the 10,000 assuming a 1 cm^2 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.

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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)

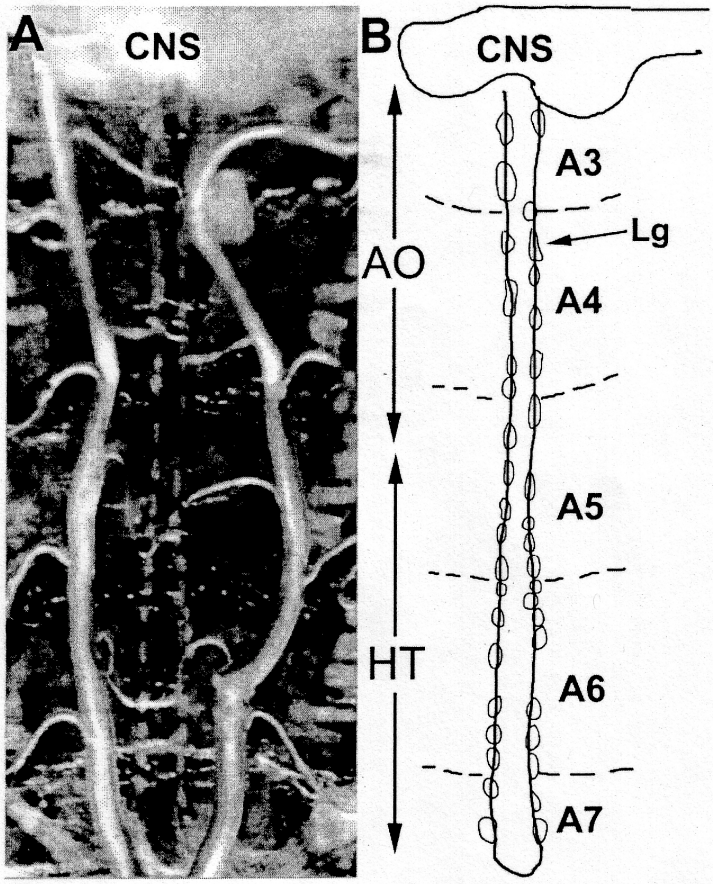


Figure 1. The heart tube is exposed by pinning the dorsal surface of the animal down and approaching the heart by a ventral dissection (A). For illustrative purposes a schematic of the photographed preparation is shown in B. Note the lymph nodes (LN) that line the outer edges of the aorta (AR) and heart (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 cm^2

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.

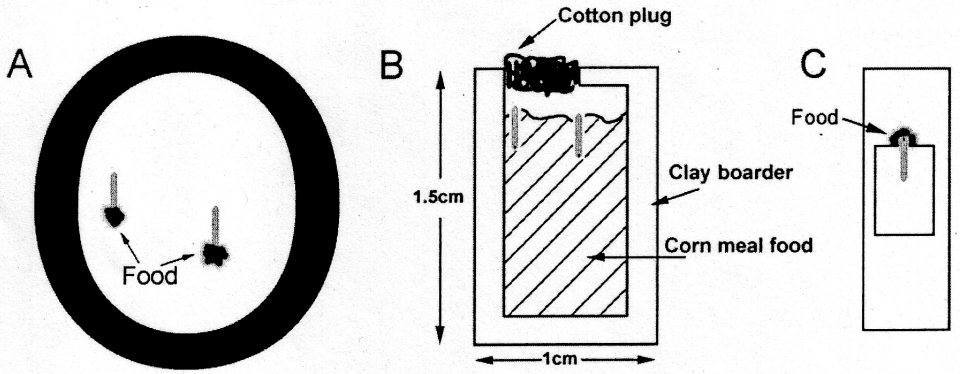


Figure 2. The unrestrained methods to monitor heart rate are shown in the "feeding trough" approach (A) and the "ant farm" approach (B). To restrain larva to one location the ventral side is super glued to a glass cover slip (C). Food is covering the head in A and C. The trachea are seen on the dorsal 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 art 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 (± 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.

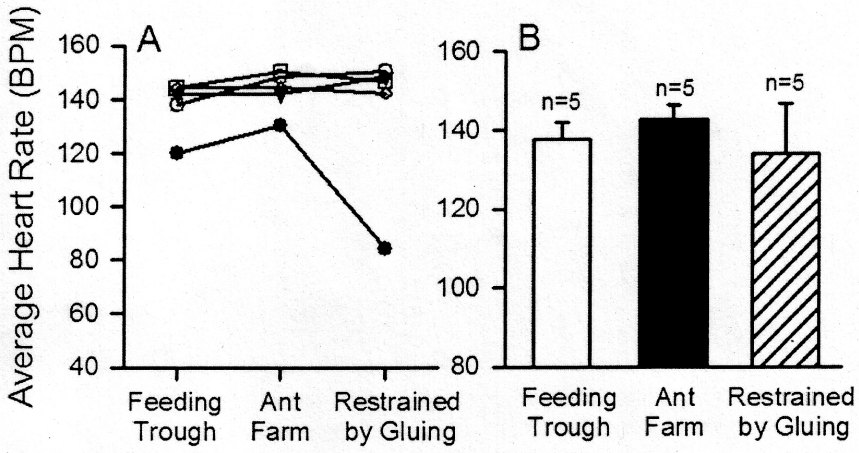


Figure 3. The heart rates, as beats per minute (BPM) for five animals as they were sequentially moved from the feeding trough to the ant farm and to super glue are shown (A). The average rate for all five animals for the five minute periods (± SEM) is shown in B.

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).

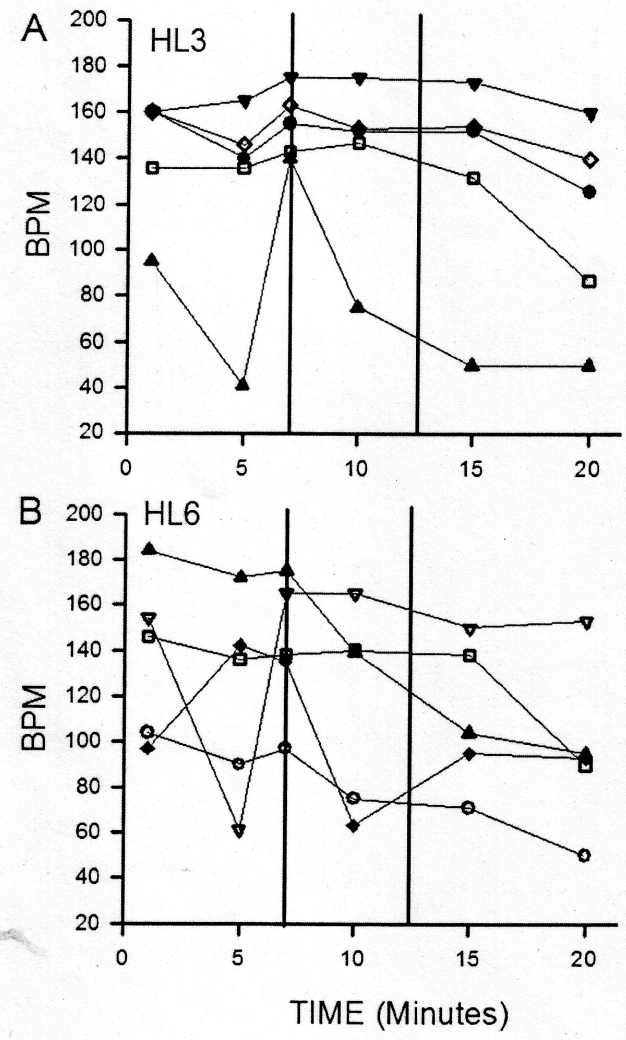
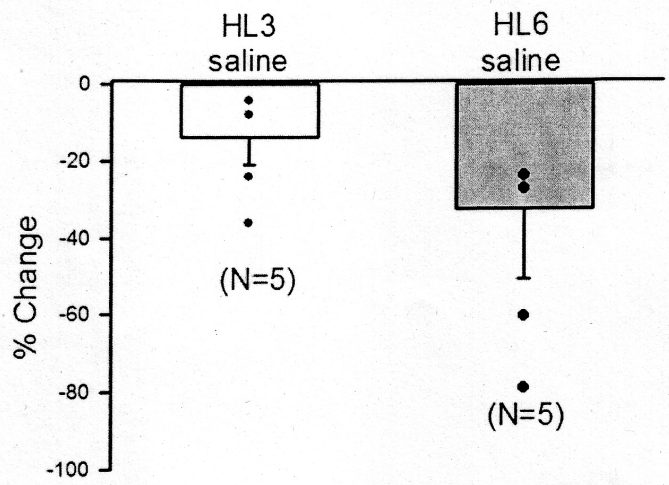


Figure 4. The heart rate was recoded during exposure to HL3 or HL6 saline during various time points. The bath was exchanged either with to HL3 (A) or HL6 (B) at 7 and 12 minutes as denoted by the vertical lines.

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 ± SEM are shown.

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87

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