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Acyrthosiphon pisum (Harris) in response to
alarm pheromone and vibration

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ESCAPE BEHAVIOUR OF THE
PEA APHID ACYRTHOSIPHON PISUM (HARRIS)
IN RESPONSE TO ALARM PHEROMONE
AND VIBRATION

by

JOHN MICHAEL CLEGG, B.Sc., M.Sc.

A thesis submitted to the Faculty of
Graduate Studies and Research in partial fulfilment
of the requirements for the degree of
Master of Science

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April 1981

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ABSTRACT

Pea aphids respond to the threat of a predator most effectively by walking away or by falling from their host plant. Among the stimuli producing escape behaviour are vibration and alarm pheromone emitted by captured nearby aphids. Using these stimuli separately and consecutively, this study found that there was no evidence that different escape tendencies were heritable, and no evidence that individual aphids have characteristic escape behaviours. On the contrary, the amount of alarm pheromone influenced the particular response or method of escape: the more pheromone, the more likely an effective and dramatic escape. Vibration preceding alarm pheromone stimulation greatly increased responsiveness to pheromone, and aphids were much more likely to respond if they were feeding on stems than on the undersides of leaves. Some combinations of stimuli appear to interact and need not be simultaneous.

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I. INTRODUCTION

The family Aphididae (Homoptera), known popularly as aphids or plantlice, comprises a large group of small soft-bodied insects that suck the sap from the stems or leaves of plants. They have a pear-like shape in which the head, thorax, and abdomen are not so distinct as in most insects. Their antennae are fairly long, and they have a pair of cornicles, or siphunculi, on the postero-dorsal surface of the abdomen.

Many aphid species have a complex life-cycle, overwintering as eggs. In spring the eggs hatch to give rise to females that reproduce parthenogenetically and give birth not to eggs but to living young. Several all-female generations, without wings, may precede the birth of winged females which migrate to a different host plant where the sequence of apterous parthenogenic generations continues. Near the end of the season there is an alate sexual generation -- males and females -- which migrate to the original host plant, mate, and leave eggs over the winter.

The reproductive potential of aphids is very impressive. The particular species used in this study, the pea aphid Acyrtosiphon pisum (Harris), for example, has four nymphal instars before reaching adulthood, in temperatures of about 20°C, at an age of about seven days. For the next two weeks it gives birth to 4 to 9 young per day. From experiments on five strains of A. pisum, Frazer (1972) calculated the doubling time for populations without

predation or lack of resources to range from 1½ to 2 days. This rate of reproduction leads to typically populous aphid colonies which spend most of their time standing still with stylets inserted into the host plant, feeding. At first sight, aphids seem to be a group that has risked its whole survival strategy in rapid reproduction to the exclusion of all other tactics.

As one would expect of a group of crowded, apparently sedentary and unprotected insects, they attract predators, notably coccinellid beetles, syrphid fly larvae, and lacewings, as well as parasites like braconids and chalcids (Borror, DeLong and Triplehorn 1976). Dixon (1958) noted the general impression about aphids and their predators by quoting Imms (1947, p. 129), who wrote that the prey of coccinellid beetles consisted

of Aphids and related small insects which are all helpless sedentary and thin-skinned creatures that almost seem to invite the attention of any predator that comes along.

More recently, Wigglesworth (1972) remarked, in a section on 'reflex bleeding' in insects, that the siphunculi of aphids allow wax-laden blood cells to escape; by its context it is apparent that he considers this as a response, perhaps a safety response, to increased abdominal pressure caused by handling or attack.

The truth, however, is that aphids are by no means so passive under the threat of predators. Evans (1976), for example, in studying the role of predator-prey size ratio on the efficiency with which the

bug Anthocoris nemorum captures the pea aphid, showed that with relatively small predators the aphid has a chance approaching 100% of escaping after being touched, either by kicking, walking, or dropping.

The function of the cornicles is defensive, too; Dixon (1958) and Dixon and Stewart (1974) reviewed the history of their perceived function. Early ideas that the droplets of liquid that emerge from the cornicles assist in removing excretory products were finally put to rest by Dixon and Stewart (1974) when they blocked the ends of the cornicles of a group of sycamore aphids, Drepanosiphum platanoides, with drops of nail varnish and found that in comparison with an untreated group this caused no change in survival, reproductive rate, or weight.

Another early idea that the waxy cornicle secretion, which appears when an aphid suffers attack by a predator, was used for 'waxing' a predator -- smearing the droplets onto the predator's head so that on hardening the wax immobilizes the moving parts it covers -- received experimental confirmation when Dixon (1958) took droplets obtained by poking aphids and smeared them on the heads of first instar larvae of the coccinellid Adalia decempunctata as soon as they had caught the leg of an aphid (Microlophium evansi). This waxing caused more aphids to escape than the similar control daubing with a droplet of water. Waxing made little or no difference when a larva had caught an aphid by its body. Dixon also observed various instar and adult combinations of beetle and aphid, noting how often aphids that escaped pulled without waxing and how often they both pulled and waxed.

Unfortunately the data presented do not show whether 'waxing' meant merely secretion of the cornicle fluid or its successful spreading onto the beetle. Nor do they indicate whether or not waxy daubing affected the escape rate.

Dahl (1971: cited by Nault et al. 1973) discovered that the smell of cornicle secretions repels other aphids. This was independently confirmed by Kislow and Edwards (1972), who also discovered that in many cases the secretion produced by one species of aphid was repellent to some other species. The indication of repulsiveness was that a fragment of paper containing the secretion, when placed on an aphid-infested leaf, caused many or most aphids to move away from it. Edwards et al. (1973) identified the repellent substance to be trans- β -farnesene in Myzus persicae and stated that W.S. Bowers had informed them that the same chemical was also the alarm pheromone for Macrosiphum rosae.

In their report of this work, Bowers et al. (1972) did not explicitly identify M. rosae as the specific source of the pheromone they analysed, but found that synthetic trans- β -farnesene caused the usual aversive behaviour when presented to ten species of aphids. Later, Bowers et al. (1977), noting that trans- β -farnesene was broadly interspecific only in the aphid subfamilies Aphidinae and Chaitophorinae, identified the alarm pheromone in Therioaphis maculata (Drepanosiphinae) as germacrene A, which when synthesized acted as alarm pheromone for only those aphid species in the subfamily (not all) that were interspecifically responsive to each

other's natural pheromones. Aphidinae and Chaitophorinae aphids did not respond to germacrene A.

Wohlers (1980) suggested that trans- β -farnesene is not the only chemical controlling escape behaviour in Acyrtosiphon pisum. In comparing responses to naturally obtained cornicle secretions and various concentrations of synthetic trans- β -farnesene, he found that aphids ran further and were more likely to drop in response to the secretions than to the synthetic chemical, although the total numbers of running and dropping responses were similar.

In the first paper that quantitatively explored the effects of aphid alarm pheromones, Nault et al. (1973) determined that the sense organs responsible for detecting them are on the antennae; Microsiphum persicae had a single sensorium on the 6th antennal segment, while Acyrtosiphon pisum and A. solani had, in addition, a secondary sensorium on the 4th segment. They observed aphid behaviour in response to droplets of pheromone picked up on the end of a glass rod from the cornicles of other aphids. If one or more of a group of five aphids moved from its feeding site, the particular droplet was said to be biologically active. The percentage of secretions meeting this criterion was between 62 and 76 for the three aphid species tested. Although this figure has been often quoted since (e.g., Roitberg and Myers 1978), it is meaningless because it ignores the possibility of aphids being unresponsive to perfectly good pheromone: in fact, to test five

aphids at a time recognizes this possibility, and the presumed (certainly unremarked) absence of 'all five aphids or none' responding confirms it. That is, if it were pheromone (not aphids) that was active or inactive, one would expect either all five or none to respond. Experiments on the duration of alarm pheromone's effectiveness after secretion showed residual potency for up to 30 or 45 minutes for the two species tested; however, the data were presented in a strange way: the numbers 0,1,2,3 were ascribed (in order) to no response, moving of appendages while remaining at the feeding site, walking, and dropping. These numbers they then averaged (which is treating ordinal measurement as interval measurement (Stevens 1951)), to produce the aphids' 'average response'.

This alarm pheromone does not of course serve as a defence for the attacked aphid, but adds to the stimuli that lead other aphids to escape or avoid predators; such behaviour may be classed as altruistic. Dixon (1958) gives a diagram showing the interactions that may occur when a coccinellid approaches an aphid (Microlophium evansi) to try to catch it. As the beetle approaches, the aphid may wave its antennae, remove its stylets from the plant, kick rhythmically, or simply continue feeding. If it has removed its stylets, it may walk away or drop off the plant (or do one and then the other). Any of these things may happen also if the aphid is touched. If it is captured, it may succeed in kicking away the predator (more likely when the predator is not larger than the aphid), or in pulling itself away; or it may put cornicle secretion onto the predator's head and possibly be released.

Aphids may perceive a predator's approach, and their response varies with the size or kind of predator, as well as with their own size (instar). Dixon (1958) discovered that adult Microlophium evansi were much more likely to drop off the plant at the close approach of an adult coccinellid Adalia decempunctata than of a second instar. On the approach of small instars (first to third) of the beetle, aphids often kicked at them and succeeded in making them change direction; they did not kick but tended to walk or drop on the approach of fourth instar or adult beetles. Dixon further discovered that although first instar coccinellids remain the same size, their walking speed increases with age. Older larvae were more successful in the ratio of first-instar aphids caught and eaten to those escaping, while the aphids themselves responded differently: proportionately more dropped and fewer walked on encounter with the older larvae. Dixon included in his definition of encounter both close approaches and contact or consumption, so unfortunately it is not possible to separate the extent to which the different aphid responses are stimulated by signals received prior to contact with the predator from the stimuli of the attack itself.

Bray-Brodsky (1980) studied the reactions of the pea aphid Acyrtosiphon pisum to the approach or attack of two different predators, the larva of the syrphid fly Syrphus corollae and the adult coccinellid Adalia bipunctata. Her interest was in the outcome of 'direct confrontations' between predator and prey, but these were not defined. It appears to include both close approach and direct contact, and it is unfortunate that data are not given

separately for cases with and without actual touching. At three different temperatures adult aphids were more likely to drop when approached by the beetles than by the fly larvae, and less likely to walk.

From these studies (Dixon 1958 and Bray-Brodsky 1980), there are hints that aphids can distinguish different predators from the vibrations they make on the plant as they move about on it. If vision were a primary source of information, then there would be only speed, not appearance, to distinguish different ages of the same beetle instar. I have not found a report of any study which allows the predator to approach its prey along something not physically connected to the plant on which the aphid stands. Such a study could rule out vision as the primary sensory input concerning predators. Smell also could contribute, and could be controlled for in experimental study.

We have seen that alarm pheromone stimulates a number of behaviours in nearby aphids. Disregarding behaviours involving actual contact with a predator, these can be arranged in a continuum from no response to the most extreme and most immediately effective:

- O: nothing
- A: waving antennae or moving legs
- B: withdrawing stylets from plant and taking one step back
- R: walking (also called running)
- D: dropping

where

- O must precede A, B, R or D;
- A need not precede B, R or D;
- B must precede R or D;
- R sometimes precedes D.

Why does the aphid not simply drop and make sure of its escape?

Roitberg and Myers (1978) discuss this at length and point out that the most appropriate response in any threatening situation must be a balance between many factors: (a) the time spent not feeding until a new site is reached; (b) the risk of desiccation on the ground; (c) the possible inability to find the same or another host plant; (d) the chance of not being found or captured by a predator while remaining at the feeding site; (e) the chance of avoiding or escaping a predator by moving elsewhere on the same plant. The relative extent of these risks changes with the life stage of the aphid (smaller instars walk more slowly on or off the plant, dry out more quickly on the ground, are more likely to be obstructed on the ground, and offer a smaller target to a predator); the risks also change with the temperature and humidity, and with the type of predator (adult coccinellids move faster than syrphid fly larvae). Roitberg and Myers (1978) tested several predictions arising from these considerations, using various instars of two populations of Acyrtosiphon pisum, one from a damp temperate region (Vancouver, B.C.) and one from a hot dry region (Kamloops, B.C.). They simulated the threat of a predator by (a) vibration from prodding the plant, near the aphids, with microforceps; (b) alarm pheromone, by holding an aphid with microforceps close to the test aphids and squeezing it until it secreted pheromone; (c) simultaneous vibration and pheromone. They found some of their expectations confirmed about the responsiveness of different instars: for example, a greater proportion of adult and fourth instar aphids responded to alarm pheromone than of younger instars. However, responses to

pheromone with simultaneous vibration (which were substantially more frequent than to pheromone alone) were greater for first instars than for adults, with fourth instars exhibiting intermediate behaviour. Second and third instars behaved similarly to first instars. Greater dropping frequency in young instars was contrary to expectation, but for the aphid itself the hazards of dropping have to be balanced against their greater ease of capture and reduced chances of escape once they have been physically encountered by a predator. In comparing the Vancouver and Kamloops populations (in the same laboratory conditions), fourth instar and adult Kamloops (hot, dry) aphids were less likely to drop, run, or back-up in response to pheromone alone, though to the combined pheromone-vibration stimulus the two populations showed similar response distributions. One presumes that the alarm pheromone was obtained from the same population that was being tested, though the authors do not state this; which will turn out later to be a matter of some interest.

The same authors (Roitberg and Myers 1979) compared the escape responses of the same two aphid populations to threat from a real predator, the ladybird beetle Coccinella californica; they scored the frequency of back-up, run, and drop responses to 'direct confrontations' with the beetle, without, however, defining what this meant -- whether it included, or excluded, being touched, or the criteria for excluding responding aphids not confronted. Only approaches from the aphid's front, not its back or side, were considered. The results were that the Kamloops (hot, dry) aphids

were less liable to run or drop and more liable to back-up than the Vancouver aphids. It is not possible to completely interpret the meaning of these data because of the exclusion of 'no response' by the experimental protocol: that is, the reader cannot tell if the Kamloops aphids are altogether less likely to respond or if only their choice of response varies, or some combination of these. In the same paper, the authors describe experiments on survival time off the plant of the two populations, at two high temperatures (37.5°C and 42°C) and in moist and dry conditions. Although Kamloops aphids survived longer at the lower temperature in a moist environment, in the other three combinations there was no difference in the time to onset of paralysis (which is followed by death); this shows that the Kamloops aphids do not have any changed ability to withstand their dry conditions which could have been used to adjust the trade-off in the risk associated with different escape responses.

✓ In their 1978 paper, Roitberg and Myers tested aphids (only from the Vancouver population) for the repeatability of their individual escape responses to alarm pheromone stimulation. They exposed aphids once each on two consecutive days to an aphid provoked to give a cornicle secretion. Of those that dropped on the first day, a higher number than expected dropped also on the second day — expectation being based on the various response frequencies on the first day. They concluded therefore that some aphids were 'droppers', noting that droppers and non-droppers appeared among the offspring of the same mother. Such a behavioural polymorphism, if

heritable, could clearly be a basis for the development of populations adapted to different conditions such as those in Vancouver and Kamloops. This idea gave rise to the initial direction of the experiments constituting this thesis: Are different escape-response tendencies heritable? Another support for a hypothesis of heritability comes from Wiener and Capinera (1979), who reported that some groups of Schizaphis graminum responded differently to a synthetic analogue of alarm pheromone; they suggested the possibility of different genetic or phenotypic responsiveness.

Bray-Brodsky (1980) briefly studied the heritability question by selecting the offspring of aphids (A. pisum) that had run once or dropped once when approached by a predator. On the basis of the distribution of offspring's responses to a single predatory threat, she concluded that running and dropping were not inherited. However, a single response in each of only two generations is a weak basis on which to support belief in what is essentially a null hypothesis. Only a very high heritability could have been detected in as short a study as this.

This heritability question is of interest not only theoretically but also practically. Several authors already cited offer, perhaps rather casually, the suggestion that synthetic alarm pheromone could prove useful for controlling aphid pestilences: its delivery would provoke dropping and so on, leading to increased mortality and reduced fecundity, and the quick decomposition and biological nature

of the pheromone would not entail any undesirable environmental consequences. However, this suggestion would be of severely limited value if responses were heritable, because it would not be long before selection pressures against the more extreme escape responses rendered the technique useless.

One may wonder if selection is possible in parthenogenetic generations, because there can be no recombination of genes from different individuals. In most aphid species there is a single sexual generation once a year, which will tend to preserve a degree of heterozygosity lasting through the non-sexual generations. According to White (1973), meiosis is incomplete during parthenogenetic reproduction in that there is no reduction division, but it seems that genetic recombination by crossing-over occurs. This allows change in chromosome composition without new genetic material and provides a possible explanation for the response to selection for winglessness in parthenogenetic lines of Myzodes persicae.

Another kind of characteristic behaviour is mentioned by Roitberg et al. (1979), who studied the influence of predators on the dispersal of pea aphids between plants. They noticed that after dropping to the ground, some aphids walked about with high turning rates waving their antennae, and at a walking speed slower than that of other aphids that walked roughly in a straight line; they called these searchers and runners, respectively. Runners were more likely to finish up on a different plant from the one they had left. The authors claimed that they had thus found two behaviour types

amounting to biotypes; however, since they made no repeat tests of the aphids that searched or ran on single occasions, there is no justification whatever for believing that their behaviour is consistent within individuals -- 'searching' could as likely be caused by landing on the head instead of the legs!

There do exist, however, biotypes in the species Acyrtosiphon pisum; Frazer (1972) discussed earlier papers on different biotypes separable by body size, feeding injury, and reproductive potential. He went on to distinguish others according to demographic and other life-table statistics which varied with the plant species on which the aphids lived.

To conclude this introduction: this study started out with the intention of determining whether any of the escape responses of pea aphids to predator threat simulated by vibration, alarm pheromone, or both, were heritable. In the light of results described in succeeding chapters, this direction changed towards investigating some physical factors concerned in the understanding of aphids' escape responses; but it is more appropriate to describe this shifting emphasis as the story unfolds.

II. CHOICE OF PROBABILITY LEVEL FOR STATISTICAL SIGNIFICANCE

Virtually all introductory books about inferential statistics take some trouble to explain the meaning of tests for statistical significance. Briefly, they explain that the data obtained in a study should be thought of as material for allowing a decision between whether the numbers observed are merely chance variations under a 'null hypothesis', or if they arise from an 'alternative hypothesis'. Because of random variation in almost all bodies of data, it is always impossible to accept or reject either hypothesis with certainty. The general purpose of any inferential statistical test is to establish the probability that the numbers obtained could have arisen by mere chance under the null hypothesis. If this probability is low, then we decide that chance was not responsible. The criterion for low is known as α : if the probability P of chance figures is lower than α , we reject the null hypothesis and accept the research hypothesis. Whatever α we choose, however, there is always the possibility that we make an erroneous decision: the null hypothesis may indeed be true even though we have rejected it, or, conversely, the research hypothesis may be true even though we have accepted the null hypothesis. There is no way out of this dilemma. Conventionally, α is taken as 0.05, 0.01 or 0.001, but many other levels are also used. The point that textbooks stress is that a value for α must be chosen before a test is made, and when P is determined, it is either above or below α .

A common practice in scientific papers is to calculate P and show it as here ≤ 0.05 , there ≤ 0.01 , and so on. This is quite improper: α must be chosen in advance, and you must abide by the decision that P then forces on you. Another practice is to attach phrases to the numbers, like 'highly significant' ($P \leq 0.01$), or 'very highly significant' ($P \leq 0.001$). These are completely meaningless: P is either significant, or it is not. This practice, moreover, induces some people to think that significance has reference to the conclusions, not just to the statistic.

The point of this argument, for present purposes, is that before starting the work reported here I adopted an α of 0.05 for any statistical test that might be appropriate, and I will report the result of any test as $P \leq \alpha$ (significant) or as not significant.

(Explicit support for some of the arguments above is given by Lindquist (1953, p. 92).)

III. CULTURE METHODS

This Chapter describes the general procedures for handling and culturing the aphids and plants used in the experiments reported below. Details of procedures peculiar to the experiments will appear in appropriate places later.

Aphids

An existing stock of pea aphids (Acyrtosiphon pisum (Harris)) supplied all aphids used in these experiments. It consisted of twelve flower pots 125 mm high of standard shape and upper diameter 127 mm, each containing about fifty pea plants (Pisum sativum, Alaska variety); each week the pots of peas were replaced with fresh pots containing seedlings about 30 mm high, to which a proportion of the aphids was transferred. By the end of each weekly period the plants were almost completely covered with aphids. From this culture I removed adult aphids as required to produce aphids which would later be the desired age for the various experiments.

All peas grew in a mixture of equal parts of peat, perlite, sand, and clay loam.

Production of Aphids for Experiments

In the laboratory conditions (see below) adult aphids gave birth to between three and ten offspring per day. It was therefore

simple to obtain enough aphids of a given age, by placing a number of adults onto a pot of pea plants, or into a covered petri dish of diameter 140 mm containing two or three pea seedlings (with shoots between 40 and 100 mm) with their roots covered by a wet piece of paper towel. The adults could be removed after three or four hours if I wanted no more than one, or maybe two, young from each mother, or after 24 hours if the number of young per mother did not matter. I would move the young either to a previously uninhabited pot of pea seedlings, or to single seedlings in vials. If the mothers had been breeding for 24 hours, then the offspring were assumed to be 12 ($\pm$ 12) hours old at this time.

Pots of peas and aphids were thenceforth kept in aphid-proof cages (210 x 210 x 330 mm high) in a growing room, while vials supporting aphids were covered by translucent ventilated covers and placed in racks in the same room.

The Growing Room

Pots of peas without aphids grew in a greenhouse under natural and artificial light; any plants supporting aphids grew in a room with a controlled environment including artificial sunlight operating on a cycle of 16 hours on and 8 hours off. This room was the site of all experiments. Outside the cages and covers, the intensity of the light at bench level varied between 2000 and 6000 lux at different places.

The temperature in the room typically varied between 23°C and 26°C in a cyclical fashion with a period of about 10 min. In the dark these figures dropped by 2°C. The temperature was in fact set to be constant at 20°C, but its variation and average were fairly constant throughout the months of experiments. Relative humidity remained between 23 and 45 percent.

Vials, Covers, and Racks

Single pea plants grew in small plastic vials (30 mm external diameter, 51 mm height) filled with the soil mixture. I made several racks which held each vial by the lip at its top so that a whole rack of vials could be easily watered by placing the rack over a suitable dish of water, which entered the vials through small holes in their bases. Watering every three or four days kept the peas healthy without their being plagued by fungi. Transparent plastic cylindrical covers with ventilation holes covered by organdy stood astride the vials and rested on the rack. These covers were 97 mm high and 46 mm in diameter. From time to time I pruned the plants to avoid undue disturbance to the aphids in removing and replacing covers at experiment time.

Any aphids tested had always spent at least the previous day on a single plant in a covered vial; some had spent most of their lives on it.

Brush and Spoon

The most-used item of equipment was an artist's thin, sparsely-bristled, camel-hair paint brush. This alone was sufficient to pick up, move, and deposit first-instar aphids.

By heating up a plastic teaspoon I was able to bend it so that its business end became narrower and its sides higher. This spoon was invaluable for catching larger aphids knocked off peas by the brush, and for gathering them from the bench, with the aid of the brush, to place them where necessary.

Stopwatch

For timing purposes I used a clockwork stopwatch with a centre-sweep second-hand revolving once per minute. The watch was similar in size to an old-fashioned pocket-watch and when suitably placed was easy to see while mainly attending to the aphids.

Pea-shaker

I needed a device for vibrating plants that would provide a more repeatable and constant stimulus than the manually applied poking stimulus of Roitberg and Myers (1978). While thinking of this problem I met a former acquaintance on a train, and as he was an engineer I explained it to him. He suggested attaching a piece of rigid wire to the moving part of an electromechanical relay. A

friend the next day made such a thing for me, together with the necessary circuitry and controls to vary its frequency from 3 to over 25 Hz. The shaker is illustrated in Fig. 1. The amplitude of its excursions was 1.6 mm.

A small amount of informal investigation revealed no obvious effect of different frequencies on the behaviour of aphids; I chose a frequency of 5 Hz, somewhat arbitrarily (though with the striking-speed of a vigorously searching Syrphus corollae larva in mind), for use throughout these experiments.

A general view of the equipment used in the experiments is shown in Plate 1.

Figure 1. Pea-vibrator.

A large relay is bound to a wooden handle by tape, and a wire connects the relay to its control circuitry. A rigid piece of bent wire (taken from another relay) is soldered to the moving part of the relay; its tip is the part applied to a plant to make it vibrate.

Plate 1. Experimental set-up, showing equipment.

A pruned pea plant in a vial stands in a rack, surrounded by a cardboard shelter with a perspex top for reducing draughts while permitting access and visibility. Near the stopwatch is the microcapillary tube holder with its rubber bulb. Almost off the bottom of the picture is the end of a pair of tweezers holding a barely visible paper sliver. The strange object at lower left is the pea-shaker, a large relay taped to a wooden handle; its extended point of application is at its upper far right. The shaker's on-off switch is dangling over the edge of a box holding its power supply and circuitry.

In the centre background there is a rack of covered vials. The small upright cylindrical bottle contains microcapillary tubes.

IV. VIBRATION EXPERIMENT

Purpose

The purpose of this experiment was to discover whether pea aphids have a characteristic response to a vibration stimulus that simulates the approach of a predator on the plant, and to discover if any such characteristic response is heritable.

Method and results

The experiment ran for three generations, until it became apparent that any of the sought-after effects were non-existent or so weak that they would take a very long time to find. The initial generation was a random selection from the 220 offspring of 120 adult mothers placed on pea seedlings in a petri dish for 9½ hours. Thus there were guaranteed to be very few pairs of sisters in the starting generation and therefore no undue lack of genetic variation.

The aphids were tested at age 7½, 8½ and 9½ days (±½ day) for their response to vibration on the plant. From their birthday until the first day of testing, up to five aphids lived on each pea, planted one to a vial. At testing I carefully removed the vial cover and placed the vial on a rack of its own so as to present a clearer view. If an aphid was alone on the surface of a leaf, I applied the shaker to the opposite side of that leaf. For an aphid on a stem, the point of application for the shaker was the opposite

side of the stem about 5 mm away. Each of the aphids on a plant underwent vibration separately unless there were more than one on the same leaf, or if two or more were close together on a stem. All vibrations lasted 5 seconds. Any aphid responding to a stimulus intended for another on the same plant I considered to have responded and gave it no further stimulus.

Following Roitberg and Myers (1978), I classified and defined the possible responses as follows:

- O, nothing no response
- B, back-up the aphid removes its mouthparts from the plant and takes one step backwards
- R, run the aphid walks or runs from where it had been feeding
- D, drop the aphid drops off the plant (even if it 'lands' on another part of the plant)

These responses are clearly ordered, and so if an aphid did more than one of these things, the 'biggest' one was recorded ($D > R > B > O$).

Aphids that responded were placed on plants in vials containing other aphids that had made the same responses. This way the history of the aphids was kept track of without an unmanageably large number of plants. In placing aphids on the second and third days of a generation's testing, I considered immaterial the order in which different responses occurred: for example, an aphid which dropped one day and did nothing the next (D,O) was considered the same as one making the same responses in the reverse order (O,D). On the

third testing day of the first generation I selected the aphids that had most often dropped, run, backed-up, or done nothing. Because of the shortage of aphids that did anything, I also selected one that had backed-up once and dropped once. These selected aphids, segregated according to behavioural history, spent 24 hours on covered peas in vials to produce the second generation.

The second generation underwent the same testing procedure for three successive days when $7\frac{1}{2}$, $8\frac{1}{2}$, and $9\frac{1}{2}$ days old. At the end of third day's testing, given the data then available, I was able to select aphids that had made no response for two generations, and aphids that had shown one back-up or dropping response for a second generation. (To run was so rare -- indeed there was no running at all in the second generation -- that it was impossible to continue selecting for running). These selected aphids produced the third generation according to the same procedure used earlier to produce themselves.

Examination of Table 1 shows that all interesting responses were so rare as to render unpromising any efforts to continue the search for inheritance of characteristic responses without a very laborious increase in effort.

Having a greater number of aphids in the third generation than in the previous two, I decided on the basis of its first day's data to abandon this experiment in its present form and saved the four aphids which dropped that day, they now being third-generation

TABLE 1

- (a) Frequencies of responses of aphids to vibration: the first generation (122 aphids x 3 days†).

	day 1	day 2	day 3	total
response				
O: nothing	94	95	92	281
B: back-up	10	3	5	18
R: run	2	0	2	4
D: drop	15	10	3	28

- (b) Frequencies of responses of second and third generation aphids to vibration, classified by criterion of selection in breeding. First and second generations were each tested for three consecutive days, the third generation for one day only.

Second generation (106 aphids x 3 days†)

responses of mother*	O	BB	BD	R	D	DD
number of aphids	28	24	5	22	15	12
response						
O: nothing	69	61	14	57	34	34
B: back-up	9	5	1	3	2	0
R: run	0	1	0	0	2	0
D: drop	2	3	0	4	5	2

Third generation (166 aphids x 1 day)

responses of grandmother:mother*	O:O	BB:B	D:D	DD:D
number of aphids	102	25	26	13
response				
O: nothing	94	17	23	11
B: back-up	6	0	1	0
R: run	0	0	0	0
D: drop	2	8	2	2

† not all aphids were tested each day because of death or aphid not on the plant.

* O means no response for 3 days; other letters refer to responses other than no response -- for example, BD means one back-up, one drop, and one no-response.

'droppers'. I put these four onto a flower pot full of pea plants and allowed them to be the founders of a culture of aphids. The population soon increased, and some of it I moved to a new pot of peas every week until after six weeks (five or six generations) I bred an experimental cohort by placing 132 adults on pea seedlings in petri dishes for 5 to 5½ hours, during which time they gave birth to 121 young. At the same time and in a similar manner, 132 adult aphids from the permanent laboratory culture ('control') gave birth to 124 young. It is possible that relaxed selection of the 'droppers' could have allowed regression during the six weeks, but it was worth trying the following experiment.

When these two groups of aphids were 7 days old I used them in a last attempt to see if there was any indication whatever that a tendency to drop in response to vibration could be inherited. From soon after birth they had lived four to a plant in covered vials. The experiment used the same procedures as before, with one difference. Although the plant shaker had a constant frequency, the way it was touched to the plant could easily affect the amount of disturbance given through it to the aphids. I had been aware of the possibility for influencing results to conform to hypothesis, and of the equally undesirable possibility of excessive deliberate precautions against this; I decided to avoid this by having a friend, in my absence, randomize the positions of all the vials in the racks and make a key to which were 'droppers' and which controls for my later use. Knowing that Mendel has been accused of fudging frequency data that are unusually close to expectation, it is with

some trepidation that I mention Table 2, which shows the frequencies of the various responses for the 'droppers' and controls: there was no difference between the two groups and no reason to continue this particular experiment.

Discussion

As we have seen, Roitberg and Myers (1978) used, as signals of simulated predatory threat, alarm pheromone both alone and in conjunction with vibration. One might wonder why they did not report experiments with vibration alone: because they did not think of it, or because they tried it without success? Since Bray-Brodsky (1980) found that pea aphids behaved differently in response to the perceived approach of syrphid fly larvae or coccinellid beetles, where vision, vibration, or smell could provide distinguishable stimuli, it was possible that vibration alone could have been a sufficient stimulus to escape.

It was also possible that what responsiveness there was resulted from the vibration physically loosening the aphids' hold on the plants. Whatever the explanation, it appeared more promising to proceed with a similar experiment using alarm pheromone as the stimulus.

TABLE 2

Frequencies of responses to vibration of two groups of aphids, a control group and a group descended from four 'third-generation droppers'; 120 aphids in each group, all tested once.

	control group	'droppers'
response		
O: nothing	108	107
B: back-up	5	4
R: run	0	1
D: drop	7	8

V. ALARM PHEROMONE EXPERIMENT

Purpose

A second experiment sought to discover if aphids inherit characteristic escape behaviours in response to alarm pheromone.

Method and results

Briefly, the plan was to start with a cohort of about 30 aphids of the same age, born of mothers of the same age also. I would test each aphid individually for its response to alarm pheromone twice per day for four days, at the end of which, on the basis of their responses, I would select the aphids whose offspring would form the second generation. The procedure would be repeated for as many generations as appropriate. (In the event, it took so long to test every aphid twice per day that after the first day I reduced this to once per day for the rest of the experiment.) Because the chance of verifying whether escape responses are an individual characteristic would depend partly on the actual frequency of responses, using as few as about 30 aphids per generation might well not succeed in this; though if response behaviours proved to be heritable this question would have been answered.

The starting generation was born of 62 adult aphids $10\frac{1}{2}$ ($\pm \frac{1}{2}$) days old, living on pea seedlings for 3 hours with about 20 aphids in each of 3 petri dishes. The age of the mothers was arranged to be

the same as that of the mothers of subsequent generations. This produced 58 young, of which about 40 were transferred to young peas growing in covered vials. Since it was desirable in the experiment to avoid pheromone being sensed by aphids not under test, these experimental aphids lived one per pea until the end of the experiment, except for their own offspring which were occasionally removed and were always easily distinguishable from the adults. The remainder of the 58 young were transferred to a few other vials to be kept in reserve in case of death.

To provide aphids to supply experimental alarm pheromone, a group of 24 to 30 adult aphids bred freely in petri dishes for four consecutive periods of 24 hours; by the time of the experiment, each of the offspring so produced had lived for a week in one of four pots of peas and formed a sufficient supply of adults. In this and all subsequent experiments the aphids supplying pheromone were seven days old (since birth).

To obtain alarm pheromone I removed an adult from a plant and put it in an empty petri dish. I poked from above at its thorax and abdomen with the paint brush until it erected its cornicles and emitted pheromone. This was visible as small droplets at the ends of the cornicles. Having prepared a sliver of paper towel about 20 mm x $\frac{1}{2}$ mm held in tweezers, I touched the end of the paper to the droplets, immediately absorbing them. This description makes it sound easier than it was to obtain pheromone; in fact I sometimes had to poke at an aphid for well over a minute to obtain anything,

and sometimes no amount of prodding was successful. The Roitberg and Myers (1978) method of holding an aphid in microforceps up to the experimental aphid was impractical on several grounds: (1) it is very difficult to hold an aphid with just the right pressure not to drop it or squash it; (2) it is also difficult to annoy it enough to produce alarm pheromone while holding it so carefully; (3) any time spent doing this while holding the pheromone aphid up to the experimental aphid produces uncontrolled visual stimulation and risks touching one with the other.

Any aphid once used successfully or unsuccessfully for supplying pheromone was not used again; and every alarm pheromone stimulus used was from one or both cornicles of only one aphid. It was always possible to confirm that pheromone had been transferred to a sliver of paper because of a yellowish-green coloration imparted to it. A fresh sliver was used for every trial.

Pilot experiments had failed to confirm that a three-second exposure to alarm pheromone would produce responses as frequently as Roitberg and Myers (1978) obtained. In order to increase the frequency of responses I adopted the following stimulation schedule, measured from the time of getting pheromone onto the paper sliver:

- (a) for 30 s, hold scented end of paper as close to the aphid as possible without danger of physical contact, especially with moving antennae;
- (b) remove stimulus for 30 s;
- (c) repeat stimulus as in (a);
- (d) remove stimulus and watch for response for another 30 s.

If an aphid ran before the second stimulus was finished, I followed it with the paper so that it was still getting the planned stimulus.

Exploratory experiments had shown that there is a behaviour intermediate between doing nothing (O) and backing-up (B): waving the antennae (in which I include any antennal movement during a period of interest). I therefore decided to add this category to these previously used, and to use it for the rest of this thesis. The response categories, then, were:

O: nothing
A: wave antennae
B: back-up
R: run
D: drop

These are still an ordered set in which each response may be said to include those above it. (Usually dropping follows running but sometimes immediately follows backing-up.) On any trial I recorded the first occurrence of any kind of response but scored for later analysis the most extreme one in the two-minute stimulation and observation period.

To test an aphid I carefully removed its vial cover and then moved its vial from the rack and put it on a platform under the growing lights. A white cardboard shelter (shown in Plate 1) placed around the vial, with a clear perspex sheet over its top, protected the aphid's surroundings from being disturbed by air currents that could

circulate the pheromone in unknown and unwanted ways. Half of the front face of the shelter was open for holding up the stimulus paper and for observation. The shelter could be turned upside down if needed, to allow a better view of, or access to, the tested aphid. An aphid that was not feeding with its mouth parts inside the plant, or one that was disturbed during preparation for the trial, was left or put on the soil of its vial and returned to its position on the growing rack for testing later in the evening -- provided it was by then settled and feeding.

All experiments took place in the afternoon or evening, so that each set of trials was separated by about a day; it would have been impossible for practical reasons, and unnecessarily fastidious, to have striven for an exact 24-hour experimental cycle. However, to reduce any chance of systematic time-of-day effects I tested the four racks of aphids in a different order each day so that over four days each rack had been tested first, second, third, or fourth once each.

For the first generation there were 30 aphids. Twenty-six of these were tested as described, while four of them acted as controls: their stimulus was the paper sliver without pheromone. Over the four days there was not a single response to paper alone. Four aphids from the other 26 also made no response in four days, so the empirical probability estimate that a given aphid will not respond to a significant stimulus in four trials is $\frac{14}{26} = 0.154$. The

probability that the four control aphids would not respond in four trials if the paper alone were a significant stimulus is therefore $(0.154)^4 \approx 5.6 \times 10^{-4}$. This probability is so low that I concluded that the control stimulus was ineffective and did no more control tests in this experiment.

Results in first generation

The data for the four days on which the first generation was tested are shown in Table 3, together with a daily summary of the number of each kind of response obtained. It is immediately apparent that the frequency of the responses B, R, and D -- the only ones that in nature would have any survival value -- are disappointingly low and form a poor basis for selecting the next generation. In fact, the only aphid that dropped at all ran on another day. Clearly there was nothing to be gained in selecting for runners and droppers, so I decided on the fourth day to select for characteristic responses of any kind, choosing (a) no response on any day, (b) running once, (c) backing-up once and (d) running once and dropping once, and to see if there would develop a tendency for any line of descent to behave differently from any other. The two right-hand columns of Table 3 show which aphids mothered the second generation, and how many of their daughters formed it.

Selection for the second generation took place on the fourth experimental day of the first generation. By removing all babies on the third day I was assured that by selection time all babies would be $\frac{1}{2}$ ($\pm \frac{1}{2}$) day old.

TABLE 3

Responses of first generation aphids to alarm pheromone stimulation (O: nothing; A: waving antennae; B: back-up; R: run; D: drop).

aphid number (nominal)	day number				aphids whose offspring formed the 2nd genera- tion, and reason for selection	no. of off- spring into 2nd genera- tion
	1	2	3	4		
A1	*	A	O	O		
A2	A	A	O	O		
B1	O	O	A	O		
B2	O	A	O	O		
B3	O	A	O	O		
B4	O	O	O	O	all O	2
B5	O	O	A	dead		
B6	O	O	O	O	all O	3
B7	A	O	O	*		
B8	O	O	O	O	all O	2
D1	A	A	O	O		
D2	R	A	O	O	one R	2
D3	O	O	O	R	one R	2
D4	A	R	O	O	one R	2
D5	O	O	O	*		
D6	R	O	O	O	one R	2
D7	O	B	O	O	one B	3
D8	O	O	O	O	all O	2
E1	A	O	A	O		
E2	A	O	O	O		
E3	O	A	O	O		
E4	A	O	O	O		
E5	O	O	O	A		
E6	O	O	A	O		
E7	O	O	A	A		
E8	dead†	R	D	O	one D, one R	6

total
responses

O	15	16	20	20
A	7	7	5	2
B	0	1	0	0
R	2	2	0	1
D	0	0	1	0

total

71
21
1
5
1

* not tested

† replaced with another aphid for subsequent days

TABLE 4

Responses of second generation aphids to alarm pheromone stimulation, with basis of selection in the first generation (O, nothing; A, waving antennae; B, back-up; R, run; D, drop).

aphid number	basis of previous selection	day number				
		1	2	3	4	
A1	all O	A	O	A	O	
A2	one R and one D	O	O	O	R	
A3	all O	O	R	O	A	
A4	all O	O	O	A	D	
A5	all O	A	A	A	A	
B1	one R	O	A	O	dead	
B2	one B	O	A	A	A	
B3	all O	O	O	A	O	
B4	one R and one D	O	O	O	A	
B5	one R and one D	O	O	R	O	
B6	one R and one D	A	O	R	O	
B7	one R and one D	O	O	O	A	
B8	all O	O	O	A	O	
C1	all O	O	A	O	O	
C2	all O	O	O	O	O	
C3	one R	O	R	D	R	
C4	one R	A	O	O	O	
C5	one R	A	A	A	B	
C6	one R	A	O	A	O	
C7	one R	O	O	A	A	
C8	one R	O	A	A	A	
D1	one R	B	A	O	dead	
D2	one R	O	A	R	B	
D3	one B	R	O	A	dead	
D4	one B	O	D	A	A	
D5	one R and one D	O	O	A	A	
<hr/>						
total responses:					total	
	O	18	15	9	9	51
	A	6	8	13	9	36
	B	1	0	0	2	3
	R	1	2	3	2	8
	D	0	1	1	1	3

Second generation pheromone experiment

Following the same procedures as for the first generation, each of 26 aphids was tested once per day for four days. Table 4 shows the response data together with the relevant maternal behaviours recorded previously. At the bottom of the table are the daily frequencies of each kind of response and the four-day totals. In Table 5 the total responses have been separated according to the criterion of selection in the first generation. Not only are the interesting responses disappointingly rare again, but there was only a single running aphid descended from a running mother -- hardly a sufficient foundation for a selection experiment -- and no backer-up descended from another. Moreover, there is no suggestion that doing nothing is a heritable trait, the distribution of the 0 column being similar to that of the totals.

There is, however, a difference between no-response (0) and some-response (not 0) frequencies in generations 1 and 2.

		Gen. 1	Gen. 2
response	0	71	51
	not 0	28	50

For these frequencies, $\chi^2 = 8.59$, $df = 1$, $P < \alpha$. Is the higher responsiveness in the second generation due to inheritance? The response frequencies for the second generation, according to descent, are:

		mother	
response	0	0	not 0
	not 0	19	32
		13	37

TABLE 5

Summary of responses of second generation aphids to alarm pheromone stimulation, classified according to the basis of the selection of their mothers in the first generation (O, nothing; A, waving antennae; B, back-up; R, run; D, drop).

responses of mothers	all O	one B	one R	one R and one D	total
total* responses in 2nd generation					
O:	20	3	12	16	51
A:	11	6	14	5	36
B:	0	0	3	0	3
R:	3	1	1	3	8
D:	2	1	0	0	3
no. of aphids tested	9	3	8	6	26

* each aphid was tested four times; some totals are less than four times the number of aphids because of death.

This tabulation gives $\chi^2 = 1.00$, $df = 1$, P not significant. Thus although there was a difference between the generations, it is not attributable to heredity,

I concluded that the experiment in its present form was not worth continuing. Impracticably greater numbers of aphids would have been necessary; and there was not even a hint that an aphid which backs-up, runs, or drops was more likely than any other aphid to repeat the behaviour.

The rate of significant escape response (B, R, and D) was clearly too low; in proceeding to the next experiment, on vibration with pheromone, I greatly hoped that Roitberg and Myers' (1978) finding of vibration increasing the pheromone effect would show up again and provide something to select from.

VI. EXPERIMENT ON ALARM PHEROMONE FOLLOWING VIBRATION

Purpose

This third experiment was for determining if aphids inherit characteristic escape responses to alarm pheromone stimulation that follows vibration of the host plant.

Method

In the previous experiment using alarm pheromone as stimulus it had been possible to maintain consistency of the timing of stimulation because the cycle began with the successful transfer of pheromone to paper sliver. In this experiment I intended to use a vibration stimulus shortly before testing responses to alarm pheromone. Obviously it was desirable to keep the interval constant between vibration ending and pheromone starting. As mentioned above, the time needed to get this pheromone was unpredictable, but by profiting from an earlier suggestion from Barlow (personal communication, 1980) to collect it in microcapillary tubes, I was able to solve the consistency problem. These tubes, called Microcaps, are 34 mm long and hold 1 μ L when full; one tube fits through an otherwise airtight plug in a larger tube of diameter about 8 mm with a flexible rubber bulb over its other end. By holding a finger over a small hole in the rubber and squeezing the bulb, fluid that has entered the tube by capillary action can be expressed. So instead of collecting pheromone directly onto a paper

sliver, I touched one end of a Microcap to the droplets when they appeared at the tips of the cornicles. Storing the pheromone in the tube allowed vibration of the plant to precede pheromone stimulation by a fixed amount, while the danger of evaporation was greatly reduced so as to be virtually negligible.

For no particular reason except that it was easy to do, I took to recording the amount of pheromone in the Microcap for every trial.

It was immediately apparent that there was unexpectedly large variation in the quantity obtained from different aphids; but whenever it came to less than 0.5 mm in the tube (~15 nL) I considered it too little and obtained a fresh quantity from another aphid. As before, the pheromone of one different aphid was used for every trial. We shall see later that these measurements proved interesting.

In the second generation of the pheromone-only experiment I had -- from vague speculations about why most pea aphids settle on the lower surfaces of leaves rather than on their tops or on stems, at least when there is not severe crowding -- adopted the practice of noting the position of each aphid tested. Where an aphid's appendages touched more than one of these three categories of place, I used the point of insertion of the stylets as the criterion. Again we shall see that this record proved interesting.

Although I had been following the Roitberg and Myers (1978) classification and definition of possible escape responses, except for adopting antenna-waving (A) in addition, I had noticed that

occasionally an aphid would withdraw its mouthparts from the plant but did not take the backward step that Roitberg and Myers included in their definition of backing-up; I considered this also to qualify as backing-up. When this happened there was sometimes almost no body movement at all, making it hard to detect, and indeed of uncertain detectability when an aphid was on the underside of a leaf (which is usually concave), because the mouthparts cannot be seen or 'not-seen' with confidence by the naked eye against a green background.

The protocol for giving an aphid a trial for its response to vibration followed by pheromone was as follows.

- (a) obtain pheromone in Microcap and start the stopwatch;
- (b) during a 10 s interval, (i) measure the amount of pheromone in the tube, (ii) put it on the bench, (iii) pick up the shaker and bring it close to the plant;
- (c) turn on the shaker and hold its end lightly against a (different) leaf some distance from the aphid; apply the 5 Hz vibration for 15 s while verifying that the aphid is being visibly but very slightly moved by the shaking without actually making one of the escape responses;
- (d) during a pause of 20 s, (i) put down the shaker, (ii) move the screen and perspex cover to surround the plant, (iii) expel the pheromone onto the tip of the paper sliver already held in the tweezers;
- (e) hold the tip of the sliver as close as possible to the aphid without danger of touching it, for 30 s;
- (f) a 30 s pause without stimulating the aphid, during which I wrote down the data so far for the current trial;
- (g) a repeat of step (e) (pheromone stimulation);
- (h) 45 s of further observation of the aphid.

I recorded all responses that occurred in either pheromone-stimulation period, in the gap between them, or in the 45 s follow-up period. If an aphid was running I followed it with the sliver so as to continue pheromone stimulation. Since in the end it was the most extreme response during a trial that was the major interest, an aphid's dropping marked the end of that trial.

Any aphid that did not still have its mouthparts in the plant after vibration or before the first pheromone stimulation was returned, covered, to its rack and left in place until being tested later in the same evening.

The starting generation of 28 aphids came from the daughters of 51 aphids $10\frac{1}{2}$ ($\pm\frac{1}{2}$) days old placed on seedlings in petri dishes for three hours. Each aphid grew up on individual plants in covered vials. Starting when they were 7 days old, I tested each aphid once a day for four days; after testing I removed any offspring so that there would be a minimum of possible inter-aphid stimulation arising from the pheromone test the next day, and so that when I selected for the second generation on the fourth day, only the offspring wanted were present. This schedule meant that the mothers of each generation were always 10 ($\pm\frac{1}{2}$) days old at the time of breeding.

Experiments took place in the afternoon or evening, roughly but not exactly at 24 hour intervals. To avoid possible bias from change in aphid reactivity with time of day, I tested the racks in rotating

order so that each of the four racks was tested once each in first, second, third, and fourth position.

Results in first generation; selection of second generation.

Table 6 shows the maximum response of the 28 aphids on four days. It is clear that vibration preceding pheromone is much more effective than was either stimulus alone in the previous experiments. Both running and dropping were much more common, common enough in fact to permit selection for runners and droppers to give a more direct test to the heritability hypothesis than had been possible before.

In order to avoid the danger in a selection experiment of all organisms in one group after very few generations being descendants of a single individual, I decided to maintain descendants of at least four members of the first generation in each selected group in subsequent generations. This plan was confounded by two mistakes. One of the aphids, B5, which dropped on the third day, would have been chosen as a mother of some of the second generation; after it dropped I looked away to record the datum and remove the young already on the plant. Unfortunately I then could not immediately find the adult, and while moving things around in the search I accidentally killed it. On searching the plant again, I found one baby missed earlier, and although it was one day older than planned I used it in the second generation. I found two days after the birth of the rest of the second generation that I had made another

TABLE 6

Responses of first generation aphids to alarm pheromone stimulation following vibration. Positions of aphids indicated by b, leaf bottom; t, leaf top; and s, stem. Responses: O, nothing; A, waving antennae; B, back-up; R, run; D, drop.

aphid number	day number				selected for (no. of offspring)
	1	2	3	4	
A1	As	Ds	Rs	dead	-
A2	Ob	Rb	Db	*	-
A3	Ob	Bs	Ab	Ab	-
A4	Ab	As	Rs	Rb	run (4)
A5	As	Os	Ds	As	drop (4)
A6	Ab	Ds	Ab	Rs	-
A7	Ab	Rb	Ab	Ds	-
A8	Ab	Ob	*	Rs	-
B1	Rt	Db	*	Os	-
B2	Ob	Rb	Rb	Rb	run (4)
B3	Bb	Bb	Rs	As	-
B4	Ds	Ds	Ab	Ab	drop (0+)
B5	Ab	Ds	Ds	dead	drop (1)
B6	Bs	Ab	Bb	Bb	-
B7	Bb	Bb	Ab	Ab	-
B8	Bs	Ab	Rs	Bb	-
C1	Ds	Rs	Ab	dead	-
C2	Rs	Rb	Ob	Ob	run (4)
C3	Ab	Rs	Ab	As	-
C4	Ab	Rs	Rs	Rb	run (4)
C5	Ds	Rb	Ab	As	-
C6	Ab	Ab	Ab	Ab	-
C7	Bb	Ab	Os	Rs	-
C8	At	At	Ds	Rs	-
E1	Rs	Ob	Rs	As	run (4)
E2	Db	Ab	Db	As	drop (4)
E3	Ab	Ab	Rs	As	-
E4	Rs	Rs	Db	Ds	both (1)

Total responses

O	3	3	2	2	10
A	12	8	9	11	40
B	5	3	1	2	11
R	4	9	8	7	28
D	4	5	6	2	17

* aphid responded to vibration and could not be tested with pheromone.

† see text for explanation of the zero.

mistake, for the plants that were supposed to be supporting the offspring of another dropper were, in fact, without aphids at all. Since mortality of young instars was typically very low I could not attribute this to natural causes. The result of these mishaps was that only three 'droppers' of the first generation were represented by their offspring in the second.

Without any other errors I was able to have five 'runners' represented in the second generation. The aphids whose offspring were selected, whether selection was for running or dropping, and the number of offspring used, are indicated on the right-hand side of Table 6. (One aphid that had made two runs and two drops contributed one aphid to the next generation, on the chance that subsequent responses might be interesting; but the daughter aphid made neither response when the time came and its line of descent was discontinued.)

Thus there was a second generation of 30 aphids; when they reached the age of $7\frac{1}{2}$ ($\pm\frac{1}{2}$) days they underwent the first of four consecutive days of testing, following the same procedures as for their mothers.

Results in second generation; selection of third generation.

The daily maximum response of each aphid is shown in Table 7. The frequencies of the escape responses that are potentially effective in nature -- B, R, and D -- were comparable with those of the first

TABLE 7

Responses of second generation aphids to alarm pheromone stimulation following vibration (O, nothing; A, waving antennae; B, back-up, R, run; D, drop). Positions of aphids indicated by b, leaf bottom; t, leaf top; and s, stem.

aphid number	offspring of runner (r) or dropper (d)	day number			
		1	2	3	4
A1	r & d	Ab	Ab	Ab	Ab
A2	r	Ab	Ab	Ob	Ab
A3	r	Ab	Rs	Ds	Rs
A4	r	As	Ab	Ab	As
A5	d	At	Rb	As	Ds
A6	d	Ab	Ab	Bs	Rs
A7	d	Ot	Bb	Ob	Ab
A8	r	Ab	Rb	As	Ab
B1	r	Ab	Rs	As	As
B2	r	Ds	Os	Ob	As
B3	r	Bb	Ab	Ab	Ab
B4	r	Bb	Bb	Ab	Ab
B5	r	Ab	Ab	Ob	Ob
B6	d	Ab	Ds	Rs	dead
B7	d	Ab	Ab	Ab	Ab
B8	d	Ab	Rs	Bs	Ds
C1	r	Bb	As	Rs	As
C2	r	Rs	Db	Db	As
C3	r	Rs	Rb	Db	Rb
C4	r	Ab	Db	Bs	Rs
C5	r	Rb	Rs	Rs	Rs
C6	r	As	Db	Rs	Rs
C7	d	As	Rb	Ab	Ab
E1	r	As	Bb	Ab	Ab
E2	r	Bt	Ab	Ab	Ob
E3	d	Rs	Ds	Rs	As
E4	r	Ab	Rs	Ab	As
E5	d	As	Rb	Os	Os
E7	r	Ds	Bs	As	Ab
E8	r	Ob	Ab	Ab	As

total responses

O	2	1	5	3	11
A	18	10	14	18	60
B	4	4	3	0	11
R	4	10	5	6	25
D	2	5	3	2	12

generation and again noticeably higher than in the earlier experiments with one or other stimulus only.

Selection for the third generation is portrayed in Table 8. The scheme was now to preserve the line of descent of five 'runners' and three 'droppers' of the first generation, as explained above. It was not possible to exclusively select, in the running group, runners who had not dropped, nor, conversely, non-running droppers in the dropping groups: those who had shown most of the behaviour selected for were chosen.

Results of third generation.

At the ages of $7\frac{1}{2}$, $8\frac{1}{2}$, $9\frac{1}{2}$, and $10\frac{1}{2}$ days ($\pm\frac{1}{2}$) the 31 third-generation aphids underwent the same schedule of testing as the first two generations. The escape response data are shown in Table 9.

Of the descendants of the three first-generation aphids who founded the line selected for dropping, only two dropped in the third generation, and these were sisters. It was therefore impossible to select for dropping in the other two lines; to select at random in this generation and to resume selection in fourth and later generations would have been possible but very inefficient in making progress towards positive results in this experiment, which could not handle more than about 30 aphids per generation.

TABLE 8

Selection of third generation in experiment on alarm pheromone following vibration.

First generation aphids with descent lines preserved		Criterion for selecting second generation aphids to breed	Second generation aphids selected to breed	Second generation run (R) and drop (D) responses
'droppers'	A5	only one present	C7	1R
	B5	2 daughters with most D	A5	1R, 1D
	E2	" " " " D	B6	1D
'runners'	A4	2 daughters of each with most R	E4	1R
	B2		C5	4R
	C2		A3	2R, 1D
	C4		C3	3R, 1D
	E1		B1	1R

TABLE 9

Responses of third generation aphids to alarm pheromone stimulation following vibration (O, nothing; A, waving antennae; B, back-up; R, run; D, drop). Positions of aphids indicated by b, leaf bottom; t, leaf top; and s, stem.

aphid number	offspring of runner (r) or dropper (d)	day number				grand- mother
		1	2	3	4	
A1	d	Ab	Ab	As	Ab	B5
A2	d	Bs	Rb	Os	As	E2
A3	d	As	Db	Ab	Db	A5
A4	r	Ab	Bb	Ab	Ab	A4
A5	d	As	Rs	Rs	Rs	B5
A6	d	At	Ot	Ab	Ab	E2
A7	d	As	As	Rs	Ds	A5
A8	r	Ab	Rb	Rb	Bb	A4
B1	r	Ab	Ab	Ab	Ab	B2
B2	r	Ab	As	As	As	C2
B3	r	Ab	Ab	Rs	Ab	C4
B4	r	Ab	Rb	At	Ab	E1
B5	r	Bs	Rs	Ds	dead	B2
B6	r	As	Ab	Rb	As	C2
B7	r	Rs	Rs	Rs	Rs	C4
B8	r	Ab	Ab	Ab	Ab	E1
C1	d	As	Rs	As	Ab	B5
C2	d	Rs	Ab	Bs	Ab	E2
C3	d	As	Rs	As	As	A5
C4	r	Ab	Bs	Ds	dead	A4
C5	d	Ab	Ab	Ab	Ab	B5
C6	d	Ab	Rs	Rs	Ab	E2
C7	d	Bb	Rs	Rs	Rs	A5
C8	r	Ab	Ab	Ab	Ob	A4
E1	r	Db	Ab	Rb	Ab	B2
E2	r	Ab	Ab	Ab	At	C4
E3	r	Ds	Ob	As	Ab	E1
E4	r	As	As	Ds	Ab	B2
E5	r	Ob	Ab	Ob	Ab	B2
E6	r	Os	As	Ab	dead	C4
E7	r	As	Rs	As	Rb	E1

Total responses

O	2	2	2	1	7
A	22	15	16	20	73
B	3	2	1	1	7
R	2	11	9	4	26
D	2	1	3	2	8

It was now appropriate to look at the data to see if there was any tendency for running and dropping to appear heritable: Table 10 gives the frequencies of each escape response for the two selected lines in the second and third generations. It is not necessary to do any statistical test to see that there is no relative increase in the responses for which the aphids were being selected: the only instance of a suggested departure from chance frequency is a higher number of runs made by the third generation 'droppers' - the opposite of what would occur under inheritance of escape behaviours.

Unlike in the two experiments on vibration and alarm pheromone acting alone, there were enough data in this experiment to permit examination of the possibility of escape responses being characteristic for individual aphids. Except for occasional instances of aphids that had died or could not be tested on a particular day, I had data for three consecutive response pairs (days 1 and 2, 2 and 3, and 3 and 4) for each aphid of each generation. Based on the observed frequency of responses by generation (lumping O and A together to reduce statistical problems following from small numbers, justifiable because for survival purposes they are equivalent) I calculated the expected frequency of each possible response pair, assuming that responses were independent of previous responses, by multiplying the total number of pairs by the product of the two probability estimates of the individual responses:

$$F_{a-b} = F_{b-a} = Np_a p_b$$

TABLE 10

Responses of two selected generations of aphids to alarm pheromone following vibration, according to descent from either 'runners' or 'droppers'.

descended from	2nd generation				3rd generation			
	'runners'		'droppers'		'runners'		'droppers'	
	n	(%)	n	(%)	n	(%)	n	(%)
response								
O: nothing	7	(9)	3	(9)	5	(7)	2	(4)
A: antennae	40	(50)	17	(49)	46	(63)	27	(56)
B: back-up	8	(10)	3	(9)	4	(5)	3	(6)
R: run	17(h)	(21)	8(1)	(23)	13(h)	(18)	13(1)	(27)
D: drop	8(1)	(10)	4(h)	(11)	5(1)	(7)	3(h)	(6)
total	80		35		73		48	

If selection for running or dropping were effective, the numbers marked (h) would be proportionately higher and those marked (1) proportionately lower when compared with each other in the same generation.

where F_{a-b} is the expected frequency of response b following response a;

N is the total number of response pairs in a generation's testing;

p_a is the probability of response a in a single test, etc.

I did this separately for each generation, based on data for that generation, and added the three groups of expected frequencies for comparison with those observed. Table 11(a) shows a summary of this process. If there are in fact characteristic responses for aphids, one should find that repetition of a response (B-B, R-R, etc.) would occur, on the whole, more often than the calculated expected frequency; and that pairs of differing responses would occur less often. Some of the numbers in Table 11(a) are in line with the existence of characteristic responses, others are contrary: a test of whether the observed numbers differ from the expected (regardless of which way) is the χ^2 test. It is well-known that a χ^2 test is inappropriate if there are many expected frequencies less than 5 because they tend to swell the value of χ^2 . Ignoring the bias here present, $\chi^2 = 13.58$, $df = 15$, $0.50 < P < 0.70$; i.e., even though the probability of a chance difference between observed and expected frequencies is here underestimated, there is yet no statistically significant difference. If we collapse frequencies of all pairs of identical responses (which ought to be higher than expected if there are characteristic responses), and all mixed pairs (which ought to be lower), then $\chi^2 = 0.821$ (with Yates' correction), $df = 1$, $0.30 < P < 0.50$. (See Table 11(b).) I therefore

TABLE 11

- (a) Examination for existence of characteristic escape responses: frequencies of all possible consecutive pairs of escape behaviours stimulated by alarm pheromone following vibration. (O, nothing; A, antennae; B, back-up; R, run; D, drop.)

consecutive pairs of response	total number of occurrences			total	expected*
	1st generation	2nd generation	3rd generation		
O/A then O/A	15	34	39	88	88.91
O/A then B	2	3	3	8	12.17
O/A then R	12	9	11	32	33.19
O/A then D	7	4	4	15	15.19
B then O/A	5	7	2	14	12.17
B then B	3	1	0	4	1.90
B then R	1	2	5	8	4.93
B then D	0	1	1	2	2.47
R then O/A	9	8	10	27	33.19
R then B	1	1	1	3	4.93
R then R	7	5	7	19	13.18
R then D	3	4	2	9	6.40
D then O/A	6	2	4	12	15.19
D then B	0	2	0	2	2.47
D then R	4	5	0	9	6.40
D then D	3	1	0	4	3.30

Comparing observed frequencies with those expected, $\chi^2 = 13.58$,
df = 15, $0.5 < P < 0.7$.

- (b) Summary of same-response and different-response pairs.

	total observed	expected (from Table 11(a))
consecutive pairs of same responses	115	107.29
consecutive pairs of different responses	141	148.71

$\chi^2 = 0.821$ (with Yates' correction)
df = 1
 $0.3 < P < 0.5$

- * The expected frequencies were calculated as follows: the relative frequency of each response in each generation was used to calculate the expected frequency of each consecutive response-pair type in that generation, on the assumption that successive responses were independent. The expected frequencies of each pair in three separate generations were added to give the numbers in this column of the table.

conclude that I find no evidence that pea aphids have characteristic response tendencies.

Position on plant and amount of pheromone.

I mentioned earlier that throughout the three generations of this experiment I noted the position of each aphid on its plant at the time of testing and the amount of pheromone used in each stimulus. I had not long been using the capillary tube method of collecting and temporarily keeping pheromone when it became obvious that there was a bigger variation in the quantity obtained than had been apparent from looking only at the droplets when I had been absorbing them directly onto paper slivers. The reason that this was not very noticeable is that the volume of droplets increases with the cube of their radius.

Previous analysis has shown that there was no indication of the existence of characteristic responses in pea aphids, nor of the heritability of any such tendencies. It is therefore permissible to consider the data for all aphids, regardless of generation, day number, or individual, as essentially independent: which means that there was no correlation between an aphid's responses between one day and the next, nor any correlation between related aphids in different generations.

The data for the aphid tests of the whole experiment are cast as a frequency table in Table 12, according to whether the aphid was on a stem or a leaf bottom (only nine were on leaf tops and are not

TABLE 12

(a) Frequencies of response to low, medium, and high quantities of alarm pheromone following 15 s of vibration, for aphids on leaf bottoms or stems.

position on plant amount of pheromone	stem			leaf bottom		
	low	medium	high	low	medium	high
Observed frequencies						
response						
O: nothing	2	4	2	4	10	5
A: antennae	21	16	12	24	56	38
B: back-up	5	2	4	3	9	5
no escape: O+A+B	28	22	18	31	75	48
R: run	13	23	19	5	8	9
D: drop	3	11	10	2	3	8
Expected frequencies						
O+A+B	29.07	37.00	31.05	25.11	56.82	42.95
R	10.08	12.83	10.77	8.71	19.71	14.90
D	4.85	6.17	5.18	4.18	9.47	7.16

(b) Summary tables of observed frequencies.

response x position			response x pheromone quantity			
	stem	leaf bottom		low	medium	high
O+A+B	68	154	O+A+B	59	97	66
R	55	22	R	18	31	28
D	24	13	D	5	14	18
position x pheromone quantity						
	low	medium	high			
stem	44	56	47			
leaf bottom	38	86	65			

(c) Partition of χ^2

source	χ^2	df	P
response x pheromone	6.29	4	N.S.
response x position	46.20	2	$< \alpha$
position x pheromone	4.49	2	N.S.
response x pheromone x position	3.14	4	N.S.
total	60.12	12	

tabulated), and according to low, medium, or high amounts of pheromone, the categories being defined as follows:

low	$> \frac{1}{2}$ mm and < 1 mm
medium	> 1 and < 2 mm
high	≥ 2 mm

where 1 mm represents 30 nL.

Because of small numbers in the top row (those that did nothing) and in the third row (backing-up), and because of the possibility that an aphid backing-up under a leaf might be indistinguishable from the O or A responses, the three responses O, A, and B are summed in the table and treated together as a single row ('no escape') in analysis.

I used the statistical method of partitioning of χ^2 (Winer 1962, Sutcliffe 1957) to test the significance of the relationships between position and response and between amount of pheromone and response. Row and column totals in the upper part of Table 12(a) were the basis for calculating the expected frequencies shown just underneath. Table 12(c) shows the summary table for the partition of χ^2 : there was indeed a greater readiness of aphids on stems to respond to alarm pheromone by running or dropping. Other relationships were not significant: one would not anticipate any relation between position and amount of pheromone because this was a part of the experimental procedure that was deliberately left to chance. Similarly, no third-order relationship should be expected.

The other possible connection, between response and amount of pheromone, proved also to be statistically insignificant.

Having found a statistically significant result, it is always appropriate to measure the size of the effect that has been found. For the association of response with position on the plant (summarized in Table 12(b)), the measure γ (Goodman and Kruskal 1954) is 0.61. (See Appendix for a brief discussion of γ .)

The sequence of negative findings has thus ended with the revelation that position on the plant has something to do with readiness to make an escape response. But has the χ^2 test led to the acceptance of a null hypothesis that is false -- that of no relationship between response and quantity of cornicle secretion? To select the stem data, post hoc, for low and high doses would of course be a fallacious procedure giving spurious probability estimates; however, the data suggested the hypothesis that quantity of pheromone influences the response made, so I decided to do another experiment specially to examine this 'dose-response' hypothesis.

This experiment is described in the next chapter, but to complete the present chapter it is necessary to describe a control experiment associated with the study on vibration-then-pheromone.

Control experiment: paper sliver as a stimulus following vibration.

Since aphids have eyes and possibly other sensors which could detect a sliver of paper and a pair of tweezers, it was necessary to conduct a control experiment to establish that the aphids' responses were indeed to alarm pheromone. Using 24 aphids I carried out tests for escape responses using exactly the same procedures as for the vibration-then-pheromone experiment except that in the interval between vibration and holding up the paper I put no pheromone on it. These aphids were in fact most of a fourth generation selected at the end of the third generation tests before I decided to end the experiment. I tested them twice, at ages $7\frac{1}{2}$ and $8\frac{1}{2}$ ($\pm\frac{1}{2}$) days. The data appear in Table 13: although vibration seems to have made the aphids more reactive than in the previous control experiment in Chapter III (associated with testing the effects of pheromone only), in that waving antennae and backing-up occurred, there is a clear difference between the control and experimental data that implies that alarm pheromone was the effective stimulus.

Table 13 also shows the total responses for the two days classified by where the aphid stood on the plant at the time of testing. The figures are consistent with the previous finding that aphids are more responsive on stems than on leaves.

TABLE 13

Responses of aphids to presentation of a paper sliver without pheromone, following vibration (O, nothing; A, wave antennae; B, back-up; R, run; D, drop). Position of aphid on plant given by: b, leaf bottom; t, leaf top; s, stem.

aphid number	day 1	day 2
A1	A b	A s
A4	A s	O s
A5	B s	O s
A6	A b	A s
A7	A b	A s
B1	*	A s
B2	O b	O s
B3	A t	A s
B4	A s	A s
B5	A b	A s
B6	A b	A s
B7	*	A s
B8	O b	A s
C1	O s	O s
C2	O b	dead
C3	O b	O b
C4	O b	A b
C5	A s	O s
C6	A b	O b
C8	A b	A s
*E2	O t	O s
E3	A s	O s
E4	A b	A s
E5	O b	O b
E6	A b	A s

Total responses	by day		by position		
	day 1	day 2	stem	leaf bottom	leaf top
O	8	10	8	9	1
A	14	14	17	10	1
B	1	0	1	0	0
R	0	0	0	0	0
D	0	0	0	0	0

* not tested

VII. EXPERIMENT VARYING THE AMOUNT OF CORNICLE SECRETION STIMULUS
FOLLOWING VIBRATION.

Purpose

This experiment was to test for a relation between response and the amount of cornicle secretion used as stimulus. Since the previous experiment had shown that aphids are more responsive on stems than under leaves (and since it is always possible to see exactly what they are doing there), I decided to carry out the experiment with aphids on stems only. The earlier data had indicated that a total of about 100 trials would be enough to allow rejection of the null hypothesis if similar trends recurred.

Method

I originally planned to use pheromone from two aphids on every trial in order to raise the number of 'high' pheromone quantities. In case the Nault et al. (1973) suggestion of the ineffectiveness of some secretions were true, it would have been necessary to use pheromone from two aphids for the low 'doses' too, to avoid fallaciously concluding that small doses were less effective. I obtained smaller Microcaps ($\frac{1}{2}$ λ) of about half the length of the 1 λ Microcaps, with two squeeze-bulbs. However, on the very first trial attempt, the secretion got stuck in both tubes and could not be expressed. With the larger tubes this had only happened once or twice in several hundred trials. Presumably this was caused by

hardening of the wax across smaller holes to form a plug. As the most time-consuming and unpredictable part of these experiments was obtaining pheromone at all, I immediately decided to fall back on the old procedure of using one aphid's pheromone only.

The only difference was that I poked more than previously with the brush after the first secreted droplets appeared and seemed to be able to obtain slightly more of it, on the average.

For this experiment I did not standardize the age of the mothers of experimental aphids: by now it seemed a trivial control that would not justify the extra labour and delay involved. To obtain experimental aphids and a supply of pheromone I took 70 adult aphids from the permanent culture and put 10 in each of 7 petri dishes containing pea seedlings. After about 24 hours I removed the adults to another 7 dishes containing seedlings and placed the 400 to 500 young onto the soil of two flower pots holding 20 or 30 seedlings each, where as usual they soon climbed onto the plants. I carried out this procedure daily until I had four cohorts of aphids; each cohort was to supply aphids for testing and for supplying alarm pheromone.

On the appropriate day I planted some 80 peas in covered vials, and when they had grown to be comparable in height with the covers I pruned them to minimize shaking the plant later when removing its cover. When the first cohort was $6\frac{1}{2}$ days old, I transferred one aphid to every plant in the vials; from previous data I would then

expect enough aphids for one day's experiment (20 to 30) to be on stems, which would be the only ones tested.

The procedure for the experimental trials was the same as in the earlier vibration plus pheromone experiment:

- 15 s vibration
- 20 s pause
- 30 s pheromone stimulus
- 30 s pause
- 30 s pheromone stimulus
- 45 s further observation

It happened that fewer aphids than expected were on stems on some days; to make sure I could test enough insects, I knocked some aphids off leaves onto the soil and examined them some two or three hours later to see if they were now on stems and feeding. If so, they were suitable for testing. This procedure overcame the problem.

All aphids were $7\frac{1}{2}$ ($\pm \frac{1}{2}$) day old at testing, as were those supplying pheromone. Each day after experimenting, I removed all aphids, including any babies, from the covered plants and put one of the next group, aged $6\frac{1}{2}$ ($\pm \frac{1}{2}$) days, onto each.

The experiment lasted 4 days, with the numbers of aphids tested on successive days being 21, 31, 34 and 15.

Results

I divided the quantities of cornicle secretion into low, medium, and high by assigning the lowest, middle, and highest thirds of the 100 measurements into these categories: which meant

low	≥ 0.5 mm and ≤ 1.1 mm (29 stimuli)
medium	≥ 1.2 mm and ≤ 2.1 mm (38 stimuli)
high	≥ 2.2 mm (33 stimuli)

where 1 mm represents 30 nL.

This was the first and only division of stimuli into categories. Table 14 shows the frequencies of each kind of response to the three stimulus categories. Again to avoid the inconvenience of too many small frequencies, the responses O, A, and B are collapsed into the 'no escape' category. The Table shows the expected frequencies based on row and column totals, and the appropriate inferential test is χ^2 : $\chi^2 = 11.06$, $df = 4$, $P < \alpha$.

The figures in Table 14 are those used for the inferential statistical test just mentioned, and lead to the conclusion that the quantity of secretion plays a part in determining escape responses to it. The strength of the association is given by $\gamma = 0.41$ (Goodman and Kruskal 1954). To give a purely descriptive portrayal of this relationship I divided the stimuli into five ordered categories (knowing that to repeat similar inferential tests is inappropriate), as shown in Table 15. Fig. 2 shows the same

TABLE 14

Frequencies of responses of pea aphids on stems to low, medium, and high quantities of alarm pheromone following 15 s of vibration.

Observed frequencies

		amount of alarm pheromone		
		low	medium	high
response	O: nothing	2	1	1
	A: antennae	14	14	8
	B: back-up	3	7	2
	O+A+B	19	22	11
	R: run	8	8	9
	D: drop	2	8	13

Expected frequencies

		amount of alarm pheromone		
		low	medium	high
response	O+A+B	15.08	19.76	17.16
	R	7.25	9.50	8.25
	D	6.67	8.74	7.59

$$\chi^2 = 11.06$$

$$\gamma = 0.41$$

$$df = 4$$

$$P < \alpha$$

TABLE 15

Frequencies of responses of pea aphids on stems to 5 levels of cornicle secretion quantity following 15 s of vibration (same data as Table 14).

	amount of cornicle secretion (x30 nL)				
	0.5-1.0	1.1-1.5	1.6-2.0	2.1-2.5	2.6
response					
O: nothing	2	1	0	0	1
A: wave antennae	13	10	5	4	4
B: back-up	1	4	5	1	1
R: run	6	5	3	5	6
D: drop	2	4	4	5	8

$\gamma = 0.35$

Fig. 2. Graphical representation of Table 15: relationship of response to quantity of cornicle secretion.

information graphically: the relative occurrence of each kind of response for each stimulus range.

To restate my assertion: these escape responses are ordered from nothing (O) to dropping (D). As stimulus strength increases the most likely responses change from the ineffective to the most dramatic. Although I have no data on the concentration of pheromone in droplets of different sizes, it is extremely unlikely that its absolute quantity remains the same or decreases with increasing size of droplet. I therefore conclude that the amount of alarm pheromone is a major determiner of the actual response made.

VIII. EFFECT OF PRIOR VIBRATION ON APHID RESPONSIVENESS

As we have seen, there was a troublesome lack of dramatic response to pheromone in one experiment and then a useful increase in response when a period of vibration preceded exposure to pheromone. It is appropriate to make a direct comparison to establish whether this is only an impression or a real finding.

There was no essential difference between the methods and procedures of the pheromone-only experiment and those of the vibration-then-pheromone experiment, with two exceptions: (i) cornicle secretion waited in a capillary tube for 45 seconds in the latter study instead of going directly onto the paper for use after only a few seconds. Although it is possible that an activation reaction could be occurring, no suggestion of this appears in the literature, and alarm pheromone would in nature be less useful if it were not immediately effective; it is much more likely that alarm pheromone loses potency in this delay. Any bias introduced by this procedural difference would work against revealing any excitatory function of the vibration. (ii) The observation period following the end of the second period of pheromone stimulation was 45 seconds in the two-stimulus experiment compared with 30 seconds in the pheromone-only experiment. In 14 cases over the whole vibration-pheromone experiment, the most extreme response occurred for the first time during the post-stimulus observation period. To avoid bias towards the conclusion I am about to draw, I re-scored these cases as the most extreme response shown before the start of this period.

Table 16 presents for comparison the response frequencies found in the two experiments: part (a) gives total frequencies for each generation. That there is a real difference between the two experiments is so clear that no calculations are necessary. The coefficient of association γ between response and the absence or presence of prior vibration is 0.75. Part (b) of the table separates the data of part (a) by position on the plant, omitting the few cases where aphids were on leaf tops. (As stated in the footnote, position data are incomplete for the pheromone-only experiment because I did not record position until day 2 of the second generation.) Again it is clear without calculations that regardless of position, prior vibration has a dramatic effect on the responsiveness of the aphids.

TABLE 16

Comparison of response frequencies in two alarm pheromone experiments: with and without prior vibration.

(a) Total responses

experiment	pheromone only		vibration-then-pheromone		
generation	1	2	1	2	3
response					
O: nothing	71	51	10	11	7
A: wave antennae	21	36	40	62	76
B: back-up	1	3	12	13	11
R: run	5	8	27	23	21
D: drop	1	3	17	10	8
total	99	101	106	119	121

(b) Responses according to aphids' position on plant†

position on plant	stem			leaf bottom		
experiment	pher. only	vib. then pher.			pher. only	vib. then pher.
generation	2*	1	2	3	2*	1 3
response						
O: nothing	7	3	2	3	16	7 8 4
A: wave antennae	8	9	20	23	20	29 41 48
B: back-up	2	4	3	7	2	8 9 3
R: run	4	17	19	15	1	9 4 6
D: drop	2	12	5	5	0	5 5 3
total	23	45	49	53	39	58 67 64

† data not given for aphids on leaf tops because of their paucity.

* I did not start to record position until day 2 of generation 2 in the pheromone-only experiment.

IX. DISCUSSION

To start with a review of the findings:

- (a) there was no evidence for the heritability of aphid escape responses to the threat of predatory attack simulated by (i) vibration, (ii) alarm pheromone, or (iii) alarm pheromone following vibration.
- (b) There was no evidence for the existence of characteristic escape responses in individual aphids when stimulated by vibration-then-pheromone. Significant escape responses after stimulation by each of these factors alone were too rare to justify a 'no evidence' comment on individual differences in behaviour because it was not possible, nor in practice sensible, to look hard enough for them.
- (c) The responsiveness of aphids to vibration-then-pheromone depends on their position on the plant, being higher for those on stems than on the undersides of leaves. (Too few aphids fed on leaf tops to permit any statement on their responsiveness there.)
- (d) Vibration that precedes alarm pheromone stimulation has a facilitatory effect on aphids' response to the pheromone.

- (e) The actual aphid response to alarm pheromone depends partly on its amount: as the amount increases, the probable response changes from nothing, through waving antennae and backing up, to running and then dropping. This was found for aphids on stems and is likely to be the case wherever they are on a plant.

Returning to the question of heritability of escape responses, it is of course impossible to say with certainty that something is not inherited because one can never be sure that one has applied enough selection pressure over enough generations to reveal a low but real heritability. However, before searching for this it is desirable, though not strictly necessary, to know whether the trait, in this case a behavioural one, occurs in different versions in different individuals. After the first two experiments reported here (both negative), I re-examined Roitberg and Myers' (1978) paper to look for a clue to what caused the rate of interesting response in my experiments to be substantially lower than that in theirs. As indicated in Chapter I, the basis for their finding of characteristic behaviour (the existence of 'droppers') was the testing on a second day of aphids that had dropped or not dropped the day before, in response to simultaneous vibration and alarm pheromone. Fig. 4 of their paper shows the proportions of aphids that ran, dropped, backed-up, or did nothing on the first day, giving the proportions of first-day droppers and non-droppers making each response on the second day. I converted these proportions to actual numbers of aphids and found that 5 aphids dropped on the

second day, whereas only about 3 would have been expected to drop based on the first day's data. No formal statistical test is needed to reject this as statistically significant. Their Fig. 4 does not seem to be accurately drawn, for the proportions do not always add up to unity, but as far as I can tell, the data on which their claim is based are such as I have estimated in Table 17 and summarized in Table 18. Roitberg and Myers say, in effect, that the proportions $\frac{12}{71}$ and $\frac{5}{18}$ differ significantly ($P < 0.01$) on the Dixon and Massey (1969) proportions test; but the test (Dixon and Massey 1969, p. 249) applied to these numbers gives $P \sim 0.3$ (two-tailed test). The non-droppers of the first day were also said to respond less than expected ($P < 0.05$) on the second day; but since both groups combined responded less on the second day, it is difficult to understand the meaning of this comment. Although Roitberg and Myers make no mention of comparing the frequencies of other second-day responses with expectations based on first-day frequencies -- to determine, for example, if first-day runners tended to run the next day -- one suspects that they did but found negative results and considered them not worth reporting. I concluded that Roitberg and Myers were mistaken in saying that there were 'droppers' and 'non-droppers'; once there were enough escape responses -- in the experiment on consecutive stimuli -- it became possible to test the hypothesis of characteristic responses. As we have seen, no support for this hypothesis materialized.

TABLE 17

Estimation of response frequency data in Roitberg and Myers (1978, Fig. 4).

	day 1	day 2	day 2
	all aphids	first-day non-drop- pers	first-day droppers
(a) height of bar in figure (mm) (probability 1 = 70.7 mm)			
O: no response	26.0	38.3	19.6
B: back-up	23.4	17.2	24.3
R: run	4.5	0	4.1
D: drop	11.2	3.8	19.8
total bar length (mm)	65.1	59.3	67.8
no. of aphids given in text	71	45	18
no. of aphids calculated from graph	65.4	37.8	17.3
(b) 'actual frequencies' calculated from measurements on graph			
O	26.11	24.39	4.99
B	23.49	10.95	6.19
R	4.52	0	1.04
D	11.25	2.42	5.04
(c) 'actual frequencies' scaled so that sums of probabilities equal 1			
O	28.34	29.06	5.20
B	25.50	13.05	6.45
R	4.91	0	1.08
D	12.21	2.88	5.25
(d) 'actual frequencies' of (c) rounded to nearest integer			
O	28	29	5
B	26	13	7*
R	5	0	1
D	12	3	5
total	71	45	18

* 6.45 in (c) is adjusted to 7 in (d) as the most likely correction to make the total equal 18.

TABLE 18

Expectations of escape response frequency on day 2 based on data of day 1, calculated from Roitberg and Myers (1978, Fig. 4), as shown in Table 17.

	first-day frequencies	second-day expectations		second-day results		second-day totals
				droppers	non-droppers	
		n = 18	n = 45	n = 18	n = 45	
O	28	7.10	17.75	5	29	34
B	26	6.59	16.48	7	13	20
R	5	1.27	3.17	1	0	1
D	12	3.04	7.61	5	3	8

As mentioned in the Introduction, Roitberg and Myers found differences in two populations of A. pisum in their responses to alarm pheromone. Now that the variability and significance of cornicle secretion volume are known, it appears possible that the result could be due also to differences in the nature and quantity of the secretions from the two populations, not just to their responsiveness.

The finding that the aphids were more responsive on the stems than on the underside of leaves is interesting. In nature the stem appears to be a more dangerous place: a syrphid fly larva climbing a stem moves up in a helical path as it alternately attaches itself to the stem by its mouth or the rear end of its abdomen. In my observations it was very rare for a larva so moving not to come in contact with any aphid feeding on the stem, and then only if the larva was big and moving quickly, even hurriedly. Similarly, a ladybird beetle walking on a stem is almost sure to touch some part of a feeding aphid there, as the aphid's legs extend well beyond the body at the sides and the back. So it would be advantageous for an aphid on a stem to have a lower threshold to signals representing such a threat. However, it is possible that the physical properties of the stimuli (vibration or pheromone) differ on the stem -- the amplitude and frequency of vibration in a stem almost certainly differ from those on a leaf, wherever the point of application of the stimulus is; furthermore, alarm pheromone may circulate differently near a stem than near a leaf, so as to produce higher concentrations of the warning chemicals around the antennae. A

third possible contributing factor could be a difference in an aphid's ability to see something associated with the other stimuli, though this seems less likely.

Now that we know that the amount of alarm pheromone appears to be a controlling factor in what escape response is made, it may not seem very suprising. However, because previous investigators have collected alarm pheromone on glass rods or pieces of paper (Wohlers 1980), or a needle (Dixon and Stewart 1975) or have held up the aphid itself that supplied it (Roitberg and Myers 1978), the great variability in the amount of secretion produced did not become apparent until my use of microcapillary tubes. Although most quantities obtained were in the region 30 to 60 nL or so, there was the occasional secretion as large as 150 nL; amounts less than 15 nL (a length of 0.5 mm in the tube) I considered as too little, or as a failure to obtain pheromone. This gave extremes differing by a factor of 10 or more. Assuming that the concentration of the alarming chemical is roughly independent of the amount of secretion, this is a large range that might be expected to influence the escape response, because from the point of view of a neighbouring aphid, a stronger smell tends to imply a closer threat and calls for a more dramatic response. One would expect that if stimulus strength determines response, it would do so in the way found: the stronger the smell, the more dramatic and immediately effective the response.

These influences on escape response -- aphid position on the plant and strength of alarm pheromone -- have proved to be large enough to create undesirable variance in a heredity experiment that does not control for them. There may well be other environmental, physical, or chemical factors influencing escape responsiveness that should be investigated too. One that springs to mind concerns the tendency for pea aphids to adopt a feeding position facing down when on a stem or towards the petiole when on a leaf (Bray-Brodsky 1980, and my own observations). This is believed to put the eyes and antennae in the best position to detect the approach of a predator walking on the plant, and an aphid in a different orientation might prove to be more sensitive or responsive to threat stimuli.

Before discussing the responsiveness induced by prior vibration, some informal observations are worth mentioning. In the experiments using pheromone, I had an ample supply of adult aphids living on a pot-full of peas in a cage. To get an aphid into a petri dish for prodding to get pheromone, I would hold the tea-spoon under an aphid and touch the aphid with the brush. Noticing that an aphid would often attempt to stay on the plant by walking out of easy reach of the brush, I adopted the practice of shaking all the plants after every few experimental trials; then when I touched an aphid it would be much more likely to drop at once. In fact, it would often miss the spoon, until it dawned on me that aphids did not always merely drop but often actually jumped. Although I recorded no data on this matter, the impression gained supported the experimental finding that vibration prior to alarm pheromone greatly increased

responsiveness to pheromone. Earlier exploratory experiments with real predators had been made difficult by an apparently accumulated excitability that culminated in almost all aphids running or dropping from the plant. It is interesting to consider here the work of Wohlers (1980). In one experiment he collected alarm pheromone droplets on filter-paper slivers and varied the time between collection and exposing the stimulus to pea aphids. With increasing delay times, the number of aphids running or dropping decreased to about zero at a two-minute delay. In a separate experiment under otherwise similar conditions he stimulated aphids with initially fresh cornicle secretions continuously for nine minutes, scoring the escape behaviours every minute. The number of aphids reacting continued to increase over more than five minutes. I interpret these findings as follows: the fresh pheromone raises the excitability of the aphids from the start, although the aphids do not act in unison. As the strength of the active ingredient wanes, aphid excitability continues to wax so that even when the smell must be rather faint it is enough to trigger a response. When the pheromone is allowed to fade before it is used to stimulate the aphids, it becomes too weak to raise the excitability before responses can be triggered.

If the above interpretation is correct, however, it requires a continuous pheromone stimulus. Wiener and Capinera (1979) found a habituation effect in Schizaphis graminum, whose response rate to a synthetic pheromone analogue decreased from 67% to 1% in aphid colonies that had been subjected to a weak level of the chemical for

the preceding 15 minutes. They also found that giving the alarm stimulus for 2 seconds every half-minute produced very little response after the first exposure.

This study shows that antecedent stimulation of a different kind can affect the response to present stimulation. The possible value of this to aphids in natural conditions is that when there is a substantial amount of nearby predatory activity, it is safer to escape in response to a general threat rather than to wait for an individual threat. In considering groups or clones of aphids, a great deal of disturbance and minor warnings may mean that many other aphids are being captured and the group as a whole should make its escape, the risks on the ground now being less than the risk of all being picked off one by one.

One implication of changing excitability applies to experiments designed to unravel aphids' escape responses. Clearly the treatment given to an aphid just before an experiment, such as shaking the host plant while moving it into position, should be minimized. Several previous investigators (e.g., Roitberg and Myers 1978, Nault et al. 1973) have tested aphids grouped together on a leaf; aphids' first response to alarm pheromone is often to wave their antennae, which are longer than their bodies and so have a long reach. If a responding aphid touches another, then this could change the responsiveness of the other to the stimulus under test. It is known that some aphids are very sensitive to being touched on an antenna (Sutherland 1969), and I noticed myself that once an aphid was

active the merest touch on the antenna was often enough to make it drop. Wohlers (1980), however, has experimentally investigated this question by comparing the frequency of reaction (run or drop) to synthetic alarm pheromone in solitary aphids (A. pisum) and in aphids forming colonies. Of 24 solitary aphids, 7 responded; while of 29 aphids in 3 colonies, 6 responded. Contrary to Wohlers' previously mentioned experiment, this makes it appear that there is no contagious effect, but the issue is still in doubt if we regard the 29 aphids in 3 colonies as providing 3 replicates rather than 29: the spacing of aphids and the immediacy of their response could decide whether or not there was inter-aphid stimulation on any particular occasion. Montgomery and Nault (1978), on the other hand, reported that aphids (Myzus persicae) in groups were more sensitive than solitary aphids to alarm pheromone. Unless the absence of group effects is clearly demonstrated, we should assume that the number of a group of aphids responding to alarm pheromone is not a good measure of the effectiveness of that stimulus because a single aphid reacting to the smell could transmit a disturbance, by touch, throughout the group.

Some studies already mentioned have a bearing on the interaction of different stimuli, both simultaneous and consecutive pairs. Wohlers (1980) covered some pairs of simultaneous stimuli. When synthetic pheromone and the presence of a predator were compared separately and together, the number of A. pisum responding to the combination equalled the sum of the numbers responding to each separately; it is hard to decide whether the data imply interaction between stimuli or

different aphids responding to one or the other stimulus only -- the second explanation seems less likely. All aphids responded (run or drop) when touched at the same time as they and their neighbours were stimulated with pheromone. The untouched neighbours responded more often in this experimental condition than when no aphid in the group was touched: the touched aphid must have then stimulated others. Another of Wohlers' experiments measured the possible effect of the aphids' being able to see the approach of the filter paper bearing the alarm pheromone, the control situation being one where the paper had been in place for some time and synthetic pheromone was pipetted onto it from the side out of view of the tested aphids. Responses were fewer when paper movement was visible.

Consecutive stimuli were the subject of some of Montgomery and Nault's (1978) experiments. Using M. persicae they found that aphids that had not moved after exposure to pheromone were more responsive to touch than non-exposed aphids. The touch stimulus was a touch on the head with a hand-held brush. They reported also that aphids were more easily caught by the ladybird Adalia bipunctata if they had not recently been exposed to alarm pheromone; however, although their data pointed to this conclusion, the authors' arithmetic was wrong and the null hypothesis could not be rejected at the α claimed (0.05): their frequencies of caught/not caught were 15/6 when aphids had not been exposed to the pheromone, and 4/6 when they had been. This gives $\chi^2 = 1.65$, $df = 1$, $P \sim 0.2$, not $\chi^2 = 5.31$, $P = 0.05$ as given by the authors.

Apart from having to resist the temptation to think that experiments with one species of aphid can be generalized to other species, I have reservations about the validity of Wohlers' (1980) and Montgomery and Nault's (1978) findings: as already mentioned, the practice of testing colonies of aphids rather than individuals introduces uncontrolled possibilities for confounding of data. This problem could be surmounted in part by considering a trial with a colony as a single replicate rather than as a trial for each of the n aphids present. My second reservation is that these studies have scored only running and dropping as responses; in experiments on stimuli separated in time it is preferable to establish that an aphid has made no physical response (such as backing-up) to the first stimulus before the second is given. Using manually-controlled touch as a stimulus is a procedure open to unintended bias unless special precautions are taken, such as using an assistant blind to the purpose of the experiment and to which experiment condition obtains.

In conclusion, then, if there is a heritable component in the escape behaviour of parthenogenetically reproducing pea aphids exposed to a simulated predatory threat, it contributes negligibly to the particular behaviour shown in comparison with other proximal causes such as the aphid's position on the plant, the strength of the alarm pheromone stimulus, and concurrent and preceding stimulation. An explanation of escape behaviour must include such factors and their interpretation in a natural context, and they must certainly be controlled for before any hereditary differences can be discovered.

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A search for a genetic component would probably be more profitable if it were concentrated on the earliest parthenogenetic generations that follow the sexual generation, since this seems to be the time when strains adapted to different plants develop (Sutherland 1969).

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XI. APPENDIX: GOODMAN AND KRUSKAL'S GAMMA

A test of the statistical significance of an observed effect says nothing about the size of that effect -- large, medium, or small, merely whether there is an effect. To interpret findings properly it is very desirable to have a measure of association between variables (Cohen 1965); an example of such a measure is the product-moment correlation coefficient r . In this thesis the data for which an association measure was wanted were frequency data where both classification variables could be ordered (e.g., low, medium, high), or where there were only two levels of one variable (e.g., with or without prior vibration): Goodman and Kruskal's γ (1954) is a suitable measure for such cases.

γ can vary between -1 and +1. Zero represents complete lack of association, and -1 and +1 complete negative and positive association, respectively. Imagine a set of data such as those in Table 14. Since there is a total of $N = 100$ observations, there are $100 \times 99/2$ pairs of observations which could be examined to see if response was positively or negatively associated with stimulus; however, any pair that are in the same row or column are tied on one or other variable, or on both: these ties say nothing about degree of association.

Where pairs are in different rows and columns, each pair indicates either positive or negative association. The measure γ is the difference between the numbers of positive and negative pairs expressed as a fraction of the total number of untied pairs.

As an example, let us take the data from Table 14. (The arrows indicate the arbitrary assignment of directions against which the sign of γ will be interpreted).

		low	med	high	+ →
+ ↓	O+A+B	19	22	11	
	R	8	8	9	
	D	2	8	13	

$$n_p = \text{no. of pos. prs.} = 19(8+9+8+13) + 8(8+13) + 22(9+13) + 8(13) \\ = 1478$$

$$n_q = \text{no. of neg. prs.} = 11(8+8+2+8) + 9(8+2) + 22(8+2) + 8(2) \\ = 612$$

$$= \frac{n_p - n_q}{n_p + n_q} = \frac{866}{2090} = 0.41$$

Thus of the non-tied pairs of observations, the excess of pairs showing a positive association over those showing a negative association is 0.41 of the total number of non-tied pairs.

New reference.

Cohen, J. 1965. Some statistical issues in psychological research. In B.J. Wolman (ed.), Handbook of Clinical Psychology, pp. 95-121. New York: McGraw-Hill.

END

3	0	H	0	8	H	8	2
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FIN