (Bailey and Zuk 2009; Scharf and Martin 2013; Lerch and Servedio 2021). Benefits of SSB have been described as preventing other males from accessing females, harming other males, dumping of old sperm, transferring sperm to the female via another male, and reduction of male aggression. Other adaptive explanations include the mitigation of risky male-male contest behavior (Kuriwada 2017), the assessment of rival quality (Abbassi and Burley 2012), or

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Figure 1. Couple of two *O. cornuta* males (recognizable by the white hair coats of the clypei). The mounted male had performed the courtship behavior and just starts an intromission attempt.

the establishment of dominance hierarchies as an extension of male-male competition (Lane et al. 2016). In all these cases, opponents are able to identify each other as members of the same sex and the courting male benefits. In addition, SSB is also observed due to female mimicry where some males produce key stimuli of receptive females. These “she-males” confer a fitness advantage, e.g., by provoking courtship to be wormed up by conspecifics in order to gain agility faster after hibernation and suffer less from predation, as in *Thamnophis* garter snakes (Shine et al. 2012), or by a reduced aggression and access to resources, as in *Aleochara* beetles (Peschke 1985). Alternative non-adaptive explanations for SSB, where neither the active nor the passive males benefit, include a simple spillover of courtship behavior (Rayner and Bailey 2019); the “prison effect,” where a scarcity of females induces SSB as a mere outlet of sexual stimulation (McGraw and Hill 1999; Bonnet et al. 2016); and, most importantly, mistaken identity (Bailey and Zuk 2009; Bailey and French 2012). The mistaken identity hypothesis includes cases where individuals are unable to distinguish between the sexes and fail to recognize real mates. Misidentification has been invoked to explain the majority of SSB cases observed in insects (e.g., 79 out of 100 examples listed by (Scharf and Martin 2013), supplementary material). Erroneous mountings can occur due to errors in processing sensory information or because the target individual resembles

an opposite-sex mating partner (Bailey and French 2012). In insects, pheromones deposited by opposite sex partners during a previous mating or a lack of sex-specific characteristics may account for such misperceptions (Bailey and French 2012). In particular, males that temporarily display female-like phenotypes may be misidentified by their interacting partners, as in the case of *Lariophagus* wasps (Steiner et al. 2005; Ruther and Steiner 2008).

In mason bees, as in many other solitary bees, males are protandrous and emerge before females (Ayasse et al. 2001; Paxton 2005). The sex ratio is strongly male-biased (Raw 1972; Bosch and Vicens 2005). Females of both mason bee species are monogamous and immediately receptive when they emerge from the maternal nest (Raw 1972; Bosch et al. 2008; Seidelmann 2014). Thus, protandry is a strategy to avoid missing mating opportunities (Kokko et al. 2014). Males are unable to monopolize females and engage in a race-like scramble competition for matings by patrolling nest sites and flowers to be the first to spot a virgin female (Raw 1976; Seidelmann 1999). Males bounce on any female-like objects present at rendezvous sites. However, like most other insect males, they rely on volatiles to identify an object as a potential mate (Dutzler and Ayasse 1996; Thomas 2011). Females of both mason bee species do not release specific volatile compounds that serve as sex pheromones to attract males (Seidelmann and Rolke 2019) but are identified by their virgin CHC bouquet (Ayasse et al. 2000), which changes within a

few days after emergence (Seidelmann and Rolke 2019). *O. bicornis* males are tuned to the virgin bouquet and are able to discriminate between receptive and non-receptive females (Dutzler and Ayasse 1996; Ayasse et al. 2000).

Positive contributions of SSB to fitness remain suspect in both *Osmia* species due to their reproductive biology. Although the operational sex ratio is always high, SSB is not a permanent phenomenon but is limited to the early flight season when males emerge. Strikingly, in same-sex couples, only newly emerged males are mounted, not older ones, making a spillover or prison effect implausible. The most likely explanation for homosexual mating attempts in both *Osmia* species, therefore, seems to be the mistaken identity hypothesis (Bailey and Zuk 2009; Scharf and Martin 2013). Males searching for receptive females may fail to determine the sex of newly emerged individuals. To test whether the mistaken identity hypothesis applies to SSB in *O. cornuta* and *O. bicornis*, the CHC bouquet of males was monitored during the first days of emergence from the overwintering cocoon. These CHC profiles were compared with the CHC bouquets of virgin and mature females to check for similarities and differences. The release of male-specific volatiles during the first few days also needed to be monitored. *O. bicornis* males produce ethyl (*Z*)-7-hexadecenoate (7-C16:1-EE) in a sternite gland, a substance assumed to be an antiaphrodisiac (Ayasse and Dutzler 1998; Ayasse et al. 2000). *O. cornuta* males have a similar gland, but the substances produced are still unknown. If male-specific volatiles were present at emergence, they could serve as sex-specific markers, ruling out the mistaken identity hypothesis.

2. MATERIAL AND METHODS

Cocoons of *O. cornuta* and *O. bicornis* were collected from regularly maintained populations located at the Botanical Garden of Halle/Saale (Germany, Saxony-Anhalt, 51° 29' 04" N, 11° 56' 07" E). Individuals of known age were reared from cocoons stored at 4 °C after overwintering

in the laboratory. Prior to the experiments, the bees were sexed according to the hair color of the clypeus by gently opening the cocoons at the top. To allow emergence, male cocoons of the same sex were transferred to emergency boxes and kept at room temperature (21–22 °C) with natural daylight regime. Bees emerged preferentially in the morning hours (Beer et al. 2019). Bees that have emerged before noon on the same day were transferred to rearing boxes and kept separated by date and sex. Males have been sampled on a daily basis, but females were sampled only at two age classes for reference. Bees were fed on a honey/water solution (2/1) until sampling.

Bees were freeze-killed, and the CHCs were washed off the bodies individually in small glass vials (1.5 ml) with PTFE-sealed screw-caps (Phenomenex) by adding 300- μ l (females) or 200- μ l (males) n-hexane (AppliChem). Vials were gently agitated to rinse the entire body surface with solvent and immediately stored at –21 °C to dissolve CHCs and quantitatively extract sternite glands and other possible scent glands overnight. Bodies were removed after 24 h, and extracts were stored at –21 °C until analysis. An accompanying test with different extraction times confirmed that the extended extraction time had no effect on the age-related changes in the CHC bouquet (see ESD for details). The fourth and fifth sternites of 5-day-old males, bearing an outstretched, glandular tissues on the inner side (named sternite gland thereafter), were surgically removed and extracted. This extract was compared with extracts from tergites and other parts of the body (e.g., heads) to identify the gland-specific compounds against the CHC compounds still present in the gland extracts.

Hexane extracts were analysed by GC/EI-MS (Varian Saturn 2100 T, MS-workstation 6.20; GC: Zebtron ZB5-ms column 30 m \times 0.25 mm \times 0.25 μ m, splitless injection at 220 °C, helium at 1.0 ml/min, 1 min at 100 °C, 20 °C/min to 200 °C, 5 °C/min to 300 °C, 5 min at 300 °C; MS: EI auto at 70 eV, mass range 40–650 m/z). Compounds were initially identified by searching the NIST mass spectral library search (v. 2.0a, build 2002) and validated by retention times (Kováts-index for

temperature programmed GC, calculated from an n-alkane (C8-C40, Supelco) standard for the 5% diphenyl/95% dimethyl polysiloxane stationary phase). Mass spectra were verified by pure reference substances and reference GC/MS chromatograms of previously identified CHC compounds in both species (Seidelmann and Rolke 2019). Due to the small amounts and co-elution of alkenes with double bonds in position 11 and higher, it was not possible to differentiate between these alkenes. Thus, n-alkenes with a double bond position higher than 9 were summarized as 11+alkenes. The positions of the double bonds in alkadienes, which were present in trace amounts throughout, could also not be identified. The double bond position of the two novel carboxylic esters found in the sternit gland of *O. cornuta* males was determined by methylthiolation reaction with dimethyl disulfide adducts (Dunkelblum et al. 1985) (see ESM for details). The identity of the newly discovered esters was verified by co-chromatography of pure substances. Compound quantification was based on the largest diagnostic mass fragment. All compounds present in quantifiable amounts were initially included in the evaluation. However, only those compounds present in all samples were retained for statistical analysis.

The perception of volatile compounds as the male-specific carboxylic esters was verified using an electroantennogram detector (IDAC-232, Syntech) coupled to a GC (Varian CP-3800, with PTV injector, EC-5 column (30 m × 0.32 mm × 1 μm, helium at 1.5 ml/min, 3 min at 50 °C, 50 °C/min to 200 °C, 5 °C/min to 320 °C, 5 min at 320 °C, outlet split 1:15 (FID/EAD) with 15 ml/min make-up gas; 5 μl sample at large volume injection, PTV: 0.2 min at 50 °C, 200 °C/min to 350 °C, split program: 20 at start, 0 at 0.25 min, 100 at 3 min, 10 at 4 min).

CHCs were statistically treated as bouquets, and the male-specific carboxylic esters were generally excluded from the analysis. Due to the compositional nature of the data, the analysis was based on relative proportions. To deal with potential non-linear intensity bias, e.g., due to body size-dependent total substance amounts, data were quantile-normalized (Bolstad et al. 2003) using the “preprocessCore” package (Bolstad 2016) for R (R Core Team 2021). Each normalized

peak area was centered log-ratio transformed:

$$clr(Y_{ij}) = \ln\left(\frac{Y_{ij}}{g(Y_j)}\right)$$

with Y_{ij} as the area of the peak (i) for bee (j), and $g(Y_j)$ is the geometric mean of all peak areas for bee (j). The multitude of compounds was reduced by principal component analysis (PCA, regression method), extracting all factors with an eigenvalue > 1 to handle potential problems of collinearity. Two discriminant function analyses (DA) were performed on the PCA scores to (1) discriminate newly emerged versus 6-day-old males to monitor age-related changes in the CHC and (2) to check for age-related differences in CHCs between the sexes. The similarity of samples that scattered nearby in the DA was checked by a hierarchical cluster analysis. A (dis)similarity matrix based on squared Euclidean distances (SED) was calculated for all individuals in both groups. Differences in similarity scores between individuals of one and the same or of different sex were tested using a binomial-logit generalized linear model (GLM, Wald test statistics). Peak areas of male-specific carboxylic esters were log-transformed prior to a statistical analysis. To visualize the onset of release of male specific esters, exponential rise to maximum functions $y = y_0 + a(1 - e^{(-bx)})$ were fitted. Changes of the CHC bouquet were represented by sigmoidal functions $y = \frac{y_0+a}{1+e^{(-\frac{x-x_0}{b})}}$.

ANOVA with Scheffe post-hoc test was used to test for age-related differences in the male-specific substances and age-related differences in the calculated score of the discriminant function. Values are expressed as mean ± standard deviation. The error level was set at $\alpha = 0.05$, and SPSS (IBM, v. 28) was used for statistical analyses.

3. RESULTS

Hexane washes of both species did not contain any sex-specific compounds other than the carboxylic acid esters present in older males (see below). In *O. cornuta*, two other carboxylic acid esters and two ketones were found in trace amounts (Table SI). However, they did not elicit a detectable electroantennogram response. No

other bee-specific substances were detected in the odor fractions (Kováts < 2100) of the washes.

The CHC-bouquets of newly emerged males of both species were composed of alkanes and alkenes with chain lengths of 21 to 31 carbons. In *O. cornuta*, n-alkanes accounted for a total peak area of $45.07 \pm 4.74\%$ and their corresponding alkenes for a total of $49.36 \pm 5.57\%$ (Figure 2a). However, particularly pentacosenes and in general molecules with their double bond in position 7 ($35.75 \pm 3.64\%$) dominate the alkene fraction (pos. 9: $13.15 \pm 2.25\%$; pos. 11+: $0.30 \pm 0.15\%$; pos. 5: $0.16 \pm 0.07\%$). Methyl-branched alkanes ($4.62 \pm 1.00\%$), alkadienes ($0.90 \pm 0.22\%$), and other compound (total $0.05 \pm 0.02\%$) were found only in traces or small amounts. In general, odd-numbered alkanes ($42.86 \pm 4.68\%$) and alkenes ($48.17 \pm 5.40\%$) predominated in general. Pentacosane was the prominent alkane and 7-pentacosene was the dominant alkene. The CHC composition of freshly emerged *O. bicornis* males (Figure 2b) was remarkably

different from that of *O. cornuta* (ANOVA: factors species [sp] and substance class [cl], $F_{SP \times CL} = 33.443$, $df = 6$, $P < 0.001$). The contents of alkanes ($46.62 \pm 12.24\%$) and alkenes ($52.40 \pm 11.56\%$) were in a comparable range. The double bonds of n-alkenes were located at positions 5 ($1.28 \pm 0.67\%$), 7 ($18.73 \pm 5.14\%$), 9 ($31.31 \pm 7.55\%$), and 11+ ($1.07 \pm 0.29\%$). Alkadienes (chain lengths of 29 and 31 carbons) were found in small amounts (total $0.98 \pm 0.94\%$). Carboxylic acid esters were found only in trace amounts. In general, odd-numbered alkanes ($43.73 \pm 11.37\%$) and alkenes ($50.57 \pm 11.62\%$) predominated, with pentacosane being the prominent alkane and heptacosenes the dominant alkenes, respectively.

The composition of the CHC bouquet changed in males of both mason bee species within less than 1 week after emergence from the cocoon. In *O. cornuta*, a total of 41 CHC compounds were detected in all individuals (Figure S1; a total of 50 samples from 0 to 6 days old) and reduced by PCA

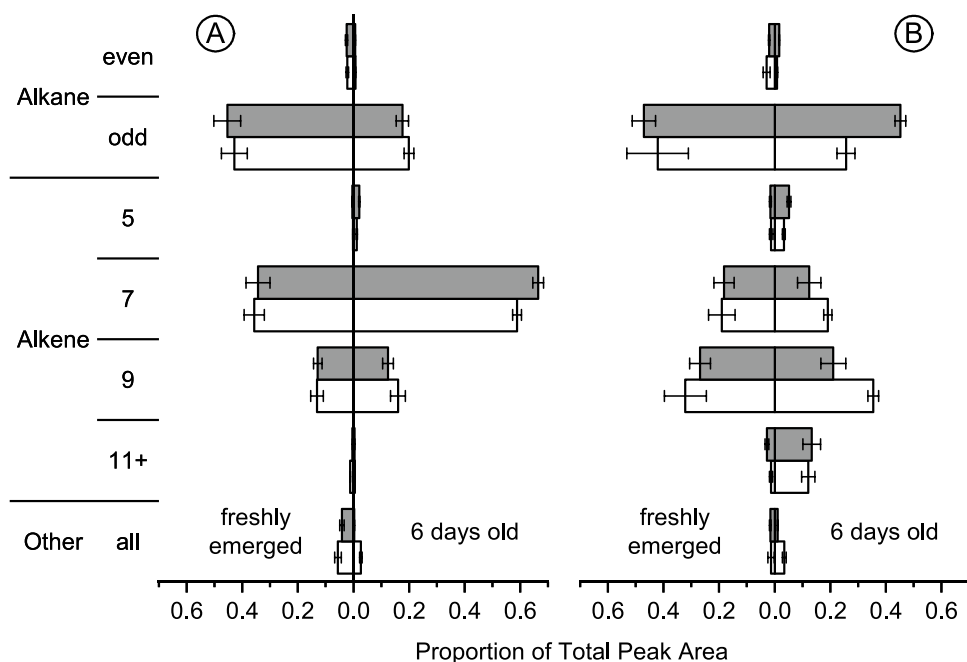


Figure 2. Composition of the CHC bouquet of *O. cornuta* (a) and *O. bicornis* (b) males (white columns) and females (dark columns). Left-handed columns refer to freshly emerged and right-handed columns to 6 days old bees; whiskers indicate standard deviations.

to 6 factors explaining 84.35% of the total variance. Newly emerged males could be distinguished from 6-day-old males by a canonical discriminant function (eigenvalue 161.177 accounting for 100% of the variance, $\lambda = 0.006$, $\chi^2 = 76.875$, $P < 0.001$). Males differed in the scores of the discriminant function depending on their age from 1 day on (ANOVA: $F_{4,50} = 81.266$, $P < 0.001$, Figure 3a). The amount of odd numbered alkanes decreased in older males (total alkanes $20.72 \pm 1.81\%$) while the prevalence of 7-alkenes continued to increase (total alkenes $76.61 \pm 1.82\%$, Figure 2a).

In *O. bicornis* males, 37 substances were detectable in all individuals (Figure S2; a total

of 67 samples ranging from 0 to 6 days). These components were reduced by PCA to 5 factors explaining 82.06% of the total variance. The scores of the canonical discriminant function modeled to distinguish freshly emerged from old males (eigenvalue 31.234 accounting for 100% of the variance, $\lambda = 0.031$, $\chi^2 = 81.616$, $P < 0.001$) and subsequently calculated for all samples differed for males up to 3 days of age (ANOVA, age as factor: $F_{5,67} = 164.334$, $P < 0.001$, Figure 3b). The changes in the hydrocarbon composition concern odd-numbered alkanes and the composition of alkenes with respect to the position of the

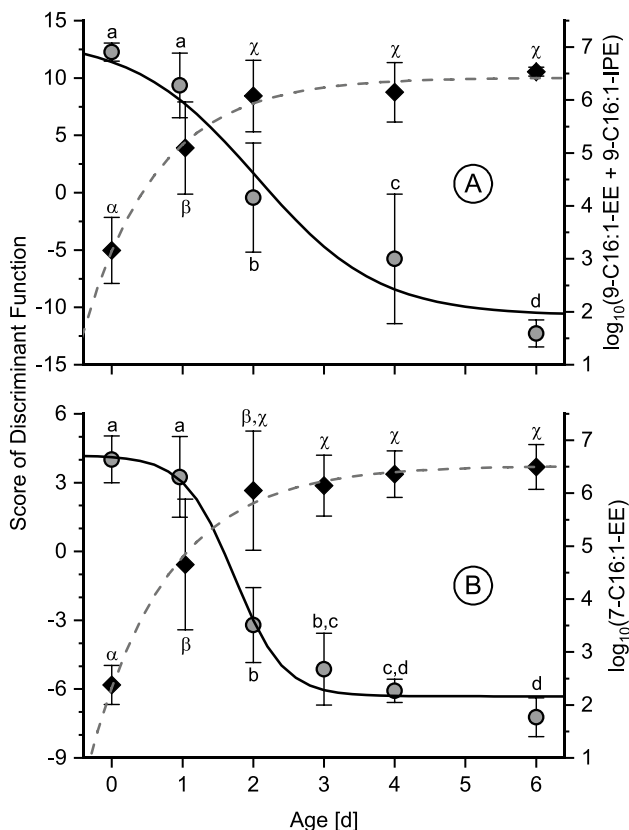


Figure 3. Shift of the CHC based discriminant function score (circles) and release of male-specific carboxylic esters from the sternal gland (diamonds) in *O. cornuta* (a) and *O. bicornis* (b) males within the first days after emergence from the cocoon. Solid lines represent four parameter logistic regression of the discriminant score (*O. bicornis*: $R^2 = 0.92$; *O. cornuta*: $R^2 = 0.87$), dashed line a three parameter exponential rise to maximum regression (*O. bicornis*: $R^2 = 0.71$; *O. cornuta*: $R^2 = 0.82$), respectively; whiskers refer to standard deviations; letters indicate homologs groups.

double bond (Figure 2a). The content of alkanes decreased to $27.23 \pm 3.16\%$, while that of alkenes increased to $69.23 \pm 2.76\%$. In contrast to *O. bicornis* females, where the dominance of 9- and 7-alkenes decreased in favor of alkenes with a double bond in position 11+ as well as in position 5, the proportion of 9- and 7-alkenes also increased in older *O. bicornis* males (Figure 2b).

The CHC bouquet of freshly emerged males of both mason bee species was quite similar to that of freshly emerged conspecific females (Figure 2). After emergence, however, the CHC profiles developed in different directions, resulting in differences in the relative amounts of alkanes and alkenes. In *O. cornuta* a PCA reduced the 41 substances of the CHCs found in both sexes to 6 factors explaining 83.97% of the total variance. Bees could be distinguished according to their age and sex by

three canonical discriminant functions (eigenvalues 109.182, 28.660, and 2.908, respectively, accounting for 100% of the variance, all $\lambda \leq 0.256$, all $\chi^2 \geq 46.334$, all $P < 0.001$). Newly emerged individuals of both sexes scattered partially overlapping (Figure 4a), whereas older individuals were widely separated. There was no difference in the similarity of CHCs of freshly emerged individuals of one and the same sex (mean $SED = 8.95 \pm 14.88$, $n = 90$) compared to individuals of the opposite sex ($SED = 11.15 \pm 14.79$, $n = 100$; binominal-logit GLM: $n = 190$, $W = 1.038$, $P = 0.308$). In *O. bicornis*, a PCA reduced the 37 substances found in both sexes to 6 factors explaining 83.26% of the total variance. A discriminant analysis extracted 3 canonical discriminant functions that significantly contributed to discriminate individuals according to their age and sex (eigenvalues 42.817, 13.692,

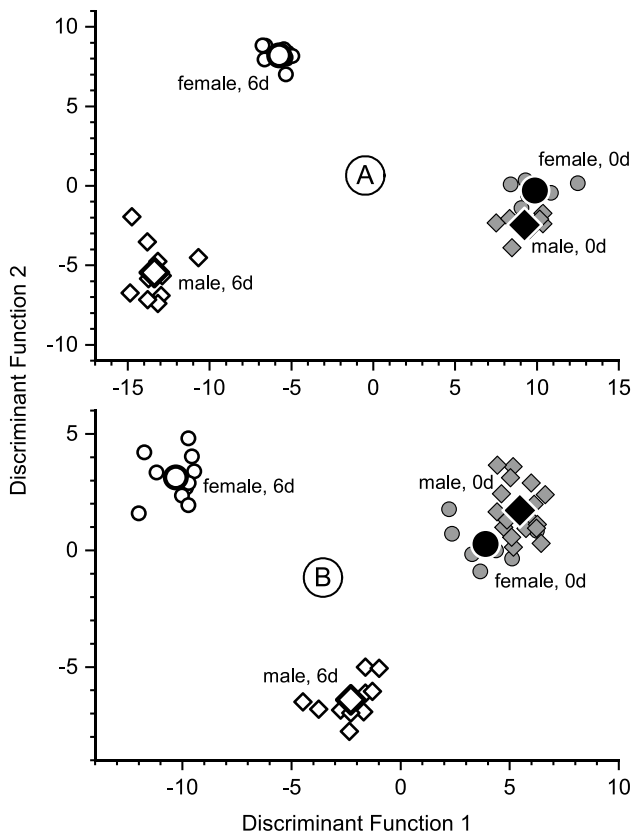


Figure 4. Scatter charts discriminating *O. cornuta* (a) and *O. bicornis* (b) individuals according to their sex and age. Circles represent females and diamonds males; filled symbols indicate freshly emerged and open symbols 6-day-old bees.

and 1.843, accounting altogether for 100% of the variance, all $\lambda \leq 0.352$, all $\chi^2 \geq 40.750$, all $P < 0.001$). As in *O. cornuta*, 6-day-old individuals were clearly distinguished by sex, while freshly emerged individuals of both sexes were scattered nearby (Figure 4b). The CHC similarity of freshly emerged *O. bicornis* individuals of one and the same sex (mean SED = 14.89 ± 11.23 , $n = 174$) did not differ from the similarity of CHCs between individuals of the opposite sex (SED = 15.87 ± 7.50 , $n = 126$; binominal-logit GLM: $n = 300$, $W = 0.729$, $P = 0.393$).

Males of both species possess a sternit gland that produce large amounts of electroantennogram-active male-specific unsaturated carboxylic acid esters, which dominated the hexane extracts of older individuals (Figures S1 and S2). A few days old *O. cornuta* males emitted two substances. The first one had a Kováts index of 1973 and was identified as ethyl-9-hexadecenoate (9-C16:1-EE, Figure S3) on the basis of the diagnostic fragments 237 m/z, 282 m/z (M^+) and its DMDS-adducts. The second substance had a Kováts index of 1997, diagnostic fragments of 237 m/z, 254 m/z, 296 m/z (M^+), a double bond also at position 9 (Figure S4), and was identified as isopropyl-9-hexadecenoate (syn: propan-2-yl hexadec-9-enoate, 9-C16:1-IPE). These carboxylic acid esters were found in 6-day-old males at levels corresponding to $14.3\% \pm 3.6\%$ and $20.8\% \pm 6.2\%$ of the total CHC peak area, respectively. Both compounds were released in a constant ratio independent of age and total amount (partial correlation, age as control variable, $df = 47$, $r = 0.963$, $P < 0.001$). *O. bicornis* males produced 7-C16:1-EE in their sternit gland (Kováts index 1968, diagnostic fragments 237 m/z, 282 m/z (M^+), double bond at position 7 verified by DMDS-adducts, Figure S5) at a peak area of about half of the sum of the total CHC ($47.1\% \pm 45.0\%$) at 6 days of age. However, these male characteristic carboxylic acid esters were not yet detectable in males of both *Osmia* species just emerging for their cocoon. Newly emerged individuals (age < 24 h) released only traces or weak amounts (Figures 3, S1 and S2). Within the first few days after emergence from the cocoon, the release of male-specific esters

increased exponentially to a maximum. The full emission range (95% of the calculated maximum) was reached within 3 days (2.87 days in *O. bicornis*, 2.44 days in *O. cornuta*; based on the estimated functions; Figure 3).

4. DISCUSSION /CONCLUSION

CHCs not only serve as a protective layer on the insect cuticle but also provide important information in intra-specific communication to identify sex, age, reproductive stage, etc. (Ginzel et al. 2020; Blomquist and Ginzel 2021; Holze et al. 2021). In this regard, CHCs are signature mixtures (Wyatt 2010) that have not necessarily evolved as pheromones but act as cues to classify individuals (Thomas 2011). The informative function is based on the plasticity in CHC composition due to the actual state of an individual (Holze et al. 2021). CHC cues play an important role in the communication system of social species (Kather and Martin 2015), but solitary bees also use CHC profiles to identify potential mates (Ayasse et al. 1999; Paulmier et al. 1999; Simmons et al. 2003; Mant et al. 2005). Also, in the two species of mason bees studied here, differences in the CHC bouquets allow a distinction between receptive and brood-active females (Seidelmann and Rolke 2019). The differences are due to changes in the mixing ratio of the same spectrum of compounds rather than the appearance or disappearance of specific compounds. In particular, the relative amounts of alkenes and the proportions of different double-bond positions change after emergence in both species, as has been observed in other solitary bees (Paulmier et al. 1999; Mant et al. 2005). Compound structure (presence and position of double-bonds and side chains) is generally more important than chain length. Insects can discriminate between compounds that differ in the position of their double bond or methyl group at a given chain length but are unable to discriminate between different homologs (van Wilgenburg et al. 2010; Kather and Martin 2015). Thus, in both *Osmia* species, the composition of alkenes may serve as a cue to identify receptive females, analogous to other

solitary bees (Paulmier et al. 1999; Mant et al. 2005; Saul-Gershenz and Millar 2006).

The monandry of females on the one hand and the pronounced male-biased sex ratio on the other hand lead to an extreme asymmetry in mate availability in both *Osmia* species (Raw 1976; Seidelmann 1999; Bosch and Vicens 2005). Males face strong competition to be the first to spot a virgin female (Thornhill and Alcock 1983; Kokko et al. 2014) and to discriminate between unmated and mated females (Thomas 2011). As females are receptive at emergence, the CHC bouquet of overwintered cocooned bees still present when they leave the maternal nest provides a reliable cue for identifying potential mates. This is especially true as females of both *Osmia* species change their CHC bouquet within a few days after emergence through an ontogenetic process that coincides with a decrease in mating readiness of young females (Seidelmann 2014; Seidelmann and Rolke 2019). Thus, it can be hypothesized that males are evolutionarily tuned to the bouquet of newly emerged individuals and use age as a proxy to discriminate between mated and unmated females (Thomas 2011). However, at the time of emergence, males of *O. cornuta* and *O. bicornis* share the same CHC pattern with conspecific newly emerged virgin females. Similar CHC phenotypes in newly emerged adults of both sexes are not uncommon and have been described, for example, in wasps (Steiner et al. 2005; Ruther et al. 2011; Hrabar et al. 2012; Todoroki et al. 2015) or beetles (Peschke 1985). As a side effect of the mate recognition system, newly emerged males may be mistaken for virgin females by mate seeking males due to their CHC bouquet of cocooned conspecifics, leading to the observed SSB in *O. cornuta* and *O. bicornis*.

SSB has always been associated with apparent fitness costs (Bailey and Zuk 2009; Scharf and Martin 2013). The most obvious cost of SSB for the two *Osmia* males involved is a missed mating opportunity. During the period of male emergence, mate recognition is not error-proof and males run the risk of erroneously mounting and courting a newly emerged male instead of a virgin female. The acceptance threshold (Reeve 1989) used by males to perceive potential mates

should maximize the fitness payoff between the cost of erroneously courting a male and the benefits of accepting a receptive female (Engel et al. 2015). The perceived cost of making an acceptance error are negligible due to the distinct protandry in both mason bee species compared to the severe fitness cost of making a rejection error due to the monandry of females and the male-skewed sex ratio. As predicted for animal species where female and male recognition cues overlap, males make permissive discrimination decisions and SSB results from an adaptive strategy to avoid rejection errors (Engel et al. 2015).

Apart from potential costs, SSB may provide adaptive benefits to one of the males involved (Bailey and Zuk 2009; Scharf and Martin 2013). Carrying a female-like CHC bouquet could be advantageous for emerging males if they are assisted by older males in cutting their way through cell partitions and debris out of the nest. However, older males do not enter nest tubes to gnaw a way to the inner brood cells to release virgin females for mating. Therefore, emerging males are unlikely to mimic virgin females through a convergent evolution of a signal to gain support under false pretenses. Consequently, the similar CHC bouquet of both sexes at emergence is probably due to primary functions that are equally relevant for both sexes (e.g., protection against infection and desiccation during diapause), as has been suggested for chalcidoid wasps (Steiner et al. 2005; Ruther and Steiner 2008).

To conclude, only newly emerged males become victims of SSB. There are no discernable adaptive benefits or apparent fitness costs associated with SSB in the two studied species of mason bees. The close similarity of CHC bouquets between newly emerged males and virgin females supports misidentification as a non-adaptive explanation for male SSB. However, a conclusive bioassay that alters the CHC signature of newly emerged males to that of older males and vice versa to demonstrate an ambiguity error is still lacking due to the complexity of bouquet modifications. Nevertheless, the mistaken identity hypothesis appears to be the most plausible explanation for SSB in *O. cornuta* and *O. bicornis*.

After emergence, the similar CHC bouquet of both sexes diverges and becomes sex-specific within a few days, as has also been found in beetles (Peschke 1985) and wasps (Steiner et al. 2005, 2007; Ruther et al. 2011). These changes in CHC composition could inadvertently result from changes in the environmental conditions of the microhabitat after exiting the cocoon and from food intake (Thomas 2011; Otte et al. 2018). The physiological state of the bees also changes with the end of hibernation and the onset of reproduction. The biosynthesis of hydrocarbons depends on physiological traits, and hydrocarbon signatures will reflect transitions in the hormonal state (Bagnères and Blomquist 2010). In addition, numerous CHC biosynthetic genes appear to show a strong expression bias in a given sex, leading to pronounced sex-specific differences in CHC profiles despite sharing the same genes (Holze et al. 2021). The observed differences in alkene levels are likely due to differentially regulated desaturases in the sexes (Wicker-Thomas and Chertemps 2010). The rapid alteration of the CHC bouquet within a few days is beneficial for both sexes. Males benefit by reducing the cost of courting an unreceptive female or engaging in SSB. Similarly, females benefit by reducing the likelihood of being harassed by unwanted courting males (Ayasse et al. 2001; Paxton 2005; Thomas 2011).

In addition to changing the CHC bouquet, males begin to emit large amounts of sex- and species-specific carboxylic acid esters that dominate the volatile bouquet of individuals after 2 to 3 days. These compounds may serve as a courtship inhibiting pheromone or abstinon (Schlein et al. 1981; Vuts et al. 2015) in mature males, preventing homosexual attacks. Males of both mason bee species will approach and attempt to grasp any object of apparent bee-like shape and size at rendezvous sites such as flowers, nests, and basking sites (Raw 1976; Seidelmann 1999). Apparently, they are not able to identify an object as a conspecific virgin female from a distance but have to bring their antennae into close proximity. Males are often seen touching or even briefly grasping mature females at flowers or when approaching their nests (personal

observations). However, due to the scramble competition polygyny (Seidelmann 1999), also male-male encounters are frequent at rendezvous sites. Esters, which are more volatile than CHC compounds, could act as an olfactory flag to announce the sex of a male from a distance, thus preventing contact and capture by a competitor. *O. bicornis* males avoid dummies treated with 7-C16:1-EE (Ayasse et al. 2000). However, as the esters are produced in a sternit gland, but self-marking by leg brushing to smear the substance on the body surface was not observed, the function of the substances remains questionable. Esters could also be used to mark flight paths on patrolled flowers, as described in bumblebees (Ayasse and Jarau 2014), or they could even serve several functions at once (Vuts et al. 2015), which have not yet been identified.

SUPPLEMENTARY INFORMATION

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AUTHOR CONTRIBUTION

The author bears the sole responsibility for study conception and design, material preparation, data collection and analysis, and preparation of the manuscript.

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DATA AVAILABILITY

The data are available from "Share_it - Open Access und Forschungsdaten-Repository der Hochschulbibliotheken in Sachsen-Anhalt" <https://opendata.uni-halle.de/handle/1981185920/112778>; DOI: <http://dx.doi.org/10.25673/110823>.

CODE AVAILABILITY

Not applicable.

DECLARATIONS

Ethics approval Not applicable. No humans or vertebrates were involved. The study was approved by a species protection exemption, Landesamt für Umweltschutz Sachsen-Anhalt, certificate RL-0590.

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