Metabolic response of Platynota stultana pupae during and after extended exposure to elevated CO₂ and reduced O₂ atmospheres

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Received 8 May 2000; accepted 15 August 2000

Abstract

The metabolic response of Platynota stultana pupae to elevated CO₂ and reduced O₂ atmospheres was measured using microcalorimetry. Initial measurements at 20°C immediately upon placement in controlled atmosphere indicated a decrease in metabolic heat rate (MHR) of 27, 45, 56, 56, and 72% in an atmosphere of 5, 10, 20, 40, and 79% CO₂, respectively, and a decrease of 20, 50, 66 and 100% under 6, 2, 1, and 0% O₂. With extended exposure to controlled atmospheres, MHR increased under 5, 10, and 20% CO₂ and 6 and 2% O₂; however, the increase was greater and occurred more rapidly with lower CO₂ and higher O₂ concentration. The MHR at 40 and 79% CO₂ remained at the initial reduced level for 8 and 6 days, respectively, then decreased with longer exposure. The MHR of pupae held under 1 and 0% O₂ remained at the initial reduced level for 22 days. Upon transfer to air, the MHR of pupae increased from the reduced levels and then decreased. When the MHR decreased by no more than 30%, as a result of controlled atmosphere treatment, the pupae still developed into adults. However, when the MHR decreased by more than 50%, the energy supply was insufficient and the pupae died. Pupa mortality was comparable between 5% CO₂ and 6% O₂, and 10% CO₂ and 2% O₂. The MHR was reduced less under 20% CO₂ than under 2 or 1% O₂; however, the pupae were more susceptible to 20% CO₂ than 2 or 1% O₂. These and other data indicate an increased toxicity of high CO₂ over low O₂ atmospheres that may be related to an increase in membrane permeability as a result of CO₂ treatment. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Carbon dioxide; Controlled atmosphere; Metabolic heat rate; Microcalorimetry; Oxygen

1. Introduction

Controlled atmospheres with elevated CO₂, reduced O₂, or their combinations can be used to control insects in harvested commodities during storage or for quarantine treatment prior to export shipment (Mitcham et al., 1997). The mode of action of controlled atmospheres on insects was reviewed by Fleurat-Lessard (1990), but many questions remain. Lack of information on the mode of action of controlled atmospheres has rendered the development of controlled atmosphere treatments costly and time consuming (Carpenter et al., 1993). If we can understand the physiological and biochemical responses of insects to controlled atmosphere treatments 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.

Platynota stultana Walsingham (Lepidoptera: Tortricidae) is an economic pest of many horticultural crops, including apple, apricot, avocado, bushberries, celery, citrus, eggplant, grape, lettuce, melons, peach, pepper, plum, prune, strawberry, tomato and walnut, and is a quarantined pest for table grapes shipped from California to Australia. Zhou et al. (2000) found the response of Platynota stultana pupae immediately upon exposure to reduced O₂ atmospheres (below 8% O₂) to be metabolic arrest. Anaerobic metabolism was initiated at <2% O₂. Metabolic arrest was also observed under 5–79% CO₂. However, the metabolic responses immediately under exposure to CO₂ and reduced O₂ do not necessarily reflect the responses under longer exposures. AliNiazeez (1971) found that introduction of up to 16% CO₂ severely depressed the respiration rate of Tribolium confusum adults during the initial hours of exposure, but subsequently the respiration rate returned to normal within 24 h.
Because treatment duration is one of the main factors that determine the efficacy of controlled atmosphere treatments, it is important to understand the metabolic response of insects under extended treatments. For example, if the metabolism can return to normal under an atmosphere, it is likely that the atmosphere would not impose significant stress on the insects. Our objectives were to understand the metabolic response of insects both under and following an extended exposure to a range of elevated CO₂ and reduced O₂ concentrations. Mortality responses were correlated with the metabolic responses to provide some insights into the modes of action of elevated CO₂ and reduced O₂ atmospheres.

2. Materials and methods

2.1. Experimental insects

*P. stultana* were reared on a lima bean-based diet in an incubator at 27°C with a photoperiod of 16:8 h (L:D) (Yokoyama et al., 1987). The 1–2 day old female pupae were selected for experiments because their metabolism was stable in this age range.

2.2. Calorimetry measurements

Rates of metabolic heat production were measured with Hart Scientific model 7707 differential scanning calorimeters with isothermal and temperature scanning capabilities (Hart Scientific Inc., Provo, Utah). The isothermal operating mode was used to measure metabolic heat rates (MHR) at a certain 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 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 MHRs of the samples.

2.3. Initial atmospheric modification in the ampoules

Appropriate amounts of air, CO₂, N₂, and O₂ were mixed using metering valves to produce the desired atmospheres of 5, 10, 20, 40, and 79% CO₂ (all in 21% O₂) and 6, 2, and 1% O₂ (balance N₂). The gas concentrations of the mixtures were analyzed by gas chromatography (model 211 Carle Instruments, Anaheim, Calif.). The gases flowed through a plastic bag (about 3 l when fully inflated) at a constant rate of 600 ml/min after being first bubbled through water to obtain >90% relative humidity (RH). The bag, placed in a temperature controlled room (20°C), had an inlet, an outlet, and a sealable side. Open ampoules containing pupae and ampoule lids were placed on sticky tape in the middle of the bag. The open side of the bag was folded and sealed by clamping two 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 concentration, which was confirmed by drawing samples from the bag and analyzing them by gas chromatography. The lids within the bag were used to seal the ampoules in the bag, and the sealed ampoules were then taken out of the bag and placed into calorimeter cells for MHR measurement.

2.4. Metabolic heat rate measurement under extended controlled atmosphere treatments

Four pupae were weighed and placed in each ampoule. The MHRs under air were first measured. The ampoules were then opened and an appropriate atmosphere was added to the ampoules as described above. The MHRs under the atmosphere were then measured. The ampoules, still sealed, were put back into the bag. After the atmosphere in the bag was established as described above, the ampoules were opened within the bag. In this manner, the pupae were held under the atmosphere for an extended period. The atmosphere in the bag was analyzed daily. The MHRs of the pupae under the atmosphere were measured daily during the first 5 days and were then measured every 2 days. For each measurement, the ampoules were sealed again within the bag, removed from the bag, and placed into calorimeter cells. After measurement, the ampoules were returned to the bag while still sealed, and were opened within the bag after the atmosphere in the bag was re-established, as described above. During the entire treatment period, the pupae remained under the treatment atmosphere. After the last measurement under the atmosphere, the ampoules were opened in air, and the MHRs under air were measured immediately. The pupae were then held in air for recovery and the MHR during recovery was measured daily for 2–5 days. The treatment, measurement, and recovery were all performed at 20°C.

2.5. Mortality test

The mortality of pupae under 5, 10, 20, 40, and 79% CO₂ (all in 21% O₂) and 6, 2, 1, and 0% O₂ at 20°C was tested. The atmospheres were bubbled through water to obtain >90% RH. The gas concentrations inside the jars were sampled daily during treatment and analyzed by gas chromatography.

Thirty pupae were placed in a cup with a mesh top.
The cup was placed in a 1000 ml jar through which the atmosphere continually flowed at 150 ml/min. The range of treatment times for each atmosphere at each temperature was determined by preliminary tests, and corresponded with treatment times that should result in 10–100% mortality. After treatment, 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 three times.

2.6. Metabolic response after controlled atmosphere treatment

The MHR of pupae under 20% CO₂ at 20°C was measured daily for 5 days as described above, and the heat rate during recovery at 20°C in air was measured periodically for 12 days.

The MHR of pupae in air immediately and 1 day after various durations of 79% CO₂ treatment was measured. Pupae (24) were placed in a cup and treated under 79% CO₂ at 20°C as described for the mortality test. Immediately after treatment, 12 pupae were randomly chosen and divided into three ampoules. After the MHR was measured, the pupae were dried in an 80°C vacuum oven for at least 1 day to obtain the dry weight. The remaining 12 pupae were placed in a 27°C incubator to recover for 1 day. After 1 day, the heat rate was measured, and the pupae were dried on the second day.

2.7. Statistical analysis

The data for MHR immediately and 1 day after 79% CO₂ treatment were analyzed by ANOVA [GLM, SAS Institute (Anon, 1989)]. Means for significant effects were separated by t-test (LSD).

3. Results

3.1. Metabolic response under controlled atmosphere

The MHR of pupae held in air at 20°C increased over time [Fig. 1(A)] and eclosed on day 7. The MHR of pupae in air prior to exposure to 5, 10, 20, 40, and 79% CO₂ and 6, 2, and 1% O₂ were 126, 150, 137, 139, 129, and 124, 125, 136 μW, respectively (data not shown).

Reductions in MHRs were detected in all insects placed under controlled atmospheres [Fig. 1(A)]. Initial measurements immediately upon placement in controlled atmosphere indicated a decrease in metabolic rate of 27, 45, 56, 56, and 72% under 5, 10, 20, 40, and 79% CO₂, respectively. The decline in MHR upon placement under controlled atmosphere was greater with higher CO₂ concentration, with the exception of 20% CO₂, under which the immediate decrease in metabolic rate was similar to that under 40% CO₂. The MHR immediately upon exposure to reduced O₂ atmospheres decreased more at lower O₂ concentrations. Immediately after placement in controlled atmosphere, the MHR was reduced by 20, 50, 66 and 100% under 6, 2, 1, and 0% O₂ at 20°C. All data points represent the average of 3–4 replications. (E) eclosed the next day; *transfer to air.

With extended exposure to controlled atmosphere, the metabolic rate increased under 5, 10, and 20% CO₂ and 6 and 2% O₂; however, the increase was greater and occurred more rapidly with lower CO₂ and higher O₂ concentration [Fig. 1(A)]. The MHRs under 5, 10, and 20% CO₂ were similar on days 1–5; however, the rapid increase of heat rate was delayed by 1 day under 10% CO₂ as compared with under 5% CO₂, and the rapid increase of heat rate did not occur under 20% CO₂.

Under 20% CO₂ and 2% O₂, the increase in MHR was
very gradual, and only reached 150 μW after 18 days and 170 μW after 15 days, respectively, as compared to a peak of 260 μW after 6 days for pupae under air. After the peak in MHR at 15 days in pupae held under 2% O₂, the MHR decreased. Pupae held in air, 5% CO₂ and the peak in MHR at 15 days in pupae held under 2% CO₂ and slowly under 40% and 79% CO₂. The heat rate of pupae previously held under 20% CO₂ and 2 and 1% O₂ and slowly under 40% and 79% CO₂. The heat rate of pupae previously held under 40 and 79% CO₂ was only 55 and 22 μW, respectively, immediately after transfer to air. The MHR of two of the replicates previously under 40% CO₂ decreased rapidly to 0 μW in air, while the remaining sample increased from 50 μW upon transfer to air to 100 μW 3 days later and then decreased (data not shown).

The MHR following exposure to 79% CO₂ for 0 to 10 days was also determined. The MHR immediately after 79% CO₂ treatment increased when treatment duration was extended from 0 (no treatment) to 1 and 3 days, and did not change further when the treatment duration was extended from 3 to 7 days (Fig. 2). However, when the treatment was extended to 10 days, the heat rate decreased below the initial heat rate. The heat rate response one day after treatment was similar to that immediately after treatment, except that the heat rate 1 day after treatment started to decrease after 5 days of treatment. In addition, after 7 and 10 days of CO₂ treatment, the metabolic rate was significantly lower 1 day after removal from the treatment atmosphere than it was immediately after removal from the treatment.

3.2. Response upon transfer to air from controlled atmosphere

Upon transfer to air after 14–18 days under the CO₂ atmospheres, the MHR increased 65, 75, 30, and 12 μW for pupae held under 10, 20, 40 and 79% CO₂, representing a 32, 48, 112, and 120% increase, respectively [Fig. 1(A)]. Pupae were held under low O₂ atmospheres for 22 days. Upon transfer to air, the MHR increased 20 and 58 μW for pupae held under 2 and 1% O₂, representing a 21 and 181% increase, respectively [Fig. 1(A)]. However, following the immediate increase in MHR upon transfer to air, the MHR decreased rapidly in pupae previously held under 20% CO₂ and 2 and 1% O₂ and slowly under 40% and 79% CO₂. The heat rate of the latter two of the replicates previously under 40% CO₂ decreased rapidly to 0 μW in air, while the remaining sample increased from 50 μW upon transfer to air to 100 μW 3 days later and then decreased (data not shown).

The MHR following exposure to 79% CO₂ for 0 to 10 days was also determined. The MHR immediately after 79% CO₂ treatment increased when treatment duration was extended from 0 (no treatment) to 1 and 3 days, and did not change further when the treatment duration was extended from 3 to 7 days (Fig. 2). However, when the treatment was extended to 10 days, the heat rate decreased below the initial heat rate. The heat rate response one day after treatment was similar to that immediately after treatment, except that the heat rate 1 day after treatment started to decrease after 5 days of treatment. In addition, after 7 and 10 days of CO₂ treatment, the metabolic rate was significantly lower 1 day after removal from the treatment atmosphere than it was immediately after removal from the treatment.

3.3. Mortality response

Mortality refers to the proportion of pupae that did not eclose during the treatment or during recovery in air at 27°C. The insecticidal efficacy of CO₂ increased greatly from 5 to 20% CO₂ at 20°C [Fig. 1(B)]. However, increasing the CO₂ concentration from 20 to 40% only moderately increased efficacy, and increasing CO₂ concentration from 40 to 79% CO₂ did not increase efficacy, and reduced mortality at shorter exposure times. Mortality was less than 10% after 6 days of treatment with 10% CO₂, but increased to 70% when the treatment was extended to 10 days. Extending the 10% CO₂ treatment to 16 days did not further increase mortality. The pupae that eclosed after 10, 12, and 16 days of treatment with 10% CO₂ eclosed while under the treatment atmosphere. There was essentially no mortality after treatment with 5% CO₂ or 6% O₂; most pupae eclosed on day 10 or 8, respectively, while under the atmosphere.

The insecticidal efficacy of reduced O₂ was greater at lower O₂ concentrations [Fig. 1(B)]. Five days of treatment with 2% O₂ resulted in <10% mortality, but the mortality increased with exposure times. Mortality after 14 days of treatment with 2% O₂ was 97%. All the pupae that eclosed after 14 days of treatment had eclosed while under the 2% O₂ treatment. Two to 3 days of treatment with 1% O₂ did not cause much mortality, but the mortality increased to 93% after 8 days of treatment.

Fifty percent mortality was obtained after approximately 8.5, 2.5, 1.5, and 2.5 days under 10, 20, 40, and 79% CO₂, respectively, and after approximately 10, 4.5 and 1.5 days under 2, 1, and 0% O₂, respectively [Fig. 1(B)]. Complete mortality was obtained after approximately 5, 4 and 5 days under 20, 40 and 79% CO₂, and after 2 days at 0% O₂. Comparing the efficacy of elevated CO₂ and reduced O₂, 5% CO₂ and 6% O₂ were comparable, as were 10% CO₂ and 2% O₂. The efficacy of 20, 40, and 79% CO₂ was greater than that of 1% O₂, but less than that of 0% O₂.
3.4. Metabolic response during and following exposure to a lethal CO₂ treatment

When pupae were exposed to 20% CO₂ for 5 days at 20°C, a treatment that resulted in 100% mortality [Fig. 1(B)], the heat rate increased gradually during the treatment (Fig. 3). Upon transfer to air, the MHR increased by 60% and was higher than the initial heat rate. The MHR continued to increase during recovery in air, but after 8–12 days the heat rate decreased.

4. Discussion

The increase in MHR of pupae over time in air reflected the developmental process of pupae during metamorphosis. During metamorphosis the larvae tissues are destructed and the adult tissues are constructed. Odell (1998) correlated an increase in respiration rate of Manduca sexta pupae and Tenebrio molitor pupae during the last 60% of pupal development with an increase of mitochondria index that indirectly reflects the amount of active tissue in the pupae. With more adult tissues constructed, there will be more metabolically active tissues and thus higher metabolic demand. It took 1–2 day old P. stultana pupae 7 days to develop into adults at 20°C.

4.1. Pupal development under controlled atmospheres

When the MHR of pupae decreased by no more than 30%, as a result of high CO₂ or low O₂ treatment, the pupae still developed into adults, but showed an increased time to eclose. The MHR decreased by 19% under 6% O₂ and by 27% under 5% CO₂, and the pupae developed into adults in 8 and 10 days, respectively. The slower developmental rate with a slightly reduced metabolic rate under mild atmospheres (6% O₂ or 5% CO₂) was similar to slower development at low temperatures where metabolism is also reduced. Pupal development was also indicated by a rapid increase of MHR under 6% O₂ or 5% CO₂, reflecting an increase in metabolically active tissues during development (Odell, 1998). These mild atmospheres had no insecticidal effects.

The rapid increase in MHR under 10% CO₂ after day 6 also indicated that pupae developed under 10% CO₂. Although none of the pupae used for measurement of metabolic response eclosed under the atmosphere, 30% of pupae used for the mortality tests eclosed by day 10 under the atmosphere, which indicates that the pupae developed under 10% CO₂. The high MHR on day 10 seemed to suggest that the pupae developed into adults, but for some reason could not eclose. Our observation that the pupae that could not eclose under the treatment also could not eclose upon transfer to air suggests that some damage had already occurred to the pupae. The decrease in MHR after day 12 further supports this notion.

Pupae under 2% O₂ developed slowly, as indicated by a relatively rapid increase in metabolism from day 8 to day 15. However, as under 10% CO₂, none of the pupae used for measurement of metabolic response eclosed under 2% O₂, but 3% of the pupae used for the mortality tests eclosed on day 14 under the atmosphere, which indicates that the pupae developed under 2% O₂. Also, as under 10% CO₂, the MHR under 2% O₂ started to decrease after a peak. The decrease in metabolic rate was greater with longer exposure times, suggesting that more cells were significantly damaged or killed with longer exposure. That the metabolism only increased slightly after transfer to air, and then decreased rapidly during recovery in air also suggested that many cells died while under the atmosphere. It is interesting to note that the mortality resulting from exposure to 2% O₂ was also comparable to that of 10% CO₂.

There seemed to be no pupal development under 20, 40, and 79% CO₂ and 1% O₂ since no rapid increase of heat rate was observed under these atmospheres. There was a significant increase (30 µW) in metabolism during the first day of treatment under 20% CO₂ and a gradual increase in metabolism over the long-term exposure. However, these increases did not seem to be related to pupal development since no pupae ever eclosed under the atmosphere. In addition, no pupae eclosed after a 5 day treatment at 20% CO₂. Our results suggest that the pupae adjusted their metabolism during the first day of exposure after an initial ‘over’ reduction of metabolism. Such adjustment also occurred under 10% CO₂; the MHR increased by 10 µW during the first day of treatment. The MHR under 40% and 79% CO₂ remained at the same level of reduction for 6–8 days and then decreased further. The further decrease of metabolism
under these atmospheres suggests that pupal cells were dying, which was also supported by the observation that the MHR after transfer to air was far below the initial rate in air and there was no recovery of metabolism during recovery in air.

The pupal development pattern under different atmospheres can provide some insights into how the pupae used energy under reduced energy supply. Insects use energy mainly for maintenance, movement, and growth. Since pupae do not move greatly, especially under high CO₂ or low O₂ atmospheres, they mainly use energy for maintenance and growth. Under 6% O₂ and 5% CO₂, the metabolism was decreased by 20–30%. However, a 30% decrease in metabolic rate did not stop the pupae from developing, suggesting that the pupae had enough energy for both maintenance and growth. When metabolism was reduced by 40–50% under 10% CO₂ or 2% O₂, the pupae still had enough energy for both maintenance and growth, although growth was reduced. Slower development under 10% CO₂ and 2% O₂ indicated that much less energy was used for growth. However, when the metabolism was reduced by more than 50% (under 40 and 79% CO₂ and 1% O₂), the energy supply was probably not enough for maintenance. Under these conditions, the pupae must stop all growth-related activities and limit their energy use for basic maintenance such as maintaining membrane potentials. The further decrease in MHR under prolonged exposure to 40 and 79% CO₂, indicating that cells were dying, suggests that the energy supply under 40 and 79% CO₂ was not enough for basic maintenance.

### 4.2. Effects of low O₂ versus elevated CO₂ atmospheres

Whether pupae had extra energy for growth under a reduced metabolism seemed to depend on the type of atmosphere. Under reduced O₂, pupae still had energy for growth under a more reduced metabolic rate. However, under elevated CO₂, the pupae did not have energy for growth even under a less reduced metabolic rate than with low O₂. For example, the metabolism of pupae under 20% CO₂ was about 37% lower than untreated pupae after 1 day in the treatment, which was a smaller decrease in metabolic rate than under 2% O₂. However, pupae under 2% O₂ gradually developed while pupae under 20% CO₂ did not. With a similar magnitude of decrease in metabolic rate, it seemed that pupae under reduced O₂ had less energy shortage than did those under elevated CO₂. In a second example, the level of reduction in metabolism was similar under 1% O₂ and under 79% CO₂; however, it seemed that pupae under 1% O₂ had less energy shortage than under 79% CO₂ because the MHR under 1% O₂ was sustained much longer than under 79% CO₂. Furthermore, based on the lower mortality, pupae under 1% O₂ seemed to have less energy shortage than under 40% CO₂, even though the metabolic rate of pupae under 1% O₂ decreased more than under 40% CO₂. It appears that elevated CO₂ affected energy use by more than just reducing metabolism. Comparing the developmental pattern under 10 and 20% CO₂ further supports this notion. The metabolism of pupae under 20% CO₂ adjusted back after 1 day to close to that under 10% CO₂. However, the pupae under 10% CO₂ developed while the pupae under 20% CO₂ did not.

Elevated CO₂ may affect pupal energy use in several ways. It has been found that high CO₂ reduces NADPH production (Friedlander et al., 1984) and inhibits the biosynthesis of glutathione (Friedlander and Navarro, 1984). It is possible that the pupae could not develop under elevated CO₂ because biosynthetic pathways were inhibited by CO₂ and such inhibition was more severe under higher CO₂ concentration. Based on this hypothesis, low concentrations of CO₂ such as 5 and 10% CO₂ would not significantly inhibit the biosynthetic pathways since pupae continue to develop and eclose under these atmospheres, although more slowly. However, CO₂ concentrations above 20% inhibited biosynthetic pathways; therefore, pupae could not develop even when they had extra energy for growth. Although this hypothesis can explain the developmental pattern under elevated CO₂, it cannot explain the much higher toxicity of 20% CO₂ compared with that of 10% CO₂ and 2 and 1% O₂ since it is not clear that inhibition of biosynthesis is related to pupal survival.

Under a similar degree of reduction in metabolism, pupae under elevated CO₂ seemed to have less energy than under reduced O₂. Therefore, it is likely that under elevated CO₂, the energy supply is lower and/or the energy demand for basic maintenance is higher. The lower energy supply could result from an inefficient production of ATP under elevated CO₂. CO₂ has been considered an uncoupler of phosphorylation, similar to 2, 4-dinitrophenol (Fanestil et al., 1963). Therefore, even with a similar metabolic heat dissipation, pupae under elevated CO₂ may generate less ATP than under reduced O₂. Friedlander and Navarro (1979) found that ATP content and energy charge in the tissues of Ephestia cautella pupae decreased more under 79% CO₂ than under 1% O₂ after 1 day of treatment. The higher energy demand for basic maintenance could result from an increase in permeability of membranes, which results in a requirement of additional energy to maintain membrane potentials. If the energy demand for basic maintenance becomes higher under elevated CO₂, then the pupae probably will have much less extra or no energy for growth.

### 4.3. Metabolic arrest under low O₂ and elevated CO₂ atmospheres

Perhaps the mode of action of low O₂ can provide clues as to the mode of action of CO₂ because the main
effect of both types of atmosphere is to reduce metabolism. The reduction of metabolism, also called metabolic arrest, has been proposed as a major strategy used by animals to cope with hypoxia (Herreid, 1980; Hochachka, 1986; Weyel and Wegener, 1996). It lessens the pressure on organisms to initiate anaerobic metabolism, which would require very high rates of anaerobic glycolysis and thus lead to rapid exhaustion of carbohydrate reserves while toxic end products accumulate (Hochachka, 1986; Weyel and Wegener, 1996). However, metabolic arrest, when decoupled from membrane functions, has been thought to be the cause of hypoxic/anoxic toxicity (Hochachka, 1986). According to Hochachka, 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 the cytosol activates phospholipases A$_1$, A$_2$, and C, leading to increased membrane phospholipid hydrolysis. The cell and mitochondria membranes become further permeable, causing cell damage or death.

### 4.4. The role of membrane permeability

However, such failure of membrane function does not occur at all, or develops slowly, if the initial membrane permeability is low. Therefore, Hochachka (1986) stressed that the real survival tool for organisms under hypoxia/anoxia is the coupling between metabolic arrest and low permeability of membranes. Ionic concentration gradients do not fall to their thermodynamic equilibrium in tissues of ectothermic anaerobes at lower ATP turnover rates under hypoxia; however, the ion concentration gradients are rapidly lost in hypoxia-sensitive tissues. Therefore, it has been proposed that the higher tolerance of ectothermic anaerobes to hypoxia, as compared with that of higher animals, is attributable to the lower membrane permeability of ectothermic anaerobes (Hochachka, 1986).

Insects in general are hypoxia-tolerant organisms. Our observation that most *P. stultana* pupae can develop to eclosion after 6 days of treatment with 2% O$_2$ supports this classification. It also suggests that the permeability of the cell membranes of *P. stultana* pupae is low. As a result, the energy needed to maintain membrane potentials and the energy used for basic maintenance are also low. This would explain the availability of extra energy for growth under 2% O$_2$ when the metabolism was reduced by about 50%. Even under 1% O$_2$ when the metabolism was reduced by 67%, the pupae sustained this level of metabolism for about 22 days although there was no development. It appeared that about 30% of normal metabolism was needed for basic maintenance, which could mean that it takes about 30% of normoxic energy supply for ion pumps to maintain the ion gradients. Therefore, when the energy supply was maintained above 30% of normal, the pupae could probably still maintain their membrane potentials if they use all their energy supply for this purpose. The pupae probably can survive a long time with >30% of normal metabolism, as shown by the metabolic pattern under 2 and 1% O$_2$.

It seems that the susceptibility of an organism to reduced energy supply depends on the degree of reduction in energy supply and the permeability of membranes. The lower the energy supply, the more susceptible the organism. The more permeable the membranes, the more energy needed to maintain membrane potential and thus the more susceptible the organism. The metabolic response and mortality response of *P. stultana* pupae under reduced O$_2$ concentrations clearly showed that the pupae were more susceptible when the energy supply was lower under lower O$_2$ concentrations.

### 4.5. Effect of CO$_2$ on membrane permeability

However, the metabolism under 20% CO$_2$ was reduced less than under 2 and 1% O$_2$, but the pupae were more susceptible to 20% CO$_2$ than to 2 and 1% O$_2$. In addition, when metabolism was further reduced under CO$_2$ levels above 20%, the susceptibility did not increase accordingly. These observations seem to suggest that the permeability of membranes was increased by high CO$_2$ concentrations. Maintaining the membrane potential required more energy, and the metabolism under 20% CO$_2$, although higher than that under 2% O$_2$, could not satisfy the energy demand for maintaining a leaky membrane. As a result, the pupae were very susceptible.

Although no direct evidence has been found to support the hypothesis that high CO$_2$ can increase the membrane permeability, it has been found that CO$_2$ can increase intracellular Ca$^{2+}$ concentration by decreasing pH (Lea and Ashley, 1978). According to Hochachka (1986), a high concentration of Ca$^{2+}$ in the cytosol can cause the cell and mitochondrial membranes to become more permeable, indirectly suggesting that high CO$_2$ can increase membrane permeability. It was also observed that high CO$_2$ caused the *P. stultana* pupae’s body fluid to leak out, suggesting that the insects’ membrane systems were affected by high CO$_2$ (Zhou et al., 2000). Also, Friedlander (1983) found a 25% reduction in the ratio of pyruvate to lactate under hypercarbia, indicating a change in the redox potential and a lesion in the electron transport chain, presumably by a modification in the permeability of mitochondrial membranes. In contrast, Sears and Eisenberg (1961) suggested that CO$_2$ decreased the permeability of cell membranes.

The increase in intracellular Ca$^{2+}$ concentration as a result of high CO$_2$ concentrations (Lea and Ashley, 1978) may directly contribute to its higher toxicity as compared with that of reduced O$_2$. It has been suggested
that high concentrations of Ca$^{2+}$ in the cytosol is an intermediate step of the mode of action of hypoxia/anoxia (Hochachka, 1986). These data indicate that CO$_2$ can lead to this intermediate step by other means in addition to reducing metabolism and thus is more toxic than low O$_2$.

4.6. Relationship between metabolic response and mortality

Although the metabolic response patterns under different atmospheres provide insight into the mode of action of controlled atmospheres, there seems to be no direct correlation between the metabolic response while under an atmosphere and the mortality response to that atmosphere. For example, the MHR under 40 or 79% CO$_2$ remained the same over the first 5 days, but the mortality increased drastically over this period of time. Similar observations can be made for other atmospheres. Therefore, it appears that mortality cannot be predicted by the metabolic response under that atmosphere. However, it should be noted that when the MHR started to decline under an atmosphere, such as after 6–8 days in 40 or 79% CO$_3$, this is a clear indication that 100% mortality was achieved, and probably at a shorter treatment time. It seems that the pupae were already dying while under the atmosphere treatments.

The metabolic response in air after controlled atmosphere treatment also did not correlate with mortality responses. For example, the metabolic response immediately after 3 and 5 days at 79% CO$_2$ was similar, but the mortality response was different. A 5-day treatment at 79% CO$_2$ gave 100% mortality, and the metabolic response, while not different after a 3 or 5 day treatment, did begin to decrease after a 7 day treatment, when measured 1 day after removal from controlled atmosphere.

The finding that the MHRs after CO$_2$ treatment were higher than the initial heat rate and increased with treatment duration may indicate that the pupae’s metabolism increased in response to physiological injury. When the length of treatment was longer than that required for 100% mortality, the metabolic rate after treatment decreased as more damage occurred during the controlled atmosphere treatment. It seems that physiological injury from insecticidal controlled atmospheres may stimulate metabolism following transfer to air until the injury becomes so severe that metabolism is reduced. The effective treatment duration may occur near this point of change in post-treatment metabolism.

The pupal mortality in this paper is defined as the proportion of pupae that did not eclose either during the treatment or during recovery in air. This is a practical definition for insect control practices. However, the pupae did not die immediately after CA treatment. The MHR in air immediately after 5 days of 20% CO$_2$ increased by 60% and was higher than the initial heat rate, even though mortality tests showed that complete mortality was obtained with 5 days at 20% CO$_2$. The increase in MHR during recovery seems to suggest that the pupae were still capable of developing, but could be the result of physiological injury. The MHR eventually decreased suggesting that the pupae could not develop normally because of controlled atmosphere injury and would eventually die. The slightly different metabolic responses of different pupae samples during recovery may reflect variability in tolerance of individual pupae to controlled atmosphere treatments.

5. Conclusions

These results have shown that atmospheres of $\geq$20% CO$_2$ or $\leq$1% O$_2$ at 20°C would be effective for control of P. stultana pupae. The data also demonstrate the increased efficacy of elevated CO$_2$ over reduced O$_2$ atmospheres. The developmental pattern under elevated CO$_2$ and low O$_2$ atmospheres and the mortality response of the pupae to these atmospheres indicated that the pupae had a greater energy shortage under elevated CO$_2$ than under reduced O$_2$ despite a similar decrease in metabolic rate. It appears that elevated CO$_2$ affected energy use by more than just reducing the metabolic rate. These data support those studies that have suggested that CO$_2$ has an effect on membrane permeability (Friedlander, 1983) and may uncouple oxidative phosphorylation (Fanestil et al., 1963).

While a direct correlation between the MHR pattern under controlled atmosphere and upon transfer to air and pupal mortality was not observed, a reduction in MHR of greater than 50% was associated with pupal mortality. Additional work with other lifestages of P. stultana and other insect species is necessary to determine if responses to controlled atmospheres are general or lifestage and species specific.

Acknowledgements

We thank Veronique Bikoba and James Shannon for their technical assistance in the research. We also thank Bill Biasi, John Church, and Lisa Neven for consultations, and Adel Kader, Tiffanie Simpson, and Tayfun Agar for editing this manuscript. This project was supported by USDA NRI grant #97-35316-4884.

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