CHAPTER 3

CONCENTRATION-DEPENDENT RESPONSES TO ODORANTS

INTRODUCTION

Chick embryos and chicks show physiological and behavioural responses when they are exposed to odorants (see Chapter 1, pages 2-8). On day 20 of incubation, embryos demonstrate increases in heart rate, bill clapping and head shaking following the presentation of amyl acetate, cineole, formic acid or dichloroethane odours (Tolhurst and Vince, 1976). Several studies have shown that chicks are able to detect and respond to odorants, as discussed in Chapter 1, but there is no method available to test the chicks' responses to graded concentrations of odour easily and reliably. Therefore, the aim of the experiments reported in this chapter was to determine whether 1-day-old chicks alter their behaviour consistently in response to different concentrations of odour. It was considered particularly important to design a species-specific test that would simulate natural exposure to odours in the newly hatched chick. As chicks peck readily at small conspicuous objects (Hogan, 197), a test based on pecking coupled with odorant presentation was developed. A method of repeatedly delivering odours at various concentrations was designed.

The test involved presenting the chick with a bead that was attached to a sample cup. The odours were delivered by applying the odorant to a piece of cotton wool within the sample cup (referred to as static olfactometry, see Chapter 2, page 34). This procedure has been used as a test of olfaction in a range of mammalian species, including rats (Moulton, 1960) and primates (Laska and Hudson, 1993). The amount of odour presented was measured as the final volume of liquid odorant applied to a sample cup after dilution with a solvent. Ideally, the concentration of an odour should be measured as the exact number of molecules that reach the olfactory epithelium. However, this is

difficult to achieve in practice, and virtually impossible to determine in a freely moving animal that is allowed to breathe (sniff) normally (Moulton, 1975). Therefore, the focus of the present experiments was to compare the chicks' responses to several concentrations of odour using a standardised method of presentation, rather than to determine the exact number of odou · molecules required to elicit the response.

The pecking responses of chicks were measured following the presentation of a small bead coupled with odour. The following were scored; the number of chicks that pecked the bead, the number of times that chicks pecked the bead and the latency for chicks to peck the bead. The first of these measures provides quantal or "all-or-none" data and is the simplest way to assess the chick's response. The number of pecks and the latency to peck provide continuous or graded data.

Another behaviour that was observed in preliminary trials, using this method of odour presentation, was head shaking. This behaviour is used as a measure of olfactory responsiveness in the chick (Tolhurst and Vince, 1976; see Chapter 1 pages 3-4). Therefore, bouts of head shaking following the presentation of odour coupled with the bead were also measured. This behaviour can also be measured as the absolute number of chicks responding, the amount of responses, or number of times they respond and the latency to respond.

The relationship between the concentration of an odour and its perceived intensity can be measured in terms of three biological variables (Tucker, 1963; Patte et al., 1975). These are the threshold, the supra hreshold slope and the saturation point. As these variables represent the responses to different concentrations of an odour they are measured separately. The differences between these variables and the different methods for measuring them are addressed below.

The threshold for responding to an odour is defined as the lowest concentration of the odour that produces a response. This can be determined using several different methods. For example, the olfactory detection threshold is determined in humans by presenting subjects with a series of odour concentrations and asking the subjects to

indicate verbally whether they have detected the odour (Doty and Kobal, 1995). The threshold for detection of an odour varies from subject to subject (Stevens et al., 1988) and, if the detection threshold to a particular odour is obtained from a number of subjects, a mean detection threshold value can be calculated. Testing subjects that are unable to provide a verbal response means that the odour threshold has to be determined using either a physiological or a behavioural response. These techniques may involve monitoring changes in electrical activity of the olfactory bulb, heart rate and respiration rate following the presentation of odours at differing concentrations (Neuhaus, 1963; Wenzel, 1967; Wenzel and Sieck, 1972). For example, Walker et al. (1986) used a cardiac conditioning paradigm to determine absolute odour thresholds in adult pigeons. This involved presenting a bird restrained in a light- and sound-attenuated chamber with a known concentration of odour paired with electric shock. Sequentially lower concentrations of odour were then presented. These birds had an absolute threshold in the range of 0.31-29.80 ppm for n-amyl acetate and 0.11-2.59 ppm for n-butyl acetate.

Behavioural responses obtained from operant conditioning procedures have also been used to determine odour thresholds in birds. For example, Stattleman et al. (1975) trained pigeons, chickens and quails (Colinus virginianus) to peck a key in response to the odour of pentane, heptane or hexane and found that they all had odour thresholds in the range of 0.3-9.0 ppm. This is just one of a number of studies that have used behavioural responses to establish odour thresholds (i.e. Michelsen, 1959; Henton, 1969; Henton et al., 1969). Although this provides evidence that chickens have perceptual abilities similar to pigeons (Stattleman et al., 1975), there are several limitations to this procedure. For example, this method has been used for adult animals only and requires a considerable amount of training before testing. Thus, it is not a suitable method for obtaining responses to odour in young birds.

It was considered possible that the quantal data obtained in the present experiment could be used to determine the odour detection threshold. The concentration of odour to which 50 percent of individuals respond is defined as the detection threshold, as indicated earlier for humans (Doty and Kobal, 1995). Figure 3.1 shows that a plot of the

frequency of positive responses against the logarithm of stimulus concentration results in a sigmoid-shaped curve with the asymptotes of the curve at frequencies of 0 and 100%. The detection threshold is equivalent to the inflection point of the curve. However, as it is not possible to determine if the chick has detected the odour unless it demonstrates a response, the term 'response threshold' is preferred here. The 'response threshold' would be equivalent to the ED_{50} (50% effective dose) that is determined from quantal-dose-response curves in pharmacological dose-effect experiments (Craig and Stitzel, 1986).
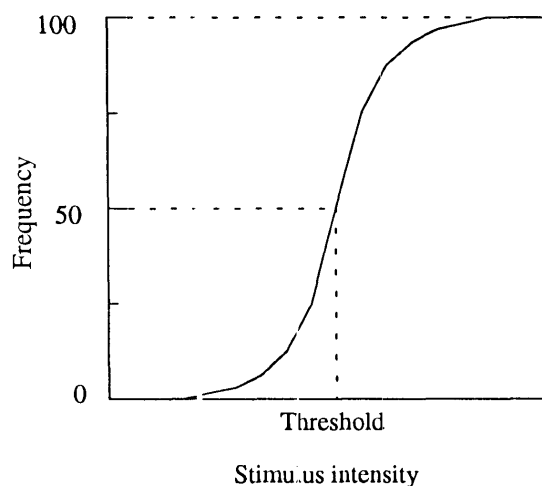

Figure 3.1   The relationship between frequency of responding and the physical intensity of a stimulus is depicted. The curve is a sigmoid-shape with asymptotes at frequencies of 0 and 100%. The intensity of a stimulus that evokes a response in 50% of individuals is said to be the response threshold.

A graded response, such as the number of times the chick performs an act, is likely to provide more information about suprathreshold responses (e.g. Pryor et al., 1970; Doty and Kobal, 1995). In humans, for example, positive responses to suprathreshold concentrations of odour have been found to increase (until a saturation point is reached, see later) according to a power function (Engen, 1965; Stevens, 1970; Moskowitz et al., 1976; Berglund et al., 1986; Sauvageot, 1987). However, this relationship has been established using a psychophysical approach, in which, human subjects are required to assign numbers to different odour intensities (magnitude estimation; Berglund et al., 1971) and thus may not be directly applied to data arising from the present experiments.

An attempt was made in the present experiments to determine if chicks show suprathreshold responses and, if so, to describe these responses using a mathematical equation.

The maximal suprathreshold response (saturation point) depends on the physical components of the stimulus, such as t1e number of odorant molecules that can occupy a unit volume of air. It also depends on physiological aspects of the individual, such as nasal air-flow dynamics and the number of available receptors or neurones that can be stimulated by the odorant. When no further response can be evoked with increasing stimulus intensity, the saturation point has been reached. Suprathreshold responses may also be described in terms of the concentration that results in 50% of the maximal response. The ED_{50} can also be determined from a graded-dose-response curve (Craig and Stitzel, 1986). In the experiments of this thesis the term EC_{50} (50% effective concentration) is used in preference o ED_{50} to distinguish between a pharmacological dose and odour concentration, althou gh in every other respect the two terms could be used synonymously.

There may, therefore, be several ways of describing a chicks' responses to an odour. To establish whether there is a relationship between odour concentration and the chicks' responses, and whether these conform to the concentration-response relationships described above, chicks were presented with different concentrations of iso-amyl acetate. This odorant was chosen as it has been used in olfactory tests in chicks (Tolhurst and Vince, 1976, Vallortigara and Andrew, 1994), other avian species, such as pigeons (Henton, et al., 1969), as well as in mammals (Slotnick and Schoonover, 1992). Some of the chicks were tested in one trial only (Experiment 3.1), while others were tested in a series of trials (Experiment 3.2). By testing chicks repeatedly, a within-subject response may be established to the different concentrations of odour. This may provide a more reliable range of responses.

There may, however, be a number of problems associated with testing repeatedly. It is possible that chicks presented with odour in a series of trials may respond differently

to those tested once only, due to the effects of repeated testing. Also, by presenting odour coupled with a visual stimulus it is possible that the repeated presentations of the visual stimulus could cause visual habituation. Therefore, beads of different colours were used in each of the trials. As repeated presentations of different concentrations of odorant are known to affect the response threshold and the suprathreshold responses depending on the order with which they are presented, at least in humans (Pangborn *et al.*, 1964), the effects of the order of presentation were examined in Experiment 3.2. Once the task testing olfaction had been established, other chicks were tested with different odorants (allyl sulfide and eugenol) to determine whether this method provided consistent results (Experiment 3.3).

EXPERIMENT 3.1: CONCENTRATION-RESPONSES USING SINGLE EXPOSURES TO THE STIMULUS

The aim of this experiment was to establish a method for presenting chicks with different concentrations of *iso*-amyl acetate and to determine graded responses to increasing concentrations of odorant.

Methods

Thirty-six chicks (20 males and 16 females) were used. Incubation and housing conditions were according to the methods described in Chapter 2 (pages 24-25).

Stimulus

The visual components of the testing stimulus and the testing cage were identical to those described in Chapter 2 (described on pages 28-31 and illustrated in Figures 2.3 and 2.4, pages 29 and 30). The bead colours used in this experiment were red, dark blue, light blue, yellow, light green and dark green (see Figure 2.3, page 29). Different bead colours were chosen to match subsequent repeated tests and because half of these chicks were tested in a further five trials in Experiment 3.2 (see page 61). A white bead was used during the training trials.

Preparation of odorants

Five different concentrations of *iso*-amyl acetate were used. The highest concentration of odorant used undiluted *iso*-amyl acetate (100% v/v), and undiluted 70% ethyl alcohol (the solvent) was used for the control stimulus. Log_{10} dilutions were prepared in the solvent and 10 µl of each solution was applied to a clean piece of cotton wool inside a sample cup. The concentration of odorant is expressed as the volume of *iso*-amyl acetate in the sample cup after dilution (i.e. 10^{-3}, 10^{-2}, 10^{-1}, 1 or 10 µl *iso*-amyl acetate). Each sample cup was placed into a sealed vial to which it was returned between trials and each stimulus was discarded after it had been used in six trials.

Testing procedure

Initially, each chick received two training trials with a white, unscented bead presented for 20 s. Ten minutes after the second training trial, each chick was presented with a differently coloured bead together with odour for 10 s. Chicks were assigned a different combination of bead colour and odour intensity assigned by a Latin-square design, such that no two chicks received the same combination. Thus, scores for this experiment were obtained by each chick receiving only one testing trial.

For each trial the number of bouts of head shaking, the number of pecks directed at the bead, the latency to the first bout of head shaking and the latency to first peck were recorded from the video tapes according to the procedure described in Chapter 2 (pages 37-39). Each trial was coded so that the experimenter was not aware of the volume of *iso*-amyl acetate in the sample cup.

Results

The scores for the amount of head shaking and pecking were tested for normality with the Kolmogorov-Smirnov Goodness-of-fit test and both measures were found to be highly skewed (head shaking: $z=4.31$, $P<0.001$; pecking: $z=1.85$, $P<0.005$). Furthermore, a standard logarithmic or square-root transformation (Martin and Bateson, 1994) was unable to sufficiently correct these right-skewed data. Thus, these data were analysed using the non-parametric statistical procedures outlined in Chapter 2 (see page

41). As the training trials were used to familiarise the chicks with the testing apparatus, the results of these trials will be presented first, followed by results of the testing trial.

Training trials

The mean responses during each of the two training trials are presented in Table 3.1.1. There were no significant differences between the first and second training trials for either the number of bouts of head shaking (Wilcoxon signed ranks test: $z=0.59$, $P=0.55$), latency to the first bout of head shaking ($z=0.70$, $P=0.48$), amount of pecking ($z=0.44$, $P=0.66$) or latency to first peck ($z=1.46$, $P=0.14$).

Table 3.1.1 Mean ± SEM head shaking and pecking responses during the training trials

	Number of head shaking bouts	Latency to first head shaking bout	Number of pecks	Latency to first peck
Training trial 1	0.22 ± 0.11	18.58 ± 0.63	2.89 ± 0.48	11.15 ± 1.20
Training trial 2	0.14 ± 0.06	19.09 ± 0.46	2.67 ± 0.36	9.23 ± 1.14

The chicks were divided into six separate groups for the presentation of different concentrations of odour (n=6 per group). Thus, the chicks' responses in the training trials were also analysed according to these groupings. There was a significant difference between these groups in the number of pecks made at the bead during the second training trial (Kruskal-Wallis test: $KW=11.30$, $df=5$, $P=0.046$). However, no significant differences were found between groups for the number of pecks during the first training trial ($KW=5.96$, $P=0.31$), the latency to peck (Trial 1: $KW=6.68$, $P=0.25$; Trial 2: $KW=1.09$, $P=0.96$), the latency to shake the head (Trial 1: $KW=3.56$, $P=0.61$; Trial 2: $KW=1.16$, $P=0.95$) or the number of head shaking bouts (Trial 1: $KW=3.56$, $P=0.61$; Trial 2: $KW=1.13$, $P=0.95$).

Responses to odorant in the testing trials

The results for the testing trial are presented in three sections, the first dealing with the results for the head shaking, the next covering the results for pecking responses and the third reporting on the possible relationship between these two behaviours.

Head shaking responses

The number of bouts of head shaking made by each chick during the testing trial is presented in Table 3.1.2. Each chick was presented with a different combination of visual and olfactory components during the testing trial. Thus, the data are organised according to the colour of the bead and the concentration of odour presented. Fifty-eight percent of chicks shook their heads during the testing trial. There was an increase in the number of chicks shaking their heads, as well as an increase in the number of head shaking bouts with increasing concentration of odour. The increase in the number of bouts of head shaking with increasing odour concentrations was significant (KW=18.52, P<0.005). There was, however, no significant effect of bead colour on the number of bouts of head shaking (KW=1.87, P=0.87). Thus, head shaking behaviour was affected by presenting different concentrations of odour and did not appear to have been influenced by the differently coloured beads. There was a clear concentration-dependent increase in the head shaking response.

Table 3.1.2 Head shaking responses by individual chicks to differently coloured beads coupled with the various concentrations of iso-amyl acetate

	Concentration of iso-amyl acetate (μl)					
Bead colour	0	10^{-3}	10^{-2}	10^{-1}	1	10
red	0	0	0	1	1	2
yellow	2	0	0	1	1	4
light green	1	0	0	1	4	3
dark green	0	3	1	0	1	3
light blue	0	0	0	0	2	3
dark blue	0	0	2	1	2	2

Each of the values tabulated above indicates the number of bouts of head shaking given by a single chick (n=36). For example, the chick presented with a red bead and the 0 μl stimulus did not shake its head, whereas the chick presented with a red bead and the 10 μl stimulus shook its head twice.

The mean ± SEM number of head shaking bouts, for each concentration of *iso*-amyl acetate, is presented in Figure 3.:.1. In this figure the data are presented as the concentration of odour (as the volume [µl] of odour applied to the sample cup), on the abscissa (logarithmic scale), and the number of bouts of head shaking, on the ordinate (linear scale). *Post hoc* analysis with the Wilcoxon-Mann-Whitney test revealed that the number of bouts of head shaking was significantly greater to both 1 and 10 µl of *iso*-amyl acetate than to the control stimulus ($z=2.09$, $P=0.04$; $z=2.81$, $P<0.01$, respectively). The amount of head shaking increased between 10^{-1} and 1 µl of odour. The amount of head shaking did not appear to reach a maximum, as there was no upper asymptote.
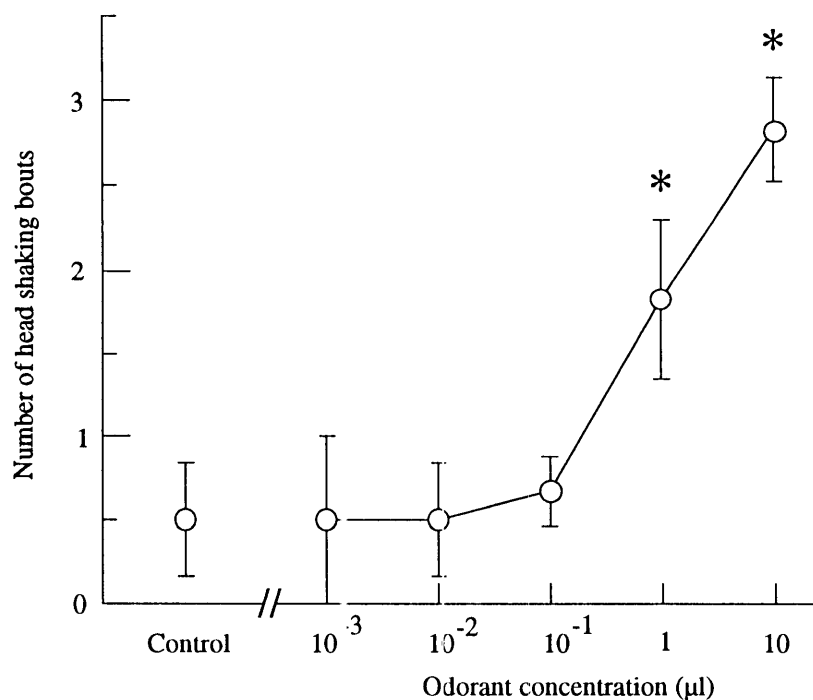

Figure 3.1.1 The mean (± SEM) number of bouts of head shaking given by chicks presented with different concentrations of *iso*-amyl acetate is plotted. Chicks were presented with odorous stimuli coupled with differently coloured beads, each tested in one trial only. Mean scores annotated with an asterisk indicate a significant difference compared to the response obtained from chicks that were presented with the control stimulus ($P<0.05$, Wilcoxon-Mann-Whitney test, n=6 chicks per data point).

The numbers of bouts of head shaking were analysed using a curve fitting regression analysis (SPSS^X/windows) to determine the nature of the relationship between odour intensity and suprathreshold responses as either a linear, logarithmic or power function. As the logarithm of zero is not defined, unity was added to each of the head shaking scores in response to stimuli that were above the response threshold (10^{-1}, 1 and 10 µl of odour). As these data were normally distributed (Kolmogorov-Smirnov goodness-of fit test: n=18, z=1.02, P=0.26), they could be examined by regression analysis. This analysis revealed that the number of bouts of head shaking were most effectively described by either a power function ($F_{2,16}$=23.41, P<0.001, r=0.77) or by a logarithmic function ($F_{2,16}$=20.43, P<0.001, r=0.75) rather than a linear equation ($F_{2,16}$=12.55, P<0.01, r=0.66). The exponent for the power function (0.19) and the logarithmic function (0.47) indicated the slope of the line on log-log or log-linear coordinates, respectively.

The latency to the first bout of head shaking is presented in Figure 3.1.2. There was a significant effect of odour concentration on the latency to the first bout of head shaking (KW=14.11, df=5, P<0.05). Chicks presented with the control stimulus shook their heads after a significantly longer delay than those presented with either 1 or 10 µl of iso-amyl acetate (z=1.96, P=0.05; z=2.45, P=0.01, respectively). There were no significant differences between the latency to respond to the presentation of lower amounts of iso-amyl acetate and the latency to respond to the control stimulus. Figure 3.1.2 shows that there was a consistent decrease in the latency to shake the head with increasing concentrations of odorant.

There was a strong negative relationship (Spearman rank-order correlation: r_s= -0.84, P<0.001) between the latency to the first bout of head shaking and the number of head shaking bouts. Thus, a decrease in the latency to shake the head was associated with an increase in the number of bouts of head shaking.
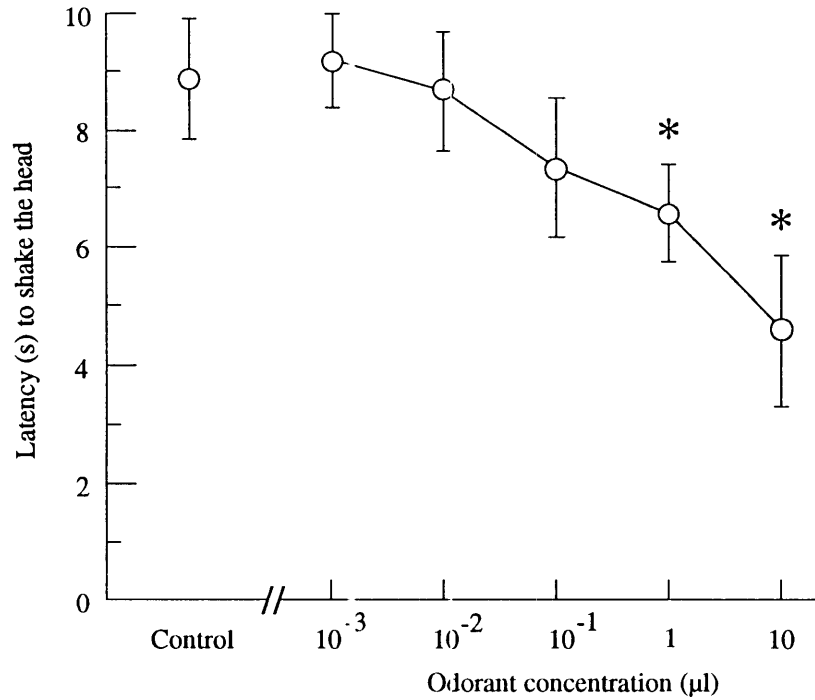
Figure 3.1.2 The mean (± SEM) latency to the first bout of head shaking by chicks presented with different concentrations of iso-amyl acetate is presented. See Figure 3.1.1 for details of presentation.

The numbers of chicks which shook their heads at least once in the trial are presented in Figure 3.1.3. If a chick shook its head, it was assigned a score of 1 and, if it did not respond, it was assigned a score of 0. There was a clear concentration-dependent increase in the number of chicks shaking their heads with increasing concentrations of odour. All chicks shook their heads when 1 or 10 μl of iso-amyl acetate were presented and thus there was a maximum value (equivalent to the upper asymptote of the sigmoid curve, see Figure 3.1). The inflection point of the sigmoid shaped curve on log-linear co-ordinates (see Figure 3.1) coincides with 50% of chicks responding and this point is used to obtain the response threshold. Using this method the response threshold is at $10^{-1.5}$ μl of iso-amyl acetate (indicated by the vertical unbroken line in Figure 3.1.3). However, this method does not account for the baseline level of responding (referred to as noise) to the control stimulus, which is above zero. Thus, the dashed line in Figure 3.1.3 indicates the lower asymptote set at the baseline level above zero and, using this method, the response threshold was at 10^{-1} μl of odorant.
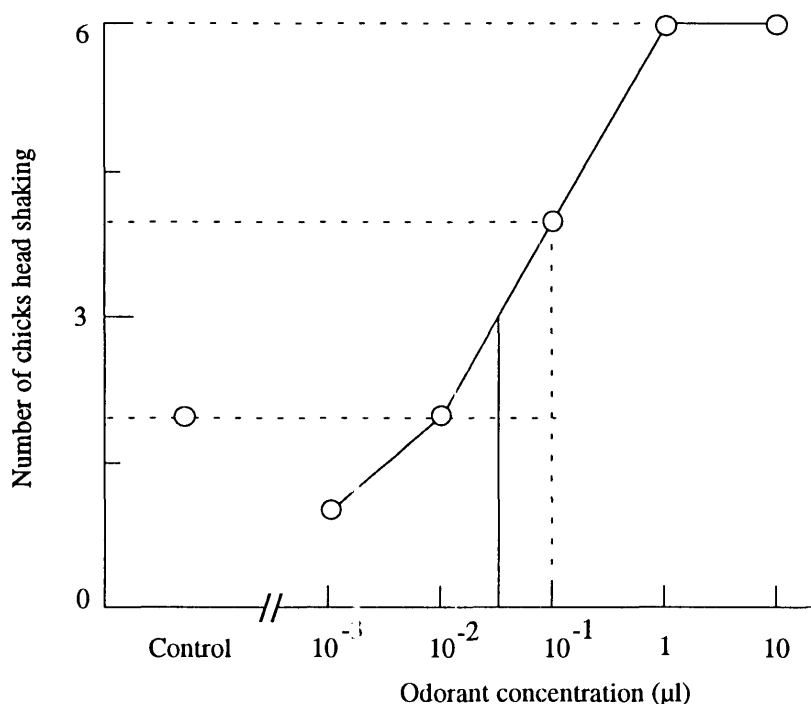
Figure 3.1.3 The number of chicks that shook their heads to the presentation of different concentrations of iso-amyl acetate. The response threshold has been calculated according to two different methods (see text). The unbroken line indicates the response threshold based on half of the maximum number of chicks responding. The dotted line incorporates the baseline level of responding (noise) to determine the response threshold.

Pecking responses

The number of pecks at the differently coloured beads coupled with different concentrations of *iso*-amyl acetate is presented in Table 3.1.3. Ninety-two percent of chicks pecked at the bead during the testing trial but there was a significant effect of presenting the different concentrations of odour (KW=16.08, P=0.007) on the amount of pecking. There was no significant difference between the number of pecks to the differently coloured beads (KW=8.73, P=0.12). Thus, pecking behaviour was affected by presenting different concentrations of odour but not significantly by differently coloured beads. However, it must be stated that the amount of pecking at a yellow bead tended to be less than that at a dark green or a dark blue bead (Wilcoxon-Mann-Whitney test: z=1.94, 0.10>P>0.05), and it was significantly less than at a light blue bead (z=2.68, P=0.008).

Table 3.1.3   Pecking responses)y individual chicks to differently coloured beads coupled with various concentratio is of iso-amyl acetate

	Concentration of iso-amyl acetate (µl)					
Bead colour	0	10^{-3}	10^{-2}	10^{-1}	1	10
red	4	7	9	4	2	1
yellow	0	4	3	2	5	0
light green	0	12	6	4	3	3
dark green	4	12	9	5	5	2
light blue	9	9	6	6	6	4
dark blue	4	6	5	6	9	1

Values indicate the number of peck: at the bead by a single chick (n=36) and are tabulated as for Table 3.1.2.

The results for the mean nur iber of pecks at the bead are presented in Figure 3.1.4.B. The data are presented on semi-logarithmic plots, as for the head shaking responses plotted in Figure 3.1.1. Post hoc analysis with the Wilcoxon-Mann-Whitney test revealed that chicks pecked significantly more when 10^{-3} µl of iso-amyl acetate was presented than when either the control stimulus (z=2.13, P=0.03) or 10 µl of odour (z=2.82, P<0.005) were presented. The chicks' responses to odour concentrations above 10^{-3} µl of odour were not significantly different from that obtained when the control stimulus was presented. Although there was a suppression of pecking with increasing odour concentration, the concentration-response curve for pecking was not as clear as that of head shaking. Despite this trend, the latency for the first peck was not significantly affected by odour concentration (KW=1.17, df=5, P=0.95; see Figure 3.1.4.C) and there was only a weak negative relationship (r_s=-0.33, P=0.046) between the latency to peck and the number of pecks. Furthermore, in contrast to the head shaking results, the number of chicks pecking the bead was maximal to the presentation of all concentrations of iso-amyl acetate (see Figure 3.1.4.A).
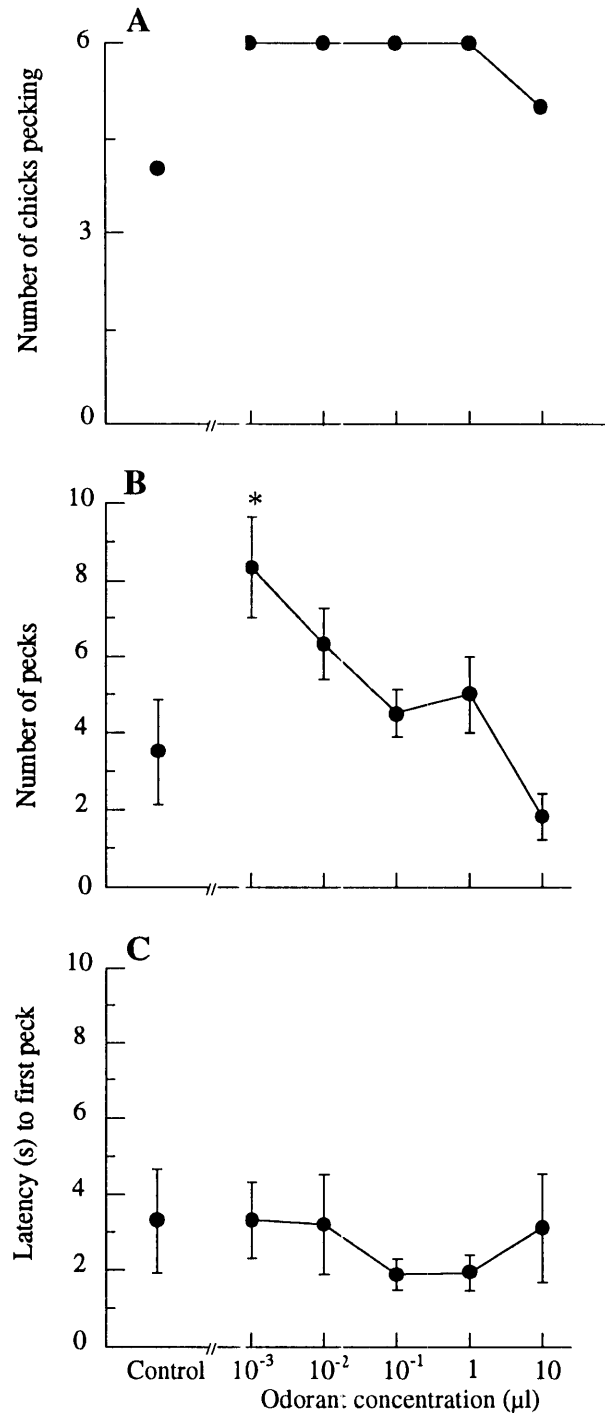
Figure 3.1.4 The number of chicks pecking (A), the mean (± SEM) number of pecks (B) and the mean (± SEM) latency to first peck (C) at beads coupled with different concentrations of iso-amyl acetate. Chicks were presented with odorous stimuli coupled with differently coloured beads in one trial only. The data are presented as in Figure 3.1.1. Mean scores annotated with an asterisk indicate a significant difference compared to the responses obtained from chicks that were presented with the control stimulus (P<0.05, Wilcoxon-Mann-Whitney test, n=6 chicks per data point). Note that no SEM are included in (A) as these data indicate the total number of chicks pecking.

Relationship between head shaking and pecking

An additional way to describe the chicks' response to odour was to examine the relationship between pecking and head shaking. There was a mild negative relationship between the number of pecks directed at the bead and the number of bouts of head shaking (Spearman rank order correlation: r_s= -0.42, P=0.01) but there was no relationship between the latency to peck the bead and the number of bouts of head shaking (r_s=-0.20, P=0.23).

To determine whether head shaking precedes pecking or vice versa the responses of each chick and at each of the concentrations of odour were allocated to one of four possible categories. These included (1) pecked at the bead but did not shake the head, (2) pecked at the bead and then shook the head, (3) shook the head but did not peck at the bead or (4) shook the head and then pecked at the bead. The results of this analysis are shown in Table 3.1.5. There were no occurrences of a chick shaking its head and then pecking at the bead, only two out of a total of 36 (6%) chicks shook their heads without pecking at the bead and in 53% of cases the chick pecked at the bead and then shook its head. Therefore, with increasing odour concentration more chicks shake their heads but only after pecking the bead.

Table 3.1.5   Sequence of pecking and head shaking responses to various concentrations of *iso*-amyl acetate

Category	Concentration of *iso*-amyl acetate (μl)					
	0	10^{-3}	10^{-2}	10^{-1}	1	10
1. peck only	4	5	4	2	0	0
2. peck then shake head	1	1	2	4	6	5
3. shake head only	1	0	0	0	0	1
4. shake head then peck	0	0	0	0	0	0

Values indicate the number of chicks responding (n=6 chicks per concentration).

Discussion

Testing separate groups of chicks each with a different concentration of *iso*-amyl acetate resulted in concentration-dependent changes in head shaking and pecking. The amount of head shaking increased with increasing odour concentration, whereas the amount of pecking increased at the lowest concentration of *iso*-amyl acetate (10^{-3} μl) and decreased with increasing concentrations of odour. Head shaking behaviour produced the clearer results as there was a concentration-dependent change in the number of chicks responding, the latency to respond as well as the number of bouts of head shaking. For pecking, concentration-dependent changes were found for the number of pecks at the bead but not the number of chicks pecking or the latency to first peck.

There was a clear concentration-response curve for the number of chicks shaking their heads and this was similar to the sigmoid-shaped curve of Figure 3.1. However, there was a low level of head shaking in the absence of odour, i.e. during the training trials as well as to the control stimulus. Therefore, it was necessary to allow for the low level of 'noise' by adjusting the baseline and then recalculating the response threshold. As shown in Figure 3.1.3 there was only a slight increase in the response threshold value when the baseline level of responding was taken into consideration.

The relationship between suprathreshold concentrations of *iso*-amyl acetate and head shaking was best described by either a power or a logarithmic function. As the responses were compared over only three different concentrations, it is doubtful whether any useful interpretation can be drawn from these equations. However, it can be noted that the exponent found for the power function is comparable with published values obtained from psychophysical tests using *iso*-amyl acetate (0.25; Patte *et al.*, 1975).

While there was a clear change in the number of head shaking bouts and the latency to head shake to suprathreshold concentrations of *iso*-amyl acetate, there did not appear to be an upper limit for responding. That is, the response did not reach asymptote at the highest concentration of odour delivered. Thus, it was not possible to determine accurate EC_{50} values for these data.

It was not possible to calculate the number of odorant molecules required to elicit the response, or the effective stimulus concentration. The method of presenting the odours was kept relatively constant although the exact concentration of odour in the air surrounding the sample cup was not measured. Furthermore, the concentration of odour delivered by static olfactometry is directly affected by factors such as the temperature, humidity, vapour pressure of the odorant, diffusion coefficient of the odour in air, as well as convection currents within the testing cage (including those created by the chicks movements).

The results from this experiment suggest that there is an inverse relationship between pecking and head shaking. That is, the stimulation of head shaking is matched with a suppression in pecking. However, it appears that pecking and head shaking are elicited by different stimuli. It is possible that pecking and head shaking responses are linked, such that a chick will shake its head only after it has pecked. However, this explanation does not seem plausible given that the delay for chicks to peck the bead was relatively invariant to each concentration of odour, whereas the latency to shake the head decreased with increasing concentrations of odour.

Thus, the first peck(s) appears to be elicited by the visual cues. The chick then responds to the odour and this results in a suppression of pecking and the stimulation of head shaking, at the higher concentrations of odour. For example, if we look at the chick's response to a high concentration of odour, such as 10 µl, then the occurrence of each behaviour can be expressed diagrammatically. In Figure 3.1.5 the chick is presented with the bead and sample cup (to which 10 µl of iso-amyl acetate has been added). The odour diffuses into the air surrounding the sample cup. It is likely that an odour gradient exists, such that the strength of odour decreases with increasing distance from the sample cup. When the odour, coupled with the bead, is introduced into the testing cage, the concentration of odour may be very low in the air surrounding the chick, but the chick can see the coloured bead. Thus, the motivation to peck at the bead would be based solely on visual cues. This explanation seems likely as 33 out of 36 chicks pecked the
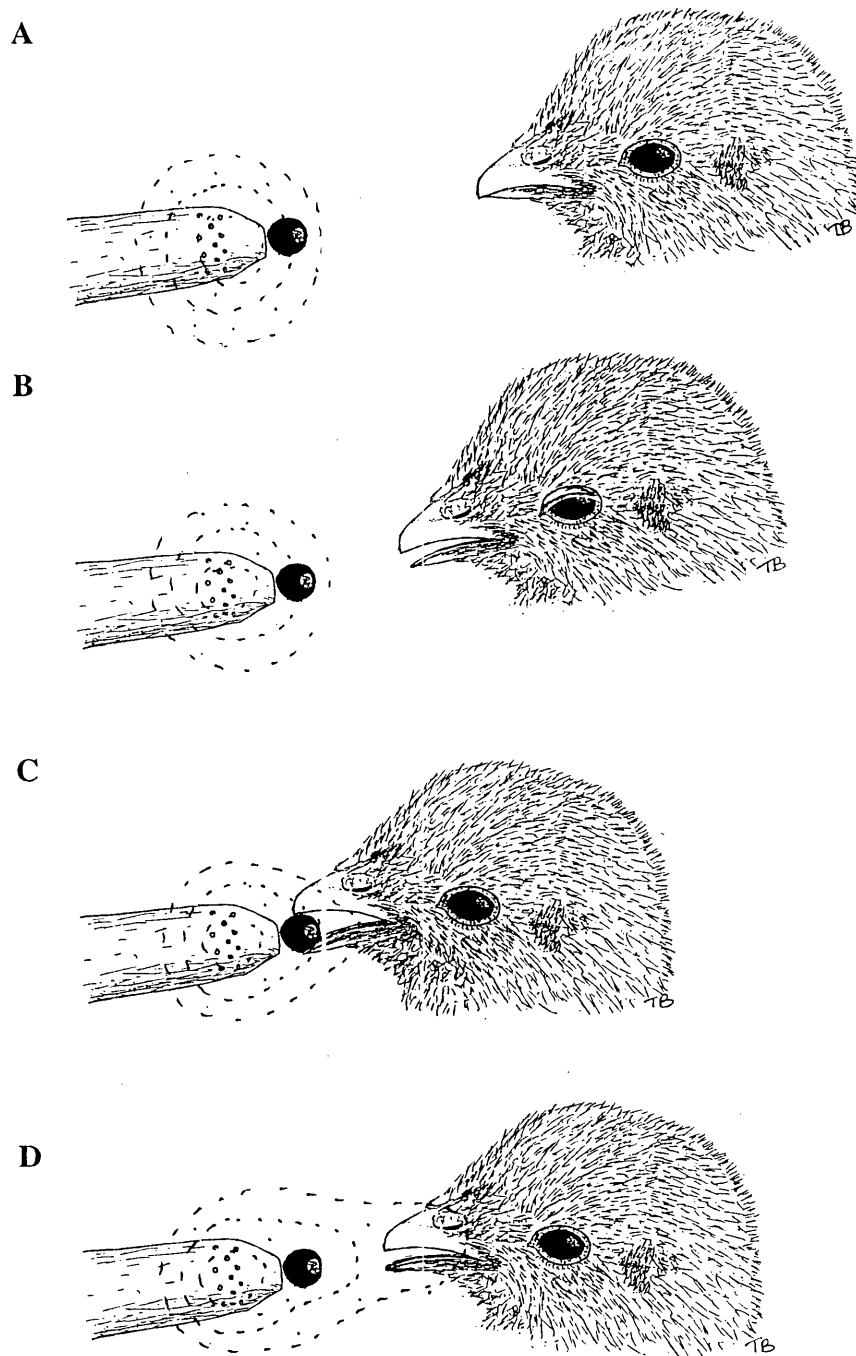
Figure 3.1.5 Diagrammatic representation of the pattern of odorant dispersal when the chick is exposed to the stimulus. It is likely that the gradient of odour concentration decreases with increasing distance from the source and this is indicated by the dashed circles. The chick may not initially detect the odour as the concentration of odour around the chick's nostrils is too low when the chick decides to peck (A) or during the ballistic pecking motion (B). It is possible that the odour does not reach the nasal cavity at a high enough concentration to elicit head shaking until either during (C) or after (D) the chick pecks the bead (see text for full explanation).

bead at least once and of those that shook their head (21) 90% did so only after they had pecked the bead. The odour may not reach the nasal cavity at a high enough concentration to elicit head shaking until the chick is close to the bead, during or after the chick has pecked the bead. Thus, it appears that in the present olfactory test head shaking is elicited solely by odour whereas pecking is elicited by visual and possibly odour cues.

EXPERIMENT 3.2: CONCENTRATION-RESPONSES USING REPEATED EXPOSURES TO THE STIMULUS

The aim of this experiment was to determine if a concentration-response curve to iso-amyl acetate could be obtained by testing the same chicks in a series of trials with differently coloured beads and various concentrations of odour. The order of presentation of odour concentrations affects odour thresholds, at least in humans (Pangborn et al., 1964), and it may be that prior exposure to the various concentrations of odour affects the chicks' responses. Therefore, separate groups of chicks were presented with odour in either an ascending, random or descending series of odour concentrations.

Methods

After each chick had been tested once only in Experiment 3.1, 18 chicks were selected for a further five bead presentations. Twelve of the chicks used were those tested previously with either the control stimulus or a stimulus that contained 10 µl of iso-amyl acetate. The remaining six chicks were selected at random. These three groups of six chicks were tested with different orders of presentation of iso-amyl acetate. The groups previously tested with the control stimulus (i.e. ethyl alcohol) received odour presentations in ascending concentrations. The group that had previously been tested with 10 µl of iso-amyl acetate received descending concentrations and the third group received a random presentation of odour concentrations. The stimuli used were prepared as described in Experiment 3.1 (see page 48) and included 10, 1, 10^{-1}, 10^{-2} and

10^{-3} μl of *iso*-amyl acetate (made up to 10 μl in 70% ethyl alcohol). The control stimulus contained 10 μl of the solvent.

Results

Presenting odorant concentrations in ascending, random or descending order

The responses during the training trials have already been presented on page 49. There were no significant differences during the training trials in the responses obtained from the three groups of chicks used in this part of the experiment (Kruskal-Wallis test: $P>0.10$ for each comparison).

The results for head shaking during the testing trials are presented in Figure 3.2.1.A, C and E. Only 4 out of the 18 chicks tested shook their heads when they were presented with the control stimulus (0% *iso*-amyl acetate) and there were clear concentration-dependent increases in the number of chicks shaking their heads with each method of presentation (see Figure 3.2.1.A).

There were no significant differences (Kruskal-Wallis test: $P>0.05$) between the latency or number of bouts of head shaking or pecks when the three methods of stimulus presentation (ascending, random or descending order of odour concentration) were compared (see Table 3.2.1). The mean (± SEM) head shaking scores obtained for each group of chicks is illustrated in Figure 3.2.1.C. There were clear concentration-dependent increases in the number of bouts of head shaking with each method of presentation. There was a tendency for chicks presented with odour concentrations in random order to shake their heads less than those presented with odour in either ascending or descending order at 10 μl of odour only (Wilcoxon-Mann-Whitney test: $z=1.95$, $P=0.051$). The decrease in the latency for head shaking and the increase in the number of bouts of head shaking with increasing odour intensity did not depend on the order of presentation (see Table 3.2.1 and Figures 3.2.1.C and E).
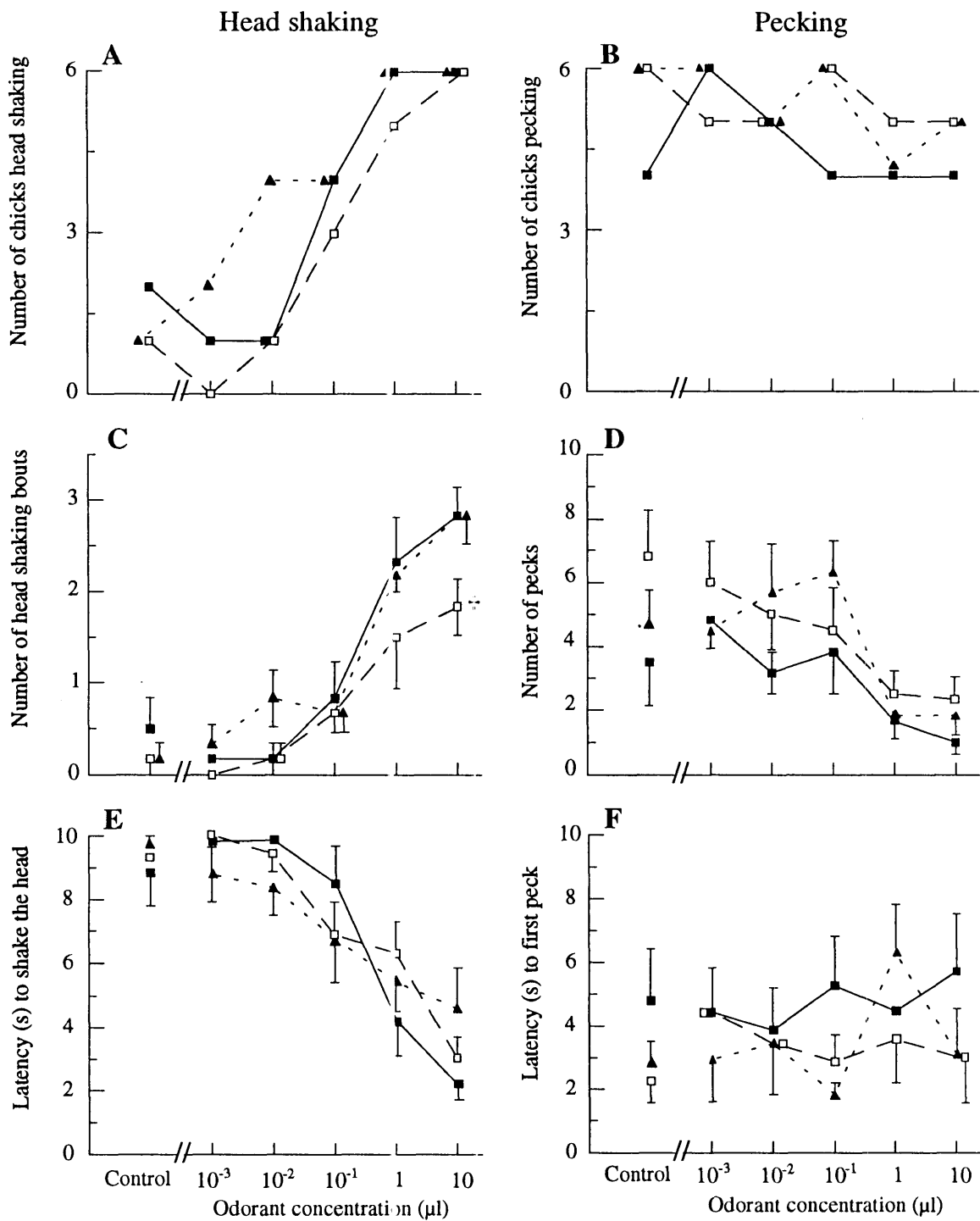
Figure 3.2.1 Head shaking (left panels) and pecking (right panels) responses of chicks presented with concentrations of *iso*-amyl acetate in different orders. The scores are presented as the number of chicks shaking their heads (A), the number of chicks pecking at the bead (B), the mean (± SEM) number of head shaking bouts (C), the mean (± SEM) number of pecks (D), the mean (± SEM) latency to the first bout of head shaking (E) and the mean (± SEM) latency to first peck (F). Chicks were presented with odour concentrations in ascending (■, unbroken line), random (□, dashed line) or descending (▲, dotted line) order (n=6 chicks per presentation method). Wilcoxon-Mann-Whitney test, † 0.10> P>0.05.

Table 3.2.1   Values of the Kruskal-Wallis statistic (KW) comparing the chicks' responses to the presentation of odorant concentrations in ascending, random or descending order

Category	Concentration of *iso*-amyl acetate (μl)					
	0	10^{-3}	10^{-2}	10^{-1}	1	10
number of head shaking bouts	0.82	2.27	4.63 †	0.04	2.61	5.26 †
latency to shake head	0.60	2.52	4.51	1.13	1.70	1.68
number of pecks	2.64	2.31	3.04	1.94	1.03	2.32
latency to first peck	1.41	1.38	1.26	3.43	1.00	1.51

Analysis was performed by Kruskal Wallis test, $df=2$, n=6 per group, † $0.10 > P > 0.05$.

The number of chicks that pecked the bead and the latency to first peck was relatively invariant across the various odour concentrations (Figures 3.2.1.B and 3.2.1.F). The results for the number of pecks at the bead are presented in Figure 3.2.1.D. There was no suppression in the amounts of pecking to the lower amounts of odour (10^{-3} to 10^{-1} μl) but the response decreased following the presentation of 1 and 10 μl of *iso*-amyl acetate. The pecking response was not significantly affected by the method of odour presentation (Table 3.2.1).

The relationship between odour concentration and the chicks' responses was also described by the response threshold and the EC_{50} values according to the methods determined in Experiment 3.1. The preferred method incorporates the baseline level of responding and assumes that the highest value obtained is at maximum. It was possible to calculate a response threshold value for head shaking only. The EC_{50} values for the latency to shake the head, the number of bouts of head shaking and the number of pecks could be determined because the level of responses approached values which were maximal (number of head shaking bouts) or minimal (latency to shake the head and number of pecks). These values are presented in Table 3.2.2.

Table 3.2.2 Response threshold a nd EC_{50} values for the different methods of odorant presentation

	Order of presenting *iso*-amyl acetate concentrations		
	Ascending	Random	Descending
Head shaking			
Response threshold	$10^{-0.7}$ (NA)	$10^{-1.15}$ (NA)	$10^{-2.2}$ (NA)
EC_{50}: number	$10^{-0.5}$ ($10^{-1.0}$ to $10^{-0.2}$)	$10^{-0.7}$ ($10^{-1.0}$ to $10^{-0.1}$)	$10^{-0.4}$ ($10^{-0.5}$ to $10^{-0.3}$)
EC_{50}: latency	$10^{-0.4}$ ($10^{-0.7}$ to $10^{-0.2}$)	$10^{-0.4}$ ($10^{-1.2}$ to 2)	$10^{-1.1}$ ($10^{-1.4}$ to 1)
Pecking			
Response threshold	NA	NA	NA
EC_{50}: number	$10^{-0.5}$ ($10^{-2.4}$ to $10^{-0.3}$)	$10^{-1.2}$ ($10^{-2.7}$ to $10^{-0.5}$)	$10^{-0.5}$ ($10^{-0.7}$ to $10^{-0.3}$)
EC_{50}: latency	NA	NA	NA

Values indicate the volume (µl) of *iso*-amyl acetate applied to the sample cup after dilution. The range of EC_{50} values is indicated in parenthesis. NA indicates that a value (or range) could not be calculated.

The number of chicks pecking the bead (indicated as the response threshold in Table 3.2.2) and the latency to first peck could not be used for the calculations as these responses were invariant to the various concentrations of odour. The most noticeable difference among the three methods of odour presentation was found for the response threshold value, as determined from the absolute number of chicks shaking their heads. Chicks presented with odour concentrations in a descending order had a lower response threshold than chicks presented with odour concentration in ascending order. An intermediate response threshold value was obtained for chicks presented with odour concentrations in random order. Despite this difference, the EC_{50} values for the latency to shake the head, the number of bouts of head shaking and the number of pecks at the bead was within the error range for each method of odour presentation.

Relationship between head shaking and pecking in a series of trials

To determine whether head shaking proceeds pecking or vice versa the responses were pooled for each method of odour presentation to increase the sample size used for comparison. The responses were allocated to one of the four possible categories

outlined in Experiment 3.1 (see page 57). A fifth category for non-responders was included for these data as the chicks did not respond on three out of the 108 testing trials (N=18). The results are shown in Table 3.2.3.

Table 3.2.3 Sequence of pecking and head shaking responses by chicks tested in a series of trials with various concentrations of iso-amyl acetate

	Concentration of iso-amyl acetate (μl)					
Category	0	10^{-3}	10^{-2}	10^{-1}	1	10
1. peck only	14	14	10	7	1	0
2. peck then shake head	2	2	5	8	11	13
3. shake head only	2	0	1	2	5	4
4. shake head then peck	0	1	0	1	1	1
5. no response	0	1	2	0	0	0

Values indicate the number of chicks responding. These data were pooled for chicks tested repeatedly with odour concentrations in ascending, random or descending order (N=18).

There was a shift in the chicks' responses from 'peck only' to 'peck then shake head' with increasing concentrations of iso-amyl acetate and the transition between these two response categories occurred at 10^{-1} μl of odour. The chicks shook their heads and then pecked the bead on four trials only (4%) and on 14 trials the chicks shook their heads without having pecked at the bead (13%). Furthermore, a chick pecked at the bead and then shook the head in 38% of the trials. Therefore, chicks tested with increasing odour concentration in a series of trials are more likely to shake the head only after pecking the bead.

Discussion

The main finding of this experiment is that similar concentration-response curves were obtained by testing chicks repeatedly compared to testing chicks once only (cf. Experiment 3.1). The most consistent results were obtained for head shaking, which increases with increasing concentration of iso-amyl acetate. Furthermore, the response

threshold value for chicks presented with odour concentrations in an ascending or random order of presentation was the same as chicks tested once only. Although the pecking response was much more variable over the lower concentrations of odour, there was a consistent suppression of the response at the higher concentrations.

It could be argued that head shaking behaviour is a result of stimulation by odour alone, whereas the decision to make the first peck is determined as a result of visual stimulation. This seems plausible given that there was no effect of odour concentration on the latency to peck or on the number of chicks pecking. The results from Experiment 3.1 indicated that chicks that shake their heads only do so (90% of the time, see Table 3.1.5, page 57) after they have pecked the bead. In the present experiment, when they were tested in a series of trials, chicks that shook their heads did so after pecking the bead on 70% of the trials. Although, some of the chicks may have detected the odour before pecking the bead most did no. The slight differences in the number of chicks that pecked may have been due to an increase in sensitivity as a result of repeated exposures to the stimulus. Alternatively, if the chick perceived the *iso*-amyl acetate odour as aversive then it may have been responding to specific aspects of the stimulus that had been paired with the odour (possibly the sample cup used in each trial).

There were no statistically significant differences between three different orders of presentation of odour concentration (ascending, random or descending). However, chicks presented with odour concentrations in a descending order had a lower response threshold than chicks presented with odour concentrations in either ascending or random order. Therefore, prior exposure to a high concentration of odour affected the chicks' subsequent responses to the same odour. Pangborn *et al.* (1964) reported that, at least for human subjects, the method of stimulus presentation affects the odour detection threshold. By contrast, Henton (1959) and Henton *et al.* (1969) found that the method of stimulus presentation did not affect odour thresholds obtained for pigeons. In these studies, adult pigeons were trained to discriminate between clean and odourised air. The odour thresholds were determined only after a stable discrimination level of key pecking had been obtained.

It is possible that the chicks in the present experiment become more sensitive to the *iso*-amyl acetate odour over the repeated trials or, alternatively, they learn certain aspects about the odour coupled with the bead. This would depend on whether the 10 min inter-trial interval was long enough to prevent adaptation of the receptors to odour enabling the chicks to detect stepwise lower concentrations of *iso*-amyl acetate on each presentation (Cometto-Muñiz and Cain, 1995a). It is possible that chicks tested repeatedly respond to the presentation of a high concentration of odour as aversive and, therefore, they were more responsive to the lower concentrations of *iso*-amyl acetate. However, as noted above, the majority of chicks shook their heads after they pecked the bead, indicating that they are exposed to a suprathreshold concentration of odour at this point. Whether the chicks learn about an odorant coupled with the visual cues of the bead is examined further in Chapters 7 and 8. The aversiveness of an odorant is also addressed in these chapters.

Comparing the chicks' responses to odorant in one trial and a series of trials

The responses from chicks tested in a series of trials were compared with the responses obtained from chicks tested in one trial only (Experiment 3.1). These results are presented in Figure 3.2.2. The broken lines in the figure indicate those tested in one trial only (n=36); the unbroken lines indicate chicks tested in a series of trials (n=6 chicks receiving an ascending order of presentation).

There were no differences in the head shaking scores of chicks tested once or in a series of trials. The number of chicks shaking their heads was almost identical. Furthermore, there were no differences in the latency or number of head shaking responses to suprathreshold concentrations of odour. The number of head shaking bouts increased in the same way for chicks tested in either a series of trials or once only, confirming that testing with a series of odour presentations did not alter the head shaking responses to suprathreshold stimuli.
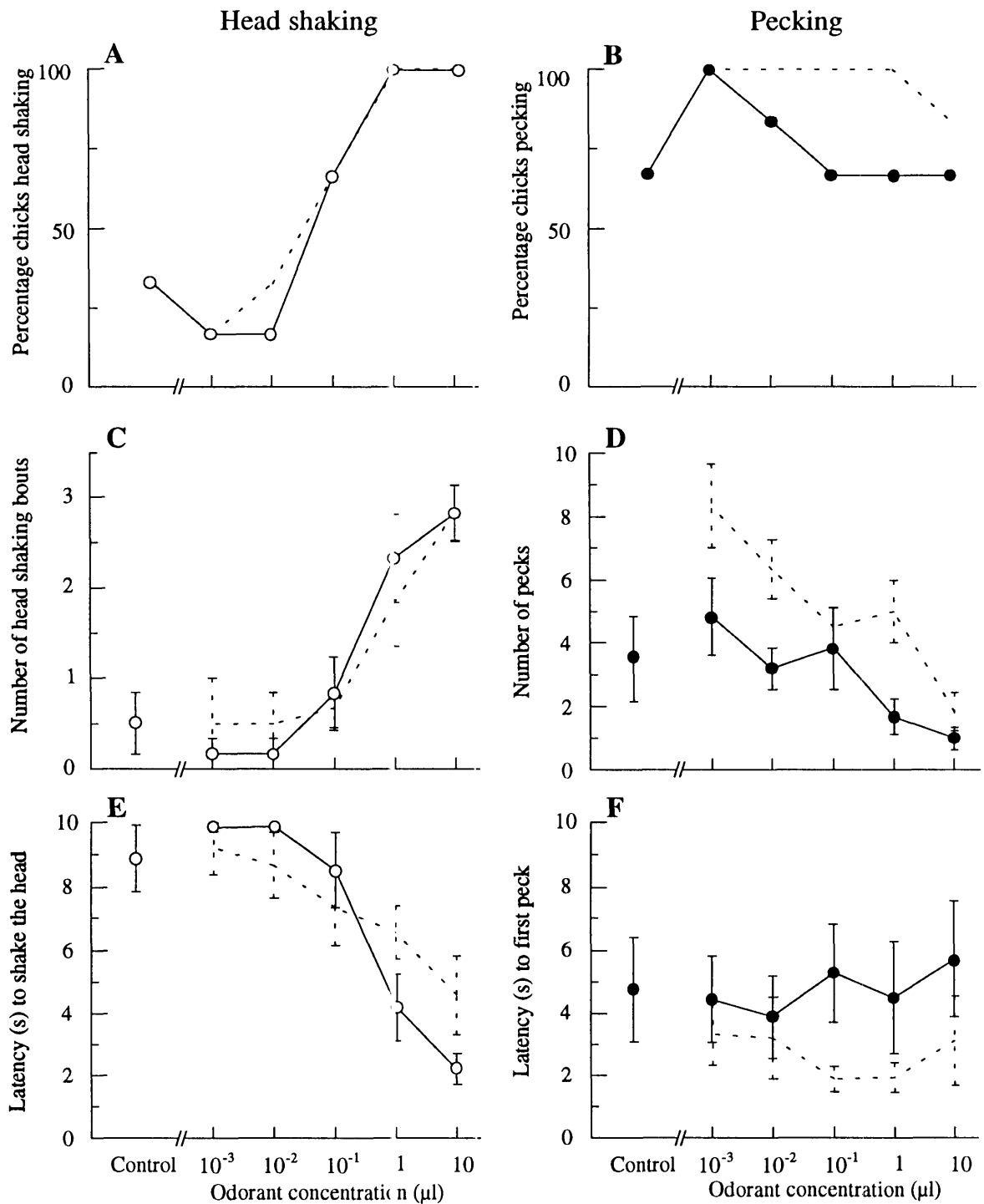
Figure 3.2.2 Comparison between the responses obtained from chicks tested in one trial only (broken lines, from Experiment 3.1) or in a series of trials (unbroken lines this experiment). The results have been separated for the head shaking (A, C, E) and pecking (B, D, F) responses. For each behaviour the results are presented according to the percent of chicks responding (A, B), the mean (± SEM) number of times they responded (C, D) and the mean (± SEM) latency to respond (E, F).

The only clear difference between the two methods of testing (once only or on a series of trials) was evident for the number of times the chick pecked the bead. While the same pattern of pecking was evident, those chicks tested on a series of trials demonstrated, overall, fewer pecks than chicks tested on one trial only. This result is not unexpected as, although the colour of the bead was changed on each trial, the chicks are likely to have habituated to other aspects of the test (Andrew and Brennan, 1983), such as the sample cup and glass rod.

The response threshold and the EC_{50} values obtained from chicks tested once only or in a series of trials are presented for comparison in Figure 3.2.3. The majority of the values for head shaking were within the range of 10^{-1} and 1 µl of *iso*-amyl acetate irrespective of the method of odorant presentation, whereas the suppression of pecking tended to occur at lower concentrations of odorant but differed with different presentation techniques. However, the responses of those chicks presented with odorant concentrations in a descending order were consistently different from the values for chicks tested once only. For this group (descending), the response threshold for head shaking was at least one log dilution lower, and the range of values for the EC_{50} (number of pecks and number of head shaking bouts) was considerably smaller, compared to the other methods of odorant presentation. By contrast, the response threshold values for chicks presented with odorant concentrations in either an ascending or random order appeared to reflect the responses of chicks tested once only. Thus, the latter two orders of odorant presentation (ascending and random) appear to be the more suitable method with which to use to obtain concentration-dependent responses following repeated presentations of *iso*-amyl acetate.
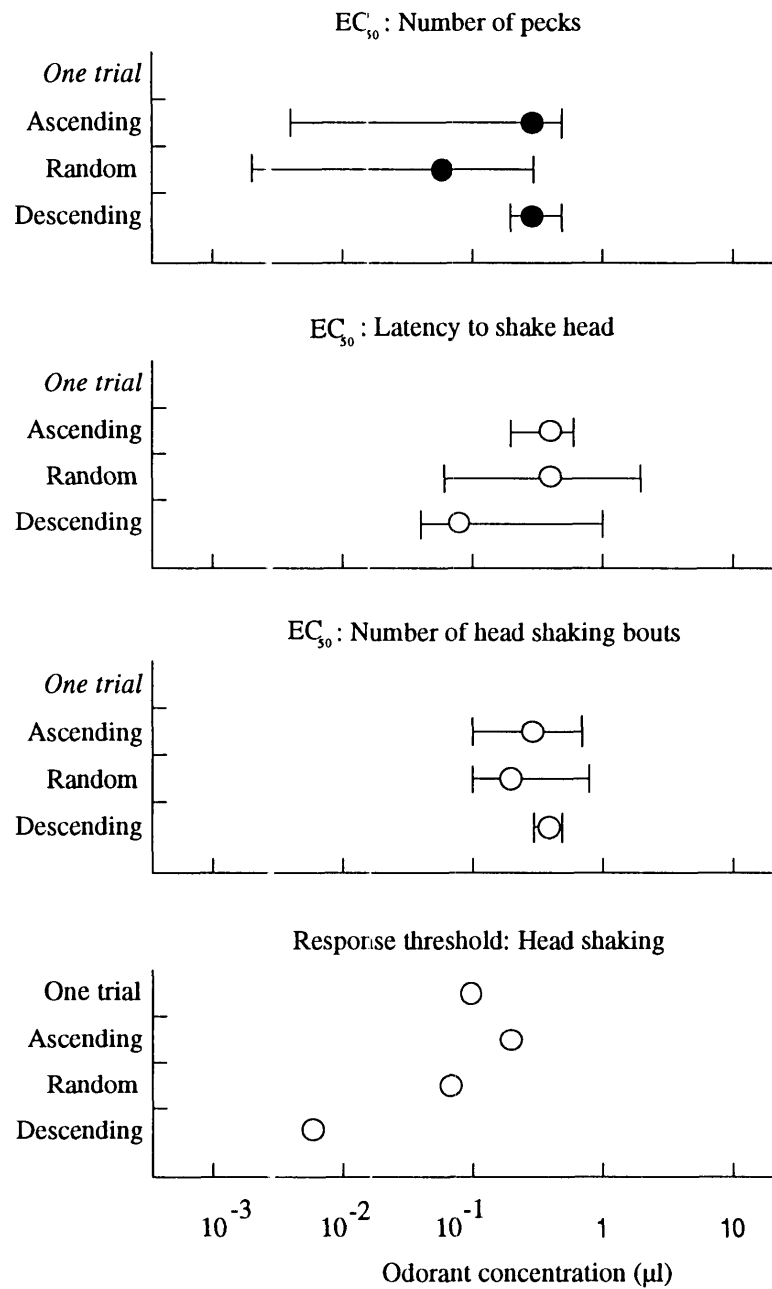
Figure 3.2.3   **Relationship between *iso*-amyl acetate concentration and the head shaking (○) and the pecking (●) responses.** Each of the values was determined from an adjusted baseline (described in text). The data are presented for each method of odour presentation, ascending, random or descending, as well as from those chicks tested on one trial only. Note that only the response threshold could be determined for head shaking for each method of odorant presentation.

EXPERIMENT 3.3: CONCENTRATION-RESPONSES TO *iso*-AMYL ACETATE, ALLYL SULFIDE AND EUGENOL

The aim of this experiment was to compare the chicks' responses to different odorants. Allyl sulfide and eugenol were presented as well as *iso*-amyl acetate as a within experiment control. The previous experiment showed that chicks presented with a descending series of odour concentrations had a lower response threshold than those presented with odour concentrations in ascending or random order. As the focus of this experiment was a comparison of the chicks' responses to different odours, rather than across methods of presentation, the chicks were all presented with an ascending series of odour concentrations. The ascending order of presentation was selected to minimise potential effects of repeated exposures to the same odour.

Methods

Eighteen chicks (8 males and 10 females not previously exposed to the bead or odorant) divided randomly into three groups were used to compare responses to *iso*-amyl acetate, allyl sulfide and eugenol. Ten minutes after the second training trial the chicks were tested in a series of six trials with graded concentrations of either *iso*-amyl acetate, allyl sulfide or eugenol. The stimuli were prepared as described in Experiment 3.1 (see page 48) and included 10^{-3}, 10^{-2}, 10^{-1}, 1 and 10 µl of each of these odours (made up to 10 µl in 70% ethyl alcohol). The control stimulus contained 10 µl of the solvent. As noted above, the odour concentrations were presented in an ascending series of concentrations.

Results

Training trials

The chicks' responses during the training trials are presented in Table 3.3.1. There was a significant difference among the three groups (grouped according to the odour that would be presented during the testing trials) in the amount of pecking during the first training trial (Kruskal-Wallis test: $K\mathit{W}=5.85$, $P=0.05$), but this difference was not evident

in the second training trial. There were no other significant differences between these groups in either of the training trials ($KW<2.5$, $P>0.28$ for each comparison).

Table 3.3.1 Mean ± SEM responses during the training trials

Group ‡	Number of head shaking bouts	Latency (s) to shake the head	Number of pecks	Latency (s) to first peck
Training trial 1				
1	0.7 ± 0.5	14.9 ± 3.3	0.7 ± 0.3 [a]	11.7 ± 3.8
2	0.2 ± 0.2	18.3 ± 1.7	2.0 ± 1.1 [ab]	10.9 ± 3.4
3	0	20	2.8 ± 0.6 [b]	11.9 ± 2.0
Training trial 2				
1	0.3 ± 0.2	17.6 ± 2.1	2.5 ± 0.7	9.7 ± 2.4
2	0.2 ± 0.2	19.7 ± 0.3	1.2 ± 0.4	9.8 ± 3.2
3	0	20	2.5 ± 0.8	7.7 ± 2.8

‡ The chicks are grouped according to the odour that they would be presented with during the testing trials; iso-amyl acetate (1), allyl sulfide (2) or eugenol (3).

Separate analyses for each measure compared the responses between each group during each trial: Kruskal-Wallis test, $df=2$ n=6 per group. Values with different superscripts are significantly different to each other Wilcoxon-Mann-Whitney test, $P<0.05$.

Responses to iso-amyl acetate in a series of trials

These data repeated those found in a previous experiment (3.2). The number of chicks shaking their heads increased with increasing concentrations of odour, reaching a maximum level at 1 and 10 µl of iso-amyl acetate (see Figure 3.3.1.A). There was a significant effect of iso-amyl acetate presented in an ascending series of concentrations, on the number of head shaking bouts (Friedman test: $F_r=21.95$, $df=5$, $P<0.001$, Figure 3.3.1.C) and on the latency to shake the head ($F_r=18.40$, $P=0.003$, Figure 3.3.1.E). Calculation of the response threshold and the EC_{50} values assumed that the response was at a maximum to the higher concentrations of odour, while the baseline level of responding was used as the minimum response level. These values, as well as the range of calculated values including the response threshold and EC_{50}, are reported in Table 3.3.2. The response threshold for head shaking, at $10^{-1.4}$ µl of odour, was slightly
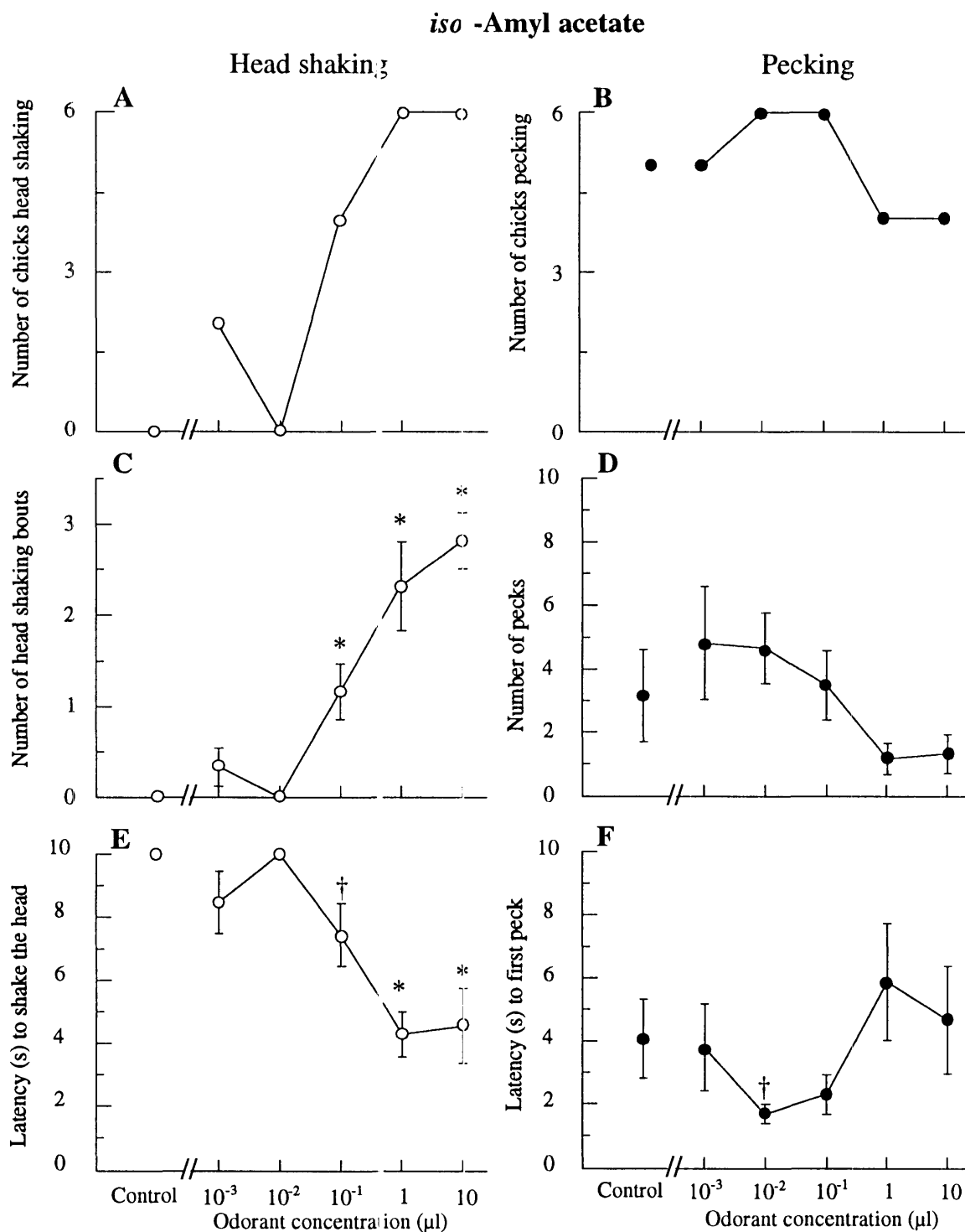
iso -Amyl acetate

Figure 3.3.1 This figure presents the head shaking (O) and pecking (●) scores by chicks presented with *iso*-amyl acetate concentrations in ascending order. The two upper panels show the number of chicks displaying head shaking (A) and pecking (B). The middle two panels show the mean (± SEM) number of head shaking (C) or pecking (D) responses, while the lower two panels show the mean (± SEM) latency to shake the head (E) and the latency to first peck (F). Annotated means indicate a significant difference compared to the response obtained when chicks were presented with the control stimulus († 0.10>P>0.05, * P<0.05, Wilcoxon signed ranks test, n=6 scores per point).

lower than that observed in the previous experiment (see Table 3.2.2, page 65) but did not differ significantly. The EC_{50} for the latency to shake the head and the EC_{50} for the number of bouts of head shaking (see Table 3.3.2) fell within the range of those observed in the previous experiment (see also Table 3.2.2).

Table 3.3.2 Response threshold and EC_{50} values for each of the odorants used

	Odour		
	Iso-amyl acetate	Allyl sulfide	Eugenol
Head shaking			
Response threshold	10^{-14} (NA)	10^{-14} (NA)	NA
EC_{50}: number	$10^{-0.7}$ ($10^{-1.0}$ to $10^{-0.4}$)	$10^{-1.3}$ ($10^{-1.5}$ to $10^{-1.2}$)	NA
EC_{50}: latency	$10^{-0.8}$ ($10^{-1.2}$ to $10^{-0.6}$)	$10^{-1.3}$ ($10^{-1.6}$ to $10^{-0.8}$)	NA
Pecking			
Response threshold	NA	1 (NA)	NA
EC_{50}: number	$10^{-0.7}$ ($10^{-1.5}$ to $10^{-0.5}$)	$10^{-1.5}$ ($10^{-2.3}$ to $10^{-1.2}$)	NA
EC_{50}: latency	NA	NA	NA

Values indicate the volume (μl) of *iso*-amyl acetate at which the responses occurred. NA indicates that a value (or range indicated in parenthesis) could not be calculated.

The results for the pecking responses are also presented in Figure 3.3.1 (panels B, D and F). The number of chicks that pecked and the latency to peck the bead (F_r=2.62, P=0.76) was invariant with the different concentrations of *iso*-amyl acetate. A response threshold could not be determined from the quantum of chicks pecking the bead (Figure 3.3.1.B). There was no significant effect of presenting graded concentrations of *iso*-amyl acetate on the amounts of pecking (F_r=7.69, P=0.17; Figure 3.3.1.D), although they followed a pattern similar to that obtained in Experiment 3.2 (see Figure 3.2.1, page 63). The EC_{50} value for the amounts of pecking was estimated to be at $10^{-0.7}$ μl of *iso*-amyl acetate. This is similar to that observed in the previous experiment, falling within the range of EC_{50} values indicated in that experiment (see Table 3.2.2, page 65).

Responses to allyl sulfide in a series of trials

The results for chicks presented with an ascending series of allyl sulfide concentrations are presented in Figure 3.3.2. The responses from chicks presented with *iso*-amyl acetate are also included in the figure for comparison (indicated by the broken line; the data is the same as in Figure 3.3.1). The number of chicks shaking their heads increased with increasing concentrations of allyl sulfide, reaching a maximum level at $10^{-1.0}$, 1 and 10 µl of allyl sulfide. There was also a significant effect of the various concentrations of allyl sulfide on the number of bouts of head shaking ($F_r=18.38$, $df=5$, $P=0.003$) and on the latency to shake the head ($F_r=19.93$, $P=0.001$). The response threshold and EC_{50} values were within the same range for the number of head shaking bouts and the latency to shake the head and are presented in Table 3.3.2.

The absolute number of clicks pecking the bead decreased at the highest concentration of allyl sulfide delivered (10 µl, see Figure 3.3.2.B). Therefore, for pecking, the response threshold was calculated at 1 µl of allyl sulfide. There was a significant effect of allyl sulfide concentrations on the latency to first peck ($F_r=11.10$, $P=0.05$; Figure 3.3.2.F). There was a significantly longer delay to peck beads coupled with $10^{-3.0}$ µl of odour than beads coupled with 10 µl of odour (Wilcoxon signed ranks test: $z=2.20$, $P=0.03$). Despite this increase in delay an EC_{50} could not be calculated for the latency to first peck as the range covered the responses to the control stimulus through to 1 µl of odour. The number of pecks at the bead also varied for chicks presented with the various concentrations of allyl sulfide ($F_r=16.05$, $P=0.007$; Figure 3.3.2.D).

Responses to eugenol in a series of trials

The results for chicks presented with eugenol in an ascending series of concentrations are presented in Figure 3.3.3. As in Figure 3.3.2, the responses from chicks presented with *iso*-amyl acetate are included in the figure for comparison (the data indicated by the broken line is the same as in Figure 3.3.1). The number of chicks
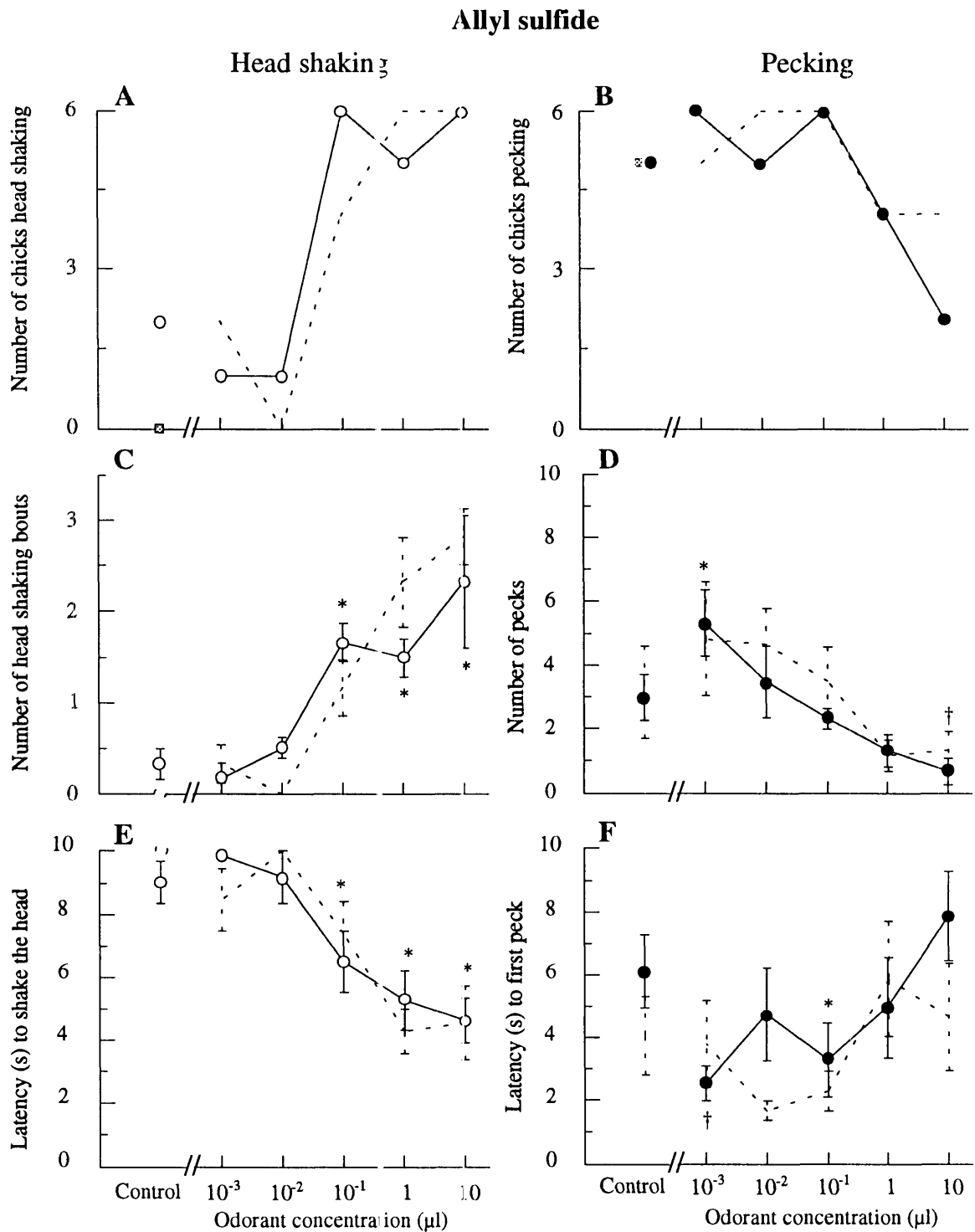
Allyl sulfide

Figure 3.3.2 This figure presents the head shaking (○) and pecking (●) scores by chicks presented with allyl sulfide concentrations in an ascending order. Data are presented as in Figure 3.3.1. Annotated means indicate a significant difference compared to the response obtained when they were presented with the control stimulus († 0.10>P>0.05, * P<0.05, Wilcoxon signed ranks test, n=6 chicks). For comparison, the broken line represents the scores from chicks presented with iso-amyl acetate (the same data as in Figure 3.3.1).
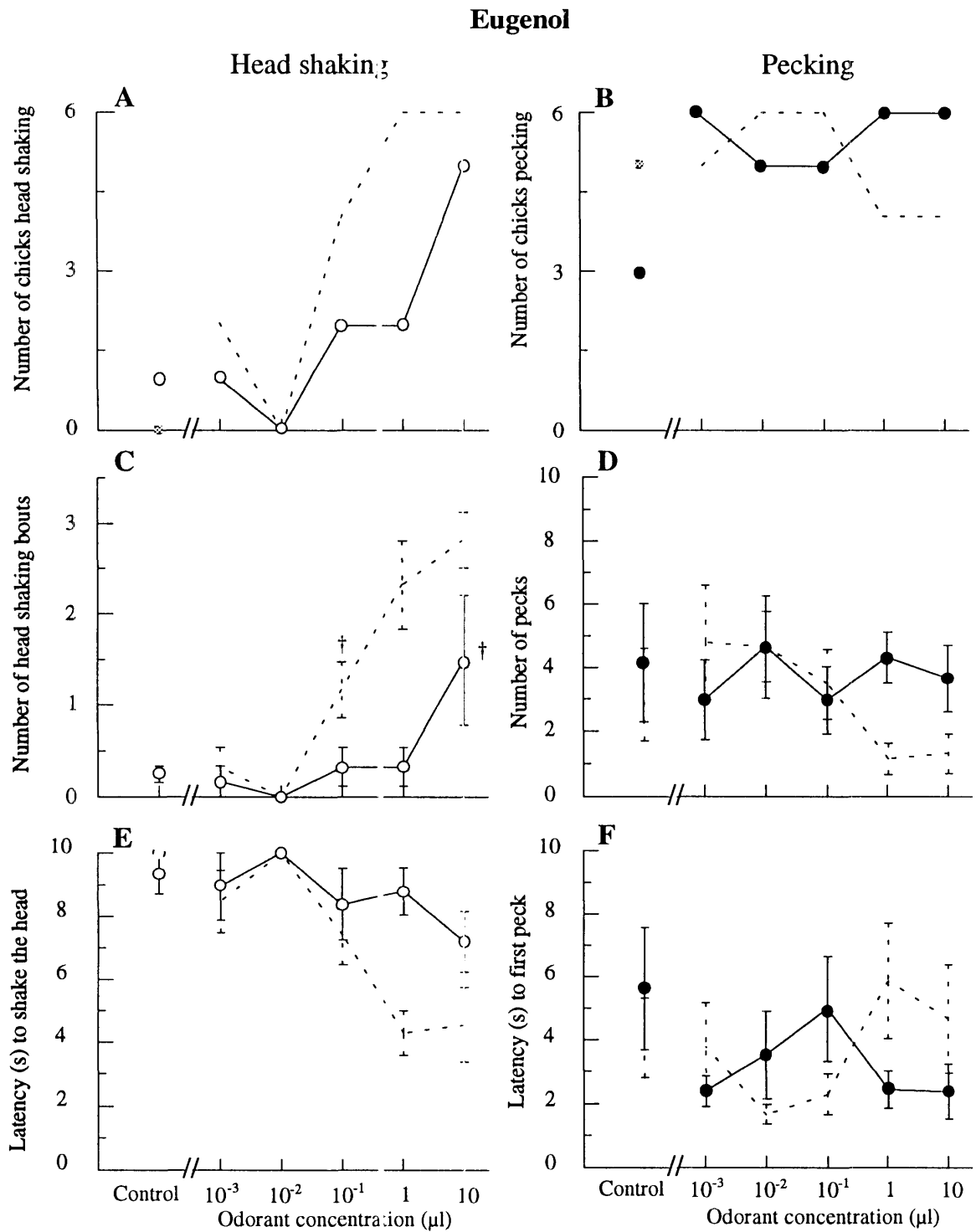
Eugenol

Figure 3.3.3 This figure presents the head shaking (O) and pecking (●) scores by chicks presented with eugenol concentrations in an ascending order. The data are presented as in Figure 3.3.2. For comparison, the broken line represents the scores from chicks presented with *iso*-amyl acetate (the same data as in Figure 3.3.1).

shaking their heads increased at the highest concentration of eugenol only (10 µl; Figure 3.3.3.A). Thus, the response threshold could not be determined although it is likely to be greater than 10 µl of odour. There were no concentration-dependent changes in the latency to shake the head (F_r=7.26 P=0.20; Figure 3.3.3.E) or in the number of bouts of head shaking (F_r=7.93 P=0.16; Figure 3.3.3.C and see also Table 3.3.2, page 75). However, there was a tendency for chicks to shake their heads more to 10 µl of eugenol than to the control stimulus (Wilcoxon signed ranks test: z=1.83, P=0.07). No EC_{50} value could be determined for the latency to shake the head, although the number of head shaking bouts began to increase at the highest concentration of eugenol delivered. No threshold of EC_{50} values could be calculated for pecking to this odour (see Table 3.3.2). Furthermore, the pecking scores were invariant to the various concentrations of eugenol (number: F_r=2.33 P=0.80; latency: F_r=4.62 P=0.46; see Figures 3.3.3.B, D and F).

Comparing the responses to different odorants

There were significant differences between the chicks' responses to the various concentrations of iso-amyl acetate, allyl sulfide and eugenol. The results from these analyses are presented in Table 3.3.3. There were similar concentration-dependent changes in the chick's head shaking and pecking responses to the odours of allyl sulfide and iso-amyl acetate and there were no significant differences between the responses (Wilcoxon-Mann-Whitney test: z<1.5, P>0.14 for each post-hoc comparison). However, the responses to eugenol, as shown in the Figure 3.3.3, were relatively invariant with increasing concentrations and differed from responses obtained from chicks presented with iso-amyl acetate. Chicks shook their heads more when they were presented with $10^{-1.0}$ (Wilcoxon-Mann-Whitney test: z=1.90, P=0.06), 1 (z=2.66, P=0.008) or 10 µl (z=1.97, P=0.048) of iso-amyl acetate than those presented with corresponding concentrations of eugenol. These results also indicated that the higher concentrations of eugenol did not suppress pecking to the same extent as iso-amyl acetate or allyl sulfide at 1 (z=2.43, P=0.02; z=2.30, P=0.02) or 10 µl (z=1.75, P=0.08; z=2.31, P=0.02) of odour.

Table 3.3.3 Values of the Kruskal-Wallis statistic (KW) comparing three different odorants

	Concentration of odorant (µl)					
Category	0	10^{-3}	10^{-2}	10^{-1}	1	10
number of head shaking bouts	2.3	0.6	2.0	7.5 *	8.4 *	5.1 †
latency to shake head	2.1	0.6	2.0	2.4	9.2 *	4.1
number of pecks	0.1	2.8	0.8	0.4	7.9 *	6.2 *
latency to first peck	0.8	0.3	3.6	0.8	1.4	5.5 †

Analysis was performed by Kruska-Wallis test, $df=2$, n=6 per group, † $0.10 > P > 0.05$, * $P < 0.05$. Separate groups of chicks were presented with the odours of either iso-amyl acetate, allyl sulfide or eugenol in an ascending series of odour concentrations.

Discussion

This experiment demonstrates that presenting different concentrations of odour leads reliably to concentration-dependent responses. It also shows that chicks show greater sensitivity to iso-amyl acetate and allyl sulfide than to eugenol, indicating that the test is sensitive to the characteristics of the odour as well as its concentration. The concentration-response curve for head shaking and pecking following the presentation of iso-amyl acetate described in Experiment 3.2 was repeated in this experiment. In addition, a similar concentration-response curve was found for head shaking and pecking elicited by the various concentrations of allyl sulfide.

Chicks displayed equal threshold sensitivity to allyl sulfide and iso-amyl acetate, the response thresholds for head shaking being at a concentration of $10^{-1.4}$ µl of each odour. However, the EC_{50} values for allyl sulfide were slightly lower ($10^{-1.3}$ µl) than those found for iso-amyl acetate ($10^{-0.7}$ µl). When graded concentrations of eugenol were presented repeatedly to the chicks the level of head shaking began to increase at only the highest concentration (10 µl) of odorant delivered. It is possible that the chicks responded to eugenol solely as a pleasant odour as high concentrations of eugenol did

not suppress pecking or, conversely, the chicks did not detect eugenol at concentrations less than 10 µl. For this odour the inverse association of pecking and head shaking is not evident. Thus, there was differential sensitivity to the odours used with chicks being most sensitive to allyl sulfide and *iso*-amyl acetate and less sensitive to eugenol.

The differences in sensitivity to the odours of *iso*-amyl acetate, allyl sulfide and eugenol, suggested by the differing response thresholds, may be due to the differing vapour pressures of these odours at the ambient temperature (presented for 25°C in Table 2.1, page 32). As odour volatility increases, for example from a vapour pressure of 0.03 mm Hg for eugenol to 8.54 mm Hg for allyl sulfide, the response threshold to the odour decreased. This is addressed further in Chapter 5. The explanation for the difference in odour sensitivity may also lie in the relative involvement of the trigeminal and olfactory systems in the chicks' responses to an odour. For example, eugenol is said to be a relatively pure olfactory stimulant, at least in humans (Doty *et al.*, 1978), although this has not been established in animals (Myers and Pugh, 1985). By contrast, *iso*-amyl acetate vapour is known to stimulate olfactory as well as trigeminal receptors in birds (Henton *et al.*, 1969; Walker *e* *al.*, 1936).

The detection of airborne chemicals, such as amyl acetate or butyl acetate, is primarily carried out by the olfactory system but the trigeminal system also responds to volatile stimuli, albeit at higher concentrations (Schumake *et al.*, 1969; Mason and Silver, 1983; Walker *et al.*, 1986). The ophthalmic branch of the trigeminal nerve has free nerve endings in the nasal cavity and, as discussed in Chapter 1 (see pages 19-20), these are involved with the detection of odours. Thus, higher concentrations of *iso*-amyl acetate may stimulate trigeminal neurones as well as olfactory neurones, and these systems may be involved in generating behavioural responses, whereas, responses to eugenol may be initiated only by olfactory stimulation. However, based solely on the concentration-dependent changes in behaviour, it is not possible to rule out the involvement of the trigeminal system in the chicks' responses to odorants.

This experiment demonstrates that presenting chicks with odour together with a bead is suitable for obtaining concer.tration-dependent changes in behaviour for a number of different odours. Furthermore, the test is robust enough to produce repeatable results, and sensitive enough to reveal differential responses to different odours.

GENERAL DISCUSSION

The olfactory test used in these experiments assessed the responses that naive 1-day-old chicks give to odours. The chicks were familiarised with the testing apparatus and the experimenter to reduce potential fear responses that are known to be associated with handling and by placing the chick in a novel environment (Jones and Waddington, 1992). This is a simple familiarisation procedure and can be contrasted to the 60-100 training trials used to meet the criterion for conditioning and the further 100 or more trials used to obtain the sensitivity to odours used in the conditioned suppression techniques in adult birds (Henton *et al.*, 1969; Stattleman *et al.*, 1975; Walker *et al.*, 1986). It, therefore, provides a suitable task with which to obtain responses to odorants without the need for extensive training.

The suppression of pecking and stimulation of head shaking to the higher concentrations of *iso*-amyl acetate and allyl sulfide may indicate that the chick perceives the odorant as aversive. This seems likely as head shaking is readily evoked by an aversive taste, which is also associated with a suppression of pecking (Cherkin, 1969), or by aversive visual stimuli (Andrew, 1975b). The chick may respond to a novel odorant as aversive also. Eight-day-old chicks demonstrate neophobic reactions, including a longer latency to feed and shorter duration of feeding bouts, following the presentation of food that has been scented with a novel odour, such as orange oil (Jones, 1987a). Despite this, the interpretation that chicks respond to the presentation of a novel odour as aversive may be specific to feeding. For example, 1-day-old chicks not previously exposed to orange oil do not preferentially approach or avoid a dish containing litter treated with orange placed at one end of the home-cage compared to a dish containing litter treated with water at the other end (Jones and Gentle, 1985). Furthermore, chicks

that have been reared over litter treated with orange oil for the first 7 days post-hatching develop a preference for that odour when tested in an otherwise novel situation at 7 or 8 days of age (Jones and Gentle, 1985). Therefore, it may be that in the present experiments, pecking and head shaking are simply due to the novelty of the odorant, rather than to it being an aversive stimuli *per se*.

There was a low level of head shaking to unscented stimuli presented during the training trials, as well as to the control stimulus. It was unlikely that the baseline level of head shaking to the control stimulus was due to stimulation by the odour of the solvent (Experiment 3.2) or to odours present in the testing cage. Head shaking is part of the usual behavioural repertoire of Burmese red junglefowl chicks by 48 h post-hatching (Kruijt, 1964). For example, Hogan (1965) reported that Burmese red junglefowl chicks, during the first week post hatching, display bouts of head shaking when they are presented with a meal worm. In his study, the rate of head shaking in the presence of the meal worm was the same before, during and after the meal worm was presented to the chick indicating that this behaviour was not elicited by odour from the meal worm. Instead, Hogan (1965) refers to head shaking as an irrelevant movement (see also Chapter 1, page 3), and he suggests that head shaking is likely to be a transitory behaviour as it occurs during or at he end of a period of fixating. Thus, the calculation of the response threshold and $EC_{5(}$ values for head shaking in the present experiments accounted for the baseline level of responding in order to establish the chicks' responses to odour.

In contrast to the head shaking scores, the number of chicks pecking the bead at least once was constant at every concentration of *iso*-amyl acetate, approximately 93% of the chicks pecked at least once. This suggests that the decision to peck may be elicited primarily by the visual cues, whereas head shaking is elicited primarily by odour alone. It is noted that head shaking may also be elicited by visual (Andrew, 1974; 1975a; 1975b) or auditory stimuli (Kruijt, 1964) but, as mentioned above, the amount of head shaking to a scented, compared to an unscented, bead increases markedly. Since the olfactory test used in this chapter relies on pecking, the involvement of visual responses

could be seen as a potential drawback. However, under natural conditions the chick would be exposed to the odour and taste of potential food items, as well as harmful substances, as a result of exploratory pecking towards conspicuous visual objects. Furthermore, pecking serves to position the nostrils at a fixed proximity to the source of the odour. Indeed, if studies in olfaction are to be based on species-specific behaviours, they require, to some extent, the inclusion of visual components of the test, such as those involved with approach (Vallortigara and Andrew, 1994) or feeding (Jones, 1987a) responses. Moreover, the linking of olfaction and visual components is not limited to the specific behaviours of the chick. For example, many mammalian species, including cats (De Boer, 1977) and primates (Liska and Hudson, 1993), use vision during overall exploration of the surrounding environment, whereas olfactory cues are used to obtain more detailed information of specific objects, such as food or conspecifics. Thus, presenting chicks with beads coupled with odour stimuli has the advantage of simulating natural behaviour. Presumably this is why the test can be performed so easily.

CONCLUSIONS

Presenting chicks with odour coupled with a coloured bead at which they can peck is a suitable method with which to obtain behavioural responses to odours. Presentations of odour, using only one trial per chick, resulted in concentration-dependent responses from chicks that were naive to the test odour. These results suggest that changing the colour of the bead used on each trial allowed reliable concentration-response curves to be generated from chicks tested on a series of trials. The differences in response to the odours of iso-amyl acetate, allyl sulfide and eugenol may be due to the intensity or the quality of the odour.

CHAPTER 4

CONTROLLING FOR REPEATED PRESENTATIONS
OF VISUAL AND VOLATILE STIMULI

INTRODUCTION

Experiments reported in the previous chapter demonstrated that chicks, tested in one trial or in a series of trials, show concentration-dependent responses to odours. Repeatedly testing chicks produced similar results to those from chicks tested once only. It would be advantageous if this method could be used throughout the remainder of the thesis, as this would enable a within animal comparison and reduce the number of chicks required. However, one limitation with repeated testing is that chicks show a marked habituation of the pecking response after only a few presentations of a bead of a single colour (Andrew and Brennan, 1983; Andrew, 1991). Therefore, the aim of the experiments reported in this chapter was to determine whether habituation of responses to the visual stimuli was prevented by presenting unscented stimuli together with differently coloured beads on each trial.

The first experiment (4.1) examined the chicks' responses to the presentation of a bead of the same colour (red) to establish that habituation can occur in the bead test reported throughout this thesis. Dishabituation for responding was also investigated in Experiment 4.1. Chicks were presented with beads of different colours, however, instead of also presenting graded concentrations of odorant with the differently coloured beads, as used in Chapter 3, the chicks were presented with unscented stimuli.

It is possible that the solvent used to dilute the odours used in Experiment 3.1 might have affected the chicks' responses. Thus, a stimulus containing only the vehicle, 70% ethyl alcohol, was presented to a third group of chicks in a series of trials. Results from this group of chicks were then compared with the responses obtained from chicks

presented with unscented stimuli (i.e. with cotton wool only). To determine whether there is habituation to a suprathreshold intensity of odour when it is presented together with beads of different colours a further group of chicks was exposed repeatedly to iso-amyl acetate at the highest concentration (10 µl) used in Experiment 3.1.

The final experiment reported in this chapter examined whether the responses of chicks to odour presentation were due to stimulation of receptors within the nasal cavity. To this end, two separate groups of chicks were tested with the odorant after their nostrils had been occluded (Experiment 4.2).

EXPERIMENT 4.1: PRESENTING ODORANTS IN A SERIES OF TRIALS; CONTROLLING FOR HABITUATION

This experiment was designed to determine whether chicks habituate to the bead and odorant and if habituation of response was prevented by presenting the odorant with a differently coloured bead on each trial.

Methods

Twenty-four chicks (13 males and 11 females that had not been previously exposed to the bead) were incubated and housed as in Chapter 2 (see pages 24-25). The chicks received two training trials with a white bead attached to an unscented sample cup, as described in Chapter 2 (page 37). The procedure used during the testing trials also followed the general procedure outlined in Chapter 2 (see page 37).

One group of six chicks was then presented individually with a red bead attached to an unscented stimulus (a piece of cotton only). A second group of six chicks was also tested individually with unscented stimuli but one of six differently coloured beads as used in each trial. The colour of the bead used in each trial was allocated according to a Latin-square design. As in Chapter 3, the bead colours used were red, dark blue, light blue, dark green, light green and yellow. Another group of six chicks was tested individually with 70% ethyl alcohol (used as the solvent in Experiments 3.1, 3.2 and 3.3) and each chick in a fourth group was presented with 10 µl of iso-amyl acetate. Thus,

each chick received six trials; the first group was presented with a red bead in each trial, while the latter three groups were presented with differently coloured beads in each trial.

These data were analysed usirg non-parametric statistics (see Chapter 2, page 41). As these experiments were primarily concerned with examining the affects of repeated testing on the chicks' responses, li ies of best fit were also calculated for each of the measures. Furthermore, the absolute number of chicks shaking their heads or pecking the bead was analysed using a no 1-parametric binary analysis for repeated measures (Cochran Q test; Siegel and Castellan, 1988). This test determines whether the proportion of responses (coded as 0=no response, 1=respond, as in Chapter 3) changes over repeated trials, and thus was an appropriate statistical procedure for analysing these data.

Results

Training trials

The mean (± SEM) head shaking and pecking scores (mean no.) during the training trials are presented in Table 4.1.1. There were no significant differences between the responses obtained from each group of chicks during either of the training trials but

Table 4.1.1 Mean ± SEM head shaking and pecking responses during the training trials for each group of chicks that would be presented with unscented stimuli or a stimulus that was scented with 70 % ethyl alcohol or 10 μl of *iso*-amyl acetate

Stimuli to be presented during testing	Number of head shaking bouts		Number of pecks	
	Training trial 1	Training trial 2	Training trial 1	Training trial 2
Beads of the same colour:				
Unscented	0	0.17 ± 0.17	2.50 ± 0.76	2.67 ± 1.09
Differently coloured beads:				
Unscented	0	0	2.83 ± 0.83	1.17 ± 0.54
70% Ethyl alcohol	0	0	0.50 ± 0.22	0.67 ± 0.33
10 μl *iso*-Amyl acetate	0.17 ± 0.17	0.50 ± 0.22	2.00 ± 0.93	2.83 ± 0.48
KW ‡	3.00	6.90	5.89	6.40
P	0.39	0.08 †	0.12	0.09 †

‡ Analysis was performed using separate Kruskal-Wallis tests for each measure during the two training trials, n=24, df=3. Differences between groups approached significance during training trial 2 only, † 0.10>P>0.05.

differences between the scores obtained during the second training trial approached significance (Kruskal-Wallis test: 0. 0>P>0.05). The results from these analyses are also presented in Table 4.1.1. While the chicks were allocated randomly to each of the groups, those that would be presen ed with 10 µl of iso-amyl acetate had a tendency to shake their heads more than chicks that would be tested with stimuli that were unscented or contained the solvent. Those tha; would be presented with the solvent had a tendency to make fewer pecks at the bead than chicks in the remaining groups.

Responses to repeated presentations of a bead of the same colour

The mean (± SEM) head shaking and pecking scores (number responding, mean no. and latency) for chicks tested with a red bead attached to an unscented sample cup are illustrated in Figure 4.1.1. A line of best fit was calculated for each of the responses, over the six consecutive trials, and t ese are also included in the figure. There was a low level of head shaking during several of the testing trials (trials 2, 3 and 4) but there was no significant effect of the repeated presentations on the number of chicks shaking their heads (Q=5.56, P=0.35; Figure 4.1.1.A), the number of bouts of head shaking (Friedman test: F_r=1.43, P=0.92; Figure 4.1.1.C) or on the delay to shake the head (F_r=1.31, P=0.93; Figure 4.1.1.E). The repeated presentations resulted in a significant decrease in the number of chicks that pecked (Q=13.85, P=0.017; Figure 4.1.1.B), a significant suppression in the number of pecks (F_r=13.55, P=0.02; Figure 4.1.1.D) and a significant increase in the delay before the first peck (F_r=11.98, P=0.035; Figure 4.1.1.F), which was most evident by the fifth trial. Thus, there was marked habituation of the pecking response following repeated presentations of a red bead.

Responses to presentations of beads of different colours

Figure 4.1.2 shows the responses of chicks presented with unscented stimuli together with the differently coloured beads. There were no significant effects of repeatedly testing chicks on either the head shaking (number responding: Q=3.00, P=0.70; mean no: F_r=0.64, P=0.99; latency: F_r=0.64, P=0.99; Figures 4.1.2.A, C and E) or the pecking scores (number responding: Q=4.00, P=0.55; mean no: F_r=4.76, P=0.45;
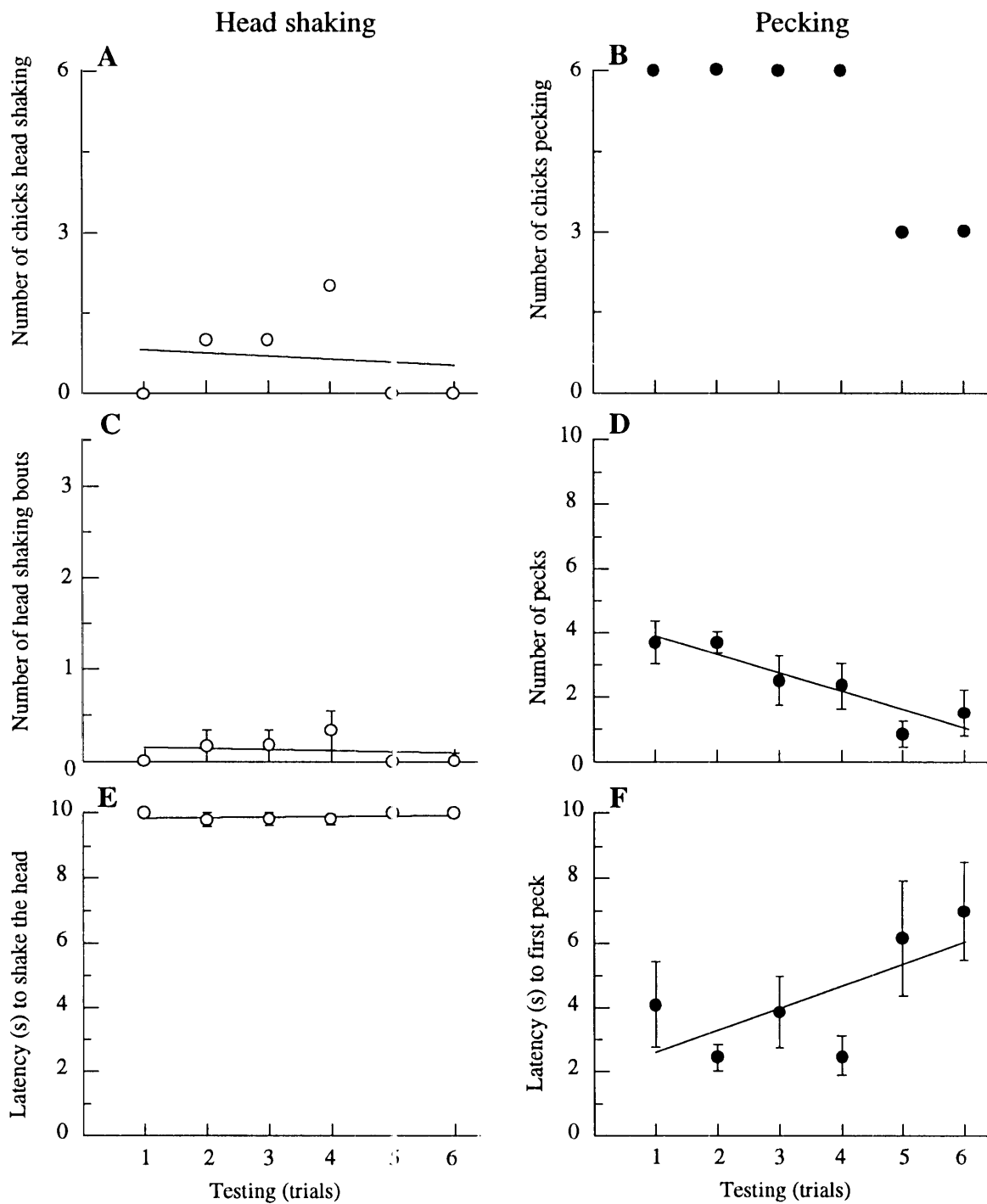
Figure 4.1.1 Mean (± SEM) responses are depicted for the presentation of a red bead during six consecutive trials. Lines of best fit were calculated and are presented in the figure. The scores are presented as the number of chicks shaking their heads (A), the number of chicks pecking (B), the mean number of head shaking bouts (C), the mean number of pecks at the bead (D), latency for the first bout of head shaking (E) and the latency to first peck (F). Mean values without error bars had SEM that were so small that they are not evident and as there were only two values in (B) a line of best fit was not calculated. There were significant effects of the repeated trials on the pecking responses ($P<0.05$) but not on the head shaking responses.
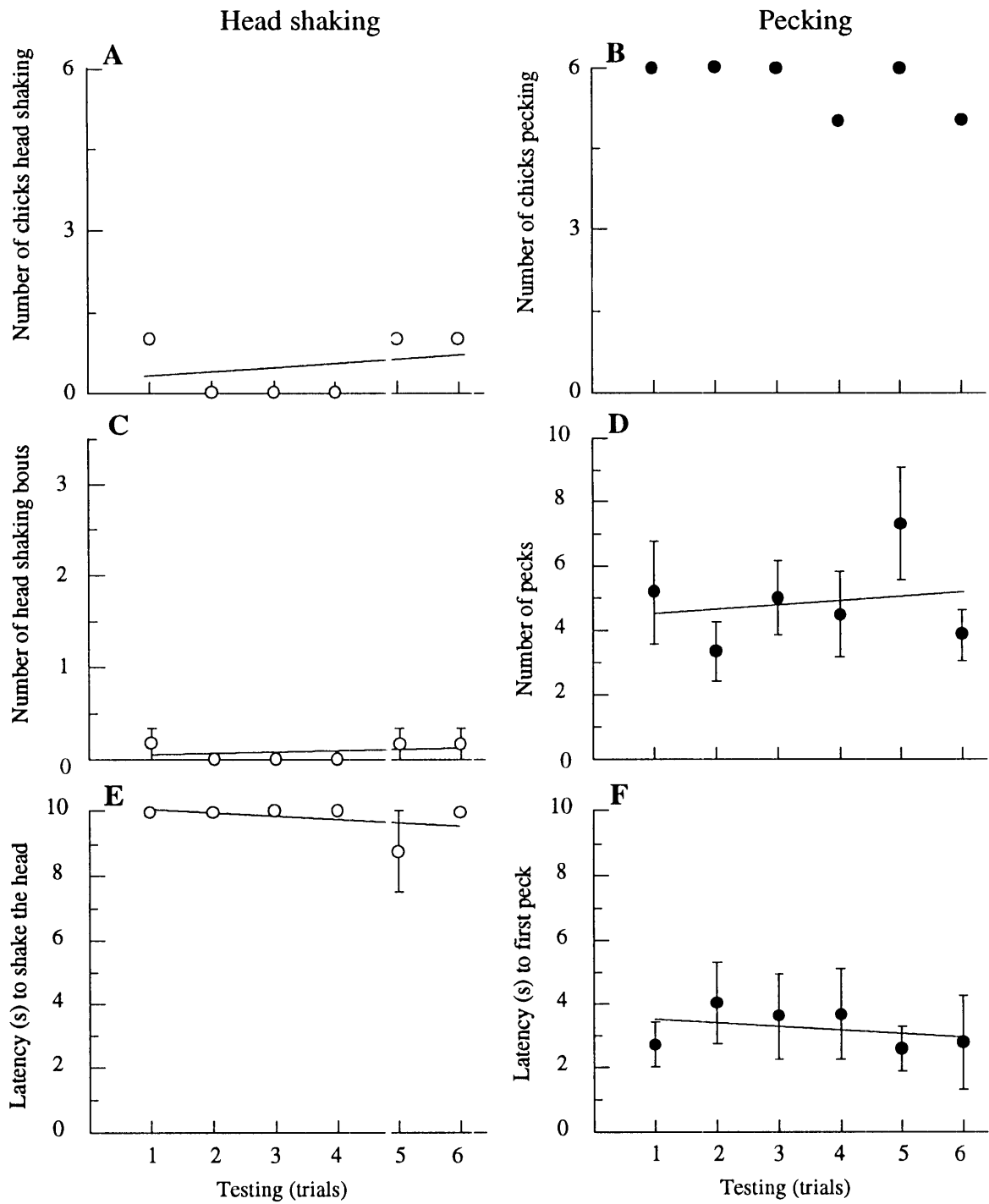
Figure 4.1.2 Mean (± SEM) responses are depicted for the presentation of unscented stimuli presented together with differently coloured beads during six consecutive trials. The data are presented as in Figure 4.1.1. As there were only two values in (B) a line of best fit was not calculated. There were no significant effects of the repeated trials on the pecking or head shaking responses ($P > 0.05$).

latency: $F_r=1.45$, $P=0.92$; Figures 4 1.2.B, D and F). This is a marked contrast to the results presented in Figure 4.1.1 and indicates that habituation of pecking was prevented by changing the colour of the bead on each trial.

Responses to repeated presentations of volatile stimuli

The responses of chicks tested with stimuli that contained the solvent, presented together with the differently coloured beads, are presented in Figure 4.1.3. As for unscented stimuli (presented together with beads of different colours), there were no significant effects of repeated testing on either the head shaking (number responding: $Q=6.25$, $P=0.28$; mean no: $F_r=1.43$, $P=0.92$; latency: $F_r=1.48$, $P=0.92$) or the pecking responses (mean no: $F_r=2.67$, $P=0.75$; latency: $F_r=6.45$, $P=0.26$). It can be noted that there was a tendency for an increase in the number of chicks pecking the bead although this did not approach significance (number responding: $Q=9.76$, $P=0.08$). Therefore, no significant habituation of the head shaking or pecking responses occurred across testing trials.

There was also no effect of repeated testing on the responses of chicks presented with stimuli containing 10 µl of iso-amyl acetate (head shaking: number responding: $Q=2.73$, $P=0.74$; mean no: $F_r=1.05$, $P=0.96$; latency: $F_r=4.14$, $P=0.53$; pecking: number responding: $Q=2.30$, $P=0.81$; mean no: $F_r=6.90$, $P=0.23$; latency: $F_r=1.07$, $P=0.75$). These data are presented in Figure 4.1.4.

There was a similar tendency, although it was not significant, for chicks presented with either the solvent or 10 µl of iso-amyl acetate to show a decrease in the latency to first peck across testing trials, which was not apparent for chicks presented with unscented stimuli. However, to facilitate statistical comparison between the responses of chicks presented with unscented stimuli and stimuli that contained the solvent or 10 µl of iso-amyl acetate, the repeated measurements were averaged over the six trials. This was possible as there were no significant differences between the responses within each group and enabled a single datum point to be obtained for each chick (Figure 4.1.5). For this
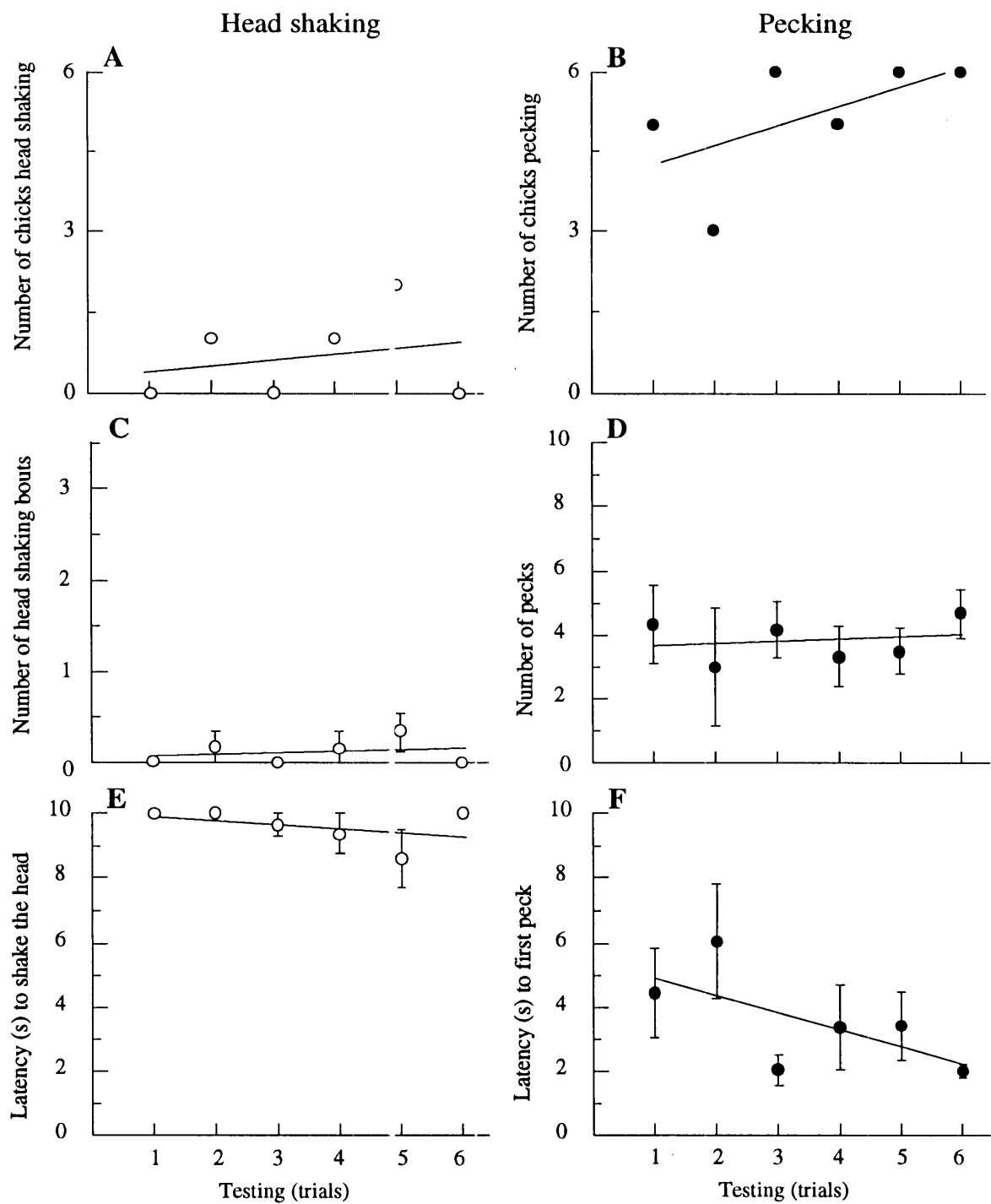
Figure 4.1.3 Mean (± SEM) scores for pecking and head shaking (number responding, number of responses, and latency) are depicted for the presentation of stimuli scented with the solvent during six consecutive trials. Data are presented as in Figure 4.1.1.
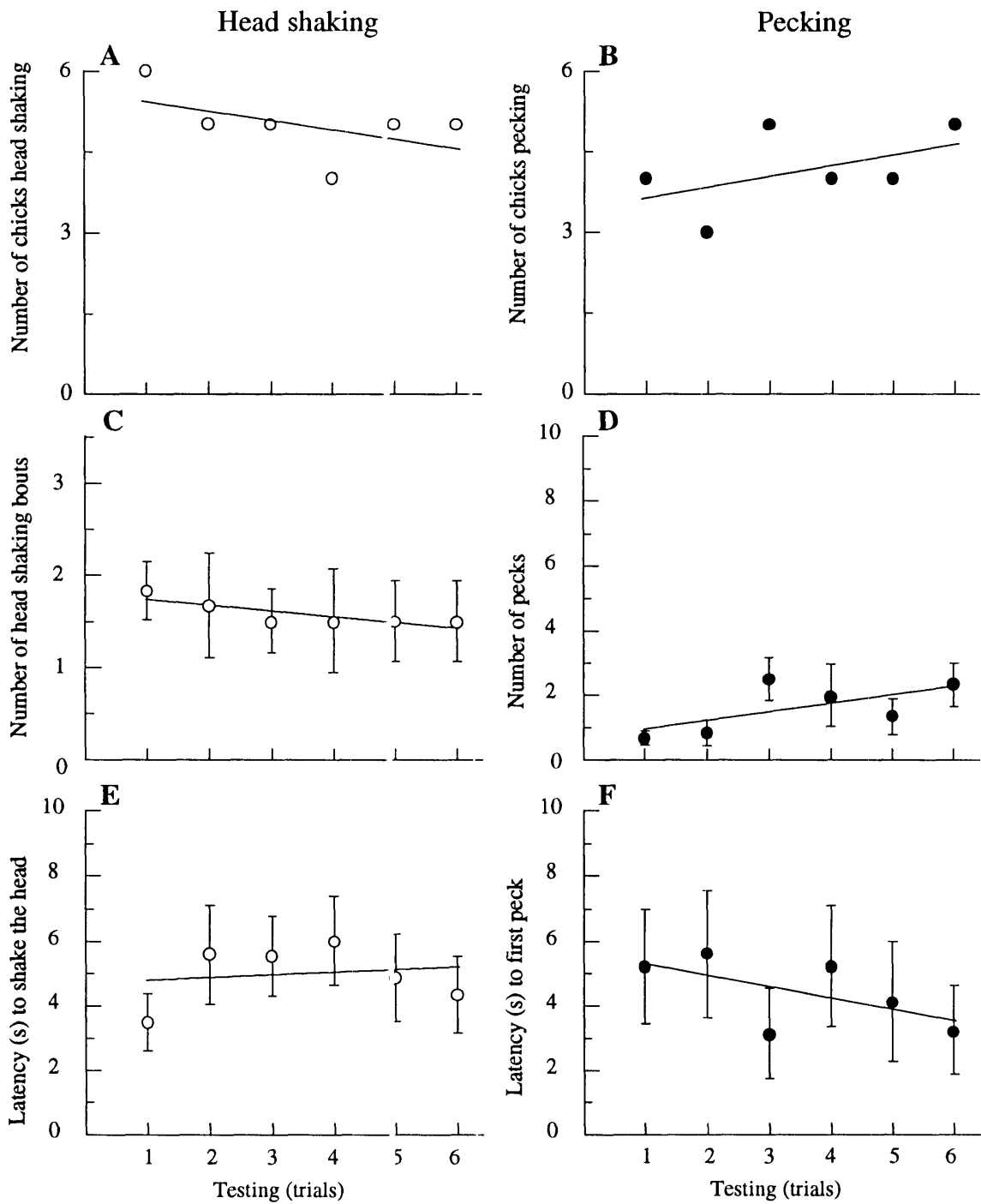
Figure 4.1.4 Mean (± SEM) scores for pecking and head shaking (number responding, number of responses, and latency) are depicted for the presentation of stimuli scented with 10 µl of *iso*-amyl acetate during six consecutive trials. Data are presented as in Figure 4.1.1.
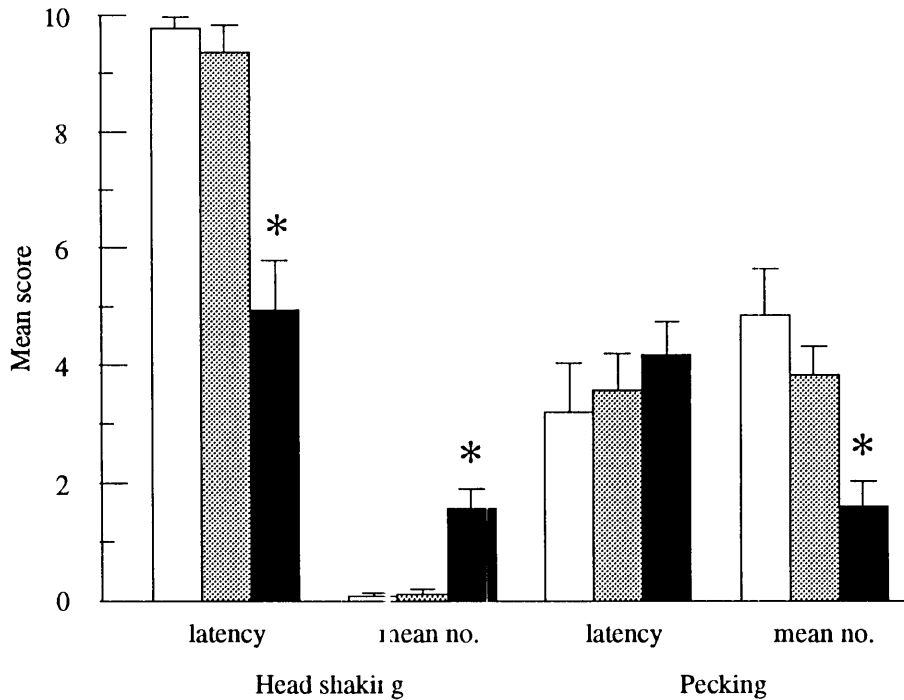
Figure 4.1.5 Mean (± SEM) of the average responses obtained from chicks tested in a series of trials with unscented stimuli (□), or stimuli that contained the solvent (▨), or 10 μl of iso-amyl acetate (■). Means annotated with an asterisk were significantly different from those chicks tested with unscented stimuli (Wilcoxon-Mann-Whitney test: $P<0.05$; n=6 chicks per group). Note that results for chicks presented with the solvent did not differ significantly from those presented with unscented stimuli.

comparison, the responses (mean no. and latency) obtained from the three groups of chicks presented with the differently coloured beads on each trial were used.

There were significant effects of the odorant (unscented, the solvent or iso-amyl acetate) on the latency to shake the head (KW=11.41, P=0.003), the number of head shaking bouts (KW=12.13, P=0.002) and the number of pecks at the bead (KW=8.65, P=0.01). Post hoc analysis with Wilcoxon-Mann-Whitney tests revealed that, compared to those tested with unscented stimuli, chicks presented with 10 μl of iso-amyl acetate made fewer pecks overall (z=2.57, P=0.01), shook their heads more (z=2.92, P=0.004) and had shorter latencies to shake their heads (z=2.90, P=0.004). The responses of chicks presented with stimuli containing the solvent were not significantly different from those tested with unscented stimuli (z<0.9, P>0.20 for each comparison) nor were there

no significant differences between their latency to peck the bead ($KW=0.92$, $P=0.63$). Thus, even when the results from the six trials were averaged, there was no significant difference between the responses of chicks presented with the solvent or stimuli that was unscented.

The colour of the bead used on each trial was randomised according to a Latin-square design, such that each chick was presented with a differently coloured bead on each trial. The results from chicks presented with unscented stimuli as well as the solvent were pooled so that the responses to each of the bead colours could be directly compared. The mean ($\pm$ SEM) number of bouts of head shaking and pecks at each of the differently coloured beads is presented in the table below (Table 4.1.2). There was no significant effect of the bead colour on either the head shaking or pecking scores. Although not significant, it can be seen that numerically higher amounts of pecking were made at the red and blue beads, compared to the yellow and green beads.

Table 4.1.2 Mean $\pm$ SEM head shaking and pecking responses to differently coloured beads

Bead colour	Number of head shaking bouts	Number of pecks
red	0	4.92 ± 1.16
yellow	0.17 ± 0.11	3.50 ± 0.68
light green	0.08 ± 0.08	3.50 ± 0.69
dark green	0	3.58 ± 0.48
light blue	0.17 ± 0.11	5.17 ± 0.90
dark blue	0.17 ± 0.11	5.42 ± 1.03
F_r	1.04	2.78
P	0.96	0.73

The means tabulated above indicated the responses of chicks presented with either unscented stimuli or stimuli that contained the solvent. These groups were pooled (n=12) for this comparison. Separate analysis with the Friedman test (F_r) indicated that there was no significant effect of bead colour on the chicks' responses.

Discussion

Changing the colour of the bead on each trial prevented the habituation of pecking that occurred during repeated presentations of a bead of the same colour. Therefore, testing chicks in a series of trials is as effective as testing each chick once only, provided that beads of different colours are used. Also, the amount of head shaking was unaffected by repeatedly presenting unscented stimuli together with either the same or differently coloured beads. Thus, while head shaking can be evoked by visual stimuli (Andrew, 1975a; 1975b; Andrew and Brennan, 1984), the present results suggest that the bead colours which were used in these tests evoked similar, low levels of head shaking.

These results also show that the solvent (70% ethyl alcohol) used to dilute the odorant in the experiments reported in Chapter 3 did not affect the chicks' responses, even though the ethanol solvent is unlikely to be completely odourless. Indeed, although humans detect ethanol as having only a slight odour (Geldard, 1972), it is nevertheless perceived as having a fruity quality (Williams and Rosser, 1981). It is possible that, due to the high evaporation rate of ethanol compared to *iso*-amyl acetate, there was less solvent in the sample cup at the time of testing and this may have resulted in very low concentrations of odour in the air surrounding the sample cup. Although it is also possible that there was an interaction between the solvent and *iso*-amyl acetate at the lower concentrations of odour that were used in Experiments 3.1, 3.2 and 3.3, the present results indicate that diluting odorants with ethyl alcohol is unlikely to alter responses during testing, validating the methods used in Chapter 3.

There was no habituation of the chicks' responses to the repeated presentation of suprathreshold concentrations of *iso*-amyl acetate odour. While there was a tendency for the amounts of head shaking to decrease slightly after the first two trials, this was not significant. Moreover, these changes were very slight and the responses did not alter to any great extent over the last four trials. It seems likely that the suppression of pecking and stimulation of head shaking indicate that the chick perceives a suprathreshold concentration of odorant as aversive. However, if this were the case, one would have

expected the chicks to avoid the stimulus. For example, in the one-trial passive avoidance learning task (Cherkin, 1969; Ng and Gibbs, 1991) a chick is trained to avoid pecking at a bead of a specific colour (such as red) that has been coated with the bitter-tasting substance methyl anthranilate and, when the chicks are re-tested they avoid pecking at a red bead but will continue to peck at a differently coloured bead (such as blue). In contrast, the present results show that chicks continue to peck at a "scented" stimulus if the visual properties (colour) of the bead are changed.

The absence of habituation when the bead colour is changed in successive trials might be explained by the way in which the chick perceives visual and olfactory cues. For example, the chicks may associate the colour of the bead with the odour. By changing the colour, but not the odour, the chicks may perceive it as a different "stimulus". Thus, the chicks would peck and shake the head, at about the same frequency, during each trial (addressed further in Chapter 9). An alternative explanation is that the pecking response of the chick is elicited by the colour of the bead and not the odour. Therefore, changing the colour of the bead prevents visual habituation despite the repeated presentation of the same odour.

The chick may not perceive the visual and odour cues at the same time. It may be that the chick's first peck at the bead is in response to the visual cues alone. That is, the chick may not be exposed to a suprathreshold concentration of odour until after it has pecked the bead (or during the peck). Therefore, the chick may not be able to sample the presence or absence of odour on later trials unless it pecks at the bead. This explanation seems likely as there was no effect of the stimulus on the latency to peck the bead. This result is in agreement with those of Experiment 3.1 (see pages 59-61) when it was found that pecking largely precedes head shaking. There were, however, significant effects of the odorant, compared to stimuli that is unscented, on the amount of pecking, the latency to shake the head and the number of bouts of head shaking. Thus, the absence of habituation to the odorant may be due, indirectly, to the use of differently coloured beads. It may be that the chick habituates to an odorant if it is repeatedly presented with a bead of the same colour. The effect of repeated presentations of odour

coupled with beads of the same colour is considered further in Chapter 7 (see pages 183-189).

EXPERIMENT 4.2: OCCLUDING THE CHICK'S NOSTRILS AND THE RESPONSE TO ODORANTS

Although several studies have shown that responses to odour are abolished by occluding the chick's nostrils (Tolhurst and Vince, 1976; Jones and Gentle, 1985; and see Chapter 1, pages 2-3 and 7) it is possible that the concentrations of odorant used in the experiments reported in Chapter 3 stimulated receptors outside the nasal cavity. To determine whether the odorants presented to the chicks stimulated receptors (either olfactory or trigeminal) within the nasal cavity, or chemoreceptors in the eyes or mouth, one group of chicks was tested with various concentrations of *iso*-amyl acetate diluted in the solvent. This group of chicks had had the nares occluded with a wax preparation before the odorant was presented.

Responses to odorants mediated by chemoreceptive systems outside the nasal cavity are more likely to occur if a higher concentration of *iso*-amyl acetate were presented. However, this was examined by presenting various concentrations of undiluted *iso*-amyl acetate to an additional group of chicks that had also had the nares occluded. It was also of particular interest to determine whether occluding the chick's nostrils with a wax preparation and then removing the wax (unblocking) damaged the nasal cavity and altered the chick's responses to odour. Therefore, this group of chicks was also tested with a single concentration of *iso*-amyl acetate after the wax had been removed.

Methods

Fourteen chicks (9 males and 5 females) not previously exposed to the bead or odorant were used in this experiment. One group of six chicks was tested with log dilutions of *iso*-amyl acetate, prepared in exactly the same way as described in Experiment 3.1 (see page 48), and were presented in an ascending series of odour

concentration (as in Experiment 3.2). Ten minutes before the training trials the chicks' nostrils were occluded with a wax preparation according to the procedure outlined in Chapter 2 (pages 39-41). Although occluding the nares prevented the chicks from inspiring air into the nasal cavities, it did not prevent them from breathing. The chicks' nostrils remained occluded until the end of testing. The responses obtained from this group of chicks were compared with the responses of chicks that also received odour concentrations in an ascending order but did not have their nostrils occluded (reported in Experiment 3.2).

Chicks in another group (n=8), which also had the nares occluded before testing, were presented with various suprathreshold concentrations of iso-amyl acetate. For this group undiluted odorant was presented at three concentrations (1, 10 and 100 μl), as well as an unscented stimulus (0 μl; containing a clean piece of cotton wool only) in four testing trials. To compare the responses to iso-amyl acetate from the same birds, they received one additional testing trial; the wax preparation was removed (referred to here as unblocked) and they were presented with 10 μl of iso-amyl acetate. The bead colours used were red, light blue, dark blue, light green and dark green, and were allocated according to a Latin-square design.

The non-parametric statistical procedures used to analyse these data are detailed in Chapter 2 (page 41).

Results

Training trials

Chicks tested with both nostrils occluded demonstrated similar amounts of pecking during the training trials as chicks that did not have their nostrils occluded (cf. Experiment 4.1; see Tables 4.1 1 and 4.2.1). There was, however, a tendency for chicks with both nostrils occluded to peck more than chicks that did not have their nostrils occluded during the first training trial (Wilcoxon-Mann-Whitney test: $z=1.95$, $P=0.051$; n=24 chicks with open nostrils and 14 with occluded nostrils) but not the second training trial ($z=1.17$, $P=0.24$). Occluding the nostrils resulted in

Table 4.2.1 Mean ± SEM head shaking and pecking responses during the training trials for chicks with occluded and open nostrils

Group	Number of head shaking bouts		Number of pecks	
	Training trial 1	Training trial 2	Training trial 1	Training trial 2
Occluded nostrils	0.71 ± 0.16	0.93 ± 0.25	4.29 ± 0.95	3.00 ± 0.77
Open nostrils	0.04 ± 0.04	0.17 ± 0.08	1.96 ± 0.39	1.83 ± 0.37

The data tabulated above presents the responses of chicks with occluded nostrils (n=14, this experiment) and those which did not have their nostrils occluded (n=24, Experiment 4.1) during the training trials. Note that the means for chicks with open nostrils were averaged and thus the values are not the same as those in Table 4.1.1.

a significant increase in the number of bouts of head shaking during both of the training trials (Trial 1: $z=4.01$, $P<0.001$; Trial 2: $z=3.12$, $P=0.002$).

Concentration-responses by chicks with both nostrils occluded

The responses of chicks that were tested with both nostrils occluded and presented with the odorant concentrations in an ascending order are presented in Figure 4.2.1. Approximately 50% of chicks shook their heads at least once during each of the testing trials. Thus, these chicks had a higher baseline level for head shaking but head shaking levels did not alter when increasing concentrations of odour were presented. Occluding both of the chicks' nostrils did not result in a significant effect of odour intensity on the latency to shake the head (Friedman test: $F_r=2.26$, $df=5$, $P=0.81$) or the number of bouts of head shaking ($F_r=0.48$, $P=0.99$).

There were no effects of odorant concentrations on the number of chicks that pecked at the bead, nor were there any significant differences in the responses to each concentration of odour for either the latency to peck ($F_r=7.90$ $P=0.16$) or the number of pecks ($F_r=3.81$, $P=0.58$). These results indicate that occluding the chicks' nostrils prevents the chick from responding to odour.
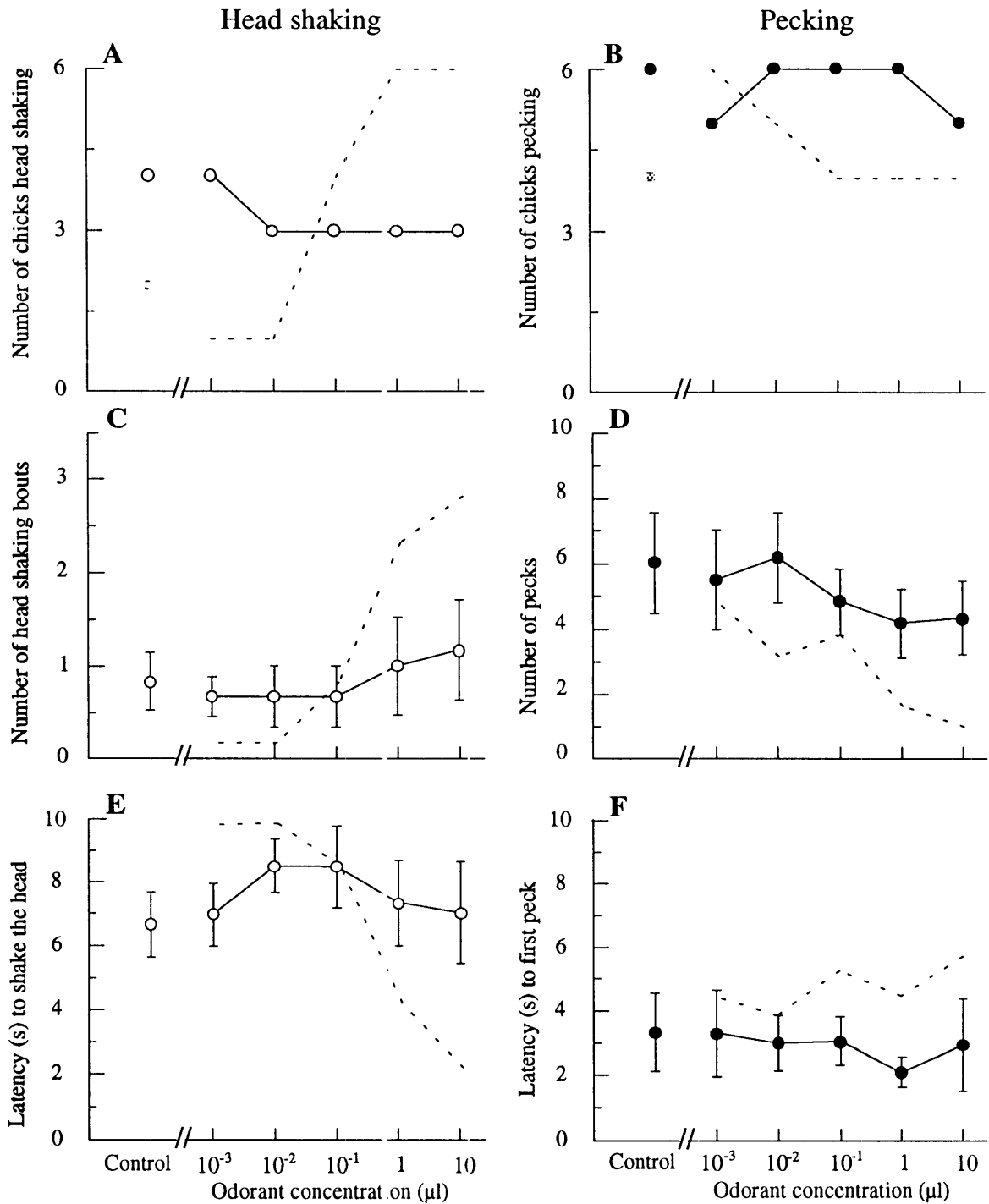
Figure 4.2.1 Mean (± SEM) number of responses by chicks tested with both nostrils occluded with a wax preparation and presented with graded concentrations of iso-amyl acetate. The scores are presented as the number of chicks shaking their heads (A), the number of chicks pecking at the bead (B), the mean number of head shaking bouts (C), the mean number of pecks (D), the mean latency to the first bout of head shaking (E) and the mean latency to peck (F). For comparison, the broken line indicates the responses from chicks that did not have their nostrils occluded and were tested with equivalent odour concentrations presented in an ascending order (from Figure 3.2.1). There were no significant effects of the odorants' concentration on the chicks' responses.

Effect of unblocking the chick's nostrils on the responses to iso-amyl acetate

Figure 4.2.2 shows the responses of chicks with occluded nostrils presented with different volumes of undiluted *iso*-amyl acetate. Approximately 50% of chicks shook their heads to an unscented stimulus as well as to the two lowest concentrations of odorant delivered (1 and 10 μl). Although 6 out of the 8 chicks shook their heads to the presentation of 100 μl of odorant this increase was only slight (see Figure 4.2.2.A). There were no significant effects of odour intensity on the number of bouts of head shaking ($F_r=2.29$, $df=2$, $P=0.51$; Figure 4.2.2.C) or the latency to shake the head ($F_r=4.20$, $P=0.24$; Figure 4.2.2.E). However, it can be noted that there appeared to be an increase in the number of head shaking bouts, as well as a decrease in the latency to shake the head, with increasing concentrations of the odorant, although these were not significant.

The number of chicks that pecked the bead was maximal with each presentation of the undiluted concentrations of odorant (see Figure 4.2.2.B). There were no significant differences between the pecking responses to each concentration of odour (mean number: $F_r=2.63$, $P=0.45$; latency: $F_r=1.16$, $P=0.76$; Figures 4.2.2.D and 4.2.2.F).

The chicks then had the wax removed from their nostrils and were presented with 10 μl of *iso*-amyl acetate in a fifth testing trial. These data are indicated by the shaded points on Figure 4.2.2. During this trial each chick shook its head and pecked the bead at least once. The latency to shake the head appeared to decrease when the chicks were tested with unblocked nostrils although this did not reach significance (Wilcoxon signed ranks test: $z=1.68$, n=8, $P=0.09$). Unblocking the nostrils resulted in a significant increase in the number of bouts of head shaking ($z=2.20$, $P=0.03$) and a significant suppression of pecking ($z=2.52$, $P=0.01$). There was, however, no effect of unblocking the nostrils on the latency to the first peck ($z=1.12$, $P=0.26$).
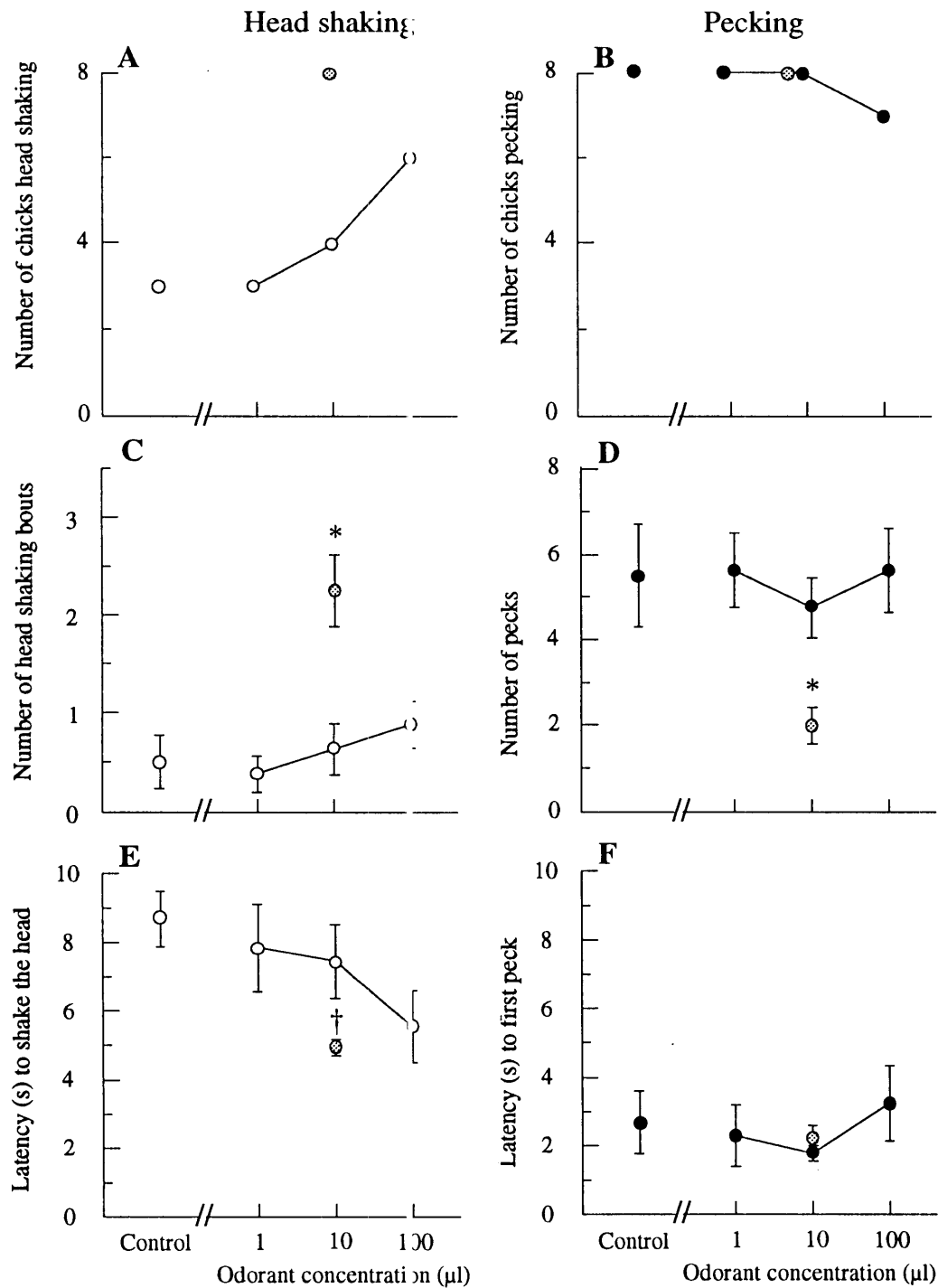
Head shaking

Pecking

Figure 4.2.2 Head shaking (left panels) and pecking (right panels) responses of chicks tested with both nostrils occluded and presented with suprathreshold concentrations of iso-amyl acetate. Scores are presented as the number of chicks shaking their heads (A), the number of chicks pecking at the bead (B), the mean number of head shaking bouts (C), the mean number of pecks (D), the mean latency to the first bout of head shaking (E) and the mean latency to peck (F). The chicks (n=8) were tested repeatedly and presented with odour concentrations in random order. The shaded symbols (⊗) indicate the same measures from the same group of chicks tested in an additional trial with their nostrils unblocked (Wilcoxon-Mann-Whitney test, n=8, † 0.10>P>0.05, * P<0.05).

Discussion

Iso-amyl acetate vapour stimulates receptors within the nasal cavity as chicks with occluded nostrils did not show concentration-dependent changes in behaviour when presented with the various concentrations of *iso*-amyl acetate. This finding supports previous studies (Tolhurst and Vince, 1976; Jones and Gentle, 1985) indicating that receptors outside the nasal cavity, such as those in the eyes or mouth, do not contribute to odour perception. However, the variance associated with the number of bouts of head shaking appeared to increase slightly with increasing concentrations of diluted odorant, as well as slight increases to undiluted odorant, suggesting that there was also some stimulation of receptors by the odorant outside the nasal cavity. It is known that odours can stimulate receptors of the ocular mucosae in humans (Moncrieff, 1955) which leads to irritating or pungent sensations (Cometto-Muñiz and Cain, 1991), although eye irritation thresholds are several orders of magnitude higher than those found for odour thresholds (Cometto-Muñiz and Cain, 1995b). The extra ocular muscles as well as the nasal and oral cavities of birds are supplied by the trigeminal nerve (Breazile and Yasuda, 1979; see Figure 1.3, page 15). Although nasal trigeminal chemoreception contributes to the perception of odours (Mason and Silver, 1983; Bang and Wenzel, 1985) it is unlikely that *iso*-amyl acetate, at the concentrations used in the present experiment, stimulated either the ocular or oral trigeminal receptors to any great degree, as the responses to this odorant were abolished when the nares were occluded. However, it had to be assumed that, in the intact animal, nasal trigeminal chemoreception was involved in the chicks' responses to an odorant.

Applying the wax preparation to the chicks' nostrils did not appear to have altered the chick's sensitivity to the single odorants. There was a three-fold increase in the number of bouts of head shaking and a suppression of pecking following the removal of the wax. Moreover, these results suggest that pecking is elicited by the visual cues alone as the latency to peck at the bead and the number of pecks were not affected by occluding the nostrils, whereas there was a suppression in the number of pecks at the bead following the removal of the wax preparation. After the chicks' nostrils were

unblocked the presentation of 10 µl of *iso*-amyl acetate elicited similar responses as those obtained from chicks that did not previously have their nares occluded. Thus, it is unlikely that occluding the chicks nostrils affects their sensitivity to odours and this formed the basis for unilateral naris occlusion reported in Chapter 6.

CONCLUSIONS

This experiment demonstrates that habituation of the pecking response is prevented by changing the colour of the bead on each trial. The lack of habituation in response to odour indicates that testing the same chicks in a series of trials, with graded concentrations of odorant, confirms similar results found in chicks tested with odorant in one trial only. Thus, by testing a chick repeatedly the number of chicks needed to be tested can be reduced, and comparisons can be made within the same animal.

There did not appear to be an effect of bead colour on the head shaking or pecking responses. The *iso*-amyl acetate vapour stimulated receptors within the nasal cavity as chicks with occluded nostrils did not show concentration-dependent changes in behaviour when presented with the various concentrations of *iso*-amyl acetate. It is, however, assumed that several intranasal chemoreceptors may be involved in the responses to an odorant. Thus, the test was suitable for screening the chicks' responses to a range different odorants and this is presented in the following chapter.