CHAPTER 6

A computerized odor delivery system for arbitrary time-varying concentrations and mixtures

6.1 Abstract

The behavior of a sensory system is only as rich as the set of stimuli it is faced with. Sensory physiologists are thus faced with the challenge of generating a set of stimuli as rich as possible in a controllable manner. As compared to vision and hearing, where computer screens and synthesizers provide great control and flexibility, the study of olfaction has suffered from a relative lack of flexible odor delivery systems. Here, I present a computerized odor delivery system capable of delivering arbitrary concentrations controlled in real time, binary mixtures in arbitrary ratios, and the potential to deliver arbitrary discrete or continuous stimulus waveforms.

6.2 The need for a novel odor delivery system: Features

There have been many successive improvements in the design of odor delivery systems (Tucker, 1963; Kauer, 1974; Dravnieks, 1975 and references therein; Kauer and Shepherd, 1975; Meredith, 1986; Vigouroux et al., 1988; Perritt et al., 1993) –often called olfactometers rather than olfactogens, a nomenclature that appears no more appropriate than calling a computer screen a photometer. For the purpose of our experiments, though, none of them possessed all of the features we required. In addition, our system is relatively inexpensive, particularly for users who already have a computer.

Concentration in liquid does not equate concentration in vapor

Some, though not all (see references above), of the odor delivery systems in use control the concentration delivered only indirectly, by selecting among flasks each of which has a liquid solution of the odorant in a solvent, such as mineral oil, at a different concentration. While varying the concentration in the solution certainly changes the concentration of the vapor in equilibrium above it, the dependence is not quite straightforward, and depends both on the volatility of the odorant and the nature of solute-solvent interactions. Indeed, varying the concentration in a solution can change the concentration in the vapor with a log linear relationship or a more complex one, depending on the solvent used (Brockerhoff and Grant, 1999). Changing the concentration in the solution by tenfold can change the concentration in the vapor by 1500-fold. The situation is even less desirable when the odor solution is placed on a filter paper, since the filter paper acts as a chromatographic column to some degree, separating solute from solvent, and thus making the concentration of the odor in the vapor phase more independent of the quantity of odorant introduced, to the extreme that, if separation is complete, the concentration of the odor in the vapor phase will be the odorant's vapor pressure, independently of the quantity of odorant introduced[1]. For direct control of the concentration in the vapor phase, and especially for its quantification, gaseous dilution is preferable over liquid dilution.

Short-term plasticity mandates repeatability across trials

The concentrations delivered by systems which vary the concentration of a solution in filter paper are notoriously time dependent (Brockerhoff and Grant, 1999). The recent discovery that odor responses in the antennal lobe of the locust undergo plasticity in the timescale of a few trials (Stopfer and Laurent, 1999) requires, for the study of such a system, a delivery system known to present repeatable stimuli across trials. In order to achieve that, the system must achieve a steady state before delivery begins, a property shared only by continuous flow systems.

The capability to deliver arbitrary concentrations

Given that we were interested in the coding of odors at varying concentrations, control of the con-

1. As long as the quantity is enough to ensure the headspace of the container can be saturated in odor.

centration was a critical requirement of our system. A majority of previous studies had focused on the *differences* in neuronal responses for different concentrations. Given that one of our interests lies in understanding how the olfactory system achieves *invariance* to concentration, we were especially interested in the ability to vary concentration continuously until we encountered a change in a neuron's response. Many of the odor delivery systems previously described, however, allow only a few discrete concentration steps. The system described here allows dilution to virtually any concentration value in between the minimum and maximum allowed, its resolution limited by the computer's ability to control voltage: a 12-bit card then allows control to better than 1/1000th of the dynamic range, and a 16-bit card provides for better than 1/16,000th. As described below, this ability to deliver similar yet distinct concentrations turned out to be critical for the discovery of abrupt transitions in neuronal responses to concentration.

Real-time online stimulus choice

Related to the ability to delivery arbitrary concentrations was our need to select the concentrations online during an experiment. Thus, while a simple system consisting of delivering the air above one of several odorant flasks pre-diluted in the liquid phase allows the selection of concentrations as close or as different as desired, the choice of concentrations must usually be done *before* the experiment begins. In finding an olfactory threshold or an abrupt transition in response, though, it is paramount to be able to adjust the concentration steps dynamically during the experiment. Controlling the stimulus using a computer allowed for real-time stimulus control.

Stationary vs. non-stationary flow

One of the problems with the simple odor delivery systems used in many previous studies of olfaction is that the pulsed (discontinuous) nature of the flow through the odorant flasks, combined with the small volume of the flask, cause the odor concentration during even short, 1-second-long pulses non-stationary. This occurs because in the period between odor pulses, the air in the flask reaches equilibrium (in the case of a pure liquid odorant, it becomes saturated with odor), but because the volume of odor delivered during a pulse is greater than the capacity of the flask, the initial phase of high concentration odor is followed by a subsequent phase of a lower concentration, whose concentration is determined not by thermodynamics but rather by the dynamics of a process out of equilibrium. This introduces an uncontrolled and unmeasured temporal dimension to the stimulus which can confound the origin of temporal patterns in neuronal responses.

Our system solves this problem by reaching a steady-state flow before the beginning of the first pulse and by bubbling incoming air through tall enough columns of liquid odorant so that the air emerges saturated in odor regardless of the amount of time since the previous pulse.

The composition of the vapor of solutions of mixtures is not stable over time or concentration

Because more volatile components evaporate more rapidly than less volatile ones, solutions of mixtures produce varying headspace compositions depending on the concentration of the solution and the length of time during which the components of the mixture are allowed to evaporate (Brockerhoff and Grant, 1999). At high concentrations, the composition of the headspace above the solution was almost identical to that of the solution, but as the concentration of the solution decreased, the proportions of the more volatile compounds decreased, until only the least volatile component was left, the more volatile compounds having evaporated soon after the solution was made.

The system presented here avoids these problems; because each odorant is kept undiluted and in large volumes, and the headspace of each odorant is allowed to reach saturation to ensure its composition is identical to that of the source.

Long-term stability in concentration delivered

Finally, previous systems that utilize a liquid dilution on a filter paper have the additional disadvantage that they lack long-term stability in the concentration delivered, given that the odorant quantities are relatively small compared to the amount delivered in a single series of pulses and that the fact that they operate out of equilibrium makes odorant quantity a factor in the concentration reached in the finite amount of time of a puff. This probably causes a slow decline in the concentration which accumulates over trials, an effect that is particularly harmful when studying plasticity with a relatively slow timecourse (see Chapter 9, for example).

Ensuring that the height of the column of liquid odorant is kept well above that required for saturation, combined with the large quantities of odorant used in each of our bubblers, achieves a constant concentration independent of the exact amount of odorant in the bubbler at any point in time. In other words, the concentration of the odorant coming out of each of our bubblers (see below) depends on a thermodynamic equilibrium rather than being dependent on the length of time for which the air stream has been allowed to be in contact with the odorant.

6.3 Design

Mechanical artifact prevention

A diagram of our odor delivery system is shown in Fig. 6.1. Air at a pressure of approximately 30 PSI is filtered with a charcoal filter and, if desired, dried by passage through anhydrous calcium sulphate (Drierite, Xenia, OH). This air is then separated into three streams. One of the streams provides pure air, at a flow rate equal to that of the odor-carrying stream (see below), to the animal in between odor pulses, ensuring that the animal is exposed to constant air flow. An electromagnetic

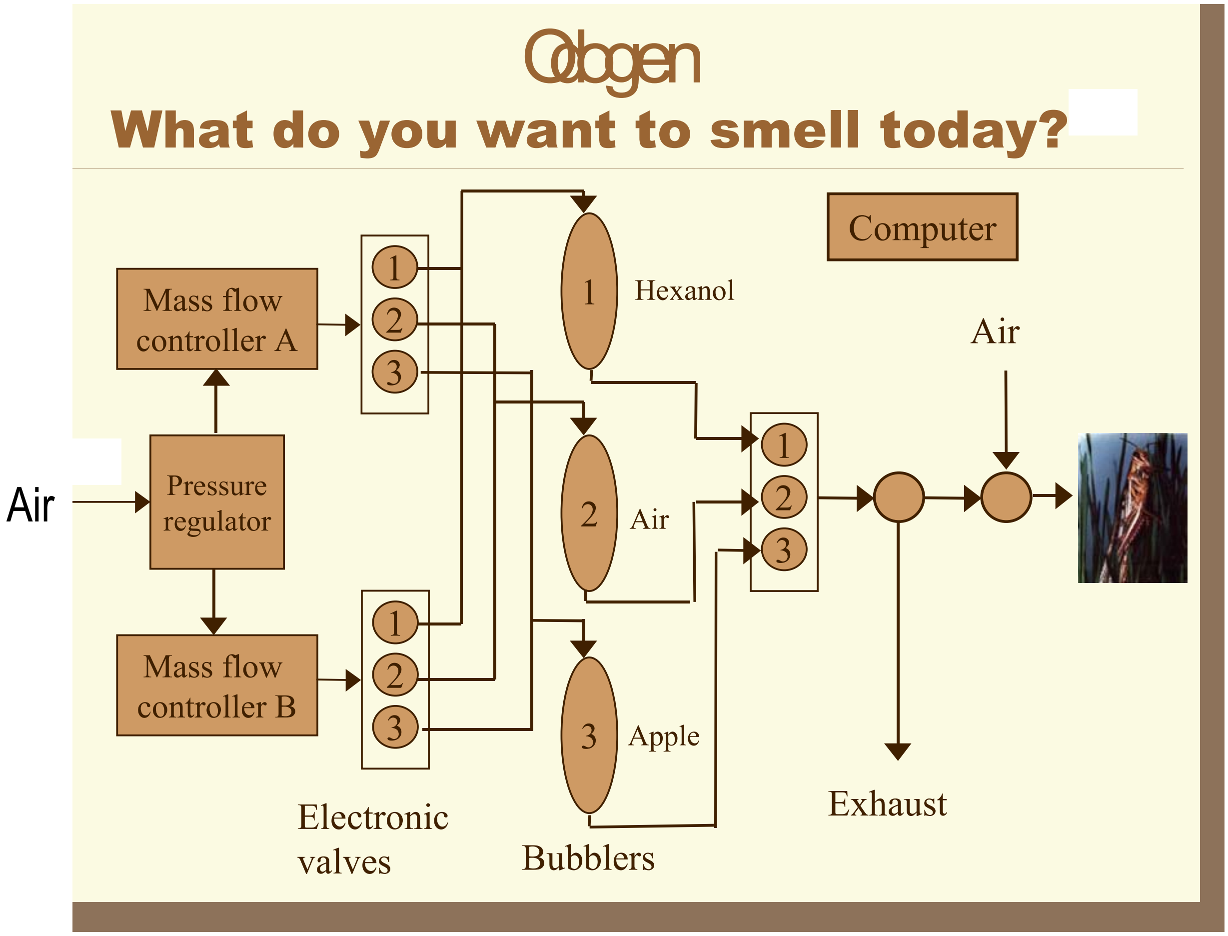

Figure 6.1. Diagram of the odor delivery system. For simplicity, only 2 of 7 odors are shown.

valve (Pneutronics or Clippard) switches between this stream and the odor-carrying stream. This eliminates the mechanosensory component of odor puffs[1].

Gaseous dilution to generate arbitrary concentrations of one of several pure odors or a combinatorial diversity of binary mixtures

The other two air streams each go through a separate mass flow controller (MFC) (Pneutronics divi-

1. A brief and smaller mechanical stimulus may persist due to the switching time of the valve switching between the stream carrying odor and that carrying air. This was reduced by placing a widening nozzle downstream of the valve to low-pass filter the stream reaching the animal.

sion of Parker Hannifin, Hollis, New Hampshire; or Unit Instruments, Kinetics division of USFilter, Dublin, Ireland), calibrated for flows between 0 and 1 liter per minute (lpm). We have verified their accuracy to at least 1 part in 100 of full scale (Figs. 6.6-6.7). Flows of less than approximately 0.02 lpm, though, are limited by inability of the air flow to form bubbles at the bottom of the bubbler under the weight of the liquid odorant column (see below). Each MFC works by setting the desired flow rate using an input voltage, supplied by the computer, and then using a feedback loop to adjust the size of an orifice within until the flow, defined as the mass flowing per unit time, measured within the MFC, equals the desired flow rate.

After exiting the MFCs, each of the air streams enters a separate diverting manifold which consists of eight electronic valves (Pneutronics or Clippard Minimatic, Cincinnati, Ohio), each of which leads to a bubbler with a different odor –one of the bubblers is empty to allow the use of pure air as one of the components of the binary mixture to ensue. The system can readily be expanded to accommodate more than seven odor components by adding bubblers and replacing the manifolds with larger ones consisting of more valves. Of the eight valves in each manifold, one is open at any given time to determine the odor that the corresponding air stream will carry.

Each of the eight bubblers was made out of glass (Rick Gerhart, Caltech Glassblowing Shop) and consists of a cylinder of ~1.5 cm diameter and ~40 cm height, with a sphere of ~5 cm diameter at the top. The bubblers are filled with undiluted liquid odorant up to a level that ensures that the odorant fills the bubbler during flow without overflowing. The air stream enters the bubbler at the bottom of the liquid column of odorant and exits it at the top, saturated in the odor. The height of the bubblers was designed so that it exceeds the height of odorant required for saturation of the air (Christine Chee-Ruiter, personal communication; Brett Doleman and Eric Severin, personal communication). This height can be measured by mass loss experiments, in which the bubbler is weighed at periodic intervals of time, increasing the height of the odorant column at the end of each interval until the mass loss during the constant intervals ceases to increase with the height of the column, demonstrating that additional fluid height no longer contributes to increased concentration of the odor in the outgoing air stream due to saturation.

Upon exiting the bubbler[1], the stream of odorized air travels through Teflon tubing to prevent absorption of the odor by the tubing walls. As an additional measure to reduce purging time, the distance between the odor selector and the target was minimized. The two streams, each with a different odor or with air and each flowing at a potentially different rate, enter a selector manifold of eight valves with eight inputs –one from each bubbler— and one output. This output leads to a glass mixer (Caltech Glassblowing Shop), where both streams are fully mixed.

In our experiments, the two flow rates are regulated so that their sum remains constant at all times, ensuring the flow of air to the animal remains at a constant rate despite variations in its odor content. The proportion of the total flow going through each of the two streams regulates the concentration of the odor in air, or in the case of binary mixtures, the relative concentrations of the two components.

Controlling concentrations as fractions of an odorant's vapor pressure has the advantage that different odorants have approximately equal thermodynamic activities at the same fraction of the corresponding vapor pressure (Ferguson, 1939; see Chapter 2). Substances at the same activity have approximately equal effectiveness as odorants, as measured by olfactory detection thresholds (Mullins, 1955; see Chapter 2).

Constant flow to eliminate non-stationarities

The output of the mixer flows through an electromagnetic valve that switches the odor stream from flowing to the animal during odor pulses to flowing to an exhaust tube in between them (Fig. 6.2). This design serves the purpose of making the flow through the bubblers continuous over the time

1. It is important to seal each of the connections in the air path (e.g., with Teflon tape), particularly downstream of the MFCs, since minute leaks can alter the flow rate and concentration of the odor delivered.

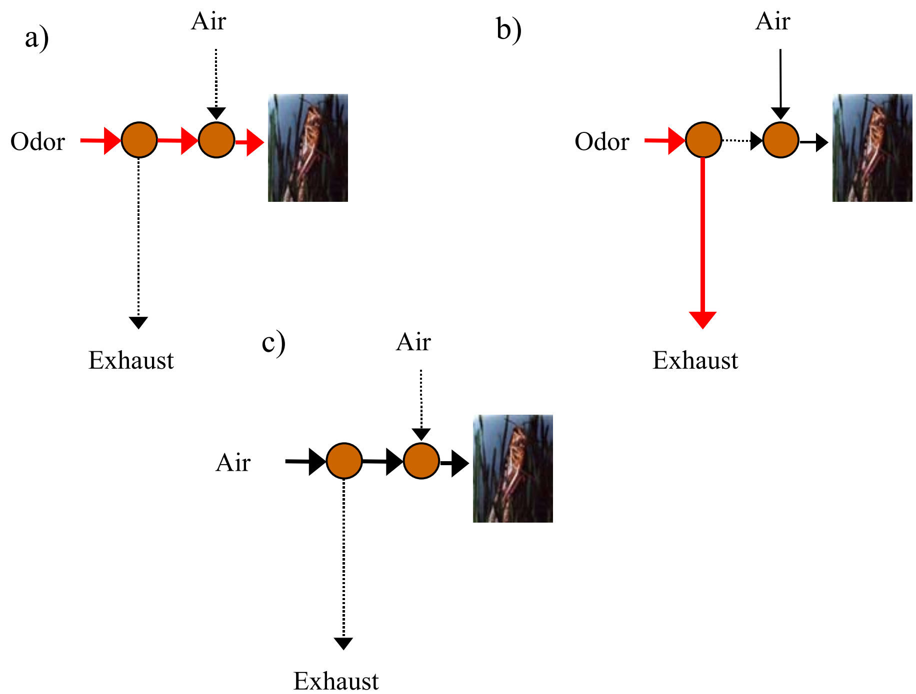

Figure 6.2. Design for the prevention of mechanical artifacts and to obtain continuous flow.
a) Circulation during odor pulses. **b)** Circulation during inter-pulse interval and during pre-circulation at the beginning of an odor series. **c)** Circulation during odor purging at the end of an odor series.
Red arrows indicate the flow of odorized air. Continuous black arrows indicate the flow of clean air. Broken arrows indicate airways not in use.

during which a given odor concentration is delivered, rather than pulsed with the delivery to the animal. This in turn serves to make the odor pulses homogeneous (Fig. 6.3).

Purging to prevent hysteresis

In an earlier design, I employed separate nozzles for each odor. This had the advantage of avoiding any contamination of tubing with a previously used odor, but had the disadvantages that i) the different angle of approach of each nozzle contributed to a difference between responses to different odors besides the one due to odor identity (M. S. Wehr and A. Bäcker, unpublished observations),

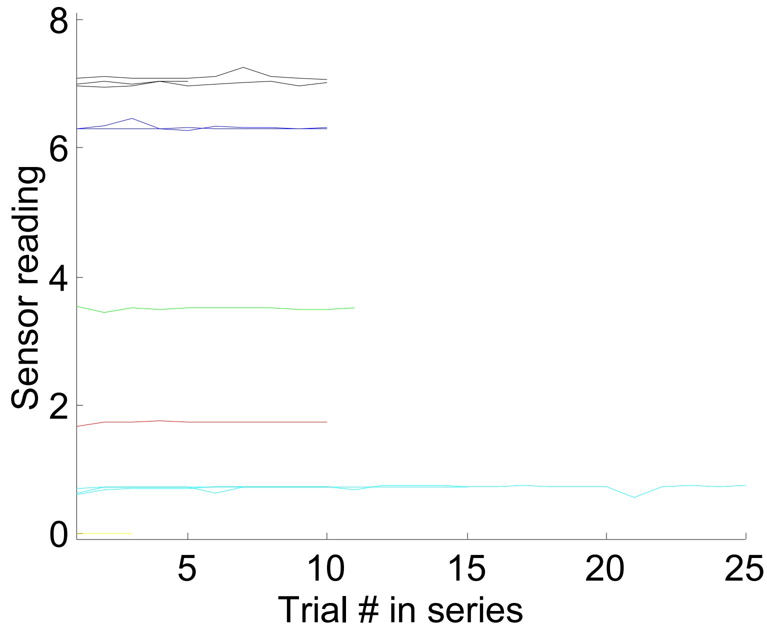

Figure 6.3. There is no hysteresis across trials: the concentration delivered is independent of the trial number in the series for each concentration (each represented by a different color). Each color represents a different concentration. Superimposed lines of the same color represent different series at the same concentration. Each point represents the mode of the sensor readings during the odor response for one trial, which correspond to the plateau level reached by the sensor for the trial.

and ii) that odors could not be mixed to deliver a blend. The first problem was solved by including a step motor to rotate the nozzles so that the active nozzle was always in the same position. This had the disadvantage of introducing a noisy electromagnetic device in the proximity of the target, though –inconvenient if the target is an animal from which electrophysiological recordings are being made. Furthermore, the need for purging was not avoided if we were to vary the concentration, and it would have been impossible to have a separate line for each concentration if we wanted the ability to delivery an arbitrary number of concentrations.

I thus opted for the present design, and implemented a purging system that proved to prevent any

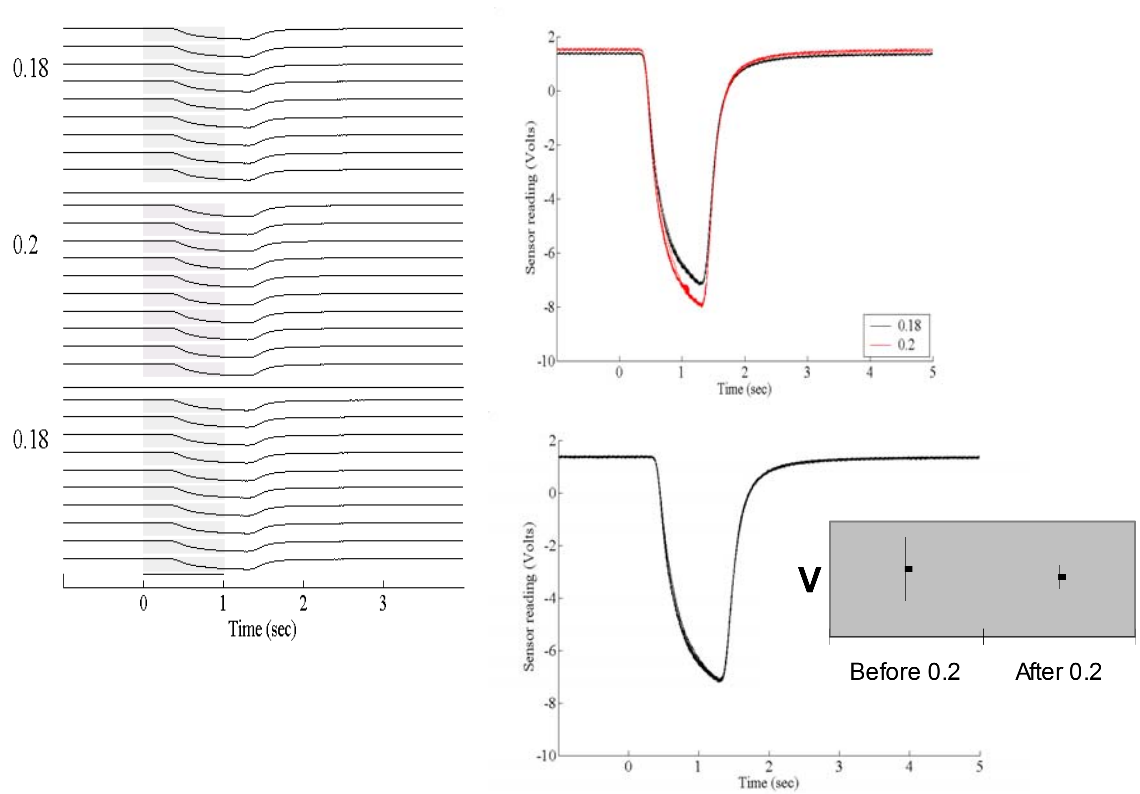

Figure 6.4. Timecourse of 1 sec long odor pulses shows no hysteresis across consecutive trials or nonconsecutive series. The sensor exhibits a negative signal in response to CO_2 . **Left:** The gray boxes show the period during which the pulsing valve was on, illustrating the delay between the switch and the sensor downstream. **Right, top:** Overlaid traces for ten trials of each of concentrations of 0.18 and 0.2 are perfectly discriminable. **Right, bottom:** CO_2 tracer delivers undistinguishable traces before and after the delivery of a higher concentration ($p>0.8$, Wilcoxon rank-sum test). All 20 traces are overlaid. Inset: Mean and standard deviation of mean CO_2 reading before and after delivery of a different concentration overlap.

hysteresis or contamination across odor series (Fig. 6.4 and Fig. 6.6). In between odor series, when the odor or the concentration is changed, the part of the system downstream of the odor selector, which is common to all odors, is purged by flowing clean air through the nozzle to the animal for approximately 30 seconds. Then, before the beginning of a new stimulus series to the animal, the new odor or concentration is pre-circulated to the exhaust for another 30 seconds to reach a steady state after purging the air in the system. This pre-circulation does not reach the nozzle. Instead, clean air flows through the nozzle before and in between the odor pulses, purging its small volume completely in between pulses so that all pulses are identical to each other. This design dictated that the valves that switch air flow between the animal and exhaust, and between clean and odorized air, be located close to the target in order to minimize the volume downstream of them.

Computer control and the capability to deliver arbitrary stimulus waveforms

All of the parameters of the system are controlled by a Macintosh personal computer using an analog and digital input/output card (National Instruments, Austin, Texas), custom-made multichannel current amplifier and indicator cards[1], Labview (National Instruments) and a software program, Odomix, written by the author for this purpose and available upon request[2]. These parameters include the flow rate for each of the mass flow controllers[3], which dictate the concentration and composition of the odor delivered as well as the flow rate of the overall stream, the selection of odor(s) in the blend delivered[4], the duration and frequency of the odor pulses[5], the number of pulses in each series, and the purging times. A separate program can be used to deliver continuously varying stimulus waveforms, a capability that could do much for our understanding of the processing of more natural odor plumes while retaining the control and understanding of the stimulus which is harder to

1. The current drawn by the valves exceeds the current sourcing capability of the computer card's digital input/output lines. In order to solve this, I constructed a Darlington circuit for each valve. This circuit used the current drawn from the computer card as a switch to turn on a circuit that drew on an autonomous current source to drive the valve. Eight of these current amplifiers were placed in each of several printed circuit boards, which also included LEDs to signal which valve was active at any point in time. Similar cards may be commercially available (SBX/TTL module, Pneutronics).
2. Email: abacker@alum.mit.edu.
3. Each MFC is controlled by one analog output line.
4. Each electromagnetic valve is controlled by a digital TTL output line (two valves per bubbler).
5. The two switching valves near the nozzle are controlled by two TTL timer lines for accurate timing control.

achieve with natural uncontrolled odor sources. This can be achieved by delivering the desired waveform as a control voltage to one of the MFCs and a complementary waveform to the other MFC to keep total flow constant, keeping the pulsing valves continuously in the odor delivery configuration (Fig. 6.2a).

The use of the computer to control the stimulus sequence also serves to keep an electronic record of the entire stimulation history[1], and could be used to program entire automatic experimental sequences. Of course, it also allows for real-time stipulation of the concentration or blends desired given the responses observed.

6.4 Testing

The performance of the system was tested using a portable gas chromatography mass spectroscopy (GCMS) system, a tracing system with a capnograph or CO_2 detector (Godart, Holland), polycaprolactone/carbon black (80:20 wt/wt) composite polymers (Lonergan et al., 1996) that change their resistance in direct response to odorants, and an insect brain.

Timecourse of the signal

To evaluate the timecourse of the odor pulses, one of the mass flow controllers was fitted with a cylinder delivering a mix of carbon dioxide in air (Fig. 6.5). The mixture of that line was mixed with the stream of the other mass flow controller, carrying pure air. The nozzle was connected to the input of a carbon dioxide sensor that works by shining light through the gas in the sensor and measuring the

1. In addition to keeping the stimulation history in a file in the computer, the stimulus specification for each series of odor pulses is encoded by the computer with a series of fast TTL pulses and output concurrently with the stimulus itself, for storage with electrophysiological data in a digital tape recorder (Biologic, France).

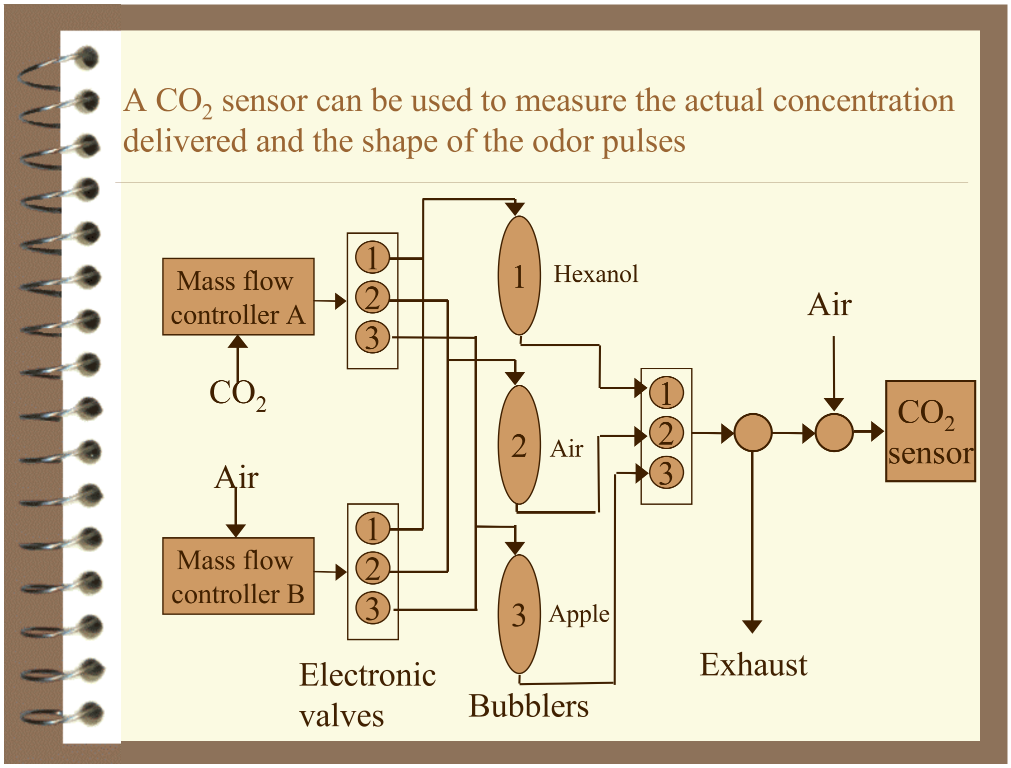

Figure 6.5. Using carbon dioxide as an odor tracer.

amount of absorption at a frequency characteristic of CO_2. The shape of the signal, filtered through the properties of the CO_2 detector, thus observed is shown in Fig. 6.4a. This timecourse was independently verified with carbon black composite polymers (Lonergan et al., 1996) that change their resistance in direct response to odorants.

Repeatability across trials

The repeatability of the odor pulses delivered can be seen in Fig. 6.4 and Fig. 6.7. The standard deviation of the mode concentration measured during pulses varied from 4.7% of the signal at a concentration of 2% of saturation to 0.6% at a concentration of 20% of saturation[1].

Repeatability across series

In order to verify the effectiveness of the purging procedure, the repeatability comparing identical stimuli delivered before and after the delivery of a different concentration was measured using the CO2 sensor. To ensure that the readings obtained for any concentration were repeatable and that the differences observed between different concentrations were not caused by hysteresis, I presented a series of 10 trials at a concentration of 18% of saturation, followed by 10 trials at 20%, and then another 10 trials at 18%. The variability across series of the same concentration (variance/mean for all 20 trials from both series at 18% = 2.7×10^{-4}) was well below the mean difference between readings for different concentrations: when decreasing the specified concentration from 20% to 18%, the mean readings decreased by exactly 10.0% (Fig. 6.4).

As an independent test to verify the return of the odor signal to baseline after the delivery of an odor pulse, GCMS was used to measure the odor concentration directly before, during and after the delivery of several odorants. The GCMS device acted as a low pass filter, but within approximately one minute the concentration reported decreased back to the baseline registered before the odor pulse (Fig. 6.6).

Linearity

Besides being repeatable, it is important that the concentrations delivered be both discriminable from each other and predictable. Figure 6.7 shows that the system's response is linear and that the

1. Concentrations above 20% saturated the CO_2 detector at the gain used and were thus excluded from the analysis. The trend observed, though, was that the fractional error consistently decreased with increasing concentrations.

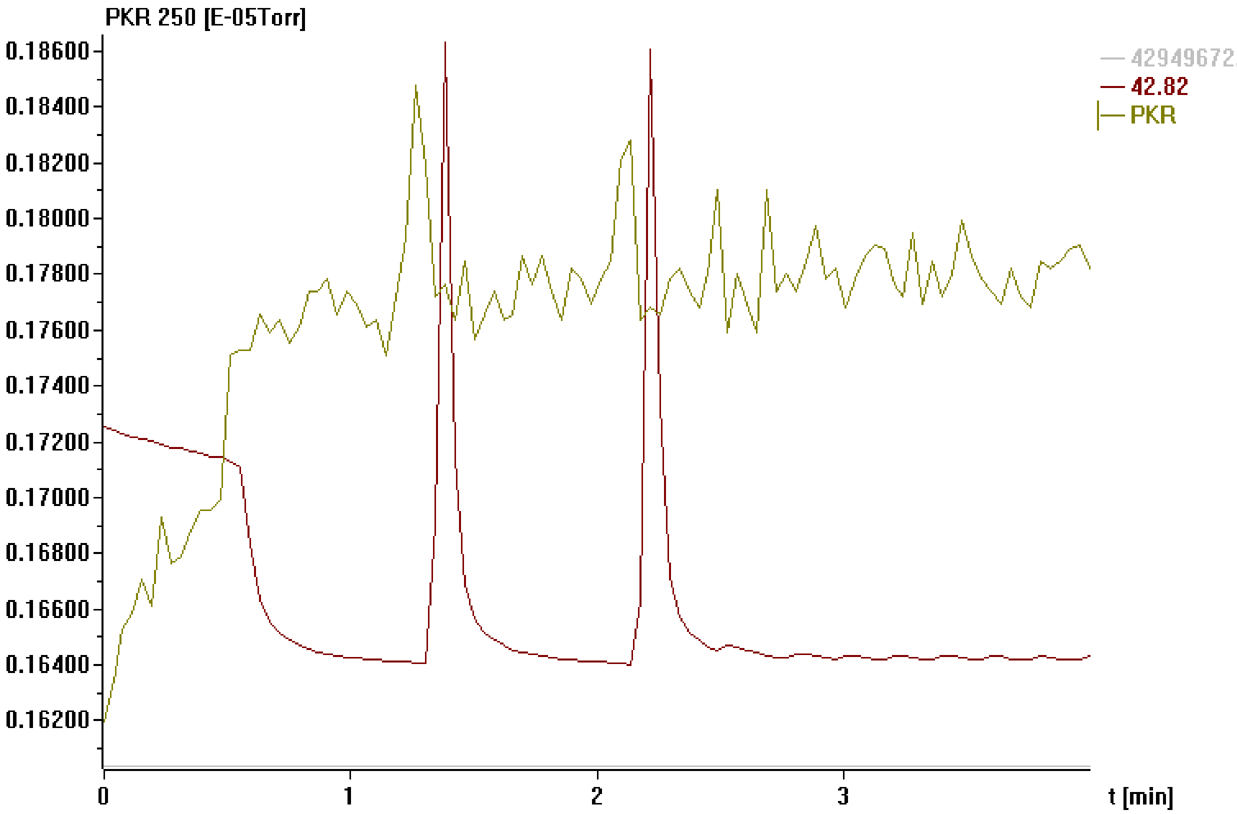

Figure 6.6. GCMS tests directly for hysteresis: Concentration returns to baseline after purging following an odor pulse (red trace).

concentration is highly repeatable and highly discriminable from others, complying with both of these requirements, even when the concentrations compared are close together (see also Fig. 6.4b).

Physiological relevance of small concentration changes

Finally, I show an application of the system that serves both to demonstrate once again the reliability of the system, and equally importantly, to illustrate that the small concentration changes that this system is capable of generating are relevant to physiology and important to understand the olfactory

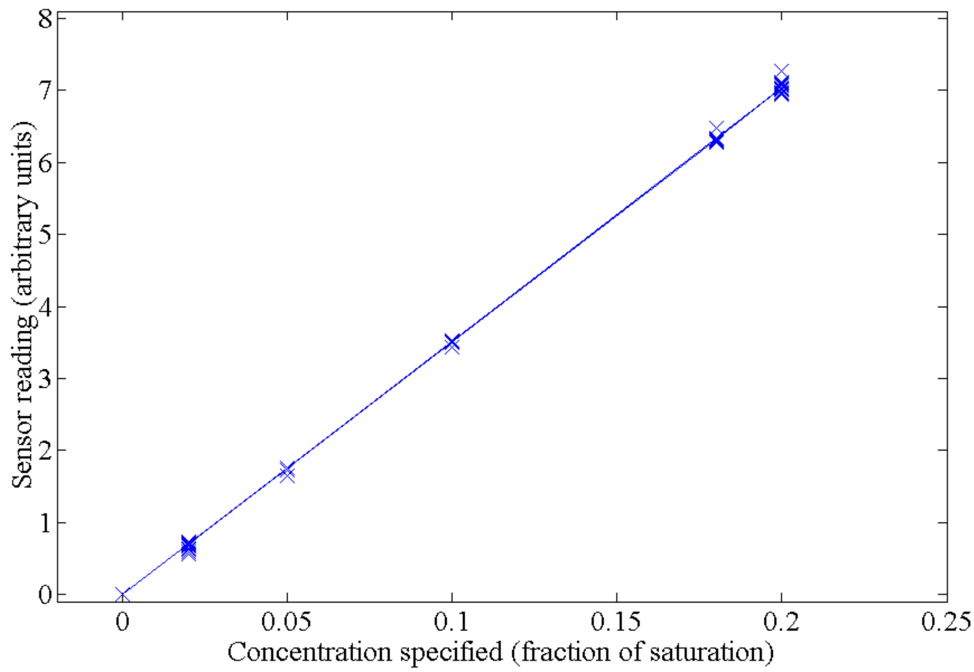

Figure 6.7: Linearity of the system. Each point represents the mode of the sensor readings during the odor response for one trial.

system. Intracellular recordings were performed of single projection neurons (PNs) in the antennal lobe of adult live awake locusts as described previously (Laurent and Davidowitz, 1994; Laurent et al., 1996). The PN shown in Fig. 6.8 responded vigorously and repeatedly to citral at a concentration of 28% of saturation, but did not respond to the same odor at 27% of saturation[1]. Such an abrupt threshold can only be found if the concentration can be specified to arbitrary values online, adjusting it dynamically toward the threshold as a neuron's responses are monitored in response to varying concentrations.

1. Figures 6.5a and 6.6 independently confirm that the odor delivery system is well able to deliver well-discriminable concentration differences in that magnitude range.

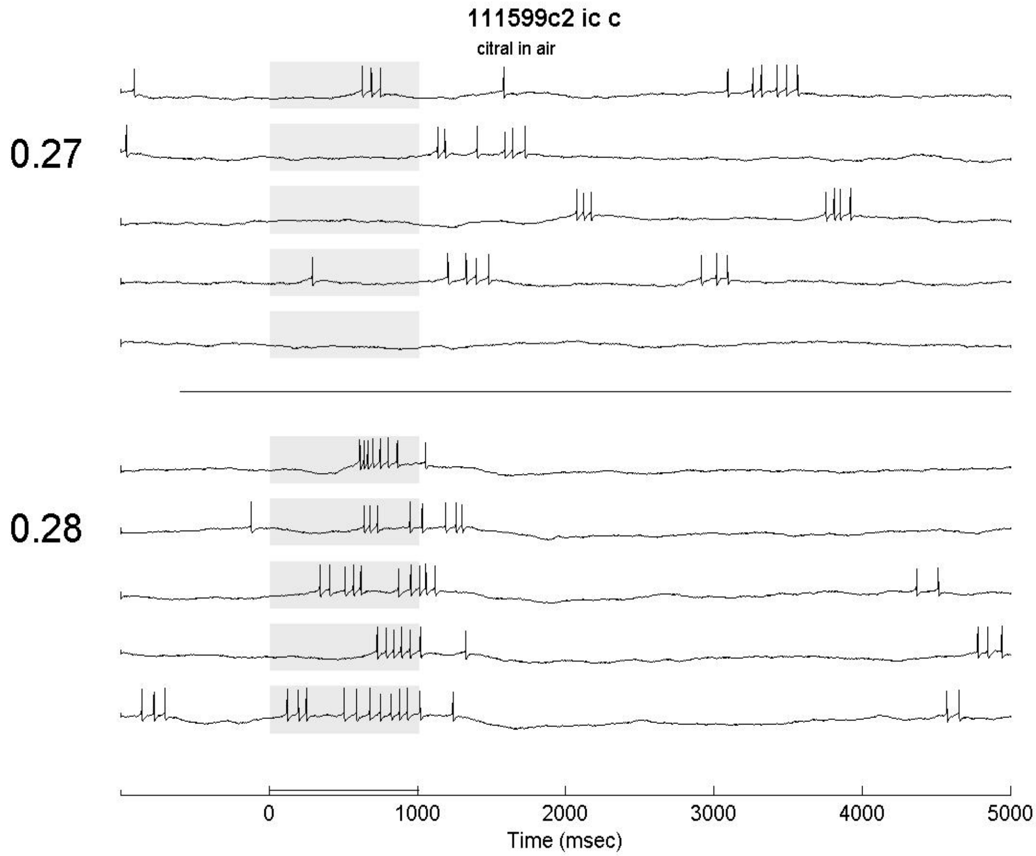

Figure 6.8: Relevance of small concentration changes for the insect olfactory system: The response of a projection neuron in the antennal (olfactory) lobe of the locust to citral at 27% of saturation and 28% of saturation.

6.5 Closing remarks

It is my hope that the use of systems such as the one described here will lead to the study of responses to continuous plume-like odor waveforms and blends of varying compositions to understand the processes that mediate the striking and at present unpredictable differences between the responses of the olfactory system to a blend versus those to their individual components.

6.6 Acknowledgements

Special thanks go to Ari Hershowitz, who contributed to an early version of the system, Yingzhong Tian, who helped in its construction, Tim Heitzmann of Caltech's Bioelectronics shop for help with the Darlington circuits, Christine Chee-Ruiter and Tom Meade for providing her expertise, Greg Sotzing for providing carbon black composite polymers, and Peter Green and Hui-Ming Hung for invaluable help with GCMS testing.