

Monitoring carbon dioxide production by *Drosophila* larvae.

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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

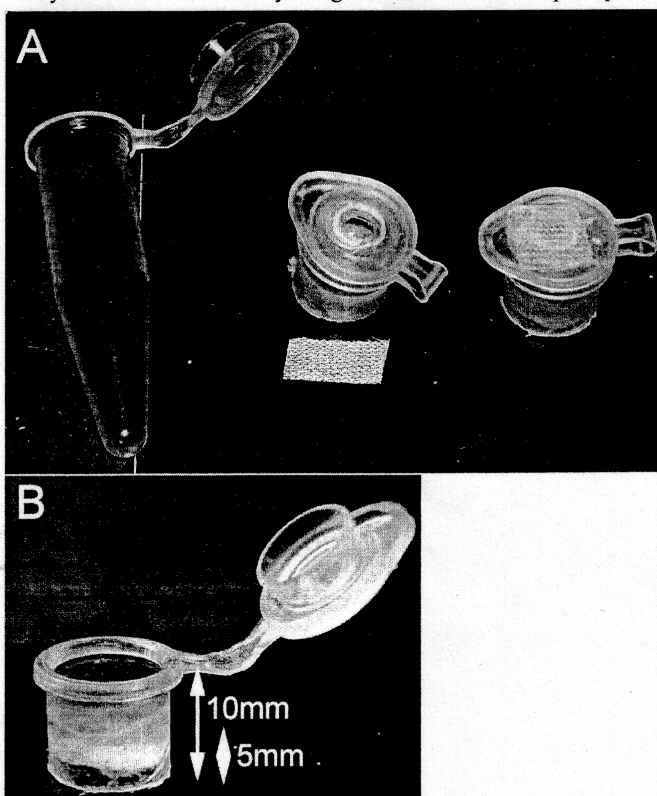


Figure 1. Schematic in the construction of the subchamber. (A) 1.5 ml microcentrifuge tube is cut and a hole is made within the lid. Plastic mesh is cut and glued with super glue to the lid. Sylgard is filled in the base and cured at 65°C over night. (B) One needs approximately 0.5 cm of space from the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

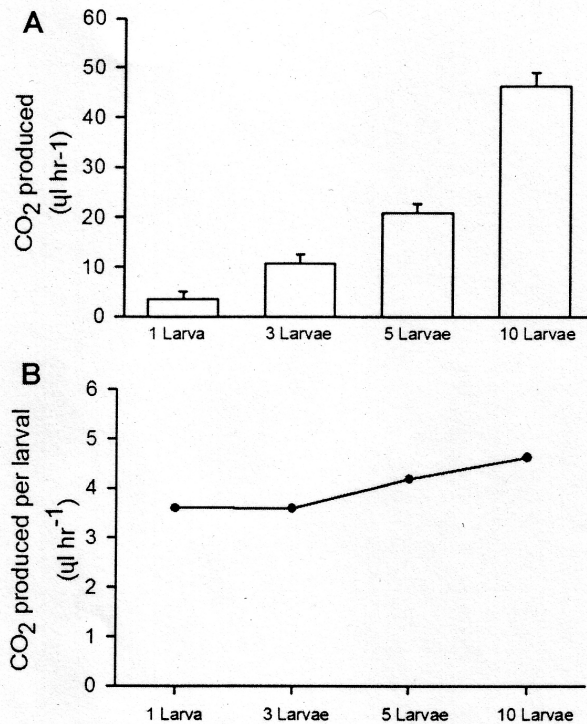


Figure 2. (A) Measures for CO₂ as μl hr⁻¹ in controls (food containing subchambers), single larva, 3 larvae, 5 larvae, and for 10 larvae are reported as a mean (± SEM). Each of the three values for the three trials are shown. (B) Determining the CO₂ produced per larva for each condition indicates that 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 = $100 \mu\text{mol s}^{-1}$, CO_2 = 450 ppm CO_2 , 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 $\mu\text{moles CO}_2/\text{sec/leaf area}$. 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 $\mu\text{moles/sec/area of leaf}$ (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.



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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)

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