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Short Communication

Use of scanning calorimetry and microrespiration to determine the effects of Bt toxin doses on *Pandemis* leafroller (Lepidoptera: Tortricidae) metabolism

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ABSTRACT

Differential scanning calorimetry and microrespiration were used to determine the effects of the biopesticide *Bacillius thuringensis* (Bt) toxin on the metabolism of *Pandemis* leafroller, *Pandemis purusana* (Kearfott). The metabolic heat rate, CO_2 evolution and O_2 consumption of 2nd and 3rd instars following a 2 h exposure to different concentrations of Bt toxin administered through artificial diet were measured from day 0 to 3. Survival (mortality) was recorded from day 0 to 4. The metabolic heat rates of the Bt challenged larvae were significantly lower than those of the untreated controls. Whereas CO_2 evolution and O_2 consumption were not significantly different between the treated and controls, except for larvae challenged at the highest dose of Bt on day 2. However, the calorespiromic ratios, kJ/mole O_2 and kJ/mole CO_2, of all Bt challenged larvae were significantly lower than untreated controls. The respiratory quotient CO_2/O_2 was lowest for larvae challenged at the two highest doses. Mortality of the larvae followed a normal pattern, with the highest mortality at the highest doses and lowest at the lowest doses. Differential scanning calorimetry can be useful in identifying larvae with compromised metabolism as a result of Bt exposure.

KEYWORDS: *Pandemis purusana*, metabolism, differential scanning calorimetry, microrespiration, Bt toxin

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INTRODUCTION

Since the beginning of the 'pesticide' era, bioassays have been the mainstay of assessing the effectiveness of a pesticide [1, 2, 3]. Normally these bioassays are labor-intensive, require large numbers of insects, and can last from days to weeks. Although nothing will replace the bioassay as the final indicator of pesticide efficacy, there may be a more efficient method to screen large numbers of pesticides before selecting the most promising candidates for the bioassays.

For several years, differential scanning calorimetry and microrespiration have been used to determine the effects of temperature and controlled atmospheres on insect metabolism [4, 5, 6]. Recent literature searches have indicated that this approach could be used to determine the sub-lethal effects of pesticides [7, 8, 9].

Up until the development of microcalorimetry methods, insect respiration rates have been determined using a Warburg apparatus [10]. Measurements of respiration rates using the Warburg apparatus were difficult to obtain for large insects, and nearly impossible for small insects (< 30 mg) [11]. As time progressed, microrespiration and calorimetry technologies were improved to allow small-scale applications possible. Differential scanning calorimetry is a method by which metabolic heat is recorded at either static or changing (scanning) temperatures. Units used for insects typically include multiple ampoules for testing several insects at one time,

and making replications and direct comparisons relatively easy [12].

Pandemis leafroller, *Pandemis purusana* (Kearfott) is a common pest of apple trees in the Pacific Northwest [13]. Conventional pesticide control often included azinphosmethyl, spinosad, and neonicotinyls [14, 15]. With the loss of many conventional chemical pesticides and increasing concerns of growers over exceeding Minimum Residue Levels (MRLs) on apples, alternative biological control agents such as the endotoxins from *Bacillius thuringensis* (Bt) have become progressively more popular [16, 17, 18].

The goal of this research was to determine whether differential scanning calorimetry and microrespiration could be useful to determine the metabolic effects and ultimate fate of insects challenged with a biopesticide.

MATERIALS AND METHODS

Insects

Pandemis leafroller pupae were obtained from the colony originating from the Washington State University Tree Fruit and Extension Research Laboratory in Wenatchee, WA in April 2014. The pupae were kept in a 1.89 L screen cage (constructed from 2.5 L bleach jugs). Each cage contained a 30 ml plastic cup with a dental cotton wick 5 cm in length protruding through the lid, that contained 25 ml of a mixture of fresh honey water (20 ml honey + 2 g Fabco in 80 ml H_2O) to supply nutrition and water to emerging adults. The rearing conditions of *Pandemis* leafroller were 24.4 °C ± 2 °C, 16:8 light:dark (L:D) photoperiod photoperiod and 40% relative humidity (RH). Wax paper was placed around the inner walls of the cage as an oviposition substrate. After 5 days of oviposition, egg masses were gently removed with soft forceps, and placed into a 30 ml cup containing 20 ml of artificial diet (Multi-Species Pre-Mix, Southland, Inc., Lake Village, AR). The second instars were removed from the diet cups and placed into fresh diet cups in groups of 3 to 5 larvae per cup. The larvae were kept under the rearing conditions either until the desired stage for the experiment, or until pupation, at which point the pupae were removed from the diet cups and placed into a screen cage to allow for adult emergence, mating and oviposition in order to continue the colony.

Bt toxin

The commercial Bt toxin Deliver™ (Certis USA, LLC, Columbia, MD) was used for this study. The label states that the active ingredient is a Cry1Ac endotoxin from the *Bacilus thuringiensis kurstaki* strain AS-12. The recommended application rate is 0.56 to 2.24 kg/ha. The stock solution used to obtain the desired doses used in this study was: 0.5 g Deliver™ in 100 ml H_2O. This is a 5 x 10^{-2} dilution of the product. The recommended field application spray rate is 3 x 10^{-4} g/ml when spraying at a rate of 1.12 kg/ha using a 3741.6 L/ha spray rate. The doses consisted of control = water, dose 1 **(D1)** = 1.25 x 10^{-3} g/ml, four times the recommended field spray rate, dose 2 **(D2)** = 6.25 x 10^{-4} g/ml, four times the recommended field spray rate, dose 3 **(D3)** = 6.25 x 10^{-6} g/ml, 0.02 times the recommended field spray rate and dose 4 **(D4)** = 6.25 x 10^{-7} g/ml, 0.002 times the recommended field spray rate.

Bioassay

This procedure was developed from a combination of Bruner and Smith [16, 17] and Smirle *et al.* [15]. A total of 400 µl of either water (control) or dilutions of the Bt toxin was evenly distributed across the surface of 50 ml of artificial diet in a 100 ml volume cup using a disposable plastic spreader. A total of 5 cups were treated at each dose. The cups were kept in a fume hood for 45 minutes to allow for the liquid to absorb into the diet. A total of five 2nd instar *Pandemis* leafroller were placed on each diet cup, allowing for 25 larvae to be treated at each dose of Bt toxin. The tests were repeated 4 times over the span of 6 weeks. The larvae were held in the treatment cups for 2 hr, after which they were gently removed with a paintbrush and placed on fresh Bt-free diet for the remainder of the bioassay period (4 days). The larvae were assessed at each day of the bioassay period for survivorship.

Differential scanning calorimetry

A 600000 multi-cell differential scanning calorimeter, MC-DSC Model (TA Instruments, Inc. New Castle, DE, USA) with two sets of 1.0 ml Hastelloy® ampoules, 11 mm diameter by 5 mm deep, was

used for the measurement of metabolic heat rate. The MC-DSC has a detection limit of 0.2 μW and baseline repeatability of 2 μW. The MC-DSC was operated with the MC-DSCRUN (v. 2.9.10, TA Instruments © 1996, 2007) program.

On each day of the bioassay period, the 2nd and 3rd instars from each treatment dose were removed, individually weighed on a microbalance, and placed into a DSC ampoule, after which the lid was sealed. Three individual ampoules were placed into individual wells within the DSC. There were 3 readings of each treatment for each day of the bioassay. The bioassays were replicated 4 times, with a total of 12 readings for each treatment x day combination.

Heat rate data were recorded at 23 °C for 2400 s (40 min) after a 600 s (10 min) equilibration step. Following the recording of heat rates in the DSC, the ampoules were removed from the DSC and the larvae were transferred individually into 2 ml gas-tight vial with self-sealing rubber lid inserts (Agilent Technologies, Santa Clara, CA. Vials part # 5182-0714, caps # 5182-0717) to allow for atmospheric gas extraction with a gas-tight syringe. Each vial containing an individual larva was sealed and held at 23 °C for 45 min. Following the holding period, a total of 100 μl headspace gas was removed from individual vials using a gas-tight glass syringe (SGE Analytical Science, Victoria, Australia. 100R-V-GT, #005279) and injected into the gas chromatograph for assessment of O_2 and CO_2 levels.

Gas chromatography

Carbon dioxide and oxygen levels for individual larvae were determined using an Agilent 7890B series gas chromatograph with a thermal conductivity detector (TCD) and a GS-Carbon PLOT column (Agilent 113-3133). On each day of the operation, gas standards were run in triplicate to develop standard curves for O_2 and CO_2. Standard gases were obtained from Oxarc, Inc. (Pasco, WA) and included compressed air, 100% N_2, 10% CO_2, 10% O_2, and a combination 0.1% CO_2 and 15% O_2. The split flow injector was set to 250 °C. The oven temperature was set to increase from an initial temperature of 35 °C to a final temperature of 220 °C at a rate of 15 °C/min. The flow rate in the column was 15 ml/min with 10 ml/min H_2 and the remaining make-up gas (N_2) set to 5 ml/min. The N_2, CO_2, and O_2 peaks were eluted in the first 5 min after sample injection.

Statistics

All DSC data were initially analyzed using the NanoAnalyze program (V.2.2.0 Copyright © 2005, 2011 TA Instruments, New Castle, DE) to obtain heat rates of individual larvae. These data were subsequently analyzed using Excel (V.14.0.6112.5000, Microsoft Office Standard 2010) to calculate average heat rates, CO_2 evolution and O_2 consumption, as well as calorespirometeric ratios and respiratory quotients. Averages, standard deviations of the mean, and standard error of the mean were calculated using Excel. ANOVAs, multiple and one-way ANOVAS, were performed using Sigmaplot (for Windows Version 12.3, © 2011 Systat Software, Inc, San Jose, CA).

RESULTS AND DISCUSSION

Mortality

The mortality of *Pandemis* leafroller larvae (Fig. 1) was significantly affected by dosage ($F_{4,58} = 25.335$, $P < 0.001$) and days after treatment ($F_3 = 37.921$, $P < 0.001$). There was no significant dose x day interaction ($F_{12} = 0.778$, $P = 0.674$). The larvae in the controls showed the lowest mortality, followed by, in order, larvae challenged with doses of 6.25×10^{-7} g/ml, 6.25×10^{-6} g/ml, 6.25×10^{-4} g/ml, and 1.25×10^{-3} g/ml.

Metabolic heat rate

The metabolic heat rates of larvae in the controls were significantly higher than all the Bt challenged larvae ($F_{4,58} = 4.964$, $P < 0.002$) (Fig. 2). There was no day x main effect interaction ($F_3 = 1.150$, $P = 0.338$). The most noticeable differences were found in larvae, 2 and 3 days post-treatment.

Respiration

There were no significant differences among larvae in treatments with respect to CO_2 evolution ($F_{4,58} = 0.293$, $P = 0.881$) (Fig. 3). Nor were there any significant differences among larvae in treatments with respect to O_2 consumption ($F_{4,58} = 0.720$, $P = 0.582$) (Fig. 4). The respiratory quotient (RQ) (moles CO_2/moles O_2), an indicator of the conversion of carbon dioxide as a function to oxygen

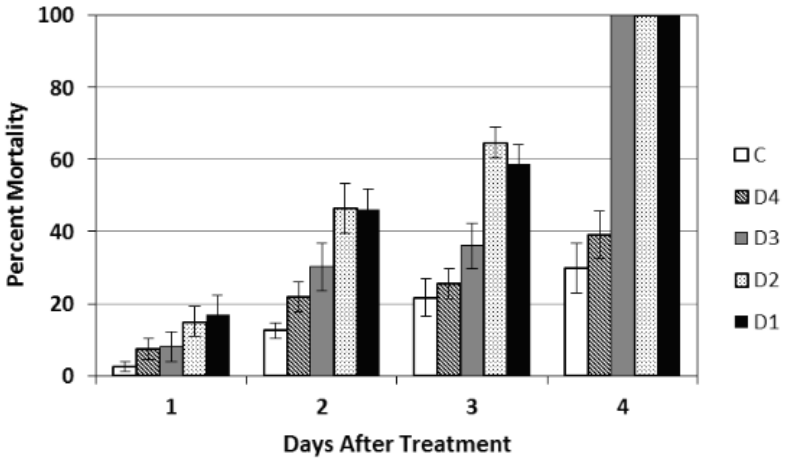

Fig. 1. The percent mortality of *Pandemis* leafroller larvae in relation to treatment dosage and days after Bt challenge. **C** = water; **D1** = 1.25 x 10^{-3} g/ml, **D2** = 6.25 x 10^{-4} g/ml, **D3** = 6.25 x 10^{-6} g/ml, **D4** = 6.25 x 10^{-7} g/ml.

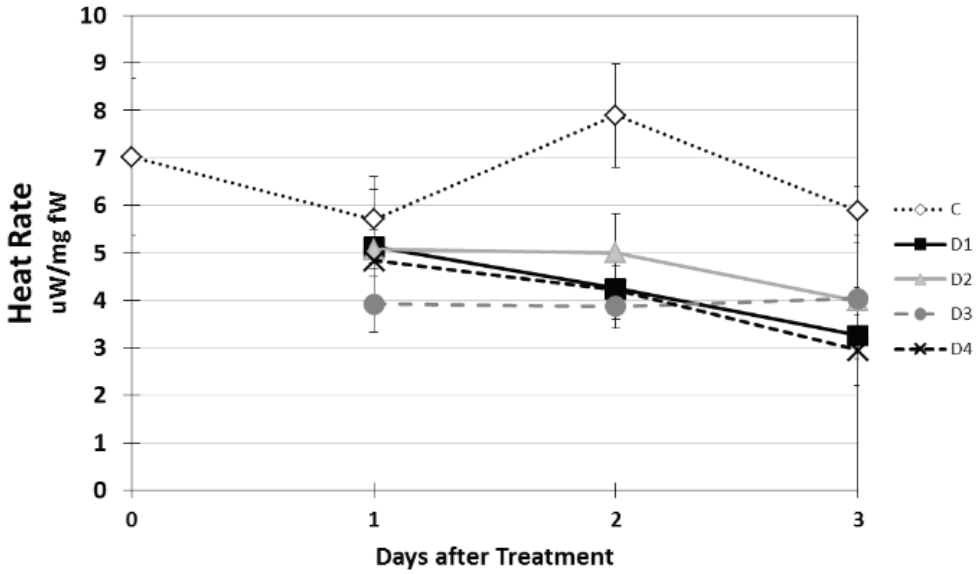

Fig. 2. Comparison of metabolic heat rate of *Pandemis* leafroller larvae in relation to treatment dosage and days after Bt challenge. **C** = water; **D1** = 1.25 x 10^{-3} g/ml, **D2** = 6.25 x 10^{-4} g/ml, **D3** = 6.25 x 10^{-6} g/ml, **D4** = 6.25 x 10^{-7} g/ml.

consumption (Fig. 5), showed that the larvae treated at the two highest doses (6.25 x 10^{-4} g/ml and 1.25 x 10^{-3} g/ml) produced less CO_2 in relation to the O_2 consumed. RQ values at 1 are indicative of carbohydrate catabolism and RQ values of 0.8-0.9 are indicative of protein catabolism, while RQ values of ~0.6-0.7 indicate lipid catabolism. These data show that less of the oxygen consumed by the larvae is converted into metabolic heat (See Figs. 2 & 6) [12].

Calorespiromic ratios

The calorespiromic ratio for oxygen (kJ/mole O_2) (Fig. 6) for all Bt challenged larvae were significantly below Thorton's Rule of 455 kJ/mole CO_2 for aerobic metabolism as compared to the untreated

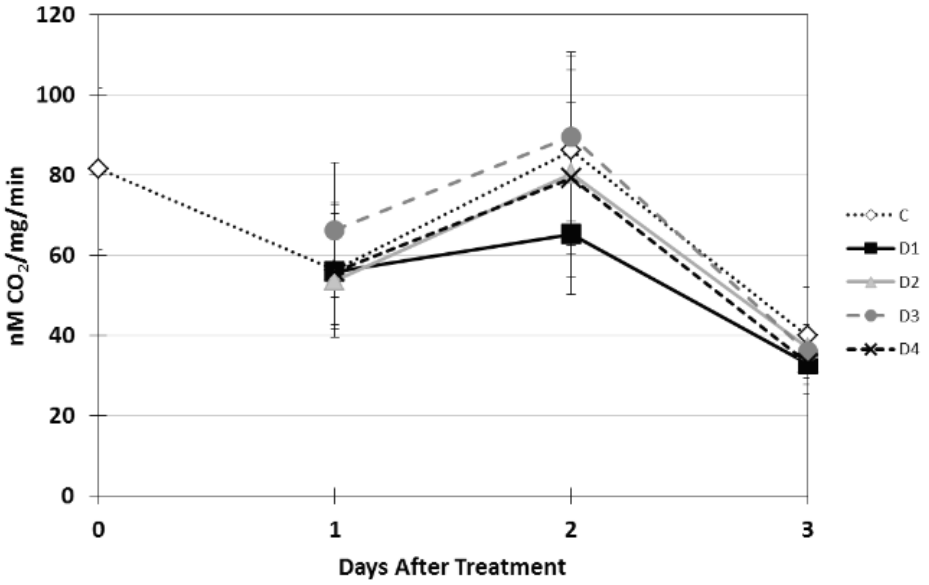

Fig. 3. Nanomoles of CO_2 produced by *Pandemis* leafroller larvae in relation to treatment dosage and days after Bt challenge. **C** = water; **D1** = 1.25 x 10^{-3} g/ml, **D2** = 6.25 x 10^{-4} g/ml, **D3** = 6.25 x 10^{-6} g/ml, **D4** = 6.25 x 10^{-7} g/ml.

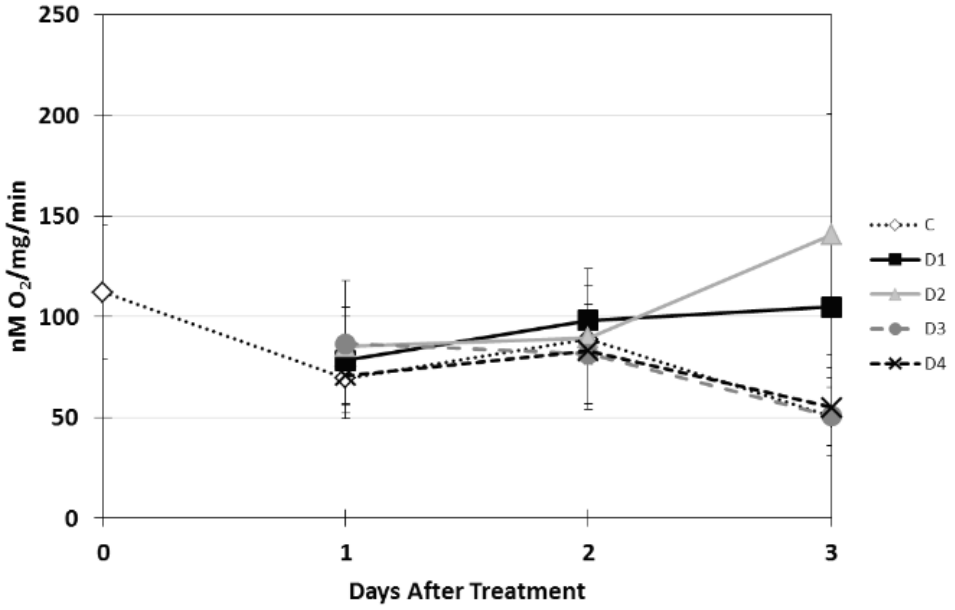

Fig. 4. Nanomoles of O_2 consumed by *Pandemis* leafroller larvae in relation to treatment dosage and days after Bt challenge. **C** = water; **D1** = 1.25 x 10^{-3} g/ml, **D2** = 6.25 x 10^{-4} g/ml, **D3** = 6.25 x 10^{-6} g/ml, **D4** = 6.25 x 10^{-7} g/ml.

control larvae. R_q/R_{O2} ratios below 470 kJ/mol O_2 indicate that the oxidation reactions are not a complete description of the overall chemistry of the system [12].

The calorespiromic ratio for carbon dioxide (kJ/mole CO_2) (Fig. 7) indicates that larvae treated at the higher doses of Bt toxin were well below Thorton's Rule (455 kJ/mole CO_2) for aerobic

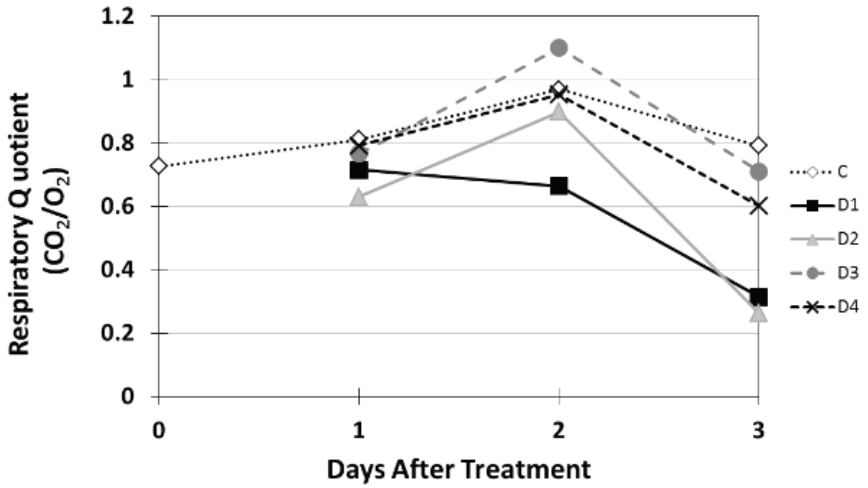

Fig. 5. Respiratory quotient, RQ, calculated as moles CO_2/moles O_2 of *Pandemis* leafroller larvae in relation to treatment dosage and days after Bt challenge. **C** = water; **D1** = $1.25x10^{-3}$ g/ml, **D2** = 6.25×10^{-4} g/ml, **D3** = 6.25×10^{-6} g/ml, **D4** = 6.25×10^{-7} g/ml.

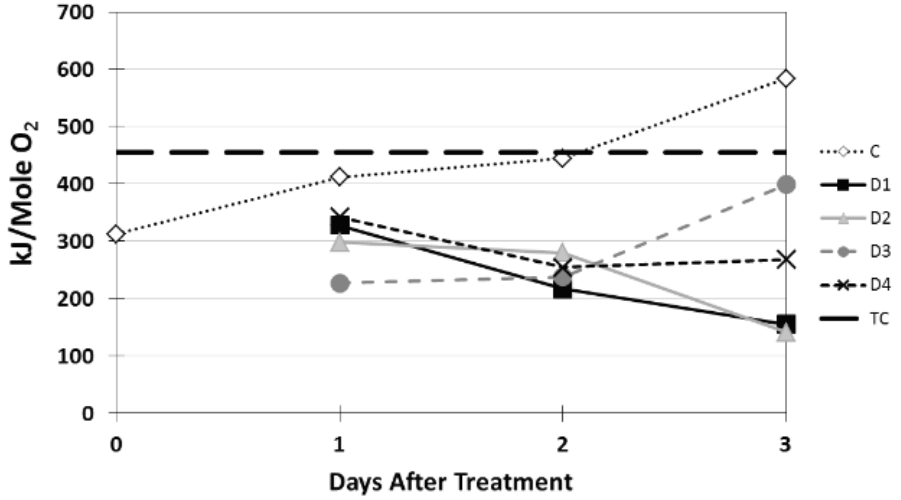

Fig. 6. Comparison of the calorespiromic ratio (heat rate/moles O_2) of *Pandemis* leafroller larvae in relation to treatment dosage and days after Bt challenge. The horizontal dash line indicates the value of Thorton's Constant (455 kJ/mole O_2). **C** = water; **D1** = 1.25×10^{-3} g/ml, **D2** = 6.25×10^{-4} g/ml, **D3** = 6.25×10^{-6} g/ml, **D4** = 6.25×10^{-7} g/ml.

metabolism as compared to untreated control larvae, demonstrating that the larvae were in an anaerobic metabolic state.

CONCLUSION

We were able to correlate the changes in metabolic heat rate, calorespiromic ratios, and respiratory quotient with respect to the dose of Bt toxin and duration after exposure in *Pandemis* leafroller larvae.

1. Metabolic heat rates of all Bt challenged *Pandemis* leafroller larvae were lower than those of untreated controls.
2. The amount of O_2 consumed (Fig. 4) versus the amount of CO_2 produced (Fig. 3) was much lower in larvae challenged at the two highest doses of Bt (Fig. 5), indicating a reduction in the conversion of metabolic resources into usable energy needed to

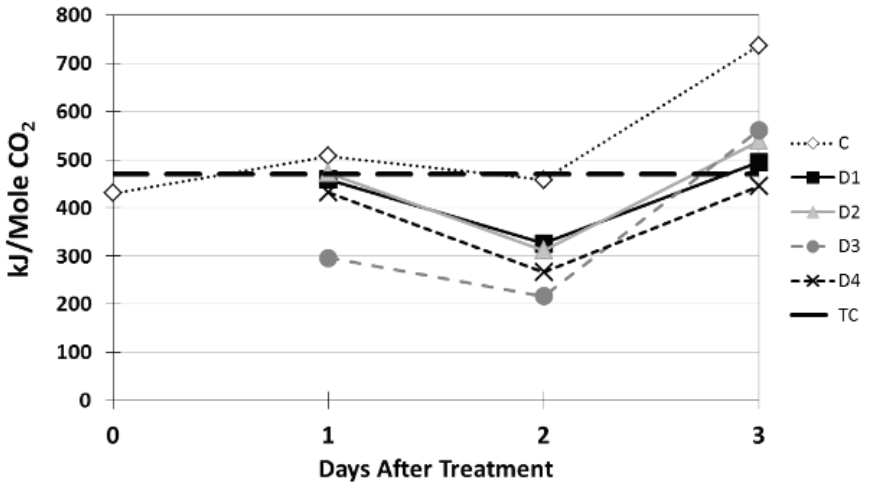

Fig. 7. Comparison of the calorespiromic ratio (heat rate/moles CO_2) of *Pandemis* leafroller larvae in relation to treatment dosage and days after Bt challenge. The horizontal dash line indicates the value of Thorton's Constant (470 kJ/mole CO_2). **C** = water; **D1** = 1.25 x 10^{-3} g/ml, **D2** = 6.25 x 10^{-4} g/ml, **D3** = 6.25 x 10^{-6} g/ml, **D4** = 6.25 x 10^{-7} g/ml.

support metabolism for normal growth and development (Fig. 7).

3. The use of differential scanning calorimetry with microrespiration may be useful in developing a rapid diagnostic for determining lethal and sub-lethal effects of biopesticides on Lepidoptera.

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CONFLICT OF INTEREST STATEMENT

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