March 1976

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ABSTRACT: An attempt was made to identify the olfactory cues produced by Douglas-fir seeds which attract deer mice (Peromyscus maniculatus) to the seeds. The olfactometers used are described, and the merits of different statistical analyses of the data are discussed. The odors produced by whole Douglas-fir seed and by the endosperm were preferred among the fractions tested to date. Deer mice were repelled by Douglas-fir turpentine, cedar oil, and, to a lesser degree, one extract.

Since the first attempts at artificial reforestation of logged or burned forests by direct seeding of coniferous species, birds and small mammals have been a major problem. In western Oregon in 1909 four thousand acres were sown (presumably hand-broadcast) to Douglas fir, Sitka spruce, and other conifers (Black, 1969). After near-complete failure (measured by a seedling count the following spring), an attempt was made to control the depredating animals. Then a second attempt at direct seeding failed. No large-scale seeding of Douglas fir was tried again until the late 1940's, when some new chemicals and methods were developed to control the seed eaters.

That the consumption of conifer seed by animals poses a threat to the success of natural and artificial reseeding has been well documented (Gashweiler, 1967; Hooven, 1958; Spencer, 1954). Pregermination losses of seeds from all biotic factors, including vertebrates, invertebrates, molds, and fungi, often range from about 50 to 100 percent (Boyer, 196A; Laurence and Rediske, 1962). In the 75,000-acre Tillamook burn in western Oregon, 68 percent of the area was considered inadequately restocked (below 20 percent) by a massive reseeding operation (Black, 1969). Seed loss is clearly an economic liability.

A number of vertebrate species are involved in the depredation of conifer seeds. Among the most common are Juncos (Junco oregonus), tree squirrels (Tamiasciurus sp.), ground squirrels (Spermophilous sp.), chipmunks (Tamias and Eutamias sp.) , shrews (Sorex and Blarina sp.), and deer mice (Peromyscus maniculatus). Deer mice, considered the most important vertebrate predator of conifer seeds because of their abundance, ubiquity, and voracity, were the subject of this investigation. They are reported to eat 200 to 350 seeds per night in captivity (Hooven, 1953, 1958; Packham, 1970).

Success in preventing seed depredation has been moderately good from both toxic baits and seed treatments (repellents). Most of these compounds, however, are no longer registered for this purpose.

This paper seeks efficacious seed protection in another direction. It has been shown that deer mice use olfactory cues to locate conifer seeds (Howard and Cole, 1967; Howard, Marsh and Cole, 1968). If the chemical or chemicals providing these olfactory cues can be identified, many new and innovative control techniques might then be attempted. Possible applications of this knowledge are discussed later.

Howard and Cole (1967) and Howard, Marsh and Cole (1968) demonstrated that deer mice detect conifer seeds through olfactory cues -- primarily if not exclusively. Single seeds buried one to three inches deep under peat moss in petri dishes were readily located and dug up by deer mice. Controls of empty petri dishes eliminated bias from tactile or human-odor cues. In identical tests under subdued light (0.25 foot-candles) and total darkness, detection did not significantly differ. This eliminated the possibility that visual cues were important in seed detection.

Since deer mice use olfaction to detect conifer seeds, this study attempted to identify the olfactory cues involved. Some of the applications of knowledge gained about these olfactory cues are presented by Radwan (1970). He states that, once the active olfactory components are isolated and identified, it might be possible either to extract and remove

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them or mask them chemically to make them unavailable to rodents. He also believes that an increased understanding of the nature of chemicals that attract seed-eaters will shed new light on the mechanisms of repellency in these animals. One further application of such knowledge might be the addition of attractive olfactory chemicals to toxic rodent baits. This would reduce the amount of bait needed for control, thereby lowering environmental contamination from toxic rodenticides.

METHODS

0lfactometers

01factometers were designed to determine the relative attractiveness of various conifer fractions to deer mice. Room temperature was maintained at about 70°F. There were no windows, and the tests were conducted in total darkness. Six separate olfactometers were maintained in the room.

Each olfactometer consisted of a galvanized metal cylindrical tub with a 46-inch inside diameter and 23 inches deep (Fig. 1). This test arena was elevated on a plywood platform, supported by three pipe legs, 21 inches high. On the floor of each olfactometer were three sensing stations in a circle two feet in diameter in the center of the olfactometer. They were equidistant from each other and midway between the wall and center of the test arena, minimizing positional bias and making a mouse less likely to cross them in exploring the sides of the chamber of attempting to escape.

Each sensing unit was 6.25 inches in diameter. Circles of plexiglass served as the base of each unit. It had 104 brass washers elevated 1/4 inch on plastic risers and held in place with bolts (Fig. 2). The elevation is necessary to prevent short circuits caused by mouse urine or damp feces. Previous prototypes failed because of this problem. There were 1/8-inch gaps between the washers to allow fecal material to drop through. The washers form a grid of positive and negative electrodes wired so that the mouse will complete the low-voltage and very-low-amperage circuit when it walks on the unit to investigate odor emitted at the center. The sensing units were designed to be plugged into receptacles on the floor of the olfactometer for each test. They are easily removable and washable.

When a mouse completes a circuit by walking on a unit, the relay closes and activates a second circuit, which activates a Mecury C6-23 event counter connected to each of the sensing units. The event counter tallies the total number of contacts made (or circuits completed and broken) at each of the units. This provides a rapid readout for comparing the distribution of activity among the three sensing units. Each sensing unit is also connected to a pen-event recorder (the data from the event recorder will be published later).

The odor-producing substances to be tested are placed in a double wire container in the center of each sensing unit. Mice attracted to the odor must walk onto the sensing unit to get to the source of the odor. The mice are not rewarded since they cannot obtain food at the odor-emitting stations.

Test Animals

The test animals used in the olfactory preference determinations were laboratory-reared deer mice (Peromyscus maniculatus), progeny of deer mice trapped in the vicinity of Mount Shasta, California. The laboratory breeding colony was established a number of years ago. New stock was field-trapped and added to the existing colony in the summer of 1973.

Only adult animals (90 days and older) were tested. Forty-eight hours before a test the mice were placed individually in plastic cages and provided with approximately 100 Douglas-fir (Pseudotsuga menziesii) seeds (seed lot is Lorane SPA #202-093-252-1.0) in a small glass bowl. No laboratory chow was offered from that time until completion of the test. The mice were checked 24 hours later to ascertain whether they had eaten the seeds.

About 3% of the deer mice completely refused the Douglas-fir seeds. These individuals, considered atypical, were excluded from the tests and replaced by mice of the same sex and age. Even most deer mice reared in the laboratory have an inherent preference for Douglas-fir seeds.

Materials

Samples F-229-119-IVA and IVB were obtained as follows: the Douglas-fir seed was

Figure 1.

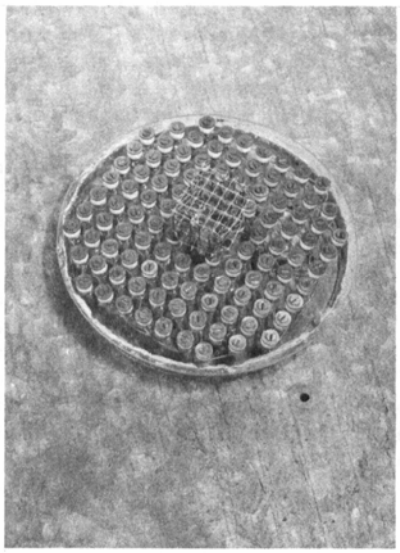

Figure 2.

Figure 1. Six olfactometer arenas made from galvanized metal cylindrical tubs with a 46--inch inside diameter and 23 inches deep.

Figure 2. One of three sensing units that are spaced equidistant from each other within each olfactometer. The sensing unit is 6.25 inches in diameter, has 104 brass contacts elevated 1/4 inch on plastic risers providing alternate positive and negative electrodes. The odor-producing substance is placed in the double wire container mounted in the center of each unit.

ground in a blender with ice water, the slurry was distilled under vacuum, and several fractions were trapped. Fractions IVA and IVB were collected at atmospheric pressure with additional heating. Sample F-370-61 was not heated. It was an ethanol extract of seeds ground in a blender, the extract filtered and ethanol removed by evaporation. F-229-l19-IVA was cooked, though less so than IVB.

The Douglas-fir turpentine certainly, and possibly the cedar oil, are products of steam distillation. The major terpenes present are limonene and beta-pinene; also present are higher molecular weight components such as sesquiterpenes and possibly di- and tri-terpenes; this is part of another investigation by Stern et al.

Procedure

To condition the animals to a larger area, more like what they will encounter in the olfactometer, and to force them to search for their food, which they have never had to do before, 24 hours before each test six mice were placed in two large cages, 18 x 36 x 18 inches, three males in one and three females in the other. This segregation should lessen the possibility that contamination by sexual pheromones might alter normal feeding behavior later during the test. The Douglas-fir seed was mixed in about 1/4 inch of sawdust on the floor of these large cages.

After the 48-hour conditioning period the six mice were transferred to the six olfactometers. At 4:45 p.m., one animal was placed in the center of each unit, the olfactometer circuits were turned on, the door to the room was closed, and lights were turned off. The room was not entered until 8:15 the next morning 15 1/2 hours later, when the tests were terminated.

Each experimental odor-producing substance was tested for three nights, but always using different mice each night. The odor was supplied at one of the three stations in each unit. The other two stations had no odors supplied and served as controls. Each

night the odor was rotated clockwise to the next of the three stations to compensate for any possible position bias. Each mouse was used on only a single night to prevent habituation to the units or odors. The replacement for each unit was of the same sex previously in that unit. For each test odor the three-night test with the six olfactometers yielded 18 sets of sensing-station data from 18 different naive mice.

The cabinet housing the relays and recording devices was outside the room to prevent mouse behavior being influenced by any noise produced by the clicking of relays. Additionally, the researcher could watch the event counters and pen recorders without disturbing the mice.

After each one-night test the sensing units were unplugged from the floor of the units, removed and scrubbed thoroughly with a bristled brush in hot water containing detergent (Ajax All Purpose Cleaner, liquid) and then dried in an air jet if to be used that night (otherwise, they were allowed to drain dry, which takes about 24 hours). The olfactometers were sponged out each day with hot water and detergent, and rinsed with clean water. Every third day or between each test if a different odor was used, they were cleaned even more thoroughly.

RESULTS

The data generated by the olfactometer tests were analyzed by several methods. One was descriptive, using the ratio of the activity at the odor station to that for the two odor-free controls as described in Table). The other methods were statistical. One statistical method was the one-way analysis of variance, as programmed by the Health Sciences Computing Facility, University of California, Los Angeles, in their BMD01V program. Two others were Scheffe's method and least-significant difference.

When the ratio is used, it controls for the response of hyperactive individuals (outliers). That is, if an individual mouse has a high value at an odor station simply because it was more active than the average mouse, a comparison of that activity with the control stations gives a truer picture of the overall situation. This method of analysis also serves as a check on the practice of removing outliers. Table 1 shows that the preferences varied widely for the different odors used. The deer mice showed relative indifference to some seed components. Two fractions (F-370-61 and F-229-119-IVA) were just slightly more attractive than the odor-free control stations. Fraction F-229-119-IVB, on the other hand, was somewhat less attractive than the controls.

Table 1. Attractant and repellent ratios of responses of 18 deer mice per sample to sensory stations emitting different odors derived from Douglas-fir seeds and cedar oil.

Sample	Preference Ratio*
	Attractant if <1
Douglas-fir endosperm	0.5980
Whole Douglas fir	0.5797
F-370-61	0.8853
F-229-119-**I**VA	0.9369
Douglas-fir seed hulls	0.9465
	Repel lent if >1
F-229-119-IVB	1.2424
Cedar oil	2.0965
Douglas-fir turpentine	2.3837

*Ratios equal 1/2 the number of times the mice responded to two odor-free control sensory stations divided by their responses to the single odor station in each olfactometer.

The results of the ratio analysis were essentially the same as those of analysis of variance (ANOV), though some differences were made more evident by the use of ratios. The repellent response to Extract F-229-1**1**9-IVB, as compared with others, and the difference in preference for the Douglas-fir endosperm (hulled seed) over the seed hulls themselves are

contrasts that are more apparent when the ratio of odor source to the control is used. Neither of these was proven significant (5% level) by the ANOV methods. It is believed, however, that the comparisons are still valid, and would become significant (even by ANOV techniques) if more replications had been undertaken.

Unfortunately, the ANOV techniques are not applicable to what the authors believe are the most important data, i.e., the ratios of activity between the control and odor stations. Therefore, the preceding ANOV calculations used only the data from the odor stations themselves. The controls were completely ignored. The result is that much valuable data was ignored.

With unequal sample sizes Scheffe's method provides an appropriate statistical way of comparing all possible pairwise combinations of means. Using Scheffe's method, the differences significant at the 5% level are those which compare the whole Douglas-fir seed with: 1) Douglas-fir turpentine; 2) cedar oil; 3) fraction F-378-61; and 4) fraction 229-119-IVB. The difference between the Douglas-fir endosperm and turpentine was also significant at the 51 level. This does not tell the whole story, however. Scheffe's method has broad application and consequently requires a greater difference between means for proof of significance.

Another method, known as least-significant difference (LSD), is appropriate for the comparisons that a researcher has in mind before the start of an experiment. One must limit the number of comparisons made with the LSD method, however, because the chance of finding a difference that appears significant but actually is not increases with the number of comparisons made. With these limitations in mind the LSD method was applied to several comparisons of interest. The whole Douglas-fir seed was compared with all other odors and found different from all at the 5% level of significance. The Douglas-fir endosperm was compared with all other compounds and found different from all but the Douglas-fir seed hulls and extract F-229-119-IVB. More definitive responses were seen with the remainder of the test materials. Both Douglas-fir turpentine and cedar oil were visited less than the controls by a factor of two. This indicates an apparent repellent response.

DISCUSSION

It is recognized that the method of obtaining these samples could account for the mouse preferences. Any heating of the large amount of oil obtained from the seeds leads to the formation of oxidation products associated with off-odors or noxious odors. F-370-61 was not heated, and F-229-119-IVA was cooked less than IVB. The repellent effects of turpentine, and possibly cedar oil, may also be off-odors developed during heating of distillation.

The volatiles from Douglas-fir seed seem to reflect an overall composition like that of needles, bark, and cortex, with differences due to the quantity of each component. In seeds some of the major components identified so far are hexanal, non-2,4-dienal, and isomers of 2,4-decadienal (Stern, Teranishi and Marsh, in press), all of which are presumed to be autoxidation products of fatty acids. An example of autoxidation is the formation of dienal isomers from linoleic and other unsaturated acids; in this case the decadienal formed is not unpleasant (Patton, Barnes and Evans, 1959).

Another possible explanation for the results is that the turpentine and cedar oil were the strongest of the odors tested. Perhaps they were powerful enough to elicit pain or discomfort in the mouse's chemoreceptors. Some contend that a repellent response exists only as a result of pain or as a cue of some unpleasant experience or sensation (Shultz and Tapp, 1970).

Another possible explanation for avoidance of those odors relates to their source. Both Douglas-fir turpentine and cedar oil had odors to humans very reminiscent of the wood of those trees. In fact, the turpentine smelled quite similar to the "pine" shavings which serve as litter material for these mice in the breeding colony. The mice may have learned to associate this odor with their bedding material. If these are indeed wood odors, the mice perhaps identified them quickly as inedible matter and spent the rest of their time in searching elsewhere. The odor of pine shavings was not tested.

An additional explanation of the responses relates to the mouse's apparent dislike for cedar seeds. Several authors report that different types of cedar seed are not well liked by rodents, so that control of depredations is usually not necessary for cedar seeds

(Schopmeyer and Helmers 1947; Issac, 1930; Moore, 1940; Fowells, 1956). This fact, if an innate characteristic, would tend to explain the lack of interest that mice have in the cedar odor. Feeding studies are needed to see whether consumption is reduced significantly by treating seed with one or both of these extracts. If so, they might be used as nontoxic seed protectants.

These results answer some of the theoretical questions. It is obvious from the data that no Douglas-fir fraction analyzed to date is as attractive to the mice as the whole seed, but that might be altered if different concentrations were tested. (Later studies suggest that pressed oil from seed is more attractive than whole seed.) The present results lead one to believe that the olfactory cue is complex and that Douglas-fir seed may be most attractive when all or most components of the seed are present or when a combination of several fractions is mixed in precise ratios.

There are other possible interpretations. Perhaps only a single discrete volatile serves as the cue and was not captured in the tested fractions. That is not likely, however, because, even when animals are unprepared or contraprepared to make an association between stimulus and response, such association can be established by repeatedly rewarding whenever the association is made (Seligmann, 1970). Furthermore, such specialization in acceptability of a discrete odor stimulus would be hard to explain from the standpoint of evolutionary adaptive significance.

Another interpretation is that one volatile is the primary cue and others, chemically similar to it, are active as cues through the process of generalization. Generalization refers to the phenomenon of stimuli slightly different from the primary one eliciting the response associated with the primary cue (Manning, 1972). This interpretation fits the observations well because it explains not only the fact that the whole seed is the best attractant, but also that some compounds resemble more closely the primary volatile than others. A great deal more experimentation (now in progress) with more finely divided and structurally identified compounds would be necessary to defend or refute any of the above hypotheses. This represents only a relatively small portion of a much more comprehensive study of volatile fractions of Douglas-fir seed now in progress. These data will be published at a later date.

ACKNOWLEDGMENTS

This project has been supported by Contracts 53500-CTI-45(N) and -CT4-3(N) of the Bureau of Land Management, Portland, Oregon. The Douglas-fir seeds were provided by the District Office of Bureau of Land Management in Eugene, Oregon. The volatile fractions were isolated at the Western Regional Research Laboratory, ARS-USDA, Albany, California. A portion of these data are from the senior author's Master's thesis. Test facilities were made available by the Institute of Ecology, University of California, Davis.

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