

Olfactory orientation of the truffle beetle, *Leiodes cinnamomea*

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Abstract

Although the truffle beetle, *Leiodes cinnamomea*, inflicts substantial damage to the ripe stage of fruiting bodies of the economically important black truffle (*Tuber melanosporum*), it is not attracted by ripe truffle odours. Rather, male beetles are attracted to infested truffles only in the presence of female beetles, suggesting that the former employ a pheromone to locate truffles over short distances. In contrast, female beetles show no attraction to infested or uninfested truffles, suggesting that they employ other cues, possibly linked to odours emitted by truffles prior to the ripe stage. We hypothesize that the chemical composition of truffle volatiles changes over the life of the truffle fruiting body, being attractive to insects early on and to mammals just prior to decomposition.

Introduction

Volatiles are important cues for many arthropods. Many, if not most, insects use odours to find food and mates or to avoid predators and competitors. The food of arthropods is selected to produce as few volatiles as possible to reduce the probability of being detected and eaten. However, volatiles may be indispensable for some life stages. For example, moths produce sex pheromones to attract mates, but these volatiles are used by the parasitoids that attack the moth eggs (Noldus, 1988). Hence, volatiles are likely to be produced only when they are essential in the life-history of an organism or when they are an inevitable by-product of some other essential process, and whenever produced, they might be used by other species.

Truffles are famous for producing strong odours that are even distinguishable by the human nose. Several species of European truffle of the genus *Tuber* are highly sought after for their culinary properties, and are known to be attractive to mammals such as dogs, pigs, wild boar, and humans (Genard et al., 1986; Talou & Kulifaj, 1992). It is thought that the chemical compounds responsible for such attraction have evolved as a means of attracting mammals that disperse the spores of these underground-dwelling fungi (Genard et al., 1986), suggesting that production of the odour is essential for the truffle. In contrast to the recognition of ripe truffle odours by certain mammal species, it is

not known how specialized arthropods living in or around truffle fruiting bodies may use sensory cues to recognize truffles, or whether these insects potentially act as dispersal agents for truffle spores.

The black truffle, *Tuber melanosporum* Vittadini (Tuberales), is one of the most highly valued in the *Tuber* genus. Despite a sizeable geographical range (it is mainly found in Spain, Italy, and France; Bertault et al., 1998), its fine-grained distribution is fragmented, due mainly to soil structural constraints (Callot & Jaillard, 1996). It grows in semi-cleared areas, in association with several tree species.

The phenology of the black truffle is briefly as follows: primordium formation occurs from late April to late June. The capophore (i.e., adult stage) appears in late July and continues to grow until late October. Mature ascocarps are produced from November until March (Callot, 1999). Biochemical synthesis of the truffle's aromatic compounds (together with melanisation) only starts when capophore growth is complete (Talou & Kulifaj, 1992). The main chemical compounds involved in the chemical bouquet emitted by the black truffle have been identified (Pacioni et al., 1990), and used to create an artificial aroma especially designed to train dogs in truffle-seeking (Delmas et al., 1987).

One of the specialized fungivores attacking truffles, the beetle *Leiodes cinnamomea* Panzer (Coleoptera: Staphylinoidea, Leiodidae) is univoltine, with a European distribution. It is known to feed on several species of truffle (*Tuber* spp.), and completes its life cycle in or near the fungal fruiting body (e.g., Arzone, 1970; Newton, 1984). Pupae are observed

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through October to November, and imagoes from November through March (Arzone, 1970). Adults are observed to lay their eggs near the truffle fruiting body, giving rise to large-mandibled juvenile beetles of different ages within a given truffle between the months of January and April (Arzone, 1970, A. Arzone, pers. comm.). Little is known concerning how these beetles pass the summer months, or about their eventual dispersal behaviour.

Both larvae and adults of *L. cinnamomea* feed on the truffle fruiting body (A. Arzone, pers. comm.), but most economic damage is done by the developing larvae. What little information is available suggests that most truffle farms in France are little affected by the beetle (P. Réjou, pers. comm.). The ecological factors leading to beetle infestations are unknown.

Given their specificity for *Tuber* spp., and the strong smell produced by ripe truffles, we hypothesized that truffle beetles use these odours to find truffles. By 'ripe' we mean that they are attractive to the trained dogs employed by farmers to locate truffles, and emit typical truffle odours that are appreciated by humans.

Materials and methods

Truffle and truffle beetle collection

All study sites were located within 50 km of Périgueux, France (00°43'08"W, 45°11'06"N). Two species of truffles were available (*Tuber melanosporum* and *Tuber brumale*), but experiments were mainly done with *T. melanosporum*.

Experiments were conducted over two periods. In 1999 (25–29 January), truffles with or without beetles were collected with the aid of a trained dog by a truffle farmer. The truffles were stored together in a refrigerator, whereas the beetles were kept together outdoors in a container with soil. From 8 to 12 January 2001, truffles were obtained from

several farms. Truffles with associated soil and beetles were stored separately in small plastic jars or boxes.

Prior to the olfactometer experiments, the beetles to be tested were collected from the soil and/or the truffle and stored individually in small tubes (1999: glass tubes: 7–9 mm, 5–7 mm long; 2001: 1.5 ml polypropylene micro-centrifuge tubes), closed with a moist cotton wool plug. Because the beetles used in experiments were collected from the field, we had no knowledge of their age.

In 1999 we were unaware of any clear morphological difference between male and female beetles, but we noted that roughly half of the individuals had arced hind legs (see inset of Figure 1). This characteristic was registered for each individual without recourse to the performance of that individual in the olfactometer experiment. In 2001, the gender of all the beetles was determined using a stereoscope, and it was confirmed that the males had arced hind legs with one or more 'teeth', while those of females were straighter and without dentations. We were therefore able to assess the gender of the beetles in 1999 based on their hind-legs after all the experiments had been completed. Because assessing the sex of the beetles disturbed them to some extent, we only did this after the experiments in 2001. It was therefore impossible to test the response of equal numbers of males and females per odour source. In 1999, only some replicates with uninfested and infested truffles were done. More replicates of the same experiments were carried out in 2001. The differences in results between the two years were not significant (χ^2 : $P > 0.05$), the results were therefore pooled.

Olfactometer set-up

In 1999, the olfactometer consisted of pieces of transparent plastic tube (5 mm diameter, Rauclair®) connected by a plastic junction (5 cm, 5 mm inner diameter). The base tube was

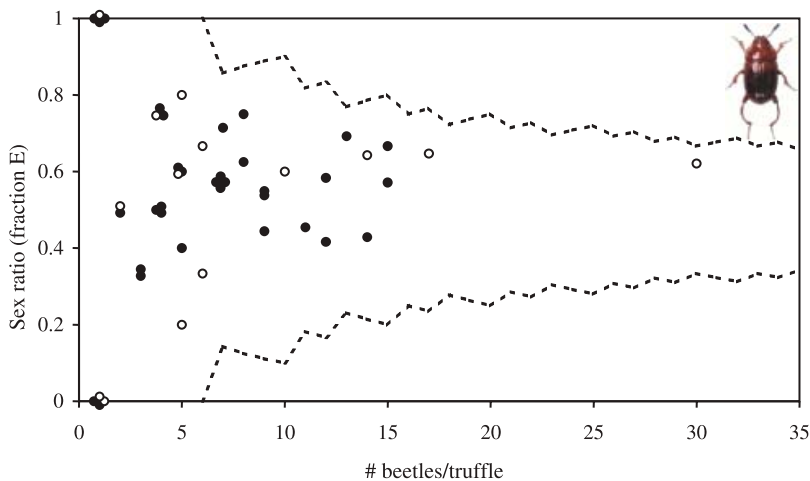


Figure 1 Sex ratio of truffle beetles per truffle. The fraction of females as a function of total number of beetles per truffle is shown. Closed circles refer to truffles of the species *T. melanosporum*, open circles concern *T. brumale*. Sex ratios inside the area delineated by the two broken lines do not differ significantly from a ratio of 0.5 (binomial test).

27 cm long, the arms 29 cm. The hose of the arms was connected directly to the odour source. In 2001, we used small glass Y-tubes (internal diameter 5 mm, external diameter 7 mm; length of arms: 5 cm; de Kogel et al., 1999). Each arm was extended with a 12.5 cm long piece of plastic hose (as above). This was then connected with a small piece of silicone hose (9 mm diameter) to a further plastic hose of the same diameter as described above. This hose was in turn connected to the odour source. The base tube was extended with a piece of plastic hose of 22 cm, which was connected by a small piece of silicone hose to a further plastic hose, leading to a vacuum pump. Air coming from the pump was led outdoors to avoid contamination of the indoor air. The air entering the set-up came from the room and was not filtered.

In 1999, the odour source was placed in the middle vial of a series of three glass vials (50 ml, Duran 29/32), connected to each other through their air inlet and outlet, the first vial (counted from the Y tube) serving as a collection vial for tested beetles, while the third (upwind) vial contained demineralized water to humidify the air. One of the arms was baited with an odour source, whereas the corresponding vial of the other arm was empty. In 2001, the odour sources were put in the first of a series of two of such vials, where the second contained demineralized water. In both years, the last vial was connected to a flow meter with needle valves (Brooks Sho-rate™, maximum capacity 20 l h⁻¹ at 1 bar and 20 °C). The air flow in both arms was kept equal and at a rate of ca. 10–15 l h⁻¹. The olfactometer was shielded from daylight and was illuminated by a fluorescent lamp from the ceiling. The olfactometer was positioned under the lamp so that both arms were illuminated in a similar fashion.

Beetles were introduced individually by disconnecting the tube at the base of the Y-tube at the silicone connection tube and allowing the beetle to walk from its unplugged vial into the olfactometer. Subsequently, the tube was reconnected, thus establishing airflow from the series of vials, through the arms and the base of the olfactometer to the pump, and the observation was started. Each beetle was observed for a maximum of 5 min or until it ended up in the collection vial (1999) or at the end of one of the arms (2001), after which it was scored as having made a choice. After this, the hose was disconnected from the silicone connection and the beetle was removed from the system. At normal walking speed, it took the beetles less than ca. 2 min to walk upwind to the collection jar (1999) or the end of the arm (2001), so each beetle could walk up one arm, return to the base, and still have enough time to reach the end of the other arm. Most beetles, however, did not turn, and walked straight up to the end of one arm. Depending on the supply of beetles, we tested 20–30 beetles

per replicate, and the odour sources were connected to the opposite arm of the olfactometer after each five beetles tested to correct for unforeseen asymmetries in the experimental set-up. All replicates of an experiment were done with a different truffle and group of beetles to avoid pseudoreplication. However, we deliberately re-tested some individuals with the same odour source to check for consistency of choice, while connecting the odour source to the opposite arm after each five runs. For these tests, the beetles were re-tested immediately. After each replicate, the olfactometer was cleaned with detergent and rinsed with tap water and then demineralised water.

Differences in numbers of beetles between the control odour source were tested using a two-sided binomial test with $H_0 = 0.5$ (Siegel & Castellan, 1988). Several different odour sources were used: uninfested truffles, truffles with attached soil and beetles, infested truffles with attached soil, soil from truffles (which had a strong truffle smell), soil and beetles from which the truffle was removed, beetles only (with some moist cotton wool to provide them with water and hiding places), and an artificial truffle odour (Canitruffe, Trufarome, EURL, Cahors, France), which is used for training truffle dogs. Truffles used in the olfactometer experiments were always *T. melanosporum*, unless stated otherwise.

Results

Number and sex ratio of beetles per truffle

Infested truffles harboured as few as one beetle, and up to as many as 30 (Figure 1). Although the two truffles with the highest number of beetles were both *T. brumale*, there was no significant difference in the numbers of beetles between the two truffle species (Mann–Whitney U-test, $n_1 = 34$, $n_2 = 14$, $U = 231$, $P = 0.88$). The sex ratio per truffle was never significantly different from 0.5 (Figure 1), but the overall female sex ratio was 0.6, which is significantly different from 50% (two-sided binomial test, $P = 0.004$).

Repeated testing of individual beetles

To ascertain that the olfactometer was suitable for studying the response of the truffle beetles to odours, we tested several beetles repeatedly using the same odour source (infested truffle without beetles). We re-tested three males and one female, and the three males were significantly attracted by the truffle odour, while the female was neither attracted nor repelled (Figure 2). None of the four individuals showed any preference for the left or right arm of the olfactometer (binomial test, $P > 0.24$). This shows that the choice of attracted individuals was persistent, while the individual that was not attracted chose randomly for either of the two arms. Hence, the Y-tube olfactometer was suitable for studying the response of truffle beetles to odours.

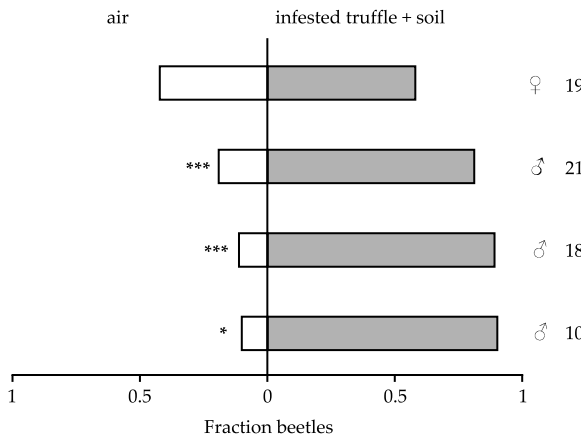


Figure 2 The fraction of choices of four individual truffle beetles (three males, one female) for an infested truffle (*T. melanosporum*) in a Y-tube olfactometer. Asterisks next to the bars indicate significant difference from a ratio of 0.5; *P < 0.05; **P < 0.01; ***P < 0.005, two-sided binomial test. Numbers to the right of the bars are the number of times each beetle was tested.

Attraction of beetles towards odours of truffle and conspecifics

When offered a choice between ambient air and an uninfested *T. melanosporum* truffle, the beetles showed a significant attraction to air in one out of three replicates (Figure 3). This was due to a significant repulsion of males (Figure 3); females were not significantly attracted or

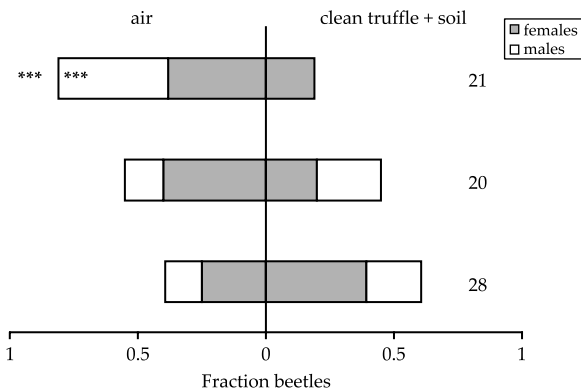


Figure 3 The fraction of truffle beetles that either chose ambient air (left bars) or the odour of a uninfested truffle (*T. melanosporum*) with associated soil (right bars) in a Y-tube olfactometer. Results are shown of three independent replicates, each with a different set of beetles and a different odour source. Grey bars refer to the response of female beetles, white bars to that of males. Asterisks inside the grey bars refer to significance of female preference, inside the white bars to the significance of male preference, and outside bars refer to significance of females and males combined; *P < 0.05; **P < 0.01; ***P < 0.005, two-sided binomial test. Numbers to the right of the bars are the number of beetles tested per replicate.

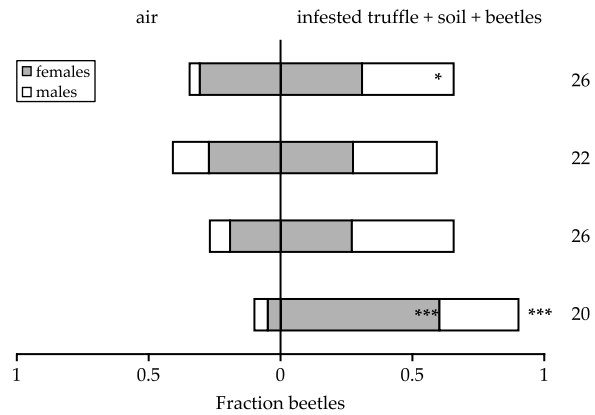


Figure 4 The fraction of truffle beetles that either chose ambient air (left bars) or the odour of an infested truffle (*T. melanosporum*) with associated soil and beetles (right bars) in a Y-tube olfactometer. Results are shown of four independent replicates, each with a different set of beetles and a different odour source. See legend to Figure 2 for further explanation.

repelled in either of the three replicates. Pooled results also did not show significant attraction or repulsion (binomial test on pooled results, males plus females: P = 0.168, males and females: ns). Hence, beetles are not attracted to uninfested truffles.

Female and male beetles were confronted with a choice between air and infested *T. melanosporum* with the soil, and the beetles that were found with it in the field. The odours from the truffle appeared slightly attractive (Figure 4); although the results of the separate replicates were not significant in most cases, the overall result was significant (binomial test on pooled results, males plus females: P = 0.007, males: P = 0.00027, females: P = 0.049). One truffle with soil and beetles was significantly attractive to females (lowest bar of Figure 4), while another was attractive to males (highest bar of Figure 4). The significant attraction of males is further evidence that the Y-tube olfactometer was suitable for studying the response of the beetles to odours.

A test of air and infested *T. melanosporum* with soil but without the associated beetles showed the opposite pattern: one out of four truffles was significantly attractive to females (second bar of Figure 5) and overall, truffles were slightly attractive to females (binomial test on pooled results, males plus females: P = 0.0052, males: ns, females: P = 0.012).

When mixtures of male and female beetles (ratio 6 ♀ : 9 ♂) were offered as odour source, they were significantly less attractive than air in two out of three replicates, and in those cases mainly for males (Figure 6, lower three bars). Overall, males were repelled significantly by groups of males and females (binomial test on pooled results of

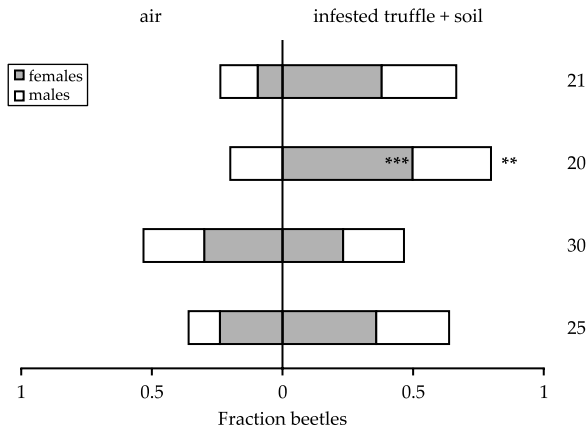


Figure 5 The fraction of truffle beetles that either chose ambient air (left bars) or the odour of an infested truffle (*T. melanosporum*) with associated soil (right bars) in a Y-tube olfactometer. Results are shown of four independent replicates, each with a different set of beetles and a different odour source. See legend to Figure 2 for further explanation.

lower three bars, males plus females: $P = 7.8 \times 10^{-5}$), while females were not. We subsequently offered odours of males or females only, but found no attraction or repulsion (Figure 6, upper two bars).

We then tested air against soil and beetles from which the truffle was removed, and found a significant overall attraction in one out of two replicates (Figure 7). The overall response was also significant (binomial test on pooled

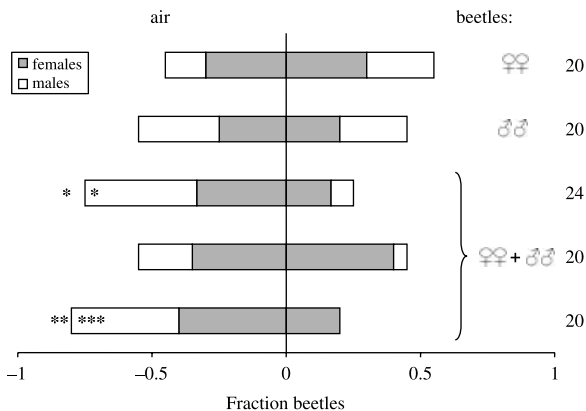


Figure 6 The fraction of truffle beetles that either chose ambient air (left bars) or the odour of conspecific beetles (right bars) in a Y-tube olfactometer. Results are shown of five independent replicates, each with a different set of beetles and a different odour source. The lower three bars refer to experiments where the odour source contained 6 ♀ : 9 ♂, the two top bars are results of experiments with either 15 ♀ or 15 ♂. See legend to Figure 2 for further explanation.

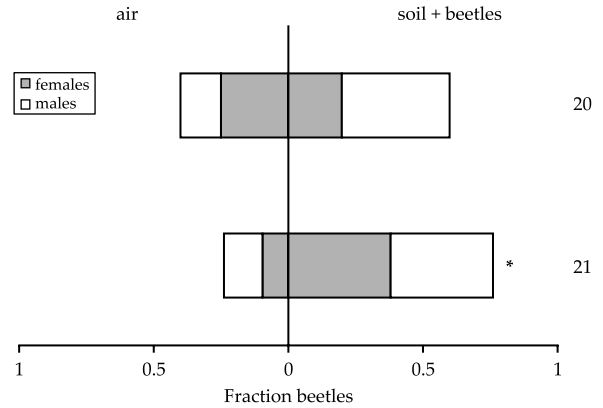


Figure 7 The fraction of truffle beetles that either chose ambient air (left bars) or the odour of soil from an infested truffle (*T. melanosporum*) with associated beetles (right bars) in a Y-tube olfactometer. Results are shown of two independent replicates, each with a different set of beetles and a different odour source. See legend to Figure 2 for further explanation.

results, $P = 0.014$, males and females: NS). In one replicate, we also tested the soil from which beetles and the truffle were removed, but we found no significant attraction, although the soil elicited a truffle odour that was easily distinguished by the human nose.

As a final test, we offered beetles a choice between an artificial truffle odour (Canitruffe) and air, and found a slight, non-significant attraction to this odour (Figure 8). Furthermore, we tested the odours of one other, sympatric truffle species, *T. brumale*, with associated beetles and soil, and found that this was attractive to females (22 out of 29

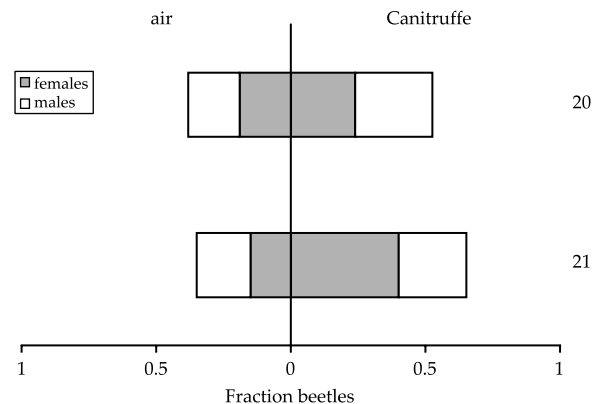


Figure 8 The fraction of truffle beetles that either chose ambient air (left bars) or an artificial truffle odour (Canitruffe, right bars) in a Y-tube olfactometer. Results are shown of two independent replicates, each with a different set of beetles and a different odour source. See legend to Figure 2 for further explanation.

were attracted, $P = 0.004$) as well as males (18 out of 22 were attracted, $P = 0.002$).

Because the truffle beetles employed were collected from both *T. melanosporum* and *T. brumale*, we also tested whether the response of beetles depended on their origin (for those odour sources that were tested with individuals of both origins), but found no significant difference in any of the comparisons (two replicates with uninfested *T. melanosporum* + soil, one replicate with infested *T. brumale* + soil + beetles, three replicates with infested *T. melanosporum* + soil, one replicate with infested *T. melanosporum* + soil + beetles, two replicates of soil (from *T. melanosporum*) + beetles, Fisher's exact test, two-tailed, $P > 0.05$).

Discussion

Overall, adults of the beetle *L. cinnomnea*, although inflicting substantial damage to the fruiting bodies of the black truffle in the larval stage of the insect, are not attracted to ripe truffle odours. However, some individuals showed persistent attraction when tested repeatedly, showing that the odours can be perceived by the truffle beetles and that the olfactometer is a suitable set-up for studying the response of these beetles to volatiles. While uninfested truffles were not at all attractive to female *Leiodes*, in one out of four cases, the combination of an infested truffle with soil and beetles (Figure 4) or an infested truffle with soil (Figure 5) was attractive. This is surprising, since truffles produce a distinctive odour, and it was expected that truffle beetles, especially females, would use these odours to locate truffles that are buried in the soil and would be hard to detect otherwise. Pacioni et al. (1991) used traps baited with various volatile compounds of the truffle odour, and recaptured truffle beetles in a trap baited with dimethyl sulphide, the main compound of the volatile blend of *T. melanosporum* and *T. brumale* (Pacioni et al., 1990, 1991). However, the total population size of truffle beetles in these studies was unknown or not reported, so it is unclear how many beetles of the total population were attracted to these compounds. Attractiveness could therefore be as low as in our experiments.

There are several possible explanations for the absence of attraction of truffle beetles to truffle odours. For instance, the physiological state of the truffle beetles may affect their response to truffle odours. Although we did not conduct treatments to investigate how starvation may have affected beetle behaviour, it is difficult to justify why, for example, the starvation level of beetles would affect their choice. The more likely reason for adult beetles to be attracted to truffles is reproduction. Since we encountered all stages of juvenile beetles inside the truffles, it is likely

that some of the adults we found were either reproducing, or had recently reproduced. We tentatively conclude that the physiological state of the adult beetles was not the main cause for the lack of beetle attraction to truffles.

It is possible that there was a variation in the quality of individual truffles and that truffle beetles can use odours to discriminate between high and low quality truffles. In this case, truffles of good quality would have become infested in the field, whilst the uninfested truffles that we tested were of lower quality and therefore unattractive. This would indicate that infested truffles should be more attractive than uninfested ones, which is indeed what we found (Fisher's exact test on pooled results of uninfested truffles with soil (Figure 3) vs. infested truffles with soil (Figure 5): $P = 0.0272$; pooled results of uninfested truffles with soil and beetles vs. infested truffles with soil and beetles: $P = 0.0121$). However, even infested truffles are not tremendously attractive; only ca. 65% of all beetles are attracted (Figures 4 and 5). It is possible that attractive truffles become less attractive upon attack, because beetles themselves act as repellents. Our results are consistent with this, but beetles mainly repel males and not females (Figure 6). If beetles are indeed unattractive, then infested truffles should become attractive when the beetles are removed from them. This seems to be the case for females, but not for males: overall, females are significantly attracted by beetle-damaged truffles with soil but without beetles (66% of the females are attracted, Figure 5), and slightly less by truffles with soil and beetles (overall, 62% of the females are attracted, Figure 4). For males, the opposite is the case (overall, 60% of the males were attracted to infested truffles with soil only (Figure 5) while 82% were attracted to infested truffles with soil and beetles, Figure 4). This is remarkable because males were mainly repelled by beetles only (lower three bars of Figure 6). However, soil plus beetles were not repellent to males or females (Figure 7). It therefore appears that females are to some extent attracted to infested truffles with beetles removed, which seems to support the idea that, at the time of our study, most uninfested truffles are uninfested because they are unattractive, while attractive truffles have already been found by female beetles, and subsequently attract males. These truffles may have been highly attractive before, but may have lost some of their attractiveness due to the beetle infestation, which may cause the truffle to produce other volatile blends.

However, there are arguments against this explanation. First, artificial truffle odour is also unattractive (Figure 8). Note however, that it is unknown whether the headspace analysis of truffle odours (on which the synthesis of artificial truffle odour is based) involved uninfested attractive truffles, uninfested but unattractive truffles, infested specimens, or a mix of the three. If artificial truffle odours

resemble the volatiles emanating from infested truffles, this suggests that dogs may be trained to find infested rather than uninfested truffles. Second, Pacioni et al. (1991) recaptured most beetles in traps baited with artificial truffle odour from December to January, a period coinciding partly with the period of the year in which we did our experiments. It is possible that their study site and ours differed with respect to the timing of truffle maturation and beetle dispersal. This is corroborated by the fact that we found adult beetles and larvae of various stages in all infested truffles, suggesting that the truffles had been infested some time prior to our study, possibly by female beetles that were previously attracted to the truffles. Moreover, it is unclear how large a fraction of the total beetle population was captured in the study by Pacioni et al. (1991), hence the attractiveness of the traps may have been as low as the attractiveness of the truffles in our experiments.

Another explanation for the unattractiveness of uninfested truffles and the low attractiveness of infested truffles is that truffle beetles actually favour *T. brumale* over *T. melanosporum*, and only infest *T. melanosporum* when the densities of uninfested *T. brumale* are low. The initial replicate with infested *T. brumale* seems to suggest that this truffle species is indeed more attractive than the other species, but further research, including choice experiments, are clearly needed. Analysis of the volatiles of both truffle species has shown that there are small differences in volatile composition (Pacioni et al., 1990), but it is still unclear how persistent these differences may be.

We hypothesize that the chemical composition of truffle volatiles changes over the life of the truffle fruiting body, being attractive to insects early on and to mammals just prior to decomposition. Further research should verify this, and would help in understanding the cues involved in the searching behaviour of truffle beetles. This could prove an essential step towards the development of control measures against truffle beetles, such as odour-baited traps.

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