No evidence for an inbreeding avoidance system in the bumble bee *Bombus terrestris*

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Abstract – Inbreeding is caused by the mating of closely related individuals and may produce a decrease in the fitness of offspring and have deleterious consequences for adults. In haplodiploid social Hymenoptera inbreeding has a further negative effect due to the production of unviable or sterile diploid males. As a consequence, mechanisms of inbreeding avoidance would be expected to evolve. In this study, we investigated the mating choice between related (inbred) or unrelated (outbred) gynes and males of *Bombus terrestris* reared in laboratory conditions by performing cage and tunnel experiments. Not only did we find no mating preference for related or unrelated partners (mating success 41.55 ± 3.7 and $39.69 \pm 4.4\%$, respectively), but the mating latency was even shorter in inbred (6.97 ± 0.6 min) than in outbred matings (8.74 ± 0.8 min). We hypothesize that in wild populations of *B. terrestris*, the lack of incest avoidance could be compensated by tolerance of high levels of inbreeding.

Bombus terrestris / inbreeding / incest avoidance / kin recognition / mating preference

1. INTRODUCTION

In animal populations, inbreeding is caused by the mating of closely related individuals and may trigger the phenomenon of inbreeding depression, characterized by increased homozygosity and the consequent expression of deleterious recessive alleles (Charlesworth and Charlesworth 1987; Barrett and Charlesworth 1991; Lacy et al. 1993; Charlesworth and Willis 2009). In haplodiploid hymenopterans, inbreeding has further negative consequences due to their particular mechanism of sex determination, which follows the single-locus complementary sex determination system

(sl-CSD) (Van Wilgenburg et al. 2006). In social species, as a result of inbred mating, there is a variable probability, depending on the kinship, that queens share one of the two alleles for the sex-determining locus with males (e.g., 50% of probability in sibling matings). When a sibling mating occurs between brother and sister sharing the same sex allele, 50% of the fertilized eggs result homozygous at the sex-determining locus and develop into unviable or sterile diploid males (Ross and Fletcher 1986; Liebert et al. 2004; Whitehorn et al. 2009a). In the case of nephew-niece matings, the percentage of diploid males in the progeny is 37.5% (Duchateau et al. 1994), while in nephew-aunt matings it reaches 75% (Gosterit 2016).

Studies on wild populations of bumble bees showed that many species suffer from the consequences of inbreeding, mostly due to geographic

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isolation and small population size (Darvill et al. 2006; Ellis et al. 2006; Whitehorn et al. 2011; Darvill et al. 2012). The costs associated with inbreeding in bumble bee populations would suggest the evolution of inbreeding avoidance mechanisms. Polyandry, social recognition, and dispersal of individuals from their natal group are among the most common and efficient systems to avoid inbreeding in social insects (Pusey and Wolf 1996; Tabadkani et al. 2012), but they are only clearly represented in a few bumble bee species. Only a few bumble bee species show polyandry (e.g., *Bombus hypnorum*, *B. bifarius*, *B. californicus*, *B. frigidus*, *B. huntii*, and *B. rufocinctus*), while most of them are monandrous (Estoup et al. 1995; Schmid-Hempel and Schmid-Hempel 2000; Brown et al. 2002). The mechanism of incest avoidance through kin recognition has been described in several social hymenoptera, such as *Apis mellifera* (Getz and Smith 1986), some species of halictine bees (Buckle and Greenberg 1981; Smith and Ayasse 1987), polistine wasps (Gamboa et al. 1986; Liebert et al. 2010), and several species of ants (Keller and Passera 1993; Keller and Ross 1998). The role of kin recognition in inbreeding avoidance has been studied in a few bumble bee species. Foster (1992) observed the presence of nestmate recognition and mating avoidance in *B. frigidus* and *B. bifarius* but not in *B. californicus* and *B. rufocinctus*. Whitehorn et al. (2009b) observed a longer mating latency between siblings compared to non-siblings in gynes and males of *Bombus terrestris*, and interpreted it as evidence for kin recognition for inbreeding avoidance. Despite this, inbred mating occurred anyway.

Mate recognition in bumble bees passes through both behavioral and chemical features (Baer 2003; Ayasse and Jarau 2014). Male bumble bees of different species can display four kind of pre-copulatory sexual behaviors to find and attract females: perching, territoriality, nest surveillance, and scent-marking and patrolling (Brown and Baer 2005; Goulson 2010). *B. terrestris* males display scent-marking and patrolling as pre-copulatory sexual behavior to find and attract females (Brown and Baer 2005; Goulson 2010). This is the most common mating location mechanism in bumble bees and consists in males patrolling along paths where they scent-mark

objects to attract gynes (Ayasse and Jarau 2014). In addition, males of *B. terrestris* may have a very broad mating range (up to 9.9 km; Kraus et al. 2009), which could also contribute to avoiding inbreeding.

B. terrestris is one of the most widespread and abundant bumble bee species in the West Palaearctic region (Rasmont et al. 2008) and queens of this species are monandrous (Schmid-Hempel and Schmid-Hempel 2000). However, the above-mentioned behavior of male patrolling cannot be observed in small experimental flight cages (Djegham et al. 1994; Sauter and Brown 2001). Previous studies showed that mating success in confinement can be influenced by several factors, such as temperature, photoperiod, adult age and size, male dimension, and experience (Tasei et al. 1998; Kwon et al. 2006; Amin et al. 2007; Amin et al. 2010; Amin et al. 2012; Gosterit and Gurel 2016). Evidence indicates that wild populations of *B. terrestris* can tolerate high levels of inbreeding, since they are able to spread as an invasive species originating from few adult individuals (Schmid-Hempel et al. 2007). In addition, inbreeding does not seem to negatively affect the immune response of adults in confined conditions (Gerloff et al. 2003). Although some studies on captive colonies of *B. terrestris* demonstrated that inbreeding can affect colony initiation and colony growth (Gosterit 2016), and that less workers and males were produced after some generations of inbreeding (Beekman et al. 1999), in other studies inbred colonies grew at similar rates or performed even better than outbred colonies, despite the production of diploid males (Duchateau et al. 1994; Gerloff and Schmid-Hempel 2005).

In this study, we compared the mating occurrence, mating latency, and copulation duration between related and unrelated bumble bee gynes and males under experimental conditions not tested previously. Our aim was to verify whether a mating preference toward the unrelated partner exists as a mechanism to avoid incest in *B. terrestris*. When such a mechanism could be demonstrated, this would facilitate mating procedures in the artificial breeding of bumble bees, avoiding the checking of the parental origin of mating pairs.

2. METHODS

2.1. Study species and rearing conditions

Second-generation laboratory colonies of *B. terrestris* ($n = 25$) were reared from commercial colonies (Bioplanet srl, Cesena, Italia) following Bogó et al. (2017). Subsequently, during the phase of male and gyne production, the obtained colonies were maintained at 25 ± 1 °C and $60 \pm 10\%$ relative humidity (RH), in continuous darkness, and fed ad libitum with fresh frozen pollen and sugar syrup. We daily removed newly emerged gynes and males and we put them in separate plastic boxes of $25 \times 15 \times 14$ cm³ (up to 20 in the same box), according to their gender, date of emergence, and colony of origin. We obtained a total of 2215 gynes and 3550 males. Gynes and males were kept in the same climatic room of the colonies, and fed ad libitum with fresh frozen pollen and sucrose syrup until they were used for the mating experiment or discarded.

2.2. Mating tests design

For the mating tests, we used 1–10-day-old gynes and 5–25-day-old males, since these are the respective age ranges at which they meet sexual maturity (Tasei et al. 1998; Amin et al. 2012). In order to obtain a sufficient number of sexuals, we did not choose only the optimal mating ages, namely 7 days for queens and 12 days for males (Duchateau and Marien 1995; Duvoisin et al. 1999), instead we included the entire period of sexual maturity, which in our experience was suitable for mating. We used a total of 517 gynes and 1115 males sampled from gynes and males produced by the second-generation colonies (Online Resource 1). We avoided to mate gynes and males derived by colonies originated by sister queens but we cannot exclude that founder queens were related in some way (e.g., they derived from a common family line in the commercial breeding). When possible, we selected medium- to large-sized gynes and males (visual estimation) and we always avoided using small males, since in *B. terrestris* the size of males has a positive impact on mating success (Amin et al. 2012). Bumble bees were sampled from the colonies,

marked on the thorax with a color tag according to the colony of origin, and placed in groups with a gyne:male ratio of 1:2, to assure a higher mating propensity (Kwon et al. 2006; Amin et al. 2010). The total number of individuals in the groups ranged between 12 and 62, according to the number of gynes and males of the proper age interval present at the same time in the same colonies.

We conducted two different mating tests: in the first (“type 1”), gynes belonged to only one colony; in the other (“type 2”), gynes belonged to two different colonies. Even though the final mating choice should rely on queens (Whitehorn et al. 2009b), we included the “type 2” test to account for possible choices made by males. For each mating type, we added both related “R” (i.e., belonging to the same colony) and unrelated “U” (i.e., from a different colony) males to test the mating preference based on the relationship (Figure 1). In each mating session, the gyne:male ratio was 1:2.

Each mating test was performed in two different environmental conditions: (i) inside a wooden flying cage ($40 \times 40 \times 75$ cm³) with mesh walls; (ii) inside a net tunnel ($4 \times 2 \times 2$ m³) in more natural conditions (Bergman and Bergström 1997). In both cases, we furnished fresh frozen pollen and sucrose syrup for the whole test duration. Cage tests were carried out in a climate room, maintained at 20 ± 1 °C and $55 \pm 5\%$ RH (following Amin et al. 2010), in natural daylight. Tunnel tests were conducted outdoors, at a temperature of 26–29 °C and RH of 50–70%. We performed a total of 47 mating sessions: 28 of type 1 (17 in cage and 11 in tunnel) and 19 of type 2 (10 in cage and 9 in tunnel).

2.3. Response measures

Mating sessions started when gynes and males were released together in the cage or tunnel, and they were terminated after 1 h, irrespective of the mating success. For the whole duration of the test, we constantly observed individuals and recorded the precise moment when copulation began, in order to calculate mating latency (namely the time elapsed between the start of the mating session and the start of copulation). As soon as mating began, each mating pair was removed from the cage or tunnel and transferred to an individual

Figure 1. Test design of the two types of mating. The subscript letters (A and B) indicate the bumble bees provenience from the same or from different colonies. *R* related, *U* unrelated.

transparent plastic box ($12 \times 9 \times 9 \text{ cm}^3$), where it was constantly observed to record the moment of copulation ended, in order to calculate copulation duration. For each couple, we recorded the colony of origin of the gyne and male, and whether they were related or unrelated. For each mating session, we also calculated the mating rate as the number of mating pairs on the number of gynes that entered the session.

2.4. Data analysis

Data were firstly tested for normality (Shapiro-Wilk test). When data were not normally distributed, we used non-parametric statistical tests. Proportions were transformed using arcsine-square-root. Differences in mating rates, timing of mating latency, and copulation duration between the two environments (cage and tunnel), the two types of mating (type 1 and type 2), and between related and unrelated bumble bees were analyzed with three-way ANOVA followed by Tukey post-hoc test. The influence of the maternal colony of gynes and males on mating success and in partner choice was tested using the non-parametric Kruskal-Wallis *H* test. Statistical analyses were performed with STATISTICA software. Power of statistical tests was analyzed with post-hoc power analysis (conventional small, medium, and large effect sizes set at $f = 0.1$, $f = 0.25$, and $f = 0.4$, respectively) using G*Power 3.0 software. Power values greater than 0.8

indicated adequate power (Williams et al. 2001). Data are shown as mean $\pm$ SE.

3. RESULTS

3.1. Mating rates and partner preference

Without considering the relationship (i.e., related or unrelated) between gynes and males, we obtained a total mean mating rate of $82.2 \pm 0.02\%$ (number of matings = 409), ranging from $73.1 \pm 4.33\%$ (number of matings = 95) in the tunnel with mating type 2 to $86.6 \pm 3.25\%$ (number of matings = 107) in the cage with mating type 1.

Factors “Environment,” “Type,” and “Relationship” and the pairwise interactions of these three factors had no significant effect on the mating rates, but there was a significant effect in the interaction among the three factors together (Table 1, Figure 2).

Post-hoc power analysis showed that the statistical test was able to detect only large effects ($f = 0.01$, calculated power = 0.15; $f = 0.25$, calculated power = 0.63; $f = 0.4$, calculated power = 0.96).

3.2. Measurement of mating phases

The three-way ANOVA showed that factors “Environment” (tunnel = $12.10 \pm 0.817 \text{ min}$; cage = $3.83 \pm 0.413 \text{ min}$), “Relationship” (unrelated = $8.74 \pm 0.746 \text{ min}$; related = $6.97 \pm 0.644 \text{ min}$), and their interaction had a significant effect on the

Table 1. Three-way ANOVA table of the effects of “Environment” (cage or tunnel), “Type” (I or II), and “Relationship” (related or unrelated) on mating rate

	df	F	P
Environment	1, 86	2.446	0.122
Type	1, 86	0.287	0.593
Relationship	1, 86	0.375	0.542
Environment*Type	1, 86	0.091	0.763
Environment*Relationship	1, 86	0.140	0.710
Type*Relationship	1, 86	0.084	0.773
Environment*Type*Relationship	1, 86	6.972	0.010

mating latency, while the factor “Type,” its interaction with “Environment” and “Relationship,” and the three factors together had no effect on mating latency (Table II, Figure 3).

The same analysis on the mating duration showed that factors “Environment” (tunnel = 21.79 ± 0.509 min; cage = 31.15 ± 0.512 min), “Type” (type 1 = 25.50 ± 0.543 min; type 2 = 27.90 ± 0.662 min), and the interaction among the three factors had a significant effect on the mating duration, while the factor “Relationship” and the pair wise interactions of the three factors had no effect on this parameter (Table III, Figure 4).

Post-hoc power analysis showed that both statistical tests were not able to detect small effects ($f = 0.01$, calculated power = 0.51; $f = 0.25$, calculated power = 0.99; $f = 0.4$, calculated power = 1).

3.3. Effect of the maternal colony

The maternal colony of gynes did not influence either the mating success ($H_{22} = 32.20$, $P = 0.074$)

or the partner choice ($H_{22} = 32.17$, $P = 0.075$). The maternal colony of males influenced the mating success ($H_{24} = 39.86$, $P = 0.022$) but had no effect on partner choice ($H_{18} = 20.91$, $P = 0.284$).

4. DISCUSSION

4.1. Partner preference and incest avoidance

The main outcome of this study is the absence of a mating preference between related and unrelated partners of *B. terrestris* under controlled conditions. In our study, the related pairs were represented mostly by brother-sister pairs, but the presence of nephew-aunt pairs could not be excluded. However, workers usually start to lay eggs late in the colony cycle, and considering the time required for development and achievement of sexual maturity they would rarely meet gynes of the same colony. As a consequence, the frequency of nephew-aunt pairs is expected to be much lower than that of brother-sister pairs.

Figure 2. Mean ($\pm$ standard error) of related (R) and unrelated (U) matings rates in the two environments (cage and tunnel) and in the two mating types (I and II); n number of mating sessions.

Table II. Three-way ANOVA table of the effects of “Environment” (cage or tunnel), “Type” (I or II), and “Relationship” (related or unrelated) on mating latency

	df	F	P
Environment	1, 401	151.727	< 0.001
Type	1, 401	0.873	0.351
Relationship	1, 401	5.879	0.016
Environment*Type	1, 401	5.496	0.020
Environment*Relationship	1, 401	1.222	0.270
Type*Relationship	1, 401	1.896	0.169
Environment*Type*Relationship	1, 401	1.118	0.291

Previous research on the same species inferred the existence of a mating preference towards non-siblings based on a shorter mating latency, interpreted as a higher mating propensity, compared to mating with siblings (Whitehorn et al. 2009b). However, a double choice mating test was never attempted with this species. In a mating-choice test similar to that of Whitehorn et al. (2009b), Foster (1992) found that gynes and males of *B. frigidus* and *B. bifarius* seemed to recognize nestmates of the opposite sex with a reduced inbreeding rate (although sibling matings were not totally avoided), while *B. californicus* and *B. rufocinctus* did not show an inbreeding avoidance system. The author hypothesized that this variability among species could be linked to the different pre-copulatory behavior: *B. californicus* and *B. rufocinctus* show a nest surveillance behavior to find females, but since they do not survey their own nest they do not need nestmate recognition cues; *B. frigidus* and *B. bifarius*, which have a patrolling behavior, can

frequently encounter their sibling gynes and therefore they are advantaged by an incest avoiding system. Following this hypothesis, *B. terrestris*, which is a patrolling species, should have a similar nestmate avoidance system, but this is not consistent with our results. Male marking pheromones represent the main mating location mechanism in bumble bees. Despite this, there is no evidence about their use as an inbreeding avoidance system, since they have been demonstrated to be species- and subspecies-specific (Rasmont et al. 2005; Coppée et al. 2008), and there are no studies about a possible nestmate specificity. In *B. terrestris*, a nestmate recognition system mediated by cuticular hydrocarbons is used to mark nest entrance and signal nest identity (Rottler et al. 2013), to prevent nest invasion by social parasites (Martin et al. 2010; Blacher et al. 2013), and to reduce the phenomenon of drifting workers (Zanette et al. 2014). Although insect cuticular hydrocarbons may also play important roles in short-distance and male-female contact communication (Kuo

Figure 3. Mean ($\pm$ standard error) mating latency in related and unrelated matings in the two environments (cage and tunnel) and the two mating types (type 1 and type 2); *n* number of mating pairs. Different letters denote statistical differences at $\alpha = 0.05$.

Table III. Three-way ANOVA table of the effects of “Environment” (cage or tunnel), “Type” (I or II), and “Relationship” (related or unrelated) on mating duration

	df	F	P
Environment	1, 395	170.212	< 0.001
Type	1, 395	9.999	0.002
Relationship	1, 395	1.272	0.260
Environment*Type	1, 395	0.367	0.545
Environment*Relationship	1, 395	0.067	0.796
Type*Relationship	1, 395	0.059	0.809
Environment*Type*Relationship	1, 395	5.565	0.019

et al. 2012), no studies have been performed on bumble bees to detect their possible role in incest avoidance.

In our study, cage conditions have probably prevented all kind of odor discrimination, due to the tight confinement and high number of individuals. By contrast, in tunnel conditions, the flying area was wide enough for a recognition based on colony odors (cuticular recognition), allowing discrimination between nestmate and non-nestmate (Liebert et al. 2010). Even though we did not provide enough time to males to display patrolling and scent marking, the tunnel size allowed the performance of other pre-copulatory behaviors, such as queen approaching and inspecting by males and queen reaction to mating attempts (Bergman and Bergström 1997; Djegham et al. 1994; Whitehorn et al. 2009a). However, neither in cages nor in tunnels was any nestmate recognition system to avoid incest observed. Therefore, we hypothesize that *B. terrestris*

does not use cuticular odor cues to prevent inbred mating, contrarily to what has been observed in some primitively eusocial bees (Smith and Ayasse 1987), in wasps (Liebert et al. 2010), and even in other insect species (Lihoreau et al. 2007; Lihoreau and Rivault 2010). Concerning scent marking performed by male bumble bees, further studies in larger tunnels could better clarify their possible role in kin recognition systems.

Amin et al. (2012) observed an influence of adult male characteristics, such as body weight and leg length, on mating rates. Although we did not directly evaluate the impact of these parameters, we observed an influence of male maternal colony on mating success, which in turn can determine these features. Contrarily, it seems that adult characteristics were not decisive in mating preference, since neither the maternal colony of gynes nor that of males influenced the choice among related and unrelated partners.

Figure 4. Mean ($\pm$ standard error) copulation duration in related and unrelated matings in the two environments (cage and tunnel) and the two mating types (type 1 and type 2); n number of mating pairs. Different letters denote statistical differences at $\alpha = 0.05$.

4.2. Mating phases duration

The duration of copulation can be used by insects to adjust their mating investment in relation to their relatedness (Tabadkani et al. 2012). In bumble bees the long copulation duration is used by males to manipulate paternity in their own interests: it promotes effective sperm transfer and allows the application of the so-called “mating plug,” a secretion produced by male accessory glands, which is applied to the female genital tract to prevent gynes from re-mating (Baer et al. 2000, 2001; Brown and Baer 2005). The mean values for copulation duration found in our study are consistent with previous reports for *B. terrestris* (Duvoisin et al. 1999), and the fact that no differences were found between inbred and outbred matings indicates the lack of a differential investment by males in response to gyne relatedness. The shorter copulation durations observed in tunnel conditions compared to the cage may be due to the different temperatures, which were about 20 °C in cage and up to 29 °C in tunnel. This higher temperature is known to have a negative effect on mating success (Amin et al. 2010) and could have shortened copulation duration. Moreover, the mating duration was lower in type 2 than in type 1 matings, probably because of a higher competition in the latter mating type since both gynes and males derived from two different colonies.

Mating latency showed a different pattern between inbred and outbred matings, but in an opposite manner to what we expected. Whitehorn et al. (2009b) observed a longer latency in sibling than in non-sibling matings and interpreted this result as showing the existence in *B. terrestris* of a kin recognition system to avoid incest. On the contrary, we found a longer latency in outbred than in inbred matings. The most likely explanation for this unexpected result is that mating latency is not directly correlated to mating preference. Instead, mating latency could be influenced by other factors, such as the mating environment. In fact, we observed a shorter latency in cage matings than in tunnel matings, probably because in tighter spaces males are advantaged in finding gynes.

4.3. Why not avoid incest?

Our results strongly suggest that *B. terrestris* is a species in which inbred matings are not avoided, similarly to *B. californicus* and *B. rufocinctus* (Foster 1992). However, these two species show a pre-copulatory nest surveillance behavior which minimizes nestmate encounters, and *B. californicus* has a polyandric strategy that further reduces the risk of inbreeding. Conversely, *B. terrestris* is monandrous and has a patrolling pre-copulatory behavior which does not exclude inbred matings.

Since in some cases inbreeding implies serious fitness consequences in bumble bee colonies (Gerloff and Schmidt-Hempel 2005; Whitehorn et al. 2009a; Gosterit 2016), we would expect the existence of other mechanisms to avoid mating with close relatives, such as adult dispersal. Queen dispersal has not been studied in *B. terrestris*, although an indirect indication comes from its alien colonization of Tasmania, which was estimated at 300 km in about ten generations, indicating a high dispersal capacity (Schmidt-Hempel et al. 2007). However, the particular context of this expansion is perhaps not representative of the dispersal process in the native range of the species. Recent studies showed that males of *B. terrestris* have a wide flight range, which can represent an effective strategy for increasing population size and reduce the risk of inbred mating (Kraus et al. 2009; Wolf et al. 2012). On the other hand, in Italy and other European countries, *B. terrestris* is the most abundant and widespread species (Rasmont et al. 2008). This high abundance acting as dilution factor in decreasing the possibility of mating with related individuals may be another key factor in incest avoidance.

Another factor to consider is that we used bumble bee colonies of commercial origin, which probably underwent several generations of artificial breeding. This could have eventually caused a selection toward a population which does not avoid incest, since enclosure conditions reduce the need to increase fitness. The possible contribution of this factor cannot be easily determined, because most mating studies found in literature have also been carried out on artificially reared populations.

Finally, the lack of an incest avoidance system in *B. terrestris* could not be interpreted as an

evolutionary gap, since inbreeding could have positive effects on parents' fitness by increasing the representation of parental genes in future generations (Kokko and Ots 2006). In addition, retaining the possibility of inbred mating could represent a protection measure in the case of transitory isolation of the population. Together with the high inbreeding tolerance observed in *B. terrestris*, the possibility to perform indiscriminately inbred and outbred mating, exploiting the advantages of both strategies, can represent one of the key factors for the ecological success of this species.

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AUTHORS CONTRIBUTION

GB and LB conceived this research and designed experiments; GB, NdM, AF, MG, and LB participated in the design and interpretation of the data; GB, LB, NdM, and LZ carried out experiments and analysis; GB, NdM, AF, MG, LZ, and LB wrote the paper and participated in the revisions. All authors read and approved the final manuscript. Data availability

A summary of the data that support the findings of this study is available in Figures. 2, 3, and 4 and throughout the text. All other data are available from the corresponding author on reasonable request.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest The authors declare that they have no conflict of interest.

Aucune preuve d'un système d'évitement consanguin chez le bourdon *Bombus terrestris*

***Bombus terrestris* / consanguinité / évitement de l'inceste / reconnaissance de la famille / préférence d'accouplement**

Keine Evidenz für ein Inzuchtvermeidungsprinzip bei der Hummel *Bombus terrestris*

***Bombus terrestris* / Inzucht / Inzestvermeidung / Verwandtschaftserkennung / Paarungspräferenz**

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