SUB-LETHAL EFFECTS OF CONTROLLED ATMOSPHERES ON INSECTS: DETECTION AND QUARANTINE IMPLICATIONS

A. CARPENTER,[1]* I.A.N STRINGER,[2] M.A. POTTER[2] AND C.W. VAN EPENHUIJSEN[1]

[1]*Food Industry Science Centre, Crop & Food Research, Palmerston North, New Zealand [*e-mail: carpentera@crop.cri.nz]*
[2]*Ecology Group, Institute of Natural Resources, Massey University, Palmerston North, New Zealand*

ABSTRACT

Data on the sub-lethal effects of controlled atmospheres (CAs) on black field crickets (*Teleogryllus commodus*) and green peach aphid (*Myzus persicae*) are presented. Literature on the sub-lethal effects of CAs on other insects is briefly reviewed. The sub-lethal effects of CAs range from reduced metabolic rate, through altered life history parameters (egg number, egg viability, length of developmental and adult phases), to loss of ability to transmit viruses, and loss of ability to survive more than a few days after treatment.

The sub-lethal effects of CAs are usually only detected when they are specifically designed experimentally to do so. For many commodities that could be disinfested with CAs for quarantine purposes, the effective treatment is close to levels that would damage the commodity, although this depends on the durability of the commodity to be treated. For treatments that have such narrow windows of opportunity, natural biological variation will eventually cause treated, non-viable, insects to be detected on the treated produce. Until more is known about the mode of action of CAs in causing insect death, and the occurrence of sub-lethal effects that can be predicted and detected, it is unlikely that CAs will be acceptable as quarantine treatments.

INTRODUCTION

Despite efforts to develop controlled atmospheres where carbon dioxide (CO_2) levels are elevated or oxygen (O_2) levels are reduced, or both, as quarantine treatments (e.g. reviews by Klag 1986; Ke and Kader 1992; Carpenter and Potter 1994; Banks and Annis 1990; Carpenter 1995, 1997), regulators have been slow to accept such treatments. The reasons for this are complex and can perhaps be conceptualised as the binary -live/dead- paradigm where regulators prefer simple decisions about whether the organism is alive or dead, rather than complicated (or high risk) decisions about viability, as viability is not subject to detection by simple inspection. The problem is compounded by the lack of any clear understanding of the mechanisms involved in insect mortality when they are treated with CAs. Thus the reliability of the treatment and the limits of its reliability are undefined at a biochemical or physiological level (Klag 1986).

The strengths and limitations of methyl bromide are well known. There are published dosage schedules for quarantine problems on many commodity types (e.g. Bond 1984).

The binary paradigm is common to most non-fumigant alternatives to methyl bromide. With methyl bromide the pest is dead – does not move, or if it moves, a leg even slightly, it's alive. Insects without apparent appendages, such as scale-insects, pose particular problems for quarantine inspectors. After exposure to CAs, heat, or irradiation, an insect might move, perhaps fairly freely, and still be sterile or otherwise non-viable. Considerable effort has gone into defining this live, but non-viable state for some insects such as tephritid fruit flies after they have been irradiated (Hallman 1999), and a little into heat-treated pests (Thomas and Mangan 1995). Very little has been published on the sub-lethal effects of CAs, except incidental observations on crickets by Stevenson and Hurst (1995), codling moth (Soderstrom *et al.,* 1991) and navel orangeworm (Storey and Soderstrom 1977). A more comprehensive work by Markwick *et al.,* (1998) explored the effects of sub-lethal atmospheres on first, third and fifth instar light-brown apple moth, a species that is more tolerant of CAs than the other leafroller larvae commonly found on New Zealand apples. In this work the effects were mostly on life-history parameters such as number of fertilised eggs laid.

The work at the Volcani Institute in Israel, (Donahaye and Navarro 2000) which showed that insects could develop resistance to CO_2 and O_2, while not directly related to sub-lethal effects, may well involve similar biochemical and physiological phenomena.

Sub lethal effects

A part of the problem is that sub-lethal effects lie on a continuum, from little effect on viability and the ability to breed effectively, to complete inability to breed. An insect might simply survive, possibly feeding, or just stay quiescent for some time.

Why does it matter?

The combinations of time and CO_2 concentration required for death in insects as a result of treatment with CAs are extreme. Long exposure times (Lill and van der Mespel 1986), high CO_2 or low O_2 concentrations, or both (Potter *et al.* 1994; Carpenter *et al.* 1996; Carpenter 1995, 1997) or elevated temperature, (Carpenter *et al.* 1996; Carpenter 1995, 1997; Markwick *et al.,* 1998) may be required.

Controlled atmosphere treatments are costly to develop and maintain. The more insecticidal they are, the more likely they are to damage perishable commodities. Long treatments are a barrier to trade as commodities need to be held for extended periods, certainly too long for produce such as apples and asparagus to be treated in transit, although the durables such as cereal and legume grains offer more manageable challenges.

Sub-lethal effects of CAs, a short history:

The sub-lethal effects of CAs were foreshadowed when entomologists began to notice that anesthesia with CO_2 or N_2 affected the subsequent viability of the insect. In some cases, the response is due in part to the presence of a virus infection. More commonly, it is due to biochemical or physiological effects that are not well understood. Carbon dioxide anesthesia reduces insect feeding, virus transmission rates, rate of weight gain, and metabolic rate (Brooks 1957; Sylvester and Richardson 1963; Edwards and Patton 1965; Woodring *et al.*, 1978; Tamaka 1982). It can have long term effects on insect behaviour and life history parameters (Berger and Zeyen 1987).

Insecticidal mode of action of controlled atmospheres

Many workers (Donahaye and Navarro 2000; AliNiazee 1972; Friedlander and Navarro 1979) have identified phenomena associated with mortality in insects treated with CAs. The phenomena described, range from loss of all ATP, (Fourney *et al* 1991) through changes in, and up to loss of all trehalose (Donahaye and Navarro 2000). Simple descriptions of such phenomena have not given us sufficient understanding of how CAs kill insects and thus no theoretical basis from which to approach the question of sub-lethal effects. However, we have not advanced our understanding of the mechanisms leading to insect death from CA treatment since the early work of Gilmore (1941) and Carlson (1960) who both felt that the build up of toxic by-products was the cause of mortality, although Gilmore (1965) postulated that the effect might be through the electron transfer chain.

Benefits from understanding sub-lethal effects

If we can predict and explain the sub-lethal effects of CAs on insects, we can begin to work towards implementation of CA and quarantine methods with regulatory authorities. A key issue for all novel disinfestation methods where a moribund or otherwise non-viable insect might be present is how a regulator can be sure that the insect has been treated and is thus non-viable. Until we understand the processes that lead to sub-lethal effects, how they vary between taxa, and how to detect them, we will remain dependant on fumigants for quarantine treatments with the associated issues of residues, occupational health, damage to the environment and adverse effects on produce quality.

More formally, once we can predict when sub-lethal effects will occur, we will be able to demonstrate the likely quarantine risk. We will now review our own research against other peoples' results to define the key issues relating to the understanding of sub-lethal effects and how they affect development of new quarantine treatments.

THE EMPIRICAL DATA

In our research on the biochemical basis of insect mortality from CA treatments we have used *Teleogryllus commodus* (black field cricket) as a model because it is a large insect that is very sensitive to CAs. It is also easily maintained, living on a wide

range of fresh produce and grain foods. Our other key model has been *Myzus persicae* (green peach aphid), which is a clonal organism. For both these insects moribund individuals, apparently non-viable, but technically still alive as they moved appendages, were observed during other research (Stevenson and Hurst 1995; van Epenhuijsen *et al.* 2001). Stevenson and Hurst (1995) did not design their experiments to detect sub-lethal effects, so they could only report the occurrence of "comatose" or moribund insects in passing. We carried out a study on the effects of the level of CO_2 (0-20%) plus 1% O_2 on second instar *T.!commodus* that was designed to detect both acute and sub-lethal (chronic) effects (Stringer *et al.* unpublished data).

The Cricket Model:

Stevenson and Hurst (1995) investigated the relationship between time of exposure, CO_2 level and the mortality of adult *T. commodus*. They recorded the presence of comatose crickets incidentally when recording the mortality data. Calculated exposure times for 50 and 90% mortality are given in Table 1.

TABLE 1

Calculated exposure times (h) for 50 and 99% mortality in black field crickets at various levels of CO_2, all with 20% O_2, except the treatment marked with * which had 12% O_2. Figures in brackets are standard errors of the data, (d.f. =16). (Reproduced from Stevenson and Hurst 1995)

CO_2 Level	LT_{50}	LT_{99}
18	32.81 (1.38)	62.83 (3.25)
30	22.74 (1.56)	57.50 (4.76)
57	17.46 (0.79)	31.72 (1.92)
57*	18.51 (1.17)	42.38 (3.52)

The data on the occurrence of comatose crickets from the same experiments are shown in Fig. 1. The frequency with which comatose crickets occurred increased rapidly between 9 and 18% CO_2, and it also increased rapidly from 2 to 4 h exposure. The highest frequencies of comatose crickets were found at: 18% CO_2 for 8, 12 and 24 h exposure; 30% CO_2 for 4, 8, 12, and 24 h exposure; at 57% CO_2 with 20% O_2 for 8 and 12 h exposure; and at 57% CO_2 with 12% O_2 for 4, 8, 12 and 24 h exposure. The 48 h exposure caused high mortality in all treatments with 18% CO_2 and above, and when mortality was above about 60%, the frequency of comatose crickets decreased rapidly. Stevenson and Hurst defined 'comatose' as the ability for insects to move appendages when prodded but inability to walk. 'Live' crickets were defined as those able to walk at the moment of assessment. They recognised two types of comatose crickets: for 0-18% CO_2, the comatose crickets moved all their limbs in a spasmodic way; at higher CO_2 concentrations the crickets mostly just moved their maxillae.
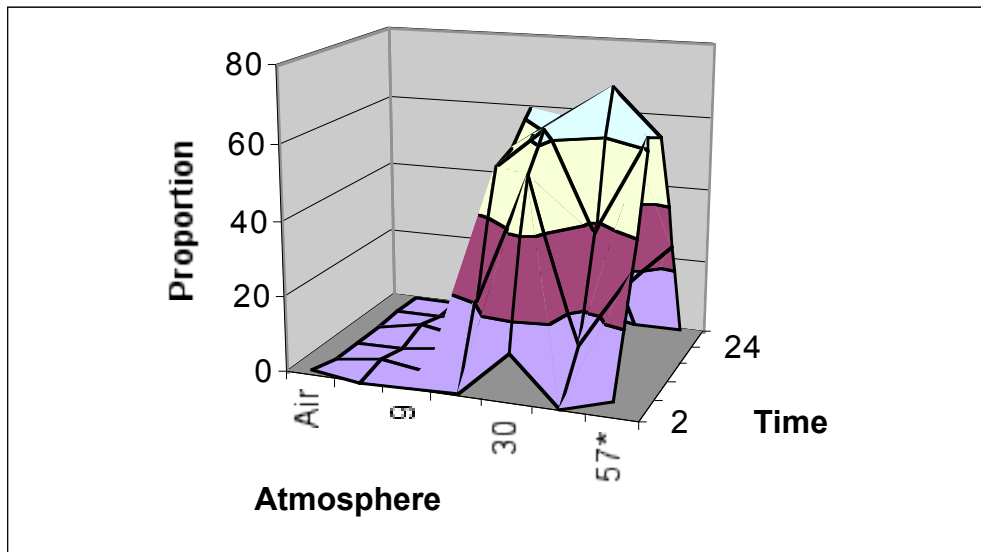
Fig. 1. A response surface showing the frequency of occurrence of comatose crickets as a function of time (hours) and concentration of carbon dioxide. Data represented from Stevenson and Hurst (1995).

In their research, Stevenson and Hurst (1995) did not retain comatose crickets to determine their eventual fate. Nor did they keep live crickets from each treatment to determine their viability. Also, we would now prefer the term 'moribund' to 'comatose'.

Stringer *et al*., (ms in preparation) carried out similar experiments, but instead of concentrating on the obvious phenomena of mortality and the moribund state, they took live, treated, second instar crickets, and reared them to maturity. The aim was to determine the effects of sub-lethal or chronic exposure to CAs on a range of key life-history parameters. They used CAs, all with 1% O_2, and with 0, 5, 10 or 20% CO_2. They found that more insects were dead when assessments were carried out 24 h after treatments had ended than when assessments were carried out 1 h after treatment. Mortality estimates from data collected 24 h after treatment had ended are given in Table 2.

The estimated times in hours to achieve the target mortality levels for the second instar crickets were much shorter than they were for the adult crickets in the study by Stevenson and Hurst (1995). This may possibly be attributed to the more insecticidal levels of O_2 used by Stringer *et al*. (ms in preparation), but may also be related to the different instars tested, and the fact that the test animals were drawn from different populations.

TABLE 2

Exposure time (hours) to achieve 50 and 95% mortality of crickets. Data in brackets are 95% confidence limits. All treatments included 1% oxygen

% CO_2	LT_{50}	LT_{95}
0	17.8 (2.74)	82.8 (23)
5	12.54 (0.8)	36.4 (6.9)
10	10.25 (3.3)	27 (17.2)
20	7.28 (0.8)	24.69 (8.7)

To determine the sub-lethal or chronic effects of CAs, second instar crickets were exposed for approximately the LT_{50} exposure times (18-19 h) and the survivors were then reared using standard methods. The atmospheres tested were air, 1% O_2+ balance N_2, and 5% CO_2 +1% O_2 + balance N_2. The results for nymphal development parameters are summarised in Table 3 and for adult development and reproductive parameters in Table 4.

TABLE 3

Nymphal development parameters for second instar cricket nymphs treated with LT_{50} rates of controlled atmospheres

Atmosphere	Initial number	Number 1 h after treatment	Mean nymphal duration	Median nymphal duration	Last nymph (d. from start)	% surviving to adult
Air	120	120	111 d	142 d	229	40
1%O_2+5%CO_2	112	52	68.5 d	85.5 d	116	56
1%O_2	170	118	49 d	58.1 d	135	41.2

From Table 3 we can see that the CO_2 + O_2 treatment was the most insecticidal: but the O_2 in N_2 had the most effect on mean and median nymphal duration, as well as the percentage of nymphs surviving to become adults. Both CAs had similar effects on the number of days from treatment until the first males and females reached the adult stage (Table 4). Both male and female life spans were shorter for crickets treated with the O_2 atmosphere than for the CO_2 + O_2 atmosphere. Both CA treatments produced adult crickets that had longer life spans than the control treatment (air). The CO_2 + O_2 CA reduced the total number of eggs laid per treatment by 52% and the O_2 in N_2 CA reduced the total egg number by 37%. Females treated with CO_2 + O_2 produced more than double the mean number of eggs per female than did those in air or the O_2 in N_2 atmosphere. The viability of the eggs in air and the CO_2 + O_2 atmosphere was broadly similar, and was almost double that for the females treated with the O_2 in N_2 atmosphere. However when the number of offspring per adult female is calculated for the O_2 + N_2 treatment one observes that the number of offspring produced per treatment was about half that of the other two treatments.

TABLE 4

Adult development and reproductive parameters for crickets treated with controlled atmospheres as second instar nymphs. (NB- only one of two replicates in the carbon dioxide treatments actually produced viable adults and only one of three replicates in the oxygen alone treatment produced viable adults)

Atmosphere	Males	Females	First male	First female	Male lifespan	Female lifespan	Total eggs laid	Eggs per female	% hatch	No. of progeny
Air	20	28	155 d	135 d	67.6 d	76.5	14350	512.5	32.5	4664
$1\%O_2$ $+5\%CO_2$	9	6	94	86	116.9 d	127.1 d	6933	1155.5	29.5	2045
$1\%O_2$	14	20	92	92	103 d	71 d	9015	450.7	54.9	4949

From this analysis, it can be seen that the sub-lethal effects of CAs on crickets are complicated and can affect a range of population parameters that would affect the ability of a pest to invade new territories.

The aphid model:

From existing information (Carpenter 1997), we hypothesised that if an insect was treated with a range of CAs (0-95% CO_2 at a standard 5% O_2), and at a temperature that did not stress the animal, for an exposure period that gave about 100% mortality at the high stress end, and 0% mortality at the low stress end of the range, we would be able to identify the conditions that lead to the occurrence of moribund aphids. *M.!persicae* was used as the test species. Moribund aphids occurred mostly between 20 and 65% CO_2. No moribund aphids lived for 12 days after the treatment ended (van Epenhuijsen *et al* 2001). The data are summarised in Fig. 2.

CONCLUSIONS

Detecting and predicting sub-lethal effects

Few data have been reported where a researcher has recorded the sub-lethal effects of a CA if they had not specifically set out to find it. Exceptions to this are works by Soderstrom *et al*. (1991), Stevenson and Hurst (1995) and Storey and Soderstrom (1991), all of who recorded information of sub-lethal effects (reduced mating success, reduced oviposition, and reduced egg viability) of CAs incidental to other work. In experiments where it was expected to find sub-lethal effects none were found (Carpenter and Koolaard in prep), even though the test insects, a pseudococcid mealy bug and a lygaeid bug are in the same insect order as the green peach aphid. Donahaye, Navarro and co-workers (Donahaye 1993; Friedlander and Navarro 1979; Donahaye and Navarro 2000) found sub-lethal effects of controlled atmospheres in

stored product pests (*Tribolium castaneum and Ephestia cautella)* as they were able to induce resistance to both CO_2 and O_2 in both species..

Markwick *et al* (1998) found that light-brown apple moth larvae treated at the first instar showed no sub-lethal effects. Treated at the third instar the main effects were on total numbers of eggs and number of fertile eggs produced. Treated as fifth instars survival to pupation was reduced, as was female pupal weight. Sex ratio was skewed showing that females were more affected than were the males. Total eggs and fertile eggs were reduced significantly. The frequency of deformed pupae increased, a phenomenon that has also been observed in insects treated sub-lethally with heat (Thomas and Morgan 1995).

Mitz (1966) has produced the only wide ranging discussion of the potential effects of CO_2 on living organisms, yet no one has taken up the challenge to develop hypothesis-driven data sets to answer the questions and ideas posed in this seminal work.
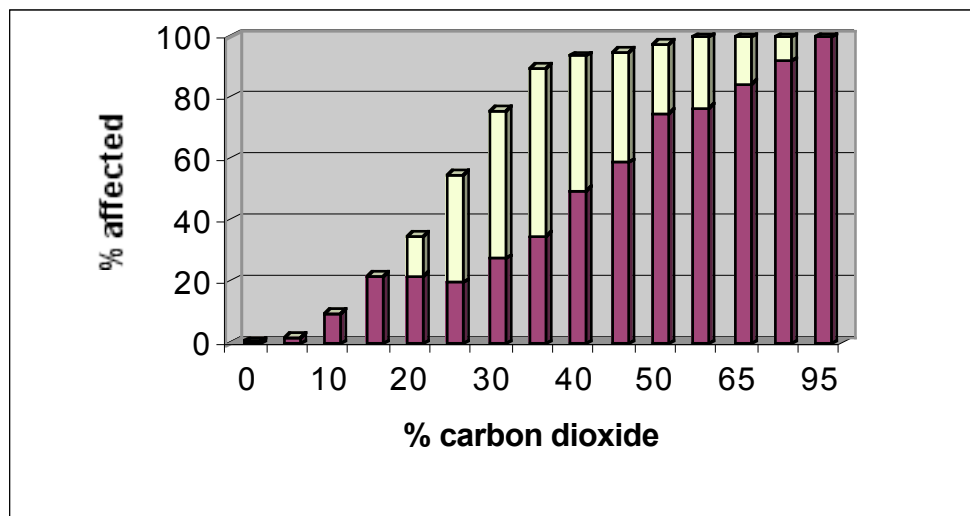

Fig. 2. Occurrence of mortality and frequency of moribund aphids as a function of carbon dioxide level after 40 hours treatment at 20ºC. Insects assessed 2 days after treatments ended. Data represented from van Epenhujsen *et al.,* (2001). Dark portions of the bars represent dead aphids and the pale portion represents moribund aphids.

Quarantine issues
Novel quarantine treatments may have to fit the narrow window between insect death, and commodity damage. To ensure that there is a practical window of opportunity for a novel disinfestation treatment, the probability that a live, chronically affected individual will be detected, may need to be relatively high. What is an inspector to do if he/she is confronted with a live insect in a supposedly treated produce? If the produce has been treated according to an accepted schedule then the

insect may be passed as non-viable. If the produce has not been subject to an agreed and audited quarantine treatment, the insect has to be regarded as a bio-security threat. If the mode of action of CAs was understood properly, it might be possible to develop a quick test for viability of the organism that could be applied in practice. Until the processes of death and sub-lethal effects of a treatment are sufficiently characterised, an acceptable quick test cannot be developed. We are still some way from this point, even for pests on which a reasonable body of information has already been published.

In studies on irradiation, Williamson *et al.* (1985) showed that when the level of contamination of a commodity with a pest was high, the inherent bell-shaped distribution of biological responses ensured that there were enough survivors to cause a bio-security problem. There is no reason to expect other outcomes from tests of CAs.

The state of the art: effects of CAs on insects
There are some aspects of the effects of CAs on insects that are clearly true:

> Insects can develop resistance to controlled atmospheres (Bond and Buckland 1979; Donahaye and Navarro 2000; Wang *et al.* 2000).

> Insect populations that are resistant to, or tolerant of, treatment with CAs, can have increased body size, reduced metabolic rate, increased enzyme levels (carboxyl esterase, superoxide dismutase, succinic dehydrogenase, and catalase) (Bond and Buckland 1979, Donahaye and Navarro 2000, Wang *et al.* 2000).

> CAs have a diverse range of sub-lethal effects, although the cause(s) of sub-lethal effects is/are not known.

> A number of biochemical and physiological phenomena undergo change in insects that are treated with CAs (e.g. enzyme levels, polysaccharide levels, water relations, nervous integration, metabolic rate, inhibition of glycolytic energy production), but none of these observed phenomena can yet be linked to the cause(s) of insect death through a predictive hypothesis.

> For every treatment of a given statistical reliability, there must be a corresponding part of the treated population that has received a sub-lethal dose.

We know a reasonable amount about the changes that occur in insects as a result of treatment with CAs, yet we do not seem to know why these changes occur. At a basic physiological level there are links between groups of observed phenomena, but the complete picture is yet to be elucidated. Until such understanding develops, it is unlikely that CAs will be accepted as quarantine treatments.

ACKNOWLEDGEMENTS

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