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Metabolic response of *Platynota stultana* pupae to controlled atmospheres and its relation to insect mortality response

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Abstract

The metabolic responses of *Platynota stultana* pupae to reduced O_2, elevated CO_2, and their combinations were investigated using microcalorimetry, and mortality of pupae under elevated CO_2 atmospheres was correlated with metabolic responses. The metabolic heat rate decreased slightly with decreasing O_2 concentration until a critical O_2 concentration (P_c) below which the heat rate decreased rapidly. The P_c increased with temperature. The percentage decreases of metabolic heat rate were comparable to the percentage decreases of O_2 consumption rate (RO_2) at 10, 8, 6, and 4% O_2, but were smaller at 2 and 1% O_2. The metabolic heat rate decreased rapidly at 20% CO_2 relative to 0% CO_2, with little to no further decrease between 20 and 79% CO_2. The percentage decreases of RO_2 under 20 and 79% CO_2 at 20°C were comparable to the percentage decreases of metabolic heat rates. The additive effects of subatmospheric O_2 and elevated CO_2 levels on reducing metabolic heat rate were generally fully realized at combinations of $\leq$5% CO_2 and $\geq$4% O_2, but became increasingly overlapped as the O_2 concentration decreased and the CO_2 concentration increased. The high susceptibility of pupae to elevated CO_2 at high temperature was correlated with high metabolic heat rate. The metabolic responses of pupae to reduced O_2 concentrations included metabolic arrest and anaerobic metabolism. The net effect of elevated CO_2 on the pupal respiratory metabolism was similar to that of reduced O_2; however, mechanisms other than the decrease of metabolism were also contributing to the toxicity of CO_2.

Keywords: Carbon dioxide; Metabolic heat rate; Microcalorimetry; Oxygen; Respiration rate

1. Introduction

Controlled atmospheres (CA) with elevated CO_2, reduced O_2, or their combinations can be used to control insects (Carpenter and Potter, 1994; Mitcham et al., 1997b). Above 20%, CO_2 can cause significant insect mortality in proportion to CO_2 concentration. Reducing O_2 to less than 3% can be insecticidal, and efficacy increases as O_2 is reduced to lower concentrations. The combined effects of elevated CO_2 and reduced O_2 are less clear; some studies have shown additive effects while others have not (Fleurat-Lessard, 1990; Soderstrom et al., 1991). Temperature greatly affects the efficacy of CA; higher efficacy is usually achieved at higher temperatures (Banks and Annis, 1990; Carpenter and Potter, 1994). There have been few physiological or biochemical explanations for these mortality responses. Lack of such knowledge has rendered the development of CA treatments costly and time consuming (Carpenter et al., 1993). If we can understand the physiological and biochemical responses of insects to CA and can relate such responses to mortality, then we might be able to develop physiological or biochemical models to determine effective treatments instead of relying on empirical mortality tests.

Hypotheses have been proposed as to how invertebrates and higher animals respond to low O_2 environments (Herreid, 1980; Hochachka, 1986; Weyel and Wegener, 1996). An organism is described as a metabolic regulator if its O_2 consumption is independent of ambient O_2 concentrations and as a metabolic conformer if its O_2 consumption is dependent upon ambient O_2 concentrations. No species is a perfect regulator over the entire range of O_2 tensions; it becomes a conformer when the ambient O_2 concentration is below a critical level (P_c). A "good" regulator would have a low P_c. The

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P_c varies with many factors, including the organism's metabolic demand. The P_c decreases at lower metabolic demand. It has been proposed that as a metabolic regulator, an organism regulates O_2 consumption at reduced O_2 tensions by behavioral and/or physiological compensations which guarantee that the O_2 concentration in the tissue does not decrease. The physiological compensation could include respiratory compensation such as increasing ventilation, circulatory compensation such as increasing the rate of blood perfusion, or the use of respiratory pigments (Herreid, 1980).

However, as a metabolic conformer at below P_c, an organism experiences hypoxia (insufficient supply of O_2 to tissues). It has been proposed that animals mainly use two strategies to cope with hypoxia: anaerobic metabolism and metabolic arrest (Hochachka, 1986; Weyel and Wegener, 1996). Anaerobic metabolism can temporarily compensate for energy insufficiency of oxidative phosphorylation. However, this strategy would require very high rates of glycolysis and thus lead to rapid exhaustion of carbohydrate reserves while toxic end products accumulate. Metabolic arrest, that is, reducing ATP turnover (demand) and thus reducing metabolic rate, is thought to be a better strategy. It lessens the pressure on the organism to initiate anaerobic metabolism. However, because reduced ATP turnover also means reduced energy use for ion transport across the membrane, the effectiveness of this strategy requires low membrane permeability.

These drawbacks of the two strategies, especially that of metabolic arrest, have been thought to be the cause of hypoxic/anoxic toxicity (Hochachka, 1986). According to Hochachka (1986), reduced O_2 consumption leads to a decreased rate of ATP production. As a result of energy insufficiency, the membrane ion pumps fail, leading to K^+ efflux, Na^+ influx, and membrane depolarization. The voltage-dependent Ca^{2+} gates are then opened, causing Ca^{2+} influx. The high concentration of Ca^{2+} in cytosol activates phospholipases A1, A2, and C, leading to increased membrane phospholipid hydrolysis. The cell and mitochondrial membranes become more permeable, causing cell damage or death.

Do these general hypotheses apply to insects? If so, can some aspects of these hypotheses explain the effects of elevated CO_2? It has been proposed that the effects of hypercarbia on insects probably do not exclude the effects of hypoxia (Fleurat-Lessard, 1990) because high CO_2 can prevent insects from using O_2 (Navarro, 1975). However, this latter point is controversial because others have observed that the O_2 consumption rate of insects is not reduced by elevated CO_2 levels with 21% O_2 present (Edwards and Batten, 1973). As to the combined effects of elevated CO_2 and reduced O_2, it appears that the influence of the proportion of CO_2 becomes more important as the O_2 content is increased above 1% and the contribution to mortality by CO_2 action increases as the low O_2 effect becomes marginal (Banks and Annis, 1990).

Our objectives were to address these questions by studying the metabolic responses of *Platynota stultana* pupae (an important pest on many horticultural commodities) to various levels of reduced O_2, elevated CO_2, and their combinations. Specifically, metabolic heat rates, indicative of the overall metabolic rates of an organism (Loike et al., 1981; Criddle et al., 1988), and respiration rates were measured under various atmospheres. In addition, mortality tests were performed under some atmospheres and the mortality responses were correlated with metabolic responses.

2. Materials and methods

2.1. Experimental insects

Platynota stultana was reared on a lima bean-based diet in an incubator at 27±0.5°C, 85% RH with a photoperiod of 16:8(L:D) h (Yokoyama et al., 1987). The 1–2 d old female pupae were selected for experiments because there was little variability in metabolic rate within this age group.

2.2. Calorimetry measurements

Rates of metabolic heat production were measured using differential scanning calorimeters with isothermal and temperature scanning capabilities (model 7707, Hart Scientific Inc., Provo, UT). The isothermal operating mode was used to measure metabolic heat rates at a given temperature. Each calorimeter has three measuring cells and one reference cell, allowing three samples to be measured simultaneously in one machine. Samples were placed in ampoules with an internal volume of 1.05 ml. The heat rates were measured continuously until they were stabilized to constant rates indicating that the samples and chamber had attained a steady state (approximately 45 min). The constant heat rates were corrected with baselines measured using empty ampoules. The corrected heat rates were the metabolic heat rates of the samples.

2.3. Controlled atmosphere set-up in the ampoules

Appropriate amounts of air, CO_2, N_2, and O_2 were mixed using metering valves to produce the desired atmospheres. The gas concentrations of the mixtures were analyzed by gas chromatography (model 211, Carle Instruments, Anaheim, CA). The gases flowed through a plastic bag (about 3 liters when fully inflated) at a constant rate of 2 liters/min after being first bubbled through water to obtain >90% relative humidity (RH). The plastic bag had an inlet, an outlet, and a sealable

side. Open ampoules containing pupae were placed on sticky tapes in the middle of the bag, with lids beside. The open side of the bag was folded and sealed by clamping two thin and narrow plates on the folded area. The outlet of the bag was clamped temporarily to inflate the bag with the mixed gases. Then the clamp on the outlet was released and the gas in the bag was pushed out. This process was repeated 4–5 times until the gas concentration in the bag reached the correct level, which was confirmed by drawing samples from the bag and analyzing them by gas chromatography. The ampoules in the bag were then sealed with their lids through the bag. The sealed ampoules were then taken out of the bag and placed into calorimeter cells for metabolic heat rate measurement.

2.4. Metabolic response of pupae to controlled atmospheres

The number of pupae per ampoule varied with the test temperature (10 at 10°C, 4 at 20°C, and 2 at 30°C) to allow the total measured heat rates at different temperatures to be close to each other. The metabolic heat rates under air were first measured. The ampoules were then opened and an appropriate atmosphere was added to the ampoules as described above. The metabolic heat rates under CA were then measured. After measurements the pupae were dried in an 80°C vacuum oven for at least 24 hours to obtain their dry weights. The percentage decrease of metabolic heat rate under an atmosphere was calculated.

2.5. Respiration measurement

The O_2 consumption rate (RO_2) and CO_2 production rate (RCO_2) of pupae under various O_2 concentrations were obtained by measuring the volume of O_2 (VO_2) consumed and the volume of CO_2 produced (VCO_2) at a given time in a closed syringe. Thirty pupae were weighed and placed in a 20 ml syringe without its needle. The syringe, along with the plunger and a small rubber septum, were placed in a plastic bag that was connected to a constant flow of a desired gas mixture as described above. When the correct concentration of the gas in the bag was established, the plunger was pushed into the syringe, leaving 18 ml of volume. Then the rubber septum was put on the tip of the syringe (where a needle is usually mounted) to seal it. These operations were performed through the plastic bag while it was sealed and the gas was flowing through it. Immediately after the sealed syringe was taken out of the bag, three 1-ml gas samples were taken from the syringe through the rubber septum and analyzed simultaneously for O_2 and CO_2 using an infra-red gas analyzer (model PIR-2000R, Horiba Instruments, Irvine, CA). The syringe, now having a volume of 15 ml, was placed in a temperature controlled room. The gas concentrations in the syringe were analyzed again after 2 hours. The VO_2 and VCO_2 were calculated from the change between the initial and final O_2 and CO_2 concentrations. The pupae were then dried in an 80°C vacuum oven for at least 24 hours to obtain pupal dry weight. The RO_2s under various CO_2 concentrations were also measured as described above.

2.6. Mortality test

The mortality of pupae was tested under 20, 40, and 79% CO_2 at >90% RH (all added to 21% O_2, balance N_2) at 10, 20, and 30°C. Using flow boards, constant flow of gas mixtures at a rate of 150 ml/min passed through a 1 liter treatment jar where test pupae were placed. The gas concentrations inside jars were sampled daily during treatment and analyzed by gas chromatography.

Controlled atmosphere treatments were conducted in controlled temperature rooms maintained at 10, 20, and 30°C. Thirty pupae were placed in a cup with a mesh top. The cup was placed in a jar through which an atmosphere was passed. The range of treatment times for each atmosphere at each temperature was determined by preliminary tests, and corresponded with treatment times during which 10 to 100% mortality was expected. After treatments the pupae were transferred to an incubator at 27°C and 80–90% RH. Adult eclosion or lack thereof was observed after 2 weeks to determine mortality. All treatments were replicated at least 3 times.

2.7. Statistical analysis

The data for the percentage decrease of metabolic heat rate, respiration rates, and respiration quotient (RQ) were analyzed by ANOVA (GLM, SAS Institute, 1989). Means for significant effects were separated by t-test (LSD). Response surfaces were fitted for the percentage decrease of metabolic heat rates under the combinations of reduced O_2 and elevated CO_2. A separate probit curve for each treatment level was fitted with mortalities as the dependent variables and treatment duration as the independent covariates (PROC PROBIT, SAS Institute, 1989). The fitted probit curves were used to calculate LT_{99} values.

3. Results

3.1. Temperature

The metabolic heat rate under air (21% O_2/0.03% CO_2) was 1.2, 3.7, and 7.5 μW/mg at 10, 20, and 30°C, respectively. The Q_{10} between 10 and 20°C was approximately 3, and was 2 between 20 and 30°C.

3.2. Reduced O_2

The metabolic heat rate decreased with decreasing O_2 concentration (Fig. 1). At all three temperatures, the decrease was slight until a critical O_2 concentration below which the decrease became rapid. The critical O_2 concentrations were higher at higher temperatures, being 6% O_2 at 10°C, 8% O_2 at 20°C, and 10% O_2 at 30°C (Fig. 2(A)). Temperature had slight but significant effects on the heat rate decreases (Fig. 2(A)). The percentage decreases were slightly higher at higher temperatures at 6, 4, 2, and 1% O_2. But at 10 and 8% O_2 the percentage decreases at 10°C were higher than at 20 or 30°C. ANOVA results for reduced O_2 concentrations were highly significant ($P<0.0001$) for O_2 concentration, temperature and O_2 concentration×temperature.

The O_2 consumption rate (RO_2) at 20°C decreased slightly with decreasing O_2 concentration down to 8% O_2, and then decreased rapidly (Fig. 3). The percentage decreases of RO_2 at 20°C, which were 11, 15, 25, 41, 77, and 83 at 10, 8, 6, 4, 2, and 1% O_2, respectively, were comparable to the percentage decreases of metabolic heat rate under various O_2 concentrations at 20°C except at 2 and 1% O_2, where the percentage decreases of RO_2 were about 10% higher (Fig. 2(A)). The percentage decreases of CO_2 production rate (RCO_2), which were 9, 19, 21, 33, 58, and 70 at 10, 8, 6, 4, 2, and 1% O_2, respectively, were comparable to those of metabolic

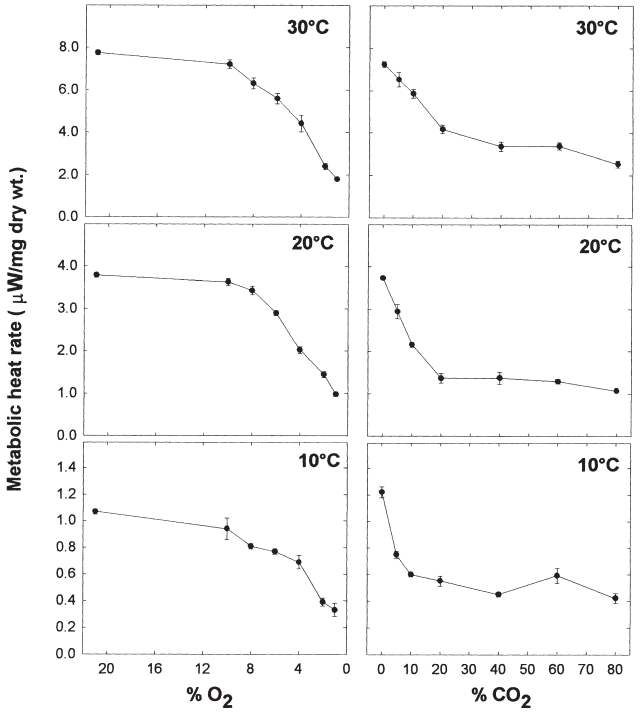

Fig. 1. Metabolic heat rate (μW/mg dry wt.) of 1–2 d old *Platynota stultana* female pupae under various O_2 concentrations(with 0% CO_2) and various CO_2 concentrations (with 21% O_2) at 10, 20 and 30°C. Vertical bars represent standard errors.

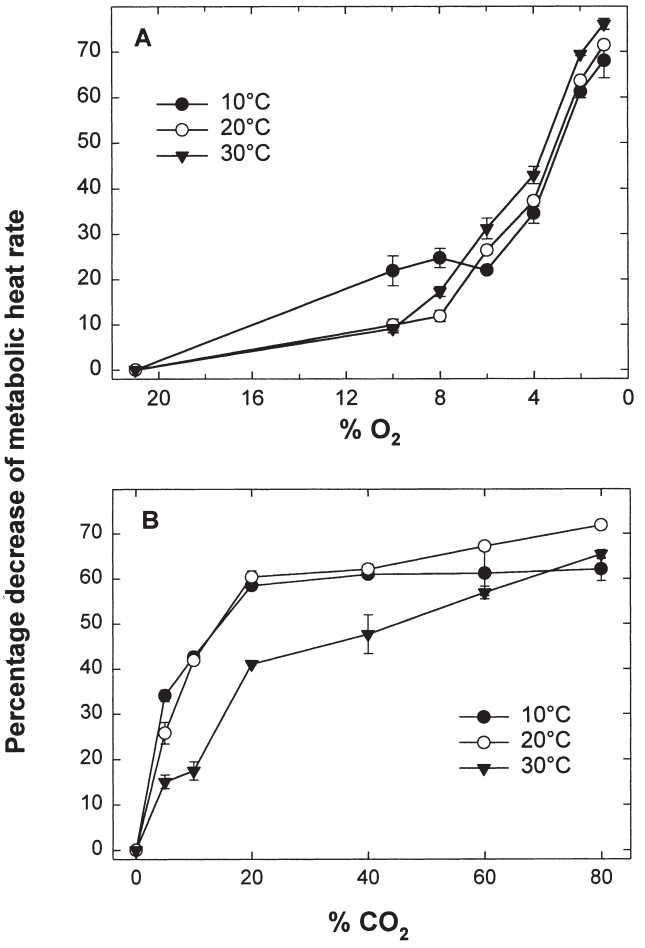

Fig. 2. The percentage decrease of metabolic heat rate of 1–2 d old *Platynota stultana* female pupae under various O_2 concentrations (with 0% CO_2) (A) and various CO_2 concentrations (+21% O_2) (B) at 10, 20 and 30°C. Vertical bars represent standard errors.

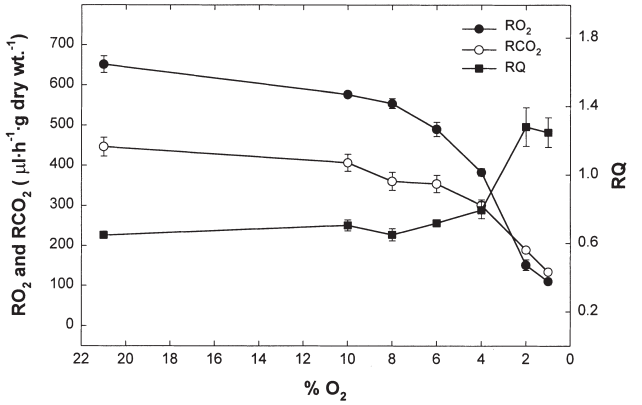

Fig. 3. O_2 consumption rate (RO_2) (μl·h^{-1}·g^{-1} dry wt.), CO_2 production rate (RCO_2) (μl·h^{-1}·g^{-1} dry wt.), and respiratory quotient (RQ) of 1–2 d old *Platynota stultana* female pupae under various O_2 concentrations(with 0% CO_2) at 20°C. Vertical bars represent standard errors.

heat rate under all O_2 concentrations (Fig. 2(A)). The respiratory quotient (*RQ*) showed no significant change between 21 and 4% O_2, with values between 0.65 and 0.80. However, the *RQ* increased significantly to about 1.3 when O_2 concentration was reduced to 2 and 1% (Fig. 3). ANOVA results for various O_2 concentrations were highly significant ($P<0.0001$) for RO_2, RCO_2, and *RQ*.

3.3. Elevated CO_2

The metabolic heat rate decreased rapidly between 0 and 20% CO_2 (Fig. 1), with a 60% decrease at 10 and 20°C and a 40% decrease at 30°C under 20% CO_2 (Fig. 2(B)). Further decrease of metabolic heat rate between 20 and 79% CO_2 was slight. At 10°C, there was no further decrease of metabolic heat rate between 20 and 79% CO_2. At 20°C, the metabolic heat rate further decreased under 60 and 79% CO_2, with a 72% decrease at 79% CO_2. At 30°C, the metabolic heat rate continued to decrease from 20 to 79% CO_2, but at a much slower rate than that from 0 to 20% CO_2. The percentage decreases of metabolic heat rate at a certain CO_2 concentration were generally lower at 30°C than at 10 and 20°C, which were mostly similar except at the two ends of the CO_2 spectrum (Fig. 2(B)). ANOVA results for elevated CO_2 concentrations were highly significant ($P<0.0001$) for CO_2 concentration, temperature and CO_2 concentration×temperature.

The RO_2 at 20°C (660 $\mu l \cdot h^{-1} \cdot g^{-1}$) decreased by 62% at 20% CO_2 (250 $\mu l \cdot h^{-1} \cdot g^{-1}$) and by 73% at 79% CO_2 (185 $\mu l \cdot h^{-1} \cdot g^{-1}$). The percentage decreases of RO_2 were comparable to the percentage decreases of metabolic heat rate under the same CO_2 concentrations (Fig. 2(B)).

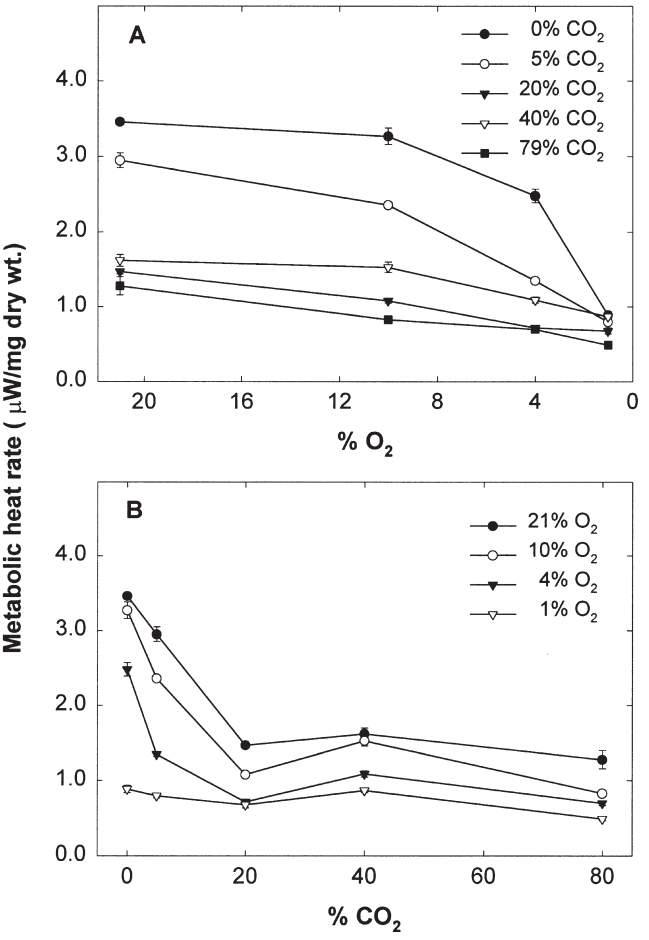

Fig. 4. Metabolic heat rate (μW/mg dry wt.) of 1–2 d old *Platynota stultana* female pupae under combinations of reduced O_2 and elevated CO_2 at 20°C; (A) effects of reducing O_2 concentration at various CO_2 concentrations; (B) effects of increasing CO_2 concentration at various O_2 concentrations. Vertical bars represent standard errors.

3.4. Combinations of elevated CO_2 and reduced O_2

Reducing O_2 concentration at 20°C decreased metabolic heat rate further at all CO_2 concentrations (Fig. 4(A) and Table 1). However, the effects of reduced O_2 were smaller at higher CO_2 concentrations (Table 2). The effects of elevated CO_2 on metabolic heat rates varied with O_2 and CO_2 concentrations (Fig. 4(B), Table 1). At 4% O_2 or higher, metabolic heat rate decreased rapidly between 0 and 20% CO_2 and there was little further decrease between 20 and 79% CO_2. At 1% O_2, only 20 and 79% CO_2 decreased the metabolic heat rate further. The additional percentage decreases in metabolic heat rate contributed by elevated CO_2 were generally smaller at lower O_2 concentrations (Table 3). At 10% O_2, all CO_2 concentrations showed their full effects, with the additional percentage decreases similar to those at 21% O_2. At 1% O_2, however, there was little additional effect of CO_2 (Table 3).

The response surface of the percentage decrease of metabolic heat rate fitted with a polynomial of term 3 showed that the full additive effects on reducing metabolism mostly occurred at combinations of ≤5% CO_2 and ≥4% O_2 (Fig. 5). The combined effects of reduced O_2 and elevated CO_2 became increasingly overlapped as the O_2 concentration decreased and the CO_2 concentration increased.

3.5. Mortality responses to elevated CO_2

Temperature had a dominant impact on the mortality responses; the higher the temperature, the more susceptible the pupae (Fig. 6, Table 4). However, the effect of temperature varied with CO_2 concentration. At 20% CO_2, lowering temperature from 20 to 10°C increased LT_{99} greatly (Table 4). At 79% CO_2, however, lowering temperature from 20 to 10°C did not change LT_{99} significantly. CO_2 concentrations affected mortality, but the specific effects were dependent on temperature. Forty and 79% CO_2 were more effective than 20% CO_2 at all three temperatures; however, 79% CO_2 was not more

Table 1
Pecentage decrease of metabolic heat rate of 1–2d old *Platynota stultana* female pupae under combinations of CO_2 and O_2 at 20°C[a]

% O_2	% CO_2 0	5	20	40	79
21	0.0 d, E	25.8 d, D	58.8 d, B	52.3 d, C	69.0 d, A
10	5.7 c, E	34.2 c, D	68.1 c, B	57.9 c, C	75.2 c, A
4	29.6 b, D	61.3 b, C	77.8 b, A	68.3 b, B	79.5 b, A
1	76.6 a, C	77.4 a, C	80.1 a, B	74.2 a, D	84.7 a, A

[a] Within each column, mean differences are indicated by lower case letters (*t*-test). Within each row, mean differences are indicated by upper case letters.

Table 2
The additional percentage decrease of metabolic heat rate caused by reduced O_2 when 1–2d old *Platynota stultana* female pupae were under various concentrations of CO_2 at 20°C

% O_2	% CO_2 0	5	20	40	79
21	0.0	0.0	0.0	0.0	0.0
10	5.7	8.4	9.3	5.6	6.2
4	29.6	35.5	19.0	16.0	10.5
1	76.6	51.6	21.3	21.9	15.7

Table 3
The additional percentage decrease of metabolic heat rate caused by elevated CO_2 when 1–2d old *Platynota stultana* female pupae were under various concentrations of O_2 at 20°C

% CO_2	% O_2 21	10	4	1
0	0.0	0.0	0.0	0.0
5	25.8	28.5	31.7	0.8
20	58.8	62.4	48.2	3.5
40	52.3	52.2	38.7	−2.4
79	69.0	69.5	49.9	8.1

effective than 40% CO_2 at 20 and 30°C. Increasing CO_2 concentration from 20 to 79% CO_2 greatly improved efficacy at 10°C, but not at 20°C.

An atmosphere of 40% CO_2+21% O_2 at 20°C caused high mortality at short treatment durations, e.g., above 40% mortality with only 3 hours of exposure (Fig. 6). This atmosphere also caused the pupal body fluid to leak out immediately during exposure. This body fluid leakage phenomenon did not occur under 20 and 79% CO_2 (+21% O_2), 0 to 21% O_2, or even 40% CO_2+1% O_2.

4. Discussion

4.1. Reduced O_2 concentrations

The O_2 consumption rate of *Platynota stultana* pupae decreased slightly with decreasing O_2 concentration until a critical concentration point (P_c) below which the decrease became rapid. This O_2 consumption pattern is typical of that of invertebrates in response to decreasing environmental O_2 concentrations (Herreid, 1980). The pupae regulated their O_2 consumption between 21 and 8% O_2 at 20°C, probably by increasing ventilation. The pupae were metabolic regulators at this O_2 range. However, the pupae became metabolic conformers at below 8% O_2 when increased ventilation could not compensate for O_2 insufficiency. It is interesting to note that the pupae's P_c was lower at a lower temperature (6% O_2 at 10°C) and higher at a higher temperature (10% O_2 at 30°C). This is in accordance with the generalization that P_c is higher at higher metabolic demand (Herreid, 1980). The metabolic heat rates of the pupae, which indicate metabolic demand, were much lower at lower temperatures.

When the pupae could not compensate for the O_2 insufficiency in their tissues at below P_c, they started to experience hypoxia. Of the two strategies, metabolic arrest and anaerobic metabolism, that an organism uses to cope with hypoxia (Herreid, 1980; Hochachka, 1986; Weyel and Wegener, 1996), it seemed that metabolic arrest was the main strategy used by *Platynota stultana* pupae. With the decreasing O_2 consumption at below P_c, the pupal total metabolism, as indicated by metabolic heat rates, decreased accordingly; the percentage decreases of metabolic heat rate were comparable to the percentage decreases of O_2 consumption rates at 10, 8, 6, and 4% O_2. The *RQ* at these O_2 concentrations, with a range of 0.65 to 0.80, did not differ significantly with each other and with that at 21% O_2, suggesting that the pupae were still using lipids as their metabolic substrates

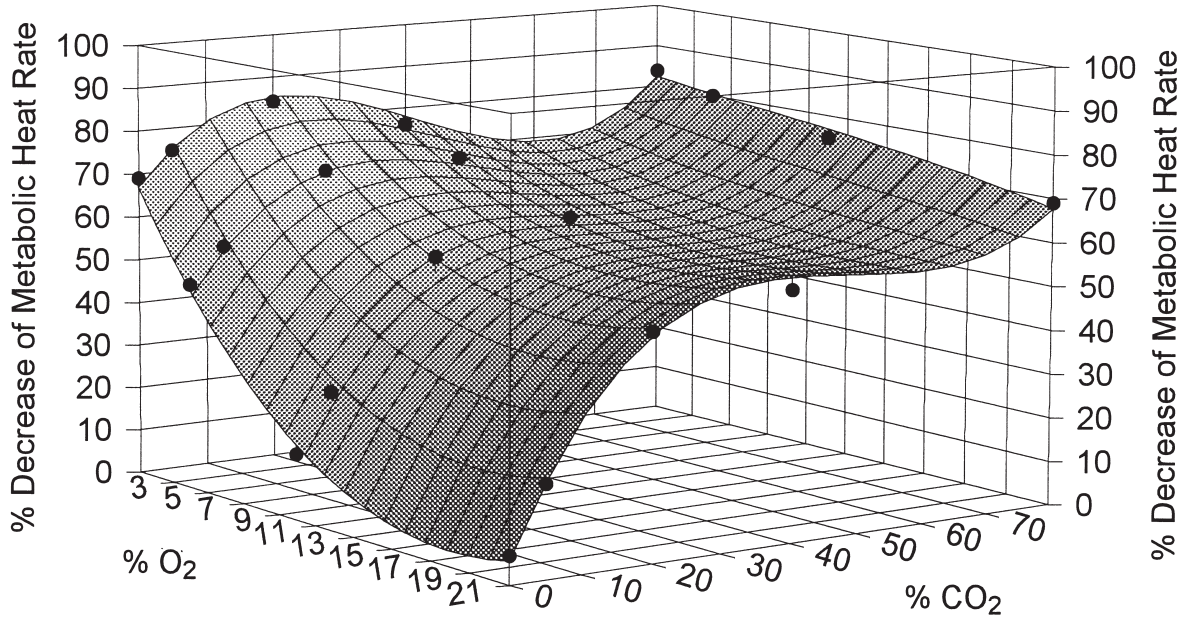

Fig. 5. The response surface of the percentage decrease of metabolic heat rate under combinations of reduced O_2 and elevated CO_2 at 20°C, fitted with a polynomial of term 3, $z=a+bx+cy+dx^2+ey^2+fxy+gx^3+hy^3+ixy^2+jx^2y$, with z denoting percentage decrease, x denoting percentage O_2, and y denoting percentage CO_2. a=74.9022, b=−8.2590, c=1.8208, d=0.2561, e=−0.0614, f=0.1412, g=−0.0010, h=0.0005, i=−0.0006, j=−0.0029. r^2=0.9941, *DF adj* r^2=0.9875, *FitStdErr*=2.4656, *Fstat*=186.8371.

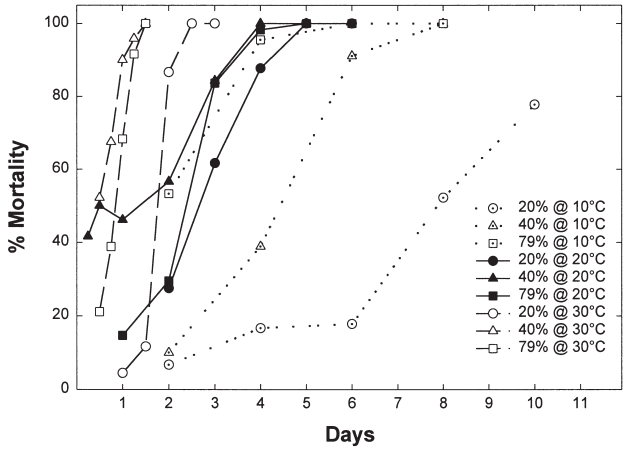

Fig. 6. Percentage mortalities of 1–2 d old *Platynota stultana* female pupae under 20, 40, and 79% CO_2 (+21% O_2) at 10, 20, and 30°C. Means represent 3 to 4 replications.

Table 4
Probit LT_{99}s (95% fiducial limits) in days for 1–2 d old *Platynota stultana* female pupae under 20, 40, and 79% CO_2+21% O_2 at 10, 20, and 30°C[a]

% CO_2	30°C	20°C	10°C
20	2.4 (2.1–3.7)	5.3 (4.9–5.8)	15.8 (13.1–21.5)
40	1.5 (1.3–1.7)	5.9 (4.5–9.9)	7.6 (7.0–8.3)
79	1.6 (1.5–1.7)	4.3 (3.8–5.5)	4.5 (4.0–5.3)

[a] Pearson's X^2s were small ($p>0.10$) except as follows: $p<0.001$ for 30°C+20% CO_2, and 20°C+40% CO_2; and $p<0.05$ for 10°C+20% CO_2 and 20°C+80% CO_2; only 20°C+40% CO_2 showed a lack of fit due to high mortality at short treatment times.

and that the pupae did not initiate anaerobic metabolism. When O_2 concentration was reduced to 2 or 1%, the percentage decrease of metabolic heat rate was less than the percentage decrease of O_2 consumption, suggesting that the metabolic arrest could not match the decrease of oxidative phosphorylation. Anaerobic metabolism must be initiated to compensate for the shortage of energy. This was confirmed by the increased *RQ* (1.3) at 2 or 1% O_2. This O_2 concentration at which anaerobic metabolism was initiated can be denoted as P_a (called anaerobic compensation point in plant literature).

That insects use metabolic arrest to cope with hypoxia and anoxia was also observed on *Locusta migratoria* and *Manduca sexta* adults by Wegener and Moratzky (1995). The metabolic heat rates of *L. migratoria* and *M. sexta* did not change between 21 and 2% O_2 at 20°C, but decreased by 30–40% at 1% O_2, 60–75% at 0.5% O_2, and 95–96% at 0% O_2. The initiation of anaerobic metabolism by insects at very low O_2 tensions was also observed by Navarro and Friedlander (1975), who found that the lactate levels in *Ephestia cautella* pupae (6 mg/100 ml hemolymph) did not change when the O_2 concentration was reduced from 20 to 3% at 26°C, but rose suddenly at below 3% and reached 288 mg/100 ml hemolymph at 1% O_2.

From the above analysis we make the following hypothesis about the metabolic response of *P. stultana* pupae to reduced O_2 concentrations. When O_2 tension is above P_c, the insects can regulate their metabolism at

close to normal levels by accelerated ventilation. This O_2 range does not affect the insects except that high ventilation may cause water loss at high temperature and low humidity. However, at O_2 tensions below P_c when sufficient O_2 cannot be supplied to the tissues and thus ATP generation is reduced, the insects lower their metabolism; that is, they reduce metabolic demands. At the O_2 range between P_c and P_a, the reduced oxidative respiration is probably sufficient to satisfy the reduced energy demand and thus anaerobic metabolism is not necessary. This O_2 range would probably not threaten the insects' survival. At O_2 tensions below P_a, the reduced oxidative respiration is not sufficient to satisfy the reduced energy demand. Anaerobic metabolism must be initiated to supplement the energy demand. Both the accumulated anaerobic end products and the very low metabolism impose stress on the insects (Hochachka, 1986). This O_2 range (below P_a) appears to be the insecticidal range.

Recent reviews of the use of controlled atmospheres for the control of insect pests (Banks and Annis, 1990; Mitcham et al., 1997b; Carpenter and Potter, 1994) have concluded that the O_2 level needs to be below 3% to be effective; and in most cases, it needs to be below 1% for rapid kill. These O_2 levels (below 3%) seem to coincide with P_a, the O_2 level at which anaerobic metabolism is initiated. It appears that empirical data support our proposition about the relationship between P_a and the toxic O_2 level.

It is important to point out that this relationship should not imply that anaerobic metabolism is the sole cause of hypoxic toxicity. The very low energy supply is probably the main cause of hypoxia toxicity, as proposed by Hochachka (1986). The low energy supply under hypoxia/anoxia has been confirmed by ATP measurements. The ATP concentration of the whole tissues of *Ephestia cautella* pupae decreased by 30% after exposure to 1% O_2 for 24 hours at 26°C (Friedlander and Navarro, 1979). The contents of ATP in the flight muscle of *L. migratoria* adults dropped to 1% of normal during 2 hours of anoxia; the ADP contents was also decreased to levels below normal while AMP accumulated 20 fold (Weyel and Wegener, 1996).

4.2. Elevated CO_2 concentrations

Our data clearly showed that elevated CO_2 concentrations prevented insects from using O_2 even with 21% O_2 present. The O_2 consumption rate of *Platynota stultana* pupae decreased by 62% in 20% CO_2+21% O_2 and by 73% in 79% CO_2+21% O_2 at 20°C. Similar observations have been made with other insect species. The O_2 consumption by *Ephestia cautella* pupae was significantly reduced by hypercarbia (Navarro, 1975). In crickets it was indicated that high CO_2 pushed respiration into anaerobic pathways (fermentative metabolism) even with 20% O_2 present (Kerr et al., 1993). The rate of respiration of *Tribolium confusum* adults, as measured by CO_2 output, was severely depressed during initial hours of exposure to elevated CO_2 concentrations (Aliniazee, 1971). However, there seem to be exceptions to this generalization. Edwards and Batten (1973) observed that the O_2 consumption rate of house flies did not decrease in 33% CO_2+21% O_2 compared with that in air. But this observation is in contradiction Edwards (1968) that high CO_2 inhibited in vitro succinic dehydrogenase in the gut tissues of *Heliothis zea* larvae, which suggests that O_2 consumption should be depressed by high CO_2 because the main metabolic pathway of oxidative respiration is inhibited.

Because elevated CO_2 prevents insects from using O_2, it appears that the net effect of elevated CO_2 on the insect respiratory metabolism is similar to that of reduced O_2. Both reduce oxidative phosphorylation even though the target sites of the two types of atmospheres may be different; reduced O_2 limits a substrate (O_2) of respiratory metabolism, whereas elevated CO_2 inhibits respiratory enzymes such as succinic dehydrogenase (Edwards, 1968). Reduced oxidative phosphorylation leads to reduced ATP generation. This has been demonstrated by Friedlander and Navarro (1979), who found that high CO_2 causes a decrease in ATP levels and the energy charge in insect tissues. It is likely that insects use the same strategies to cope with energy shortages caused by hypercarbia as those used to cope with energy shortages caused by hypoxia: metabolic arrest and/or anaerobic metabolism (Hochachka, 1986; Weyel and Wegener, 1996). That the strategy of metabolic arrest is used by insects in response to hypercarbia is supported by our observation that the total metabolism of *Platynota stultana* pupae decreased at elevated CO_2 concentrations and that the percentage decrease of metabolism, as indicated by metabolic heat rate, was comparable to the percentage decrease of O_2 consumption rate at various CO_2 levels. The insects probably reduce or cease most growth-related biosynthetic activity and limit their energy use to survival needs such as maintaining membrane potentials. That high CO_2 reduces NADPH production (Friedlander et al., 1984) and inhibits the biosynthesis of glutathione (Friedlander and Navarro, 1984) seems to support this notion. Although it was not clear from our data that the pupae initiated anaerobic metabolism under elevated CO_2, this effect has been shown by other researchers. Kerr et al. (1993) suggested that in crickets high CO_2 atmospheres induced anaerobiosis even with 20% O_2 present. Navarro and Friedlander (1975) observed that lactate rose in *Ephestia cautella* pupae exposed to 80% CO_2+20% O_2.

The metabolism of *Platynota stultana* pupae decreased rapidly as the environmental CO_2 concentration was elevated to 20%, with a 60% decrease at 20°C. Further decrease was slight when CO_2 concen-

tration was elevated from 20 to 79%. Since respiratory enzymes are inhibited by CO_2 (Edwards, 1968), this quantitative response seemed to indicate that the capacity of respiratory enzymes was increasingly inhibited by increasing concentrations of CO_2, but after a point more CO_2 did not further inhibit the capacity. It is interesting to note that empirical mortality data have shown that toxic levels of CO_2 are generally above 20% (Banks and Annis, 1990; Mitcham et al., 1997a; Carpenter and Potter, 1994).

4.3. Temperature

The normal metabolic rate of *Platynota stultana* pupae tripled from 10°C to 20°C and doubled again from 20°C to 30°C, reflecting the huge impact of temperature on insect metabolism. Temperature also has a slight but significant effect on the metabolic response of insects to both reduced O_2 and elevated CO_2, but the effect seemed to differ between reduced O_2 and elevated CO_2. The percentage decrease of metabolism by a given low O_2 concentration was higher at higher temperatures, whereas the percentage decrease of metabolism by a certain elevated CO_2 concentration was lower at higher temperatures. However, it is interesting to note that the response patterns with varying O_2 or CO_2 concentrations at different temperatures were similar.

4.4. Relationship between metabolic response and mortality response to elevated CO_2

Three trends have been observed regarding the mortality response of *Platynota stultana* pupae to elevated CO_2: (1) the pupae were more susceptible to CO_2 treatment at higher temperatures; (2) the effects of temperature varied with individual CO_2 concentration; and (3) CO_2 concentration (above 20%) affected mortality, but the specific effects were temperature dependent. The higher susceptibility at higher temperatures seemed to correlate with higher metabolism. However, it is interesting to note that the metabolic response to elevated CO_2, as indicated by the percentage decrease of metabolism, was only slightly different at 10, 20 and 30°C. In fact, the percentage decreases at 30°C were less than the percentage decreases at 20 and 10°C. It appeared that it is not the relative percentage decrease of metabolism but the absolute decrease of metabolism that was related to susceptibility. To illustrate, if we accept that metabolism can be represented by the unit of metabolic heat rate, then the absolute decrease of metabolism by 20% CO_2 was 2.2 μW/mg at 20°C (a 60% decrease of the normal metabolism of 3.7 μW/mg). The absolute decreases of metabolism were 3.1 at 30°C and 0.7 at 10°C. It is likely that it is the absolute decrease of metabolism that causes energy shortage, which would have to be compensated from the same ATP pool. Because the absolute decrease of metabolism is much lower at 10°C than at 20 or 30°C, it would take longer to use up the ATP pool at 10°C than at 20 or 30°C. Therefore, it seems that the insect susceptibility is related to the absolute decrease of metabolism. However, this correlation cannot explain the observation that the efficacy of 79% CO_2 differed little at 10 and 20°C.

The efficacy of 40 and 79% CO_2 was higher than that of 20% CO_2 at all three temperatures. But there was no difference between 40 and 79% at 20 and 30°C, while 79% was more effective than 40% at 10°C. Similar findings have been obtained with other insect species. The LT_{95}s for codling moth eggs at 25°C were 3.6, 1.3, 1.4, and 1.6 d at 20, 40, 60, and 80% CO_2 in air (Soderstrom et al., 1991), suggesting that the efficacy was not enhanced above 40% CO_2. The mortality of New Zealand thrips adults did not increase when CO_2 concentration was increased from 40 to 60% at 24°C (Carpenter et al., 1998). Recent reviews have concluded that there was no enhancement of insect mortality above 40–60% CO_2 (Banks and Annis, 1990; Carpenter and Potter, 1994). Our data show that this conclusion is mostly applicable to temperatures such as 20 and 30°C. At 10°C, increasing CO_2 concentration from 40 to 79% increased mortality of *Platynota stultana* pupae. The increased efficacy of CO_2 concentrations above 40–60% at low temperatures was also observed at 0°C on Pacific spider mites (Zhou and Mitcham, 1998). The metabolism of *Platynota stultana* pupae decreased rapidly from 0 to 20% CO_2, but further decreases were slight between 20 and 79% CO_2. The minor enhancement of mortality between 40 and 79% CO_2 at 20 or 30°C could be related to the slight further decrease of metabolism. However, the higher efficacy of 40% CO_2, compared with that of 20% CO_2, was not correlated with a similar percentage decrease of metabolic rate. In addition, although the efficacy of CO_2 increased greatly from 20 to 79% CO_2 at 10°C, the percentage decrease of metabolism showed no difference at this concentration range. It seems that mechanisms other than the decrease of metabolism were contributing to the toxicity of CO_2. For example, 40% CO_2 at 20°C caused *Platynota stultana* pupae's body fluid to leak out, suggesting that the insects' membrane systems were affected. Because CO_2 can increase intracellular Ca^{2+} by decreasing pH (Lea and Ashley, 1978), it is likely that although the metabolism cannot be further reduced by CO_2 concentration above 40%, elevating CO_2 concentration can further decrease pH and thus cause intracellular Ca^{2+} to rise more and faster, leading to cell damage or death (Hochachka, 1986). The greater efficacy of higher concentrations of CO_2 at low temperatures could be related to the higher solubility of CO_2 in tissues at low temperatures (Yacoe, 1986).

It is important to point out that the metabolic responses presented in this report were immediate responses, which do not necessarily reflect the responses

under extended exposure to reduced O_2 or elevated CO_2 concentrations. It is interesting to note that the percentage decreases of metabolism are comparable between 2% O_2 and 20 or 40% CO_2 and between 1% O_2 and 79% CO_2. If other modes of action in addition to the decrease of metabolism are contributing to CO_2 toxicity, then the elevated CO_2 concentrations should be more effective than their comparable reduced O_2 concentrations.

4.5. Combinations of elevated CO_2 and reduced O_2

Empirical studies on the additive effects of combinations of elevated CO_2 and reduced O_2 on insect mortality have yielded mixed results. Some observed additive effects (Calderon and Navarro, 1979; Krishnamurthy et al., 1986) while others did not (Soderstrom et al., 1991; Mitcham et al., 1997a). However, it seems that these different results are probably, in most part, attributable to the different ranges of gases used; additive effects were mostly observed at milder gas combinations such as 5–15% CO_2+2% O_2, while absence of additive effects was mostly observed at more severe gas combinations, such as $>$40% CO_2+0 to 0.5% O_2. These mixed results in mortality are probably related to metabolic responses. The additive effects of combinations of elevated CO_2 and reduced O_2 on the decrease of metabolism of *Platynota stultana* pupae were almost fully realized at combinations of $\leq$5% CO_2 and $\geq$4% O_2. However, the combined effects became increasingly overlapped as O_2 concentration decreased and CO_2 concentration increased.

Assuming that the decrease of metabolism is the main mode of toxicity, the observations that the additional decreases of metabolism contributed by reduced O_2 were smaller at higher CO_2 concentrations and that the additional decreases of metabolism contributed by elevated CO_2 were smaller at lower O_2 concentrations suggest that reducing O_2 concentrations at high concentrations of CO_2, such as 40–79%, would not enhance mortality nor would elevating CO_2 concentrations at $<$1% O_2 concentrations. This information should reduce the amount of empirical testing required for development of insecticidal controlled atmosphere treatments.

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References

Aliniazee, M.T., 1971. Effect of carbon dioxide gas on respiration of the confused flour beetle. Journal of Economic Entomology 64, 1304–1305.

Banks, H.J., Annis, P.C., 1990. Comparative advantages of high CO_2 and low O_2 types of controlled atmospheres for grain storage. In: Calderon, M., Barkai-Golan, R. (Eds.), Food Preservation by Modified Atmospheres. CRC Press, Boca Raton, pp. 93–122.

Calderon, M., Navarro, S., 1979. Increased toxicity of low oxygen atmospheres supplemented with carbon dioxide on *Tribolium castaneum* adults. Entomologia Experimentalis et Applicata 25, 39–44.

Carpenter, A., Potter, M., 1994. Controlled atmospheres. In: Sharp, J.L., Hallman, G.J. (Eds.), Quarantine Treatments for Pests of Food Plants. Westview Press, Boulder, pp. 171–198.

Carpenter, A., Kerr, S., Lill, R., Irving, D., Corrigan, V., Cheah, L.H., 1993. Recent developments in the use of controlled atmospheres for postharvest disinfestation. In: Proceedings of Australasian Postharvest Conference, Gatton, Queensland, Australia, September, pp. 395–401.

Carpenter, A., Wright, S., Lash, P., 1998. Response of adult New Zealand flower thrips (Thysanoptera: Thripidae) to high carbon dioxide and low oxygen atmospheres at various temperatures. Bulletin of Entomological Research 86, 217–221.

Criddle, R.S., Breidenbach, R.W., Lewis, E.A., Eatough, D.J., Hansen, L.D., 1988. Effects of temperature and oxygen depletion on metabolic rates of tomato and carrot cell cultures and cuttings measured by calorimetry. Plant Cell and Environment 11, 695–701.

Edwards, L.J., 1968. Carbon dioxide anaesthesia and succinic dehydrogenase in the corn earworm, *Heliothis zea*. Journal of Insect Physiology 14, 1045–1048.

Edwards, L.J., Batten, R.W. II, 1973. Oxygen consumption in carbon dioxide anesthetized house flies, *Musca domestica* Linn. (Diptera: Muscidae). Comparative Biochemistry and Physiology 44A, 1163–1167.

Fleurat-Lessard, F., 1990. Effect of modified atmospheres on insects and mites infesting stored products. In: Calderon, M., Barkai-Golan, R. (Eds.), Food Preservation by Modified Atmospheres. CRC Press, Boca Raton, pp. 21–38.

Friedlander, A., Navarro, S., 1979. The effect of controlled atmospheres on carbohydrate metabolism in the tissue of *Ephestia cautella* (Walker) pupae. Insect Biochemistry 9, 79–83.

Friedlander, A., Navarro, S., 1984. The glutathione status of *Ephestia cautella* (Walker) pupae exposed to carbon dioxide. Comparative Biochemistry and Physiology 79C, 217–218.

Friedlander, A., Navarro, S., Silhacek, D.L., 1984. The effect of carbon dioxide on NADPH production in *Ephestia cautella* (Wlk.) pupae. Comparative Biochemistry and Physiology 77B, 839–842.

Herreid, C.F., 1980. Hypoxia in invertebrates. Comparative Biochemistry and Physiology 67A, 311–320.

Hochachka, P.W., 1986. Defense strategies against hypoxia and hypothermia. Science 231, 234–241.

Kerr, S.B., Carpenter, A., Cheah, L.H., 1993. Mode of action of novel disinfestation techniques. AgriTech 93, 112.

Krishnamurthy, T.S., Spratt, E.C., Bell, C.H., 1986. The toxicity of carbon dioxide to adult beetles in low oxygen atmospheres. Journal of Stored Products Research 22, 145–151.

Lea, T.J., Ashley, C.C., 1978. Increase in free Ca^{2+} in muscle after exposure to CO_2. Nature 275, 236–238.

Loike, J.D., Silverstein, S.C., Sturtevant, J.M., 1981. Application of differential scanning microcalorimetry to the study of cellular processes: Heat production and glucose oxidation of murine macrophages. In:. Proceedings of the National Academy of Sciences of the USA, 78., pp. 5958–5962.

Mitcham, E.J., Zhou, S., Bikoba, V., 1997a. Controlled atmosphere for quarantine control of pests of table grape. Journal of Economic Entomology 90, 1360–1370.

Mitcham, E.J., Zhou, S., Kader, A.A., 1997b. Potential of CA for postharvest insect control in fresh horticultural perishables: an update of summary tables compiled by Ke and Kader, 1992. In: Proceedings of the Seventh International Controlled Atmosphere Research Conference, July 13-18, University of California, Davis, California, USA, 1, pp. 78–90.

Navarro, S., 1975. Studies on the effect of alterations in pressure and compositions of atmospheric gases on the tropical warehouse moth, *Ephestia cautella* (Walker), as a model for stored products insects. Ph.D. Thesis, Hebrew University of Jerusalem, Israel.

Navarro, S., Friedlander, A., 1975. The effect of carbon dioxide anesthesia on the lactate and pyruvate levels in the hemolymph of *Ephestia cautella* (Wlk.) pupae. Comparative Biochemisty and Physiology 50B, 187–189.

SAS Institute, 1989. SAS/STAT User's Guide, version 6, 4th ed. SAS Institute, Cary, NC.

Soderstrom, E.L., Brandl, D.G., Mackey, B., 1991. Responses of *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) adults and eggs to oxygen deficient or carbon dioxide enriched atmospheres. Journal of Stored Products Research 27, 95–101.

Wegener, G., Moratzky, T., 1995. Hypoxia and anoxia in insects: microcalorimetric studies on two species (*Locusta migratoria* and *Manduca sexta*) showing different degrees of anoxia tolerance. Thermochimica Acta 251, 209–218.

Weyel, W., Wegener, G., 1996. Adenine nucleotide metabolism during anoxia and postanoxic recovery in insects. Experientia 52, 474–480.

Yacoe, M.E., 1986. Effects of temperature, pH, and CO_2 tension on the metabolism of isolated hepatic mitochondria of the desert uiguana, *Dipsosaurus dorsalis*. Physiological Zoology 59, 263–272.

Yokoyama, V.Y., Miller, G.T., Harvey, J.M., 1987. Development of oriental fruit moth (Lepidoptera: Tortricidae) on a laboratory diet. Journal of Economic Entomology 80, 272–276.

Zhou, S., Mitcham, E.J., 1998. Sequential controlled atmosphere treatments for quarantine control of Pacific spider mites (Acari: Tetranychidae). Journal of Economic Entomology 91, 1427–1432.