

# THE POTENTIAL FOR STORED-PRODUCT INSECTS TO DEVELOP RESISTANCE TO MODIFIED ATMOSPHERES\*

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**Abstract-** Laboratory selections for resistance of *Tribolium castaneum*, adults to low oxygen concentrations (LOC) and high carbon dioxide concentrations (HCC) have shown that this species is capable of developing low but significant levels of resistance to the respective modified atmospheres (MAs). Also resistance obtained in adults was partially transferred to the eggs and larvae. Studies on the modes of action of MAs, and the mechanisms of resistance in *T. castaneum*, indicate that the LOC resistant strain selected at 0.5% O<sub>2</sub> was better able to survive anoxia than the non-selected strain. However, metabolism during exposure to the LOC was mainly aerobic. Possibly mortality occurs when aerobic metabolism can no longer be maintained and is superseded by anaerobic metabolism with accumulation of toxic end-products. For exposure to HCC, death was the result of desiccation, and exhaustion of triglyceride (TG) energy reserves, and not by prolonged narcotic effect of anaesthesia, or the accumulation of toxic end-products. The resistant strain had greater TG reserves and was able to control water loss during exposure. Tests for susceptibility of both the LOC and HCC selected, and a non-selected strain of *T. castaneum* to methyl bromide and phosphine failed to reveal cross-resistance with these fumigants.

## INTRODUCTION

The controlled or modified atmosphere (MA) method of stored-product insect control is firmly established and based on much laboratory and field work. Although commercial application has so far been limited in scope, it has been used as a treatment for bulk grain, and more recently for bagged grain (Annis, 1990), while its influence on grain quality has also been studied (Banks, 1981; Gras and Basson, 1990).

The mode of action of MAs on insects depends largely on the atmospheric gas composition used. The method of flushing and maintenance using carbon dioxide (CO<sub>2</sub>), involves purging the air until a CO<sub>2</sub> concentration of usually not less than 60% CO<sub>2</sub> is reached, leaving 8% oxygen (O<sub>2</sub>), with the balance nitrogen (N<sub>2</sub>) and rare gases. Insects exposed to this atmosphere will suffer the stress of hypercarbia and moderate hypoxia. Use of catalytic O<sub>2</sub> convertors enables O<sub>2</sub> concentrations to be reduced to about 1% with a concomitant increase in CO<sub>2</sub> concentration to 10-14%. This produces hypoxia, possibly anoxia, and moderate hypercarbia. Flushing the storage

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container with N<sub>2</sub> may produce O<sub>2</sub> concentrations of < 1%, resulting in either hypoxia or anoxia.

Resistance of stored-product insects to contact insecticides and fumigants has been well documented. Therefore the question as to whether these insects are able to develop resistance to alternative control measures is pertinent. The first suggestion of the possibility that stored product insects would develop resistance to MAs was made by Banks and Annis (1977). Navarro and Calderon (1979) warn of situations that may arise where insects survive around leaks in incompletely sealed structures. Bond and Buckland (1979) were the first to show that stored product insects have the genetic potential to develop resistance to MAs, when they obtained a three fold increase in tolerance to CO<sub>2</sub> by *Sitophilus granarius* (L.) after selecting for seven generations. Similar results were obtained by Navarro *et al.* (1985) for resistance of *S. oryzae* to hypercarbia by selection over 10 generations.

A long-term study of the development of resistance by laboratory strains of *Tribolium castaneum* (Herbst) to hypoxia and to hypercarbia was carried out by us recently (Donahaye, 1990), and included investigations into the mechanisms of resistance. This required that the extensive studies already published on the influence of hypoxia, anoxia and hypercarbia on insects and stored product insects in particular, be focussed on the potential for stored product insects to survive MA treatments. My findings are presented in the light of this information.

#### **Adaptation of insects to anoxia and hypoxia in nature**

Although the origin of insects is believed to be from some aquatic ancestors with little aerobic O<sub>2</sub> available, most terrestrial insects are less adapted to anoxic conditions and are rapidly rendered immobile by lack of O<sub>2</sub>. While certain insects have developed "exploitative adaptation strategies" that enable them to invade anoxic environments (Hochachka and Somero, 1973), others appear to have developed "compensatory adaptations" that enable them to compensate for temporary absence of O<sub>2</sub> by maintaining high capacities for anaerobic generation of ATP though their survival depends on ultimate return to aerobic conditions (Pasche and Zachariassen, 1973). Other cases of tolerance to hypoxia revealed a continuation of aerobic respiration at low O<sub>2</sub> tensions (Rumbo, 1979), while certain lepidopterous larvae were shown to maintain normal levels of O<sub>2</sub> uptake at a level of 1% O<sub>2</sub> (Buck and Keister, 1955). In the light of these findings it may be anticipated that selection for resistance to hypoxia in stored product insects would most likely result in a similar form of compensatory mechanism.

#### **Adaptation of insects to hypercarbia in nature**

Carbon dioxide at high concentrations acts as a narcotic in most insects. However, in nature some insects develop in CO<sub>2</sub>-enriched atmospheres. This is the case of certain elaterid and scarabaeid larvae living in deeper layers of the soil, where CO<sub>2</sub> concentrations are high and O<sub>2</sub> concentrations low. The special case of *Gastrophilus* larvae living in the stomach of the horse is another example (Levenbook, 1951). In all these cases hypoxia occurs concomitantly with hypercarbia, so that adaptation is of a dual nature and the individual role of hypercarbia is not clear.

#### **Laboratory induced resistance of a strain of *Tribolium castaneum* to hypoxia**

Selection of *T. castaneum* over 40 generations to produce a strain resistant to a MA composed of 99.5% nitrogen (N<sub>2</sub>) and 0.5% O<sub>2</sub> at 95% relative humidity (RH) was carried out by Donahaye (1985; 1990a); this was termed the LOC selected strain. At the 40th generation the LOC-selected adults had a resistance factor of 5.2 at the LT<sub>50</sub> level in comparison with the laboratory strain from which the LOC strain had been selected.

It was shown that the strain that developed resistance to LOC (hypoxia), was also more resistant than the non-selected strain to anoxia. This may have been due to better adaptation to anoxic conditions by more successful maintenance of energy charge, or removal of toxic end products of glycolysis, although this was not proved experimentally. However, the physiological and biochemical adaptation of the insect to 0.5% O<sub>2</sub> was mainly one by which the insects were able to survive by continuing to carry out aerobic metabolism. The natural tolerance of the non-selected strain to this low O<sub>2</sub> tension was very great, and the adaptation of the selected strain was extraordinary. At 1% O<sub>2</sub> the insects were active, and the LT<sub>50</sub> of the non-selected strain was more than 10 days. It was for this reason that at the outset an O<sub>2</sub> tension of less than 1% was chosen in spite of the difficulty in accurately maintaining such low O<sub>2</sub> concentrations over long time periods.

In a review article on hypoxia in marine invertebrates, Herreid (1980) develops on the classic viewpoint that aerobic animals are either metabolic conformers - if their O<sub>2</sub> consumption varies directly with environmental partial pressure of O<sub>2</sub>, or they are metabolic regulators - if their O<sub>2</sub> consumption is independent of this factor. According to the current view, regulators and conformers form two ends of a spectrum of response. Clearly, if mortality of *Tribolium* is caused at the point where a state of aerobic metabolism in hypoxia can no longer be sustained and is superseded by anaerobic metabolism, then any compensatory mechanism rendering the LOC-selected strain more of a regulator and less of a conformer, will increase its potential for survival. Physiological compensation may include a lower metabolic rate, or reduction in metabolic activity during hypoxia, thus leading to energy and O<sub>2</sub> conservation (even at 0.5% O<sub>2</sub> limited movement of insects could be observed throughout exposure). Matheson and Parsons showed a positive correlation in *Drosophila* between metabolic rates and mortality due to 100% CO<sub>2</sub> (anoxia accompanied by hypercarbia), and negative correlations between body weights and both mortalities and metabolic rates. These correlations were also shown to exist for *S. granarius* laboratory-selected for resistance to methyl bromide (Upitis *et al.* 1973), and resulted in greater body weight, extended life cycle and lower respiratory rate of the resistant strain. Also for the LOC selected strain it was shown that the pupal and adult stages were significantly heavier, oviposition rate was lower, and respiration rates in air were significantly lower than those of the non-selected strain. Possible physiological compensation during exposure that were not investigated, may be by increased O<sub>2</sub> transport, including maintenance of tracheal pumping, transfer across cell membranes, or transport in the hemolymph by increased heart pumping.

It was evident that some anaerobic metabolism does also take place, as witnessed by increased lactate concentrations after 24 and 48 h, and also by polysaccharide concentrations that gradually dropped during exposure. However, exposure to LOC did not appear to have a marked desiccation effect, in contrast to the findings for exposure to HCC described below. This is contrary to evidence in the literature of the diuretic effect of anoxia (Woodring *et al.* 1978) and evidence in *Drosophila* that resistance to anoxia is associated with resistance to desiccation (Parsons, 1971). The cause of death by exposure to LOC was not elucidated. If it occurs as a result of inability of the insect to continue aerobic metabolism, then several studies undertaken on the effect of anoxia on *Tribolium* spp. are pertinent. Our findings on exposure to 100% N<sub>2</sub> revealed survival of both strains as 7.4 and 5.8 times shorter than at 0.5% O<sub>2</sub>, with LT<sub>95s</sub> of 30.3 and 15.5 h for the selected (F22) and non-selected strains respectively. These are confirmed by the findings of AliNiazee (1972) but differ from those of Kennington (1966) who found that in *Tribolium confusum*, anaerobic metabolism at a relatively efficient level is present with high survival at 27°C after 72 h exposure. He states that an oxygen debt mechanism does not seem to operate since he found no evidence of significantly greater O<sub>2</sub> use immediately after O<sub>2</sub> deficiency. Also there was a lack of CO<sub>2</sub> production during O<sub>2</sub> deficiency indicating that the insect was not continuing to live on O<sub>2</sub> already present in the tissues. Kennington presented two hypotheses on mortality due to O<sub>2</sub> deprivation: (i) the beetles are injured by an

accumulation of toxic products such as lactic acid and other materials; and (ii) materials critical to the support of anaerobic metabolism become exhausted. He found that the oxygen deficient process is irreversible. Our findings on exposure of both strains of *T. castaneum* to anoxia confirm that there is a rapid accumulation of lactic acid.

In a study of the biochemical effects of anoxia on *T. confusum* pupae, Kennington and Cannell (1967), showed that lipids were not used as a reserve of energy during anoxia and that they were mobilized extensively- as soon as the insect was reintroduced into air. This may mean that carbohydrate stores had been depleted by glycolysis during anaerobiosis and, on return to air, fat reserves served as the principal store. Lactic acid production presented two patterns: there was significant removal of accumulated lactic acid by ordinary aerobic oxidative processes when the anoxic pupae were returned to air; and there was a significant drop in lactic acid concentration after 72 h exposure to anoxia compared with 48-h anoxic pupae, indicating that lactic acid was being converted or removed in a manner not dependent on atmospheric oxygen. This decrease in lactic acid beyond 48 h suggests that it was being reduced to other molecules or excreted faster than it was being produced.

In contrast, Kashi (1981) suggested that *T. castaneum* and *T. confusum* do incur an oxygen debt. To repay this, the metabolic activity may speed up when O<sub>2</sub> becomes available. He summarized that there is a close relationship between the ability of insects to withstand anoxia and to resist the toxic action of phosphine. However in our experiments, exposure of both the LOC-selected and non-selected strains to phosphine and also methyl bromide failed to reveal any enhanced resistance to these fumigants acquired by the LOC-selected strain (unpublished results).

It is possible that in addition to the accumulation of toxic-end products, adelynic energy charge cannot be maintained. Although most of the investigations into the connection between energy charge and viability have been done on microorganisms (Ball and Atkinson, 1975) it was shown for *E. cautella* pupae (Friedlander and Navarro, 1979) that both hypoxia and hypercarbia caused a marked reduction in the amounts of adeline nucleotides and energy charge.

### **Laboratory induced resistance of a strain of *Tribolium castaneum* to hypercarbia**

Selection of *T. castaneum* over 40 generations to produce a strain resistant to an atmosphere containing 65% CO<sub>2</sub>, 20% O<sub>2</sub>, and 15% N<sub>2</sub> at 95% RH was carried out by Donahaye (1985; 1990b); this was termed the HCC selected strain. At the 40th generation adults of the HCC-selected strain had a RF of 9.2 at the LT<sub>50</sub> level when compared with the laboratory strain from which the HCC strain had been selected.

For the strain resistant to HCC, the fact that O<sub>2</sub> consumption continued during exposure and also the low levels of glycolytic intermediates recorded, indicate that mortality was not an anoxia effect. This should be considered in the light of the findings of Woodring *et al.* (1978) on *Acheta domestica* who showed that CO<sub>2</sub> anaesthesia at 40% and above arrests breathing, thereby resulting in O<sub>2</sub> starvation within the body. The small size of *Tribolium* plus the relatively high O<sub>2</sub> concentration under the experimental conditions may have allowed sufficient passive diffusion of O<sub>2</sub> into its body and thereby prevented the anoxia effect. Although other workers have indicated this and additional effects of CO<sub>2</sub> on insects, including change of pH, neuro-chemical changes including its effect on the peripheral and central nervous system (Fuseau-Braesch and Nicolas, 1981) and cardiac arrest (Sillans and Biston, 1979) - no suggestions have been made that these are the cause of insect mortality.

In this study it was shown that the non-selected strain was unable to control water loss. Mobilisation of triglyceride (TG) reserves was rapid and was almost exhausted within 96 h, indicating that the attempt to mobilize water reserves was insufficient to prevent desiccation.

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tended to increase consumption of glycogen and also to reduce the total amount of adenine nucleotides and the energy charge (Friedlander and Navarro, 1979). Citrate and malate levels were also reduced by hypercarbia, indicating a reduction in activity of the Krebs cycle. Friedlander *et al.* (1983) reported a decrease in ribulose 5-phosphate levels due to a reduction in activity of 6-phosphogluconic acid dehydrogenase in *E. cautella* pupae exposed to hypercarbia. The inhibition of the pentose phosphate shunt results in reduction of reducing power in the form of NADPH.

More recently, Friedlander and Navarro (1984) showed that exposure of *E. cautella* pupae to hypercarbia leads to decrease in levels of glutathione. There are numerous implications in the suppression of glutathione by CO<sub>2</sub> if this is truly the case, because of the complex role of glutathione in the conservation of enzymes, biological membranes and neurotransmitter release. Furthermore, Friedlander (1984) underlines the importance of the fact that the energy factor is reduced under hypercarbia. Since during nerve activation (synaptic transmission) re-acetyllisation of choline to acetyl-choline occurs in the presence of ATP, he proposes that the hindering of acetylcholine production under hypercarbia is liable to cause neurotoxic damage.

In conclusion, the continuing process of selection of *T. castaneum* adults at the 40th generation, both for the strain selected for resistance to HCC and that selected for resistance to LOC, is clearly polyfactorial in nature. This may be viewed in the light of the basic strategies of biochemical adaptation to environmental stress as laid out by Hochachka and Somero (1973). They state that since the metabolic activities of organisms are strictly dependent upon macromolecules (enzymes and nucleic acids), adaptational processes ensure that macromolecular functions occur at the correct rates and are of the proper type to permit the vital process to continue satisfactorily. Three mechanisms are given by which the general goal may be attained: the types of macromolecules present may be changed, the amounts or concentrations may be adjusted, or the functions of the macromolecules may be regulated in adaptive manners.

From the above, the inherent difficulties in pinpointing the physiological and biochemical differences between the selected and non-selected strains become clear.

Lastly, what are the implications of laboratory selection of insects to modified atmospheres, on the practical use of MAs as an alternative insect control method in commerce? The chosen laboratory MAs were different from those used in real-life situations. However, rates of development of resistance were similar to those recorded for laboratory induced resistance to fumigants. There are many similarities between MA resistance and resistance to methyl bromide in the laboratory (Monro 1964, Uptis *et al.* 1973), which was also polyfactorial in nature, and in which it was also shown that resistance attained by the adult was in part conferred to immature stages. Resistance to phosphine appears to be different, in that curves straightened and steepened as tolerance increased (Monro *et al.* 1972, Saxena and Bhatia, 1980) and homozygous characteristics were acquired.

It may therefore be reasonable to assume that in situations where MA treatment becomes standard control practice, the rate at which resistance to MAs will develop will be similar to that occurring for MB.

Several other factors may accelerate the development of resistance to MAs in practice, namely:

a) The fact that most MA technologies produce concomitant conditions of hypoxia and hypercarbia so that selection may be simultaneous for both factors, plus selection for resistance to desiccation.

b) The greater energy reserves and lower metabolic rates of the resistant strains may lead to higher survival under conditions of starvation. This may be of great value for survival of residual infestations when storage structures are empty.

c) The laboratory studies indicated that resistance obtained in the adult stage was also conferred to other stages.

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In sharp contrast, the HCC-selected strain was able to maintain a water balance over a much longer time period. Thus, if CO<sub>2</sub> acts on the nerves of the spiracular muscle (Burkett and Shneiderman 1967), then it is possible that the resistant strain is able to overcome this and close its spiracles, while the susceptible strain could not. Furthermore, TG reserves per insect were significantly greater in the HCC-selected strain and their mobilisation was slower, indicating a better control mechanism for the maintenance of water balance.

An examination of weight loss during exposure to HCC showed that even though exposure was carried out at 95% RH, precisely to avoid the desiccation factor, the insects were nonetheless susceptible to desiccation. Our findings indicate that the water content of the dead insect in equilibrium with 95% RH is approximately 25%, whereas the live insect needs to maintain a water content of 55%.

At the time of complete mortality, insects of the HCC selected strain had lost approximately 25-30% of their total dry weight, comprising about 95% of their total TG reserves and from 6 to 12% dry weight from other sources. This is in contrast to the non-selected strain which had lost only ~12% of its dry weight at the time of complete mortality which included ~86% of its TG reserves.

It is suggested, therefore, that eventual mortality of the selected strain was due to exhaustion of energy reserves, and that an important aspect of the selection process is one which favors those insects that have built up greater reserves and expend them more slowly. The other resistance mechanisms, as suggested earlier, are likely to be compensatory mechanisms against the physiological and biochemical effects of hypoxia and hypercarbia, and spiracular control, or possibly other adaptations limiting water loss.

All aspects of the mode of action of CO<sub>2</sub> on insects are not yet clear. Carbon dioxide may be termed a narcotic in that it produces symptoms in insects that are similar to most fumigants and anesthetics, namely, induced inactivity, followed by a reversal of these symptoms when returned to the normal atmosphere. The mode of action of these "indifferent narcotics" has been the subject of many studies, particularly with reference to man. Miller (1961) and Pauling (1961) concurrently proposed general theories on the mode of action of anaesthesia, both based on the production of hydrate microcrystals or "icebergs" by the anaesthetics. Previous hypotheses were summarized by Miller in the light of the gas hydrate theory and possible mechanisms of narcosis were proposed, including the possibility that the anaesthetic exerts its effect on the mechanism of permeability change in the nerve tissue during conduction. The effect could be on acetylcholine esterase, the receptor protein or the storage protein. From general considerations Miller states that it is apparent that the major effect of gaseous anaesthetics is on the transmission at synapses, including neurons and neuromuscular junctions.

More recently Sillans and Biston (1978, 1979) reported that CO<sub>2</sub> causes heart arrest in larvae of *Bombyx mori* and so showed that in this case the effect is at the nerve level. Since the hydrate of CO<sub>2</sub> is carbonic acid (HCO<sub>3</sub>), the result is an increase in acidity. In this context it was shown by Baker and Honerjager (1978) that CO<sub>2</sub> causes changes in membrane permeability by changes in pH within the cell. Sillans and Biston (1979) concluded that CO<sub>2</sub> may act in three ways: indirect influence on the membranes of cells by raising the acidity; direct influence by dissolving within the bilipid layer of the membrane and in so doing, affecting the shape and position of proteins which in turn affect the movement of ions and transmitters; and the development of micro crystals of hydrates, as proposed by Pauling (1961). In spite of the above findings, the question as to whether mortality is caused directly by the prolonged anaesthetic effect or by some other biochemical lesion has yet to be clarified.

Navarro and Friedlander (1975) showed that CO<sub>2</sub> anaesthesia of *E. cautella* pupae caused an increase in lactate level, indicating a steadily increasing stress on the organism with increased CO<sub>2</sub> concentration, although lactic acid concentrations were not as great as those caused during O<sub>2</sub> depletion. With *E. cautella* pupae hypercarbia

POSSIBILITE DE DEVELOPPEMENT D'UNE RESISTANCE AUX ATMOSPHERES  
MODIFIEES PAR LES INSECTES RAVAGEURS DES DENREES STOCKEES

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RESUME

Des études en laboratoire portant sur la sélection de la résistance d'une souche d'adultes de *Tribolium castaneum*, *Sitophilus granarius* et *S. oryzae* aux concentrations élevées de dioxyde de carbone (HCC) et sur la résistance d'une souche de *T. castaneum* aux basses concentrations en oxygène (LOC) ont montré que ces espèces sont capables de développer des niveaux de résistance peu élevés, mais significatifs, aux atmosphères modifiées (MAS). D'autre part, la résistance obtenue chez les adultes de *T. castaneum* a été en partie transférée aux oeufs et aux larves. Des études portant sur le mode d'action des MAS et sur les mécanismes de résistance chez *T. castaneum* indiquent qu'aux HCC, la mort est causée par la dessiccation et la pénurie des réserves de triglycérides (TG) et non par un effet narcotique prolongé dû à l'anesthésie ou à l'accumulation de substances toxiques de fin de chaîne métabolique. La souche résistante possède de plus grandes réserves de TG et est capable de maîtriser le manque d'eau pendant l'exposition.

La souche résistante au LOC sélectionnée pour 0,5 % de O<sub>2</sub> s'est montrée plus apte à survivre à l'anoxie que celle qui n'avait pas été sélectionnée pour cette concentration. Cependant, le métabolisme, pendant l'exposition à ces LOC, est principalement aérobique. Il est possible que la mort n'apparaisse que lorsque le métabolisme aérobique ne peut plus être maintenu et se trouve dépassé par le mécanisme anaérobique avec accumulation de toxines. Des mesures de la sensibilité de ces deux souches, sélectionnées pour résister, soit aux LOC, soit aux HCC, envers le bromure de méthyle et la phosphine, ainsi qu'une souche de *T. castaneum* non sélectionnée n'ont pas permis de découvrir de résistance croisée avec ces fumigants. Nous suggérons que l'alternance entre MA et fumigation pourrait retarder le développement de la résistance des insectes ravageurs à ces deux traitements.