

Technique Notes



Monitoring activity of *Drosophila* larvae: Impedance and video microscopy measures.

Cooper, Ann Simone, and Robin L. Cooper¹. Department of Biology, University of Kentucky, Lexington, KY 40506-0225; Key Words: circadian, technique, insect, behavior; ¹To whom correspondence should be addressed; Phone: 859-257-5950; Fax: 859-257-1717; [Email: RLCOOP1@pop.uky.edu](mailto:RLCOOP1@pop.uky.edu)

It is known that adult *Drosophila* show circadian patterns in movement and feeding behavior (Saunders, 1997). Circadian cycles in adults can be entrained by exposure to light as early as the embryo stage. In addition, entrainment for a circadian cycle can be induced in larval stages. The neurons responsible for this are controlled by the larval optic nerve (Bolwig's nerve) (Hassan *et al.*, 2000; Helfrich-Forster *et al.*, 2002; Malpel *et al.*, 2002). As far as we know there has yet to be a report on measures of circadian rhythms in *Drosophila* larvae, possibly because of the difficulty in such measurements.

To examine circadian rhythms in larval stages we used various approaches to image larvae from the 1st instar (1mm in length) to 3rd instar (4-5mm in length). Problems in monitoring larvae arise in that they burrow in their food. If the food is too deep one can not project white light through the food. In order to maintain the health of the animals over prolonged periods in examining patterns of movement, such as circadian rhythms, the animals must have access to food and be maintained in a humid environment. In addition, larval stages from the 1st to early 3rd instar are negative phototactic, while the late 3rd, usually referred to as the wandering 3rd, are positive phototactic. If food is present the larvae tend not to exhibit locomotion but to continually eat in a static location. Thus, the black mouth hooks and entire head are readily seen moving back and forth while the animals eat. The mouth hook movement is a common behavioral bioassay for *Drosophila* larvae (Neckameyer, 1996). If no food is provided the larvae will crawl over the surface thus making it hard to define its territory for monitoring. If Petri dishes are used the larvae crawl on the walls as well as on the lids. Thus, movements within different planes of focus make optical recording procedures difficult to use over an extended period of time. Additionally, the required humidity produces condensation on the surfaces of the containers. With uneven layers of food the small larvae are also readily lost visually for periods of time.

In this report, we present a technique termed the "Ant Farm Technique" of two glass plates (microscope slides; 75 x 25 mm; J. Melvin Freed Brand) narrowly spaced (1 to 1.5 mm) apart by a thin layer of larvae food (*e.g.* moist corn meal- a modified version of Lewis, 1960; Appendix) so that the larvae are able to be visualized within one plane of focus. To prevent larvae from crawling out from the edges of the two plates of the glass modeling clay is used. The larvae are very susceptible to dying if the carbon dioxide is not allowed to escape the chamber. The use of cotton on one edge is sufficient for gas exchange. Problems arise with a level platform since larvae become trapped in the moist food and the meniscus of the water and separate the chamber preventing exchange of gases with the outside. We have found that by slightly tilting the platform at 20 to 45 degrees the larvae remain, the majority of the time, with their head pointed downward and their tail containing the spiracles out above the food or within an air passage in the food layer.

This "Ant Farm Technique" also allows video imaging within a single plane with food of uniform thickness. Since in this configuration the larvae tend not to crawl rapidly throughout the food, but instead to eat and gradual move around in the 2D plane. Thus, the rapid head movements are readily observed and can be used as a desired behavioral trait to monitor for determining activity patterns. Larvae tend to eat continuously throughout their larval stages albeit at various rates. If monitoring the mouth hook movements is specifically desired then video microscopy will suffice with this arrangement. We have monitored instars from the 1st to the 3rd wander stage without disruption in this manner. White light was used 24 hours a day by use of a light box projecting light through the food. The light box used a 15 W neon light and was kept at a 30 cm distance from the glass plates. The microscope (adjustable zoom 0.67 to 4.5; World Precision Instrument; Model 501379) was tilted in order to remain parallel to the tilted glass plates. A 0.3× base objective and tube objective 0.5× were used to gain enough spatial resolution and magnification to cover a 1 cm by 2 cm rectangle. A mounted camera through a trinocular mount was used (Mintron, MTV; World Precision Instrument). The ambient temperature was maintained at 20°C with good circulation.

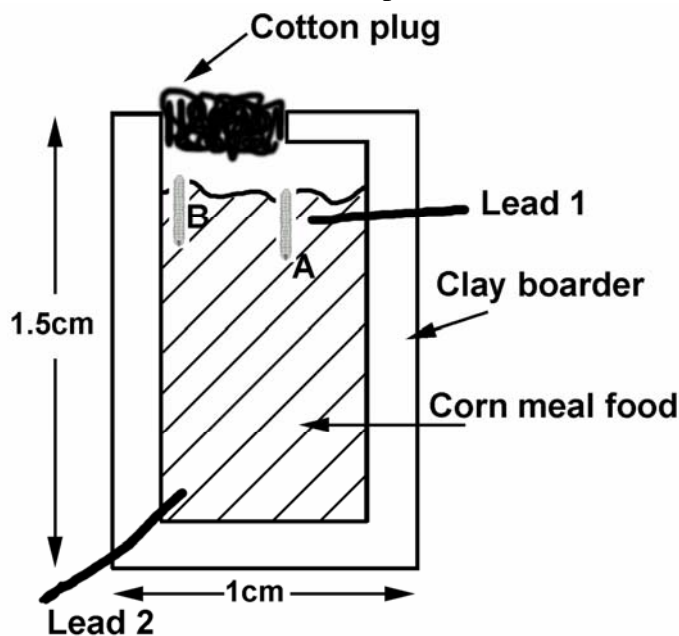


Figure 1. Schematic of the "Ant Farm" chamber. Two glass slides sandwich the clay border with the wires held firmly in place. The food is kept moist by the liquid tight borders with the exception of allowing gas exchange through the cotton plug at the top of the chamber.

Possibly this procedure could work when viewing in red light to examine if circadian behaviors existed in what the animal would perceive as a dark cycle. With our current video microscopy setup we did not have enough sensitivity to detect body wall or the mouth hook movements in red light. Thus, we developed an electrophysiological measure of dynamic resistance, also commonly referred to as an impedance measure, to determine any body movement from crawling to head movements for eating. Any slight change in resistance of the media induced by the animal's movement is able to be detected. With this recording arrangement, while the apparatus remains in total darkness, we were able to avoid even using red light. Studies are now underway to monitor circadian patterns with this technique (Cooper *et al.*, 2005).

Two insulated silver wires (diameter 0.005 inches and with the coating 0.008 inches; A-M systems, Inc., Carlsburg, WA) were placed through the modeling clay so the tips were exposed to the corn meal solution. These two wires need to span the chamber to insure a measure if the larvae moved within the chamber (Figure 1). The output of the impedance detectors (UFI, model 2991) was recorded on-line to a PowerMac 9500 via a MacLab/4s interface (ADInstruments). All events were measured and calibrated with the MacLab Chart software version 3.5.6 (ADInstruments, Australia) with an acquisition rate set at 400 points/sec. The deflections in the baseline can be calculated as activity over time. The impedance measures have been used in the past successfully to monitor heart rate in intact crayfish (Listerman *et al.*, 2000).

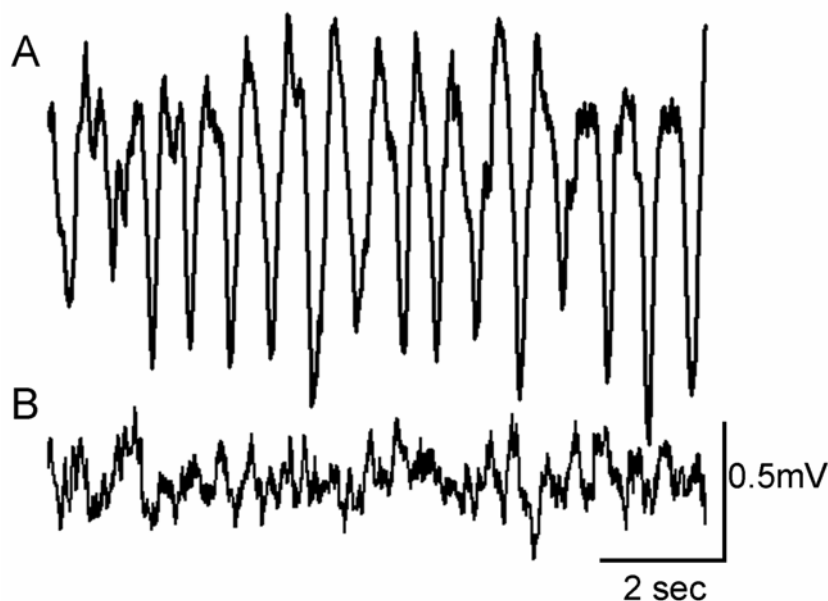


Figure 2. Impedance measures when the larva is close (A) to a lead (Position A in Figure 1) and far (B) from a lead (Position B in Figure 1). The entire trace is 10 seconds in duration.

Several larvae or single larva can be placed into a given chamber. However here we wanted to directly link movements of a larva with the impedance signals. As a larva approaches close to a lead, the deflection of the signal increases (Figure 2). To insure accurate measure of movements to impedance signals, carbon dioxide was used to anesthetize the larva while in the apparatus. When the larva ceased all movements the impedance measures flat lined. As the larva recovered and started to move and resume eating the impedance signals were also revived. This procedure can also be designed for many parallel units of single or multiple larvae for high throughput in screening *Drosophila* larvae in relation to mutants and drugs mixed with the food. A

disadvantage in this technique is that the leads must remain in electrical contact through the media.

Appendix: *Drosophila* food: Water (17 l), agar (93 g), cornmeal (1,716 g), inactive yeast (310 g), sucrose (517 g), dextrose (1,033 g), phosphoric + propionic acid mix (164 ml distilled water to 836 ml of propionic acid. Add 917 ml distilled water to 83 ml of phosphoric acid. Combine the two diluted acid solutions to produce the acid mix -use 200 ml), and 1.6 vol tegosept in EtOH.

Acknowledgment: Funding was provided by Kentucky Young Researchers Program at the University of Kentucky (A.S.C.).

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Monitoring carbon dioxide production by *Drosophila* larvae.



Cooper, Robin L.¹, and D. Nicholas McLetchie. Department of Biology, University of Kentucky, Lexington, KY 40506-0225; Key Words: circadian, technique, insect, behavior, respiration; ¹To whom correspondence should be addressed; Phone: 859-257-5950; Fax: 859-257-1717; [Email: RLCOOP1@pop.uky.edu](mailto:RLCOOP1@pop.uky.edu)

Adult insects vary metabolic rates throughout stages of development as indicated by patterns in their movement, feeding behavior, and growth (Balderrama *et al.*, 1992; Economos and Lints, 1985; Van Voorhies *et al.*, 2004). The production of carbon dioxide (CO₂) is a common non-invasive procedure to index rates of metabolism of insects and vertebrates. Also variation in CO₂ is a bioindex to measure circadian cycles in insects and mammals (Barrozo *et al.*, 2004; Stupfel *et al.*, 1995). As far as we know there has yet to be a report on measures of circadian rhythms in *Drosophila* larvae by any technique. The lack of such studies might be due to the difficulty in such measurements for single animals that will burrow in food and continuously move in order to eat.

Our goal is to examine circadian rhythms in larval stages of *Drosophila*. In doing so, we devised a method to monitor whole body metabolism by monitoring CO₂ production using a gas analyzer with a sensitivity range in fractions of a part per million of CO₂. Most commercially

available gas analyzers for animals are not designed for such small measurements of single insect larva such as *Drosophila* that are only 1 mm to 4 mm in length. In addition, to keep the larvae healthy during measures, required for developmental studies or examining circadian patterns, the larvae must have access to food and be maintained in a humid environment. One also

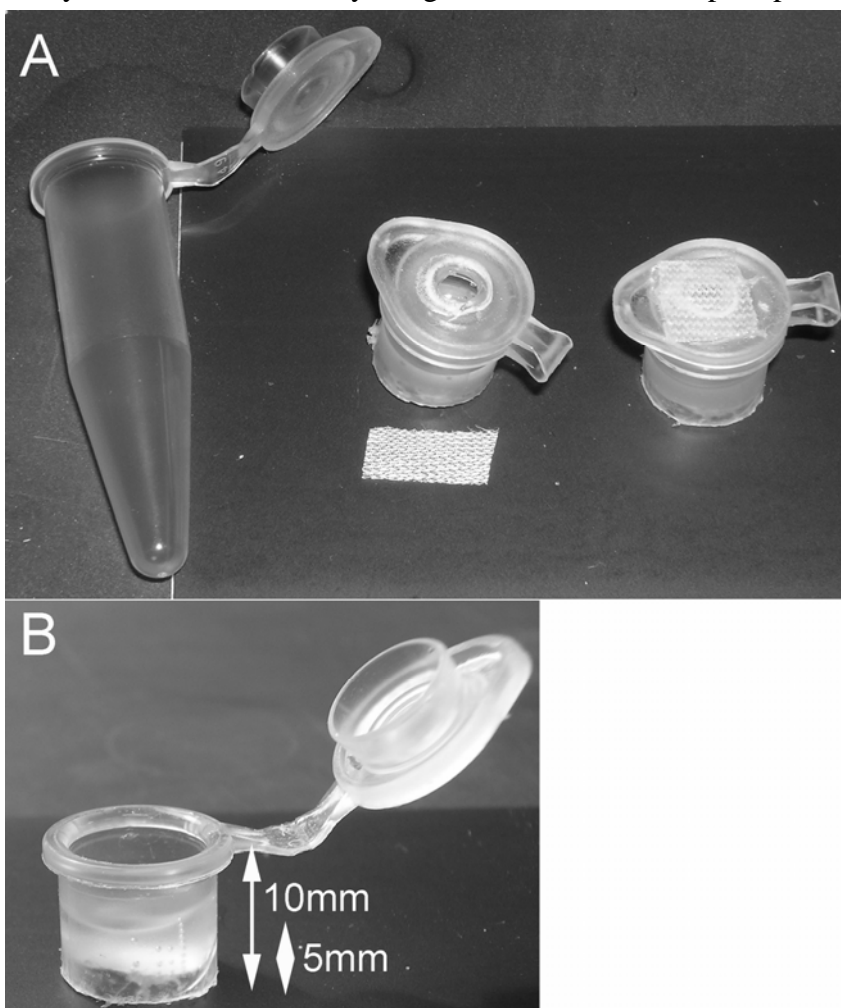


Figure 1. Schematic in the construction of the subchamber. (A) 1.5 ml microcentrifuge tube is cut and a hole is made within the lid. Plastic mesh is cut and glued with super glue to the lid. Sylgard is filled in the base and cured at 65°C over night. (B) One needs approximately 0.5 cm of space from the top of the tube to the Sylgard. A length of 1 cm is about the limit to fit within the compartment of the standard "leaf chamber".

requires a means of controlling the light cycle, since it can have an impact on development as well as circadian rhythms. Commercially available gas analyzers designed to monitor CO₂ utilization in plants provide the sensitivity needed for monitoring single larva of *Drosophila*. In addition, light intensity, humidity and temperature as well as CO₂ are regulated in some commercially available instruments for monitoring plant gas exchange.

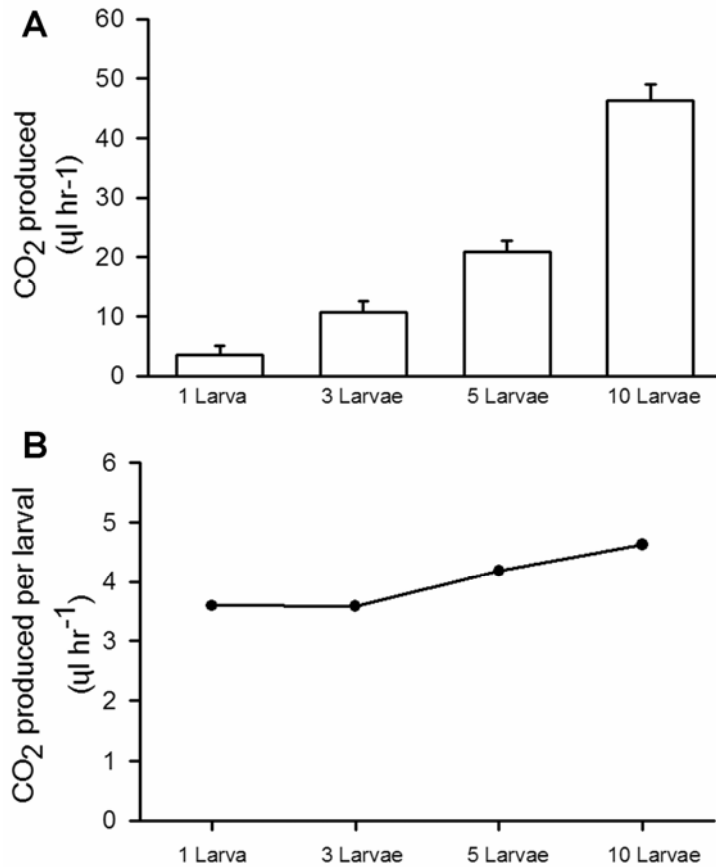


Figure 2. (A) Measures for CO₂ as µl hr⁻¹ in controls (food containing subchambers), single larva, 3 larvae, 5 larvae, and for 10 larvae are reported as a mean (± SEM). Each of the three values for the three trials are shown. (B) Determining the CO₂ produced per larva for each condition indicates that only a slight rise in metabolism occurs when larvae are maintained as a group.

In this report, we present a technique using the LI-COR model LI-6400 (LI-COR Biosciences, 4421 Superior St., Lincoln, NE. 68504, USA) that is designed to be portable for field studies and have a CO₂ monitoring sensitivity range of 0-3000 µmol mol⁻¹. The CO₂ is measured by a non-dispersive infrared gas analyzer. This instrument is also capable of regulating CO₂, humidity, temperature, air flow, and lighting conditions. However, the leaf chambers are not designed to maintain

animals from moving into the monitoring devices or out of the region for directed air flow. Thus, we designed a sub chamber which fits well within the standard the leaf chamber to contain the larva and food (Figure 1). The sub chamber is an inexpensive design that can easily be produced in any laboratory setting. A standard micro centrifuge tube (1.5 ml) is trimmed to 1 cm in length. The cut end is placed in a baking clay and the bottom filled with Sylgard (184, Dow Corning Corp., Midland, MI, USA) that is cured (hardened) by placing the tube and clay within an oven (65°C) overnight. Unlike wax or soft clay, the baking clay hardens preventing the Sylgard from leaking during its curing process. A space of 0.5 cm from the top of the tube is left for placing food and the animals. A hole in the lid of the tube is made with a soldering iron and a fine plastic screen mesh is super glued over the hole. The lip of the lid to open the top and the hinge of the lid is used to hold the sub chamber between the gaskets of the leaf chamber. The hole allows for gas exchange, the net prevents the larvae from escaping, and the ability to open and close the sub chamber allows for ease of sample preparation.

Wild-type (Canton S) of *Drosophila melanogaster* were maintained at 25°C on a standard cornmeal medium. Only early 3rd instar larvae were used (Li *et al.*, 2002). We ran five conditions to determine the feasibility of monitoring CO₂ with the current experimental design and instrumentation. In all the tubes, 0.25 grams of fresh corn meal for food was placed. Larvae were

raised on a standard corn meal medium (a modified version of Lewis, 1960; Appendix 1) as a food source. Triplicates without larva (control), single larva, 3 larvae, 5 larvae, and 10 larvae were prepared. The tubes were left undisturbed for 30 minutes prior to gas analysis. Controls were used to detect any baseline gas exchange between the corn meal and air.

The integrated software and display panel built within the instrument allows real time graphical as well as digital readings of pertinent variables. Set conditions were flow rate = $100 \mu\text{mol s}^{-1}$, $\text{CO}_2 = 450 \text{ ppm}$, temperature = 27°C , humidity ranged from 57% to 44%, no light. Tubes were randomly placed in the leaf chamber. CO_2 coefficient of variation was allowed to stabilize (2 to 5 min). Each sample was assayed every 2 minutes for the triplicate recording. The instrument outputted CO_2 gas exchange as a variable named "Photo" which was in units of $\mu\text{moles CO}_2/\text{sec}/\text{leaf area}$. Conversion of these units to units of $\mu\text{liters CO}_2/\text{hr}$, which are the units typically used in the respiration literature, are detailed in Appendix 2. The mean respiration rate of these triplicate recordings was used in the analysis. The average of the controls was subtracted from the experimental groups.

As the number of larvae increase CO_2 production increased. The means (\pm SEM) are: single larva $3.5983 \mu\text{l hr}^{-1}$ (± 1.539), 5 larvae $10.7485 \mu\text{l hr}^{-1}$ (± 1.8704) and 10 larvae $20.9291 \mu\text{l hr}^{-1}$ (± 1.7896). There was consistency in the values within the triplicates ran on each sample and among the three trials for each treatment. Figure 2A shows the mean of all three trials for each condition. When calculated on a per larva basis, larvae in the higher density produced approximately the same CO_2 as for larvae in the lower density (Figure 2B).

We have shown clearly that CO_2 can be measured for *Drosophila* larvae with the use of an instrument designed originally for botanical research in photosynthesis and respiration. We have not examined the possibilities of using the instrument for metabolic correlation or circadian patterns by varying light or other environmental conditions. The instrument used in this study has several different types of chambers available, including chambers with built-in software controllable light sources and transparent chambers. So there are various possibilities to regulate light and correlate with continuous metabolic activity of the animals. This will allow circadian measures by metabolic activity to be monitored. Many experimental perturbations in the environment and food sources can be implemented while following the changes induced by single *Drosophila* larvae. There is a growing need for such measures as there is a number of mutations associated activity in *Drosophila*. There is also a need in the field to use bioindices for monitoring whole animal metabolism to assess better the effects of particular mutations. The procedures presented herein can also be used for other insects in their larval as well as adult forms.

To control the level of ambient CO_2 within the environment of the chamber small canisters (12 g pure liquid CO_2 cylinder) are available to use. However the use time is about 6 to 8 hours so, if prolonged periods are being monitored or if one did not want to disturb the gas flow in the chamber while conducting experiments, an adaptor is available from the company to hook larger gas cylinders with much longer usage times.

Appendix:

1. *Drosophila* food: Water (17 l), agar (93 g), cornmeal (1,716 g), inactive yeast (310 g), sucrose (517 g), dextrose (1,033 g), phosphoric + propionic acid mix (164 ml distilled water to 836 ml of propionic acid. Add 917 ml distilled water to 83 ml of phosphoric acid. Combine the two diluted acid solutions to produce the acid mix -use 200 ml), and 1.6 vol tegosept in EtOH.

2. Calculation using the LI-6400. The variable "PHOTO" is in units of $\mu\text{moles}/\text{sec}/\text{area of leaf}$ (in this conversion the area is calculated to be for a meter²). To convert μmoles to μliters the value is multiplied by 22.41 (*i.e.* 22.41 liters in 1 mole or 22.41 μliters in 1 μmole). To convert seconds to hours the product is multiplied by 3600. To remove area of the leaf one must divide the product by

the 10,000 assuming a 1 cm² area is entered into the software for a given sample. If a different area is entered in for area of leaf then 10,000 is divided by the area entered into the software. This conversion provides the units of "μliters/hr".

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Monitoring heart rate in *Drosophila* larvae by various approaches.



Dasari, Sameera, and Robin L. Cooper¹. Department of Biology, University of Kentucky, Lexington, KY 40506-0225; Key Words: technique, insect, behavior, pharmacology, mutations; ¹To whom correspondence should be addressed; Phone: 859-257-5950; Fax: 859-257-1717; [Email: RLCOOP1@pop.uky.edu](mailto:RLCOOP1@pop.uky.edu)

With a number of mutational lines in *Drosophila* which alter larval development and cell-cell signaling, there are increasing quests for biological assays of function and sensitivity assays to exogenously induced compounds. In addition, since it is now well accepted that developmental defects in *Drosophila melanogaster* have correlative significance in mammals (Giudice, 2001; Hirth and Reichert, 1999) more diverse biological assaying abilities are required to examining physiological topics under various conditions. Measures of heart rate in larval *Drosophila* have been used for a number of years to assay the effects of biogenic amines, peptides and pharmacological agents (Johnson *et al.*, 1997, 2000; Nichols *et al.*, 1999; Zornik *et al.*, 1999). Since the larval *Drosophila* heart is myogenic (Dowse *et al.*, 1995; Johnson *et al.*, 1997), as in mammals, possibly developmental defects common to both systems could be investigated more readily in *Drosophila* because of the short developmental time, easy rearing conditions, and mutational manipulability. In this report we present three ways which add to the diversity of approaches to recording heart rate within intact larval *Drosophila*. In addition, we introduce two physiological salines (HL3 and HL6) that have yet to be examined on *in situ* preparations of the larval *Drosophila* heart.

The *Drosophila* heart is also referred to as the dorsal vessel. It is a continuous tube extending from the last abdominal segment to the dorso-anterior region of the cerebral hemisphere. The heart is divided into anterior aorta and posterior heart (Figure 1; Rizki 1978). The tracheal movements can readily be seen moving in unison with each heart beat. This occurs because the heart pulls on the ligament attachments which the trachea are connected. Thus, the movement of the trachea are commonly used to monitor *Drosophila* larval heart rate because of the clear contrast of the tracheal structures as compared to the translucent heart (Miller, 1985; Johnson *et al.*, 1997; Nichols *et al.*, 1999; White *et al.*, 1992).

In order to visualize the beating larval heart directly through a microscope or a projection of an image through a microscope, the larva must remain still enough to obtain counts of the beats. Restraining the larvae could also introduce stress to the animal which would undoubtedly alter the physiological responses one might be trying to assess, such as the sensitivity to introduced compounds or screening mutational lines related to heart function. Other arthropods (*i.e.*, crayfish)

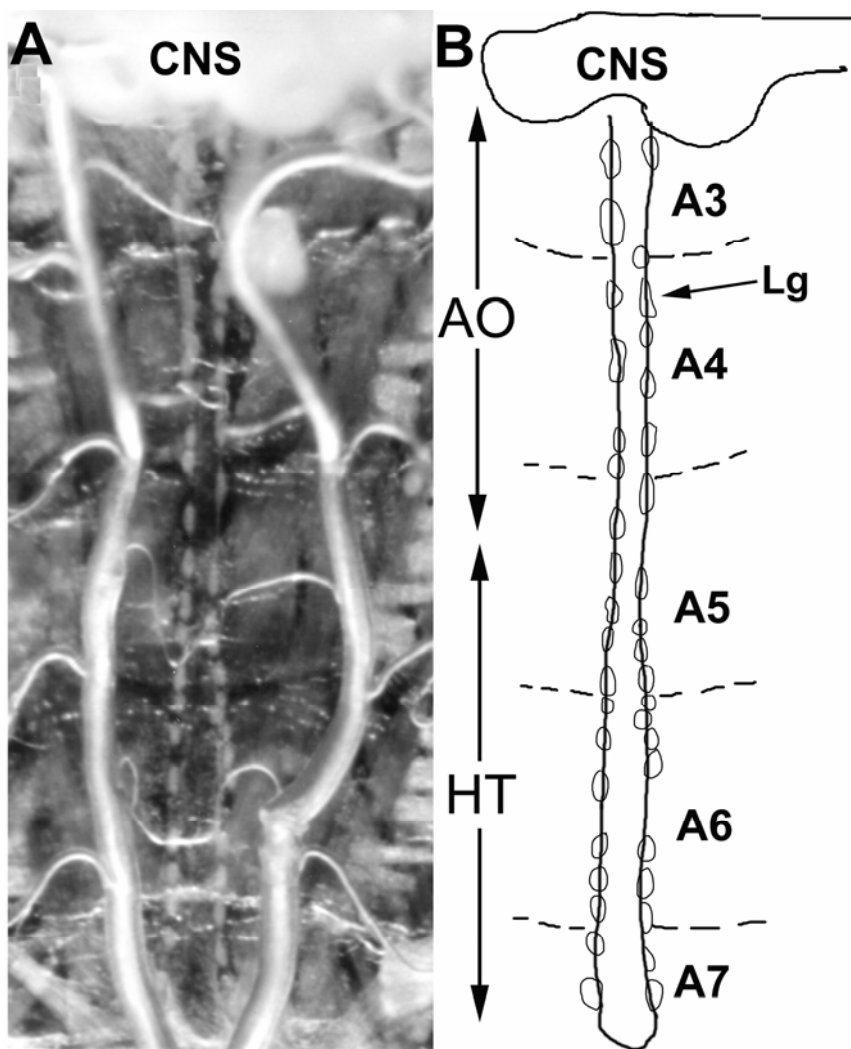


Figure 1. The heart tube is exposed by pinning the dorsal surface of the animal down and approaching the heart by a ventral dissection (A). For illustrative purposes a schematic of the photographed preparation is shown in B. Note the lymph nodes (LN) that line the outer edges of the aorta (AR) and heart (H) allow one readily to see the longitudinal boundary of the heart and aorta.

are known to be sensitive to stress and have altered responsiveness to neuro-modulators (Page and Cooper, 2004). To examine if restraining larval *Drosophila* by adhering the animal to one location would alter heart rate, we developed two means by which to record heart rate in unrestrained animals for comparison to a restrained conditions. In addition, we developed a means to follow an individual over extended lengths of time in unrestrained conditions that could be used to assess pharmacological agents introduced in the diet or to examine various times in development in mutational lines.

The first unrestrained method is to place a few drops of a dilute food mixture over the animal's head. This method we refer to as the "feeding trough" approach (Figure 2A). The second unrestrained method is referred to as the "ant farm" approach (Figure 2B). This technique consists of two glass plates spaced apart by a thin layer of larvae food. The larvae are able to be visualized within one plane of focus. This technique is explained in more detail in

this issue of *Drosophila* Information Service (Cooper and Cooper, 2004) in an electrophysiological approach to monitor larval movements. Here we use a visual assessment at high magnification as to view the two trachea on the dorsal aspect of the larva. This "Ant Farm" technique allows video imaging within a single plane with food of uniform thickness. Since in this configuration the larvae tend not to crawl rapidly throughout the food, but instead to eat and gradually move around in the 2D plane, the glass plates can be moved as to keep the larva in focus while viewing under high magnification. In the "feeding trough" and "ant farm" approach a microscope with an adjustable zoom (0.67 to 4.5; World Precision Instrument; Model 501379) was used. A 2× base objective and tube objective 0.5× was used to gain enough spatial resolution and magnification to cover a 1 cm²

area. A mounted camera through a trinocular mount was used (Mintron, MTV; World Precision Instrument). The ambient temperature was maintained at 20°C.

The third approach consisted of restraining the larva to one location by super gluing the ventral aspect of the larvae to a glass cover slip (Figure 2C). A modified approach that is commonly used to restrain larvae consists of using double stick tape on a glass slide and placing the ventral side of the larva to the tape (Baker *et al.*, 1999). However this approach does not work well if one wants to feed a larva over time, since the moisture of the food is pulled by capillary action along the body wall of the animal, which results in the tape losing its adhesiveness to the animal. We found that placing a thin line of super glue and waiting until it becomes tacky would allow us to place the ventral side of the larva into the glue as it was drying. Thus, the animal can not move and the posterior end remains elongated for good visualization of the dorsal aspect. The head of the larva is placed far enough over the edge of the glass slide as not to allow the drying glue to be pulled toward the head. The head and spiracles remain free from glue. With use of super glue the animal can eat and even be covered in a moist solution while remaining adhered to the glass cover slip.

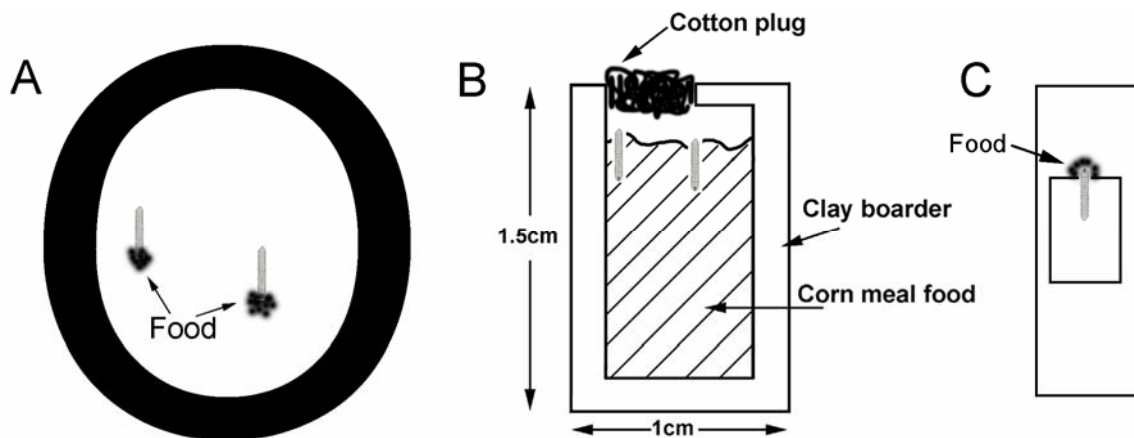


Figure 2. The unrestrained methods to monitor heart rate are shown in the "feeding trough" approach (A) and the "ant farm" approach (B). To restrain larva to one location the ventral side is super glued to a glass cover slip (C). Food is covering the head in A and C. The trachea are seen on the dorsal view and can be monitored readily for movements related to heart rate. In the restrained approach, the cover glass is placed on top of the glass slide. This allows light to pass through the animal from the ventral side. Also the lip of the cover glass to the glass slide provides a space to avoid gluing the mouth as well as providing a means to hold the food over the animals head without covering the caudal part of the body.

To examine how these three approaches affected the heart rate of animals, five larvae were individually housed in food and tested sequentially through the three aforementioned approaches. A larva was placed in a plastic Petri dish (diameter 5.4 cm) and a line of food was placed in front of the animal's head (*i.e.*, the feeding trough). Five to ten minutes were allowed to pass before recording heart rate. Five minutes of heart rate were obtained on VHS tape. Sometimes the animals move into the food and the monitoring of heart rate would be paused until the beats could be clearly observed. The animal was then placed in the "ant farm" that contained the same food content. Likewise ten minutes were allowed for acclimation, followed by five minutes of monitoring the heart rate. After this procedure, one side of the glass was removed from the ant farm to remove the larva and place it on a patch of super glue which was partially cured. When the larva was adhered well (<1 minute) the

head of the animal was covered with food as in the feeding trough procedure. After a ten minute period five minutes of heart rate were monitored. The individual heart rates for each condition are shown in Figure 3. Each point represents an average number of beats per minute (BPM). Note each animal is individually graphed (Figure 3A) as well as a mean (\pm SEM) for the group of five animals (Figure 3B). One larva had a drastic drop in heart rate after being glued, possibly due to gluing effects; however, the majority of animals did not show a large alteration in heart rate among the three conditions.

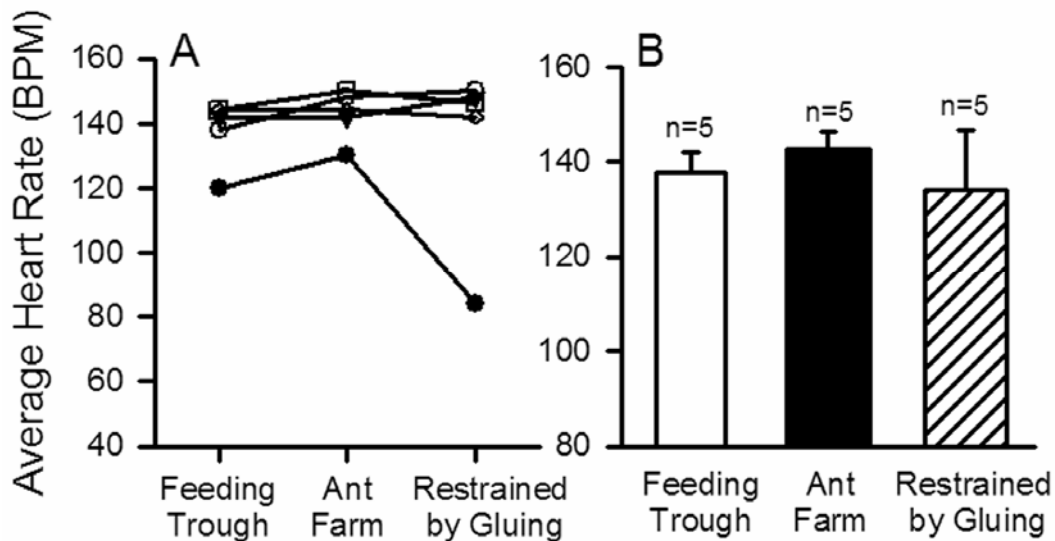


Figure 3. The heart rates, as beats per minute (BPM) for five animals as they were sequentially moved from the feeding trough to the ant farm and to super glue are shown (A). The average rate for all five animals for the five minute periods (\pm SEM) is shown in B.

The exposed hearts of *Drosophila* larvae were used to test the relatively new physiological salines that were initially developed to record synaptic responses at neuromuscular junctions in larval *Drosophila*. These physiologically based salines have yet to be used to examine their feasibility for monitoring heart rate in exposed preparations. The HL3 (hemolymph-like 3 saline) was designed based on measurement of ions by ion sensitive electrodes using pooled larval hemolymph (Stewart *et al.*, 1994). The HL6 is a modified HL3 saline with the addition of various amino acids (Macleod *et al.*, 2002). The pH of the salines was adjusted to 7.2 immediately prior to experimentation. The pH tends to drift upward in these salines over extending periods of time (*i.e.*, a day). Five preparations were dissected as described previously (Gu and Singh, 1995; Nichols *et al.*, 1999). In brief, the animal is pinned with its dorsal surface down and making a longitudinal cut along the length of the animal. The internal organs are carefully moved to one side and removed. Care is taken not to damage segmental nerves or the central nervous system (the larval brain). With this approach the heart is readily observed along the length of the semi-intact larvae. As seen in Figure 1 the heart tube can be readily observed for counting contractions. Preparations were exposed to HL3 or to HL6 during the dissection. The preparations were monitored initially and after 5, 10, 15 and 20 minutes. In HL3 dissected preparations a fresh exchange of the HL3 bath occurs at 7 and 12 minutes (see vertical lines in Figure 4A). The average heart rate was taken during the first five-minute period and the period from 15 to 20 minutes and a percent change was determined (Figure 5). Preparations dissected in HL6 did not show any initial contractions of the heart ($n = 5$, $p > 0.05$ non-parametric). However if the HL 6 saline is exchanged to HL3 the heart recovers. After two minutes in HL6, the bath was

exchanged to HL3 for another two minutes followed by switching the bathing media back with HL6. Upon switching back to HL6 the beating is robust initially followed by a decline. The bath was exchanged at 7 and 12 minutes with HL6 (see vertical lines in Figure 4B). The switch to HL6 at seven minutes resulted in a slight burst in the heart rate (Figure 4B). As for the HL3 observations, the average heart rate was taken during the first five-minute period and the period from 15 to 20 minutes and a percent change was determined (Figure 5).

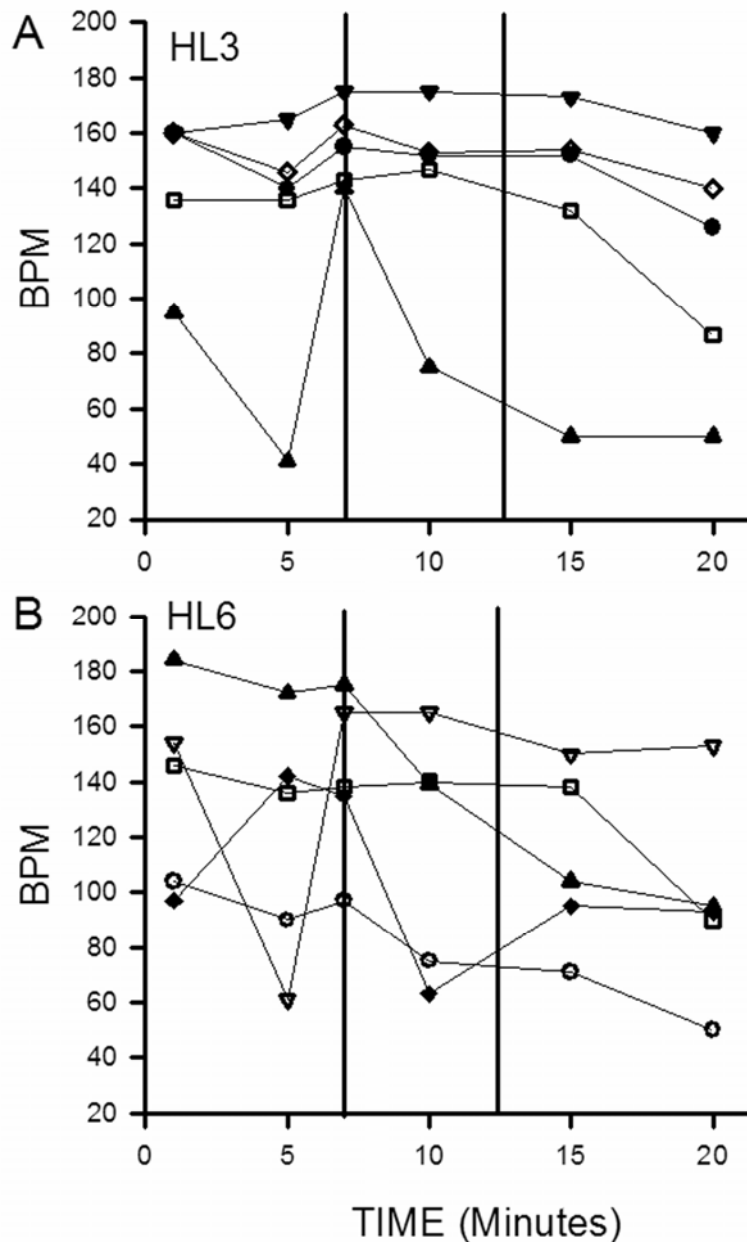
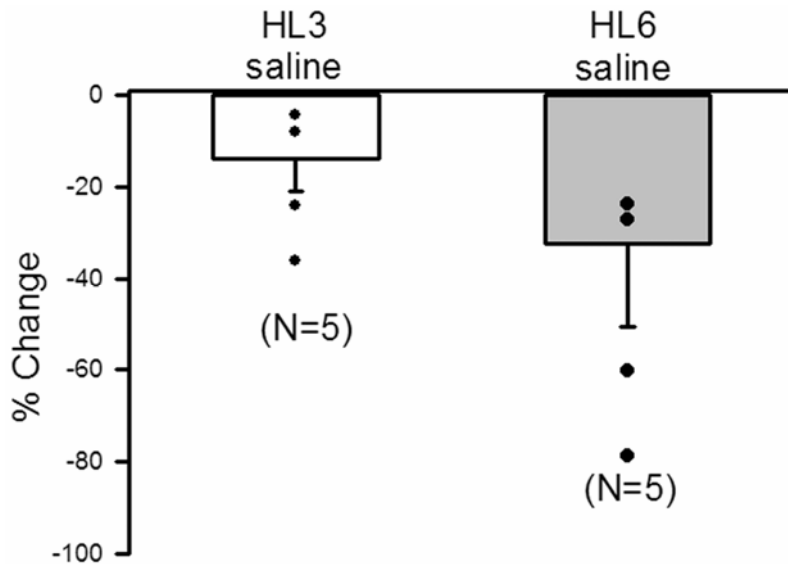


Figure 4. The heart rate was recoded during exposure to HL3 or HL6 saline during various time points. The bath was exchanged either with to HL3 (A) or HL6 (B) at 7 and 12 minutes as denoted by the vertical lines.

Previous reports have shown the effect of ion composition and pH on larvae heart rate. A pH at 7.1 produces a steady beat (Papaefthimiou and Theophilidis, 2001) and salines with higher potassium content produce a higher beat frequency (Gu and Singh, 1995). A solution commonly used in physiology of *Drosophila* referred to as Jan and Jan solution (Jan and Jan, 1976) was previously shown to produce a lower heart rate than other insect salines as well as to the rates we report for the HL3 saline.

Depending on the questions to be addressed in relation to heart function in *Drosophila* larvae, we feel that the techniques described in this report provide the field with additional options to examine effects of compounds introduced in the animals diet for acute as well as chronic studies in non-restrained intact larvae as well as intact restrained larvae. Consequences of heart function in mutational lines can also be readily assessed using the non-restrained approaches. Restraining intact larvae with super glue provides one with the freedom

of not having to track a moving larva, but as we have shown most of the time the rates are very comparable between the unrestrained and restrained approaches. Considering that introduced compounds in the diet might be altered or not even be taken up across the gut, the *in situ* approaches might be most suitable for addressing direct effects on the function of the heart. In addition, in the dissected preparations the central nervous system can be removed to assess if it has any role in modulating the heart rate during exposure to various pharmacological agents (Dasari and Cooper,



2004). High throughput screening is also possible to assess multitudes of pharmacological agents or mutational screens with these approaches presented.

Figure 5. The average heart rate was taken during the first five-minute period and the period from 15 to 20 minutes and a percent change was determined. The mean change and the \pm SEM are shown.

Acknowledgment: We thank Dr. Ruthann Nichols (Univ. of Michigan) for helpful comments regarding the optical means of monitoring heart rate in larvae.

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"Marker removal" screen to generate an improved wing disc GAL4 driver.



Park, Hyochun, and Kevin Edwards. Department of Biological Sciences, Box 4120, Illinois State University, Normal, IL 61790, USA.

Introduction

The GAL4-UAS system allows one to test for interactions between gene constructs that would be lethal if expressed constitutively (Brand, *et al.*, 1994). The *Drosophila* wing is a particularly

appealing place to study interactions among genes responsible for patterning, signal transduction, growth, and morphogenesis, since the wing is not required for viability and it provides a flat, two-dimensional pattern that is easily quantified. To perform interaction studies in the wing, one can combine a wing disc-specific GAL4 driver with two or more *UAS*-regulated responder transgenes (e.g., Guichard, *et al.*, 1999). The phenotypes of flies with the driver and each responder, alone and in combination, are then compared.

To identify which constructs are present in a given fly, one may rely on the *miniwhite* gene that marks most *UAS* transgenes. Since the pigmentation provided by *miniwhite* is additive, flies with one versus two responders can be distinguished by scoring the intensity of eye color (e.g., yellow versus orange). Unfortunately, this approach is nearly impossible for two of the most commonly used wing-specific drivers, *A9-GAL4* and *P[GawB]Bx^{MS1096}* (hereafter, "*MS1096*"; Capdevila and Guerrero, 1994; Marquez, *et al.*, 2001). Both of these wing drivers are located on the X chromosome and express *miniwhite* very strongly, resulting in near wild-type red eyes. When *A9-GAL4* or *MS1096* is present, it drowns out the additional *white* activity provided by typical *UAS* responder transgenes, making it difficult to detect the responders. We solved this problem by generating a derivative of *MS1096* that lacks *miniwhite*. The derivative, *MS1096_w*, is functionally indistinguishable from the original *MS1096* in terms of strength and specificity of GAL4 expression, but it produces no eye pigment and thus allows for accurate scoring of other *white*-marked constructs.

Results and Discussion

The *w* derivative of *MS1096* was obtained using an F₁ "marker removal" screen. Transposase gene *delta2-3* was used to induce imprecise excisions (actually failed break repairs; Engels, *et al.*, 1990) of the *MS1096 P[GawB]* element, and the excisions were screened for those that inactivate *miniwhite* but leave the *GAL4* gene intact. The screen is diagrammed in Figure 1. Mosaic *w¹¹¹⁸*, *MS1096* males bearing *CyO*, *H[Pdelta2-3]* were crossed to *FM7c/+*; *UAS-Pez*, and the female progeny that carried *FM7c* but not *CyO* (*MS1096*/FM7c*; *UAS-Pez* or *+*) were examined. *MS1096*-driven overexpression of *Pez*, which encodes a tyrosine phosphatase, leads to small wings (Edwards, *et al.*, 2001, and in preparation). Thus, these progeny primarily consisted of red-eyed flies, in which the *miniwhite* marker in *MS1096* was unaffected, and flies with normal-sized wings, in which *UAS-Pez* was absent or the *GAL4* region of *MS1096* was excised. We screened for rare F₁ females with light eye color that retained the small-wing phenotype indicative of *MS1096* driving *UAS-Pez*. (Note

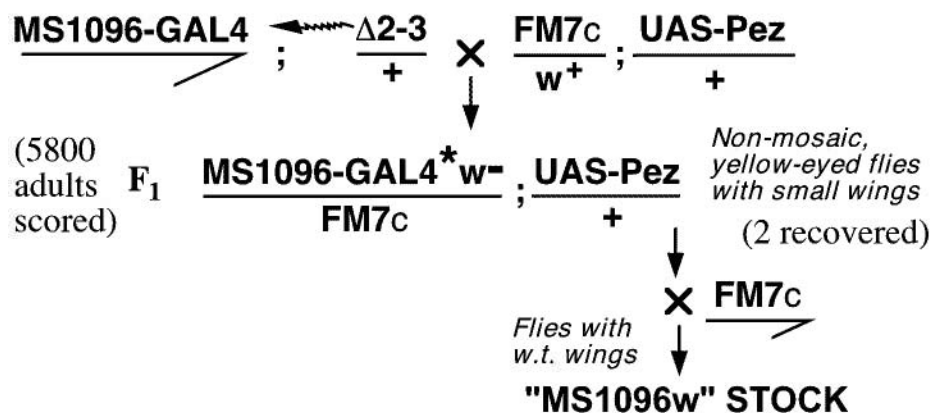


Figure 1. Schematic diagram of the "marker removal" screen (see text for specific genotypes).

that any *UAS* responder, such as *UAS-Egfr*, could have been used in place of *UAS-Pez*, so long as it produced fertile adults with a scorable phenotype.) 5800 F₁ flies were scored, of which ~1/16 were of the appropriate genotype for the screen, and two balanced females were identified in which *MS1096** was lacking *miniwhite* yet positive for GAL4 activity. The

positive females were crossed to *FM7c* males, and non-*UAS-Pez* progeny were selected to make stocks. One line, named *MS1096w*, was retained, re-isogenized, and characterized.

MS1096w hemi- and homozygotes have white eyes, indicating that *delta2-3* activity has completely inactivated *miniwhite*. To determine if *MS1096w* still functions as a strong, specific wing driver, we crossed it to several responders with known *MS1096* phenotypes. In all cases *MS1096* and *MS1096w* were indistinguishable. An example is shown in Figure 2. Both *MS1096w/+* and *MS1096/+* females, each driving one copy of *UAS-Egfr*, display extra wing vein material and distorted vein pattern characteristic of excess EGF signaling (e.g., Guichard, *et al.*, 1999).

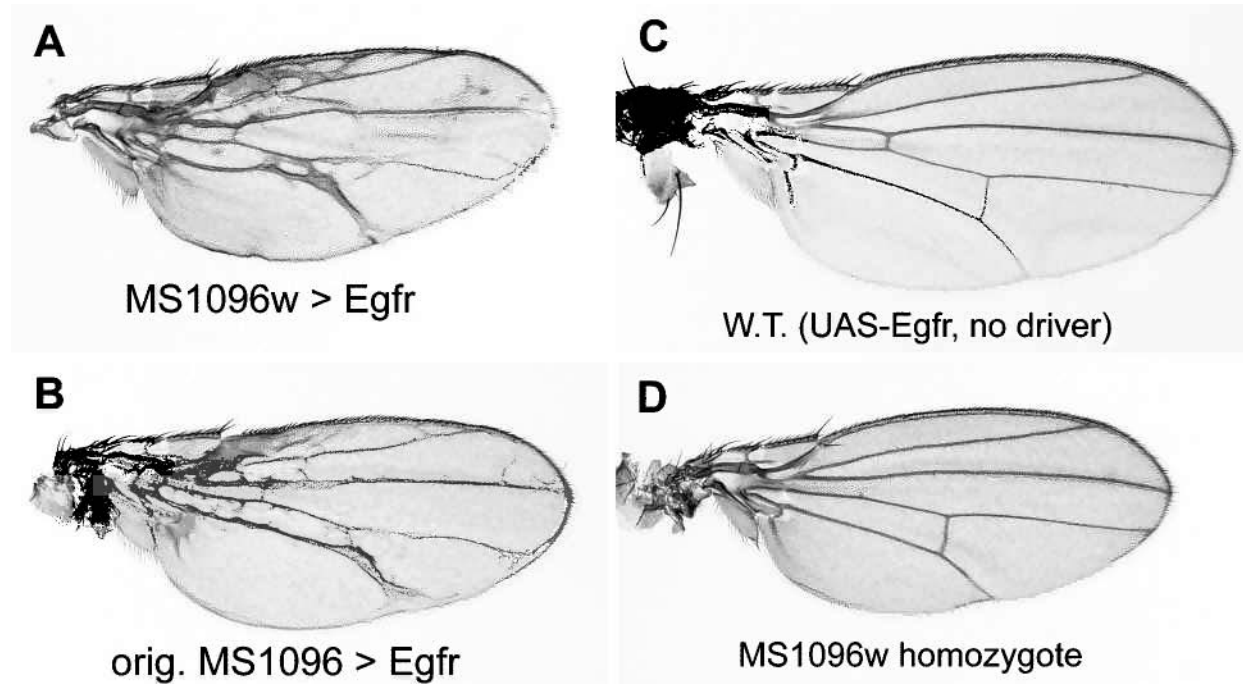


Figure 2. Wing phenotypes of *MS1096w*. A, C; Progeny of a cross between *MS1096w/FM7c* and *UAS-Egfr*. A, *MS1096w/+*; *UAS-Egfr*. C, *FM7c/+*; *UAS-Egfr* (wild type). B, *MS1096/+*; *UAS-Egfr*. A-C raised at 26°C. D, *MS1096w* homozygous female with anterior crossveinless phenotype.

The *MS1096* insert was shown to be located in the second intron of *Beadex/dLMO* (*Bx*), and it acts as a mild, loss-of-function *Bx* allele (Milan, *et al.*, 1998). Thus *MS1096* males exhibit mild vein defects even with no responder present. To determine if the *MS1096w* lesion has altered this phenotype, we examined wings of *MS1096w* males and homozygous females. *MS1096w* does not fully revert the *MS1096* phenotype, as would be expected for a precise excision, confirming that part of *P[GawB]* remains in *MS1096w*. Loss of *miniwhite* does not worsen the *MS1096* phenotype either, suggesting it is an internal deletion of *P[GawB]* (or an external deletion of limited extent, that does not eliminate *Bx* expression). However, *MS1096w* slightly differs from *MS1096*: most *MS1096w* mutants have a normal vein pattern, and the most common defect is loss of the anterior crossvein, seen in ~30% of mutants (Figure 2D, compare to Milan, *et al.*, 1998; and Brentrup, *et al.*, 2000). Thus it appears the deletion of *miniwhite* has slightly altered *Bx* expression. Both *MS1096w* and its progenitor are fully recessive, and so for use as wing drivers they should be employed only in a heterozygous state (unless the goal is to study interactions with *Bx*).

In conclusion, we describe a simple, efficient F_1 screen that could be adapted to remove *miniwhite* from any GAL4 or *UAS* transgene, provided the GAL4 or *UAS* portion can produce a dominant phenotype that does not interfere with fertility. We used this screen to obtain a w^+ derivative of *MS1096*, named *MS1096 w* , which has been submitted to the Bloomington Drosophila Stock Center. Since *MS1096* is such a powerful driver, *miniwhite* is usually not required to detect its presence. Our derivative thus allows scoring of multiple transgenes in an *MS1096* background.

Materials and Methods

Flies were reared on Jazz-Mix (Fisher) at room temperature unless stated. *MS1096* was a gift from Dr. S. Newfeld. *UAS-Pez* was generated in our lab (K. Vadali, C. Weddle, and K. Edwards, in preparation). All other stocks were obtained from the Bloomington Drosophila Stock Center. Wings were mounted in Euparal and photographed with an Olympus DP10 digital camera mounted on a Leica MZ9.5 dissecting microscope.

Acknowledgments: This work was funded by NIH R15-GM62185 to K.E.

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pUASTET: A dually inducible Gal4 – Tet-activator transformation vector.



Mohler, Jym. Barnard College, 3009 Broadway, New York, NY 10027 USA; Phone: 212 854-4381, email: jmohler@barnard.edu

The UAS-Gal4 activation system of Perrimon and Brand (1993) has proven to be an extremely robust system for tissue-specific activation of specific genes in *Drosophila*, largely because of the wide array of Gal4 driver lines that have been developed. In contrast, mammalian expression systems tend to rely on the Tet-activator system of Gossen and Bujard (1992), in which a synthetic activator consisting of the *E. coli* TetR protein fused to the VP16 activation domain is used to activate expression from genes under the control of a multimerized *tet* operator (*tetO*). The Tet-activator system has the advantage that it can be regulated by the administration of tetracycline or doxycycline, which binds to the synthetic activator. Two forms of the synthetic activator have been developed (Gossen *et al.*, 1995), that are either inactivated by doxycycline (tTA) or activated by doxycycline (rtTA), such that the target genes can be regulated in either way by the administration of doxycycline. Construction of similar transformants for *Drosophila* indicated that the Tet-activation system can also be used in *Drosophila*, where gene activation can be regulated simply by the addition of tetracycline or doxycycline to the feeding media (Bello *et al.*, 1998; Bieschke *et al.*, 1998). This inducible system seems especially advantageous for the study of adult physiology and behavior.

A major limitation in the use of the Tet-activation system in *Drosophila* is the limited number of driver lines available to drive Tet-responsive constructs, in contrast to the plethora of lines available for the Gal4 system. In this report, I describe a new transformation vector, pUASTET,

responsive to both Gal4 and Tet-activators. Genes cloned into pUASTET can be used with either Gal4 or tTA/rtTA driver lines to exploit the full versatility of both systems.

Construction of pUASTET:

The multimerized tet-operator (*7xtetO*, Gossen and Bujard, 1992) was amplified by PCR from 7T40 (a *Drosophila* Tet-responsive vector, Bieschke *et al.*, 1998) using primers containing *SphI* sites near their 5'-ends (GGGGGGGCATGCTTCGTCTTCAACAATTCCCTCGAG and GGGGGGGCATGCTACACGCCTACTCGACCCGGGTACCGAG). The recovered PCR fragment was digested with *SphI* and inserted into the unique *SphI* site in pUAST (Brand and Perrimon, 1993), just upstream from the multimerized UAS binding sites in that vector. The resulting construct has multimerized *tetO* and UAS binding sites upstream of a minimal *hsp70* promoter driving transcription of a polylinker region with four useful cloning sites: *EcoRI*, *BglII*, *NotI/EagI* and *XbaI* (see Figure 1; because the fragment containing the multimerized tet-operator retained flanking restriction sites *KpnI* and *XhoI*, these sites are not generally suitable for cloning in the polylinker region.)

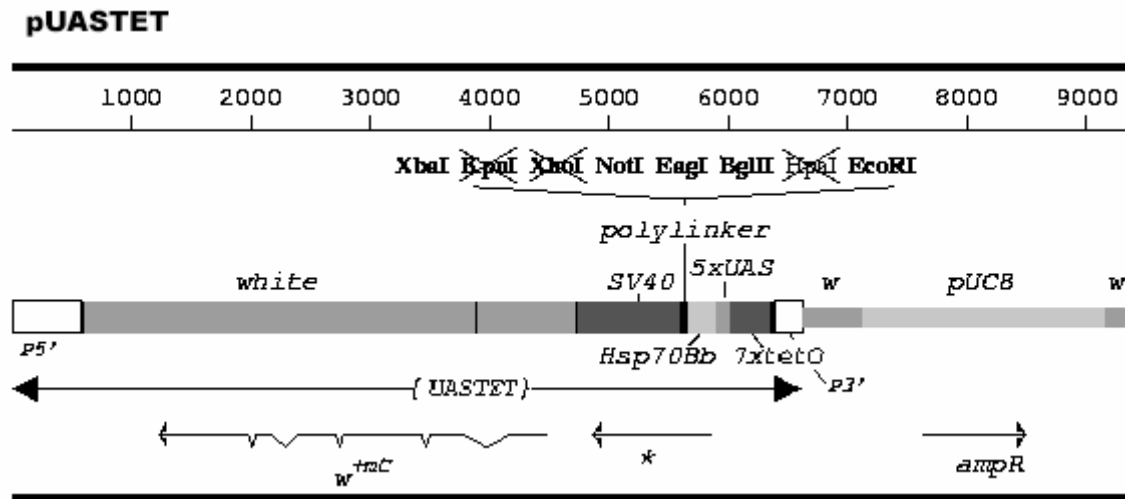


Figure 1.

Tests of pUASTET:

The *lacZ* (β -galactosidase) gene, removed from pCaSpeR- β -Gal, was inserted between the *KpnI* and *XbaI* sites of pUASTET. This *lacZ* pUASTET construct was transformed into *Drosophila* and its expression with spatially restricted Gal4 drivers (*dll::Gal4* and *h::Gal4*) or a Tet driver (*205 = HoxA7::tTA*) was examined by *in situ* hybridization and immunohistochemistry to β -gal. Patterns and intensities of β -gal expression observed for *lacZ* pUASTET was similar to expression with the same drivers from single-responder constructs: *lacZ* pUAST (for Gal4) or *tetO-lacZ* (for tTA; Bello *et al.*, 1998).

Acknowledgments: This project was funded by an award from the National Institutes of Health, GM63500.

References: Bello, B., D. Resendez-Perez, and W.J. Gehring 1998, *Development* 125: 193-202; Bieschke, E.T., J.C. Wheeler, and J. Tower 1998, *Mol. Gen. Genet.* 258: 571-579; Brand, A., and N. Perrimon 1993, *Development* 118: 401-415; Gossen, M., and H. Bujard 1992, *PNAS* 91: 9302-9306; Gossen, M., S. Freundlieb, G. Bender, G. Muller, W. Hillen, and H. Bujard 1995, *Science* 268: 1776-1769.



Novel assay and analysis for measuring climbing ability in *Drosophila*.

Todd, Amy M., and Brian E. Staveley. Department of Biology, Memorial University of Newfoundland, St. John's, Newfoundland & Labrador, Canada, A1B 3X9; telephone (709) 737-4317; telefax (709) 737-3018; Corresponding Author: Dr. Brian E. Staveley; e-mail address: bestave@mun.ca

Introduction

The locomotor activity of *Drosophila* has been commonly used to investigate mobility in ageing *Drosophila* and especially in *Drosophila* models of neurodegenerative diseases (Le Bourg and Lints, 1992; Feany and Bender, 2000; Haywood and Staveley, 2004). Climbing ability is a commonly used assay to measure locomotor activity where a frequently applied method uses empty, standard size vials in which flies are evaluated as having the ability to climb an eight centimeter distance or not. Our novel method employs a long, thin tube in which flies are graded for various levels of climbing. Our approach to analyze climbing is based upon an index that 1) is sensitive to differences in climbing ability, and 2) uses non-linear fit curves that allow for easy comparison of data. The following describes the assay design and analysis, along with a demonstration of how the assay operates using data that were produced while investigating locomotor effects in a *Drosophila melanogaster* model of Parkinson's disease.

Drosophila Strains and Culture

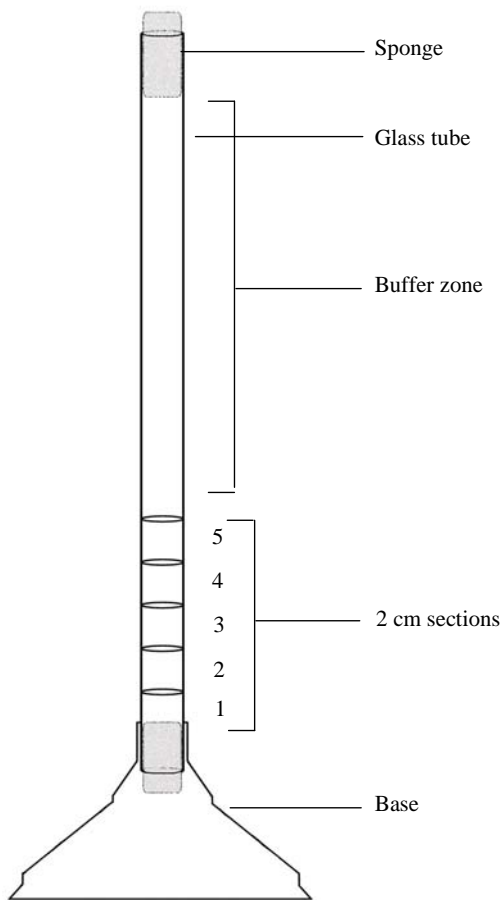
The *UAS-TH* and *UAS-Ddc* (True *et al.*, 1999) lines were generously provided by Dr. S.B. Carroll (University of Wisconsin). The double *Ddc-GAL4* driver line was generated by standard genetic means from *Ddc-GAL4³⁶* and *Ddc-GAL4^{3D}* (Li *et al.*, 2000), provided by Dr. J. Hirsh (University of Virginia). The *w¹¹¹⁸* line was provided by Dr. H. Lipshitz (University of Toronto). Individuals were generated for the assay by crossing *w¹¹¹⁸* females to males of the *UAS-TH*, *UAS-Ddc* and the double *Ddc-GAL4* lines. All flies were raised upon standard cornmeal/yeast/molasses/agar medium at 25°C in standard plastic shell vials.

Assay Design

The climbing apparatus (Figure 1) consists of a 30 cm long glass tube, with a diameter of 1.5 cm. The tube is held in place by a plastic funnel that acts as both a base for the tube and as a means for transferring flies into the apparatus. Starting from the base, the glass tube is divided into a series of five sections, each 2 cm in height (scored 1-5), with an additional buffer zone in the upper portion of the apparatus. Sponges are placed in each end of the tube to prevent escape yet allow air exchange.

Due to the natural negative geotaxis displayed by *Drosophila*, the flies climb up the sides of the apparatus after being tapped down to the bottom. Flies are allowed ten seconds to climb after being tapped down and are given a score based upon the sections reached. The flies are scored ten times (trials) per climbing session, from which a climbing index is calculated as follows:

$$\text{Climbing index} = \sum(nm) / N$$



Where n = number of flies at a given level, m = the score for that level (1-5) and N = total number of flies climbed for that trial.

For climbing analysis, fifty male progeny from each genotype under investigation are collected within 24 hours of eclosion and separated into groups of ten individuals. Starting at day one after eclosion, each group is tested for climbing ability and is continually tested every five days throughout their lifespan.

Figure 1. Schematic representation of the climbing apparatus. The apparatus consists of a 30 cm long glass tube, with a diameter of 1.5 cm. The tube is held in place by a plastic base that also acts as a funnel for transferring flies into the apparatus. The glass tube is divided into five, 2 cm sections (1-5) with a buffer zone at the top of the apparatus. The ends of the tube are plugged with sponges to prevent escape of flies.

Statistical Analysis

Data from the climbing assay was analyzed using the GraphPad Prism 4.02 program (GraphPad Software, Inc.). Climbing data were compared using curve fit

comparison in which the climbing index was subtracted from five and a non-linear regression curve fit was performed. The resulting slopes of the fitted curves for each set of data were compared with 95% confidence intervals.

The model generated for the climbing data using a non-linear regression curve fit is as follows:

$$\begin{aligned} \mathbf{5- Climbing\ index} &= \mathbf{C\ e^{Kt}} \\ \mathbf{or\ Climbing\ index} &= \mathbf{5- (C\ e^{Kt})} \end{aligned}$$

Where C = constant, K = the slope of the fitted curve and t = time in days.

Sample Analysis

As a part of ongoing experiments looking at dopamine synthesis in relation to a model of Parkinson's disease, *tyrosine hydroxylase* (*TH*) and *dopa-decarboxylase* (*Ddc*) (True *et al.*, 1999) were expressed using the UAS/GAL4 system for ectopic gene expression (Brand and Perrimon, 1993) and assayed for climbing ability. The climbing results from the double *Ddc-GAL4* flies and the *UAS-TH* and *UAS-Ddc* flies are presented (Figure 2). A comparison of the *UAS-TH* flies with the *UAS-Ddc* flies (Figure 2a) shows a small but significant difference in climbing ability. The slopes for the fitted curves (Table 1) are 0.056 and 0.046, respectively, and do not overlap within a 95% confidence interval (95% CI). In contrast, comparison of the *UAS-TH* flies with the double *Ddc-*

GAL4 flies (Figure 2b; Table 1) does not show a significant difference in climbing ability within a 95% CI with slopes of 0.056 and 0.050, respectively.

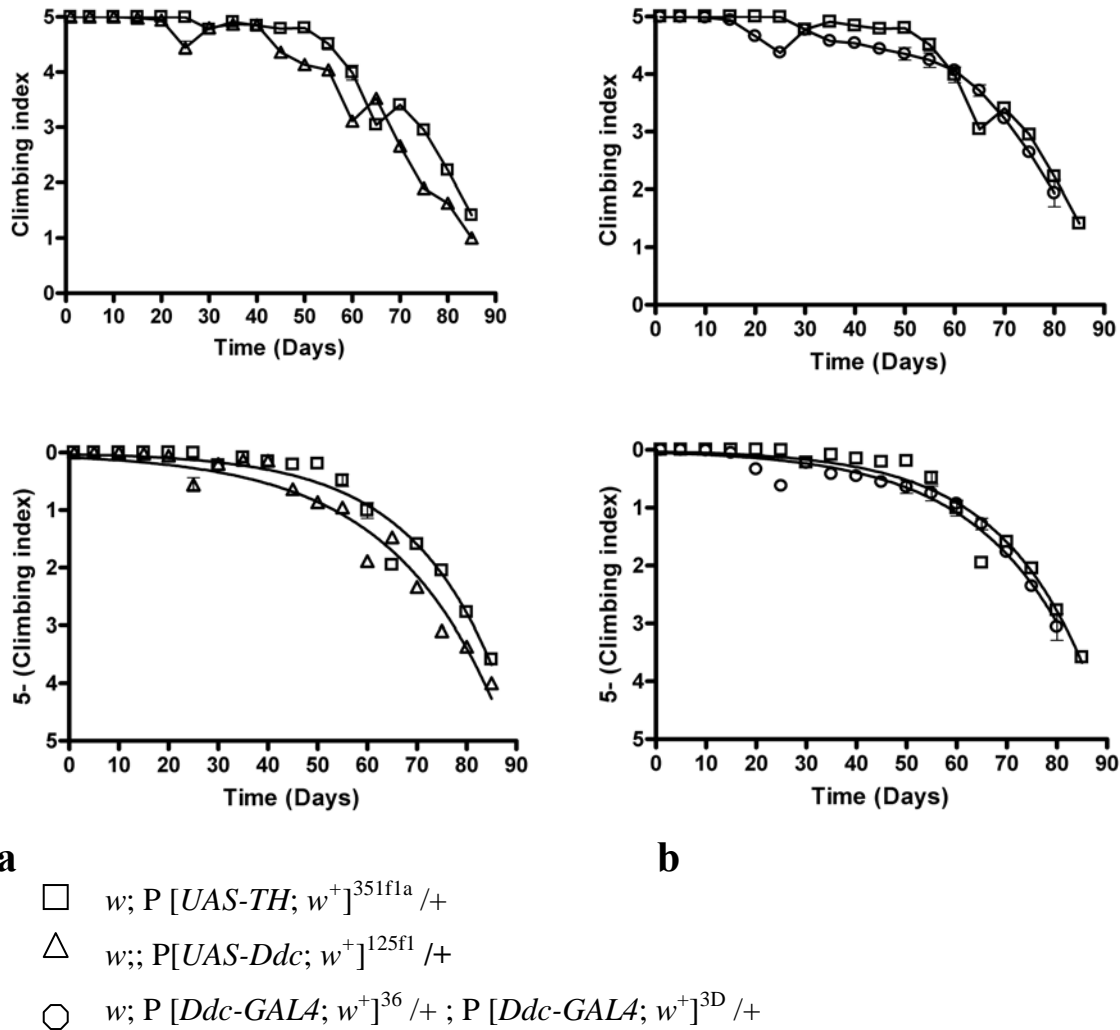
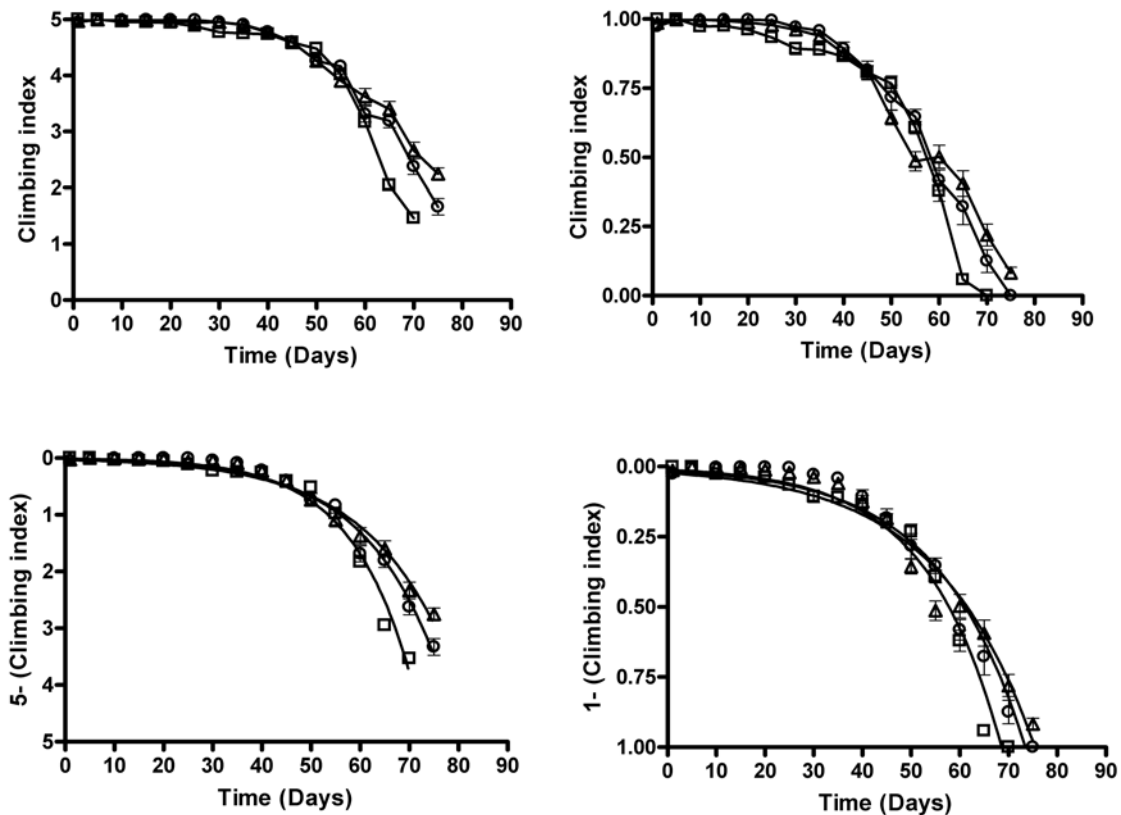


Figure 2. Comparison of the climbing ability showing curves where a significant difference is present (a) and where there is not a significant difference (b) The top graph in each is the non-fitted data where the bottom graph is the fitted curve. Error bars in the non-fitted graphs are standard error of the mean; most error bars are within the area of the data point symbol.

Table 1. Comparison of the non-linear fitted curves for climbing ability showing slopes (K), standard error (SE), confidence intervals (CI), and generated models for each of the fitted curves.

Genotype	Slope (K)	Standard error (SE)	95% Confidence intervals (CI)	Generated model (Climbing index = $5 - Ce^{Kt}$)
$w; P[UAS-TH; w^+]^{351f1a} / +$	0.056	0.0018	0.052 to 0.059	Climbing index = $5 - (0.032 e^{0.056t})$
$w;; P[UAS-Ddc; w^+]^{125f1} / +$	0.046	0.0012	0.043 to 0.048	Climbing index = $5 - (0.087 e^{0.046t})$
$w; P[Ddc-GAL4; w^+]^{36} / + ; P[Ddc-GAL4; w^+]^{3D} / +$	0.050	0.0020	0.046 to 0.054	Climbing index = $5 - (0.053 e^{0.050t})$

**a**

- $w;$; $P[UAS-TH; w^+]^{z60f2} /+$
- △ $w;$ $P[UAS-Ddc; w^+]^{16f2} /+$
- $w;$ $P [Ddc-GALA; w^+]^{36} /+ ; P [Ddc-GALA; w^+]^{3D} /+$

b

Figure 3. Comparison of graded (a) and non-graded (b) climbing methods using non-linear curve fit analysis. The top graph in each is the non-fitted data where the bottom graph is the fitted curve. Error bars in the non-fitted graphs are standard error of the mean; most error bars are within the area of the data point symbol.

Comparison to the Non-graded Method

A common method for analysis of climbing ability in *Drosophila* is a similar apparatus with a single line at the 8 cm mark (see Feany and Bender, 2000; Haywood and Staveley, 2004) in which flies are scored as being able or unable to reach the 8 cm distance. This type of assay is usually performed in empty 9.5×2.7 cm vials. The 1.5 cm tube proposed here presents less opportunity for individuals to fly in the apparatus. In addition, the non-graded method results in less sensitivity when detecting differences in climbing ability. Figure 3 shows a comparison of the graded method proposed here (Figure 3a) with the non-graded method (Figure 3b) using the non-linear curve fit analysis for each. From these graphs, it is apparent that the non-graded method is unable to detect climbing ability when nearing the end of lifespan. In the non-graded method flies appear as unable to

climb when 70-80 days old, when they are actually able to climb between two and six centimeters at this age, as shown in the graded method.

The non-graded method is also unable to discern the difference in climbing ability between the *UAS-TH* flies and the double *Ddc-GAL4* flies (Tables 2 and 3). In the graded method (Table 2), the slope of the curve for the *UAS-TH* flies has a 95% CI of 0.076-0.084, and does not overlap with the 95% CI for the double *Ddc-GAL4* flies of 0.061-0.070. In contrast, analysis of the same data using the non-graded method (Table 3) results in overlapping confidence intervals for the slopes of the *UAS-TH* and the double *Ddc-GAL4* cohorts, with 95% CI of 0.060-0.068 and 0.052-0.060, respectively. Therefore, if using the non-graded method in this analysis, the difference in the climbing ability between these two genotypes would not have been detected.

Table 2. Comparison of the non-linear fitted curves for climbing ability using the graded method showing slopes (K), standard error (SE), confidence intervals (CI), and generated models for each of the fitted curves.

Genotype	Slope (K)	Standard error (SE)	95% Confidence intervals (CI)	Generated model (Climbing index = $5-Ce^{Kt}$)
<i>w</i> ; P[<i>UAS-TH</i> ; <i>w</i> ^{+260f2} /+]	0.064	0.0019	0.060 to 0.068	Climbing index = $5-(0.012 e^{0.064t})$
<i>w</i> ; P[<i>UAS-Ddc</i> ; <i>w</i> ^{+16f2} /+]	0.050	0.0020	0.046 to 0.054	Climbing index = $5-(0.023 e^{0.050t})$
<i>w</i> ; P [<i>Ddc-GAL4</i> ; <i>w</i> ⁺³⁶ /+ ; P [<i>Ddc-GAL4</i> ; <i>w</i> ^{+3D} /+]	0.056	0.0021	0.052 to 0.060	Climbing index = $5-(0.016 e^{0.056t})$

Table 3. Comparison of the non-linear fitted curves for climbing ability using the non-graded method showing slopes (K), standard error (SE), confidence intervals (CI), and generated models for each of the fitted curves.

Genotype	Slope (K)	Standard error (SE)	95% Confidence intervals (CI)	Generated model (Climbing index = $5-Ce^{Kt}$)
<i>w</i> ; P[<i>UAS-TH</i> ; <i>w</i> ^{+260f2} /+]	0.080	0.0022	0.076 to 0.084	Climbing index = $5-(0.014 e^{0.080t})$
<i>w</i> ; P[<i>UAS-Ddc</i> ; <i>w</i> ^{+16f2} /+]	0.059	0.0024	0.054 to 0.063	Climbing index = $5-(0.036 e^{0.059t})$
<i>w</i> ; P [<i>Ddc-GAL4</i> ; <i>w</i> ⁺³⁶ /+ ; P [<i>Ddc-GAL4</i> ; <i>w</i> ^{+3D} /+]	0.065	0.0024	0.061 to 0.070	Climbing index = $5-(0.026 e^{0.065t})$

Evaluation of Assay

We propose that this novel, graded climbing assay is an accurate method for measuring climbing ability in *Drosophila*. The narrow glass tube used in the climbing apparatus is able to discourage *Drosophila* from flying upward within the tube, and, therefore, the index is a measure of climbing ability only. The narrow tube also helps to prevent flies from falling long distances during the ten second climbing trial, making it easier to score the highest point reached by each fly.

As early as by day twenty, and throughout the remainder of the assay, flies were occasionally observed to move in horizontal circles in one graded section of the tube. This usually occurred in the lowest section of the tube, and, although these flies were very mobile, their horizontal movement resulted in a low score and consequently a lower climbing index for the genotype. Horizontal movement, as well as downward climbing and complete lack of climbing, was often observed to

increase as the trials progressed from one through ten during a given session. These climbing behaviors contributed to the change in the slope of the fitted line with increasing trials as shown in Table 4. However, it should also be noted that with each trial, the 95% Confidence Interval decreases (Table 4) and indicates that with the addition of each trial the fitted slope of the line becomes more accurate.

Table 4. The effect of number of trials performed during the climbing assay on the resulting slope (K) of the non-linear fitted line and the confidence interval for climbing ability.

Trial Number	Genotype		
	$w^+; P[UAS-TH; w^+]^{z60f2} /+$	$w^+; P[UAS-Ddc; w^+]^{16f2} /+$	$w^+; P [Ddc-GAL4; w^+]^{36} /+ ; P [Ddc-GAL4; w^+]^{3D} /+$
1	K=0.10 ± 0.022	K=0.073 ± 0.015	K=0.073 ± 0.025
2	K=0.098 ± 0.012	K=0.076 ± 0.0087	K=0.079 ± 0.014
3	K=0.096 ± 0.0088	K=0.075 ± 0.0070	K=0.077 ± 0.011
4	K=0.093 ± 0.0068	K=0.074 ± 0.0072	K=0.075 ± 0.0091
5	K=0.091 ± 0.0061	K=0.069 ± 0.0072	K=0.073 ± 0.0074
6	K=0.090 ± 0.0055	K=0.066 ± 0.0062	K=0.073 ± 0.0065
7	K=0.087 ± 0.0051	K=0.064 ± 0.0057	K=0.071 ± 0.0060
8	K=0.083 ± 0.0049	K=0.063 ± 0.0055	K=0.069 ± 0.0053
9	K=0.082 ± 0.0045	K=0.061 ± 0.0050	K=0.067 ± 0.0050
10	K=0.080 ± 0.0042	K=0.059 ± 0.0046	K=0.065 ± 0.0046

Conclusions

The climbing assay is an accurate measure of climbing ability in *Drosophila*. The novel apparatus and longer testing period is more sensitive to differences as compared to the commonly used non-graded method. The number of trials was determined to be appropriate; however, concerns remain about fatigue as a result of the continuous repetition of the trials (with no break in between each trial). This is a problem in both the proposed graded and non-graded methods and may be resolved by providing a controlled break period between trials. Alternatively, a combination of a decrease in the number of trials and an increase in the overall number of flies tested may reduce the possibility of fatigue. In summary, the combination of our novel apparatus and analysis provides an accurate assay for climbing ability in *Drosophila*.

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DNA preparations from fly wings for molecular marker assisted crosses.

Gleason, Jennifer M., Kelsie A. Cropp, and Rebecca S. Dewoody. Department of Ecology and Evolutionary Biology, University of Kansas, Lawrence, KS, 66045, USA.

Isolation of DNA from *Drosophila* requires the destruction of the fly. To enable crosses of flies with molecularly determined markers, we have developed a protocol to isolate DNA from wings. The method is based upon the single fly DNA preparation of Gloor and Engels (1992). Although parental flies may be genotyped after a cross is established, genotyping flies before crossing eliminates the waste of time and fly food involved in producing crosses of the wrong genotypes.

Protocol

Materials:

DNA squishing buffer (Gloor and Engels, 1992)

10 mM TrisHCl pH 8.2

1 mM EDTA

25 mM NaCl

20 mg/ml proteinase K

200 μ l PCR tubes or 96 well plates

pipette tips

waterbath at 37°C

waterbath at 95°C

Procedure:

1. Anesthetize flies to be genotyped and cut the wings off using either fine-point tweezers to slice the wings or fine-point scissors to clip the wings. Remove as much of the wings as possible without killing the fly.
2. Place the fly in an individual vial to keep for a future cross. We use 95 mm \times 16.5 mm vials with approximately 4 ml of standard fly medium.
3. Place wings in a 200 μ l PCR tube, if only doing a few preps, or in a 96 well plate if genotyping many individuals.

4. Add the proteinase K to the squishing buffer to a final concentration of 200 $\mu\text{g/ml}$. Make only enough buffer with proteinase K for the immediate extractions and throw away extra buffer. Squishing buffer can be made well in advance.
5. Take up 10 μl of squishing buffer with proteinase in a pipette tip. Mash the wings with the tip, without expelling the solution, until the wings are fully shredded.
6. Expel the buffer in the tip into the tube.
5. Incubate at 37°C for 30 minutes.
6. Incubate at 95°C for 2 minutes.
7. Store at -20°C, leaving the wings in the tube. Use 2-3 μl in a PCR reaction.

Genotyping

From two wings of *Drosophila melanogaster*, we can perform 3-5 PCR reactions of similar quality to those of single fly preparations (Gloor and Engels, 1992). Survivorship of the flies after 5 days was comparable between winged and wingless flies (86% and 89%, respectively). The success of the crosses does decrease with wingless flies, as measured by the presence of larvae a week after crosses are established (Table 1).

Table 1. Success of crosses with wingless *Drosophila melanogaster*.

Female	Male	Number of crosses	Number of crosses producing progeny	Percent success rate
wings	wings	58	55	94
wings	wingless	86	56	65
wingless	wings	64	46	72
wingless	wingless	91	68	75

All flies were anesthetized for the same period of time and held for 5 days before setting up crosses.

We have successfully used this procedure for introgressing particular genomic regions of the *Drosophila sechellia* genome into *D. simulans*. Because most *Drosophila* produce courtship song by wing vibration, the procedure will not work for species that require courtship song for mating. For such species, if the male sings with only one wing, wing preps on single wings might work well, although we have not attempted such a preparation.

Reference: Gloor, G., and W. Engels 1992, *Dros. Inf. Serv.* 71: 148-149.