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Pneumatic Transport System for Associative Learning in Larval *Drosophila melanogaster*

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Abstract

A widely used associative learning technique for biological studies involves introducing Drosophila larvae to two different odorants and associating one of the odorants with a stimulus. The experimental method requires transferring the larvae back and forth between two odor chambers. Each chamber contains a different odorant and one of the chambers contains an accompanying stimulus. By introducing the larvae to the two different odorants several times, the larvae are trained to commit the odorant associated with the stimulus to memory. We created a mechatronic system for transporting Drosophila larvae back and forth between two agar trays to reduce manual labor and enable a scalable platform for associative learning and related studies. Air was chosen as the means of transporting the larvae and a chamber was constructed for housing the two agar trays. Control electronics were implemented for creating a sweeping blowing motion to transport the larvae in a gentle manner. Computer aided design (CAD) software was employed in conjunction with a 3D printer to build nozzles which help to direct airflow. Flow analysis software was utilized to model computational fluid dynamic simulations for optimizing air nozzles. In this study, a pneumatic transport system was developed and tested. The experimental results showed a 90% success rate for the transportation of larval Drosophila across the chamber and an overall decrease in transportation time by 4.8 times compared to manual transportation.

Keywords: Associative learning; Drosophila; Transport; Mechatronic; Fluid dynamics

Introduction

Associative learning is a widely used method for performing biological studies in which a new response becomes associated with a particular stimulus. *Drosophila melanogaster*, generally known as the fruit fly, is able to associate a particular odor with a reward or punishing stimulus [1-3]. Drosophila is commonly used for studying olfactory associative learning because the olfactory systems of insects assert a high degree of homology with their counterpart in vertebrates, both structurally and functionally [4-8]. In addition, they are easy to care for, have four pairs of chromosomes, breed quickly and lay many eggs. In classical conditioning (Pavlovian conditioning), a conditioned stimulus (CS) is paired to either an appetitive or an aversive unconditioned stimulus (US) [9,10]. Some researchers developed an associative learning paradigm using odorants as the CS and appetitive ("+" fructose) or aversive ("-"quinine) reinforcers as the US in larval Drosophila. Two treatment conditions are compared. In the first treatment Drosophila are trained to associate odorant A to the appetitive reinforcer and odorant B to the aversive reinforcer (A+/B-). In the second treatment, the reciprocal training strategy is applied (A-/B+). The results provide a differential preference toward the two distinct odorants among the groups. There are many different treatment options to give to the larvae, including change of surface texture, odor pairing and pretreatment with drugs including L-dopa (dopamine precursor), 3-LY (dopamine receptor inhibitor), ethanol, etc. In addition, mutant larvae are used to determine whether certain genes display divergent phenotypes. These types of experiments can show which genes or neurons are involved in this learning process. Research in this area is limited, however, due to the intense labor required to handle larval Drosophila.

The standard workflow for associative learning experiments is very labor intensive and time consuming. A set of associative learning experiments typically requires an hour of focus and delicate attention to detail. Steps 1-5 below briefly summarize the manual workflow steps.

- Step 1: Before the experiment, the experimenter must prepare two petri dishes containing two different odors, as well as the larvae that will be used in the experiment.
- Step 2: The experiment begins with the experimenter using a brush to place the larvae into one of two odor-permeated petri dishes for three minutes. After the larvae are exposed to the first odor for three minutes, the experimenter must use a brush to transfer all of the larvae to the second petri dish containing the second odor.
- Step 3: The larvae are contained in the second petri dish for three minutes and then transferred back to the first petri dish.
- Step 4: Steps 2 and 3 are repeated until the larvae have been exposed to each odor three separate times.
- Step 5: The experimenter performs a preference test by transferring the larvae to a third petri dish that contains both odors on opposite sides of the dish. The larvae are placed into the center of the dish and are given 3 min to move around the dish. After 3 min, the experimenter counts the larvae on each side of the dish and records the data.

This paper presents a mechatronic system for transporting *Drosophila melanogaster* larvae in associative learning experiments.

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The use of such a system can greatly reduce the time and effort of transporting the larvae, which is the most time and effort-intensive step of the associative learning experiment. We present a specially designed machine that permits effective transport of the larvae in a gentle manner that does not aggravate them or create a negative influence on the experiment.

Materials and methods

Computational fluid dynamics simulation

The success of the experiment is dependent on the ability of the larvae to accurately process information. If the larvae are agitated, they are more likely to incorrectly process information and produce faulty data. It is therefore essential to transport the larvae in a gentle manner that will not agitate them. Airflow was chosen as the transporting mechanism because the intensity level can be easily adjusted with pneumatic valves. The fluid dynamics of the airflow were analyzed in order to properly asses the flow of air through the chamber. SolidWorks FloXpress was used to perform flow analysis of air through a nozzle and throughout the chamber. Bernoulli's principle (Equation 1) was used to calculate the volumetric flow rate of air delivered by the air compressor.

$$\frac{\upsilon^2}{2} + gz + \frac{p}{\rho} = \text{constant} \quad (1)$$

The average *Drosophila melanogaster* larvae contains a mass m of approximately 1.4 mg [11,12]. The coefficient of kinetic friction μ between the larvae and the agar is approximately 0.1 [13]. The frictional force f required to move the larvae is equal to 1.373x10^{-6} kgm/s^2 (Equation 2).

$$f = \mu mg \quad (2)$$

Table 1 lists the fluid dynamics experimental variables and their values.

Symbol	Description	Range
v	Fluid flow speed	0-5 m^3/s
g	Acceleration due to gravity	9.81 m/s^2
z	Elevation	0 m
p	Pressure	0-413 kpa
ρ	Density	1.20-6.10 kg/m^3
μ	Coefficient of friction	0.1
f	Force	1.373E-6 kgm/s^2
m	Mass	1.4 mg

Table 1: Fluid dynamics experimental variables.

Mechanical design

Designing a pneumatic transport chamber required analysis of the most optimal flow of air through the experimental chamber. A simple chamber, a set of agar trays and a set of air nozzles were constructed from rapid prototyping technologies (Figure 1a). SolidWorks was used to create a 3-dimesional CAD model of the system (Figure 1b). The airflow through the chamber was modeled via FloXpress, a finite element analysis (FEA) function in SolidWorks, in order to determine the most effective nozzle shape. Table 2 presents the chamber components with their functions and dimensions.

Laser cutting was utilized to create a pneumatic transport chamber for performing successful associative learning experiments. Laser cutting involves the cutting of materials via a laser directed by optics and controlled by a computer. AutoCAD was used to generate a drawing of the laser-cutting pattern for the chamber (Figure 1c) and a laser cutter was used to cut the AutoCAD drawing out of 1/4-inch-thick acrylic sheets. Layers 1, 2 and 3 were bolted together and layer 4 was designed as a removable cap. The openings were placed at opposite ends in order to facilitate optimal flow of air back and forth through the chamber.

Component	Function	Dimensions (L x W x H)	Figure
Chamber	Houses agar trays and nozzles	78.7 x 50.8 x 17.8 mm	1d
Agar Tray	Holds agar surface for larvae to move on	25.2 x 38.3 x 11.7 mm	1e
Nozzle	Directs air flow	19.7 x 41.5 x 5.35 mm	1f

Table 2: Pneumatic transport system components functions and dimensions.

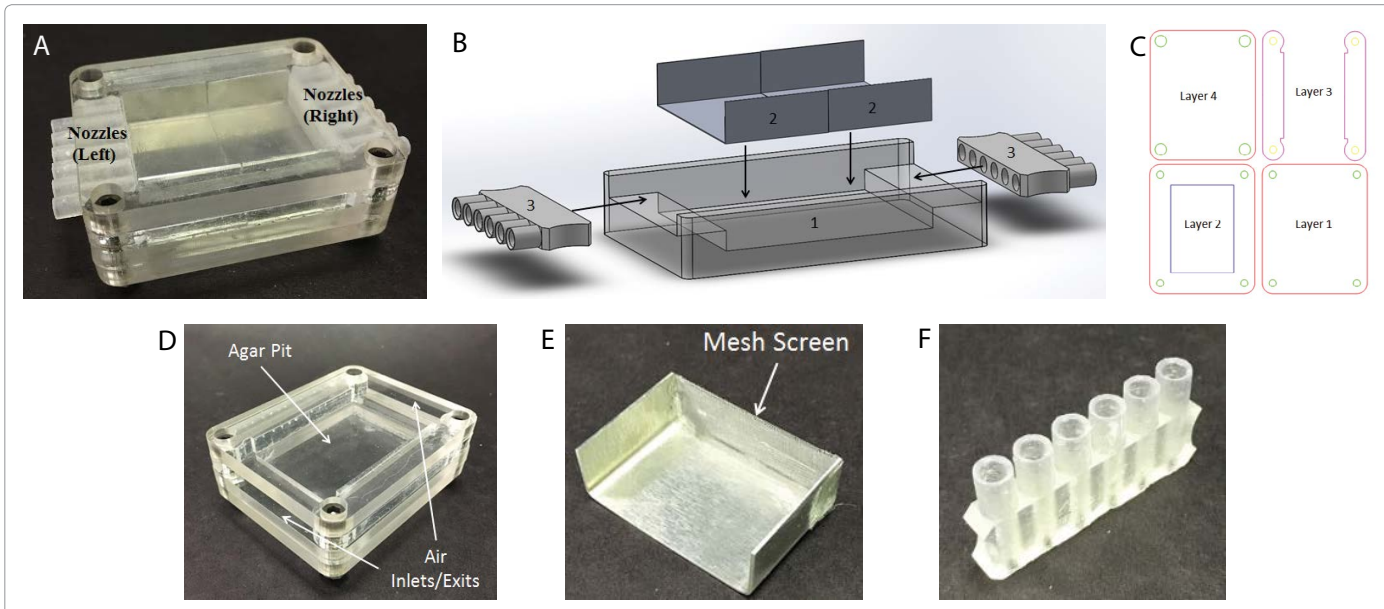

Figure 1: (a) Prototype of two-way pneumatic transport chamber with agar trays and nozzles inserted; (b) Exploded view of pneumatic transport chamber: 1-chamber, 2-agar trays, 3-air nozzles; (c) AutoCAD drawing of laser cutting pattern for pneumatic transport chamber; (d) Prototype of pneumatic transport chamber; (e) Prototype of agar tray with mesh screen; (f) 3D printed prototype of air nozzle.

A rectangular hole was inserted into layer 2 of the chamber design to create a pit intended to house two agar trays (Figure 1d).

Agar trays (Figure 1e) were manufactured from 1/16th inch thick sheet metal to a shape that fits snug into the chamber. Two sides of the trays have heights that extend through the top of layer 3 of the chamber, while the other two sides are left open. A mesh screen was placed on one of the open sides of the tray to allow air to provide a means for containing the larvae within the chamber while allowing air to pass through. The agar trays serve as containers to confine the agar gel and the larvae. The layers of acrylic used for the chamber were screwed together tightly enough to contain the liquid agar without leaking. The purpose of having two separate trays is to provide two different types of agar gel for the larvae to rest on. One gel tray contains sugar and one gel tray is without sugar. The trays can then be easily removed to handle larvae.

Air nozzles (Figure 1f) were designed to transport larvae across the chamber gently so that larvae are not aggravated and their experimental learning performance is not adversely affected. The nozzles were designed using SolidWorks. They were designed to fit the width of the chamber opening and to support a steady flow of air through the majority of the chamber in order to push the larvae past the midway point of the chamber, from one agar tray to the other. The air nozzle design contains air inlets compatible for fitting 4 mm diameter rubber tubing. In the design, six separate tubes are connected to the nozzle and each one is turned on sequentially with programming. The sequential operation of the air inlets at a finely tuned flow creates a gentle sweeping motion across the chamber rather than supplying full power to each of the inlets all at once, which might aggravate or injure the larvae.

Control electronics

Electronics were implemented in order to control the direction of airflow. Zero differential solenoid valves (model number: 2ACK-1/4-12VDC-G, valve type: Two-way normally closed, response time: <20 ms, max operating pressure: 115 psi) were used to control the flow of air to each tube connected to the nozzles. Power transistors were used as the valve drivers to enable a current to be applied to the valves. A national instruments data acquisition card was used to control the operation of the valve drivers and the data acquisition card was controlled through LabVIEW. The control box containing the solenoid valves, the valve drivers and the data acquisition card are shown in Figures 2a and 2b displays a schematic of the control electronics. The LabVIEW program utilized pulse width modulation to control the pulsing of the overall flow volume entering the pneumatic chamber. The purpose of having six valves is to create a sweeping motion without an actuating motor so that the larvae can be transported more gently across the chamber.

Results

Machine workflow

Table 3 compares the manual workflow with the machine-assisted workflow. Our objective is to improve the throughput of the red color steps so that this conditioning training can become more automatic. The machine-assisted workflow (Table 3, right column) is similar to the manual workflow except for the experiment preparation and steps 8, 9, 13 and 14. During preparation of the machine-assisted experiment, the experimenter pours agar into the machine's two agar trays. Sugar is mixed with the agar of one of the trays to create an unconditioned stimulus. Once the agar has dried, 0.10 mL of water is applied to the surface of the agar to facilitate gentle movement of the larvae upon blowing. A few larvae are placed into the chamber and the air pressure is adjusted to a level that gently moves the larvae across the agar surface. The first few larvae are discarded and 50-60 larvae are manually placed onto agar tray A with a brush to begin associative learning training. Agar tray A is placed into odor chamber A (Figure 3). After the larvae are exposed to odor A for three minutes, agar tray A is removed from odor chamber A and placed into the machine. The machine is operated to transport the larvae to agar tray B. The larvae that were not successfully transported to agar tray B (approximately 10%) are discarded in order to ensure that all of the remaining larvae have been trained properly. Agar tray B is then removed from the machine and placed into odor

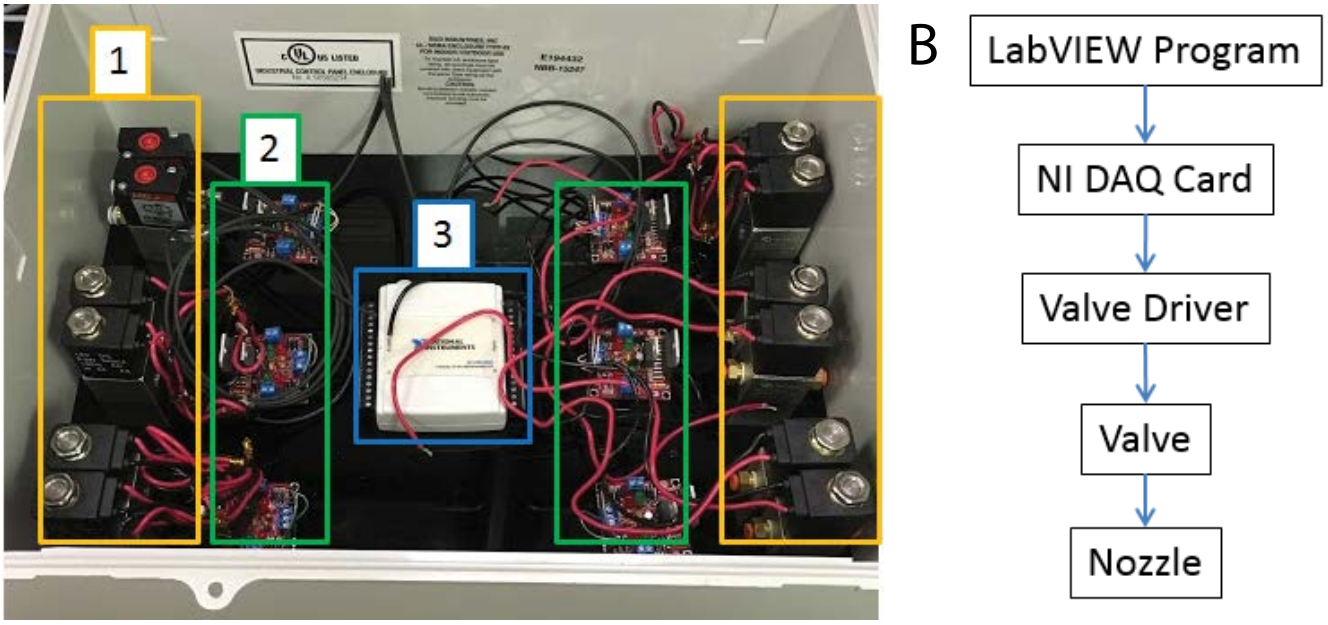

Figure 2: (a) Photograph of control electronics: 1-Valves, 2-Valve Drivers, 3-NI DAQ Card; (b) Schematic of the flow of control electronics.

	Manual workflow	Machine-assisted workflow
Step 1	Experiment preparation	Experiment preparation
Step 2	Use brush to softly touch the larvae	Use brush to softly touch the larvae
Step 3	Visually inspect to make sure the larvae stick to the brush Criteria: Yes/No · No: Go back to step 2 · Yes: Move the brush to odor chamber A	Visually inspect to make sure the larvae stick to the brush Criteria: Yes/No · No: Go back to step 2 · Yes: Move the brush to agar tray A
Step 4	Softly touch the brush to the larvae in odor chamber A	Softly touch the brush to the larvae in agar tray A
Step 5	Inspect to make sure the larvae stick to the agar Criteria: Yes/No · No: Go back to step 4 · Yes: Move the brush pick up new larvae	Inspect to make sure the larvae stick to the agar Criteria: Yes/No · No: Go back to step 4 · Yes: Move the brush to pick up new larvae
Step 6	Repeat steps 2-5 until all larvae are in odor chamber A	Repeat steps 2-5 until all larvae are in agar tray A. Place agar tray A into odor chamber A
Step 7	Cap odor chamber A to expose larvae to odor A for 3 min	Cap odor chamber A to expose larvae to odor A for 3 min
Step 8	Uncap odor chamber A and use the brush to softly touch the larvae	Uncap odor chamber A and place agar tray A into the transport chamber
Step 9	Visually inspect to make sure the larvae stick to the brush Criteria: Yes/No · No: Go back to step 8 · Yes: Move the brush to odor chamber B	Activate transport chamber and visually inspect to make sure the larvae have been transported to agar tray B Criteria: Yes/No · No: Repeat step 9 · Yes: Move agar tray B to odor chamber B and discard remaining larvae in agar tray A
Step 10	Softly touch the brush to the agar in odor chamber B	
Step 11	Inspect to make sure the larvae stick to the agar. Criteria: Yes/No · No: Go back to step 10 · Yes: Move the brush to odor chamber A	
Step 12	Repeat steps 8 - 11 until all larvae are in odor chamber B	
Step 13	Cap odor chamber B to expose larvae to odor B for 3 min	Cap odor chamber B to expose larvae to odor B for 3 min
Step 13	Uncap odor chamber B and use the brush to softly touch the larvae	Uncap odor chamber B and place agar tray B into the transport chamber
Step 14	Visually inspect to make sure the larvae stick to the brush Criteria: Yes/No · No: Go back to step 13 · Yes: Move the brush to odor chamber A	Activate transport chamber and visually inspect to make sure the larvae have been transported to agar tray A Criteria: Yes/No · No: Repeat step 14 · Yes: Move agar tray A to odor chamber A and discard remaining larvae in agar tray B
Step 15	Softly touch the brush to the agar in petri dish A	
Step 16	Inspect to make sure the larvae stick to the agar. Criteria: Yes/No · No: Go back to step 15 · Yes: Move the brush to odor chamber A	
Step 17	Repeat steps 13-16 until all larvae are in odor chamber A	
Step 18	Repeat steps 7-17 two more times	Repeat steps 7-14 two more times
Step 19	Perform preference test	Perform preference test

Table 3: Manual vs. machine-assisted experimental workflow comparison.

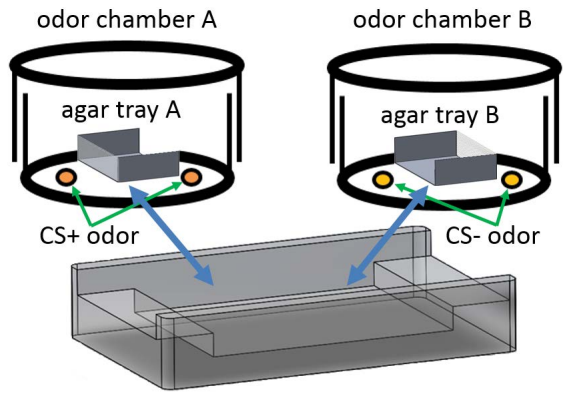

Figure 3: Schematic of associative learning experimental setup with two separate odor chambers.

chamber B (Figure 3). These steps of the machine-assisted workflow remove the hassle of manually transporting each individual larvae back and forth between petri dishes with a brush, saving a significant amount of time and effort in the performance of the experiment. After the larvae have been trained three times, the experimenter uses a brush to move the larvae manually to the middle of the preference test plate where they start in a binary choice between the CS+ and CS- odor.

Parameter	Value
Chamber volume	71.163 cm^3
Inlet flow rate	4.719 x 10^{-4} m^3/s-9.439 x 10^{-4} m^3/s
Exit pressure (atmospheric)	101.325 kPa
Temperature	293.2 K

Table 4: Flow simulation parameters.

Computational fluid dynamics simulation

Analysis of the fluid mechanics of the airflow through the nozzle and the chamber provided detailed information about the most effective nozzle design for effectively transporting the larvae across the chamber. The simulation results indicated that a circular nozzle produces the most even distribution of airflow in the y-axis. This ensures that the larvae will be blown in the direction of the x-axis, across the midline of the chamber. Figure 4 shows the SolidWorks FloXpress simulation of airflow through the nozzle and chamber at various volumetric flow rates. Table 4 displays the flow parameters used in the simulations.

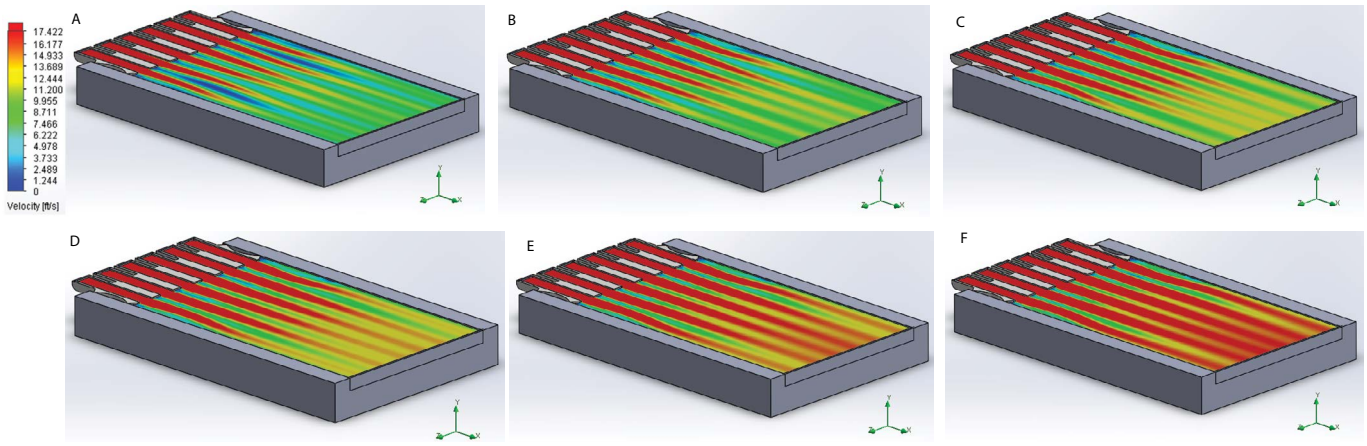

Figure 4: Solid works FloXpress air flow simulation through transport chamber at (a) 1.0 cfm (4.719 x 10^{-4} m^3/s); (b) 1.2 cfm (5.663 x 10^{-4} m^3/s); (c) 1.4 cfm (6.607 x 10^{-4} m^3/s); (d) 1.6 cfm (7.551 x 10^{-4} m^3/s); (e) 1.8 cfm (8.459 x 10^{-4} m^3/s) and (f) 2.0 cfm (9.439 x 10^{-4} m^3/s).

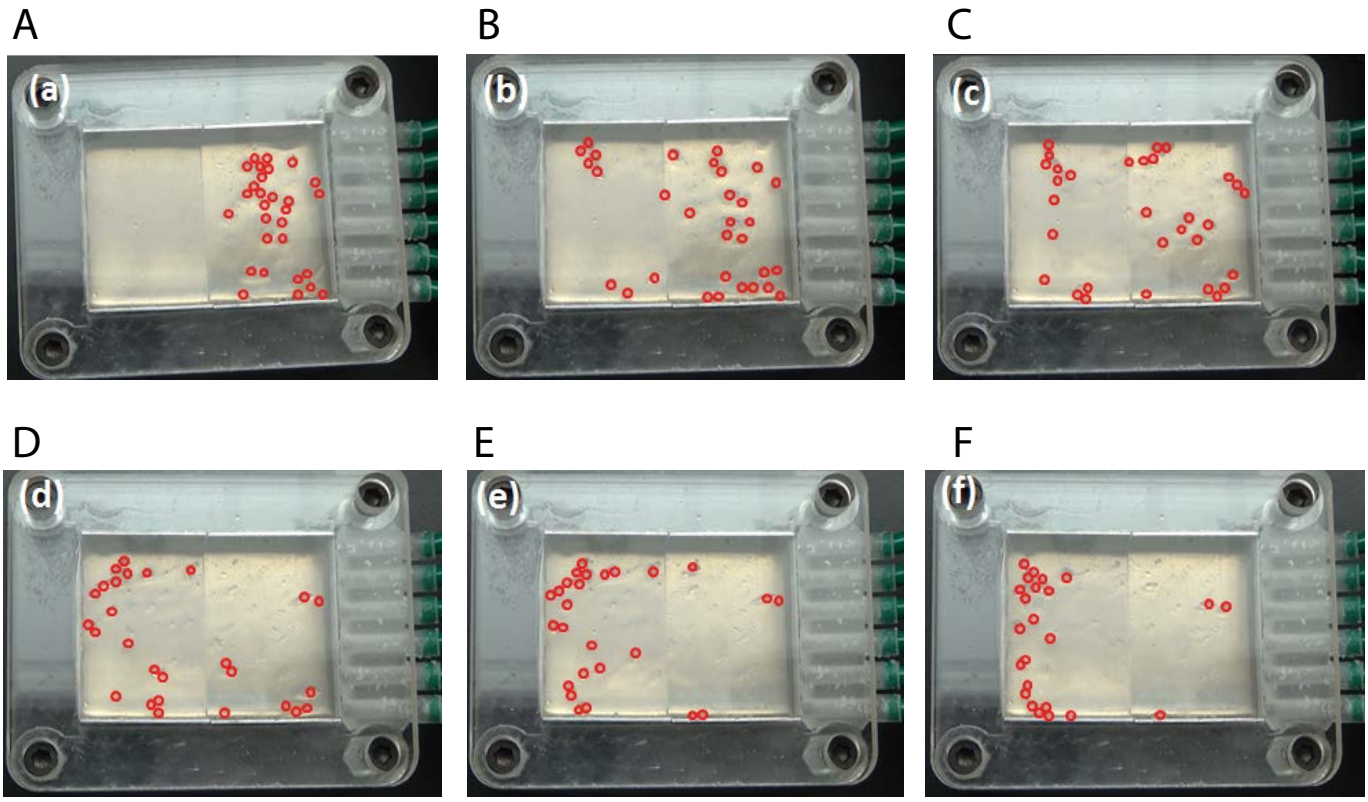

Figure 5: Progression of movement of larvae across the transport chamber at (a) 0%; (b), 20%; (c) 40%; (d) 60%; (e) 80% and (f) 100%. Red circles indicate the locations of the larvae.

Throughput quantification

The pneumatic transport chamber was tested with Drosophila larvae. The agar trays were inserted into the chamber and liquid agar was poured into the chamber. Once the agar cooled and solidified, approximately 30 larvae were placed onto the agar gel on one side of the chamber and the chamber was capped. An air compressor was used to blow the larvae from one side of the chamber to the other. Figure 5 shows the progression of movement of larvae across the chamber. Ten blowing trails were conducted and recorded. Figure 6a shows that, on average, 90% of the larvae were successfully transported across the chamber with a standard deviation of 3.55%. These results provide that such a pneumatic transport system could dramatically speed up the most time intensive step in associative learning experiments, which is classically performed via human manual labor. The elimination of human labor could allow for a significant increase in experimental throughput, providing more data for researchers.

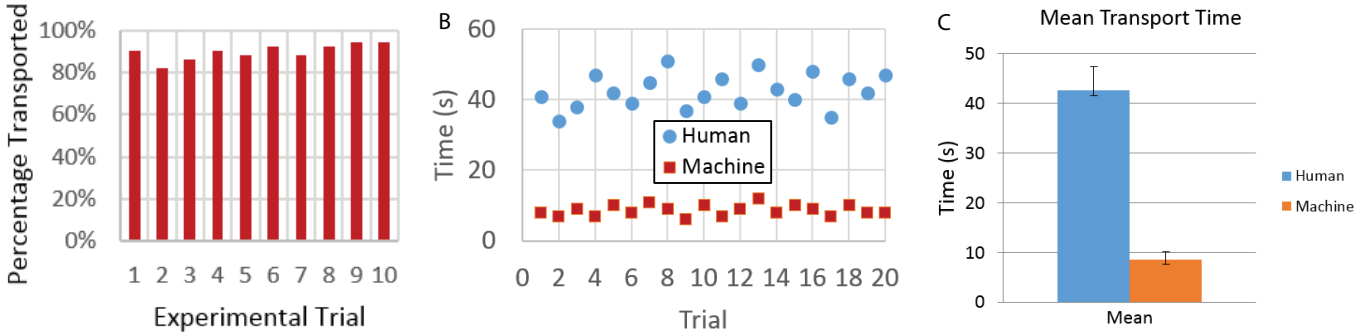

Figure 6: Pneumatic transport system experimental data showing transportation success and time. (a) Transport rate; (b) experiment time comparison; (c) mean transport time and standard deviation.

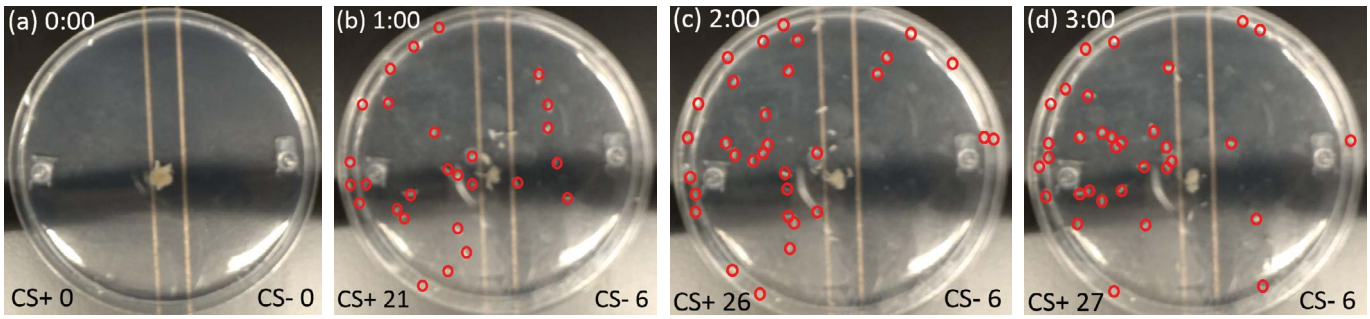

Figure 7: Preference test after three-time training with sugar-associated odor on the left side (CS+) and the control odor on the right side (CS-) at (a) 0:00 min; (b) 1:00 min; (c) 2:00 min and (d) 3:00 min.

The time taken to transport the larvae by hand was compared to the time taken to transport the larvae with the pneumatic transport chamber and the results are provided in Figure 6b. The mean transport time was approximately 43 s for humans and 9 s for the machine (Figure 6c), a 4.8 fold improvement. The standard deviation of transport time was approximately 4.7 s for humans and 1.5 s for the machine, shown as error bars in Figure 6c. An analysis of variance (ANOVA) test was also performed to investigate the null hypothesis that the means are equal. The results of the ANOVA test gave a P-value of 5.39E-28, rejecting the null hypothesis and showing a significant discrepancy in the mean values.

Associative learning

Reciprocal training associative learning experiments were performed by the experimenter to confirm that the larvae were not agitated using the pneumatic transport machine and were neurologically comparative with the manually handled larvae. We used third instar feeding stage larvae aged 5 days (± 12 h) after egg lay. Flies of the Canton-S wild-type strain were used and kept in mass culture, maintained at 25°C, 60-70% relative humidity. In the first stage of training, OCT was trained to be associated with sucrose (CS+), while AM was trained with normal agar (CS-). In the reciprocal training, AM was trained to be associated with sucrose (CS+), while OCT was trained with normal agar (CS-). Figure 7 shows images from the preference test after the first stage of three-time training using the machine. After a three-minute preference test (OCT+/AM-), there are 27 larvae on the CS+ odor side and 6 on the CS- odor side, resulting in a 4.5:1 preference towards the CS+, sugar-associated odor. The results were similar for the reciprocal training experiment (OCT-/AM+), with a 3.5:1 preference towards the CS+ side, supporting that the larvae were not agitated during blowing experiments.

Discussion

The pneumatic transport system was developed to improve the speed and throughput of associative learning experiments in larval *Drosophila melanogaster*. Rapid prototyping and computational fluid dynamics analysis were utilized to create an optimized flow chamber and computer software was implemented to control the flow of air through the chamber. The efficacy of the system was validated through a series of experiments in which larval Drosophila were transported across the chamber. Experimental results showed a 90% success rate for the transportation of larvae across the chamber and an overall decrease in transportation time by 4.8 times compared to manual transportation. These results demonstrate the successful utilization of a pneumatic transport system to automate the larvae transportation step in associative learning experiments, which is classically performed via human manual labor. The use of an automated transport system enables a scalable platform for associative learning experiments. Future work will aim to improve the pneumatic transport chamber and to automate the entire associative learning experiment. This includes improving the transport rate, incorporating image processing to track the location of the larvae throughout the experiment as well as developing a robotic arm to move the transport chamber trays from the transport chamber to the odor chamber and then back to the transport chamber.

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