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A Fully Automated *Drosophila* Olfactory Classical Conditioning and Testing System for Behavioral Learning and Memory Assessment

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Abstract

Background—Aversive olfactory classical conditioning has been the standard method to assess *Drosophila* learning and memory behavior for decades, yet training and testing are conducted manually under exceedingly labor-intensive conditions. To overcome this severe limitation, a fully automated, inexpensive system has been developed, which allows accurate and efficient Pavlovian associative learning/memory analyses for high-throughput pharmacological and genetic studies.

New Method—The automated system employs a linear actuator coupled to an odorant T-maze with airflow-mediated transfer of animals between training and testing stages. Odorant, airflow and electrical shock delivery are automatically administered and monitored during training trials. Control software allows operator-input variables to define parameters of *Drosophila* learning, short-term memory and long-term memory assays.

Results—The approach allows accurate learning/memory determinations with operational fail-safes. Automated learning indices (immediately post-training) and memory indices (after 24 hours) are comparable to traditional manual experiments, while minimizing experimenter involvement.

Comparison with Existing Methods—The automated system provides vast improvements over labor-intensive manual approaches with no experimenter involvement required during either

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training or testing phases. It provides quality control tracking of airflow rates, odorant delivery and electrical shock treatments, and an expanded platform for high-throughput studies of combinational drug tests and genetic screens. The design uses inexpensive hardware and software for a total cost of ~\$500US, making it affordable to a wide range of investigators.

Conclusions—This study demonstrates the design, construction and testing of a fully automated *Drosophila* olfactory classical association apparatus to provide low-labor, high-fidelity, quality-monitored, high-throughput and inexpensive learning and memory behavioral assays.

Keywords

Pavlovian classical conditioning; automation; olfactory; learning; memory; *Drosophila*

1. Introduction

The *Drosophila* model system has been used in classical Pavlovian conditioning studies for over 40 years, providing innumerable insights elevating the understanding of genetic, molecular and cellular mechanisms of learning acquisition and memory consolidation (Hersherberger et al., 1967; Quinn et al., 1974; Tully and Quinn, 1985; Roman and Davis, 2001; Busto et al., 2010; Kahsai et al., 2011; Perisse et al., 2013; Tabone and de Belle, 2014; Walkinshaw et al., 2015). By far the most common type of analysis involves aversive olfactory associative training and testing using a large adult *Drosophila* population (~100 flies per trial). The animals are initially trained by timed exposure to an electric shock-paired odor cue (conditioned stimulus, CS+) and an unreinforced odor cue (CS−) (Pavlov, 1927; Pitman et al., 2009; Malik and Hodge, 2014). During the testing phase, the animals are simultaneously presented with both CS+ and CS− odors, provided time to choose between the odors with a directional movement, and then the population distribution for each odor choice is recorded as a performance index (Tully and Quinn, 1985; Malik and Hodge, 2014). The learning index (LI) is typically determined immediately following the end of the training cycle (~15 minutes), and the memory index (MI) assayed at 24 hours after a spaced training paradigm. This procedure assays associative aversive conditioning, as opposed to appetitive/reward conditioning, which is quantitatively measured without the introduction of bias by the innate preference for either of the conditioned stimuli (Gerber et al., 2004; Schwaerzel et al., 2003).

For decades, the performance of these experiments has required an entirely manual and heavily labor-intensive procedure for both training and testing (Quinn et al., 1974; Tempel et al., 1983; Tully and Quinn, 1985; Tabone and de Belle, 2014). The manual experiment is performed in two phases; 1) electrical shock training and 2) choice testing of the trained flies. While specific protocol parameters vary widely across laboratories, in the standard manual methodology 50–100 flies are sequentially exposed to two matched, equally aversive odors (e.g. octanol and methyl-cyclohexanol). The first odor is conditioned by delivery of a series of 8–12 electric shocks (2.5 seconds each at 80V DC) through a copper grid every 5 seconds (2.5 seconds rest between each shock), with a second shock-free exposure to the non-conditioned odor (Tully and Quinn, 1985; Pitman et al., 2009). Shock voltage ranges from 60V to 120V DC across protocols and shock delivery may be as short as 100 milliseconds. After training, flies are tested for their ability to learn (immediately

following training) or retain memory (e.g. 24 hours following training) with a T-maze presenting both odors. Assessments can be done following a single mass training session with 7–10 training cycles or using spaced training (with 15 minutes rest interval between each cycle), but effective long-term memory consolidation at 24 hours requires spaced training (Pascual and Prat, 2001; Ge et al., 2004; Davis, 2005). Learning acquisition occurs with the utilization of pre-existing cellular components, not requiring new protein synthesis, whereas 24 hour long-term memory requires *de novo* protein translation (Tully et al., 1994; Davis, 2005; Bolduc et al., 2008).

Manually conducting these learning and memory studies is extremely labor-intensive, owing to the hands-on time on the training apparatus, the waiting periods between treatments, and animal collection/transfer between training and testing phases. Moreover, manual experiments of such length and involved manipulations are subject to considerable operator variation. Therefore, developing an automated training and testing system would greatly widen the scope of studies that could be pursued, increase precision control and allow more consistent reproduction of outcome results. Automated learning/memory studies would be a huge boon for both basic science applications and testing interventions in *Drosophila* disease models (Dubnau et al., 2003; Gatto et al., 2014; Walkinshaw et al. 2015). A semi-automated system has been designed (Murakami et al., 2010). In the present study, the aim was to improve upon this semi-automated system with a fully automated system incorporating both training and testing phases. In addition, our system includes quality control fail-safes to monitor airflow rates, odorant delivery and electrical shocks. Finally, the cost of each fully automated training and testing system was kept below \$500US using readily available commercial components. In the following sections, the design and construction of both the hardware and software components of the automated system are described in sufficient detail to allow reconstruction of the complete apparatus. Results from the final automated apparatus were compared to those obtained with the traditional manual approach to demonstrate highly effective learning and memory behavioral assessments.

2. Materials and Methods

2.1 Experimental animal rearing and behavioral testing environment

Drosophila were reared in standard fly vials on standard cornmeal/agar/molasses food in a humidity controlled incubator at 25C with a 12 hour light/dark (12:12 LD) cycle (1AM lights on, 1PM lights off). Rearing density was controlled by transferring parental animals to fresh vials every 3–4 days. Adults used for behavioral testing were staged to <5 days post-eclosion at 25C, and transferred to fresh food vials at 24 hours prior to training. All behavioral studies were at Zeitgeber time 12–19 (Lyons and Roman, 2009). All training and testing procedures were conducted at room temperature (21–23C), with dim red light illumination used to prevent any positive phototaxis bias. The testing apparatus was mounted on a flat, level surface to prevent any negative geotaxis bias (Murphey and Hall, 1969), with the linear actuator calibrated to maintain proper alignment of the centerpiece with the tubes. Odor-balanced, equally aversive odorants 3-Octanol (OCT diluted in mineral oil (Sigma-Aldrich, St. Louis, MO) at 10^{-3} M) and 4-methylcyclohexanol (MCH at 1.5×10^{-3} M) were prepared fresh daily, maintained in closed containers until use. Testing was

conducted in atmospheric humidity ranging from 30%–50% as previously reported (Malik and Hodge, 2014). Routine testing of naïve flies showed equal distribution between odors at given concentrations. Airflow was kept constant at 1500 mL/min using manostat control for all odorant delivery. Air blanks were used in both the training and testing compartments. The training tube used parallel copper wiring for 80V electrical shock reinforcement. Conductance through the training tube was automatically measured prior to each training session. The effectiveness of automated designs was compared to the standard manual T-maze assay used previously (Fig. S1; Coffee et al. 2012; Gatto et al. 2014). *Drosophila* were loaded into the training tube and presented with the conditioned stimulus odorant (CS+) together with electrical shocks and the control stimulus odorant (CS–) without conditioning (Fig. S1). Animals were then manually transferred by elevator to the testing tubes, with a T-maze choice of CS+ and CS– odorants (Fig. S1). Depending on the interval from training, behavior was quantified as a learning index (LI; 15 mins following training) or memory index (MI; 24 hrs following training) as follows: $[(CS-) - (CS+)] / [(CS-) + (CS+)]$ (Tully and Quinn, 1985). For automated trials (see below), solenoid valve, airflow, odorant and electrical shock stimulus failsafe monitors were automatically monitored.

2.2 Training tube and electric shock control circuit

Rollled copper tape with conductive adhesive of 1/8" width (Lucent Path, CA) was cut to 1/16", and then affixed at 1/16" spacing to transparency sheets sized to fit the inner diameter of the acrylic training tube. The copper tape overlapped the edge of the sheet continuing onto the back in alternating longer/shorter lengths at both the top and the bottom of the sheet. A strip of the copper tape was placed across the back connecting the longer strips to one another to create electrical continuity, and these copper crossings were reinforced with high purity, conductive silver paint (SPI Supplies, West Chester, PA). Two wires were then soldered to the grid to provide ground (GRND) and voltage (VDD). Copper pre-printed breadboards (SB400 PC Breadboard; BusBoard Prototype Systems, Alberta, Canada) were cut to size with jumper, GRND and VDD wires attached to each board. The GRND and VDD wires for the copper grid and copper board were threaded through the base of the shock tube and attached to appropriate connector pins (male, 2mm; A-M Systems, Sequim, WA) to accommodate alligator clip attachment to an electrical stimulator. An electric shock control circuit controls the strength and duration of shock delivery using a robotgeek relay. The jumper JP4_RELAY3 pin 1 is connected to the ground, pin 2 is connected to the training tube, and pin 3 is connected to a SD9 square pulse stimulator. The training tube also connects with ground. When the robotgeek relay does not receive the 5V signal, the training tube connects to two grounds. When the relay receives the 5V signal, the training tube connects to stimulator and ground to provide the electrical shocks to the copper circuit inside the training tube.

2.3 Design fabrication of the automated olfactory training and testing apparatus

Two types of apparatus were designed and tested en route to achieving the fully automated system. The first design was the "Rotating Automated Training and Testing System" (RATTS). RATTS uses two servomotors to move 3D-printed parts, coupled with airflow control to achieve automated training and testing phases. The second design was the "Linear Automated Training and Testing System" (LATTS). LATTS employs a linear actuator and

airflow control for *Drosophila* transfer between training and testing stages of the experiment. For RATTS, a Stratasys Dimension 768 SST 3D printer (Edina, MN) was used in house to print all the essential core components. The 3D printing material was acrylonitrile butadiene styrene (ABS). Design of the 3D parts in several iterations was done using the computer-aided design suite PTC creo (PTC Inc.; Needham, MA). The RATTS schematics are provided as supplementary materials. For LATTS, PTC creo was also used to design the acrylic parts, with a KEM 150W laser cutter (Trotec Inc.; Canton, MI) used for fabrication. Materials used were both optically clear and colored cast acrylic (McMaster-Carr, Elmhurst, IL). The LATTS schematics are provided as supplementary materials.

2.4 Automated odorant detection and thermal mass flow sensors

The TGS 2620 odor sensor operates based on a change of conductance in the presence of volatile odorants (e.g. 3-octanol and 4-methylcyclohexanol) and is used to measure odorant concentration. The electrical properties of the detector require that the sensor be warmed via an internal heater (Figaro, 2002). In the automated system, R_S is measured with the voltage drop over a precision resistor R_{L2} in series with R_S and R_{L1} . The sensor resistance is determined as: $R_S = VC \cdot R_S / V_{OUT} - R_{L1} - R_{L2}$ (Figaro, 2005). VC is the supply voltage of 5.0V DC, and V_{OUT} is the voltage measured over the precision resistor R_{L2} . The ratio between sensor resistance R_S and 300ppm of ethanol resistance R_0 is the sensor signal of interest to deduce 3-octanol and 4-methylcyclohexanol concentrations. The FS5 thermal mass flow sensor consists of two temperature-dependent platinum resistors on one chip. A low-ohm resistor with a small area is used as the heater, whereas the high-ohm resistor measures the reference temperature. Using a bridge circuit, the differing resistance value of the two elements leads to differential (self) heating dependent upon the applied voltage, the mass flow and the media in which the sensor is located. The measuring principle of the sensor can be used for large operation ranges; from 0.1m/s up to 100m/s. Based on the FS5 flow sensor electronic circuit datasheet, LM741 differential amplifier (Texas Instruments) and 2N2222 NPN transistor (ST Microelectronics) were used, excluding the calibration resistor. A LM7805 3-terminal positive voltage regulator (Texas Instruments) and LT1635 operation amplifier (Linear Technology Corporation) were used, including a rail-to-rail output to form voltage subtractor operational amplifier circuits in order to match the input range of the Arduino analog input.

2.5 Commercial parts for the automated olfactory training and testing apparatus

RATTS uses two servomotors (FS5106B, SparkFun Electronics Co., Niwot, CO) with airflow control to transfer staged adult *Drosophila* from training to testing phases. LATTS instead uses a linear actuator (LAC2P, Creative Werks Inc., Bensenville, IL) to achieve elevator manipulation with airflow control to move the animals. All other components used in both RATTS and LATTS designs are commercial off-the-shelf parts. There are five solenoid valves (VDW350-6G-2-01, SMC Co., Tokyo, Japan), each connected to a polystyrene odor cup containing mineral oil-diluted odorants or an empty odor cup (air blank). Three other solenoid valves regulate airflow control. Eight fittings are used; 1) straight adapter 3/8" OD (outer diameter) x 3/8" NPT (national pipe thread taper) male (51215K107, McMaster-Carr), 2) straight adapter 1/4" OD x 1/8" NPT male (51215K104, McMaster-Carr), 3) reducing connector 1/4" x 3/8" OD (5779K355, McMaster-Carr), 4)

wye 3/8" OD (5779K46, McMaster-Carr), 5) wye 1/4" ID (5463K723, McMaster-Carr), 6) cross 1/4" ID (5463K96, McMaster-Carr), 7) one-way check valve 1/4" barb (47245K27, McMaster-Carr) and 8) straight adapter 1/4" OD x 3/8" NPT male (5779K111, McMaster-Carr). All components are connected by tubing carrying compressed air in four standard sizes; 1) OD 3/4" x ID 5/8" (Vinyl Tubing, Home Depot Inc., Nashville, TN), 2) OD 3/8" x ID 1/4", 3) OD 1/4" x ID 0.170" and 4) OD 1/8" x ID 1/16". Both IDE (Arduino IDE 1.0.6, New York City, NY) and Labview (National Instruments Co., Austin, TX) are used to operate a Uno R3 board (Arduino) controlling the gating of solenoid valves for airflow through one of three odor selectors into the training tube. Both IDE Arduino and Labview also control 1) programed movement of the linear actuator (LATTs), and 2) delivery of electric shocks by regulating a square pulse stimulator (SD9 Square pulse, Grass Technologies Inc., Warwick, RI). A Taguchi Gas Sensor (TSG2620, Figaro USA Inc., Arlington Heights, IL) with high sensitivity to organic solvent vapors is employed to monitor the odorant concentration in the training tubes. The change of conductance in the sensor in the presence of odors is used to measure odorant concentration (Figaro, 2002). Two thermal mass flow sensors (FS5.A.1L. 195, Innovative Sensor Technology USA, Las Vegas, NV) in each testing tube are employed to monitor airflow rates by utilizing thermal properties of flow dynamics. Stimulation voltages are recorded with an Arduino data logger shield (Adafruit Industries, New York City, NY) with a 2Hz sampling frequency. A list of all the component parts used in the automated systems construction is shown in Fig. S2.

3. Results

3.1 Rotating Automated Training and Testing System (RATTS)

The first automated design engineered was the RATTS, which uses two servomotors to move multiple 3D printed parts (Fig. 1). Air leakage between the printed components initially made the vacuum pump suction insufficient, rendering the delivered odorant concentrations too dilute for effective training. Therefore, we introduced slots in the printed components to accommodate O-rings sealing the interfaces between the parts, in order to make the entire design airtight (Fig. 1A). The attachment port for the vacuum suction is located in the center of the RATTS assembled cylinder (Fig. 1B), since symmetric odor evacuation is critical for the system design. Odorant delivery is controlled by a series of solenoid valves (Fig. 1C). Sensors are integrated into the system to detect both vacuum-controlled airflow and odorant concentration. An air pump is used to move *Drosophila* between training, resting and testing chambers (Fig. 1C). The frequency and strength of the air movements can be independently controlled to move the animals effectively with the minimum required force. Multiple rounds of testing were required to optimize the dimensions of the 3D printed components, including the rotating cylinder (Fig. S3), filter screen (Fig. S4), non-rotating compartment (Fig. S5) and the assembly cap (Fig. S6). The supplementary figures show the optimal final dimensions determined for each printed component; with bottom, front and left side cross sectional views. A particularly tight fit is required between rotating cylinder, non-rotating central compartment and the assembly cap. The 3D printed material (acrylonitrile butadiene styrene; ABS) has to be airtight to maintain vacuum and for effective air movements of animals between each sequential chamber.

Drosophila had to be able to move freely without restrictions, and yet also be maintained in each chamber for training, resting and testing phases.

Figure 2 illustrates the RATTs flow map for shock training and learning/memory behavior testing. There are a series of regulated openings and grates to allow differential airflow and animal movement during both the training and testing phases. Coordinated rotation of the 3D printed pieces in sequence is required to progress through each experimental phase. The first step is to introduce *Drosophila* (100 staged animals) into the training tube (Fig. 2), with all subsequent steps fully automated. The animals are first trained with a series of CS+ and CS– odorant presentations in the training tube, separated by air blank treatments. The servomotors rotate the cylinder screen filter by 45° increments to regulate *Drosophila* movement (Fig. 2). The animals are moved from the training tube to the center chamber of the cylinder by an air push from the pump, and are then retained by closing the portal through screen filter rotation for a specified period of time (Fig. 2). A further clockwise 45° rotation opens portals to the testing tubes with airflow presentation of both CS+ and CS– odorants at the same time. The animals have a specified time period to choose and move toward either odor. An anticlockwise 45° rotation closes portals to the testing tubes, trapping the animals, which are then counted to determine a performance index (Fig. 2). Spaced training trials separated by 15 minute rest periods are used to assess memory. *Drosophila* are then held for 24 hours prior to the testing phase for memory consolidation (Fig. 2). We found that animals could be held in the RATTs assembly for 24 hours without significant death, and therefore that experimenter involvement is not required for either learning or memory testing.

Following extensive testing, multiple problems were encountered with the RATTs system. First, a disproportionate number of *Drosophila* remain in the RATTs cylinder centerpiece chamber instead of choosing between the odorant pathways in the testing phase. Despite multiple design rounds to optimize the component dimensions (Figs. S3–S6), the rotational requirements of the RATTs cylinder and screen filter diameters left the center chamber volume too large, compromising *Drosophila* odor detection and choice movement. This flaw could not be efficiently corrected because the RATTs rotating design prevented the cylinder diameter from being sufficiently reduced (Figs. S3–S6). Second, significant odor delivery imbalance was routinely experienced in the cylinder centerpiece chamber due to uneven airflow through the system. The airflow problem is compounded by the inability to further reduce the rotating parts of the RATTs assembly, as testing and training tube diameters dictated the minimum dimensions for all other components. Third, the RATTs rotating design prevented it from being sufficiently airtight, an absolute requirement for effective airflow animal transfer. Despite the introduction of O-ring seals, the requirement for 45° rotational changes to open/close portals introduced too much surface area to effectively control leakage (Fig. 2). Moreover, the 3D printed material (acrylonitrile butadiene styrene; ABS) remained somewhat porous even after the use of multiple sealants, which were difficult to apply to the full cylinder assembly and introduced a variable that was undesirable for scaling up to the full behavioral testing facility. Owing to these multiple limitations with the RATTs, we began a second generation design employing a totally new approach.

3.2 Linear Automated Training and Testing System (LATTS)

In order to solve the problems encountered with RATTS, a new linear design, which more closely mimics the traditional manual apparatus was developed (compare Fig. S1 and 3; Tully and Quinn, 1985). This simplified linear system effectively solved the airtight problem and allowed the centerpiece chamber size to be greatly reduced in volume (Fig. 3A). Initially, a Stratasys Dimension 768 SST 3D printer was used to synthesize the LATTS core components from the same acrylonitrile butadiene styrene (ABS) material used with the RATTS (Fig. 3B). However, the 3D component surfaces were rough, and post-print finishing was required for the 3D parts to meet the airtight requirements of the final assembly (Mireles et al., 2011). Sandpaper was initially used to smooth the 3D parts, which introduced a variable that is difficult to control for multiple test vehicles. In an improved design, the assembly components were fabricated with optically clear and colored cast acrylic, instead of using ABS. The acrylic material is transparent so that it is easy to track *Drosophila* during the experiment. The LATTS optically clear and colored-cast acrylic parts include the base unit and the moving elevator (Fig. 3C). LATTS employs a linear actuator coupled to airflow control for *Drosophila* transfer between the training and testing stages of the experiment (Fig. 3C). The linear actuator moves the base center (elevator) up and down to achieve *Drosophila* transfer between training, rest and testing stages. The three quick connectors make moving the apparatus very easy (Fig. 3C). The four bolts with wing nuts create an airtight seal without using a clamp (Fig. 3B). The apparatus does not need to be adjusted between experiments. A vacuum pump is used to pull either the odors or the air through the training and testing tubes. As with the RATTS design described above, odorant delivery is controlled by a series of solenoid valves (Fig. 3D). Flows sensors are integrated into the system to monitor airflow, and odorant sensors automatically report odorant concentration. An airflow pump (“airflow push”) is used to generate air pulses to push *Drosophila* from the training and resting tubes into the elevator (Fig. 3D).

The final LATTS design successfully automates both training and testing phases (Fig. 4). The first Arduino Uno R3 board controls the gating of eight solenoid valves, the linear actuator and the electric shock stimulator. A second Arduino Uno R3 board is used with a data acquisition device to log results from an odor sensor, two airflow sensors and stimulator sensor (Fig. 4). During the LATTS optimization phase, the aperture and connector tube sizes were modified for airtight connections throughout the apparatus, including both the training and testing tubes (Fig. S7). After testing the sealed LATTS, the focus shifted on optimizing the rate of *Drosophila* transfer from training tube to elevator. After testing with different airflow push parameters, it was found that multiple airflow pushes delivered in one full 45 psi pulse and three pulses with successively decreasing pressure gave the best transfer results. Each pulse lasted 0.20 seconds with 0.20 seconds in between each successive pulse. The LATTS electronic design is divided into 1) control of linear actuator and airflow, and 2) data logging (Fig. S8). A single transistor relay controls the linear actuator for elevator movement, eight solenoid valves regulating airflow and a digital pin driving the electrical stimulator for the CS+ odor. For data logging, the Arduino Uno R3 board employs one analog pin for the odor sensor, two pins for airflow sensors, and a fourth pin to record electrical shocks (Fig. S9–S11). A square pulse driven by a control circuit delivers a user-determined number and duration of 80 V shocks to the training tube copper mesh (Fig. S12).

Supplemental figures 13 and 14 show how to build the control circuits for the linear actuator, eight solenoid airflow valves and electrical stimulator. Several fail-safe sensors are built into the automatic assembly to track and record all elements of the training procedure. An Arduino data logger shield records data from thermal airflow sensors, odorant concentration sensor and electric shock delivery (Fig. S15–S17). All data are automatically saved to time-stamped files and transferred to a computer, and can be reviewed at any time to ensure all steps of the training procedure occurred as programmed.

Figure 5 illustrates the LATTS flowchart for learning and memory behavior. Engineering the coordinated movement of the central elevator in conjunction with appropriate airflow dynamics was the most critical part of this automated design. The linear actuator controls the elevator, to move *Drosophila* between training, rest and testing coordinates (Fig. 5). Precise timing of all events is necessary to enable the fully automatic procedure. In the learning pathway, 100 animals are introduced into the training tube for sequential presentation of the CS– odor (unconditioned) and CS+ odor (paired with programmed electrical shocks through the copper grid), spaced by air blank rest intervals deliver via the vacuum pump (Fig. 5). *Drosophila* are then moved by the air pump through the solenoid valve and into the center chamber of the elevator. Following a 2 minute rest period, the animals are then moved down the elevator by the linear actuator to the CS+/CS– T-maze choice point. Solenoid valves open to the testing tubes, and the flies are given a programmed period of time to move in either direction (Fig. 5). The valves then close and the elevator shifts trapping the animals in the two test tubes, so that the learning index can be calculated. In the memory pathway, a similar automatic sequence is followed with a couple of key differences. First, spaced training is used with a CS+ stimulus train followed by a 15 minute rest period for a programmed cycle of repeat training (Fig. 5). This spaced training paradigm is required to induce consolidated long-term memory. Second, after training the animals are held for 24 hours. This involves the addition of a resting tube for the flies to be maintained in during the post-training/pre-testing period (Fig. 5). *Drosophila* can be held in the LATTS assembly without significant death or impairment of memory performance, and experimenter involvement is therefore not required for the lengthy memory testing experiment. Following the 24 hour period, animals are automatically moved by air push and linear actuator to the CS+/CS– T-maze choice point, allowed to choose direction, trapped in the testing tubes, and the memory index calculated (Fig. 5).

3.3 The LATTS Software Interface

Both Arduino IDE and Labview applications are used to program all aspects of the automatic training and testing cycle (Fig. 6). The software interfaces operate the Arduino Uno R3 board to control the pre-odor exposure time period, training phase odorant exposure period, CS+ electric shock cycles (voltage, duration, train length and interval between repeat trains), time period to remain in the linear actuator elevator, rest period duration and T-maze choice time. Figure 6A shows the Labview computer-user interface for the learning pathway. Five separate front panel areas enable programming of all experimental parameters. The user selects the “Activate Learning Test” button to conduct the automated learning trial using the parameters coded (Fig. 6A). Figure 6B shows the Labview computer-user interface for the memory application. For this pathway, the computer interface also programs the

spaced training intervals and time period in the resting tube (Fig. 6B, boxes h and i). Box (i) sets the training phase parameter for the number of spaced training cycles to be completed. For a long-term memory experiment, 24 hours post-spaced training rest time is needed, but the rest period duration parameter can be changed in box (h). The user then selects the “Activate Memory Test” button to conduct the automated memory trial using the parameters coded (Fig. 6B). Once the full set of parameters is entered for either learning or memory pathways, they can be saved for repeat use of the program.

The software is designed to be user-friendly and as self-explanatory as possible (Fig. 6). Flexibility has been built in at all levels for user-defined changes throughout both the learning and memory pathway interfaces. Control software allows operator-input variables to define parameters of *Drosophila* learning, short-term memory and long-term memory assays. The Labview user interface environment is based on user-defined application blocks in the front panel (Fig. S18A). The Arduino IDE user interface employs code directly loaded into the Arduino Uno board to control the solenoid valves, linear actuator and electrical stimulator (Fig. S18B). Both software applications can be used independently to control the experimental trial. When the experiment parameters are to remain constant for long periods, the code can be directly uploaded directly from the Arduino IDE and the Arduino Uno R3 board, rather than using the Labview user interface. This saves the experimenter time at the start of the experiment and can be used to streamline high-throughput pharmacological and genetic screens. The system code for the Arduino IDE and the Labview front panel code can be downloaded from the web (supplemental materials show the Arduino code, Labview code and block diagram; Fig. S18–S19). It would be easy for a user to modify this code for other specific applications.

3.4 Comparison of Manual and Automated Learning and Memory Trials

For quantified comparisons with the new automated system, manual learning and memory trials were done in parallel. Alongside genetic background controls (w^{1118}), *Drosophila fragile X mental retardation 1* (*dfmr1*) mutants were tested as an established disease model with characterized defects in learning and memory (Coffee et al. 2012; Gatto et al. 2014). For each training session, 80 flies were loaded into the training tube and acclimated for 2 minutes (Fig. S1). The sequentially introduced odorants MCH and OCT were then each presented for 100 seconds with an air blank of 100 seconds in between. During exposure to the conditioned stimulus odorant (CS+), a series of twenty 80V electrical shocks of 2.5 seconds each were given every 2.5 seconds (Fig. S1). The number of conditioning shocks was increased due to reduced humidity, since high humidity adversely affected the delivery of shock by the shock tube copper gridding. The animals were then exposed to 100-second intervals of odorless airflow, unshocked odor (control stimulus, CS–) and again odorless airflow. Animals were then transferred manually to the center of the elevator and acclimated for 2 minutes in the T-maze choice point chamber (Fig. S1). Finally, the test tubes were manually opened to generate converging air currents carrying CS+ and CS– odors for 2 minutes, after which animals were manually trapped in the testing tubes, anesthetized and counted. Each ½ learning index (LI) was computed ($LI = [(CS-) - (CS+)] / [(CS-) + (CS+)]$) and then the odorants were reversed in a subsequent experiment using the same odorant cups for the full LI to be calculated as the average of the two ½ LIs (Tully and Quinn, 1985). The

manual trial LI values for genetic control averaged 0.53 ± 0.05 ($n=10$ paired $\frac{1}{2}$ LIs; Fig. 7A) and 0.18 ± 0.06 for *dfmr1* mutants ($n=6$ paired $\frac{1}{2}$ LIs). For the LATTS automated trials, the airflow was controlled via programmed opening and closing of the appropriate solenoid valves in complete absence of experimenter involvement. After training, animals were then automatically transferred to the center of the elevator apparatus by the air pump and the linear actuator then lowered the centerpiece to the testing chamber for the T-maze choice of converging air currents carrying CS+ and CS- odors. Animals were then trapped in choice tubes by automatic movement of the linear actuator. The LATTS automatic trial LI for genetic control averaged 0.44 ± 0.02 ($n=12$ paired $\frac{1}{2}$ LIs; Fig. 7A) and 0.17 ± 0.05 for *dfmr1* mutants as predicted ($n=6$ paired $\frac{1}{2}$ LIs). There was no significant difference in LI values, and the automatic system generated less variable results.

Established manual procedures were used to assay 24-hour massed and spaced long-term memory (LTM; Bolduc et al., 2008). In massed training, no rest interval is provided between the 10 training sessions (Fig. 7B). In spaced training, 15-minute rest intervals were spaced between each of the 10 training sessions (Fig. 7C). Following both forms of training, animals were held for a 24-hour rest period before testing for memory acquisition. Manual trial massed memory MI values averaged 0.20 ± 0.04 ($n=10$ paired $\frac{1}{2}$ MIs; Fig. 7B). LATTS massed memory MI values averaged 0.17 ± 0.02 ($n=10$ paired $\frac{1}{2}$ MIs; Fig. 7B). The manual trial spaced memory MI values for genetic control averaged 0.24 ± 0.01 ($n=10$ paired $\frac{1}{2}$ MIs; Fig. 7C) and 0.009 ± 0.07 ($n=8$ paired $\frac{1}{2}$ MIs) for mutants. For the LATTS automated spaced memory trials, additional programming specified 15-minute rest intervals between each of the 10 training sessions, and animals were then automatically transferred by air push (one full 45 psi pulse and three pulses with successively decreasing pressure) into the rest tube. Animals were then transferred to a fresh vial of food for the 24 hour rest period prior to automatic testing. The LATTS spaced memory trial for genetic control MI averaged 0.27 ± 0.015 ($n=10$ paired $\frac{1}{2}$ MIs) and 0.004 ± 0.06 for *dfmr1* mutants ($n=8$ paired $\frac{1}{2}$ MIs). There was no significant difference in MI values under both memory protocols compared to manual trials. Automatic sensor recordings closely and precisely monitor all training parameters during the trials. The odor sensors record characteristic voltage changes for each odor type (Fig. 7D). For the odorant concentrations used, OCT presents a voltage change of +0.3V, the air blank presents +0.25V, and MCH presents a signal of +0.39V. The airflow sensors report and movement deviation between the alternate odor cups (Fig. 7E). The reading of +3.15V represents airflow of ~1500mL/min, with minimal deviation between the odors presented. The shock sensor measurements indicate proper delivery of electrical shocks with the programed timeframe and voltage (Fig. 7F). Differential shock paradigms can be confirmed and monitored with the sensor readings. Together the sensor array provides quality-control tracking of odorant delivery, airflow rates and electrical shock training to confirm that experiments are always performed exactly as programed.

4. Discussion

A fully automated system has been developed for *Drosophila* classical olfactory associative conditioning and testing, which allows highly labor-efficient behavioral learning and memory analyses. This fully automated system provides seamless *Drosophila* transfer from training through testing phases, with experimenter involvement only to load animals at the

outset of a trial and to quantify the choice outcome at the end of the trial. This includes testing at variable time points following conditioned training, allowing assessment of immediate learning, short-term memory (STM) and long-term memory (LTM). Both massed and spaced training give results comparable to labor-intensive manual methods. This automated system also includes numerous fail-safes to ensure that odorants, airflow rates and electrical shocks are delivered correctly and consistently, allowing for experimental quality control and rapid trouble-shooting. The system was designed using inexpensive electronic devices and milled acrylic parts, making it economical and accessible to most investigators.

A semi-automated system for *Drosophila* olfactory training and testing was previously published in this journal (Murakami et al., 2010). However, costly custom-built parts were used in this earlier assembly, and the method stopped short of automating both training and testing phases of the experiment. The semi-automated system used an interface (USB-6501, National Instruments Co.) to connect both shock stimulator and odorant solenoid valves to a PC. 5V-trigger signals transmitted from the USB-6501 were amplified to 12V by a relay (PS7113-1A-A, NEC Electronics) to control the solenoid valves (Murakami et al., 2010). A Labview software application was used to control the gating of solenoid valves controlling airflow through an odorant cup and into the training tube. A semiconductor-based odor sensor (TSG2620, Figaro Co.) was employed to monitor odor concentration in the training tube with outputs recorded using a data acquisition device (USB-6212, National Instruments Co.). Our system expands on this semi-automated system by incorporating both training and testing phases, adding flow sensors to ensure consistent airflow rates, and adding a voltage sensor to ensure correct electrical stimulus conditioning. The Arduino Uno R3 board used in our fully automated system improves on the previous USB-6501 relay (Murakami et al., 2010), and the Arduino platform provides an open-source physical computing base that is widely-accessible to users, with a simple I/O board and development environment implementing Labview.

In the process of optimizing the fully automated system, a number of factors that improved olfactory conditioning and testing efficacy were encountered. Previous work adapted a pulsed odor flow protocol in which electric shocks are precisely paired with fluctuating CS+ odor pulses (Murakami et al., 2010), indicating this approach to be an improvement for conditioning over traditional constant flow protocols. However, we did not find the pulsed protocol to improve on conditioning, especially at more dilute odorant concentrations, and higher odorant concentrations impaired conditioning. We did find that closely timed odorant delivery and shock pairing is essential for optimal conditioning, as previously reported (Tanimoto et al., 2004). In the LATTS assembly, the automated odorant and electrical shock sensors indicated a delay of less than 10 seconds between shock onset and full strength odor concentration is required for the most successful tests. Delaying shock onset by >10 seconds after opening of CS+ solenoid valves consistently decreased performance indices. Moreover, precise pairing of odorant concentrations between training and testing is important due to the ability of *Drosophila* to retain odor intensity memories (Masek and Heisenberg, 2008; Yarali et al. 2009). Thus, preparing odor cups for training and testing compartments in the same time frame improved our automated trial results. The airflow rate did not have a

significant impact on performance indices so long as levels were maintained equivalent between training and testing phases of each trial, as recorded with the integrated sensors.

The airflow push for animal transfer between compartments took the greatest time in perfecting, and most notably impacted performance indices. In automated LATTS trials, high airflow rates counter intuitively impaired the movement of flies from the T-maze central compartment (LI, 60 psi airflow push, average= 0.20 ± 0.03 ; manual transfer, average= 0.49 ± 0.02); 60 and 70 psi air pushes resulted in >50% of transferred flies remaining in the center. Moreover, airflows greater than 50 psi compromised performance indices, presumably due to the high impact and concussive force of the transfer. It was found that setting the air pressure to 45 psi and programing 4 spaced air pushes yielded an optimal balance between high animal transfer rate and high performance indices. However, even this protocol appears somewhat disorienting to the transferred flies, as a T-maze choice time of 4 minutes compared to the standard 2 minutes improved performance indices in the automated tests but not in manual tests (LI, manual, 2 minutes, average= 0.51 ± 0.05 ; manual, 4 minutes average= 0.56 ± 0.1 ; automated, 2 minutes average= 0.33 ± 0.08 ; automated, 4 minutes average= 0.47 ± 0.02). With these modifications, the LATTS generated comparable performance indices to the traditional manual system, albeit with a non-significant trend to be slightly lower for massed leaning and memory. However, the time and labor savings with the automated system far outweigh the slight reduction in testing values. Future design will focus on optimizing airflow transfer in an attempt to further increase the efficacy of the automated system.

With the successful LATTS in hand, our focus will now shift to system scalability. We initially plan to generate an array of 10 LATTS to enable 5 full performance indices to be determined concurrently. This expansion will dramatically increase work-flow, allowing a minimally 5-fold increase in productivity with respect to memory trials, as currently we are only capable of performing 1 full memory assay per day with a manual station. Current manual tests require the full-day efforts of a dedicated researcher, whereas the automated trials will require a minimum of work, freeing the researcher for other purposes. This level of throughput will be essential as we explore *Drosophila* genetic interaction tests and drug trials. Tests of the *Drosophila* Fragile X syndrome (FXS) disease model (Coffee et al. 2012; Gatto et al. 2014) show the automated system will prove ideal for this application. With ever increasing needs in neuropharmaceutical development, LATTS will allow effective drug testing at multiple concentrations, and at multiple stages of development and aging. Moreover, LATTS will allow us to assess combinatorial drug therapies, which are becoming prominent, for example in treating autism spectrum disorders with challenging, wide-ranging symptomatic presentation. There is emerging evidence of beneficial adjunctive treatments with co-administration of memantine, N-acetylcysteine or cyclooxygenase-2 inhibitors with the dopamine antagonist risperidone in autism spectrum disorders (Asadabadi et al., 2013; Ghaleiha et al., 2013; Ghanizadeh and Moghimi-Sarani, 2013). However, such polypharmacy must be undertaken cautiously to maximize benefit while minimizing potential adverse interactions. Rapid, labor-efficient, cost-effective pharmaceutical screening in *Drosophila* disease models, such as the FXS model, should

prove extremely beneficial as a first-line approach to inform multi-drug therapeutic strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

STM	short-term memory
LTM	long-term memory
CS⁺	conditioned stimulus
CS⁻	control stimulus
LI	learning index
MI	memory index
LATTs	linear automated training and testing system
RATTs	rotating automated training and testing system
NPT	national pipe thread taper
OD	outer diameter
ID	inner diameter
ABS	acrylonitrile butadiene styrene

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Highlights

- Fully automated system of *Drosophila* olfactory associative conditioning and testing
- Odorant levels, airflow and electrical shock delivery all automatically monitored
- Control software allows operator-input variables to define experimental parameters
- System allows *Drosophila* learning, short-term memory and long-term memory assays
- Accurate and efficient behavioral learning/memory analyses with a minimum of labor

Fig. 1. The rotating automated training and testing system apparatus

(A) Schematics of the RATTTS 3D printed parts, depicted left to right: RATTTS cylinder, screen filter, non-rotating guide and support, and the cylinder cap. The cap is used for fixing the two servomotors, which rotate the cylinder and the screen filter. (B) Image showing the fully assembled RATTTS cylinder. The two servomotors can be seen at the top and bottom, attached to the cylinder cap. The attachment vacuum suction port with exit tube is located at the center right. (C) Image showing an expanded single unit of the RATTTS apparatus assembly, with tubing, connectors, solenoids and electrical control components.

Fig. 2. Flowchart schematic diagram for the RATS behavioral training and testing

Diagram showing the RATS training and testing flow map. 3D printed parts include the central cylinder, screen filter, non-rotating core and assembly cap. One servomotor controls the cylinder and another servomotor controls the screen filter. 45° rotation through the prescribed positions drives each stage of the experimental procedure, as detailed. Both immediate learning (2 minutes after massed training) and long-term memory (24 hours after spaced training) can be done without any experimenter involvement between introducing *Drosophila* into the training tube at the start of the experiment, and counting *Drosophila* in the testing tubes at the end of the experiment to determine the performance indices.

Fig. 3. The linear automated training and testing system apparatus

(A) Schematics of the LATTS assembly shown left to right; the LATTS elevator, left base and right base. (B) Image showing the 3D printed parts forming the base connection. The four corner bolt and wing nuts (red arrows) functioned as a clamp to make the assembly air-tight. (C) The full LATTS assembly is shown, with the linear actuator and three quick connectors mounted on a wooden board. (D) Image showing an expanded single unit of the LATTS apparatus assembly, with tubing, connectors, solenoids and electrical control components for the air-flow configuration. The letters correspond to the parts shown in the Figure 4 schematic.

Fig. 4. Schematic LATTs diagram showing the full assembly

The system is composed of an air pump, vacuum pump, electrical stimulator, two thermal mass airflow sensors, one odorant sensor, eight solenoid valves, three one-way check valves, five odor cups, the indicated different sizes of connecting tubing, and a controlling computer. The eight solenoid valves act as switches to control odorant delivery and *Drosophila* movement with airflow. The vacuum pump drives odorant and air blank (rest) airflow. The air pump provides the force to move *Drosophila* from the training tube or resting tube to the center of the elevator cylinder. The two thermal mass flow sensors chart airflow rates and the odor sensor ensures correct odorant delivery. The vinyl tubing (1/4" OD x 0.170" ID) connects to 1/4 NPT Female two-port solenoid valves 1/4" OD x 1/4 NPT Male fitting for odorant delivery.

Fig. 5. Flowchart schematic diagram for the LATTS behavioral training and testing

Schematic diagram is showing LATTS training and testing following the separate learning and memory pathways. The linear actuator controls the elevator, which moves *Drosophila* between training, resting and testing compartments. Computer controlled solenoid valves regulate the airflow for odorant delivery and animal movement in and out of the elevator. The CS+ odor is paired with a programmed series of electrical shocks applied through the copper grid in the training tube. Both massed and spaced training can be employed, with user-selected shock intensity, number and spacing. The 24-hour memory pathway includes a resting tube for fly maintenance during the post-training/pre-testing period.

Fig. 6. Operator panel of automated software application

(A) The learning pathway computer-user interface showing five separate areas to set experimental parameters. Panel (a) tracks progression through selected procedures, with a new trial set in panel (b). Panel (c) sets pre-odor exposure time. Panel (d) sets electrical shock parameters. Elevator controls and T-maze choosing times are coded in panels (e) and (f). Panel (g) programs training odorant exposure time. (B) For the memory interface, panels (h) and (i) allow additional modifiable parameters, with the post-training rest period coded

in (h) and the number of spaced training cycles selected in (i). Select “Activate Learning Test” or “Activate Memory Test” buttons to initiate the respective experimental trials.

Fig. 7. Comparison of manual and LATTs automated trials outcomes

(A) *Drosophila* were trained and tested for learning using both the traditional manual apparatus and automated LATTs in parallel. Learning index (LI) values are comparable between the two approaches, with no significant difference between manual and automatic trials. (B,C) *Drosophila* were trained and tested for massed (B) and spaced (C) 24-hour memory using manual apparatus and automated LATTs in parallel. Memory index (MI) values are again comparable. All data are presented as the mean $\pm$ SEM. (D) Odor sensor data for OCT, air blank and MCH at concentrations used. The red graph represents OCT as CS+, black shows MCH as CS+. (E) Airflow sensor data of pressure during training. Left panel shows OCT presentation, middle panel (grey) blank airflow and right MCH. (F) Shock sensor data of 10 training shock delivery with proper intervals during CS+ presentation.