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METABOLIC HEAT: A NEW WAY OF LOOKING AT HOW CONTROLLED ATMOSPHERES KILL INSECTS

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ABSTRACT

The use of calorimetry as a tool to understand the effects of controlled atmospheres (CA) on insects is briefly reviewed. A variety of data are presented to illustrate the various types of information that calorimetry can make available to researchers. The use of a calorimeter connected to a mass spectrometer to determine the occurrence of anaerobic respiration is described and reported. We conclude that calorimetry is a useful tool to simplify the experimental options when developing new insecticidal CA treatments. It can also be used for development of other treatments such as fumigants.

INTRODUCTION

Crop & Food Research began work on development of controlled atmospheres (CAs) as quarantine treatments for fresh produce in 1985 (Lill and van der Mespel 1986). Despite continuing research and development around the world, no quarantine treatments based on CAs have entered commerce. Two themes emerge from the reviews of CAs as quarantine treatments (e.g. Klag 1986; Ke and Kader 1992; Carpenter and Potter 1994; Hallman 1994), that are reinforced by recent work (Carpenter 1995, 1997; Mitcham *et al,*. 1997; Zhou *et al*., 2000; Carpenter *et al*., 2001). These are:

1. There are variations in response to CAs between taxa and between seasons for specific taxa that have not been explained.
2. The physiological and biochemical bases of the insecticidal effects have not been adequately explained, despite Mitz (1979) formulating a set of key hypotheses.

We began to question approaches to development of CAs for quarantine treatments to discover what commodity traders and regulators need to know if CAs are to be used as quarantine treatments. There appear to be six issues:

In the absence of a simple binary mortality response to CAs how reliable are CA disinfestation treatments?
Are moribund insects viable?
Are all moribund/comatose insects demonstrating the same physiological or biochemical response to CAs?
Are there commonalities between insect taxa in their responses to CAs that could be used to develop generic disinfestation systems?
What is the key composition/application of a controlled atmosphere?
How do CAs kill insects?

The last question may be the most important; the other questions may simply be sub-sets of it. We developed a set of hypotheses around the premise that understanding whole organism responses to CAs would facilitate development of better approaches to understanding the biochemical and physiological bases of why CAs might be useful as quarantine disinfestation methods. The least complex way to determine whole organism effects of a treatment on insects is to use calorimetry.

METHODOLOGY

Calorimetry simply measures heat production by the test insects, basically the sum of anabolic and catabolic activities. Over the last decade microcalorimetric methods have been developed for studying plant material (Criddle and Hansen 1999). We have recently used similar methods to determine the response of a range of insects at various life stages to changes in temperature and CAs. In addition we are developing combined calorimetry – mass spectrometry methods so that O_2 consumption and CO_2 production can be measured simultaneously with metabolic heat rates. Details of the experimental methods will be given elsewhere (Downes *et al.* 2001).

A Calorimetry Sciences Corporation (Utah) differential scanning calorimeter is used for the metabolic heat measurements. This has a sample ampoule volume of 1 mL and up to 3 samples can be run simultaneously. Although this is designated as a scanning calorimeter for most bio-calorimetry experiments it is operated in the isothermal mode, e.g. held at a constant temperature of say 25°C. The detection limit in the isothermal mode is about 2 microwatts, which is adequate for studies of small insects. Advantages of this calorimeter are the ease and speed with which the sample temperature can be changed and the rapid establishment of thermal equilibrium at the new temperature. We have found this calorimeter to be particularly suitable for the rapid evaluation of the response of insects to gross changes in conditions.

To avoid erratic thermal signals due to insects moving to the lid of the ampoule, mobile insects are confined to the bottom of the ampoule by a fine stainless steel mesh. When it is desired to change the atmosphere during the course of an experiment a modified lid is used on the ampoule. This lid has silica inlet and outlet capillaries sealed to it of the type used in gas chromatography. When it is desired to

sample the composition of the atmosphere in the ampoule by mass spectrometry an additional fine capillary of 50 micron i.d. is included. An important experimental detail is that good quality hardware such as valves should be used in the gas handling system so that, for instance, truly anoxic conditions can be achieved in the ampoule.

The mass spectrometer provides a convenient means of following the changes in the chemical composition of the atmosphere in the ampoule during metabolic heat determinations.

Problems with CA disinfestation

We had found significant variation year to year in the response of *Thrips obscuratus* (Crawford) (Thysanoptera: Thripidae), *Nysius hultoni* (White) (Coleoptera: Lygaeidae), *Myzus persicae* (L) (Hemiptera: Aphidae) and *Pseudococcus longspinis* (Targette-Tonzonii) (Hemiptera: Pseudococcidae) to a variety of CAs (van Epenhuijsen *et al*. 2001; Lill and van der Mespel 1986; Carpenter 1995, 1997; Carpenter unpublished; Potter *et al*. 1992). It was clear that if CAs were to be used as quarantine treatments then the basis of this variation needed to be understood.

The second issue was lack of a testable hypothesis that attempted to explain how CAs killed insects. The literature on this topic is challenging. Many workers have found biochemical changes in insects that have died as a result of treatment with insecticidal CAs (Carpenter *et al*. 2001). This approach where phenomena have been described has not led to a unified understanding of the processes involved when a CA is insecticidal. The published information is very useful and gives clues to the underlying processes, but we still lack a unifying hypothesis.

Fourney *et al*., (1991) found dead insects contained no ATP; did they die because they had none, or did they have none because they were dead? AliNiazee (1972), found a range of biochemical parameters that varied with treatment with anoxic atmospheres, but did not yet produce an overview that integrates the observed phenomena. Donahaye and Navarro (2000), found that after treatment with insecticidal CAs insects lacked an energy source, which in itself does not explain why the insects died as it does not show what biochemical processes were involved. Friedlander and Navarro (1979), showed that in developing insect lines resistant to CAs, the physical volume affected the dynamics of mortality. Stevenson *et al*., (unpublished) hypothesized that pupal thrips were more resistant to CAs because they had fewer spiracular openings than the larvae or adults, again suggesting a simple physical component to the mortality process.

The published record

Invertebrate eco-physiologists and entomologists have used calorimetry to test a number of hypotheses in recent years. Harak *et al*., (1999) used calorimetry to study the effects of a toxicant on the physiology of a diapausing lepidopteran pupa. Adaptation to freezing in Antarctic nematodes, in large Orthoptera, and in Curculionidae has been quite extensively studied using calorimetry (Block 1994; Ramlov *et al*. 1996; Rojas *et al*. 1992). Metabolic heat flux in pregnant viviparous

cockroaches was studied by Schultze-Motel and Greven (1998) as an approach to understanding the energetic demands of viviparity. Zhou *et al.,* (2000) studied the effects of CAs on metabolic heat production by pupae of a lepidopteran caterpillar and concluded that the method provided an insight into the relative contribution of O_2 and CO_2 to insect mortality. They also suggested that their data showed that the mechanisms involved in mortality from treatment with CO_2 were different from those from treatment with O_2.

Hypotheses

We hypothesized that under insecticidal controlled atmospheres, insect metabolic activity would decline slowly. There would be a consequential change from aerobic to anaerobic respiration, leading to changes in the ratios of metabolic heat rate to rates of O_2 consumption and CO_2 production.

We also postulated that the gross response of an organism would vary with life stage and habitat. We expected that insects living on the outside of a plant (aphids, leaf-rollers) would react differently to those living in plant tissue (codling moth) where environmental CO_2 levels would be higher and O_2 levels slightly lower than they were on the plant surface. Stored-product insects were expected to be different again, because they live in dry environments with relatively high CO_2.

We had also begun to question the cost-effectiveness of standard approaches to development of a novel quarantine treatment. For a specialty crop such as persimmon, exported from New Zealand, to quarantine-sensitive markets such as Japan, with at least 15 actionable pests present, the huge cost of empirically developing a data set that identified the most tolerant life stage of the most tolerant pest put innovation beyond reach. Could calorimetry be used as a tool to short-circuit this process?

Lastly, as a controlled atmosphere could be anything between 0 and 20 (100?)% O_2 and 0-100% CO_2, at temperatures between 0 and 45°C, it is difficult to determine which parameters to test empirically. This becomes more complex when one includes sequential CAs in the consideration, particularly for perishables in transit (Shelton and Carpenter unpublished).

Can calorimetry provide insights at the beginning of the process of developing a new quarantine treatment?

EXPERIMENTS AND RESULTS

Calorimeter output

Figure 1 shows a typical calorimeter trace. In this case codling moth pupae were the test organism, although all traces are generally similar. Values below 0 show that the sample is producing heat, that is, endothermic heat production is being measured. In this experiment where the air was replaced with CO_2 and nitrogen (N_2), metabolic heat decreased rapidly when the CA was introduced. At the end of the run the experimental atmosphere was replaced by air. The metabolic heats at the end of experimental run when the insects were returned to air were lower than at the start of

the experiment showing that the insects had been stressed in some way. The periodicity of the trace in air may be due to discontinuous gas exchange by the pupae.

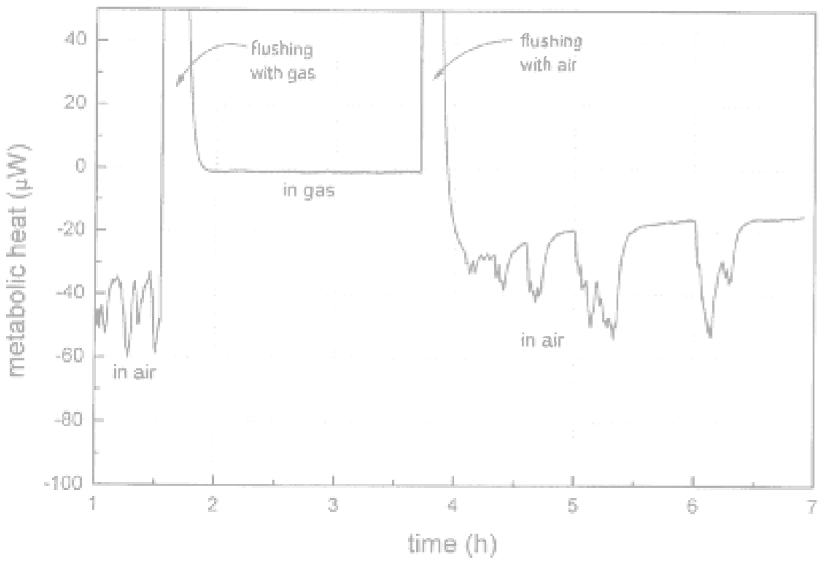


Fig. 1. Typical calorimeter trace, (codling moth pupae) under 60% CO_2 and 40% O_2. The excursions at the points where the ampoule was flushed with the CA or air are due to perturbation of the thermal equilibrium at that point.

The instantaneous concentrations of O_2 and CO_2 in the ampoule can be evaluated directly for the mass spectrometer readings. It is more difficult to measure the rate of O_2 consumption and CO_2 production by the insects, because a significant correction has to be made for the effects of the continuous withdrawal of gas from the ampoule by the mass spectrometer and replacement by gas of the original composition. We have used the Poiseuille equation to calculate the flow rate down the 2 m of capillary that connects the calorimeter to the mass spectrometer. When this correction is applied, the resultant rates of O_2 consumption and CO_2 production for insects in air in the calorimeter are very close to those expected on the basis of metabolic heat rates.

Taxonomic comparisons

The responses of *Ctenopseutis* sp. (leafroller caterpillar), *Cydia pomonella* (L) (codling moth caterpillar and pupa), *Ephestia kuehniella* (Mediterranean flour moth), *Myzus persicae* (green peach aphid), *Sitophilus oryzae (*rice weevil*)* and *Tribolium confusum* (confused flour beetle) to hypercarbic (60% CO_2) and hypoxic (0-10% O_2) atmospheres were all similar (data not presented): a very rapid switch to a low level of metabolism occurred once the atmosphere was introduced. An example of this is

shown for *C. pomonella* in Fig. 1. This general case applied to all the other taxa, except for larvae of *S. oryzae*, although this may be more of a methodological issue than a real difference. Neither the natural environment of the species nor the life stage appeared to affect the dynamics of the process. The presence of O_2 did have an impact with the base level of the atmospheres being higher when O_2 was present than it was when the atmosphere was anoxic.

Metabolic heat, metabolic substrate and respiratory gas production

The combination of calorimetry and mass spectometry was used to obtain three measures of insect respiration rate:

Φ the metabolic heat rate, and $\mathbf{R_{O2}}$ and $\mathbf{R_{CO2}}$ the rates of O_2 consumption and of CO_2 production, respectively.

The direct heat of combustion of nearly all organic compounds has a value of –455+/- 15 kJ per mole of O_2 consumed [Thornton's Rule (Criddle and Hansen 1999)], and this is the value to be expected for $\Phi/\mathbf{R_{O2}}$ for aerobic respiration unaccompanied by growth. Note that the heat of combustion per mole of organic compound can be very different. The ratio $\mathbf{R_{CO2}/R_{O2}}$ would be unity for a carbohydrate substrate under these conditions, but for other substrates it would vary depending on the average formal oxidation number of the substrate carbon.

Results are given in Table 1 for an experiment with aphids in which the original air atmosphere was replaced by 5% O_2+ 95% N_2, and then by air again. All values in the Table are instantaneous values. The reported O_2 values are a result of insect metabolic depletion of the original values. There was a large decease in metabolic heat production as the O_2 level decreased. At all three O_2 values the ratio $\Phi/\mathbf{R_{O2}}$ was close to the theoretical value of –455 kJ per mole of O_2 for the oxidation of organic substrates. In the first stage of the experiment, in partially depleted air $\mathbf{R_{CO2}}$ was similar to $\mathbf{R_{O2}}$ giving a value near unity for $\mathbf{R_{CO2}/R_{O2}}$. In the low O_2 atmosphere $\mathbf{R_{CO2}}$ was relatively large in comparison to $\mathbf{R_{O2}}$ leading to a $\mathbf{R_{CO2}/R_{O2}}$ ratio significantly greater than unity, suggesting that the metabolic pathway being utilized had changed, or, that a more oxidized substrate was being utilized. We speculate that the slightly reduced value of $\mathbf{R_{CO2}}$ at the end of the experiment was a result of the replenishment of the previously depleted pool of more oxidized substrate.

Thermal cycling

We found that cycling the temperature to which the insects were subjected, gives a rapid indication of the level of physiological stress associated with the temperatures involved. This is shown in Fig. 2. This shows that the effects of 40 and 50°C were more dramatic than were 0ºC. We believe this approach will help us understand which CAs and possibly fumigants are most effective, although the key experiments have not yet been carried out.

TABLE 1
Effect of oxygen level on the metabolism of *Myzus persicae* at 40ºC (fresh weight of aphids 0.02203 g)

Time from start of experiment – minutes	15	40	65
Oxygen concentration %	15.7	3.85	18.9
Rate of metabolic heat production μW	-519	-207	-406
Rate of oxygen consumption mol sec^{-1}	12.1 x 10^{-10}	4.4.5 x 10^{-10}	8.56 x 10^{-12}
Rate of carbon dioxide consumption	11.8 x 10^{-10}	5.86 x 10^{-10}	7.94x10^{-10}
Ratio of heat to oxygen kJ mol^{-1}	-429	-465	-474
Ratio of heat to carbon dioxide kJ mol^{-1}	-440	-353	-511
Ratio of carbon dioxide to oxygen	0.98	1.32	0.93

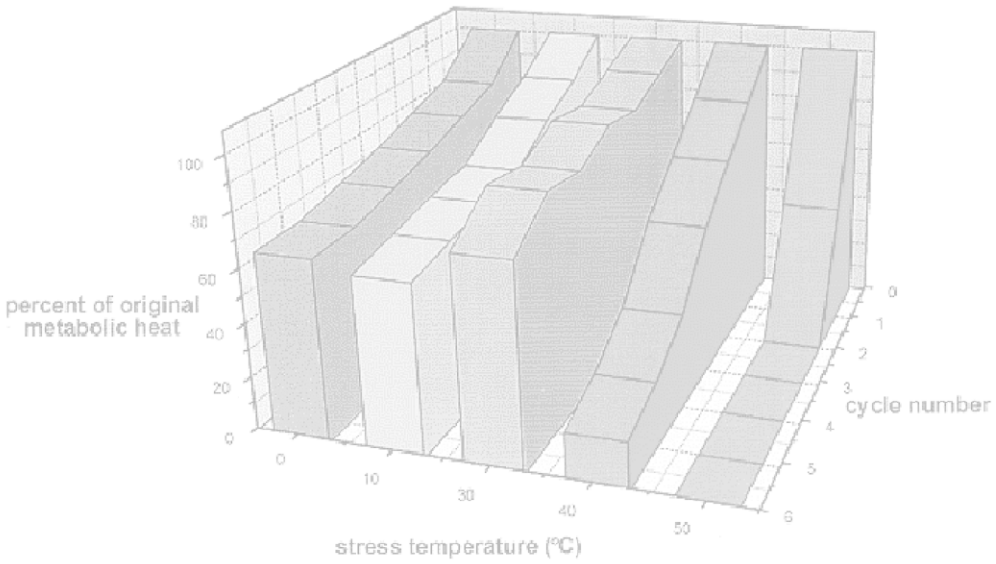


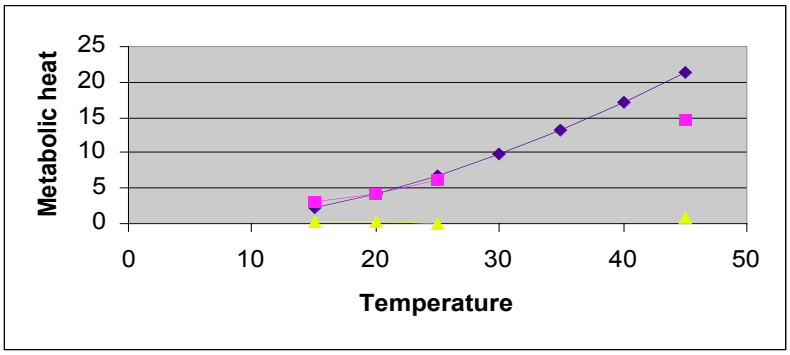
Fig. 2. Proportions of original metabolic heat rate when *Myzus persicae* were cycled between 25ºC and various experimental temperatures after allowing 30 min for the system to stabilize between changes.

Time and temperature interactions

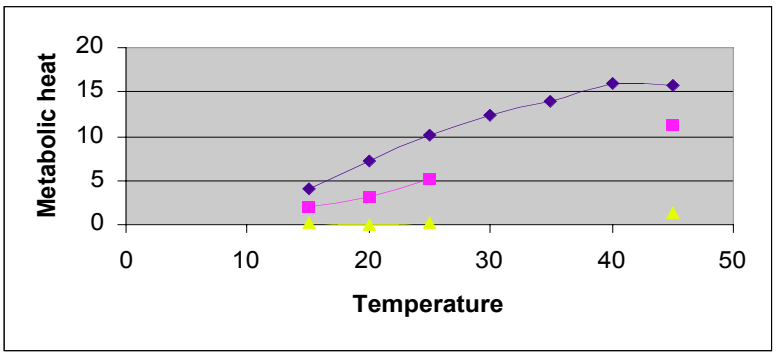
These effects, which are central to developing a novel quarantine system based on CAs, are easily explored with calorimetry. As both temperature and time increase, so does insect mortality. The effects of temperature on the responses of adult and larval confused flour beetles to air, 5% O_2 and 60% CO_2 are shown in Fig. 3. Here the

interaction between the composition of the atmosphere and temperature can readily be seen.

Adult confused flour beetle



Larval confused flour beetle



Adult rice weevil

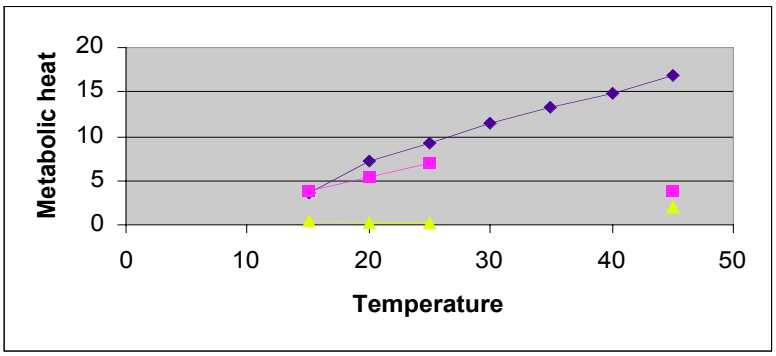


Fig. 3 Effects of varying temperature on metabolic heat production by selected insects when held in air or a hypoxic or a hypercarbic controlled atmosphere. Diamonds = air; Boxes = 5% O_2; Triangles = 60% CO_2.

In air, the metabolic heat production for all three insects was similar. At ambient temperatures, metabolic heat production by larval confused flour beetle was suppressed more by 5% O_2 than it was by the adults, and that of the rice weevil was intermediate between them. In the hypercarbic atmosphere all three experimental insects demonstrated similar and dramatic levels of suppression of metabolism. This relatively simple data set illustrates how calorimetry can be used to develop understanding of the responses of insects to CAs without extensive empirical experimentation.

Effects of anoxic conditions:
In Fig. 4 data are presented that show the relationship between the ability of *Myzus persicae* to recover its original metabolic heat levels after various time periods under anoxia. When they recovered to more than 50% of their initial metabolic heat levels they were apparently alive. Below 50% of their initial metabolic heat level, they were dead. There were some variations close to the 50% value.

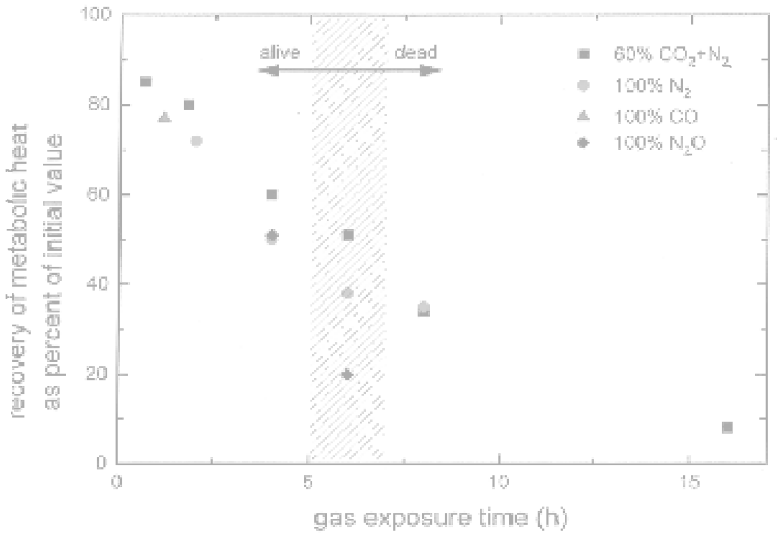


Fig. 4. Recovery of metabolic heat rate by *Myzus persicae* to different exposure periods to different anoxic atmospheres.

Effects of various CAs on insects:
We compared the effects of 4 atmospheres at 0, 20 and 40°C on Mediterranean flour moth larvae after an exposure of 1 h. The data are shown in Table 2. An O_2 level of

5%, with the balance N_2, had some effect on the metabolic heat produced by the larvae. When the O_2 concentration was 1%, the heat levels were rather lower than they were when O_2 was 5%. In 5% O_2 + 60% CO_2, with the balance N_2, the metabolic heat rates were higher than when there was 60% CO_2, with the balance N_2. In two treatments the insects died, one in which heat levels had been reasonable high and in the other very low. In another treatment they were very low without the insects dying. These data show that there are differences between hypercarbic and hypoxic atmospheres and how they affect insects, and show that metabolic heat production alone is not necessarily a good predictor of insect mortality.

TABLE 2
Metabolic heats of Mediterranean flour moth larvae in four CAs after 1 hour as a percentage of their initial metabolic heat

Temperature	5% O_2	1% O_2	5% CO_2+5% O_2	60% CO_2
0ºC	85%	66%	53%	32%
20ºC	79%	79%	87%	6%
40ºC	82%	38%	58% (died)	2% (died)

DISCUSSION

We have been able to demonstrate that under low O_2 conditions insects change from aerobic to anaerobic respiration as was predicted. Thus they reduce their reliance on standard respiratory substrates.

The recognition of a change in the nature of metabolism is dependent, in part, on the appropriateness of the correction to the mass spectrometry data. As noted above the $\square/\mathbf{R_{02}}$ ratios are in accord with the theoretical value which suggests that this is so, but a more direct method to confirm the calculated rate of gas removal from the ampoule by the mass spectrometer would be useful.

We have also shown that under anoxic or CA conditions, there is a sudden switch to a much lower metabolic level and not the detectable graduated change in metabolic rate that we had expected. That is, the change occurs within the thermal re-equilibration period of the calorimeter after the disturbance caused by changing the atmosphere.

We have shown that the insecticidal effectiveness of a CA or anoxic conditions can be estimated better by thermal cycling than by simple measurement of metabolic heat rate. The thermal cycling data indicate that an insect becomes programmed to die when its recovery metabolic heat rate in air is less than 50% of its initial metabolic heat rate in air. This suggests that under CAs and anoxic conditions, insects lose biochemical and/or physiological systems sequentially until they are unable to replace or reactivate them. From this we hypothesize that CAs do not have a single point of effect. In turn, this explains why many researchers have found such

a wide variety of biochemical phenomena associated with insect mortality when treated with CAs. This may also explain why mortality curves for groups of insects treated with CAs are extremely variable, ranging between 20 and 80% mortality.

In general, the change in metabolic heat rate was similar to all the insect species we have tested, regardless of normal habitat, or life stage. These findings differ from the observations of Hoback and Stanley (2001) who suggested that habitat had a significant effect on insect tolerance to hypoxia, with grain pests more likely to be adapted to oxygen depletion than pests of fresh commodities.

We have begun to show some of the applications of isothermal calorimetry to postharvest entomology. There are technical issues still to be solved. We still require appropriate empirical data to "ground truth" what we find from the calorimetry data. We can find what type of atmosphere is most likely to kill insects with very little effort and use this as a directive to cost-effective empirical data collection.

There are some limitations to the process: to effectively interpret data when using calorimetry, access to a significant amount of empirical data is essential to ensure that understanding of the data output is correct; for small insects groups of individuals must be tested; some experimental aspects require further development.

There are also advantages: the method is rapid; experiments are repeatable; data provide corroboration for other experiments, allowing more robust analysis of data than would otherwise be possible.

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