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A honeybee's ability to learn, recognize, and discriminate odors depends upon odor sampling time and concentration

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Abstract

Animals sample sensory stimuli for longer periods when they must perform difficult discrimination tasks, implying that the brain's ability to represent stimuli improves as a function of time. Though it is true in other senses, few studies have examined whether increasing sampling time improves olfactory discrimination. In the experiments reported here, we controlled odor sampling time with the goal of testing whether odor concentration affected a honeybee's ability to learn, recognize, and discriminate odors. We observed that increasing sampling time during conditioning and testing improved a honeybee's ability to learn, recognize, and differentiate low concentration (0.0002M) odors. For intermediate (0.02M) concentration odors, both acquisition and recognition improved when stimulus duration was longer, but discrimination was unaffected. Having longer to sample a high concentration (2.0M) stimulus also improved acquisition, but it did not affect the ability to recognize or differentiate odors. Differences in the time taken to respond to the conditioned and novel odors during the test period depended upon the difficulty of the discrimination task. Our results suggest that the sensory coding of molecular identity takes longer for low concentration odors.

Keywords

olfaction; honeybee; speed-accuracy trade-off; temporal coding; antennal lobe

Introduction

Measuring how long an animal or a person takes to respond during odor discrimination (Wise & Cain, 2000; Uchida & Mainen, 2003; Ditzen, Evers, & Galizia, 2003; Abraham, Spors, Carleton, Margrie, Kuner, & Schaefer, 2004) and measuring how stimulus sampling time affects accuracy in an olfactory discrimination task (Rinberg, Koulakov, & Gelperin, 2006) are methods that have been employed to examine how much time is needed by the olfactory system to encode odors. The premise of these experiments, used routinely in psychophysics (Helmholtz, 1880; Luce, 1986; Abraham, Spors, Carleton, Margrie, Kuner, & Schaefer, 2004), is simple: the amount of time taken during an olfactory discrimination task should reflect both how long it takes to encode sensory information and how long it takes to produce motor output. Any change in the time needed to perform a discrimination task should reflect whether

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the sensory system requires more time to produce differences in neural representations assuming that the time to produce motor output remains constant.

Studies that have adopted this rationale using various odor-discrimination tasks with rats, mice, and honeybees, have not reached the same conclusion about the time taken by the olfactory system to represent an odor's molecular identity, however (Uchida & Mainen, 2003; Abraham, Spors, Carleton, Margrie, Kuner, & Schaefer, 2004; Rinberg, Koulakov, & Gelperin, 2006; Friedrich, 2006). For example, experiments with rats and honeybees that measured response time during an olfactory discrimination task failed to observe differences in the amount of time taken to discriminate both monomolecular odors and binary mixtures (Uchida & Mainen, 2003; Ditzen, Evers, & Galizia, 2003). The response times of animals in these tasks were short even for difficult discrimination tasks (300 ms for rats and 690 ms for free-flying honeybees), leading to the conclusion that odor encoding requires little time (Uchida and Mainen, 2003, 2006). A similar study with mice found that mice also discriminate monomolecular odors quickly (200 ms), but that more difficult tasks such as binary mixture discrimination take longer to perform (Abraham, Spors, Carleton, Margrie, Kuner, & Schaefer, 2004). Measuring response time without controlling the stimulus sampling time could make it difficult to perceive differences in response times that depend on task difficulty since sampling-time requirements may vary across subjects, species, and by task (Friedrich, 2006). A means of overcoming this problem is to fix sampling time in discrimination tasks and then assess accuracy (Friedrich, 2006); when this approach is used, rats discriminate highly similar binary mixtures with greater accuracy if given longer to sample odors (Rinberg, Koulakov, & Gelperin, 2006), suggesting that more time is needed to encode information about small differences in odor ratios.

An odor's concentration produces differences in both spatial (Rubin and Katz, 1999; Johnson and Leon, 2000; Sachse and Galizia, 2002; Skiri, Galizia, & Mustaparta, 2003) and temporal (Stopfer, Jayaraman, & Laurent, 2003) aspects of odor coding. As odors of low concentration are more difficult to discriminate than high concentration odors (Cleland and Narla, 2003; Wright and Smith, 2004), one might expect that more time would be required to encode information about low concentration odors. Two previous studies of the speed-accuracy trade-off which incorporated concentration into their experiments (Ditzen, Evers, & Galizia, 2003; Abraham, Spors, Carleton, Margrie, Kuner, & Schaefer, 2004) came to different conclusions about whether concentration affects reaction time in olfactory discrimination tasks, however. If mice are trained to discriminate odors in a "go, no-go" task, they take longer to discriminate odors of different concentrations than they do to discriminate odors of different molecular identity or of different ratios in a binary mixture (Abraham, Spors, Carleton, Margrie, Kuner, & Schaefer, 2004). Free-flying honeybees, on the other hand, took the same amount of time to perform a discrimination task regardless of the concentration or molecular identity of odors (Ditzen, Evers, & Galizia, 2003). Neither study controlled stimulus sampling time across subjects.

The series of experiments described here were designed with the aim of testing whether the olfactory system needs more time to encode information about an odor's molecular identity when the odor's concentration is low. Making use of a method developed to condition individual restrained honeybees (Bitterman, Menzel, Fietz, & Schafer, 1983), we controlled the amount of time each subject was allowed to sample an odor stimulus by presenting odors as discrete odor pulses of defined durations. The honeybee is an excellent model system for testing hypotheses regarding the nature of the olfactory code because both its olfactory system and its ability to learn and discriminate among odors have been well studied (Menzel, 2001; Menzel & Giurfa, 2001). By measuring the latency to a honeybee's proboscis extension after test odor onset, we also examined whether response time varied as a function of odor concentration.

Materials and methods

Worker honeybees (*Apis mellifera carnica*) were collected from indoor flight room colonies. Each subject was chilled on ice, placed individually in a restraining harness, fed to repletion, and held at room temperature overnight. The next day, subjects were conditioned with a monomolecular odor as a conditioned stimulus (CS) and a 0.4 μ l droplet of 1.5 M sucrose solution as the unconditioned stimulus (US) in a classical conditioning protocol designed to produce appetitive olfactory learning (Bitterman, Menzel, Fietz, & Schafer, 1983; see Smith 1998 for details). Each subject received 12 conditioning trials presented at an inter-trial interval of 5 min. On each conditioning trial, a piezo buzzer sounded at 1 s prior to odor stimulus activation and immediately at the end of odor delivery to alert the experimenter to deliver the US. This allowed the delivery of the US to occur precisely such that it overlapped with CS delivery on each trial. If a honeybee extended its proboscis in response to the odor before the piezo buzzer sounded, the US was delivered directly to the proboscis without waiting for the second beep. Within 10 min after conditioning, subjects were tested in a random sequence with the conditioned stimulus (CS) and other odor stimuli (depending on the experiment, see below for details) using the same 5 min interval between test trials.

The odors used as conditioned stimuli in our experiments were the aliphatic alcohols, 1-hexanol and 1-octanol, and the ketone, 2-octanone, diluted from purity (Sigma-Aldrich, St. Louis, MO) in hexane to three different concentrations: 0.0002M, 0.02 M, and 2.0M. These concentrations have been used in several previous studies of honey bee odor perception (Bhagavan & Smith, 1997; Wright & Smith, 2004; Wright *et al*, 2005) in which we have established that the diluent, hexane, does not contribute more to the salience of the CS than the mechanosensory aspect of the stimulus (Wright & Smith, 2004). These two odors (1-hexanol and 2-octanone) were chosen because they elicit similar electro-antennogram dose-response curves from the honeybee's antennae (Wright and Smith, 2004). We designed our experiments with the knowledge that high concentration odors were easier for honeybees to discriminate than low concentration odors (Wright & Smith, 2004) so that we would have a predictable difficulty axis, in which task difficulty decreased when odor concentration increased. Each odor was used as a conditioned stimulus for different groups of subjects. Odor stimuli were delivered as discrete pulses of air mixed with odor. Five μ l of an odor solution were applied to a strip of filter paper, which was inserted into a modified, 1 ml glass syringe. We used one glass syringe per subject and changed each syringe out on every trial. To ensure that the concentration of the CS remained constant from trial to trial, we replaced the entire set of syringes with new syringes after using each for 4 trials based on our previous work which showed that the concentration of the CS was substantially depleted in the low and intermediate concentrations after 8 trials with a 4 s stimulus (Wright & Smith, 2004). Each syringe was discharged once prior to its use as a CS. Using this method, we expected that the concentration of the odor presented during an odor pulse was standardized across animals and that each stimulus' concentration remained constant for the duration of the pulse.

The syringe was positioned approximately 3 cm in front of a subject and was attached to a 3-way valve (LEE Co.) which directed airflow through the syringe. When triggered by a programmable controller (Automation-Direct), the air stream (40ml/s) was diverted via the valve through the odor syringe for a set period of time that ranged from 200 ms through 2 s (see below). Using this apparatus, a discrete odor pulse could be directed at the subject's antennae and then quickly exhausted from the conditioning arena. At the flow rate of the air stream (40ml/s), the headspace within the syringe was replaced 8 times with clean air for a 200 ms stimulus and, for a 2 s stimulus, was replaced 80 times.

Behavioral response measures

On every acquisition trial, the response of each subject was recorded as a positive response if the subject extended its proboscis during odor stimulation and before the sucrose US presentation. These data are plotted as the percentage of subjects that responded on each trial. Proboscis extension was recorded as a binary variable - extension response or not - during the test trials. A 20 s period of each test trial was also recorded using a digital camcorder for offline quantification of the latency to proboscis extension. If a subject did not respond within the 20 s period, we recorded a negative response to the test odor. We defined latency to proboscis extension as the time from the air valve opening to produce the olfactory stimulus to the first frame of full proboscis extension.

Experiment 1: Effect of sampling time and odor concentration on learning and stimulus recognition

Here, we conditioned honeybees with one of two odor stimulus durations (200 ms and 1000 ms) to examine how sampling time and odor concentration affected a honeybee's ability to learn and to recognize the conditioned odor stimulus. Each subject was conditioned to one of three different concentrations: low (0.0002 M), intermediate (0.02 M), and high (2.0 M). The combination of two stimulus durations and three concentrations produced six different groups of subjects. Three different odors (hexanol, octanol and 2-octanone) were counterbalanced as the conditioned stimulus. We predicted that decreasing odor concentration and shortening the duration would make odor stimuli more difficult to learn to associate with reward during conditioning and also more difficult to recognize during the test period. To examine odor recognition, each subject in the six different treatment groups was tested with the conditioned odor at the same concentration experienced during conditioning. Four tests were presented in random order with four different stimulus durations (200 ms, 500 ms, 800 ms, 1000 ms) on separate trials. This range of stimulus durations corresponds to the series of stimuli examined in an electrophysiological study of the locust olfactory system by Mazor & Laurent (2006).

Experiment 2: Effect of sampling time and odor concentration on discrimination and response latency

In this experiment, we examined how stimulus duration affected the ability of honeybees to recognize the conditioned stimulus relative to other, novel odors of the same concentration. We also examined whether the response time (latency to proboscis extension) varied as a function of difficulty of the discrimination task. For each group of subjects, we presented odor stimuli throughout conditioning at one of the three concentrations: 0.0002 M (low), 0.02 M (intermediate), or 2.0 M (high). Three different odors (hexanol, octanol and 2-octanone) were counterbalanced as the conditioned stimulus. Within each concentration treatment, different subgroups were conditioned at each of the following durations: 200 ms; 500 ms; 800 ms; 1000 ms; or 2000 ms. This produced a total of 15 independent treatment groups. After conditioning we tested each subject with each of the odors (1-hexanol, 1-octanol, and 2-octanone) at the same duration and concentration as the conditioned odor. The sequence of test odor presentation was randomized across subjects to minimize variability due to extinction effects.

Statistical analyses—We used one behavioral measure (proboscis extension) during conditioning and three behavioral measures (proboscis extension, latency to proboscis extension, and proboscis extension duration) during the test conditions. Proboscis extension was scored as a binary variable (yes or no) and was, therefore, analyzed using logistic regression (lreg). Multiple comparisons (mc) were performed using a least squares contrast in PROC GENMOD in the statistical software, SAS. Latency to proboscis extension was analyzed using a generalized linear model (glm) with a gamma distribution function in SAS. Specific pairwise comparisons were made using a Mann-Whitney (MW) test. Latency to proboscis

extension was only measured from subjects that responded during the test; subjects that did not respond were not included in the analysis.

Results

Sampling time and concentration affect the rate of olfactory learning

The data from the conditioning trials performed during Experiment 1 clearly show that the rate of acquisition (as measured during the first six conditioning trials) depended both on the duration of the stimulus and on stimulus concentration (Figure 1, lreg, 3-way interaction: $\chi^2_2 = 7.03$, $P = 0.030$). High concentration odors, in general, were learned at a faster rate than low concentration odors (lreg, 2-way interaction: $\chi^2_1 = 84.5$, $P < 0.001$). The rate of learning was also slower for short duration stimuli (200 ms) than for long duration stimuli (1000 ms) (lreg, 2-way interaction: $\chi^2_1 = 4.43$, $P = 0.034$). Subjects conditioned with short-duration, low (0.0002 M, Figure 1 A) or intermediate (0.02 M, Figure 1B) concentration stimuli achieved a lower asymptotic level of association during conditioning than those conditioned with long duration stimuli (low concentration, lreg, main effect, $\chi^2_1 = 10.9$, $P < 0.001$; intermediate concentration, lreg, main effect, $\chi^2_1 = 16.5$, $P < 0.001$). (The asymptotic level of association is the point at which the probability of producing conditioned responding in our subjects ceased to change during conditioning. A lower asymptote indicates that this task was much more difficult and that the association between odor and reward formed during conditioning was weaker (Mackintosh, 1974)). The rate of acquisition for the subjects conditioned with high-concentration short-duration stimuli was slower than for those conditioned with high-concentration long-duration stimuli (lreg, 2-way interaction, $\chi^2_1 = 7.20$, $P = 0.007$), but these subjects were still able to reach the same asymptotic level of association by the 7th conditioning trial (mc, $P = 0.135$). The odor used as the conditioning stimulus did not affect acquisition of the learned association (lreg, main effect, $\chi^2_1 = 16.5$, $P = 0.184$).

Longer sampling times and high odor concentrations improve stimulus recognition

Experiment 1 measured how stimulus duration during the test period affected a honeybee's ability to recognize the conditioned odor stimulus. The responses to each concentration are plotted separately for the short (200 ms, Fig. 2A) and long (1000 ms, Fig. 2B) stimuli for better resolution of the influence of concentration on the average probability of responding. If a honeybee had been conditioned with a low or intermediate concentration odor of short stimulus duration, it was less likely to respond on average to all the test stimuli than if it had been conditioned with a long duration or a high concentration stimulus (Figure 2, 2-way interaction, lreg: $\chi^2_2 = 6.98$, $P = 0.031$). We compared how test stimulus duration affected the responses of the honeybees conditioned with the low and intermediate concentration separately from those conditioned with the high concentration because of the difference in the average rate of responding of the subjects conditioned with the high concentration stimulus. For the subjects conditioned with the low and intermediate concentration stimuli of both short and long duration, the ability to recognize the odor improved as a function of the test odor sampling time (stimulus duration of test odor) (main effect, lreg: $\chi^2_3 = 9.65$, $P = 0.022$). The probability of responding was on average higher if our subjects had been conditioned with a long duration stimulus (main effect, lreg: $\chi^2_1 = 11.0$, $P = 0.001$). In contrast, the responses of subjects conditioned with the high concentration were unaffected by either the duration of either the conditioning stimulus (main effect, lreg: $\chi^2_1 = 1.74$, $P = 0.187$) or the test stimulus (main effect, lreg: $\chi^2_3 = 0.88$, $P = 0.843$). The odor used during conditioning and testing did not affect a honeybee's response during the test (lreg, main effect: $\chi^2_2 = 0.77$, $P = 0.381$).

Low concentration, short-duration stimuli are harder to discriminate

Previously, we reported that a honeybee's ability to distinguish odors of different molecular identities was strongly affected by odor concentration such that low concentration odors were

harder to distinguish (Wright & Smith, 2004). In Experiment 2, we expected to observe that stimulus duration (sampling time) would have the strongest effect on a honeybee's ability to distinguish low concentration odors of different molecular identities. Indeed, we observed that a honeybee's ability to differentiate the conditioned odor (CO) from the other test odors (DO) improved when the concentration of the test odors increased (Fig. 3 A,B,C: lreg, 2-way: $\chi^2_2 = 13.7$, $P = 0.001$). Because of the strong effect of concentration on the responses during the test, we chose to compare the responses of subjects conditioned with different stimulus durations for each of the concentrations separately. The responses of subjects conditioned with the low concentration (Fig. 3A) depended on stimulus duration (lreg, 2-way interaction, $\chi^2_1 = 7.43$, $P = 0.006$). These subjects were only able to discriminate the CO from the DO when the stimulus duration was 2000 ms long: for all other sampling times, honeybees could not discriminate the CO from the DO ($P > 0.05$ for all 4 comparisons). The response during the test did not depend upon stimulus duration for the honeybees conditioned with the intermediate (Fig. 3B) and high (Fig. 3C) concentrations, however, as all subjects could discriminate the CO from the DO for each of the different stimulus durations ($P < 0.05$ for all 10 comparisons).

As we observed in the previous experiment (Fig. 2), stimulus duration and odor concentration also affected the average probability that our subjects would respond to any of the test stimuli (Fig. 3). Subjects conditioned with short duration, low concentration stimuli had a lower probability on average of responding to any odor during the test compared to subjects conditioned with long duration, high concentration stimuli (lreg, 2-way: $\chi^2_8 = 15.6$, $P = 0.048$). Sampling times (stimulus durations) of less than 2000 ms elicited a significantly lower probability of responding if our subjects were conditioned with the low concentration stimuli (0.0002M) (lsc, for all 4 comparisons: $P < 0.001$). For the intermediate and high concentration odors, stimulus duration did not affect the average probability of eliciting a response during the test ($P > 0.05$). As in the previous experiment, the responses during the test depended only on the relative difference between the conditioned odor and the different odors, but not on which odor was used as the CS (lreg, 2-way: $\chi^2_2 = 1.07$, $P = 0.587$).

Honeybees respond faster to the conditioned odor than a different, novel odor

In Experiment 2, we used the latency to proboscis extension as a direct measure of the response time of a honeybee towards an odor during the test period. On average, the latency towards the CO was significantly shorter than the response latency to the DO (540 ms vs. 940 ms, Figure 4) (glm, main effect: $\chi^2_1 = 4.69$, $P = 0.030$), but it also depended upon the stimulus duration (glm, 2-way: $\chi^2_2 = 7.11$, $P = 0.029$) and on odor concentration (glm, 2-way: $\chi^2_2 = 21.9$, $P < 0.001$). For this reason, we compared the latency of the response to the CO to the DO for each of the different combinations of stimulus duration and concentration (15 in total). We observed that the average response to the CO was significantly faster (reflected in a shorter latency) than the response to the DO in only 3 out of the 15 cases. This occurred for the 2000 ms, low concentration stimuli (Fig. 4A) (MW, $z = -2.3$, $P = 0.022$), for the 200ms, intermediate concentration stimuli (Fig. 4B) (MW, $z = -2.8$, $P = 0.006$), and for the 200ms, high concentration stimuli (Fig. 4C) (MW, $z = -2.7$, $P = 0.006$). These three conditions were situations where we observed that the ability to distinguish the odors was difficult, but possible for a honeybee to perform (Figure 3). For these three conditions, subjects conditioned with high and intermediate concentration odors responded faster to the CO (high = 431 ms, intermediate = 401 ms) than subjects conditioned with low concentration odors (571ms), and the difference in the response time towards the CO vs the DO was greatest for the subjects conditioned with the high concentration odor (Fig. 4, glm, 2-way: $\chi^2_2 = 6.00$, $P = 0.049$). While a trend for a shorter latency towards the CO was observed for the other comparisons besides these three conditions, none of the comparisons were significantly different (MW: $P > 0.05$).

Discussion

Our results show that sampling time affects olfactory learning, recognition, and discrimination, suggesting that having longer to sample an odor improves the ability of the olfactory system to form a neural representation of an odor's molecular identity. This was especially true for the most difficult tasks in our experiments, those involving low-concentration odors. We also observed that the latency to proboscis extension, or the amount of time each subject took to decide to respond to a test odor, was faster towards the conditioned odor than the novel odor only for a subset of the experimental conditions, specifically, the most difficult (yet still achievable) odor-discrimination tasks at each odor concentration. For this same subset of difficult, achievable tasks, the reaction time towards the low concentration odors took longer than towards the intermediate or high concentration odors.

A longer stimulus yields more information

The fact that honeybees improved their ability to learn, recognize, and discriminate odors in our experiments when odor stimuli were of a longer duration implies that having longer to sample an odor stimulus yields more information about an odor's presence and its molecular identity. Even after a honeybee had mastered the task of associating an odor with reward during conditioning, its ability to recognize an odor during the test period improved when it was presented with an odor of longer stimulus duration. Our results support the hypothesis that the amount of information gained by the honeybee's olfactory system about an odor's molecular identity *increases* as a function of sampling time. Stimulus duration could potentially affect how the olfactory system represents odors in at least two ways: 1) if odor sampling time was too short, it could affect the ability of the olfactory receptor neurons (ORNs) to produce responses to odors; or, 2) stimulus sampling time could affect the ability of the honeybee's antennal lobe (AL) to represent information, if having more time meant producing a better representation of the odor stimulus.

It is unlikely that our stimuli were too short in duration to compromise the ability of a honeybee's ORNs to respond to the CS, as our subjects were able to learn to associate the CS with reward even for the shortest duration stimuli. When ORNs respond to odor, they typically produce a characteristic increase in firing rate which scales linearly with respect to odor concentration (Duchamp-Viret, Duchamp, & Chapu, 2000; de Bruyne, Foster, & Carlson, 2001). This increase in spike rate reaches a plateau approximately 50-150 ms after odor onset in honeybee (Getz & Akers, 1992), cockroach ORNs (Getz & Akers, 1997a,b; Getz, 1999), and *Drosophila* ORNs (de Bruyne, Foster, & Carlson, 2001). In the lobster, ORN responses peak 160-300 ms after odor onset even for concentrations at the limits of an animal's detection threshold (Gomez & Atema, 1995). As the spike rate does not scale to the duration of the stimulus (i.e. longer duration stimuli low concentration stimuli do not produce a higher spike rate), it is unlikely that increasing stimulus duration affects how ORNs encode odor concentration, as long as the duration is sufficient for the ORNs to plateau (e.g. 50-150ms). In fact, a modeling study suggests that lengthening the duration of the odor stimulus after this peak period does not add more information to the rate code produced by insect ORNs (Getz, 1999). Our data supports this idea, as honeybees were able to learn to associate an odor with reward even in the case where the least information was transmitted in our experiments - a 200 ms low concentration stimulus - suggesting that enough information was transmitted via the ORNs for a honeybee to recognize and respond to the 200 ms stimulus from trial to trial. It is possible in this task that their recognition of the CS was also based on a honeybee's ability to learn the mechanical aspect of the air pulse in which the odor was delivered (Wright & Smith, 2004); how much each contributed to a honeybee's ability to learn this task is unclear in our study, however.

Another possible explanation is that allowing a honeybee to have more time to sample an odor stimulus could also improve the AL's ability to produce a neural representation for the stimulus. While we could not find any reports measuring the spatial pattern of response in the AL or olfactory bulb (OB, vertebrates) to odors of varying stimulus duration, electrophysiological recordings of the slow temporal patterns of projection neurons in the locust AL have shown that stimulus duration influences the ability of the projection neurons to encode olfactory information (Brown, Joseph & Stopfer 2005; Mazor & Laurent, 2005). For example, when information input to the locust AL is limited either by shortening sampling time or lowering odor concentration, the spiking activity of projection neurons synchronizes for a shorter time and exhibits less coherence (Stopfer, Jayaraman, & Laurent, 2003; Mazor & Laurent, 2005). Stimulation with low concentration odors decreases the amplitude of the local field potentials produced by projection neuron activity and reduces the duration of synchronization of projection neurons after odor onset (Stopfer, Jayaraman, & Laurent, 2003). Furthermore, while even stimuli of 300 ms duration can be correctly classified within 300 ms after odor onset, increasing stimulus duration enhances the length of time that the AL can correctly classify stimuli when odors are presented in the range of 300-1000 ms in duration (Mazor & Laurent, 2005 - note: the concentration used by these authors is in the range of our intermediate/high concentration odors).

Task difficulty is key to revealing differences in speed of response times

Response-time experiments are the classic psychophysical paradigm for revealing the time course of neural coding (Luce, 1986), and as a consequence, most studies of the speed-accuracy trade-off in olfaction have relied on measuring response time as a means of estimating indirectly how long the olfactory system must sample an odor in order to be able to discriminate it (Uchida & Mainen, 2003; Ditzen, Evers, & Galizia, 2003; Abraham, Spors, Carleton, Margrie, Kuner, & Schaefer, 2004). As a behavioral measure, response time is an integration of stimulus sampling time, higher level brain processing time, and motor output. Whilst, as we discussed above, there are good reasons for preferring to control the sampling time, we did also measure the latency-to-response during the discrimination test period in Experiment 2. We found that under the circumstances where we observed differences amongst response times, honeybees took longer to respond to the CO when the discrimination task was harder (e.g. when the odor was of a low concentration), a result also observed in olfactory discrimination tasks with mice (Abraham, Spors, Carleton, Margrie, Kuner, & Schaefer, 2004). However, our experiments also show that the ability of our experiments to reveal differences in response times depended upon whether the task was at the limits of a honeybee's performance. For example, honeybees responded faster to the CO than to the DO during the test period only when the discrimination task was difficult but still just possible: there was no difference in the response time to CO versus DO when the discrimination task was too difficult (i.e. when our subjects could not differentiate the odors as assayed by the probability of proboscis extension), and likewise, if the task was easy for a honeybee to perform (e.g. for intermediate- and high-concentration odors of a duration longer than 200 ms), there was no difference in response time to the CO versus DO.

In studies of odor perception, the methods employed to understand how stimulus parameters influence the ability of the olfactory system to encode information are often key to the inferences generated about olfactory coding. For example, Linster, Johnson, Yu, Morse, Xu, Hincó, Choi, Choi, Messiha, & Leon (2001) found that rats could perceive differences in enantiomers when they were subjected to a differential habituation paradigm which allowed them to gain more experience with the odors they were asked to discriminate. This is in contrast to previous authors (Rubin and Katz, 1999) who were unable to show that rats could differentiate these compounds using appetitive conditioning in a 'go-no go' task. Comparison of our experiments with previous studies of the speed-accuracy trade-off also shows that the

method used to study how sampling time affects discrimination also affects the results obtained. Another study which used free-flying honeybees found that odor similarity and concentration did not affect the time taken by honeybees to discriminate odors (Ditzen, Evers, & Galizia, 2003); the discrepancies between our experiments and those of Ditzen, Evers, & Galizia (2003) can be explained by differences in the methods employed. In our experiments, we carefully controlled the stimulus presentation in order to expose our subjects to a discrete, defined sampling period. Ditzen, Evers, & Galizia (2003) could not control the sampling time of individual subjects because their subjects were free to choose which odors to sample and where to land.

Our results also imply that failing to observe a difference in response times with respect to task difficulty (as in, for example, the experiments of Uchida & Mainen (2003) and Ditzen, Evers, & Galizia (2003)) may be a consequence of constructing an olfactory discrimination task that is too easy for well-trained animals to perform. Response times can be affected by the amount by experience a subject has with visual stimuli (Gilbert, Sigman, & Crist, 2001), and this could also be true in olfaction. Olfactory perceptual learning, or changes in the way that the brain responds to odors as a function of experience (Gilbert, Sigman, & Crist, 2001; Davis, 2005), has been reported in the AL/OB of locusts (Stopfer & Laurent, 1999) honeybees (Faber, Joerges, & Menzel, 1999), moths (Daly, Christensen, Lei, Smith, & Hildebrand, 2004b), fruit flies (Yu, Ponomarev, & Davis, 2004; Sachse, Rueckert, Keller, Okada, Tanaka, Ito, & Vosshall, 2007), and rats (Ravel, Chabaud, Martin, Gaveau, Hugues, Tallon-Baudry, Bertrand, & Gervais, 2003; Martin, Gervais, Hugues, Messaoudi, & Ravel, 2004). Perceptual learning would make it possible for animals to improve their ability to recognize stimuli, and this would translate into reduced response times during olfactory discrimination tasks in a manner that would clearly depend upon the amount of training an animal received (Gibson, 1953; Gilbert, Sigman, & Crist, 2001). It might also mean that differences in discrimination times for “easy” versus “hard” tasks could diminish with training, with the possible eventual outcome that little or no difference is observed amongst response times.

Conclusions

The honeybee's olfactory system can render an odor and allow it to produce a motor reflex (proboscis extension) towards a conditioned odor within 430 ms after odor onset when it has experienced appetitive conditioning with this odor for 12 trials. This is in the range of what has been reported for rats (Uchida & Mainen, 2003) and mice (Abraham, Spors, Carleton, Margrie, Kuner, & Schaefer, 2004) when time of odor travel and motor output are subtracted from the final measured response time. We expect that this response time reflects the performance of the olfactory system when it has been tuned to its optimum performance as a result of previous experience such as appetitive conditioning. We expect that future studies which address how experience affects response time are likely to reveal that conditioning improves both an animal's response time and changes an odor representation in the olfactory system to facilitate odor encoding (Gilbert, Sigman, & Crist, 2001; Davis, 2005).

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Figure 1.

Odors of a short (200 ms) stimulus duration were more difficult to learn to associate with reward than odor stimuli of a longer duration (1000 ms). Honeybees were conditioned with one of a low (A, 0.0002M), intermediate (B, 0.02M), or high (C, 2.0M) concentration stimulus of either a 200 ms (dashed lines) or 1000 ms (solid lines) duration. For subjects conditioned with odors of a low (A) or intermediate (B) concentration the final asymptotic strength of the association (measured as the point at which the probability ceases to change) was lower for a short duration stimulus. Subjects conditioned with a high concentration, short duration stimulus exhibited a slower rate of acquisition but attained the same asymptotic level of association as those conditioned with a long duration stimulus (C). Low concentration stimuli: $N_{\text{short}} = 44$, $N_{\text{long}} =$

29; intermediate concentration stimuli: $N_{\text{short}} = 50$, $N_{\text{long}} = 30$; high concentration stimuli: $N_{\text{short}} = 30$, $N_{\text{long}} = 37$.

Figure 2.

When a honeybee has more time to sample an odor, its ability to recognize an odor improves. The probability of responding to the conditioned odor on the final trial of the conditioning period is plotted as the leftmost point on the x-axis. Honeybees were conditioned with a low (0.0002M), intermediate (0.02M), or high (2.0M) concentration stimulus of either 200 ms (A) or 1000 ms (B) in duration. The high concentration odors elicited a significantly greater average response during the test than the low (mc, $P < 0.001$) or intermediate (mc, $P < 0.001$) concentration odors. The average response to the low and intermediate concentration odors was not significantly different (mc, $P = 0.201$). For subjects conditioned with the low and intermediate concentration stimuli, recognition of the conditioned odor improved when the duration of the test stimulus increased. Subjects conditioned with the high concentration odor responded with a high probability to all the test odors, regardless of whether they had been trained with a 200 ms or a 1000 ms stimulus. Short duration stimuli: $N_{\text{low}} = 44$, $N_{\text{int}} = 50$, $N_{\text{high}} = 30$. Long duration stimuli: $N_{\text{low}} = 29$, $N_{\text{int}} = 30$, $N_{\text{high}} = 37$.

Figure 3.

The effect of sampling time (stimulus duration) on odor discrimination depends on an odor's concentration. Honeybees were conditioned with one of a low (0.0002M, A), intermediate (0.02M, B), or high (2.0M, C) concentration stimulus at one of 5 different stimulus durations (x-axis) and then tested with the conditioned odor (CO) and two other odors (DO) of the same concentration and stimulus duration. Stimulus duration had a strong effect a honeybee's ability to distinguish low concentration odors. Subjects conditioned with the intermediate and high concentration stimuli readily distinguished the CO from the DO regardless of the stimulus duration. The number of subjects for each experiment is represented by the numeric label at the base of the white bar.

Figure 4.

Latency from odor onset to proboscis extension was only shorter for the conditioned odor (CO) than the different odors (DO) during difficult, but possible discrimination tasks (* = $P < 0.05$) (A = 0.0002M, B = 0.02M, C = 2.0M). “Possible” discrimination tasks are defined by whether the probability of responding was significantly different towards the CO than the DO as in Figure 3. Latency was measured as the time from its resting position to fully extended when a honeybee responded to a test odor. The data presented here are the latency to proboscis extensions for the subjects depicted in Figure 3; however, non-responses (i.e. instances when a honeybee did not extend its proboscis towards an odor stimulus) were not included in the analysis.