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Interactions between controlled atmospheres and low temperature tolerance: a review of biochemical mechanisms

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Controlled atmosphere treatments using carbon dioxide, oxygen, and/or nitrogen, together with controlled temperature and humidity, form an important method for post-harvest sterilization against insect-infested fruit. However, in insects, the cross tolerance and biochemical interactions between the various stresses of modified gas conditions and low temperature may either elicit or block standard stress responses which can potentiate (or limit) lethal low temperature exposure. Thus, the success of such treatments is sometimes erratic and does not always result in the desired pest mortality. This review focuses on the biochemical modes of action whereby controlled atmospheres affect insects low temperature tolerance, making them more (or occasionally, less) susceptible to cold sterilization. Insights into the integrated biochemical modes of action may be used together with the pests' low temperature tolerance physiology to determine which treatments may be of value in post-harvest sterilization.

Keywords: thermal biology, stored product, pest management, biological control

INTRODUCTION

Many treatment regimes rely on one of several stresses [e.g., temperature/controlled atmospheres (CAs)] to reduce survival of insects for pest management or post-harvest disinfestation. However, current research is increasingly exploring the use of multiple, combined, or sequential stresses to act synergistically and enhance insect mortality rates, while reducing commodity damage. The phasing out of numerous chemicals previously used to sterilize fruit and other commodities has prompted renewed interest in CA treatments [reviewed by Fields and White (2002)]. Controlled atmosphere treatments usually involve augmenting temperature sterilization with either high CO_2 (18–90 kPa) or low O_2 (0–11.5 kPa; Hallman and Denlinger, 1998). These gas conditions are frequently used along with controlled humidity, and can be combined to give a high CO_2 and low O_2 environment. There has also been research into using ozone for pest control in stored produce (Tiwari et al., 2010). Cross tolerance of insects to different environmental stressors can disrupt post-harvest control measures in unexpected ways and the success of CA treatments is species-dependent, erratic and does not always result in additional pest mortality (e.g., Navarro, 1978; see Mitcham et al., 2001; Pryke and Pringle, 2008).

In general, CAs at high temperatures reduce the exposure time of fruit thereby reducing fruit damage. However, some commodities are particularly intolerant to warm temperatures (e.g., grapes and cut flowers). At low temperatures, CAs can improve pest mortality during low temperature sterilization, and sometimes shorten the treatment duration too. Whilst the effects of high temperature treatments together with CAs are relatively well documented for several insect pests (e.g., Neven and Rehfield-Ray, 2006), CA treatments coupled with low temperature exposures are not well understood. Treatments with minimal deleterious effects on fresh commodities are challenging to develop (but see **Table 1** for a list of published potentially viable low temperature CA protocols). The inconsistent results may indicate that the pre-treatments either elicit or block standard stress responses which potentiate (or limit) lethal low temperature exposure. In addition, insects may also be exposed to conditions that enhance thermal tolerance during the harvesting process prior to sterilization or during the actual CA process which could ultimately affect treatment efficacy.

This mini-review focuses on the biochemical mechanisms whereby CAs affect insect low temperature tolerance, potentially making them more susceptible (or tolerant) to low temperature sterilization protocols. While previous reviews have highlighted the need for better understanding of the mechanisms (Mitcham et al., 2006; Phillips and Throne, 2010), none have considered in detail the biochemical interactions between the various stresses of modified gas conditions and low temperature (though see Storey and Storey, 2004). Here we provide an overview of the mechanisms involved in low temperature tolerance and CA responses, both from insect physiology and pest control case studies. The aims of this mini-review are therefore (i) to briefly highlight the biochemical links between the seemingly unrelated stressors of gas and temperature, (ii) identify potential commonalities between stress pathways in order to better understand the mechanisms of low temperature mortality and CA treatments, and (iii) identify future directions for insect physiology research which might be of benefit to applied pest management.

Table 1 | Examples of insect pest post-harvest sterilization methods using low temperatures augmented with controlled atmospheres.

Ordering of exposures	CA exposure			Low temp exposure	Pest species	Life stage	Commodity or Medium	References
	CO_2	O_2	Ozone					
CO_2 → low temp	95% (20 h @ 20˚C)			1.5˚C	*Ceratitis capitata* (Diptera: Tephritidae)	3rd instar larvae	Mandarin fruit	Alonso et al. (2005)
Low temp → CO_2	95% (25˚C)			1.5˚C, 3 d	*Ceratitis capitata* (Diptera: Tephritidae)	3rd instar larvae	Mandarin fruit	Palou et al. (2008)
O_2 → low temp		1%		0˚C	*Epiphyas postvittana* (Lepidoptera: Tortricidae)	Larvae	Persimmons	Dentener et al. (1992)
					Pseudococcus longispinus (Hemiptera: Pseudococcidae)	Larvae and adults		
O_2 → low temp		0.3% (16 or 30 h @ 30˚C)		0˚C, 1 mo	*Epiphyas postvittana*	All stages	Pears	Chervin et al. (1997)
					Cydia pomonella	5th instar larvae		
					Grapholita molesta (Lepidoptera: Tortricidae)	5th instar larvae		
Low temp + O_2		0.003%		10˚C, 2 d	*Frankliniella occidentalis* (Thysanoptera: Thripidae)	Larvae and adults	Iceberg lettuce	Liu (2008)
Low temp + ozone			40 ppm (up to 48 h)	20.4 ± 0.1˚C, 65 ± 4% RH	*Tribolium castaneum* (Coleoptera: Tenebrionidae)	Larvae and adults	Grain	Holmstrup et al. (2011)
O_2 + CO_2 + low temp	45%	11.5%		0˚C	*Platynota stultana* (Lepidoptera: Tortricidae) and *Frankliniella occidentalis* (Thysanoptera: Thripidae)	All life stages	Grapes	Mitchum et al. (1997)
O_2 + CO_2 + low temp	9 or 18%	2%		0, 7, 13, or 20˚C	*Thrips obscuratus* (Thysanoptera: Thripidae)	Adults	Kiwifruit	Potter et al. (1994)
O_2 + CO_2 + low temp	3%	1.5%		0˚C, 28 d	*Epiphyas postvittana* (Lepidoptera: Tortricidae)	1st and 5th instar larvae	Apricots	Whiting and Hoy (1997)
O_2 + CO_2 + low temp → 20˚C (1 w)	2.4–2.5 or 1.0–1.1%	2.6–3.0 or 1.5–1.7%		1 or 3˚C, 31–34 w	*Quadraspidiotus perniciosus* (Hemiptera: Diaspididae)	All life stages (from natural infestations)	Apples	Chu (1992)
O_2 + CO_2 + low temp	<1%	1.5–2.0%		0 ± 0.28˚C, 95–100% RH, 13 w	*Cydia pomonella* (Lepidoptera: Tortricidae)	4th and 5th instar non-diapausing larvae	Apples	Toba and Moffitt (1991)
O_2 + CO_2 + low temp	20, 50, or 80%	2, 10, or 20%		10 or 15.6˚C, 7–10 d	*Anastrepha suspensa* (Diptera: Tephritidae)	Eggs and larvae	Agar diet	Benschoter (1987)

CA, controlled atmospheres; RH, relative humidity; mo, month(s); w, week(s); d; day(s); h, hour(s). Only studies which were effective in increasing pest mortality and/or decreasing exposure time, while not significantly damaging produce, are presented.

MOLECULAR MECHANISMS OF LOW TEMPERATURE TOLERANCE

Insect tolerance to low temperature treatments for post-harvest sterilization depends on basal low temperature tolerance, the ability to withstand or repair the stress associated with long-term low temperature exposure, or the ability to rapidly develop biochemical protection (for introduction to this literature, see recent reviews in, e.g., Clark and Worland, 2008; Doucet et al., 2009; Lee, 2010). This ability to rapidly adapt to the external stressor is a form of phenotypic plasticity, in particular, if it occurs within the organism's life-time. Both the immediate environment as well as the species' evolutionary history will determine the extent to which an individuals' response is plastic (e.g., Chown and Terblanche, 2007; Nyamukondiwa et al., 2011; Overgaard et al., 2011).

In most cases, commodities cannot withstand temperatures below freezing and are held at, or just above, 0°C for the duration of the treatment. While a large proportion of insects will enter chill coma at these temperatures, and some species or life stages could die (e.g., *Drosophila melanogaster* survives about 8 h at 0°C), those with mechanisms of enhanced low temperature tolerance are able to survive more severe exposures. However, such generalizations are complicated by a range of factors. Firstly, it is important to remember that most of these mechanisms are closely linked to one another, as well as to external factors, e.g., diet (see e.g., Shreve et al., 2007). Thus, in order for biochemical mechanisms to buffer the effects of low temperature exposure in an insect, the basic components enabling these processes need to be present, together with active metabolism. These known mechanisms of low temperature tolerance are understood in some detail and rely on interactions between genes, proteins, cryoprotectants, and the regulation of biological membranes to preserve cell structure and function (see **Figure 1** and **Table 2** for summary and synthesis).

Ultimately, the gene and biochemical responses described (**Table 2**) are all dependent on the availability of energy provided by metabolism. The "membrane pacemaker" theory of metabolism provides a link between cellular metabolism and lipid membrane bilayers. It suggests that the balance between monounsaturated and long-chain polyunsaturated acyl chains within the bilayer plays a role in establishing the metabolic rate (reviewed in Hulbert, 2003). Aging membranes will be subjected to a change in acyl

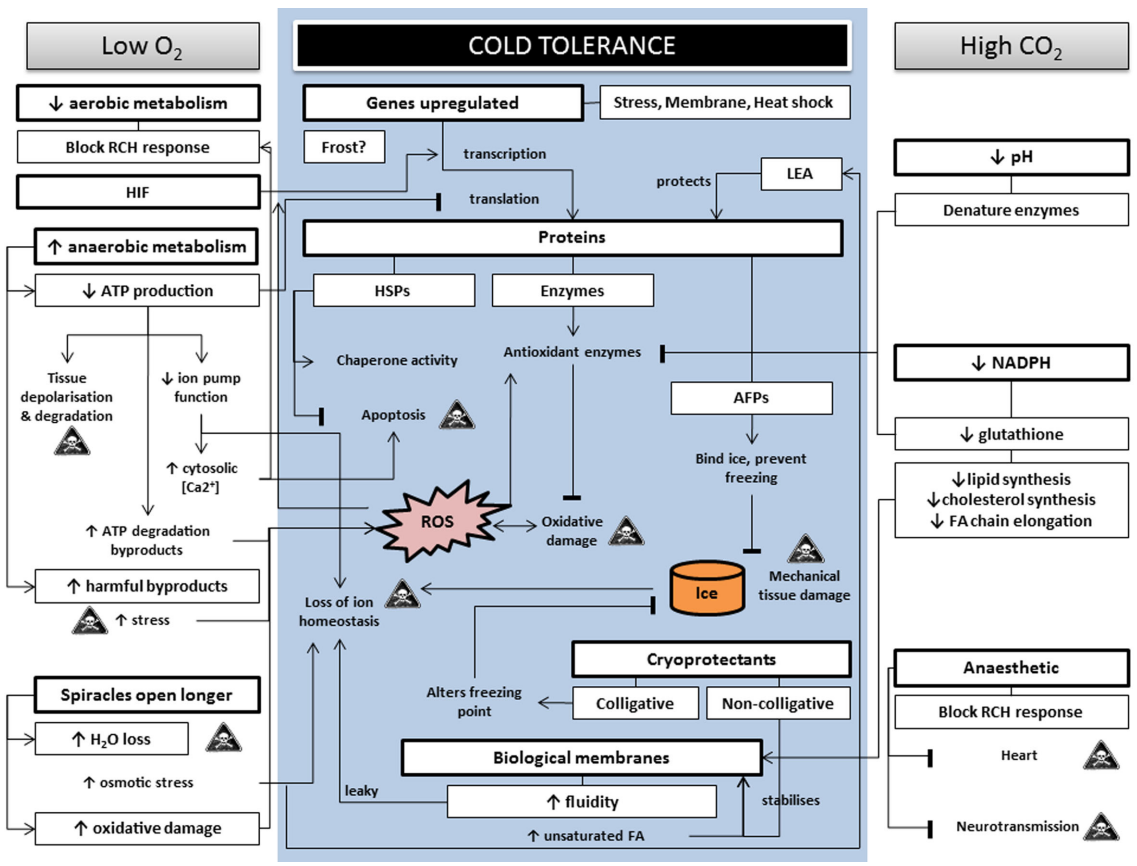

FIGURE 1 | A schematic representation of the cross tolerance between controlled atmospheres (low oxygen and high carbon dioxide) with low temperature stress. The central shaded block represents the mechanisms for survival of low temperatures. Arrows indicate preceding steps in reactions, while solid lines indicate inhibition. The "skull and crossbones" icon represents processes that are likely to lead to mortality. RCH, rapid cold hardening; ATP, adenosine triphosphate; HIF, hypoxia inducible factor; LEA, late embryogenesis abundant proteins; HSPs, heat shock proteins; AFPs, anti-freeze proteins; ROS, reactive oxygen species; FA, fatty acids; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate.

Table 2 | A summary of mechanisms of insect low temperatures tolerance.

Molecule(s)	Mode of protection	Cross tolerance (stressors)?	Species	Example reference
Senescence marker protein-30; mitochondrial acyl carrier protein 1; Frost	Unknown (with regard to cold stress)	Indirect (increased expression also associated with: heat, starvation, desiccation)*	Mainly *D. melanogaster*	Qin et al. (2005), Morgan and Mackay (2006), Sinclair et al. (2007), Colinet et al. (2010a)
Glycerol-3-phosphate dehydrogenase	Flight behavior; lipid and carbohydrate metabolism	Indirect (increased expression or allozyme variation also associated with: heat, starvation)*	Mainly *D. melanogaster*	Barnes et al. (1989), Leemans et al. (2000)
Desaturase2	Fatty acid biosynthesis and modification	Indirect (increased expression also associated with: heat, starvation, desiccation)*	Mainly *D. melanogaster*	Morgan and Mackay (2006), Greenberg et al. (2006)
Heat shock genes and proteins (HSF, HSP23, 26, 70, 83, HS-RNAω)	Molecular chaperone activity; Anti-apoptotic activity (blocks caspase-mediated apoptosis)	Yes (general stress, heat, desiccation)	Numerous insect species incl. *D. melanogaster*	Somero (1995), Feder and Hofmann (1999), Sørensen et al. (2003), Sørensen (2010), Colinet et al. (2010b), Beere et al. (2000), Jäättelä et al. (1998)
Late embryogenesis abundant proteins (LEA)	Act as antioxidants or protein and/or membrane stabilizers	Yes (desiccation)	Mainly plants	Tunnacliffe and Wise (2007), Hand et al. (2011)
Anti-freeze proteins (AFP)	Increases phase transition temperature of the membrane		Polar fish; plants; insects	Tomczak et al. (2002)
Antioxidants (e.g., SOD, catalase)	Reduces oxidative damage		Numerous insect species	Joanisse and Storey (1996), Hermes-Lima et al. (2001)
Membrane composition (fatty acids, cholesterol)	Increases membrane fluidity		*Sarcophaga bullata*; *D. melanogaster*; *Caenorhabditis elegans*	Lee et al. (2006), Overgaard et al. (2005), Murray et al. (2007), M'Baye et al. (2008)
Sugars and polyols (cryoprotectants)	Decreases freezing point; Stabilizes membranes; chaperone activity; free radical scavengers	Yes (desiccation)	Numerous insect species	Koštál et al. (2007), Overgaard et al. (2007)

**Gene information from: Candidate Genes for Climatic Stress Traits in Drosophila. A. A. Hoffmann and co-workers (http://cesar.org.au/index.php?option=com_candidate_gene.)*

composition which may in turn influence a species' lifespan. Interestingly, the carbon units in polyunsaturated acyl chains are very susceptible to oxidative damage, whilst saturated and monounsaturated acyl chains do not possess these carbon atoms (Hulbert, 2003). Molecular changes also occur in the acyl chains' packing which decreases membrane permeability (Tomczak et al., 2002). Additionally, changes in cholesterol content and temperature can result in changes in membrane fluidity by altering the phase of the lipid bilayer (M'Baye et al., 2008).

Thus, an insect's low temperature tolerance depends on interactions between the different molecular levels to ensure function and survival. The cellular mechanisms work together to limit oxidative damage and maintain the integrity of membranes and essential proteins during the low temperature exposure, as well as to buffer the potential heat-stress of returning to warmer temperatures thereafter. A breakdown in just one of these systems is potentially sufficient to stress and overwhelm the entire low temperature tolerance response, perhaps directly or indirectly resulting in insect mortality. In addition, selected tissues may be particularly susceptible to low temperature stress, with knock-on effects for the whole organism if the low temperature-sensitive tissue becomes irreparably damaged (e.g., MacMillan and Sinclair, 2011).

AUGMENTATION WITH MANIPULATED ATMOSPHERES

Pörtner (2001) proposed a theory for thermal tolerance of animals that centered round O_2 limitation as the major factor. The theory of O_2 and capacity limitation of thermal tolerance (OCLT) proposes that once aerobic capacity has been exhausted at temperatures close to critical limits, anaerobic mitochondrial metabolism begins. Although this theory was proposed for marine animals, it is plausible that O_2 limitation may induce a similar sequence of mechanisms responsible for insect low temperature tolerance. To date, little evidence supporting the OCLT has been found in insects (e.g., Klok et al., 2004; Stevens et al., 2010; but see Verberk and Bilton, 2011). Regardless, OCLT serves as an important theory which requires further testing since it has direct applicability to understanding the mechanisms of thermal death (Stevens et al., 2010; Verberk and Bilton, 2011).

In the next section, we briefly consider the cellular responses induced by low O_2 in insects, followed by responses to high CO_2 and ozone.

OXYGEN

Responses to low O_2 can be broadly classified into two classes: the regulating class and the conforming class (Hochachka, 1991; Makarieva et al., 2006). Organisms in the regulating class maintain the energy required for normal processing during the exposure by increasing glycolytic flux and using large amounts of substrates, while those in the conforming class decrease energy use, respiration rate, and substrate usage without engaging in glycolytic pathways. Organisms in the conforming class will usually survive long-term exposures to low O_2 better than those in the regulating class. Damage repair processes are ongoing in the regulating class and only occur upon return to normoxia in the conforming class; repair mechanisms are nevertheless essential for survival of hypoxia.

Insects exposed to hypoxia (or anoxia) will decrease aerobic metabolism, which has been shown to block rapid cold hardening (RCH) in the housefly, *Musca domestica* (Coulson and Bale, 1991). Similarly, Nilson et al (2006) showed that anoxia blocked RCH in *D. melanogaster* after 1 h of exposure, but had no effect at shorter times. RCH is a form of phenotypic plasticity whereby a non-lethal low temperature "shock" protects against subsequent, previously lethal, low temperature exposure (Lee et al., 1987; Lee and Denlinger, 2010). In contrast, Yocum and Delinger (1994) found that RCH is not induced under anoxic conditions in the flesh fly (*Sarcophaga crassipalpis*) indicating that these processes required aerobic conditions to occur. It is therefore clear that gas conditions may interact with low temperature stress responses in at least some species.

The decrease in aerobic metabolism caused by decreasing temperature can be accompanied by a switch to anaerobic metabolism, which can be detrimental if experienced for long periods (**Figure 1**). Increased anaerobic metabolism decreases the amount of adenosine triphosphate (ATP) production which, in turn, can depolarize and degrade tissues, increase ATP degradation and reduce the functioning of ion pumps (e.g., Chiappini et al., 2009). Byproducts of ATP degradation can react with O_2 to form reactive oxygen species (ROS) that cause oxidative damage (Weyel and Wegener, 1996), while a malfunctioning of ion pumps will increase cytosolic calcium concentrations which, if left unbuffered, can trigger apoptosis and block RCH (reviewed by Orrenius et al., 2003; Teets et al., 2008). However, during hypoxia, coprophilous beetles are able to extract sufficient O_2 from air that contained a very low concentration of O_2 (1%) without switching to anaerobic metabolism, which suggests that not all insects are affected in the same way (Holter and Spangenberg, 1997). In addition, during hypoxia insects may keep their spiracles open for longer periods in order to meet their O_2 demands (Hetz and Bradley, 2005). Opening of the spiracles can lead to an increased water loss rate, which is believed to be how low O_2 concentrations can augment a low temperature sterilization regime (Navarro, 1978). Based on studies of insect gas exchange, keeping spiracles open for long periods may result in oxidative damage (Hetz and Bradley, 2005) – possibly through the formation of ROS acting as a signaling mechanism (Boardman et al., 2011). In addition, hypoxia also stimulates hypoxia inducible factor (HIF), a transcription factor stabilized by the presence of ROS, which is necessary for the upregulation of hypoxia-responsive genes (Huang et al., 1996).

CARBON DIOXIDE

High CO_2 concentrations may decrease pH which can be detrimental to membranes and cellular function. A decrease in pH will also denature enzymes, including antioxidant enzymes needed for low temperature tolerance, especially if there are no additional heat shock proteins (HSPs) to act as chaperones. In addition, high CO_2 causes a decrease in NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) enzyme and a subsequent decrease in glutathione production (Friedlander, 1983). NADPH and the antioxidant glutathione are involved in protecting against the toxicity of ROS, while NADPH also contributes to lipid synthesis, cholesterol synthesis, and fatty acid chain elongation.

Secondly, high concentrations of CO_2 are commonly used as an anesthetic for insect handling. Identical to low O_2, CO_2 anesthesia blocked RCH in *D. melanogaster* after 1 h of exposure, but had no effect at shorter times (Nilson et al., 2006). Badre et al. (2005) investigated the mechanism underlying this response and found that in *D. melanogaster* larvae, with intact spiracles, high CO_2 caused their hearts to stop, and blocked synaptic transmission at the neuromuscular junction by decreasing the number of glutamate receptors. Further investigations showed that these effects were not due to hypoxia, low pH, or action of the central nervous system.

OZONE

Recently, there has been increased research into the use of ozone, a strong oxidizing agent, for pest control in stored products (e.g., Tiwari et al., 2010; Holmstrup et al., 2011). The mechanisms of action of ozone are attributed to an increase in ROS, together with direct deleterious reactions with proteins, DNA, and polyunsaturated fatty acids (Hermes-Lima, 2004).

IMPLICATIONS OF INTERACTIONS BETWEEN TEMPERATURE AND ATMOSPHERE

Figure 1 illustrates some of the potential biochemical mechanisms of cross tolerance of CAs with low temperature tolerance, and shows how the altered gas conditions can potentially interfere with typical low temperature tolerance mechanisms. Simply put, low O_2 decreases aerobic metabolism which, apart from directly blocking RCH responses, decreases transcription and ion homeostasis, and increases oxidative damage. Furthermore, water loss rate is increased through the opening of the spiracles, perhaps leading to increased osmotic and ionic stress, and downstream oxidative damage. High CO_2 directly affects the heart and nervous system (anesthetic effects), and decreases pH and NADPH which affect the antioxidant response and membrane functioning. Any one (or combination) of these factors may be the key reason as to why survival may be reduced under combination CA low temperature treatments. Yet some observations suggest that survival may improve under these combination treatments. Possible reasons for the improved survival may be cross tolerance between temperature and gases (where the mechanisms of either low temperature or gas tolerance are sufficiently protective against

both stresses to ensure survival) or natural tolerance to one or more of the potential stressors.

At high temperatures, heat hardening can also benefit insects by increasing survival at previously lethal high temperatures, as well as upregulating general stress responses. While high temperature responses fall largely outside the scope of this review, the physiological and biochemical changes that insects undergo to survive high temperature shock may provide insights into how they survive low temperature shock. Moreover, cross tolerance to temperature stress has been demonstrated in a variety of species (e.g., Burton et al., 1988; Chen et al., 1991; Sinclair and Chown, 2003). The heat shock transcription factor that regulates HSPs induces stress-associated genes, including some HSPs, after long-term low temperature acclimation, but typically these do not play a role in resistance to low temperature shock, RCH, or high temperature knock-down in *D. melanogaster* (Nielsen et al., 2005). Therefore, any stress of heat exposure prior to CA low temperature sterilization may inadvertently improve survival of the insect pest through cross tolerance of low and high temperatures, as well as low temperatures and desiccation stress.

INNATE TOLERANCE TO EXTREME ATMOSPHERES

Controlled atmospheres can also occur naturally in an insects' immediate environment. For example, insects living at high altitudes will experience hypoxic conditions (Frazier et al., 2006), while those that burrow underground or live in dung pats may experience both hypoxic and hypercapnic (high CO_2) conditions (Holter and Spangenberg, 1997). Thus, some pest species may be pre-adapted for survival to CAs, and it may be argued that an understanding of basic ecology and biology may be important for selecting effective treatments and understanding why certain treatments fail to increase mortality. In addition, the adaptations present in these organisms may highlight the mechanisms that are needed to ensure survival under these conditions and, in turn, reveal insights into the cellular or whole organism physiological systems responsible for mortality under altered environmental conditions.

Some insects are naturally more tolerant of changes in gas concentrations during specific life stages. For example, embryos of *D. melanogaster* are able to arrest the cell cycle at the metaphase or S-phase in response to hypoxia, and will resume normal cell cycling within 20 min of reoxygenation (Douglas et al., 2001). In addition to the cell cycle arrest, *D. melanogaster* are able to detect the O_2 changes through the accumulation of nitric oxide in sensor cells in order to change their behavior, and alter tracheal development, possibly with the aid of HIF (Wingrove and O'Farrell, 1999). Similarly, pupae of *Tribolium confusum* are able to survive 48 h of anoxia, but die if the anoxia continues beyond this point, possibly due to depletion in carbohydrate stores by anaerobic glycolysis (Kennington and Cannell, 1967).

Tolerance to gases is aided by the insects' ability to withstand reoxygenation. After anoxia, return to normoxia has been shown to cause damage in both *D. melanogaster* (Lighton and Schilman, 2007) and other vertebrate species (Hermes-Lima and Zenteno-Savín, 2002). One possible cause of increased hypoxia/anoxia tolerance may be the ability to return to efficient energy metabolism, without generating excess heat, upon reoxygenation (Wegener and Moratzky, 1995). During anoxia, ATP is degraded and the resulting byproducts can react with O_2 to become ROS (**Figure 1**). The ability of insects to recover from anoxia may be further attributed to their ability to limit the degradation of adenine nucleotides during anoxia, and re-synthesize ATP from these nucleotides during recovery (Weyel and Wegener, 1996).

CONCLUSION AND FUTURE DIRECTIONS

Reactions to similar treatments vary widely between insect species, hence treatment regimes should be investigated in a species-specific manner, based on the known physiology and biochemical responses of each species (Navarro, 1978). Species relatedness may aid generalizing thermal tolerance and its plasticity but even then should be undertaken with caution (e.g., Nyamukondiwa et al., 2011). Established thermal responses or lack thereof should be used as background information to determine which treatments may be worth testing to determine their suitability for post-harvest sterilization or control of grain pests. Furthermore, as emphasized in this mini-review, there is a large degree of overlap between the mechanisms of gas and temperature stress responses. Tolerance to these stressors depends largely on limiting oxidative damage and maintaining the integrity of biological membranes and essential proteins. The success of CA augmentation of low temperature treatments may be attributed to their ability to interfere with basal low temperature tolerance mechanisms, causing sufficient cellular and/or tissue damage to result in insect mortality, or perhaps influencing damage and repair mechanisms during recovery periods from CAs. Future work aimed at expanding the potentiating and blocking types of action in these stress pathways, and the interactions among them, will likely enhance both applied pest management, as well as understanding of whole organism physiology to extreme conditions.

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