# Postharvest phytosanitary disinfestation strategies using thermal and atmospheric stress: commodity and insect tolerances

By

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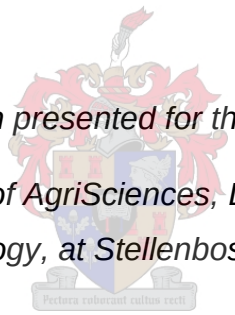

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# DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the authorship owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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# SUMMARY

South African chill sensitive horticultural products deemed for export cannot be exported using certain phytosanitary cold sterilisation regimes, without negatively affecting fruit quality. Low temperature phytosanitary treatments are required to control a variety of pests, however in some cases, cold temperature treatments are ineffective against insects that display high levels of thermal tolerance. Developing alternative phytosanitary treatments is therefore crucial, and maintaining a balance between desirable fruit quality and effective control of insect pests is an important consideration throughout the process. In the present study, the potential of two postharvest mitigation technologies were investigated to assess their potential in controlling targeted pests while maintaining fruit quality - CATTS (Controlled Atmosphere Temperature Treatment System) and ethyl formate fumigation. CATTS was investigated specifically as a potential postharvest mitigation treatment for chill sensitive plum cultivars. CATTS technology incorporates heat and atmospheric stress to control insect pests. Key phytosanitary pests of South Africa which require control include the grain chinch bug, *Macchiademus diplopterus* (Distant) (Hemiptera: Lygaeidae), the banded fruit weevil, *Phlyctinus callosus* (Schöenherr) (Coleoptera: Curculionidae) and the false codling moth, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae). In the first part of this dissertation, different temperature treatments in combination with controlled atmosphere were tested and fruit was cold-stored using two different cold storage regimes, namely standard cold sterilisation and the dual temperature regime, to examine the effectiveness of the CATTS treatments and cold storage for phytosanitary control. Finding a balance to maintain fruit quality and kill both internal and external pests proved challenging. As a pre-conditioning benefit of heat treatments was observed during the first season, treatments were aimed at enhancing this effect during the second season, to enable the fruit to withstand low temperatures for longer periods to control internal pests. The second part of this dissertation is an in-depth investigation into the physiology of *Macchiademus diplopterus*. This was conducted to provide insight into the thermo-tolerant ability of this pest, as CATTS treatments were found to be ineffective for phytosanitary control. The compositional changes that occur during aestivation were examined through biochemical (macromolecules) and molecular (soluble protein identification) analyses. These were performed on the insects before entering aestivation and during the aestivation period. To examine the biochemical compositional changes the insect undergoes during thermal stresses, insects from early and mid-aestivation were treated with different CATTS treatments and cold storage regimes (cold sterilisation and dual temperature regime). The insect mortality and macromolecule content in each aestivation period provided insight into

the different factors that influence its survival. A significant difference was observed in mortality and biochemical composition between early and mid-aestivating insects. Mechanisms identified that initiate defence and survival strategies during unfavourable conditions included heat shock protein and cryoprotectant synthesis. The high thermal tolerance of *M. diplopterus* therefore requires a different approach for phytosanitary control. The third and final part of this dissertation addresses that need. Fumigation using ethyl formate was investigated as a potential alternative to thermal treatments. The main aims were to examine, firstly, the potential of ethyl formate as a fumigant to control the *M. diplopterus*, and, secondly, the effect of ethyl formate on the fruit quality of selected stone and pome fruit cultivars. A central composite design (CCD) method was used to treat pome and stone fruit cultivars to assess phytotoxicity after fumigation. A range of ethyl formate concentrations and fumigation durations were tested in conjunction with various other factors such as pulp temperature, harvest maturity, time during the season in which the cultivar ripens and the effect of pre-ripening. No phytotoxic damage was observed on stone fruit. Pome fruit, in contrast, had a phytotoxic response, and the CCD model predicted fumigation limits for treatments. Ethyl formate fumigation is highly effective against *M. diplopterus*, providing an alternative treatment for this highly thermo-tolerant pest. Both postharvest mitigation technologies tested here provide valuable insight into the response of both the commodity and insect to the various treatments. Challenges for the application of both technologies have been elucidated, and are addressed and discussed. The research presented here represents significant steps taken towards having more effective postharvest disinfestation strategies available for phytosanitary control.

# OPSOMMING

Suid-Afrikaanse koue-sensitiewe, hortologiese produkte wat bestem is vir uitvoer, kan nie met sekere fitosanitêre koue-sterilisasie metodes uitgevoer word sonder om die vrugkwaliteit te benadeel nie. Lae-temperatuur fitosanitêre behandelings word vereis om ‘n verskeidenheid plae te beheer, maar in sekere gevalle is koue-temperatuurbehandelings oneffektief teen insekte wat hoë vlakke van termiese verdraagsaamheid toon. Die ontwikkeling van alternatiewe fitosanitêre behandelings is dus noodsaaklik en die balans tussen gewenste vrugkwaliteit en effektiewe beheer van insekplae is ‘n belangrike oorweging gedurende die proses. In hierdie studie is die potensiaal van twee na-oesplaagbestuurtegnieke ondersoek om hul potensiaal in die beheer van teikenplae, terwyl vrugkwaliteit behou word, te evalueer – CATTS (Gekontroleerde Atmosfeer Temperatuur behandelingstelsel) en etielformaat beroking.

CATTS is spesifiek ondersoek as ‘n potensiële na-oesplaagbestuurbehandeling vir koue-sensitiewe pruimkultivars. CATTS-tegnologie integreer hitte en atmosferiese stres om insekplae te beheer. Belangrike fitosanitêre plae van Suid-Afrika wat beheer vereis, sluit in die graan stinkluis, *Macchiademus diplopterus* (Distant) (Hemiptera: Lygaeidae), die gebande vrugtekalander, *Phlyctinus callosus* (Schöenherr) (Coleoptera: Curculionidae) en die valskodlingmot, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae). In die eerste deel van die studie is verskillende temperatuurbehandelings, in kombinasie met beheerde atmosfeer, getoets en vrugte was by lae temperatuur gestoor deur twee verskillende kouestoortegnieke te gebruik, naamlik standaard koue-sterilisasie en die dubbele temperatuur regime, om die effektiwiteit van die CATTS-behandelings en kouestoor vir fitosanitêre beheer te ondersoek. Om ‘n balans te vind om vrugkwaliteit te behou en beide interne en eksterne plae dood te maak, was uitdagend. Omdat ‘n kondisioneringsvoordeel van hittebehandelings waargeneem is tydens die eerste seisoen, was behandelings in die tweede seisoen daarop gemik om hierdie effek te versterk, om die vrugte in staat te stel om vir langer periodes lae temperature te kan weerstaan om interne plae te beheer. Die tweede deel van die studie is ‘n in-diepte ondersoek in die fisiologie van *Macchiademus diplopterus.* Dit was uitgevoer om insig te verskaf oor die hitte verdraagsaamheidsvermoë van die plaag, omdat daar gevind is dat CATTS-behandelings oneffektief is vir fitosanitêre beheer. Die samestellingsveranderings wat plaasgevind het tydens estivasie, is ondersoek deur biochemiese (makromolekules) en molekulêre (oplosbare proteïen identifikasie) analise. Dit is uitgevoer op die insekte voordat estivasie aanvang geneem het en tydens die estivasieperiode. Om die biochemiese samestellingsveranderinge wat die insek ondergaan

tydens termiese stres te bepaal, is insekte van vroeg en mid-estivasie behandel met verskillende CATTS-behandelings en kouestoortegnieke (koue-sterilisasie en dubbele temperatuur regime). Die insekmortaliteit en makromolekuulinhoud in elke estivasieperiode het insig gelewer in die verskillende faktore wat oorlewing beïnvloed. ‘n Betekenisvolle verskil is waargeneem in die mortaliteit en biochemiese samestelling tussen vroeë en mid-estivasie insekte. Meganismes wat geïdentifiseer is wat verdedigings- en oorlewingstrategieë inisiëer tydens ongunstige toestande, sluit in hitteskokproteïen- en kouebeskermingsintese. Die hoë termiese verdraagsaamheid van *M. diplopterus* benodig dus ‘n ander benadering vir fitosanitêre beheer. Die derde en finale deel van die studie spreek hierdie behoefte aan. Beroking met etielformaat is ondersoek as ‘n potensiële alternatief tot termiese behandelings. Die hoof doelwitte was om, eerstens, die potensiaal van etielformaat as ‘n berokingsmiddel om *M. diplopterus* te beheer en, tweedens, die effek van etielformaat op die vrugkwaliteit van bepaalde steen- en kernvrugkultivars, te ondersoek.

‘n Sentrale saamgestelde ontwerp (SSO) metode is gebruik om kern- en steenvrugkultivars te behandel om fitotoksisiteit na beroking te evalueer. ‘n Reeks etielformaatkonsentrasies en berokingstye is, in samewerking met verskeie ander faktore soos pulptemperatuur, oesrypheid, tyd gedurende die seisoen waartydens die kultivar ryp word en die effek van rypmaking, getoets. Geen fitotoksiese skade is op steenvrugte waargeneem nie. Kernvrugte, in teenstelling, het ‘n fitotoksiese reaksie getoon en die SSO model het berokingslimiete vir behandelings voorspel. Etielformaat beroking is baie effektief teen *M. diplopterus* en verskaf dus ‘n alternatiewe behandeling vir die hoogs termies verdraagsame plaag. Beide na-oesbestuurtegnieke wat hier getoets is, verskaf waardevolle insig in die reaksie, van beide die kommoditeit en insek, tot die verskeie behandelings. Uitdagings vir die toediening van beide tegnieke is toegelig en is aangespreek en bespreek. Die navorsing wat hier aangebied word, verteenwoordig beduidende stappe wat geneem is om meer effektiewe na-oes disinfestasie strategieë beskikbaar te stel vir fitosanitêre beheer.

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# NOTE

This dissertation presents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters, therefore, has been unavoidable.

Chapter 2: Paper 1 has been published in Acta Horticulturae:

**Smit, R., M. M. Jooste, and S. A. Johnson. (2018).** CATTS technology: Phytosanitary control and market expansion of chill sensitive Japanese plums for South Africa. Acta Hortic. 1194: 201–208.

Chapter 4: Paper 5 has been published in Scientia Horticulturae *(Accepted September 2019)*:

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# CHAPTER 1

## General introduction and literature review

Food security and sustainability are critical focus areas in the production and trade of fresh agricultural produce. Fruit production, in general, has become so much more than just producing products for the local market. In South Africa, it is a multi-million Rand industry, with fruit producers exporting large volumes of fresh produce to growing international markets. International trade in agricultural products carries the risk of introducing pests into importing countries, which could have a significant negative impact on the native environment and crops. Importing countries, therefore, impose quarantine or phytosanitary measures against a variety of potential pests as a safeguard against invasion of their crops by foreign pests. Regulatory requirements stipulate that postharvest phytosanitary treatments must be applied to ensure pest-free products (Neven, 2010; DAFF, 2019). These protocols are influenced by the commodity and target foreign market. Regulations usually call for zero tolerance, that is, if a single living insect is found on a commodity, the consignment will be rejected, resulting in economic losses for the industry. Postharvest phytosanitary treatments can be categorized as chemical or physical treatments (Lurie, 2001; Yahia, 2011). Chemical treatments include fumigation and insecticidal dips. The use of temperature (both hot and cold), controlled or modified atmospheres and irradiation are considered to be physical treatments. Before a phytosanitary treatment is approved, the specific protocol undergoes rigorous testing and development to ensure efficacy to control the pests while simultaneously maintaining fruit quality.

Fumigation with methyl bromide, historically the most widely used fumigant due to its efficacy against a wide range of pests at concentrations that did not negatively affected commodity quality and marketability, is no longer preferable, since its ozone-depleting properties were identified (Fields and White, 2002). Hence, in the field of quarantine entomology the current research focus is on developing effective, non-chemical postharvest disinfestation methods for phytosanitary pest control. Although effective in controlling pests, these disinfestation methods also have disadvantages in that exposure to relatively high or low temperatures, or irradiation, can easily induce fruit damage, if techniques are not applied effectively.

South African export fruit is affected by a variety of insect pest species that pose phytosanitary risks to various markets. It is crucial to ensure that continued growth of foreign markets is not deterred as a result of the rejection of consignments due to fruit infestation

by phytosanitary pests. Key phytosanitary pests associated with South African export fruit include the false codling moth, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), the grain chinch bug, *Macchiademus diplopterus* (Distant) (Hemiptera: Lygaeidae) and the banded fruit weevil, *Phlyctinus callosus* (Schöenherr) (Coleoptera: Curculionidae) (Johnson and Addison, 2008; Johnson and Neven, 2010; 2011). The false codling moth is an internal fruit pest as the larvae bore into, and feed inside the fruit. The grain chinch bug and banded fruit weevil are external pests and may be present on packed export fruit. Currently, there are exemptions related to the use of methyl bromide for phytosanitary purposes, until viable alternatives are available. However, postharvest phytosanitary treatments that target external phytosanitary pests are lacking. For internal pests, cold treatment can be highly effective and is the standard method used to control false codling moth. A cold treatment of 22-days at -0.55ºC is approved by the United States Department of Agriculture as a disinfestation treatment against false codling moth on stone fruit, grapes and citrus (USDA, 2019). However, storage under such low temperatures for extended periods can be detrimental to fruit quality. In particular, chill-sensitive stone fruit cultivars develop chilling injury under these conditions (Lurie and Crisosto, 2005).

To find alternative postharvest treatments to control phytosanitary pests, and address the problem of chilling injury due to the cold sterilisation regime, two new technologies were investigated in the present study. The potential of Controlled Atmosphere Temperature Treatment System (CATTS) technology, as well as fumigation with ethyl formate, were assessed. CATTS technology uses exposure to high temperatures, for short periods, in combination with a controlled atmosphere to disinfest fruit (Neven and Mitcham, 1996). The controlled atmosphere consists of reduced oxygen and elevated carbon dioxide levels which, in combination with the thermal stress, disrupts insect respiration and ultimately results in death. Previous studies on the effect of heated CA treatments on false codling moth larvae, grain chinch bug, and banded fruit weevil adults indicated that such treatments have the potential to control these pests (Johnson and Neven, 2010; 2011). In addition to pest control, the heat exposure applied during CATTS treatments has the potential to pre-condition the commodity to withstand prolonged cold storage better. Commercially, high-temperature postharvest treatments (e.g. hot water) are used to reduce chilling injury symptoms in products such as mangoes, avocados, cucumber and peppers (Sevillano *et al*., 2009). CATTS technology may provide a mitigation treatment that will allow phytosanitary control for chill-sensitive plum cultivars, which cannot be cold-stored for extended periods at -0.55°C. Furthermore, should high-temperature pre-conditioning reduce

chilling injury significantly, the CATTS treatments could also replace the role of intermittent warming regimes for stone fruit as a method of preventing chilling injury. Intermittent warming or dual temperature regimes have in the past resulted in temperature management difficulties during shipment in containers (Punt and Huysamer, 2005). The potential risk of chilling injury could be alleviated by using a pre-conditioning high-temperature CATTS treatment followed by single-temperature storage at 0°C.

The second technology examined in this study was the use of ethyl formate as a postharvest fumigant. Since the application of methyl bromide as a fumigant has been banned, the quest to find alternative fumigants has gained momentum. Potential alternative postharvest fumigants include carbonyl sulfide, sulfuryl fluoride, phosphine and ethyl formate or combinations thereof (Ducom and Banks, 2006; Lee *et al*., 2018). However, the advantages of ethyl formate over other fumigants, such as phosphine, lies in its ability to kill insects rapidly, as well as its ability to break down into the naturally occurring chemical compounds, formic acid and ethanol (Desmarchelier *et al*., 1998). Ethyl formate has insecticidal and fungicidal properties, and is considered to be one of the most promising fumigants to replace methyl bromide. Regarding its effect on insects, ethyl formate penetrates the insect's body through the spiracles and inhibits oxygen respiration (Simpson *et al*,. 2004; Ryan and De Lima, 2014; Lee *et al*., 2018).

Alternative technologies are crucial for our agricultural export industries to continue pest-free fruit trade, and maintain and expand international trade. The overall aim of the present study was to establish the feasibility of these two alternative technologies as postharvest mitigation treatments for fruit potentially infested with phytosanitary pests. Post-treatment insect mortality and fruit quality were determined, and in the case of the grain chinch bug, physiological aspects of the insect's biochemical makeup were investigated to better understand its apparent ability to withstand thermal treatments.

## 1.1 South African insect pests requiring phytosanitary treatment

Depending on the commodity and trading partner, a variety of insect pest species can be of quarantine concern to importing countries. The three key phytosanitary pests focussed on in the present study are the lygaeid bug *Macchiademus diplopterus* (grain chinch bug), a curculionid *Phlyctinus callosus* (the banded fruit weevil), and a tortricid *Thaumatotibia leucotreta* (false codling moth). All three species are indigenous to South Africa, have limited global distributions and consequently, are of quarantine concern to countries importing commodities from South Africa.

### 1.1.1 *Macchiademus diplopterus,* grain chinch bug

The distribution of the grain chinch bug is limited to the South Western Cape of South Africa, and appears to be related to a correlation between its life cycle and the winter rainfall areas in this region (Slater and Wilcox, 1973). A survey conducted on the distribution of the grain chinch bug indicated that the highest numbers of insect infestations occur in the areas of Ceres, Porterville and Piketberg in the Western Cape, South Africa (Johnson and Addison, 2008).

The grain chinch bug feeds and reproduces on wild grasses and cultivated grain crops such as wheat. Aestivation, defined as a prolonged period of dormancy which insects use as a survival strategy during unfavorable conditions (Storey and Storey, 2012), forms part of the grain chinch bug's seasonal cycle during the summer months. During aestivation, grain chinch bugs migrate from host plants (wheat) in large numbers to surrounding areas in seek of shelter. Sheltering sites include nearby trees, such as *Eucalyptus* trees, where aestivating bugs can be found underneath the loose bark during the summer months (Myburgh and Kriegler, 1967; Okosun, 2012). Orchards, which are near wheat fields are also likely to get infested with aestivating adult grain chinch bugs. Sheltering bugs in fruit orchards are the most problematic, as they hide within grape bunches, the navels of oranges and at the stalk ends of fruits such as peaches and nectarines. The grain chinch bug can also hide within apples and pears entering at the calyx end. However, it does not cause any damage to the fruit (Johnson and Neven, 2011). Since the grain chinch bug infests fruit during harvest, it is therefore potentially picked, packed and exported with these fruit.

### 1.1.2 *Phlyctinus callosus,* banded fruit weevil

The banded fruit weevil is indigenous to the Western Cape Province of South Africa (Barnes and Pringle, 1989). In the Southern Hemisphere, the banded fruit weevil has spread from South Africa to New Zealand, Tasmania, and Australia (CABI, 2019a). Although it has frequently been intercepted in the USA, it has not successfully established in the Northern Hemisphere.

Banded fruit weevil females lay their eggs in the hollow spaces in plant tissue and leaves (Barnes and Pringle, 1989). The larvae are soil-dwelling and feed on plant roots (Barnes, 1989). After pupation, they emerge from the soil as adults (between October and December) and move into the aerial parts of the fruit trees. Adult weevils cause damage by feeding on the leaves, bark and fruit (stalks and fruit as a whole) of grapes, apples and stone fruit

(Prinsloo and Uys, 2015). The most notable injury in vineyards generally occurs during November and December when the developing bunches of grapes are attacked. The adult banded fruit weevil can cause scarring to grapes, as well as damage to the stalks of individual berries. Damage by the adults on fruit such as apples, nectarines and plums can make the product unmarketable. The pre-harvest management strategies for controlling banded fruit weevil are continuously being improved upon, but do not eliminate the possibility of weevils in packed fruit.

### 1.1.3 *Thaumatotibia leucotreta,* false codling moth

The distribution of the false codling moth is primarily throughout sub-Saharan Africa (CABI, 2019b). False codling moth is extremely polyphagous. Avocados, beans, coffee, cotton, grapes, plums, macadamias, maize and tomatoes are amongst the many crops targeted by false codling moth (CABI, 2019b). In South Africa it affects mainly citrus, stone fruit and table grape exports.

The false codling moth can infest fruit during all stages of fruit development. If it strikes the fruit during early fruit development, fruit may ripen prematurely and drop (Donovan, 2015). Females lay their eggs on the fruit and the neonate larvae bore into the fruit, feeding on the pulp and tunneling deeper as they mature. In addition to internal damage, the external damage due to the holes on the fruit surface will expose the fruit to disease and decay. Larvae inside the fruit pose a phytosanitary risk as an internal pest in export fruit.

## 1.2 Postharvest phytosanitary treatments

Various control strategies during harvest, transport and handling of fruit are used to reduce the threat of infestation. Postharvest treatments are essentially the ‘last port of call’ in controlling phytosanitary pests.

### 1.2.1 CATTS Technology

By combining heat treatments with controlled atmospheres, the effectiveness of pest control can be accomplished in hours instead of days (Mitcham, 2007). Controlled Atmosphere Temperature Treatment System (CATTS) technology uses a combination of high-temperature treatments (for short periods) and a controlled atmosphere (CA) to control pests (Neven and Johnson, 2018). The CA, which consists of reduced oxygen and elevated carbon dioxide levels, in combination with thermal stress affects, insect respiration and ultimately results in death. The efficacy of the CA treatment for controlling insect pests will

vary with the selected treatment temperature. The higher the temperature, the faster the mortality under a given atmosphere. Insects have an increased susceptibility to CA at higher temperatures, as decreasing the availability of $O_2$ during heat stress inhibits the insects' ability to support their increased metabolic demand (Mitcham, 2007). Research conducted by Neven *et al*. (2001) on codling moth, *Cydia pomonella* L (*Lepidopteran: Tortricidae*) found that heat treated apples and pears were firmer than untreated control fruit. A significant suppression of storage scald was observed in heat treated 'Granny Smith' apples stored for 150 days. Two CATTS protocols suitable for quarantine disinfestations of codling moth and oriental fruit moth, *Grapholita molesta* (Busck) were applied to a variety of mid and late peach and nectarine cultivars by Obenland *et al*. (2005). Their research concluded that CATTS treatments did not adversely affect fruit quality for almost all the cultivars tested. The only exception occurred in fruit that had high levels of surface injury which was enhanced by the application of CATTS.

Some of the first successful CATTS treatments were developed as a postharvest tool to control codling moth larvae in sweet cherries. These treatments consisted of 1% $O_2$ and 15% $CO_2$ in $N_2$ with temperatures ramped to a target temperature of either 45°C or 47°C (Neven and Mitcham, 1996). A study conducted by Son *et al*. (2012) yielded promising results regarding CATTS treatments to control the peach fruit moth, *Carposina sasakii* Matsumura (found in Korea), in apples. During this study the *Carposina sasakii* larvae (fourth and fifth instar) did not survive after an hour CATTS treatment, with no undesirable effect on fruit quality, with regard to fruit firmness, sweetness and decay observed.

The heating rate or ramp rate is defined as the temperature change that occurs over time to reach the target temperature. This ramp rate will influence the duration of a treatment to achieve the targeted temperature. Increasing the ramp rate could potentially shorten the treatment duration for effective control. To reach the target temperature of 46°C with a heating rate of 12°C.h$^{-1}$, to control codling moth and oriental fruit moth in apples, the treatment duration was 3 h; when a faster ramp rate of 24°C.h$^{-1}$ was applied to peaches and nectarines the duration decreased to 2.5 hours (Neven and Johnson, 2018). An increase in the rate of heating facilitates larval mortality, and in turn, decreases the running time of the CATTS treatment. This is due to the anoxic effect that is created by the CATTS treatment in the insect (Son *et al*., 2012). The influence of anoxia was investigated in the flesh fly (*Sarcophaga crassipalpis* Macquart) where Yocum and Denlinger (1994), examined the insect's physiological response to temperature. They found that anoxia inhibited the

synthesis of heat shock proteins that are needed to facilitate the insects' thermal tolerance capabilities.

The effect of heating rates and CA on the mortality of the subjects in the present study, grain chinch bug, banded fruit weevil and false codling moth, has previously been examined in simulated-CATTS treatments (Johnson and Neven, 2010; 2011). Using a controlled atmosphere waterbath system, Johnson and Neven (2010) showed that the fourth instar false codling moth was very tolerant to treatments with heating rates of 12°C.h$^{-1}$and 24°C.h$^{-1}$, and could not be adequately controlled. Further research also showed that false codling moth larvae were more tolerant than banded fruit weevil and grain chinch bug adults (Johnson and Neven, 2011) at a slower heating rate. At faster heating rates, the grain chinch bug adults were more tolerant, requiring a CA treatment of more than 180 min in comparison to the 90 min required to control the banded fruit weevil. False codling moth larvae required > 2.5 hours when a faster ramp was applied, a slow heating rate required an extended duration which resulted in poor fruit quality. The banded fruit weevil adults appeared to be the least tolerant to the heated CA treatments when exposed to slow and fast heating rates. The contrasting responses between the false codling moth larvae, grain chinch bug adults and banded fruit weevil to heating rates and duration of exposure indicates the complexity of developing a treatment that will control all three simultaneously while maintaining fruit quality.

#### *1.2.1.1 Insect mortality: Heat plus Controlled Atmosphere*

Physiological functions of an insect, such as growth, metabolism and reproduction, occur optimally within certain thermal limits; outside this range, performance is reduced (Lurie and Mitcham, 2007). Exposure to low and high temperatures will influence metabolic function, and prolonged exposure at these conditions will result in damage and injury, and may eventually result in death (Lachenicht *et al*., 2010). An insect faced with variation in temperature, copes by altering behavior, phenology, adaptation on a genetic level or through a combination of these factors. The response of organisms to high-temperature stresses with regard to thermo-tolerance and heat shock proteins has been researched for many years. In postharvest treatment development, the thermo-tolerance of the insect defines the treatment temperature(s) and duration required to kill target pests.

The upper and lower critical thermal limits refer to the extreme temperatures that are lethal to insects (Terblanche *et al*., 2007). These critical thermal limits need to be considered when examining the effectiveness of heat treatments in controlling the targeted pests.

Temperatures outside the optimum range for the target pest adds to the stress. For example, the metabolic rate in air of the omnivorous leafroller moth pupae, *Platynota stultana* Walsingham (Lepidoptera: Tortricidae), tripled when the temperature was increased from 10 to 20°C, demonstrating the effect temperature has on insect metabolism (Zhou *et al*., 2000; Atkins *et al*., 1957). It has also been observed that an increase in treatment effectiveness occurs with a decrease in $O_2$ concentrations, as well as an increase in the treatment temperature up to 30°C, against arthropod pests of a range of fresh products (Mitcham, 2007). Furthermore, when the mealybug species, *Pseudococcus affinis* Maskell was exposed to a variety of $O_2$ concentrations (0.4-20.9%) and temperatures (35-45°C), the time needed for 99% mortality decreased with an increase in temperature and decrease in $O_2$ levels (Whiting and Hoy, 1997).

The location of a pest, and its various life stages on a commodity is an important consideration in treatment development. These factors influence treatment duration for effective mortality of the pest. Pests found on the product surface will be directly and immediately affected during heating, while pests located on the inside will need the temperature of the product to increase over time until the critical lethal temperature for the internal pest is reached (Armstrong and Mangan, 2007). If the least tolerant life stage of a pest is found deep inside the commodity, it may be more difficult to kill than a more tolerant life stage found on or near the surface. For example, in watermelons infested with fruit fly eggs and larvae, the eggs may be at its most tolerant stage, but the larvae that are tunneled deep into the fruit pulp may be more difficult to kill, as the surrounding pulp acts as a heat barrier and insulates the larvae (Armstrong and Mangan, 2007).

If the pest is more thermo-tolerant than the commodity other environmental factors need to be applied to reduce the pest's thermo-tolerance or increase the commodity's thermo-tolerance (Armstrong and Mangan, 2007). Low $O_2$ and elevated $CO_2$ levels are used commercially to control pests in grains and are used to retain the quality of fresh horticultural products in extended storage or transportation. An insecticidal CA treatment requires days to completely control arthropod pests, while control occurs within hours at high temperatures. However, some insect species can survive with an atmosphere containing reduced $O_2$ and elevated $CO_2$ by reducing their metabolic rate, or initiating metabolic arrest (Mitcham et al., 2006). The reduction in the metabolic rate results in a decrease in pressure on the organism to initiate anaerobic metabolism. Anaerobic metabolic processes lead to an accumulation of toxic by-products, as well as a reduction in ATP production. Higher $CO_2$ levels generally yield higher mortality rates compared to low $O_2$ levels, because it leads to a

decrease in ATP production (Mitcham et al., 2006). The variability in an insect's response to low $O_2$ and elevated $CO_2$ could be due to failure in the membrane function, which occurs more rapidly under elevated $CO_2$ than under hypoxia (Hochachka, 1986; Zhou *et al*., 2001; Mitcham *et al*., 2006). Hypoxia is defined as a situation in which the $O_2$ supply is inadequate to meet oxygen demand (Harrison *et al*., 2018). Elevated $CO_2$ levels decrease the pH in the cell which causes an increase in intercellular $Ca^{2+}$ concentrations. Higher $Ca^{2+}$ concentrations in the cytosol result in the cell, and the mitochondrial membrane, becoming more permeable, indicating that membrane permeability is increased with high $CO_2$. The failure of the membrane integrity in elevated $CO_2$ environments could, therefore, be due to insufficient energy production, as well as increased membrane permeability due to higher $Ca^{2+}$ concentrations (Mitcham *et al*., 2006). Insect mortality may also be affected by the anaesthetic effect of $CO_2$; this anaesthetic effect of $CO_2$ can decrease with an increase in temperature, as $CO_2$ is less soluble in cell fluids at higher temperatures (Mitcham *et al*., 2006). The membrane function also fails under low $O_2$ levels, due to insufficient energy supply for control over the membrane gradients.

The synergistic effect of two stresses (CA plus heat) is further enhanced by a third stress (storage at low temperature) (Mitcham, 2007). For example, a 30% higher mortality was reported for codling moth larvae with the use of the three combined stresses (elevated temperatures in combination with CA followed by low-temperature storage) compared to only using CA plus high temperature (Neven, 1994; Chervin *et al*., 1998).

#### *1.2.1.2 Fruit Quality: Heat plus Controlled Atmosphere*

Since the late nineteenth-century heat treatments in the form of hot water dips, vapour heat or hot forced air have been used to treat numerous fresh commodities. Hot water dips in 1909 were one of the earliest attempts to control tarsonemid mites in fruit (Cohen, 1967; Hallman and Armstrong, 1994; Sharp, 1994). Vapour heat was used in Mexico in 1913 to control Mexican fruit fly (*Anastrepha ludens*) (Tang *et al*., 2007). The balance between controlling a pest and causing damage to the commodity could differ with only a few degrees Celsius in some cases. This is why a multitude of different heat treatments exist for specific products (Lurie and Mitcham, 2007). Different heat treatment methods result in different heating rates and final temperature distributions in commodities, which influences the efficacy of the treatment for phytosanitary control, and the level or type of damage to fruit quality (Tang *et al*., 2007).

Peel browning, pitting and yellowing of green vegetables are signs of external heat damage to agricultural commodities. One of the most common types of damage observed is surface scalding. It was found that the degree of external browning of stone fruit increased at 52°C, with an increase in the duration of the heat treatment (from 15 to 45 min), but was reduced when the fruit was enclosed in plastic wrap, possibly due to reduced water loss and shriveling (Lurie and Mitcham, 2007). Mangoes displayed severe peel scalding with exposure to forced air heat treatment of 45°C, but no damage was observed at 43°C, indicating that there is a threshold temperature for skin injury. Extreme heat treatments can increase water loss, while milder heat treatments can cause the cuticular wax to melt and fill in microcracks and stomata, thus reducing water loss.

In citrus and nectarines, the most common symptom of internal heat damage is flesh darkening. In nectarines and peaches, heat treatment may enhance the development of flesh mealiness after storage (Lurie and Mitcham, 2007). In mangoes and papayas, heat damage causes poor colour development, abnormal softening, lack of starch breakdown and the formation of internal cavities (Lurie and Mitcham, 2007). Symptoms of cavitation include spongy tissue with air pockets, which do not appear until the fruit has ripened and starch degradation in the tissue is inhibited. Figure 1.1 illustrates the cavitation that occurs in mangoes after heat treatment.

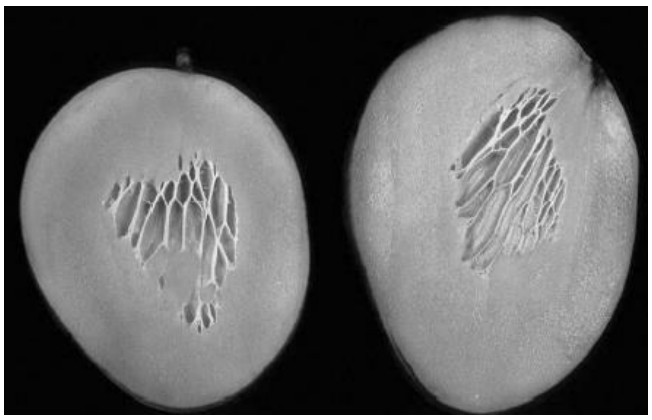


Fig 1.1. Example of cavitation in mangoes after heat treatment (Lurie and Mitcham, 2007).

Mitcham and McDonald (1993) related a change in the internal atmosphere (i.e. increased levels of $CO_2$ and decreased levels of $O_2$) to the development of internal cavities in the heat-treated mangoes. Internal heat injury does not only result in tissue damage but also involves protein denaturation or disruption of protein synthesis. Many cellular processes are influenced by exposure to heat, for example, a decline in chloroplast levels, a reduction in respiration rate, an increase in heat shock protein synthesis and an increase in electrolyte leakage (Nahar *et al*., 2013).

The use of CA as an insecticidal quarantine treatment can often result in damage to the commodity, because horticultural products vary in their tolerance to low $O_2$ and elevated $CO_2$. Any given commodity may tolerate short exposure times to such CA conditions and will only express damage after extended periods of exposure. If the concentrations exceed or do not reach the threshold concentrations that the fruit can tolerate, damage will occur. Exposure to CA atmospheres may result in various physiological disorders, such as impaired ripening of climacteric fruit (melons and plums), internal browning (in pears, apples and peaches), external browning (on lettuce) and pitting (on apples and pears) (Kader, 1986).

Although postharvest treatments for phytosanitary control can result in reduced fruit quality, as described above, pre-conditioning thermal treatments can be used to enable fruit to better withstand subsequent thermal treatments, be it for pest control or storage for extended periods of time. The best pre-treatment or pre-conditioning treatment, in general for many fruits is at 38°C, and when applied to, for example avocados, damage decreased when exposed to further thermal treatments (Lurie and Jang, 2007). This was observed in 'Hass' avocados when held in 38°C water for 60min, which increased the product's thermo-tolerance when further exposed to hot water at 50°C (Woolf and Lay-Yee, 1997).This pre-treatment significantly reduced the severity of external browning and skin hardening caused by hot water treatments. Research conducted by Park *et al*. (2018) indicated that preconditioning 'Fuyu' persimmon fruit at 30°C for 6 or 24 h lowered the incidence of storage disorders by delaying quality deterioration during cold storage . There is a correlation between the expression of heat shock proteins (HSP) and thermo-tolerance in many organisms, and it has been shown that heat stress can also condition plants to tolerate low temperatures more efficiently through the production of heat shock proteins (Lurie and Jang, 2007). Heat treatments have the ability to reduce the susceptibility of the product to chilling injury, which is high in chill-sensitive plums and complicates the current postharvest control against phytosanitary pests, such as false codling moth.

### 1.2.2 Ethyl formate as a fumigant

A fumigant is a volatile gas consisting of a chemical or a mixture of chemicals, which can kill insect pests and can penetrate commodities and food containers, reaching areas inaccessible to other pesticide formulations (Phillips *et al*., 2012; Singh, 2012). If applied correctly fumigants can deliver high levels of mortality. Factors that affect fumigation efficacy include type of fumigant, concentration, exposure time and temperature during fumigation. These factors will determine the dosage required to control the target pest and specific life

stage. For example, the egg stage of the red flour beetle (*Tribolium castaneum* Herbs) is the most tolerant to phosphine fumigation, but in contrast, the pupal stage is the most tolerant when methyl bromide is applied (Hartzer *et al*., 2010; Phillips *et al*., 2012; Gautam and Opit, 2015).

The influence of temperature during treatment is a crucial factor to consider for a successful fumigation treatment. Lower fumigation temperatures tend to make the insect pest less susceptible to the fumigant, as their respiration rate is low (Singh, 2012). Coldblooded arthropods exposed to cool temperatures (20°C and below) move and respire at a lower rate, resulting in less of the fumigant being taken up (Phillips *et al*., 2012). Fumigations at higher temperatures (25°C to 30°C) can initiate increased metabolism, which will improve the fumigant intake. Consequently, less fumigant is required when fumigating at higher temperatures, and this could potentially be more efficient. Fumigation at low temperatures will require a higher dosage, for an extended period (Singh, 2012).

Potential alternative fumigants, to methyl bromide, include carbonyl sulfide, sulfuryl fluoride, phosphine and ethyl formate (Ducom and Banks, 2006; Beckett *et al*., 2007; Lee *et al*., 2018). These alternatives have potential issues, such as extended duration of the treatments required, reduced efficacy and increased costs involved (Hansen and Johnson, 2007). Insect resistance has become a global problem when fumigating with phosphine and may therefore not be a sustainable alternative (Beckett *et al*., 2007). Ethyl formate has been used as a disinfestation treatment to control dried fruit pests since the 1920s (Simmons and Gertler, 1945). It is recognized as a GRAS (Generally Recognized As Safe) chemical and is used in commercial manufacturing of artificial flavourings for essences and soft drinks, such as lemonade (Budavari *et al*., 1989; FDA, 2014). Ethyl formate is an ester molecule which is also known as ethyl methanoate, and occurs naturally in several foods (Phillips *et al*., 2012). Ethyl formate kills insects rapidly and breaks down into natural occurring, formic acid and ethanol (Desmarchelier *et al*., 1998). The mode of action for ethyl formate control of the target insects occurs through binding with cytochrome a, and the inhibition of cytochrome c oxidase. This inhibition of cytochrome c oxidase leads to loss of cell function and ultimately, cell death through the depletion of molecular oxygen in the cells (Haritos *et al*., 2003; Ducom and Banks, 2006; Linde, 2008).

Two methods are currently being researched for the application of ethyl formate as a postharvest fumigant; the use of liquid ethyl formate (without stabilizing gas) and the use of a commercial formulation of liquid ethyl formate and $CO_2$, registered as Vapormate$^{TM}$. Vapormate$^{TM}$ approved treatments consists of concentrations ranging from 30 to 420 g/m$^3$,

and exposure times from 1 to 6 h to control a variety of pests, such as aphids, thrips, mites and mealybugs when applied to fresh commodities (such as lettuce, banana, pineapple, table grapes and citrus fruits) (Linde, 2008). However, fumigation using liquid ethyl formate, and the commercial application thereof needs further research, as Vapormate™ is very costly and this may be a limiting factor for Vapormate™ fumigations (Yang *et al*., 2017). The focus of the fumigation studies in this dissertation is, therefore, fumigation with liquid ethyl formate.

It has been observed that when targeting internal insects ethyl formate penetrates the commodity poorly compared to methyl bromide and phosphine. insects. Penetration of the fumigant refers to the movement or diffusion of gas through materials (Bond, 1984). The addition of $CO_2$ in the Vapormate™ formulation may aid with penetration (Phillips *et al*., 2012). It has been observed, however, that ethyl formate fumigation treatments are more effective against external surface pests than internal pests, such as fruit fly larvae (De Lima, 2010). This penetration limitation is amplified in commodities that contain large volumes of water. The reduced penetration characteristic of ethyl formate is due to its ability to decompose in water at a rate directly related to temperature (Phillips *et al*., 2012). A significant benefit of ethyl formate fumigation treatments, when applied successfully, is the rapid control of external insects with low residues. Research by Grout and Stoltz (2016) examined the ability of Vapormate™ to control grain chinch bug, with promising results, highlighting the potential of using ethyl formate as a fumigant for post-harvest control of external pests such as the grain chinch bug and banded fruit weevil on South African export fruit.

## 1.3 Conclusion

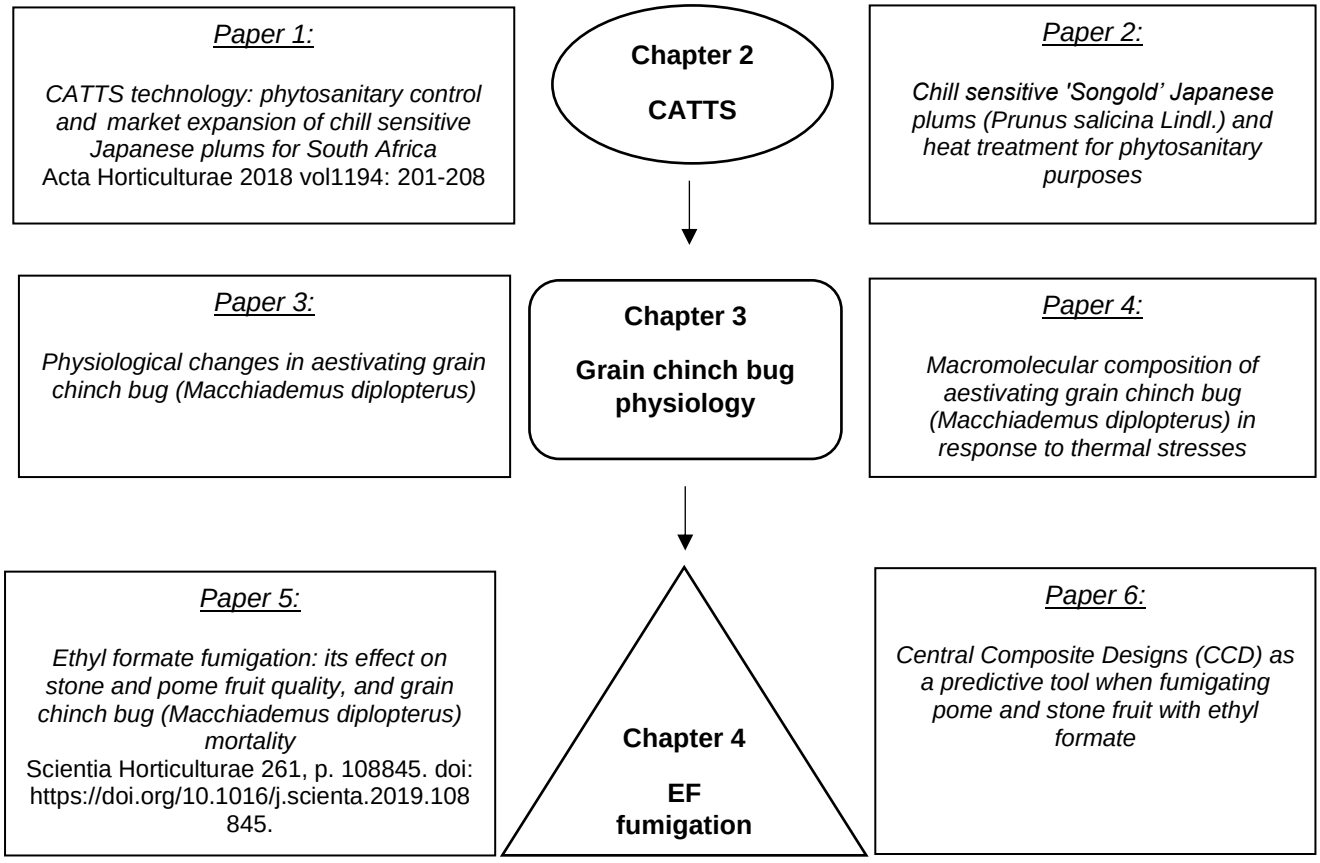
The risk of loss of trade due to interceptions of phytosanitary pests such as the false codling moth, grain chinch bug and banded fruit weevil, justifies the South African deciduous fruit industry exploring options to find effective measures to maintain and expand export markets. The ideal quarantine treatment is practically implementable, inexpensive and does zero damage to the fruit while achieving disinfestation of the associated phytosanitary pest complex. However, given the multiple factors described above that need to be taken into consideration in treatment development, there is no ‘silver bullet’, and all options must be thoroughly investigated to find workable solutions. Examination of both horticultural and entomological aspects is needed to find the balance required between these two disciplines to create successful phytosanitary treatments.

Within the overall aim of this dissertation, which is to establish the feasibility of CATTS treatments and ethyl formate fumigation as postharvest phytosanitary treatments for South African export fruit, **specific objectives** were as follows:

a) to determine the effect of CATTS treatments in combination with different cold-storage regimes on fruit quality of chill-sensitive plums, and its effect on the mortality of internal and external phytosanitary pests;
b) to examine the physiology of a key phytosanitary pest, the grain chinch bug, *Macchiademus diplopterus*, to better understand its apparent high level of tolerance to thermal postharvest treatments;
c) to determine the effect of ethyl formate fumigation treatments on pome and stone fruit quality and mortality of the grain chinch bug, *Macchiademus diplopterus*.

The research outcomes of this dissertation are presented in the following chapters comprised of academic papers addressing each of the three objectives:

- Chapter 2: Paper 1 and Paper 2 plus conclusions (objective a)
- Chapter 3: Paper 3 and Paper 4 plus conclusions (objective b)
- Chapter 4: Paper 5 and Paper 6 (objective c)




- Chapter 5 is the final chapter of the dissertation with concluding comments.

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# CHAPTER 2

## Paper 1

## CATTS technology: phytosanitary control and market expansion of chill sensitive Japanese plums for South Africa

**Abstract**

Postharvest mitigation treatment is necessary to disinfest export fruit of phytosanitary pests. Cold temperature as a phytosanitary treatment is effective against a variety of pests. However, some stone fruit cultivars are chill-sensitive and cannot be exported using the current cold sterilisation regimes. The use of CATTS (controlled atmosphere temperature treatment system) was investigated as a chemical free new technology incorporating heat and atmospheric stress to control phytosanitary pests (grain chinch bug (*Macchiademus diplopterus*), banded fruit weevil (*Phlyctinus callosus*) and false codling moth (*Thaumatotibia leucotreta*) larvae) on chill-sensitive Japanese plum cultivars (*Prunus salicina* Lindl.). CATTS treatments were followed by either the standard cold sterilisation regime for false codling moth, or dual temperature cold-storage for chill-sensitive plums. Fruit quality was evaluated at different intervals during storage. It was found that the banded fruit weevil required lower temperatures with shorter treatment durations to obtain 100% mortality compared to the false codling moth or the grain chinch bug. Both of the latter required more stress to increase the mortality rate resulting in decreased fruit quality in some cases. From the results it was evident that CATTS technology holds promise, but that the treatments used in this study must be adjusted to ensure insect mortality and satisfactory fruit quality.

**Keywords:**heat treatments, controlled atmosphere, chilling injury, phytosanitary insect pests

### 2.1.1 Introduction

Current phytosanitary treatment for Japanese plums (*Prunus salicina* Lindl.) from South Africa require a cold sterilisation period of 22 d at -0.6°C (PPECB, 2016). However, exposure of most plum cultivars to low temperatures for extended durations results in the development of chilling injury (CI). CI in plums manifests as internal browning or gel breakdown in the mesocarp of the fruit which has economic implications due to consumer dissatisfaction with the product (Taylor, 1996). The incidence of CI is of great concern to the industry, therefore, alternative postharvest mitigation treatments are needed for plums to be exported to markets with phytosanitary requirements.

Postharvest treatments using high temperatures (e.g., hot water or curing) are being used commercially on mangoes, avocados, cucumber and peppers, not only to control insects and pathogen inhibition, but also to reduce CI symptoms (Sevillano *et al.*, 2009). Factors that play an important role in the success of high temperature treatments to reduce CI are the duration of exposure to the high temperature, and cultivar.

CATTS (controlled atmosphere temperature treatment system) technology combines the effects of a short exposure to high temperature and atmospheric stress in the form of a low $O_2$ (1%)/high $CO_2$ (15%) environment to control phytosanitary pests (Neven, 2003). In the USA CATTS treatments were developed for the control of codling moth and western cherry fruit fly in sweet cherries, and codling moth and oriental fruit moth in apples, peaches and nectarines (Neven and Mitcham, 1996; Shellie *et al*., 2001; Neven *et al*., 2001; Obenland *et al*., 2005). These treatments were included in the USDA-APHIS Quarantine Treatment Manual (USDA Treatment Manual, 2015).

Studies using a water bath to simulate CATTS temperature treatments have been conducted on two plum cultivars and associated phytosanitary pests in South Africa (Johnson and Neven, 2010; Johnson and Neven, 2011). Results from these studies indicated that the water bath treatments were effective against the pests and could be applied to the fruit. However, in the present study, initial trials carried out in a laboratory scale CATTS unit during the 2014/2015 season resulted in severe internal and external damage to plum fruit while insect mortality was not satisfactory (Table 2.1.1). Consequently, the aim of this study was to better understand the effect of CATTS treatments and storage regimes on fruit quality to aid in the development of a treatment that will maintain product quality while maintaining optimal efficacy with regard to pest control.

Table 2.1.1. Initial CATTS treatments used on 'Laetitia' and 'Songold' plums in the 2014/2015 season. All treatments started with an average fruit pulp temperature of ~ 23°C. At start of treatment CA was set to 1% $O_2$ and 15% $CO_2$ and RH was controlled at ~80%.

| Cultivar | Temp ramp ($°C.h^{-1}$) | Max. pulp temp (°C) | Total run time (min) | Fruit quality | Grain chinch bug mortality (%) |
|---|---|---|---|---|---|
| Laetitia | 24 | 46 | 210 | Extensive external and internal damage | 90.8 |
| Laetitia | 24 | 46 | 240 | Extensive external and internal damage | 100 |
| Songold | 16 | 35 | 120 | Extensive external and internal damage | 86.6 |
| Songold | 16 | 35 | 120 (holding time at pulp temp of 35°C) | Internal fruit damage | 87 |
| Songold | 16 | 46 | 180 (holding time at pulp temp of 46°C) | Less internal fruit damage | 100 |

## 2.1.2 Materials and Methods

### Insects

External pests investigated were adult grain chinch bug (GCB), *Macchiademus diplopterus* (Distant) (Hemiptera; Lygaeidae) and adult banded fruit weevil (BFW), *Phlyctinus callosus* (Schöenherr) (Coleoptera: Curculionidae). The internal pest investigated was the 4th larval instar of the false codling moth (FCM), *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae). GCB were field collected in Ceres, (33°22'10"S; 19°19'24"E), Western Cape, South Africa. BFW was collected in Grabouw (34.1521°S; 19.0037E°) and Piketberg (32.9047°S; 18.7660°E), Western Cape, South Africa. FCM 4th instar were sourced from XSIT (FCM mass rearing facility) located in Citrusdal, Western Cape, South Africa.

### Fruit

'Flavor Fall' pluots were sourced from a commercial packhouse in Simondium, Western Cape South Africa. The average flesh firmness at harvest was ±44 N (measured with an 11.1 mm tip), which is within the picking window used for export (DAFF, 2015a). The samples were packed according to export standards in 4.5 kg cartons with a perforated, high density polyethylene shrivel sheet to prevent moisture loss during cold-storage. The fruit was not cooled before being transported to the laboratory. At the laboratory the fruit was stored at 23°C for 5 h before treatment.

## CATTS treatments

CATTS treatments were conducted with a laboratory scale CATTS unit (Techni-Systems, USA). The CATTS treatments and cold-storage regimes tested on 'Flavor Fall' pluots are listed in Table 2.1.2. Fruit was removed from the packaging and placed in open crates in the CATTS unit. Immediately after treatment fruit was repacked in the packaging and placed under cold-storage at -0.5°C and cold-stored according to each treatment's designated regime (Table 2.1.2).

Table 2.1.2. CATTS treatments and cold-storage regimes tested on 'Flavor Fall' pluots. Atmospheric composition in the CATTS unit was 1% $O_2$ and 15% $CO_2$ in $N_2$ with an RH of 80%. All treatments started with a fruit pulp temperature of ~23°C.

| Treatment | Treatment outline | Storage regime |
|---|---|---|
| 1 | Ramp (16°C/h) until pulp temperature was 40°C and held for 90 min | Standard cold-sterilisation regime: -0.6°C for 22 d) plus 20 d at -0.5°C (to allow stock rolling overseas) plus a simulated shelf-life of 7 d at 10°C |
| 2 | Ramp (16°C/h) until pulp temperature was 40°C and held for 180 min | |
| 3 | Ramp (16°C/h) until pulp temperature was 35°C (held for 60 min), plus ramp until pulp temperature was 45°C (held for 90 min) | |
| 4 | Same as Treatment 3 but held for 180min at 45°C | |
| 5 | Control (without CATTS) | |
| 6 | Same as Treatment 1 | Dual-temperature regime: 10 d at -0.5 °C + 10 d at 7.5°C plus 22 d at -0.5°C (commercially used) plus a simulated shelf-life of 7 d at 10°C |
| 7 | Same as Treatment 2 | |
| 8 | Control (without CATTS) | |

## Statistical layout and data analysis

A complete randomised design with 6 replicates per treatment was used. Each replicate consisted of 75 fruit (25 fruit per evaluation: after CATTS treatment, after cold storage and after cold storage plus shelf-life). Each replicate contained 40 GCBs (20 evaluated after treatment and 20 after cold storage), 20 BFWs (10 evaluated after treatment and 10 after cold storage) and 20 FCM larvae (10 evaluated after treatment and 10 after cold storage: each consisting of 5 larvae allowed to penetrate fruit before treatment, and 5 larvae contained in a perforated Eppendorf placed among the fruit in the crate).

Data were analysed using a one-way analysis of variance with STATISTICA version 10 (Statsoft, Inc., 2011). AVONA-generated P-values and the significant differences between means were determined using Fisher's least significant difference (LSD) test with a 95% confidence interval.

**Fruit evaluation**

**a) On arrival at the laboratory.**

Flesh firmness (N) (Southtrade fruit pressure tester, Model FT327, Alphonsine, Italy, fitted with an 11.1 mm tip), % Brix (digital refractometer, Atago PR-32a, Japan) and titratable malic acid (%) (automated Metrohm 719 S Titrino titrator, Herisau, Switzerland) were determined on 10 fruit per replicate per treatment. Two fruit per replicate per treatment were used to measure $C_2H_4$ (µL $C_2H_4$ $kg^{-1}.h^{-1}$) and $CO_2$ (mg $kg^{-1}.h^{-1}$) evolution.

**b) Immediately after CATTS treatment, after cold-storage and after shelf-life.**

External fruit quality (shrivel, decay and external heat damage) was determined subjectively on 20 fruit per replicate per treatment per evaluation and was expressed as a percentage of the total fruit examined. Shrivel (%) was counted when shrivelled skin extended over the shoulder of the fruit. Flesh firmness (N) was assessed on both cheeks of 10 fruit per replicate per treatment. Internal disorders (gel breakdown, internal browning and internal heat damage) were determined on 10 fruit per replicate per treatment and expressed as a percentage of the total number of fruit examined. Internal disorders were evaluated by cutting the fruit around the equatorial axis and separating the two halves of the fruit. Insect mortality was assessed at each evaluation as well as 8 h later to allow for metabolic adjustment of the insects to ambient temperature. The five FCM inoculated fruit were cut to assess larvae mortality.

## 2.1.3 Results and Discussion

Treatments 2, 3, 4 and 7 resulted in 100% mortality of BFW (Figure 2.1.1A), and Treatments 3 and 4 resulted in 100% mortality of FCM larvae (Figure 2.1.1B) immediately after the CATTS treatments. However, no CATTS treatment achieved a 100% mortality of GCB immediately after treatment (Figure 2.1.1C). The highest GCB mortality immediately after treatment was observed in Treatments 3 and 4 (70 and 71%, respectively). Treatment 5, which did not receive CATTS treatment and was stored under the standard cold sterilisation (ST) regime used for FCM commercially, gave 100% mortality for all the insects after cold-storage (Figure 2.1.1). However, the ST regime is not approved for control of BFW or GCB,

and is not feasible for application to chill-sensitive plums. BFW mortality increased with increased exposure duration to CATTS, but there was no significant difference between ST and dual temperature (DT) regimes on insect mortality. A 100% mortality rate was attained after both ST and DT for the FCM larvae with and without CATTS treatment. This result suggests that DT does not pre-condition FCM larvae to survive the cold treatment as was suspected. For GCB Treatments 6, 7 and 8 had lower insect mortalities (Treatments 7 and 8 significantly so), compared to the same treatments followed by ST storage (Treatments 1, 2 and 5). ST storage, with and without CATTS, was therefore, more effective in controlling GCB, suggesting that GCB may be pre-conditioned and have increased cold tolerance as a result of the DT regime.

Severe external heat damage manifested on all the fruit of Treatments 3 and 4 after CATTS treatment, after cold storage and after shelf life, while no external heat damage manifested in any of the other treatments (data not shown). It is suggested that the extended duration the fruit was exposed to high temperatures (35 and 45°C) caused the severe heat damage observed in these treatments.

Internal heat damage, e.g., cavitation and pit burn, is caused by excess heat which ‘cooks’ the internal tissue resulting in damage as the fruit is unable to cool sufficiently by means of transpiration (Lurie and Mitcham, 2007; De Kock, 2015). In this study pit burn incidence was observed in Treatments 1, 2, 6 and 7 after cold-storage and after shelf-life (Figure 2.1.2A). Treatment 2 had the highest incidence of pit burn after shelf life. Figure 2.1.2B shows an example of pit burn that was induced in the fruit due to CATTS treatment. Due to high levels of secondary infection due to the severe heat damage no measurements could be made for Treatments 3 and 4. We found that external and internal heat damage caused by CATTS treatments could be minimised or prevented through immediate hydro-cooling of the fruit after CATTS treatment with water at 5°C (data not shown).

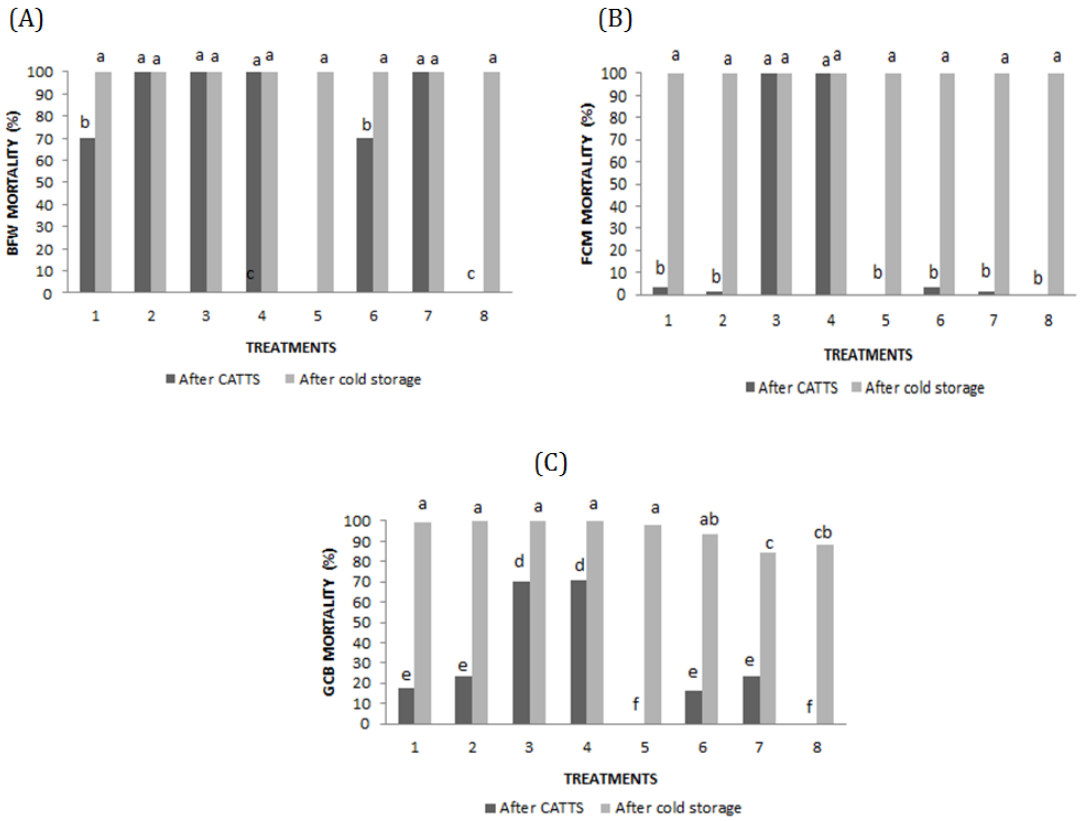
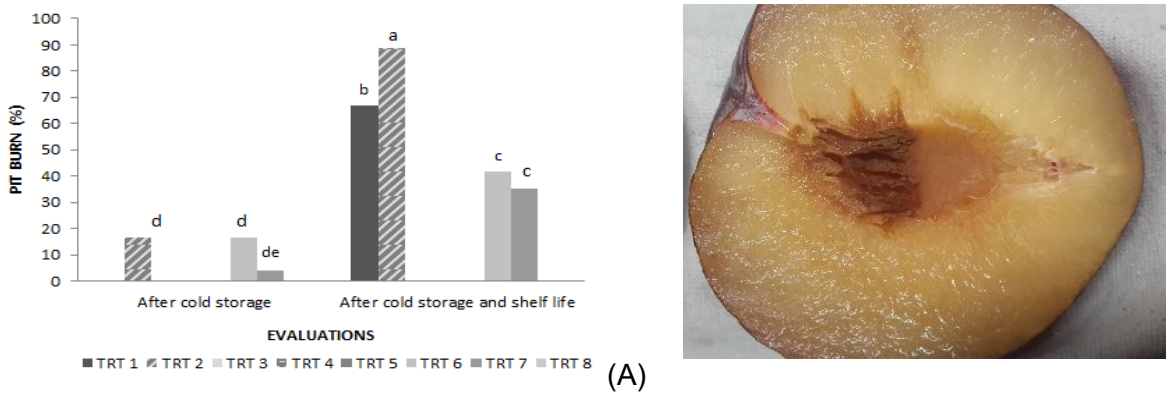



Figure 2.1.1. Percentage insect mortality after CATTS treatment and cold storage for (A) banded fruit weevil, (B) false codling moth and (C) grain chinch bug (C). BFW = banded fruit weevil; GCB = grain chinch bug; FCM = false codling moth. *Lower case letters indicate significant differences between treatments. Refer to Table 2.2 for a description of the various treatments*




(A) (B)

Figure 2.1.2. (A) The effect of CATTS treatments on the incidence of pit burn (%) in 'Flavor Fall' pluots; (B) Example of induced pit burn that occurred in 'Flavor Fall'. *Refer to Table 2.1.2 for a description of the various treatments.*

Flesh firmness generally decreased with an increase in storage time, although differences were not always statistically significant (Table 2.1.3). Fruit stored with Treatment 5 (the cold-sterilisation regime (ST)) had the least change in flesh firmness over storage time. As was expected the decrease in flesh firmness over storage time was more pronounced in fruit stored with the DT regime (Treatments 6, 7 and 8) due to the warming period. Jooste (2012)

also found that 'Sapphire' plums stored under the ST regime had higher flesh firmness compared to the DT regime. It is known that increased $C_2H_4$ production causes changes in fruit texture as it induces cell wall degrading enzymes such as polygalacturonase (Czarny *et al*. 2006., Mitcham and Lurie, 2007). However, in this study the $C_2H_4$ evolution rates were low for all the treatments and significant differences were not observed between treatments on the different evaluation dates (data not shown). However, $C_2H_4$ evolution rates were not determined during or directly after the intermittent warming period in this study. Hence, it can only be speculated that the lower flesh firmness in fruit stored under the DT regime is due to higher rates of $C_2H_4$ evolution during the warming period.

Table 2.1.3. The effect of CATTS treatment and cold-storage duration on quality of 'Flavor Fall' pluots.

| | | Evaluations (B) | | | Prob. > F[2] | | |
|---|---|---|---|---|---|---|---|
| **Examination parameters** | **Trt[1] (A)** | **Immediately after CATTS treatment** | **After cold storage** | **After shelf life** | **A** | **B** | **A×B** |
| Flesh firmness (N) | 1 | 43.61eb | 42.43eb | 42.14edf | <0.0001 | <0.0001 | <0.0001 |
| | 2 | 39.2ec | 49.0abc | 39.2eh | | | |
| | 3 | - | - | - | | | |
| | 4 | 29.4.0j | - | - | | | |
| | 5 | 43.51eb | 43.61eb | 43.02eb | | | |
| | 6 | 41.94edfg | 40.57eh | 37.83hi | | | |
| | 7 | 46.35ab | 39.30hf | 38.81hg | | | |
| | 8 | 43.41edf | 35.48k | 38.81hg | | | |
| Shrivel (%) | 1 | 18.75efg | 53.13c | 18.17d | <0.0001 | <0.0001 | <0.0001 |
| | 2 | 9.00hi | 25.0e | 5.0ji | | | |
| | 3 | - | - | - | | | |
| | 4 | 3.00ji | - | - | | | |
| | 5 | 7.29ji | 62.5b | 23.96ef | | | |
| | 6 | 10.42hgi | 59.38bc | 16.67hf | | | |
| | 7 | 10.42hgi | 79.17a | 25.76e | | | |
| | 8 | 7.29ji | 38.54efg | 11.53hgi | | | |

*[1] Values in same row followed by different letters denotes significant differences (P < 0.05) according to the LSD test. Refer to Table 2 for a description of the various treatments.*
*[2] Two-way ANOVA table with complete randomised design for Factor A (treatment) and Factor B (storage duration).*

The incidence of shrivel generally increased from harvest until the end of cold-storage, and decreased significantly during the shelf-life period in all treatments (Table 2.1.3). Although Treatments 2 and 7 received the same CATTS treatment, Treatment 2 fruit had the lowest (although not always statistically significant), and Treatment 7 the highest (although not always statistically significant) shrivel levels after cold-storage and after shelf-life. This could

be due to the difference in maturity of fruit stored under DT compared to ST, as indicated by the flesh firmness (Table 2.1.3). More mature fruit would be more susceptible to shrivel (Theron, 2015). The high shrivel levels in Treatment 7 fruit was probably caused by excessive moisture loss induced by a combination of the long holding time at 40°C (180 min) in the CATTS unit, the enhanced maturity of the fruit after the intermittent warming, and the relatively large vapour pressure deficit created during re-cooling of the fruit at the end of the intermittent warming period. Overall, shrivel levels were unacceptably high in control fruit as well as CATTS treated fruit. This result indicates that 'Flavor Fall' has an inherent problem with moisture loss during cold-storage which should be addressed with better handling and packaging protocols.

Gel breakdown incidence only occurred after cold storage plus shelf life simulation, which is typical of CI (Bramlage and Meir, 1990) (Figure 2.1.3). Treatment 6 (stored with the DT regime) had significantly higher GB levels after shelf-life compared to the other treatments. Treatment 6 differed significantly from its ST counterpart, Treatment 1. This could be due to a pre-conditioning effect of the CATTS treatment which enabled Treatment 1 to tolerate the ST storage more effectively than treatment 6 under the DT regime.

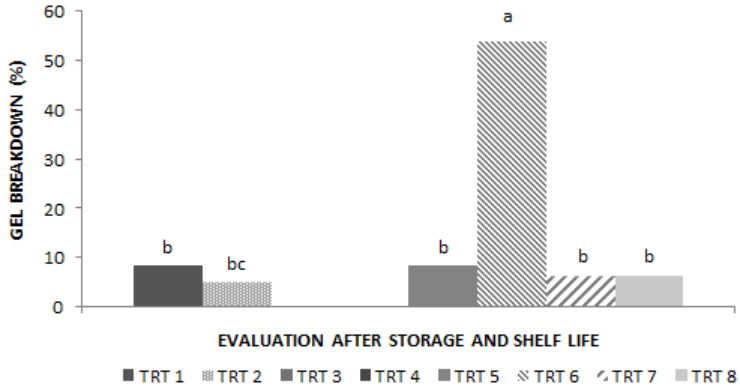



Figure 2.1.3. The effect of CATTS treatments on the incidence of gel breakdown (%) in 'Flavor Fall' pluots after storage plus shelf life simulation. *Refer to Table 2.1.2 for a description of the various treatments.*

## 2.1.4 Conclusion

The use of CATTS on horticultural products provides a non-chemical method of control of various pests and a method to reduce the products' rate of maturation and its susceptibility to CI. The tolerance of the product to these treatments should, however, be evaluated carefully as the treatments might have a negative effect on the product quality. The pre-conditioning effect observed in Treatment 1 to lower CI compared to its DT counterpart is an indicator, thereof as well as the higher flesh firmness observed in fruit stored under the

ST compared to the DT regime. CATTS treatments applied on ‘Flavor Fall’ pluots in this study did not attain a 100% mortality rate on all the insect pests tested. BFW were the most susceptible. CATTS treatment alone may not be the solution as a sole treatment for 100% mortality of GCB. A combination of CATTS treatments plus cold storage could potentially be more efficient than CATTS treatment alone for GCB. FCM required higher temperatures with prolonged exposure to CATTS to attain 100% mortality after treatment. This is not feasible as fruit quality was compromised.

It is clear that adjustments must be made to the rate of heating as well as the maximum hold temperature to prevent external and internal heat damage to the fruit. Hydro-cooling with water at 5°C immediately after treatment is strongly recommended to prevent pit burn and external heat damage incidence after CATTS treatment. The incidence of shrivel in this cultivar should be addressed with better handling and packaging protocols in order to prevent the extreme moisture loss that was observed.

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# Paper 2

# Chill sensitive 'Songold' Japanese plums (*Prunus salicina* Lindl.) and heat treatment for phytosanitary purposes

## 2.2.1 Introduction

As a pest of quarantine concern for various international markets importing fruit from South Africa, *Thaumatotibia leucotreta* (Meyrick) (Lepidoptera: Tortricidae), the false codling moth, creates many challenges with regard to phytosanitary control. Its susceptibility to cold temperatures makes cold treatment disinfestation a good option for postharvest treatment. However, this polyphagous pest affects a variety of agricultural crops, and not all commodities can withstand an approved cold treatment protocol for *T. leucotreta* of -0.55°C, or below, for 22 days (USDA, 2019). Stone fruit, particularly most plum cultivars, develop chilling injury when exposed to low temperatures for extended periods. Commercially, these chill-sensitive cultivars are stored under a dual temperature or intermittent warming regime, to protect against chilling injury, which manifests as internal browning or gel breakdown in the mesocarp of the fruit during prolonged storage at low temperatures (Taylor, 1996). South Africa exported approx. 10 million cartons (5.25 kg equivalents) of plums during the 2017/2018 season to various markets (Hortgro, 2018). The incidence of chilling injury in plums is therefore of great concern to the industry, as customer dissatisfaction with affected fruit has economic implications. As does the rejection of consignments due to the interception of phytosanitary pests such as *T. leucotreta*. Thus, it is essential to develop alternative postharvest treatments to provide phytosanitary security, and maintain fruit quality.

CATTS (Controlled Atmosphere Temperature Treatment System) technology uses temperatures and atmospheric stress by combining forced hot air with an oxygen-poor/carbon dioxide-rich environment to control target pests (Neven, 2003; Hansen and Johnson, 2007; Neven and Johnson, 2018). This mechanism of control increases the respiratory demand for the target pests, while at the same time restricting the availability of oxygen, leading to metabolic arrest and death. In the USA, CATTS treatments were developed for the codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricidae) and western cherry fruit fly, *Rhagoletis indifferens* Curran (Diptera: Tephritidae) in sweet cherries, and codling moth and oriental fruit moth (*Grapholita molesta* Busck (Lepidoptera: Totricidae) in apples, peaches and nectarines (Neven and Mitcham, 1996; Neven, 2005; Neven and Rehfield-Ray, 2006; Neven *et al.*, 2006).

Adapted from Neven (2008), a water bath system simulating CATTS conditions, was used to test the effect of heated CA treatments, comparable to those developed in the USA for codling moth and oriental fruit moth, on *T. leucotreta* (Johnson and Neven, 2010). Johnson and Neven (2011) used the same water bath system and treatment conditions to evaluate treatment efficacy on another two phytosanitary pests of South African export fruit, the grain chinch bug, *Macchiademus diplopterus* (Distant) (Hemiptera: Lygaeidae) and the banded fruit weevil, *Phlyctinus callosus* (Schöenherr) (Coleoptera: Curculionidae). Results from these studies indicated that *T. leucotreta* was more tolerant of heated controlled atmosphere treatments than codling moth and oriental fruit moth when subjected to both slow (12°C/h) and faster (24°C/h) heating rates. *T. leucotreta* was also more tolerant than *M. diplopterus* and *P. callosus* of CATTS treatments at the slower heating rates. At faster heating rates, however, *M. diplopterus* was the most tolerant. As subsequently confirmed in the previous chapter (Smit *et al*. 2018), *P. callosus* is the least tolerant to CATTS treatments.

High temperature conditions created for CATTS treatments may not only control pests, but could also protect the fruit against chilling injury. Commercially, high temperature treatments (e.g. hot water) are used as a pre-conditioning treatment to reduce chilling injury (CI) symptoms in a variety of products, such as mangoes, avocados, cucumber and peppers (Sevillano *et al*., 2009). Heat treatments can result in changes in fruit ripening through the inhibition of ethylene synthesis and cell wall degradation through changes in gene expression and protein synthesis (Picton and Grierson, 1988; Lurie *et al*., 1996; Ferguson *et al*., 2000). In response to exposure to high or near lethal temperatures the plant initiates a stress response that will induce or enhance the synthesis of heat shock proteins (HSP). Exposure to postharvest heat treatments or elevated field temperatures can, therefore, lead to HSP gene expression and HSP accumulation (Lurie and Jang, 2007). Proteins such as heat shock proteins which have been associated with thermo-tolerance plays a major part in reducing chilling injury in fruit and vegetables (Vierling, 1991; Ding *et al*., 2001). Heat shock proteins such as sHSPs (small heat shock proteins) are involved in maintaining cell membrane fluidity and integrity of fruits and vegetables when subjected to low temperatures during chilling stress (Torok *et al*., 2002; Tsvetkova *et al*., 2002; Horváth *et al*., 2008). In addition, research conducted by He *et al.* (2012) illustrated the potential of hot air treatments to alleviate CI, specifically in bananas. Their research suggested that stimulating sHSP gene expression through heat treatments can increase resistance to CI during low temperature storage. This was associated with a reduction in electrolyte leakage. Hot air treatments have also been found to induce the expression of HSP70 in grapes. Zhang *et al.* (2005) observed

a synergistic action between HSP70 and the enzymatic antioxidant system, which resulted in increased membrane integrity and CI resistance.

The pre-conditioning advantage of a heat treatment can be negated by a variety of physiological damage conditions that can manifest on a commodity due to heat stress. These include surface lesions, pitting, scalding, loss of aroma, surface and internal discoloration and early senescence (Armstrong and Mangan, 2007). Therefore, a commodity's temperature threshold (temperature range and duration within which no physiological damage occurs) is an important consideration in heat treatment development.

Based on the results of the preceding chapter (Smit *et al*., 2018), further treatment development for the pests of phytosanitary concern included in this dissertation, is focussed on *T. leucotreta* and *M. diplopterus*. Although control of *T. leucotreta* and *M. diplopterus* with treatments consisting of prolonged exposure to high temperatures resulted in poor fruit quality, a pre-conditioning effect was observed. This suggests that there is potential to use low temperature storage in combination with CATTS treatments to control the pests, and potentially the approved cold sterilisation treatment required to control *T. leucotreta* specifically, without the onset of chilling injury.

The research presented in this chapter examines the effect of CATTS treatments and different cold storage regimes to control *T. leucotreta* and *M. diplopterus* while maintaining fruit quality. The study addresses problem areas identified in Paper 1, and investigates ways of reducing the effect of heat damage. CATTS treatments have the potential to either control the target pest directly or in combination with preconditioning of the commodity, which would enable commodities to withstand low temperature treatments for phytosanitary control. The potential pre-conditioning effect of CATTS treatments will be investigated to examine its effectiveness in reducing chilling injury in chill sensitive plums.

## 2.2.2 Materials and Methods

### *Insects*

During the 2015/2016 season, aestivating adult grain chinch bug (GCB) (*M. diplopterus*) and the 4th instar of the false codling moth (FCM) (*T. leucotreta*), were included in trials. Based on observations in the first season, during the 2016/2017 season, only FCM was included in the trials. Aestivating adult GCB were field-collected in Ceres, Western Cape, South Africa (S 33:22'10"1, E 19:19'24"2). Fourth instar FCM were sourced from a FCM mass rearing facility, XSIT, located in Citrusdal, Western Cape, South Africa (S 32:5891°, E 19:0118°).

***Fruit***

During both seasons, the Japanese plum (*Prunus salicina* Lindl.) cultivar, 'Songold', was sourced, at harvest, from a commercial packhouse in Franschhoek (33°54'33.2"S, 19°06'58.4"E), Western Cape, South Africa. The samples were packed according to export standards in 4.5 kg cartons with a perforated, high density polyethylene shrivel sheet to prevent moisture loss during cold-storage. The fruit was not cooled before being transported to the laboratory. At the laboratory, the fruit was stored at 23°C for ~5 h before treatment.

***CATTS treatments***

***CATTS chamber:*** All CATTS trials were carried out in a laboratory-scale CATTS chamber manufactured by Techni-Systems (USA). The CATTS chamber is a flow through, airtight system with computerized temperature, dew point and atmosphere controls. The atmosphere inside the chamber is manipulated by injection with nitrogen, carbon dioxide and synthetic air (medical air from compressed gas cylinder), humidified by micro-misting nozzles and passed over a heater element to increase air temperature. The chamber can accommodate 2 plastic trays of fruit (58.4cm x 38.1cm x 40.6cm). Probes allow data logging of temperature changes (in chamber and within fruit), gas levels (nitrogen, carbon dioxide and synthetic air) and relative humidity.

***Season 2015/2016:*** Based on previous work (Paper 1) CATTS treatments were amended as follows: a faster ramping heat rate (80°C.h$^{-1}$), higher final air temperature (56°C), to a pulp temperature of 42°C and shorter holding periods (see Table 2.2.1).

Table 2.2.1: CATTS treatments and cold storage regimes for 'Songold' plums during the 2015/2016 season. Atmospheric composition in the CATTS unit was 1% $O_2$ and 15% $CO_2$ in $N_2$ with a relative humidity of 80%. All treatments started with a fruit pulp temperature of ~ 23 °C.

| Treatments | CATTS treatment conditions | Cold storage regime |
|---|---|---|
| **1** | 80°C.h$^{-1}$ ramp until air temp 56°C, when pulp temp reached 42°C hold 5 min | **Cold sterilisation:** -0.6°C for 22 days plus 20 days at -0.5°C plus simulated shelf life of 7 days at 10°C |
| **2** | 80°C.h$^{-1}$ ramp until air temp 56°C, when pulp temp reached 42°C hold 5 min then bring air temp down to 40°C and hold for 90min | |
| **3** | No CATTS treatment (control) | |
| **4** | Same as Treatment 1 | **Dual temperature**: 10 days at -0.5°C + 7 days at 7.5°C + 25 days at -0.5°C plus simulated shelf life of 7 days at 10°C |
| **5** | Same as Treatment 2 | |
| **6** | No CATTS treatment (control) | |

The atmospheric composition for treatments was 1% $O_2$ and 15% $CO_2$ in $N_2$, and the relative humidity was maintained at 80%. A complete randomised design with 6 replicates per treatment (Treatments 1-6) was used. Each replicate consisted of 75 fruit (25 fruit per evaluation: after CATTS treatment, after cold storage and after cold storage plus shelf-life). For each replicate 40 GCBs (20 evaluated after treatment and 20 after cold storage) and 20 fruit infested with 4$^{th}$ instar FCM (10 evaluated after treatment and 10 after cold storage) were used; larvae were allowed to penetrate fruit before treatment and infested fruit were placed among the fruit and GCB insects in the crate). Immediately after treatment fruit was hydro-cooled in ice water (0-1°C) for 30 min to reduce heat damage, packed in commercial packaging and then stored at either -0.6°C (standard cold storage regime temperature; Treatments 1, 2 and 3 (control)) or under a dual temperature regime (Treatments 4, 5 and 6 (control)), followed by 20 days at -0.5°C to make up the remainder of the commercial cold storage period for plums. Hydro-cooling was applied, as an alternative to forced air cooling, which would be used in a commercial scenario, as facilities for forced air cooling were not available close to the CATTS unit. Insect mortality was assessed immediately after treatment and after cold storage, and in both instances, again 8 h later (to allow for metabolic adjustment of any survivors). Mortality was assessed by prodding of the insects to check for any movement, indicating survival. Mortality was calculated as the percentage of dead insects relative to total treated insects for each treatment.

***Season 2016/2017:*** Based on the findings from the 2015/2016 season, controlled atmosphere was not applied in amended treatments in the second season, as fruit quality was improved when treatments were applied under regular air, instead of CA. The focus of the amended treatments was on improving fruit quality of cold-stored chill-sensitive fruit. The aim was to examine the pre-conditioning effect of heat treatments in preventing or decreasing the incidence of chilling injury during the cold sterilisation regime. The focus was therefore shifted to control internal pests only (i.e. FCM) using one specific heat treatment in combination with different cold storage regimes and the addition of SmartFresh™ to improve fruit quality. It was observed during the 2015/2016 season that harvest maturity played a major role in the fruit's susceptibility to heat damage. Less mature fruit were less susceptible to heat damage compared to more mature fruit (Fig. 2.2.1A and B).

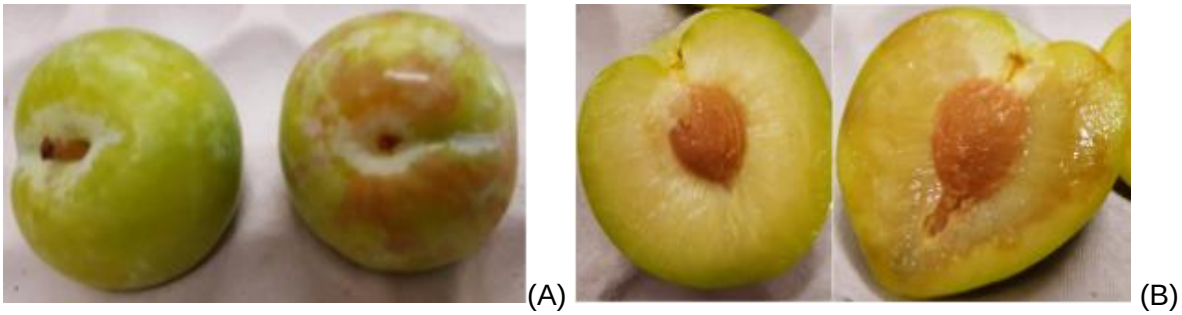



Figure. 2.2.1. External (A) and internal heat damage (B) in less mature fruit (left) and more mature fruit (right) demonstrating the influence of harvest maturity on susceptibility to heat damage.

Application of SmartFresh™ would minimise the effect of harvest maturity as a factor that would influence the products' response to heat treatments. The ramp rate (°C.h$^{-1}$) applied was determined by collecting orchard data during the 2015/2016 season. Information obtained from the orchard data indicated that plum pulp experienced a maximum ramp rate of approximately 11 to 12°C.h$^{-1}$ in the orchard. A ramp rate of 12°C.h$^{-1}$ was therefore used to reach 35°C, with the relative humidity set at 80% for all CATTS treatments. SmartFresh™ was applied at ambient temperature before treatment. A complete randomised design with 6 replicates per treatment (Treatments 1-8; See Table 2.2.2) was used. Each replicate contained 75 fruit (25 per evaluation) and 40 4$^{th}$ instar FCM (10 infested fruit per evaluation). Larvae were allowed to penetrate fruit before treatment and infested fruit was placed among the fruit in the crate.

After the heat treatments, fruit was hydro-cooled in ice water for 1 h, and then packed in commercial packaging and stored at either -0.6°C (standard cold storage regime temperature; Treatments 1, 2, 3 and 4 (control)) or under a dual temperature regime (Treatments 5, 6, 7 and 8 (control). Insect mortality was determined after treatment, and

later, after 10, 20 and 42 days in cold-storage at -0.6°C for the cold sterilisation regime. For the dual temperature regime, insect mortality was measured after treatment, after 10 days at -0.5°C, after 10 days at -0.5°C plus 10 days at 7.5°C and after 10 days at -0.5°C plus 10 days at 7.5°C plus 22 days at -0.5°C.

Table 2.2.2: Heat treatments and cold storage regimes applied to false codling moth infested 'Songold' plums during the 2016/2017 season. *Note: SmartFresh™ was applied to subsections of treatments listed*

| Treatment | SmartFresh™ treated | Temperature treatment conditions | Cold storage regime |
|---|---|---|---|
| **1** | No | Ramp 12°C.h$^{-1}$ until pulp temperature is 35°C. Hold for 5 hours | **Cold sterilisation:** -0.6°C for 22 days plus 20 days at -0.5°C plus simulated shelf life of 7 days at 10°C |
| **2** | Yes | | |
| **3** | No | Control | |
| **4** | Yes | | |
| **5** | No | Ramp 12°C.h$^{-1}$ until pulp temperature is 35°C. Hold for 5 hours | **Dual temperature**: 10 days at -0.5°C + 7 days at 7.5°C + 25 days at -0.5°C plus simulated shelf life of 7 days at 10°C |
| **6** | Yes | | |
| **7** | No | Control | |
| **8** | Yes | | |

Mortality was assessed by prodding the insects to check for any movement, indicating survival. Mortality was calculated as the percentage of dead insects relative to total treated insects for each treatment.

***Fruit evaluation***

After harvest, total soluble solids (TSS), expressed as % Brix, was determined using a temperature-controlled digital refractometer (Atago digital refractometer PR-32a, Japan). Titratable acid (TA) (%) expressed as malic acid equivalents, was determined through titration of 10 g of juice with 0.1 N NaOH to a pH end point of 8.2 using an automated titrator (Metrohm 719 S Titrino, Herisau, Switzerland). Both TSS and TA were measured using pooled juiced samples of 20 fruit per replicate per treatment. Other maturity parameters (hue angle and flesh firmness) of fruit for all trials were evaluated at harvest, after treatment, after cold storage and after cold storage plus shelf life simulation. Hue angle (°) was measured using a calibrated colorimeter (Minolta chroma meter CR-400, Japan) to quantify

the change in ground colour of the fruit. Flesh firmness (N) was measured using a Fruit Texture Analyzer (Güss Manufacturing, Strand, South Africa).

After treatment, after cold storage and cold storage plus shelf life simulation, external fruit quality parameters (shrivel, decay and external heat damage) and internal fruit quality parameters (such as internal browning, gel breakdown and internal heat damage) were determined. External fruit quality was subjectively evaluated on 20 fruit and internal fruit quality was subjectively evaluated on 10 fruit per replicate per treatment and expressed as a percentage of the total fruit examined. Shrivel was recorded when fruit had shrivelled skin which extended over the shoulder of the fruit. External heat damage can appear as peel browning, pitting and scalding. This damage to the epidermis could also result in the development of necrotic tissue, which was recorded as decay. Internal disorders were evaluated by cutting each of the 10 fruit around the equatorial axis and separating the two halves of the fruit. Chilling injury in more mature fruit manifests as gel breakdown and in less mature fruit as internal browning (Taylor, 1996). Total chilling injury was defined as the sum of gel breakdown and internal browning observed. Gel breakdown is the gelatinous breakdown of the inner mesocarp tissue surrounding the stone which can spread throughout the mesocarp tissue in severe cases, and internal browning is the brown discolouration directly beneath the skin of the fruit in the mesocarp tissue (Jooste, 2012). Internal heat damage can also manifest as darkening of the flesh, as well as cavitation (Lurie and Mitcham 2007).

**Statistical analysis of data**

Fruit quality and insect data for all trials were analysed using factorial ANOVA with STATISTICA version 13 (Statsoft Inc., 2017). ANOVA-generated P-values and the significant differences between means were determined using Fisher's least significant differences (LSD) test with a 95% confidence interval.

## 2.2.3 Results

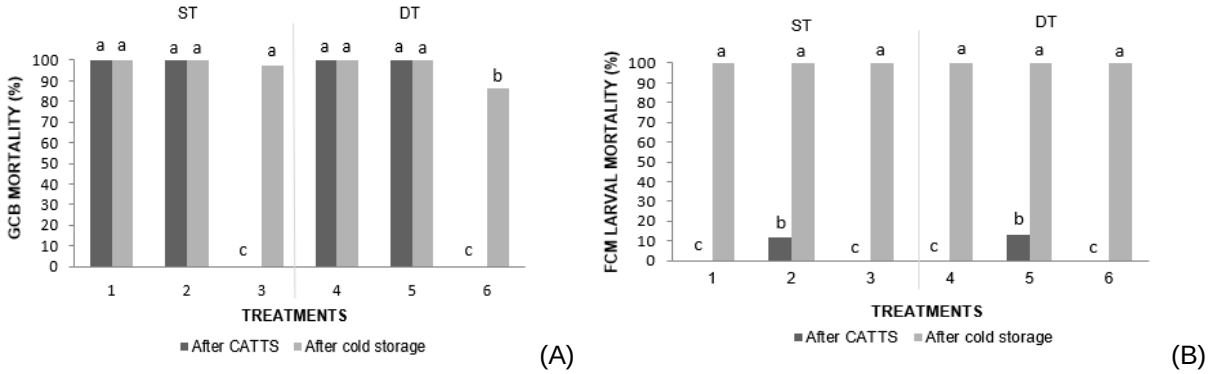
**Insect mortality**

***Season 2015/2016***

During the 2015/2016 season, the less intense of the CATTS conditions, Trt 1 and Trt 4, which had a fast and high temperature ramp, but a short holding time, yielded 100% mortality of grain chinch bug (GCB) immediately after treatment (Fig. 2.2.2A). Reassessment of mortality 8 hours after CATTS treatments did not indicate any revival of insects previously recorded as dead. Since effective mortality was achieved through the application of CATTS

treatments, the effect of the cold storage regimes on mortality of GCB, can only be seen in the controls (Trt 3 and Trt 6), where no CATTS treatment was applied. Here, the cold sterilisation (ST) regime also produced 100% mortality of GCBs, while the dual temperature (DT) regime resulted in 87% mortality. In contrast to GCB, the CATTS treatments applied here were not effective against the false codling moth (FCM) larvae.

CATTS conditions in Trt 2 and Trt 5, which were the same as in Trt 1 and Trt 4 in terms of ramping rate and target temperature, but more intense as it had a longer holding period, resulted in only 12% and 13% mortality of FCM, respectively (Fig 2.2.2B). Both the ST and the DT regimes produced 100% mortality of FCM larvae in the untreated controls (Trt 3 and Trt 6). This was expected for the ST regime, as it is the regime prescribed for phytosanitary control of FCM. The total cold storage period for the DT regime was 42 days, and despite the temperature fluctuation set in this regime, it still killed the larvae exposed to it in this study.




(A) (B)

Figure 2.2.2. Percentage insect mortality after CATTS treatment and cold storage during the 2015/2016 season for (A) grain chinch bug (GCB), (B) 4th instar false codling moth (FCM). Lower case letters indicate significant differences between treatments. *Note: Stored using cold sterilisation (ST) regime: Trt 1 = 80°C.h$^{-1}$ ramp until air temp 56°C, when pulp temp reached 42°C hold 5 min; Trt 2 = 80°C.h$^{-1}$ ramp until air temp 56°C, when pulp temp reached 42°C hold 5 min; Trt 3 = No CATTS treatment (control). Stored using dual temperature (DT) regime: Trt 4 = same as Treatment 1; Trt 5 = same as treatment 2; Trt 6 = No CATTS treatment (control)*

### *Season 2016/2017*

Heat treatments applied during the second season, based on orchard fruit temperature data in an effort to improve post-treatment fruit quality, and an extended holding period to improve FCM mortality, were effective against FCM in combination with cold storage. Heat treatments alone did not result in any mortality of FCM larvae, and only after a period in cold storage was mortality observed. Figure 2.2.3 shows the mortality of FCM larvae recorded after 10 days in cold storage for both cold storage regimes. Irrespective of the cold storage regime, or the application of SmartFresh™, high levels of mortality (57% – 90%) were

achieved in heat-treated fruit (Trt 1, 2, 5 and 6), while all larvae in the untreated controls (Trt 3, 4, 7 and 8) were still alive after 10 days in cold storage. The application of SmartFresh™ before treatment did have a significant effect on FCM larval mortality, in that, after treatment of SmartFresh™ treated fruit, mortality was significantly lower than in non-SmartFresh™ treated fruit, 73% (Trt 2) and 90% (Trt 1) respectively under the ST regime; and 57% (Trt 6) and 73% (Trt 5) respectively under the DT regime). After 20 days in cold storage, 100% mortality was observed for all treatments stored using the ST regime. The DT regime yielded 100% mortality for all heat treatments and the control yielded mortality of 78%.

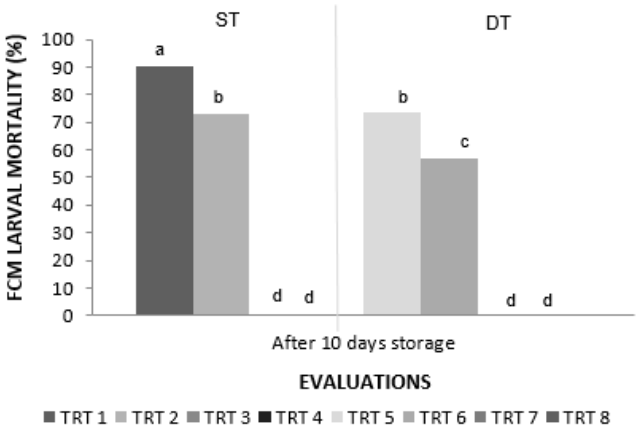



Figure 2.2.3: The effect of CATTS treatments after 10 days of cold storage on false codling moth (FCM) 4th instar mortality during the 2016/2017 season. Different lower case letters indicate significant differences between treatments. *Note: Stored using cold sterilisation (ST) regime: Trt 1 = Ramp 12°C.h$^{-1}$ until pulp temperature is 35°C. Hold for 5 hours (no Smartfresh™); Trt 2: Ramp 12°C.h$^{-1}$ until pulp temperature is 35°C. Hold for 5 hours (Smartfresh™); Trt 3 = control (no Smartfresh™); Trt 4 = control (Smartfresh™). Stored using dual temperature (DT) regime: Trt 5 = same as Treatment 1; Trt 6 = same as treatment 2; Trt 7 = same as treatment 3 (Control without Smartfresh™); Trt 8 = same as treatment 4 (Control with Smartfresh™)*

## Fruit quality

### *Season 2015/2016*

The hue angle of fruit decreased slightly (more yellow) with an increase in storage duration, as was expected. At the final evaluation point, after shelf life simulation, data showed that hue angle was not significantly affected by heat treatments. The differences observed between ST and DT storage regimes, where the DT-stored fruit appeared slightly more yellow-green, compared to the darker green ST-stored fruit, were expected (Appendix A).

Flesh firmness data of all fruit evaluated after shelf life simulation are presented in Fig 2.2.4A. Fruit subjected to Trt 4 and stored under the DT regime was significantly firmer at

40N, than all other treated fruit, as well as the controls (~ 20N), irrespective of storage regime. Fruit subjected to Trt 1 (same as Trt 4 CATTS conditions) but stored under ST was also firmer than control fruit, but marginally so.

High levels (>40%) of external heat damage were observed after CATTS treatment with a rapid initial temperature ramp and extended holding period (Trt 2 and Trt 5) (Fig. 2.2.4B). Treatments with the short holding period (Trt 1and Trt 4) exhibited no external heat damage and were comparable to control fruit (Trt 3 and Trt 6). After cold storage and after shelf life simulation, Trt 2 fruit stored using the ST regime had significantly less heat damage than Trt 5 fruit stored under DT. External heat damage manifesting as peel browning is shown in Figure 2.2.5A.

After cold storage, Trt 2 and Trt 5 (longer holding time) manifested lower incidence of shrivel compared to Trt 1 and Trt 4, and their respective controls stored for both storage regimes (Fig. 2.2.4C). However, after shelf life simulation, all treatments and controls yielded unacceptable levels of shrivel (> 10%).

After cold storage, Trt 5, stored under the DT regime had a significantly higher percentage of total chilling injury (CI), than its ST counterpart (Trt 2), 54% versus 6%, respectively (Fig. 2.2.4D). However, after shelf life simulation, total CI decreased significantly in Trt 5 fruit, and increased Trt 2 fruit. The decrease observed for Trt 5 after shelf-life, could be due to sampling error, as chilling injury is expected to increase during the shelf-life simulation. After cold storage, total CI for Trt 1 and Trt 4 did not differ significantly, but after shelf life simulation total CI increased to 58% in Trt 1 and to 14% for Trt 4.

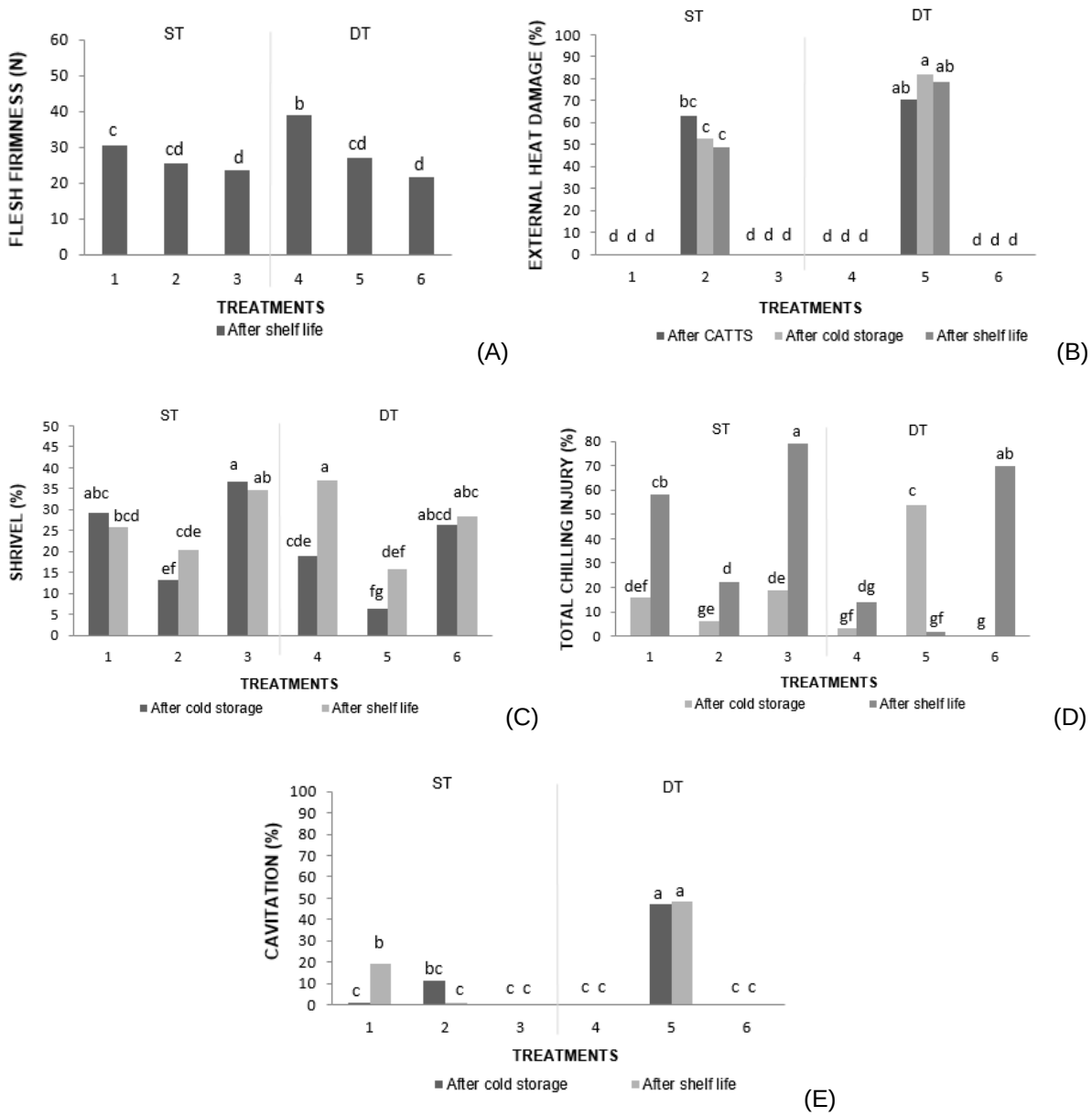



Figure 2.2.4. The effect of CATTS treatments on the (A) flesh firmness, (B) external heat damage, (C) shrivel, (D) gel breakdown, (E) cavitation in 'Songold' plums during the 2015/2016 season. *Note: Stored using cold sterilisation regime (ST): Trt 1 = 80°C.*$h^{-1}$ *ramp until air temp 56°C, when pulp temp reached 42°C hold 5 min; Trt 2 = 80°C.*$h^{-1}$ *ramp until air temp 56°C, when pulp temp reached 42°C hold 5 min then bring air temp down to 40°C and hold for 90 min; Trt 3 = No CATTS treatment (control); Stored using dual temperature regime (DT): Trt 4 = same as Treatment 1; Trt 5 = same as treatment 2; Trt 6 = No CATTS treatment (control)*

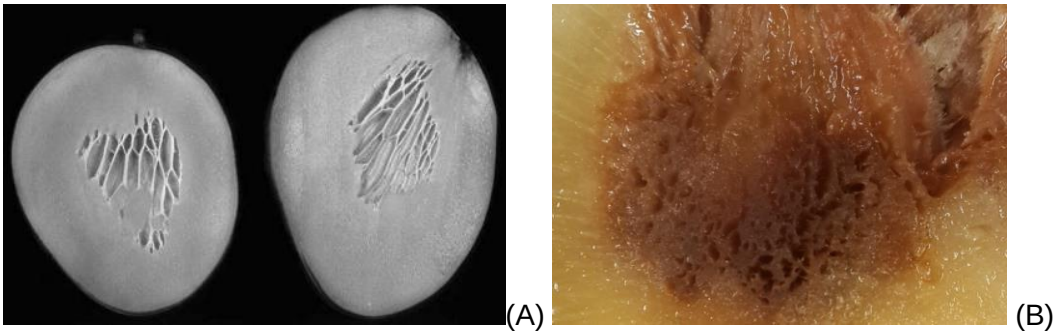
After shelf life simulation, the controls for both cold storage regimes yielded a significantly higher percentage of damaged fruit compared to the CATTS treated fruit. Trt 3 (control fruit) stored using ST, presented with 79% of total CI damaged fruit compared to 58% for Trt 1 and 22% for Trt 2. Thus, indicating a potential pre-conditioning effect of heat treatments to lower the incidence of total CI when stored using low temperature regimes. Fruit stored using the DT regime yielded a lower percentage of damaged fruit compared to the ST regime after

the shelf life simulation. The DT stored control fruit yielded 70% damaged fruit, which could possibly be due to a higher maturity of fruit resulting in a higher susceptibility to damage.

Cavitation was highest in fruit from Trt 5 and stored under the DT regime (~50%) (Fig. 2.2.4E). This was evident at the post cold storage evaluation and it remained high after shelf life. All other fruit, except Trt 1 after shelf life had very low levels of cavitation, and did not differ significantly from the controls. Figure 2.2.6B shows the cavitation observed in heat treated 'Songold' plums during the 2015/16 season.

(A) (B)

Figure 2.2.5. Peel browning (A) and flesh darkening around the stone (B) as a result of heat damage in 'Songold' plums during the 2015/2016 season.



(A) (B)

Figure. 2.2.6. Cavitation observed in mangoes (A) as a result of heat treatment (source: Lurie and Mitcham 2007) and in 'Songold' plums (B) in the present study, during the 2015/2016 season.

***Season 2016/2017***

As seen in the previous season, heat treatments did not significantly influence the hue angle when stored using ST and DT. The differences observed between ST and DT were expected and do not affect the marketability of the product (Appendix A).

For all treatments flesh firmness generally decreased with an increase in storage duration (Fig. 2.2.7A). As expected, SmartFresh™ treated fruit had significantly higher flesh firmness than non-SmartFresh™ treated fruit. This was seen in both storage regimes - after shelf life flesh firmness in Trts 2 and 4 (SmartFresh™ treated) were significantly higher than 1 and 3 (non-SmartFresh™ treated), and Trts 6 and 8 (SmartFresh™ treated), significantly higher

than 5 and 7 (non-SmartFresh™ treated). The higher flesh firmness will enable a longer shelf life duration and potentially improved quality.

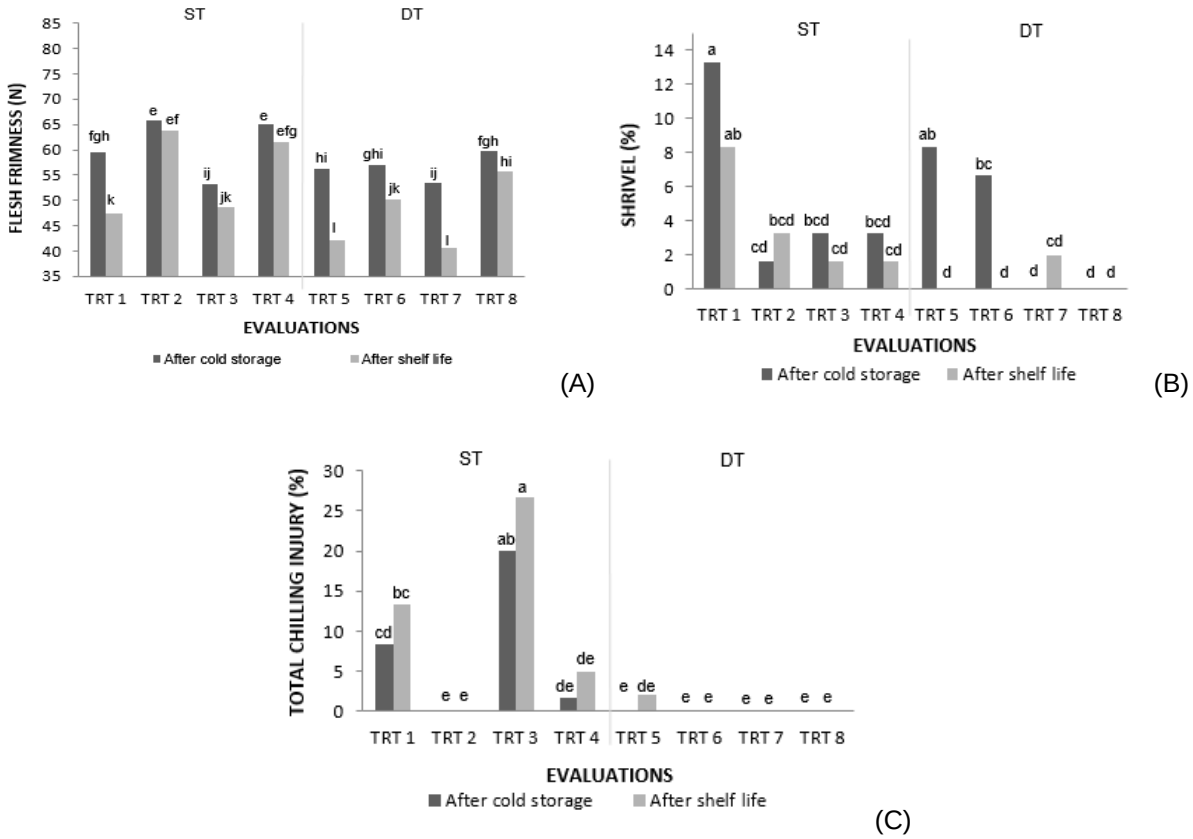



Figure 2.2.7. The effect of CATTS treatments on the flesh firmness (A), shrivel (B) and total chilling injury (C) of 'Songold' plums during the 2016/2017 season. *Note: Trt 1 - 4 = Cold sterilisation regime (ST), Trt 5 – 8 = Duel temperature (DT) regime. Trt 1, 5 = Ramp 12°C $h^{-1}$ until pulp temperature is 35°C. Hold for 5 hours (no Smartfresh™); Trt 2, 6 = Ramp 12°C $h^{-1}$ until pulp temperature is 35°C. Hold for 5 hours (Smartfresh™); Trt 3, 7 = control (no Smartfresh™); Trt 4, 8 = control (Smartfresh™).*

After cold storage and shelf life, Trt 1 (heat treatment and non-SmartFresh™ treatment, stored using ST) had significantly higher levels (12%) of shrivel than the 2- 4% of Trt 2 (heat treatment and SmartFresh™ treated, stored using ST) and both controls stored using ST (Fig. 2.2.7B). After shelf life, only Trt 1 differed significantly from all the other treatments (Trt 2 - 8). Shrivel levels of Trt 2 – 8 after shelf life simulation were below 10% which is deemed acceptable.

No external or internal heat damage was observed after treatment, after cold storage or after shelf life. This confirms the importance and benefits of an extended period of hydro-cooling fruit immediately after treatment.

Figure 2.2.7C shows the incidence of total chilling injury (CI) that was observed in 'Songold' plums, after cold storage and after shelf life. The highest levels of CI (20 – 30%) were seen

in control of untreated fruit (Trt 3). The pre-conditioning effect of the heat treatment (Trt 1) reduced CI to below 15%, but more importantly, the combination of heat and SmartFresh™ (Trt 2) resulted in zero CI. Treatment 4, which only underwent SmartFresh™ treatment, but was not heated, exhibited low levels of CI (< 5%). This further supports the effect of heating as a pre-conditioning treatment against CI. As expected, fruit stored under the DT regime had little (< 2%) or no CI.

### 2.2.4 Discussion

*Macchiademus diplopterus* is highly tolerant of the CATTS treatments applied in this study, requiring high temperatures and fast temperature ramp rates to reach their thermal limit, to the point of causing severe damage to fruit and unacceptable fruit quality. An insect's metabolism and physiology are significantly affected by the heating rates of heat treatments. Codling moth larvae in fruit may acclimatise at heating rates of 0.13-0.20°C/min in a controlled atmosphere chamber, resulting in the extended duration of the final temperature, in order to reach the same mortality at a slower heating rate (Evans, 1987; Neven, 1998a; Neven, 1998b; Tang and Wang, 2007). Thermo-tolerance research conducted by Okosun (2012) on *M. diplopterus* indicated that during aestivation, the dormant period of its life cycle when grain chinch bugs may be present on export fruit, these insects can tolerate below zero temperatures and temperatures as high as 51.5°C . Furthermore, thermal tolerance of *M. diplopterus*, for both low and high temperatures, increases as they enter aestivation, and pre-exposing *M. diplopterus* to low and high acclimation temperatures enables the insect to tolerate prolonged cold storage treatments. Consequently, the application of temperature treatments, in this case, heat treatments, to effectively control *M. diplopterus*, requires temperature conditions in postharvest mitigation measures that lead to poor fruit quality. Alternative treatments need to be investigated and developed for phytosanitary control of *M. diplopterus*. Additionally, the thermal tolerance of *M. diplopterus* requires in-depth study to better understand the challenges it causes, and develop potential ways of overcoming them to improve phytosanitary control.

*Thaumatotibia leucotreta* larvae, however, are more susceptible to heat treatments. Although heat alone did not result in larval mortality, suggesting that heat transfer from the surface of the fruit to the centre of the fruit was not efficient enough to control *T. leucotreta* alone. However, additional cold stress did bring about high levels of mortality early into the cold storage period (10 days). Thus, exposure to heat significantly reduced survival during subsequent cold storage, implying that a combination treatment would result in a shorter

cold sterilisation period (< 20 days), in comparison to what is currently used for phytosanitary control of *T. leucotreta*.

Another advantage of such a combination treatment would be preconditioning chill sensitive fruit cultivars to better withstand the cold storage period. However, heat damage as a result of treatment is an important consideration, and during the first season of the present study, the influence of harvest maturity on the product's susceptibility to heat damage was evident. Adams *et al*. (2001) observed the same in tomatoes where the fruit were more sensitive to elevated temperatures during their later maturation stage. Research conducted on mangos showed severe peel scalding with exposure to forced air heat treatment of 45°C, but no damage was observed at 43°C, indicating that there is a threshold temperature for skin injury and that fruit differed in its susceptibility due to seasonal and maturity effects (Lurie and Mitcham, 2007). Harvest maturity not only affects a products' susceptibility to heat damage but the incidence of shrivel as well. Research by Jooste (2012) found that more mature fruit had higher levels of shrivel, than less mature fruit. This is possibly due to inadequate removal of respiratory heat from the fruit. Moisture stress induces higher levels of ethylene to be produced, which accelerates senescence and membrane deterioration (Jooste, 2012). Heat stress due to high temperature ramps in combination with mixed harvest maturity yielded shrivel levels above the maximum criteria for export during the 2015/2016 season.

The introduction of SmartFresh™ during the second season (2016/2017) was used to minimise the influence of harvest maturity on the fruits' susceptibility to heat damage. Despite the lower levels of shrivel during the 2016/2017 season in the heat-treated fruit, shrivel levels were still unacceptably high in treated and control fruit. The lower shrivel levels in the heat treated fruit can be ascribed to melting of the wax layers on the fruit surface during treatment which could have sealed open lenticels and micro-wounds on the fruit surface preventing moisture loss (Woolf and Ferguson, 2000). The transpiration rate or rate of moisture loss is influenced by product factors (e.g. maturity stage) and external environmental factors (e.g. temperature). The transpiration rate can be controlled by using treatments, such as waxes, or manipulation of the environment, such as increasing relative humidity (Kader, 1985). The apparent natural high incidence of shrivel in 'Songold' plums seen here, should be addressed with better handling and packaging protocols in order to prevent the extreme moisture loss that was observed, even in untreated fruit.

Loss of flesh firmness leads to reduced fruit quality and potentially shorter shelf life expectation. Heat treatments during the first season, 2015/2016, which used short exposure times at high temperatures, produced fruit that was significantly firmer compared to controls

irrespective of which storage regime was applied. This is an indication of the ability that heat treatments have to delay ripening and extend storage life, as was also seen by Biggs *et al*. (1988), Klein (1989) and Hansen and Johnson (2007) where inhibition of ripening may be mediated by the ripening hormone ethylene. During hot air treatments of 35 to 40°C of apples and tomatoes the inhibition of ethylene synthesis occurred within hours. During the 2016/2017 season, the addition of SmartFresh™ extended the storage life more efficiently, with higher flesh firmness in heat and SmartFresh™ treated fruit for both storage regimes.

The application of SmartFresh™ did improve overall fruit quality after treatment, and addressed the effects of harvest maturity, however, it did also initially reduce the efficacy of treatments against *T. leucotreta*. This was only seen during the early stages of cold storage (< 10 days), by 20 days in cold storage, in either regime, 100% mortality of *T. leucotreta* larvae was still achieved with the low number of larvae used.

Heat retention due to the longer treatments and inadequate cooling after treatment during the first season caused severe heat damage to treated ‘Songold’ plums, both externally (peel browning) and internally (cavitation). Postharvest physiologists emphasize the importance of removing heat from harvested fruit as quickly as possible and storing them at temperatures lower than ambient to reduce postharvest decay and help with the extension of the products’ shelf life (Armstrong and Mangan, 2007). Research conducted by Anthony *et al*. (1989) observed that external browning of stone fruit increased as the heat treatment duration increased. However, this was eliminated when the fruit was enclosed in plastic wrap, which possibly reduced shrivel and moisture loss of the product. Moisture loss of a product not only leads to loss of physical weight, but also negatively affects the appearance of the product. The outer layer of the product, consisting of the epidermal cells, cuticle, stomata, and/or lenticels, regulates moisture loss (Chigwaya, 2016) and the plastic wrap would create a barrier and interfere with this regulatory function. Internal damage due to heat treatments can occur even in the absence of external damage. The most common symptoms include flesh darkening in citrus and nectarines. In mango and papayas, heat damage causes poor colour development, abnormal softening, lack of starch breakdown and the formation of internal cavities (Lurie and Mitcham, 2007). Symptoms of cavitation include spongy tissue with air pockets which do not appear until the fruit has ripened, whereby starch degradation in the tissue is inhibited (Lurie and Mitcham, 2007). During the 2016/2017 season, after adjustments were made to the application of hydro-cooling after treatment, a decrease was observed in heat damage. This was achieved by quickly

removing the residual heat within the fruit resulting in no damage to the peel or internal tissue (through cavitation).

During both the 2015/2016 and 2016/2017 seasons the potential pre-conditioning effect of heat treatments was evident with a reduction in chilling injury in heat treated fruit compared to the controls. During season 2015/2016 control fruit stored using DT displayed higher levels of injury compared to the 2016/2017 season. This could be due to the maturity of the fruit, during the 2016/2017 season a higher flesh firmness was evident indicating the fruit were less mature than those used during the 2015/2016 season. The application of Smartfresh™ during the 2016/2017 season lowered the incidence of chilling injury even more substantially, compared to the other treatments stored using ST. Applying heat treatments as a pre-conditioning treatment can potentially strengthen a product's resistance too high and low temperatures (Lurie, 1998). The development of thermal tolerance is dependent on exposure temperatures, for example 35 – 40 °C will initiate heat shock protein synthesis but 42°C and higher will reduce heat shock protein synthesis resulting in heat damage (Vierling, 1991; Ferguson *et al*., 1994; Sabehat *et al*., 1998; Ferguson *et al*., 2000). Saltveit (1991) found a correlation between heat shock proteins and thermal tolerance affecting chill sensitivity in tomatoes. The application of heat treatments for 2 – 3 days at 38°C in air, reduced the tomatoes sensitivity to low temperatures (Saltveit, 1991; Lurie and Klein, 1991; Sabehat *et al*., 1996; Lurie and Sabehat, 1997). Lipid composition studies found that a short exposure to heat can initiate processes for tissue adaption to low temperatures (Lurie *et al*., 2006). Cultivars vary greatly with regard to their susceptibility to CI. Research conducted by Jooste (2012) found that fruit that are more mature had a higher incidence of CI. The CI incidence increased with storage duration, as well as with an increase in ethylene production rates, in more mature fruit. This is due to higher levels of saturated fatty acids in the membranes of mature fruit which are less fluid (Jooste, 2012).

The research above has indicated that application of a heat treatment alone does not sufficiently control *T. leucotreta* larvae, while maintaining fruit quality. A combination treatment of heat followed by cold storage, could be more beneficial, not only as a phytosanitary control measure, but also as a method to reduce the product's susceptibility to chilling injury and reduce the rate of senescence. Research has indicated that *M. diplopterus* is highly thermal tolerant. The mechanisms for its thermal tolerance are yet unknown, and shedding light on these mechanisms could provide insight into amendments that could be made to thermal phytosanitary treatments to potentially increase their effectiveness against *M. diplopterus*. Application of CATTS treatments lowered the

incidence of chilling injury in ‘Songold’ plums stored using low temperature regimes. Overall, the heat treatment and SmartFresh$^{TM}$ combination produced fruit with no chilling injury, as well as acceptable levels of flesh firmness and good colour for marketability, but significantly reduced the mortality of FCM larvae.

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### 2.3.1 Conclusions:

### Papers 1 and 2

The aim of Papers 1 and 2 was to examine the use of CATTS on horticultural products as a non-chemical method for control of selected phytosanitary pests, as well as a potential method to reduce the product's rate of senescence and its susceptibility to chilling injury. The development of a successful phytosanitary treatment requires finding the balance of, not only controlling the targeted pest, but also minimising the damaging effects thereof on the commodity. To find a happy medium between all the various factors at play proved more difficult than initially anticipated.

Initial CATTS treatments applied to 'Flavor Fall' pluots in Paper 1, yielded poor fruit quality and unsatisfactory insect mortality of all tested pest species. During these initial trials, *Phlyctinus callosus*, the banded fruit weevil was the most susceptible to treatments. In contrast, the most challenging to control was *Macchiademus diplopterus*, the grain chinch bug. Amended treatments applied during the following season verified the ability of the grain chinch bug to tolerate various CATTS treatments. Controlling all three targeted pests, *P. callosus*, *M. diplopterus* and *Thaumatotibia leucotreta*, with one blanket CATTS treatment is therefore not a viable option.

Subsequent CATTS treatment trials, presented in Paper 2, further highlighted various factors/questions with regard to the insects and horticultural products that need to be addressed in order to develop an effective phytosanitary treatment. The physiological state of both the insect and commodity will ultimately determine if the balance for effective control can be achieved. For example, the physiological characteristics of the commodity itself (e.g. seed cavities, air pockets and seeds which heat more slowly than the remainder of the commodity) may obstruct mortality by protecting pests from heat treatments (Armstrong and Mangan 2007). The commodity could potentially protect internal pests such as *T. leucotreta* larvae, while external pests could be controlled more effectively. However, aestivating *M. diplopterus* remained difficult to control with heat treatments, and the potential effect of the insect's physiological state while in aestivation raised further research questions. The focus of treatment development therefore shifted to control the internal pest, *T. leucotreta*, and to examine the potential preconditioning effect of CATTS treatments to address chilling injury, a risk associated with the cold sterilisation treatment applied for phytosanitary control of *T. leucotreta*.

Chilling injury in plums is a consequence of prolonged storage at low temperatures and presents as internal browning and flesh translucency, which is not appealing to the consumer. The ideal quarantine treatment for chill-sensitive plums would therefore be, to minimize fruit damage and economic costs by using the maximum temperatures that the commodity can tolerate for a period that would be sufficient to kill the target pest. It has been proposed that fruit and vegetables exposed to high temperatures in the field acquire a certain level of thermo-tolerance as a result of that (Lurie and Mitcham 2007). This acquired thermo-tolerance allows the product to be resistant to thermal damage due to stresses such as temperature disinfestation treatments. This pre-conditioning phenomenon was observed in Paper 1 and 2, where CATTS treatments did reduce the incidence of chilling injury in cold-sensitive plums stored under the cold sterilisation regime for treatment of *T. leucotreta*. Temperatures of 35 - 40°C initiate the synthesis of heat shock proteins which aid in the development of thermo-tolerance, which yields this pre-conditioning effect (Vierling 1991). Some sub-tropical fruit develops thermo-tolerance when preconditioned at temperatures below 40°C, before a higher temperature disinfestation treatment (Lurie and Mitcham 2007). The expression of heat shock proteins in CATTS treated plums were investigated during trials conducted here, but these results are not included in this dissertation. Future research on these heat shock proteins will provide insight into the pre-conditioning adaption mechanism of chill sensitive products after heat treatment and cold storage.

This same phenomenon of pre-conditioning that improves thermal tolerance may be the reason for the high survival rate of *M. diplopterus* when exposed to thermal treatments. Additionally, the previously alluded to high thermal tolerance of aestivating *M. diplopterus*, may also contribute to survival after treatment. A better understanding of the physiology of *M. diplopterus*, particularly during aestivation, is needed to provide insight into why the thermal treatments used during these trials were ineffective, and whether temperature treatments are even a feasible option for this pest. The following chapter (Paper 3 and Paper 4) addresses this need.

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# CHAPTER 3

# Paper 3

# Physiological changes in aestivating grain chinch bug (*Macchiademus diplopterus*)

## 3.1.1 Introduction

The evolutionary success of insects is evident by their widespread distribution over many ecological niches. The ability of insects to survive in extreme environments, such as, at high latitudes or altitudes, indicates the evolution of biochemical strategies that reduce the negative influence of stressors, like high and low temperatures (Doucet *et al*., 2009). Adaption strategies, which include behavioural changes as well as physiological responses, play a crucial role for survival during unfavourable conditions. Some insects, such as certain species of moths (Lepidoptera) and beetles (Coleoptera), undertake long migratory flights to escape low winter temperatures. In contrast, other insects seek shelter within local areas, such as tree bark crevices, which buffer them from intolerable environmental temperature changes (Dingle, 1996; Doucet *et al*., 2009). Often this shelter-seeking strategy is coupled with physiological changes to further mitigate against stresses such as low or high temperature. These physiological changes are referred to as cold or heat hardening. Cold and heat hardening are conceptually similar, and include strategies for suppression of metabolic rate, retention of body water/ fluids, conservation and reprioritization of energy and body fuel reserves, and mechanisms to preserve and stabilize organs and cells for weeks or months to increase the insect's survivability (Hochachka and Guppy, 1987; Sejerkilde *et al*., 2003; Storey and Storey, 2012). Aestivation, a prolonged period of dormancy, is such a survival strategy, and the grain chinch bug, *Macchiademus diplopterus* (Distant) (Hemiptera: Lygaeidae) requires phytosanitary control during its aestivation period.

*Macchiademus diplopterus* is an indigenous pest of cultivated grain crops and wild grasses in South Africa and a phytosanitary pest of export fruit due to its shelter-seeking behavior as it moves into aestivation during the summer months (Myburgh and Kriegler, 1967; Slater and Wilcox, 1973; Johnson and Addison, 2008). When host plants desiccate, adult *M. diplopterus* move to surrounding shelter sites. These could include fruit orchards, in which case aestivating bugs will seek shelter in the stalk or the calyx ends of fruit and consequently pose a phytosanitary risk to trading partners (Myburgh and Kriegler, 1967; Slater and Wilcox, 1973; Annecke and Moran, 1982).

The high thermal tolerance levels of *M. diplopterus*, in particular cold tolerance, was first alluded to when export fruit that was under cold storage during transit, was intercepted with live insects, and consignments were rejected (Myburgh and Kriegler, 1967; Malumphy, 2011; Malumphy *et al*., 2012). The heat tolerance capabilities of *M. diplopterus* was observed during research conducted by Johnson and Neven (2011). Their research indicated that *M. diplopterus* was the most tolerant species of three phytosanitary pest species tested, namely *M. diplopterus, Phlyctinus callosus and Thaumatotibia leucotreta*, when exposed to heat and controlled atmosphere treatments. Research presented in Papers 1 and 2 of this dissertation, also illustrated the ability of *M. diplopterus* to withstand high-temperature controlled atmosphere (CATTS) treatments. Thermo-tolerance research conducted by Okosun (2012) indicated that aestivating *M. diplopterus* could tolerate the high and low temperatures that could be used as phytosanitary treatments, making these less viable options for control. Pre-exposure of *M. diplopterus* to low temperatures through rapid and gradual pre-cooling, also enabled the insects to tolerate prolonged cold storage treatments. Okosun (2012) noted that the physiological state of the aestivating adult *M. diplopterus* could be the contributing factor enabling the insect to be more tolerant of thermal stresses than actively metabolising insects. Additionally, Okosun (2012) proposed that *M. diplopterus* becomes more tolerant of thermal stresses as it progresses further into the aestivation phase of its life cycle.

The ability of an insect to control production, metabolism, storage and transport of energy is a key element in its ability to maintain functions at a survival level during aestivation for prolonged periods. Energy reserves are stored in animal cells as glycogen, sugars or lipids and used on demand as a fuel source and in response to changing temperatures (Steele, 1985; Storey and Storey, 1986). Lipids are a key reserve class of molecules used by insects during diapause or aestivation, as lipids contain four times the energy available per unit mass than sugars. Molecular compositional changes that enhance antioxidant defences necessary to survive an enhanced stress response, forms another crucial key to surviving aestivation. The main functions of these compositional changes are to protect the cells during dormancy, and as a defence mechanism when transitioning from a low metabolic state to normal metabolism, defending against the rapid generation of reactive oxygen species (Hermes-Lima and Zenteno-Savín, 2002; Storey and Storey, 2012). Mechanisms to protect cells and cellular function against thermal and desiccation stresses includes the production of heat shock proteins (Goto and Kimura, 1998; Goto *et al*., 1998; Clark and Worland, 2008). Heat shock proteins have also been associated with aiding in the recovery

from cold shock and are upregulated during diapause (Denlinger *et al*., 2001; Rinehart *et al*., 2006). Many heat shock proteins are water soluble proteins located in the cytoplasm and in organelles such as the nucleus and mitochondria. Heat shock proteins such as Hsp70 are localized in mitochondria, nucleus and nucleolus and are essential in the cytoplasm (Ali and Banu, 1991).

This study aims to examine the physiology of aestivating *M. diplopterus* and identify molecular and biochemical compositional changes that occur as *M. diplopterus* moves through aestivation. Identifying these changes could provide insight into the ability of the organism to become more thermo-tolerant and withstand thermal phytosanitary treatments. The role of heat shock proteins, proteins involved in energy regulation and other defense mechanisms will be examined to determine their influence on the thermo-tolerant strategies of *M. diplopterus*. The use of soluble protein extraction methods will, therefore, exclude membrane proteins and shift focus to stress associated proteins such as heat shock proteins.

## 3.1.2 Materials and Methods

### Insects

Adult grain chinch bugs (*Macchiademus diplopterus*) were field collected in Ceres, (S 33:22’10”1, E 19:19’24”2), Western Cape, South Africa from November 2016 to June 2017). A number of collections were done from before the aestivation period started, and throughout aestivation, to obtain samples of insects representative of five different stages during the aestivation period, based on calendar weeks: *1)* before the aestivation cycle started (weeks 43 to 47); *2)* early in the cycle (week 48 and 50); *3)* midway (week 11 and 13); *4)* mid to late (week 20 and 22) and *5)* late in the aestivation cycle (week 24 and 26). A total of 13 collections were conducted. Aestivating *M. diplopterus* insects were collected from shelter sites under the bark of blue gum trees (*Eucalyptus globulus* Labill.), as well as from corrugated cardboard bands tied around the base of fruit trees. Each sampling week was made up of six replicates, each containing 40 insects. After collection, insects were placed in a -80°C freezer until samples were freeze-dried to remove moisture and preserve content for analysis. Extractions from the freeze-dried samples were used to determine the levels of macromolecules (proteins, lipids, sugars and glycogen) and soluble proteins present at each sampling point over the aestivation period. A 10 mg of sample was required for analyses, which was equivalent to 10 insects, therefore 10 grain chinch bugs were used per replicate for determination of macromolecules (6 replicates; n = 60 insects per sampling

week) and soluble proteins (3 replicates; n = 30 insects per sampling week), and the excess stored for future use.

**Determination of macromolecules - lipids, sugars, glycogen and proteins**

Extraction and analysis methods for the determination of total protein, lipid, sugar and glycogen content of freeze-dried samples of *M. diplopterus* were modified from Yuval *et al*. (1998), Olson *et al*. (2000), Lee *et al*. (2004) and Yi and Jean (2011), and the detailed modified protocol is described below.

***Extraction procedure:*** A 2% $Na_2SO_4$ solution was added to a microtube containing the ten insects per replicate, an electric grinding pestle was then used to grind samples for 30 s to 1 min. Ground samples were centrifuged for 1 min at 15 000 rpm and the supernatant was used in further analyses.

***Quantification:***

*Total protein measurement:* A Bradford protein assay protocol was followed to determine the total protein content of an aliquot taken from the supernatant produced in the extraction described above. Absorbance was measured at 595 nm using a microplate reader (Varioskan, Thermo Electron Corporation).

For the determination of lipid, sugar and glycogen content, a solution of chloroform and methanol (2:1) was added to the remaining supernatant. Samples were vortexed and centrifuged at 15 000 rpm for 4 min. The supernatant was separated into three layers: sugars in the top layer (~300µl); glycogen as a viscous layer in the middle; and lipids in the bottom layer (~700µl). Each layer per sample was transferred to a separate marked microtube. The lipids and sugars were analysed immediately, and the glycogen layer was washed with methanol and stored at -20°C for later analysis.

*Total lipids measurement:* The lipid layer was evaporated using nitrogen to create a pellet. Sulphuric acid (95 – 98%) was added, and samples were heated at 90°C using a heating block, for 10 min. After cooling to room temperature, phosphoric acid with vanillin was added, and the sample was placed on a shaker for 20 min at room temperature. Absorbance was measured at 530 nm using the microplate reader.

*Sugar measurement:* The anthrone reagent (mixture of sulfuric acid and anthrone) was added to the supernatant containing the sugar layer and inverted several times, after which it was boiled for 7.5 min. After heating, the samples were placed on ice and absorbance readings were taken at 620 nm on the microplate reader.

*Glycogen measurement:* Water was added to the glycogen pellet and heated at 70°C for 20 min. Anthrone was then added to the sample and boiled for 7.5 min. After the samples had cooled to room temperature, absorbance readings were taken at 630 nm on the microplate reader.

**Determination of soluble proteins**

***Extraction procedure:*** The method of Unruh *et al*. (2008) was modified as follows for the extraction of soluble proteins. For each of the 3 replicates, 250 µl of buffer solution was added to 10 mg insects in a microtube. The buffer solution consisted of TBS, 1M EDTA (pH8), 0.1% Triton, 0.05% ß-mercapto ethanol and 1X protease inhibitor (10:1:100:1, v/v). Samples were ground using a motorized pestle for three sessions (roughly 1 min per session) and then vortexed. Samples were centrifuged for 4 min at 14 000 rpm. The supernatant was transferred to a clean tube, and 250 µl of buffer was added to the pellet. The pellet with buffer was then ground for another three rounds (roughly 1 min per round). Supernatants were combined and centrifuged for 4 min at 14 000 rpm.

***Quantification:*** Soluble protein content quantification and analysis were carried out at the Centre for Proteomic and Genomic Research (CPGR) in Rondebosch, Cape Town, South Africa. Samples were transferred to protein Lobind tubes (Sigma 666505), and cold acetone was used to precipitate proteins through overnight incubation at -20˚C. Samples were then centrifuged for 15 min at 4˚C at 21000 x g. After the supernatant was removed, the pellet was washed three times with cold acetone after which the pellets were air-dried. Protein pellets were solubilized by resuspending them in 50mM triethylammonium bicarbonate (TEAB) and 2% Sodium dodecyl sulfate (SDS) and placed at 95 ˚C for 5 min. After which samples were centrifuged for five minutes at 10000 x g. Quantification was performed using the QuantiPro BCA assay kit (Sigma QPBCA).

A total of 50 µg of protein from each sample was transferred to a protein LoBind plate (Merck, 0030504.100). The protein was then reduced using tris (2-carboxyethyl) phosphine (TCEP) which was added to a final concentration of 10mM TCEP and incubated at 60˚C for 1 h. Samples were cooled to room temperature and then alkylated with methylmethanethiosulphonate (MMTS) which was added to a final concentration of 10mM MMTS and incubated at room temperature for 15 min. HILIC magnetic beads were added at an equal volume to that of the sample and a ratio of 5:1 total protein. The plate was then incubated at room temperature on a shaker at 900 rpm for 30 min, allowing for binding of the proteins to the beads. After binding, the beads were washed twice with 500µl of 95%

ACN for 1 min. Trypsin, made up in 50mM TEAB was added at a ratio of 1:10 total protein and the plate was incubated at 37˚C on the shaker for 4 h for digestion. After digestion, the supernatant containing peptides was removed and dried down.

Samples were resuspended in 0.1% trifluoroacetic acid (TFA) before clean up by Zip-Tip (Sigma Z720070). Subsequently the samples were dried and re-suspended in an LC loading buffer: 0.1% FA, 2.5% ACN.

**Gel section digestions:** Protein content analysis of gel sections was also carried out at CPGR. Soluble protein samples that were extracted were separated in a gel matrix according to size. Larger proteins move slower through the matrix than smaller proteins. Gel bands of ~ 80 – 85kDA were cut for further analysis.

Gel bands were destained twice with 100mM ammonium bicarbonate (AmBic), 50% acetonitrile (ACN) for 45 min with agitation at room temperature. Excess liquid was removed, and the gel pieces were dehydrated with ACN and subjected to vacuum centrifugation for 5 min. Protein was then reduced by rehydrating the gel pieces in 2mM tris-carboxyethyl phosphine (TCEP), made up in 25mM AmBic, followed by agitation at room temperature for 15 min. Excess liquid was removed, and protein alkylated by covering the gel pieces in 20mM iodoacetamide (IAA), made up in 25mM AmBic, and incubating in the dark at room temperature for 30 minutes. After alkylation, the gel pieces were washed three times with 25mM AmBic at room temperature for 15 minutes with agitation. Excess liquid was removed, and gel pieces were dehydrated as before. Protein was digested by rehydrating the gel pieces in 0.02mg/ml trypsin made up in 50mM Ambic. Gel pieces were incubated on ice for 1 h, and excess liquid was removed. Subsequently the gel pieces were then covered with 50mM AmBic and digested overnight at 37˚C. After digestion, the excess liquid was transferred to a new tube, and the gel pieces were soaked in 0.1% trifluoroacetic acid (TFA) for 1 h at 37 ˚C. Excess liquid was removed and added to the first extract. Samples were then dried by vacuum centrifugation and the buffer replaced by analytical grade water. Samples were dried down once more and resuspended in 0.1 % formic acid (FA), 2 % ACN made up in analytical grade water for LCMS analysis.

LCMS analysis was conducted with a Q-Exactive quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, USA) coupled with a Dionex Ultimate 3000 nano-HPLC system. Peptides were loaded on a C18 trap column (300 µm × 5 mm × 5 µm). The trap is then switched in-line with the analytical column for loading of peptides for 26 minutes. Thereafter the trap is switched offline so as to prevent loading of hydrophobic contaminants.

Chromatographic separation was performed with a PepAcclaim C18 column (75 µm × 25 cm × 2 µm). The solvent system employed was solvent A: LC water (Burdick and Jackson BJLC365); 0.1% FA and solvent B: ACN, 0.1% FA. The multi-step gradient for peptide separation was generated at 300 nL/min as follows: time change 6 min, gradient change: 3.5 – 9% Solvent B, time change 45.5 min, gradient change 9 – 24.6% Solvent B, time change 2 min, gradient change 24.6 – 38.7% Solvent B, time change 2.1 min, gradient change 38.7 – 52.8% Solvent B, time change 0.4 min, gradient change 52.8 – 85.4%. The gradient was then held at 85.4% solvent B for 10 minutes before returning it to 3.5% solvent B for 15 minutes to condition the column. The mass spectrometer was operated in positive ion mode with a capillary temperature of 320°C. The applied electrospray voltage was 1.95 kV.

**Statistical analysis of data**

Statistical analysis of total proteins, lipids, glycogen, and sugars was analyzed using a one-way analysis of variance with STATISTICA version 13 (Statsoft Inc., 2017). ANOVA-generated P-values and the significant differences between means were determined using Fisher's least significant differences (LSD) test with a 95% confidence interval.

The peptide sequence were searched against *Oncopeltus fasciatus* downloaded from Baylor College of Medicine sequencing center. Raw files were processed using Progenesis QI for Proteomics (Non-linear Dynamics, UK) software and regulated proteins (p-value <0.05 and fold change ≥ 2), containing at least two unique peptides, were reported. Relative quantification was conducted using Progenesis QI for Proteomics (Nonlinear Dynamics, UK). Data processing included peak picking, run alignment, and normalization (singly charged spectra were removed from the processing pipeline). Relative quantification was based on four biological replicates per condition using non-conflicting peptides. A protein with a fold change ≥ 2 with a corresponding p-value of <0.05 was considered regulated. Database interrogation was performed with Byonic Software (Protein Metrics, USA) using the *Oncopeltus faciatus* database sourced from Baylor College of Medicine sequencing centre (www.hgsc.bcm.edu).

In-gel samples were processed by in-gel digestion. In-solution samples were digested using an automated HILIC magnetic bead-based workflow. In preparation for the HILIC magnetic bead workflow, beads were aliquoted into a new tube and the shipping solution removed. Beads were then washed with 250µl wash buffer (15% ACN, 100mM Ammonium acetate (Sigma 14267) pH 4.5) for one minute. This was repeated once. The beads were then resuspended in loading buffer (30% ACN, 200mM Ammonium acetate pH 4.5). The rest of

the process described hereafter was performed using a Hamilton Mass-STAR robotics liquid handler (Hamilton, Switzerland). A total of 50 µg of protein from each sample was transferred to a protein LoBind plate (Merck, 0030504.100). Protein was reduced with tris (2-carboxyethyl) phosphine (TCEP; Sigma 646547) which was added to a final concentration of 10mM TCEP and incubated at 60˚C for one hour. Samples were cooled to room temperature and then alkylated with methylmethanethiosulphonate (MMTS; Sigma 208795) which was added to a final concentration of 10mM MMTS and incubated at room temperature for 15 minutes. HILIC magnetic beads were added at an equal volume to that of the sample and a ratio of 5:1 total protein. The plate was then incubated at room temperature on the shaker at 900RPM for 30 minutes for binding of protein to beads. After binding, the beads were washed twice with 500µl of 95% ACN for one minute. For digestion Trypsin (Promega PRV5111), made up in 50mM TEAB was added at a ratio of 1:10 total protein and the plate was incubated at 37˚C on the shaker for four hours. After digestion, the supernatant containing peptides was removed and dried down. Samples were resuspended in 0.1% trifluoroacetic acid (TFA, Sigma T6508) prior to cleanup by Zip-Tip (Sigma Z720070). Thereafter, samples were dried down once more and then resuspended in LC loading buffer: 0.1% FA, 2.5% ACN. Peptides were then analyzed by LCMS, and relative quantification results were obtained.

The functions of annotated genes for *Macchiademus diplopterus* were hypothesised based on homology with gene sequences from other available sources. Annotations were provided by an online source OrthoDB (https://www.orthodb.org). Deductions are based on the recognition of gene sequence similarities which indicate shared ancestry (Kriventseva *et al*., 2019).

Venn diagrams (http://bioinfogp.cnb.csic.es/tools/venny/) were used to determine proteins in common and exclusive during the different evaluation periods. Heat maps were generated using XLSTAT-Biomed (https://www.xlstat.com/en/solutions/biomed).

### 3.1.3 Results

**Compositional changes of macromolecules (total proteins, lipids, and carbohydrates)**

Significant differences for each of the macromolecules analysed were observed for the different sampling periods (before, early and mid to late). No significant differences were observed, however, between the two weeks for each of the aestivation sampling periods (early, mid, mid-late and late). Data from the two sampling weeks for each period were therefore pooled.

The level of glycogen present in samples collected during week 44 (before aestivation) was significantly higher than in samples from all the other time points (Fig. 3.1.1). The glycogen content almost doubled from 28 µg/mg DW (dry weight) to 51 µg/mg DW from week 43 to 44, before insects were observed entering aestivation. After this increase, the level of glycogen decreased to between 6 and 12 µg/mg DW during weeks 45 to 47 (still before the insect moved into aestivation). Once the insect entered aestivation (week 48), an increase in glycogen levels occurred until mid-late aestivation (week 20 and 22), with levels reaching 42 µg/mg DW. During the late aestivation period (week 24 and 26) glycogen levels started to decrease to 29 µg/mg DW.

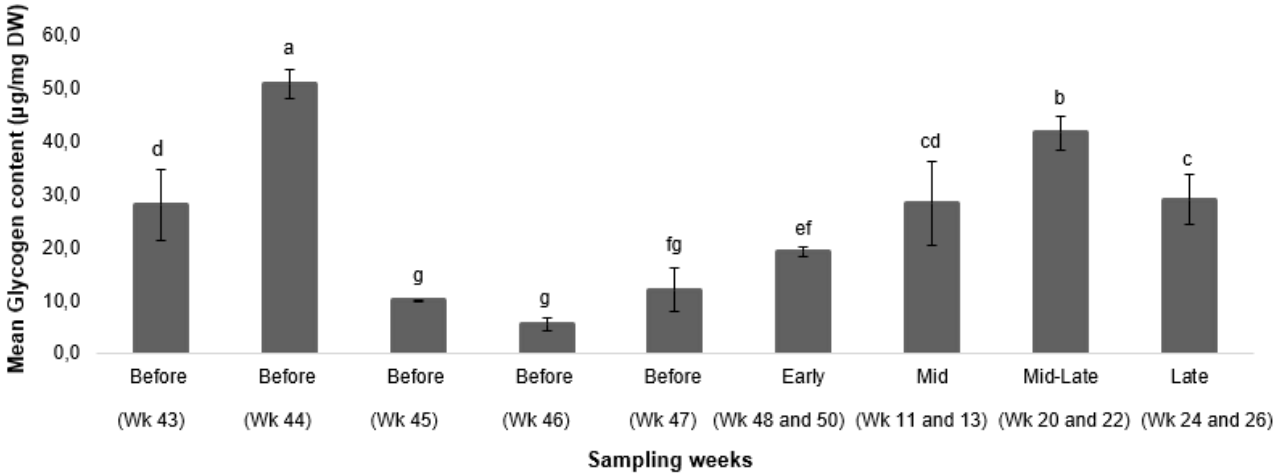



Figure. 3.1.1. Mean glycogen content ± standard deviation (µg/mg DW) of *Macchiademus diplopterus* before aestivation (weeks 43 to 47) and during the early (weeks 48 and 50), mid (weeks 11 and 13), mid-late (weeks 20 and 22) and late (weeks 24 and 26) aestivation periods (November 2016 – June 2017). *DW = dry weight*

Across the five weekly collections made during the before aestivation period, sugar content decreased significantly from 166 µg/mg DW (week 43) to 50 µg/mg DW (week 47) (Fig. 3.1.2). This was followed by a slight increase and then decrease as the aestivation period started (early (weeks 48 and 50) and mid (weeks 11 and 13)), but levels were not significantly different from weeks 45 and 46, before aestivation. By mid-late aestivation, sugar content increased significantly again to 110 µg/mg DW. A decrease in sugar content occurred from mid-late (weeks 20 and 22) to the late aestivation period (weeks 24 and 26).

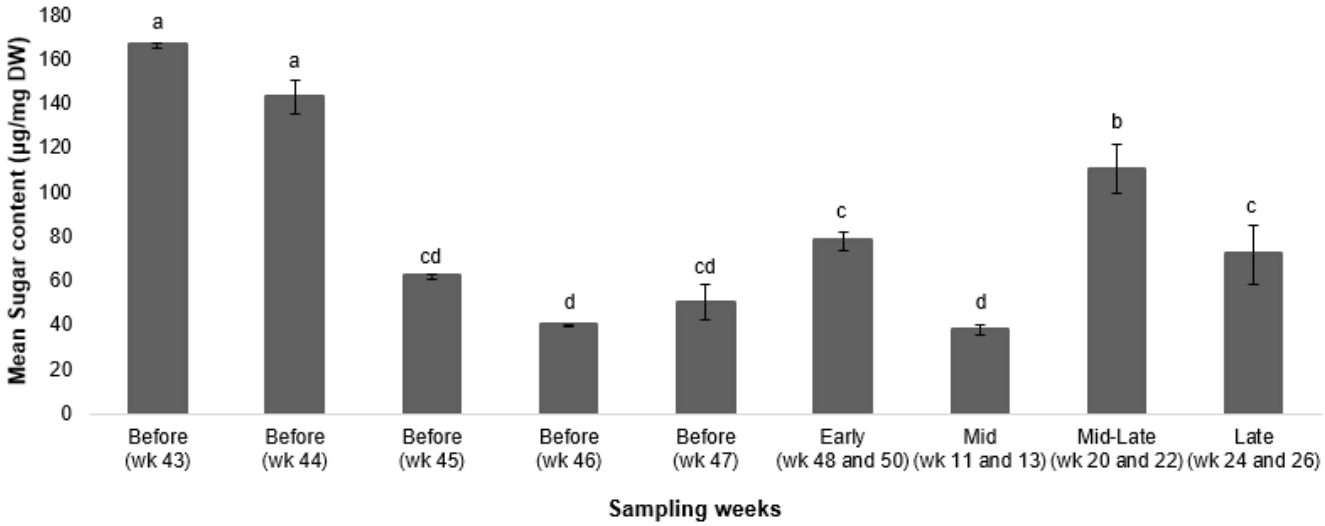



Figure. 3.1.2. Mean sugar content ± standard deviation (µg/mg DW) of *Macchiademus diplopterus* before aestivation (weeks 43 to 47) and during the early (weeks 48 and 50), mid (weeks 11 and 13), mid-late (weeks 20 and 22) and late (weeks 24 and 26) aestivation periods (November 2016 - June 2017). *DW = dry weight*

During the before aestivation period, the total lipid content increased significantly reaching a peak of 153 µg/mg DW by week 45 (Fig. 3.1.3). Lipid content started to decrease gradually as the insect progressed into and through aestivation. By mid-late aestivation (weeks 20 and 22) lipid content was 97.7 µg/mg DW. This was followed by a significant decrease in lipid content in late aestivation (weeks 24 and 26) samples at 56 µg/mg DW.

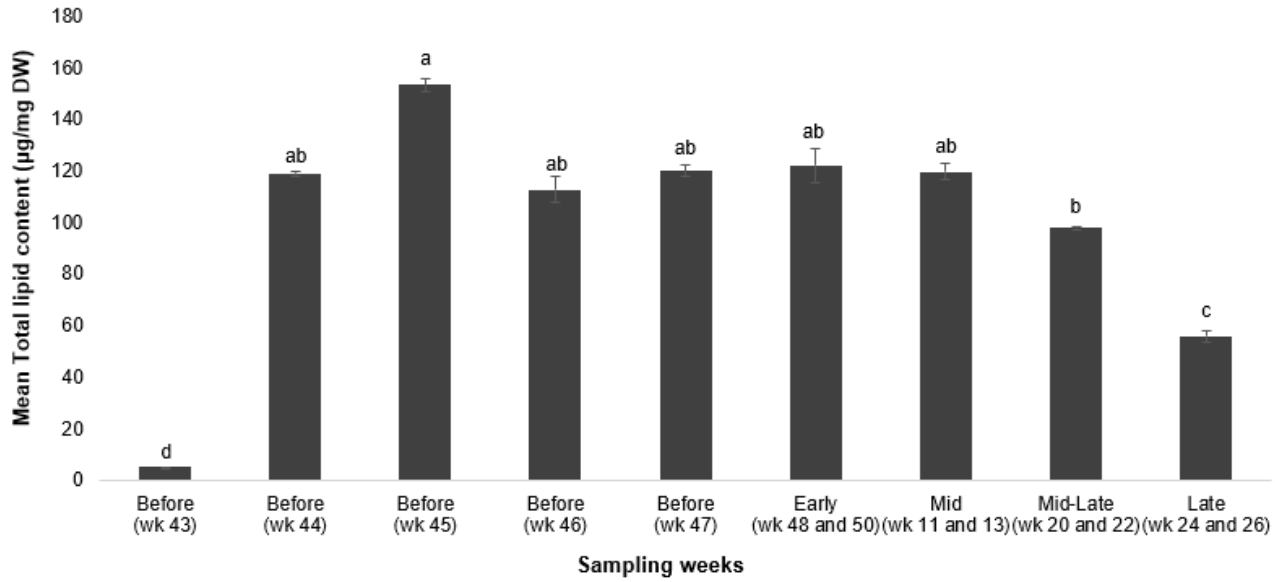



Figure. 3.1.3. Mean total lipid content ± standard deviation (µg/mg DW) of *Macchiademus diplopterus* before aestivation (weeks 43 to 47) and during the early (weeks 48 and 50), mid (weeks 11 and 13), mid-late (weeks 20 and 22) and late (weeks 24 and 26) aestivation periods (November 2016 – June 2017). *DW = dry weight*

A steady decrease in the total protein content of samples collected weekly during the before aestivation period (weeks 43 to 47) was observed, from 156 µg/mg DW to 36 µg/mg DW (Fig. 3.1.4). By early aestivation (weeks 48 and 50) the total protein content was 16 µg/mg DW. During aestivation, total protein content did increase slightly, but by late aestivation the level of protein, 24 µg/mg DW, was not significantly different from what it was at the start of aestivation (16 µg/mg DW).

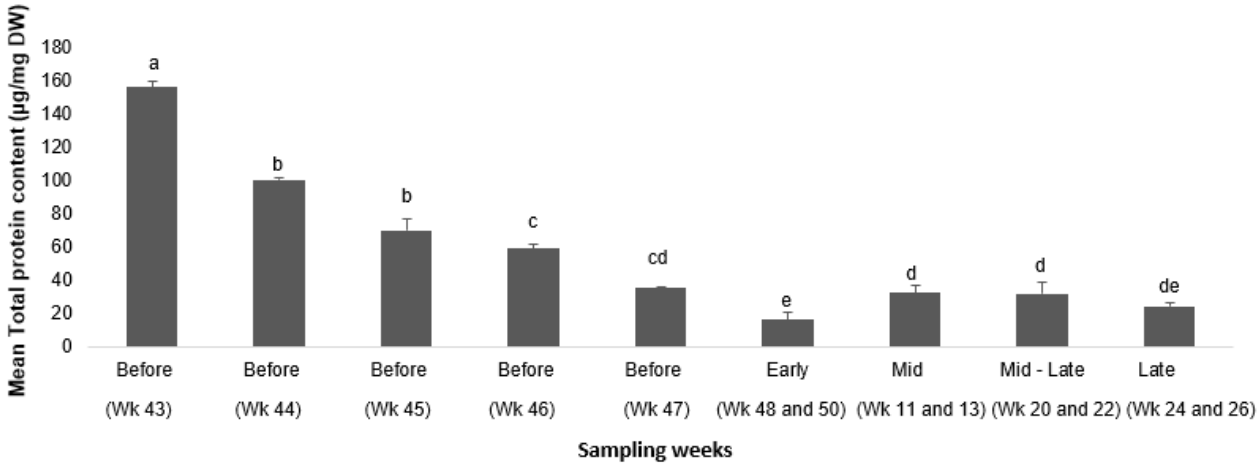



Figure. 3.1.4. Mean total protein content ± standard deviation (µg/mg DW) of *Macchiademus diplopterus* before aestivation (weeks 43 to 47) and during the early (weeks 48 and 50), mid (weeks 11 and 13), mid-late (weeks 20 and 22) and late (weeks 24 and 26) aestivation periods (November 2016 – June 2017). *DW = dry weight*

Figure 3.1.5 illustrates an overview of changes in glycogen, sugar and lipid content over the sampling period (weeks 43 to 26). The highlighted sections indicate when the most significant changes occurred. During week 45 (before aestivation), an increase in lipid content occurred, with a decrease in sugar and glycogen content. During the early aestivation period (weeks 48 and 50), the lipid content stabilized, while increases in glycogen and sugar content occurred. An increase in sugar and glycogen content was observed from the mid to mid-late aestivation periods (weeks 11 to 22), in conjunction with a decrease in lipid content. During the mid-late to late period a decrease in total lipids, sugar and glycogen content was observed.

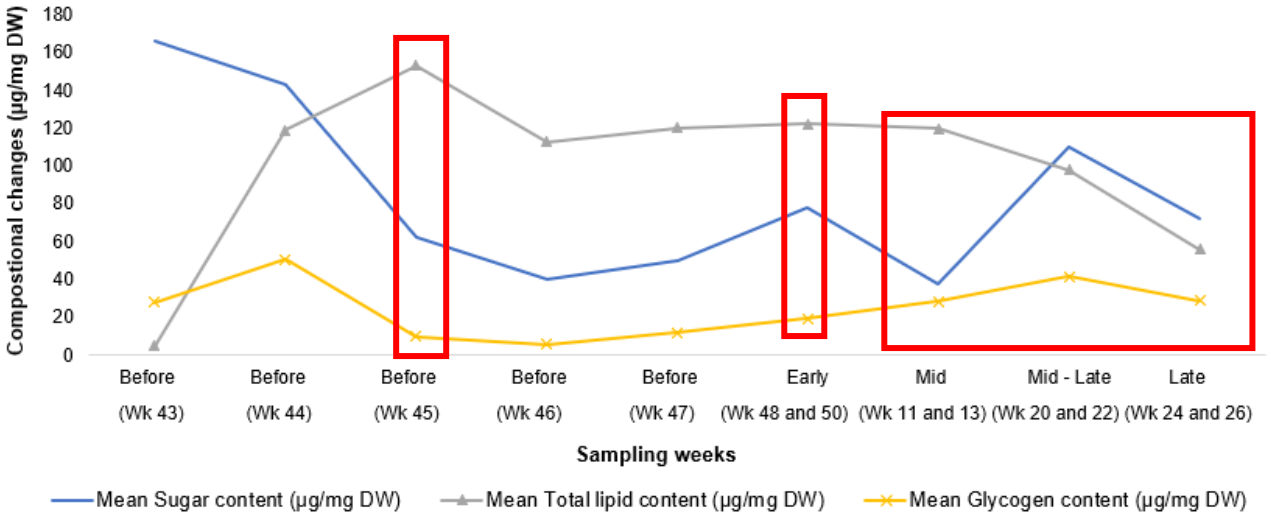



Figure. 3.1.5. Comparison of changes in total lipid, glycogen and sugar content in *Macchiademus diplopterus* samples collected before and during the aestivation cycle (November 2016 – June 2017). *DW = dry weight*

***Soluble proteins comparison:***

The soluble protein extraction method yielded different compositional profiles of different elements of proteins for each of the sampling periods (before, early until late). No significant differences between the protein elements identified for each weekly collection during the before aestivation period, as well as the two weekly collections during aestivation, were observed. Additionally, no significant differences were observed for all the samples collected during the mid, mid-late and late aestivation periods. Therefore, the data for the before aestivation period were pooled, as well as the mid, mid-late and late aestivation periods.

An overview comparing the different elements for the different sampling periods (before (pooled); early; and mid until late (pooled) is illustrated in Fig 3.1.6 as a Venn chart. The chart illustrates that 281 elements were exclusively found in before samples, 48 elements were in common between before and early samples, 38 were exclusive to early samples, 230 were common between before and mid until late aestivation samples, and 89 elements were in common across all the sampling periods (before, early until late). No elements were found to be exclusive to the mid until late aestivation period.

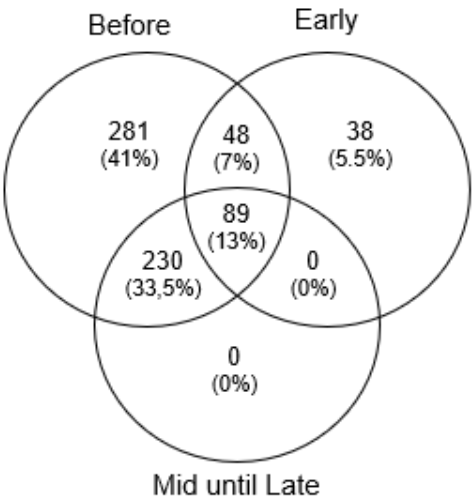
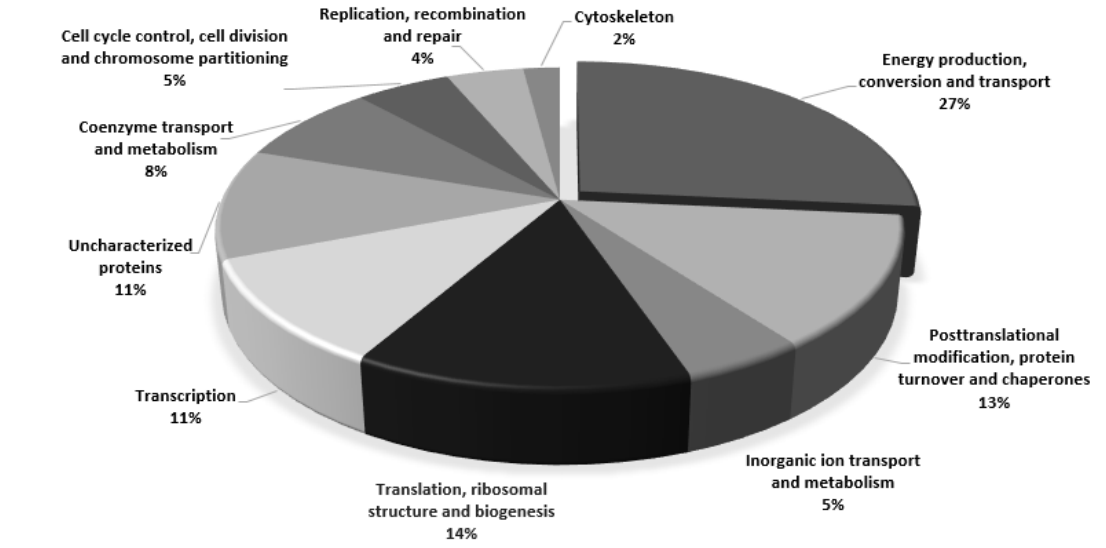
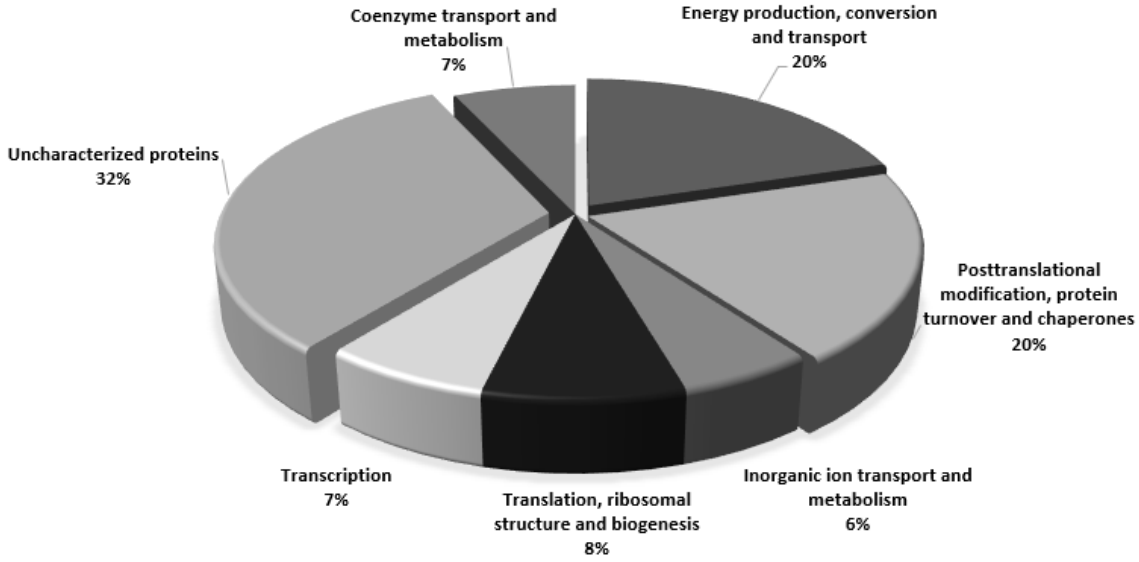



Figure. 3.1.6. Venn diagram illustrating the elements that were exclusive to, and in common between, the aestivation periods before, early and mid to late aestivation. *Note: 281 elements were exclusive to before aestivation samples, 48 elements were in common in before and early aestivation samples, 38 were exclusive to early samples, 230 were in common between before and mid to late aestivation, and 89 elements were in common during the whole sampling period.*

The deduced functions of the annotated genes for *M. diplopterus* allowed for the different protein elements present during the sampling period to be placed into functional groups. Comparison of the functional groups at different times during the sampling period indicates if an increase or decrease in activity or abundance occurred. Elements identified for each sampling period could theoretically be involved in more than one functional group. This was taken into account when calculating the percentage of each group. Figure 3.1.7 illustrates the percentage of different functional groups present in the before and early aestivation samples. A decrease in abundance was observed for various functional groups as the insects entered aestivation. The highest percentage of elements observed during the before aestivation period (Fig 3.1.7A) were involved in energy production and conversion (approx. 27%), this functional group decreased by approx. 7% in abundance as the insect entered the early stages of aestivation (Fig 3.1.7B). A 7% increase was also observed for elements involved in posttranslation modification as the insect progressed into early aestivation. The early aestivation insects contained a larger number of uncharacterized proteins (32%) compared to the 11% observed before the insect entering aestivation. Elements involved in functions such as replication, recombinant and repair, cell motility and elements involved in the cytoskeleton, which were present before aestivation, were not detected during the early aestivation period.




(A)




(B)

Figure. 3.1.7. Percentages of functional groups present in the soluble protein elements extracted from *Macchiademus diplopterus* collected before entering aestivation (weeks 43 to 47) (A) and during early aestivation (weeks 48 and 50) (B) in November and December 2016. (A) Represents functional groups from 648 elements; and (B) functional groups from 175 elements. Some gene products have more than one function. *Note: The functional group energy production and transport includes: Lipid and carbohydrate transport and metabolism. An example of elements from energy production group included Acetyl-CoA carboxylase and fatty acid synthase; Cytoskeleton = Actin; Posttranslational modification, protein turnover, chaperones = Heat shock protein 70 family and small heat shock protein HSP20*

Figure 3.1.8 illustrates and highlights selected functional groups of elements identified during the early and pooled mid to late aestivation periods. Overall, a decrease in abundance of functional groups occurred, rather than in the number of individual elements identified. A 7% increase in the abundance of elements involved in energy production and conversion took place from early aestivation (Fig. 3.1.8A) to mid-late aestivation (Fig. 3.1.8B), with a 16% decrease in uncharacterized proteins. During week 48 and 50 elements

which were high in abundance included malate dehydrogenase, which is involved in energy production and conversion.

Comparing early and mid-aestivation a 4% decrease in individual elements occurred in the posttranslational modification group, which contains proteins such as heat shock proteins. This indicates a decrease in the number of individual elements but not necessarily their individual abundance. Although the total protein content decreased significantly over time as seen under analysis of the macromolecules, the levels of the individual proteins that make up the total protein content changed over time. This is evident in the gel images generated in the soluble protein bioassays (Fig. 3.1.9). The gel image indicates two bands that became darker over time (as the insect moved into aestivation). Darkening and thickening of the bands implies that the concentration of protein increased. Gel section digestion and identification of proteins in the isolated band (~80-85kDA in size), revealed that from the early aestivation period (weeks 48 and 50) to the mid aestivation period (weeks 11 and 13), 14 elements were common to both periods, but 15 were unique to the early period, and the number unique to the mid aestivation period, increased to 46 (Fig 3.1.10). Analysis of isolated gel sections indicated that the mid aestivation samples contained a significantly higher number of heat shock proteins compared to the early aestivation samples. Indicating that although the individual elements of the posttranslational modification group decreased the abundance of individual elements increased from early to mid.

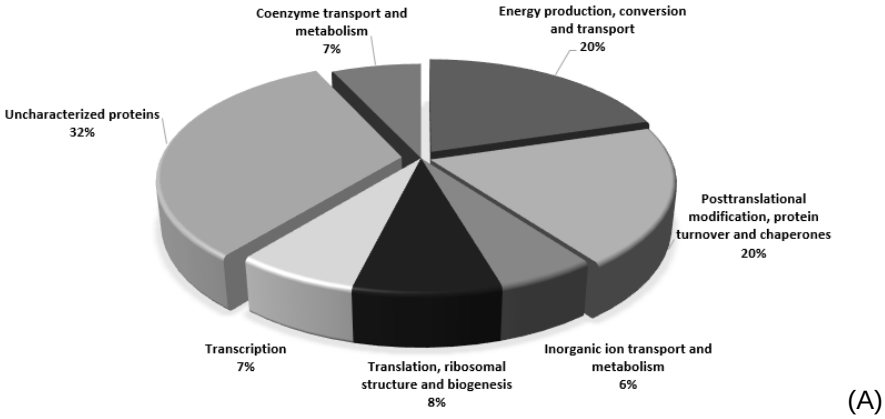



Figure. 3.1.8. Percentages of functional groups present in the soluble protein elements extracted from *Macchiademus diplopterus* during early aestivation (weeks 48 and 50) (A) and mid to late (weeks 11 until 26) aestivation functional groups from 175 elements (B) from December 2016 to June 2017 functional groups from 319 element. *Note: The functional group energy production and transport includes: Lipid and carbohydrate transport and metabolism. An example of elements from energy production group included Acetyl-CoA carboxylase and fatty acid synthase; Posttranslational modification, protein turnover, chaperones = Families of heat shock proteins 70 and 90*

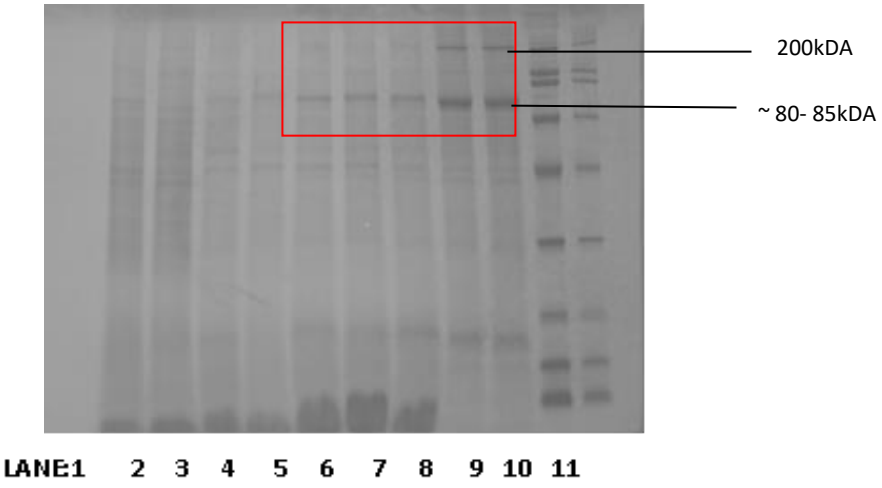



Figure. 3.1.9: Gel image of soluble proteins of *Macchiademus diplopterus* before and during aestivation (early to mid-aestivation period). *Note: Lane 1 = Before aestivation (Week 43), 2 = Before aestivation (Week 44), 3 = Before aestivation (Week 45), 4 = Before aestivation (Week 46), 5= Before aestivation (W47), 6 = Early aestivation (Week 48), 7 = Early aestivation (Week 50), 8 = Mid aestivation (Week 11), 9 = Mid aestivation (Week 13), 10 and 11 = molecular weight markers. Protein molecular weight (in daltons) are indicated on the right*

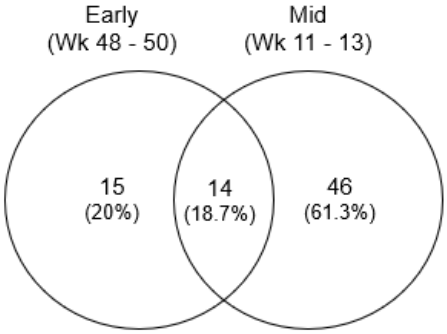



Figure. 3.1.10. Venn diagram illustrating the unique and common proteins from gel bands cut at ~ 80 – 85kDA (Fig. 3.1.9) of *Macchiademus diplopterus* samples collected during early aestivation (weeks 48 and 50) and mid aestivation (weeks 11 and 13).

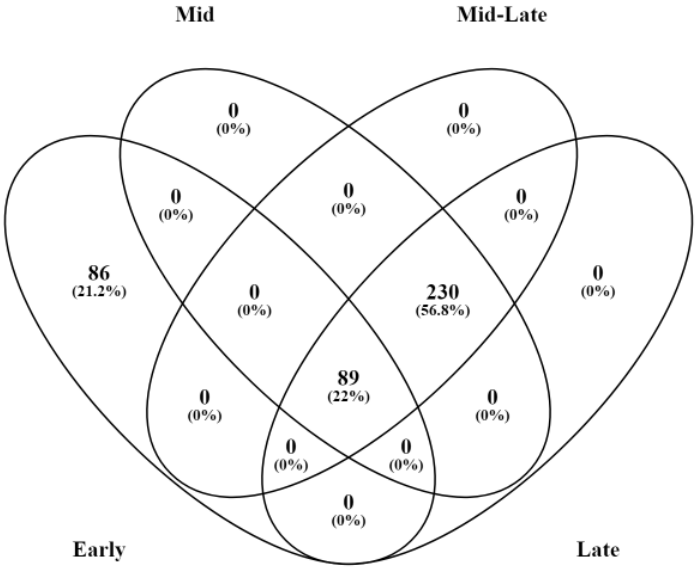



Figure. 3.1.11. Venn diagram illustrating the elements that were exclusive to, and in common between, the aestivation periods early, mid, mid-late and late aestivation of *Macchiademus diplopterus*. *Note: 86 elements were exclusive to early aestivation samples, 230 elements were common to mid, mid-late and late aestivation and 89 were in common in all four aestivation periods.*

Comparing the aestivation periods with each other revealed 86 elements unique to the early aestivation period, 230 elements in common between mid until late aestivation periods and 89 in common between all four aestivation periods with (Fig. 3.1.11). These 89 elements were also present during the before aestivation period (see Fig. 3.1.6). A heat map (Fig. 3.1.12) was generated to visualize the abundance or intensity of expression of these common elements over time, based on some of the identified proteins. These included the heat shock proteins and others involved in energy storage, synthesis, and transport.

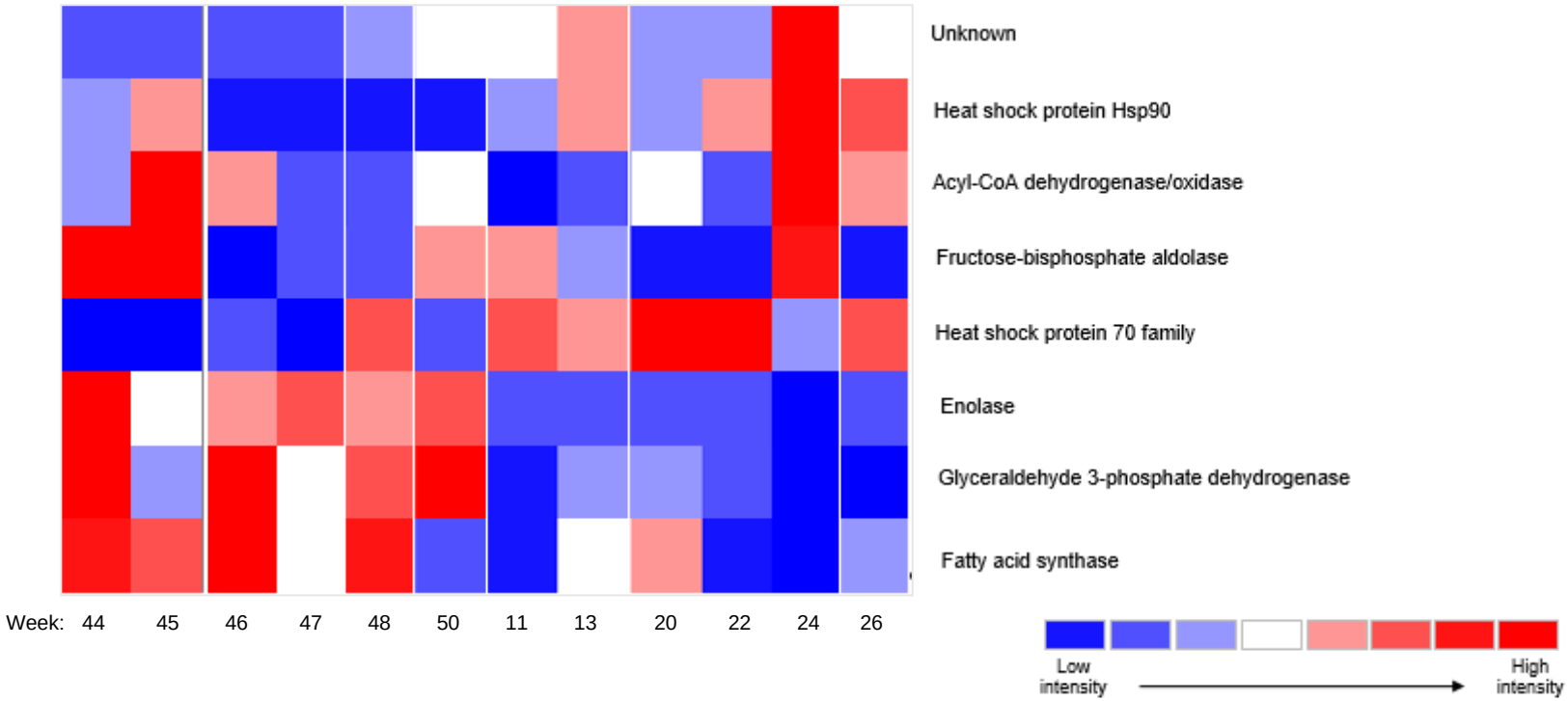



Figure. 3.1.12. Heat map illustration of intensity of expression of elements in common, identified from *Macchiademus diplopterus* samples collected before and during aestivation (weeks 44 – 26) from November 2016 to June 2017.

During the before aestivation period (weeks 44 to 47) Acyl-CoA dehydrogenase/oxidase, fructose-bisphosphate aldolase, enolase, glyceraldehyde 3-phosphate dehydrogenase and fatty acid synthase all had high levels of expression (Fig. 3.1.12). This was particular to weeks 44 and 45 for Acyl-CoA dehydrogenase/oxidase and fructose-bisphosphate aldolase, which decreased in expression after that, but had high levels of expression again by late aestivation (week 24). For the later three, enolase, glyceraldehyde 3-phosphate dehydrogenase and fatty acid synthase, high expression continued into early aestivation (weeks 48 and 50) but decreased after that. The differences in expression of these various before and during aestivation may be related to the interconversions in lipids, sugars and glycogen, presented above.

Expression of the Hsp70 family is confined to the aestivation period (Fig. 3.1.12), as very low intensity of expression occurs before aestivation. Hsp90 also increases in expression during late aestivation. In addition, an unknown protein was expressed during aestivation, and requires characterization. Of the unique heat shock proteins (not included in Fig. 3.1.12) identified during mid until late aestivation, smHsp20 families were amongst the highest in abundance. During early aestivation heat shock proteins which form part of the Hsp10 and 60 families were identified as unique when compared to mid until late aestivation.

### 3.1.4 Discussion

Aestivation involves an ancient signalling and regulatory system enabling organisms to suppress growth and development during periods in which water, nutrients, and energy levels are limited, and is considered a form of less severe dormancy, where physiological changes can be reversed rapidly (Storey and Storey, 2012). Organisms entering and maintaining a hypometabolic state during aestivation require biochemical changes that ensure conservation of energy, preservation of cell structure and function, and the ability to arouse from aestivation when conditions are favourable. Changes elucidated in the present study, in the composition of macromolecules and specific proteins in *M. diplopterus* during aestivation, provides new information on the physiology of this phytosanitary pest, and helps explain its tolerance for thermal stress, which makes temperature postharvest disinfestation treatments challenging.

Energy usage during aestivation is minimized through reduction in metabolic rate, but this also results in a reduction in the normal synthesis and degradation of macromolecules (carbohydrates, lipids and proteins). Therefore, other preservation strategies require up-regulation to ensure survival. These mechanisms include enhanced antioxidant defences

and increased chaperone proteins (e.g. heat shock proteins) to manage different stress responses (Kültz, 2005). The biochemical regulation of organisms during aestivation has two main options to regulate low metabolic activity. One is through reversible controls suppressing cell function, examples including the inhibition of enzyme and functional protein activity. The other option is through compositional changes of selected proteins due to differential transcription, translation or degradation, for example the up-regulation of the urea cycle enzymes, antioxidant enzymes, iron binding enzymes and heat shock proteins (Storey and Storey, 2007; Ramnanan *et al*., 2009; Ip and Chew, 2010; Storey and Storey, 2011). During extended periods of dormancy, the ability of oxidatively damaged macromolecules to degrade and resynthesize is low due to the suppression of these energy consuming processes. Through elevated levels of antioxidants, the oxidative damage to macromolecules could be limited, as with increased levels of elevated chaperones, which limit the accumulation of misfolded proteins during long term dormancy.

Before aestivation, *M. diplopterus* feeds on host plants and can fly to aestivation sites in which they seek shelter during the aestivation period when a low metabolic state is implemented for survival. As *M. diplopterus* moves into and through aestivation its energy reserves, with regard to glycogen, sugars and lipids change. The decrease in glycogen observed during the period before the insect entered into aestivation, could be as a result of energy usage for migratory flight to aestivation sites. A rapid decrease in glycogen could occur through either direct utilization as an energy source during a cold shock or through flight (Amiri and Bandani, 2013). This is due to the ability of glycogen and sugars to be interconverted during temperature changes. The increase in glycogen content during the early to mid-aestivation period, in conjunction with a decrease in sugar content could have been due to inter-conversion taking place at this time, and is also reflected in the changes in the functional groups and the increase of elements involved in energy production. During the mid to mid-late aestivation period, an increase in the sugar levels occurred. This could be a result of a stress response in which adaption to heat (during summer) was needed, and the production of trehalose and sugar alcohols were induced from glycogen (Storey, 1997).

The importance of body fat during dormancy is evident in the increase in lipid content in *M. diplopterus* during the weeks before the onset of aestivation. *Macchiademus diplopterus* appears to create a large lipid reserve before entering aestivation, probably as an energy source for survival during its prolonged period of low metabolic activity. The same phenomenon is seen in another Hemipteran pest of graminaceous plants, *Eurygaster integriceps* Put. (Hemiptera: Scutellidae), a member of a complex of species that make up

a group generally known as Sunn pests (Critchley, 1998). *Macchiademus diplopterus* and *E. integriceps* display similar behaviour in that the latter, also undergoes an obligatory diapause period during which it ceases to feed and remains in sheltering sites. In *E. integriceps* however, the diapause period is made up of two phases: aestivation during late summer and autumn, and hibernation during winter, collectively termed as the overwintering period (Brown, 1962a; Brown, 1962b; Critchley, 1998). Bashan *et al*. (2002) found that an increase in stored lipids in pre-diapause adult *E. integriceps* occurred in spring, which would then be used to support their energy requirements during aestivation and hibernation. During the overwintering period of *E. integriceps*, approximately 25% of the insect's stored fat reserves is consumed and a large portion of the population may die if these fat reserves are inadequate (Critchley, 1998). The higher levels of lipids within the insect's body fat are due to its higher capacity for lipid synthesis than glycogen synthesis (Amiri and Bandani, 2013). An insect that contains higher levels of lipids has a more significant energy reserve, which could also be used for post aestivation or diapause activity (Ellers and Van Alphen, 2002). During aestivation, it is crucial that the organism conserves energy and rations the use of stored fuels. It needs to retain body water and has to dispense nitrogenous end products and stabilize organs and cells for extended periods (Storey and Storey, 2012). Lipid accumulation is therefore also important in aestivation or diapause termination, which may indicate why the lipid levels in *M. diplopterus* started to decrease significantly during the late aestivation period. *M. diplopterus* has a long flight from wheat fields to aestivating sites in which the conversion of carbohydrates to lipids takes place for energy storage during aestivation. This could potentially be why there was an increase in both glycogen and lipids before entering aestivation as the insects are actively flying around, but simultaneously preparing for energy conservation.

Although macromolecule analysis indicated that total protein content of *M. diplopterus*, before and during aestivation, decreased, analysis of individual soluble proteins indicated that changes occurred that also support the hypometabolic state of aestivation.

The increase in abundance of acyl-CoA desaturase which occurred during late aestivation, coincides with the onset of winter occurs. The enzyme Δ 9 – acyl-CoA desaturase has an essential role in temperature adaptation for insects exposed to low temperatures by increasing the ratio of unsaturated to saturated fatty acids in cell membranes to maintain the liquid crystalline phase (Kayukawa *et al*., 2007). An increase in Δ 9–acyl-CoA desaturase expression, therefore, has a role in cold hardiness. The enzyme fructose-bisphosphate aldolase is involved in the glycolysis and gluconeogenesis pathways. Research conducted

by Muise and Storey (1997) on larvae of the freeze-avoiding gall moth (*Epiblema scudderiana* Clemens) indicated the suppression of fructose-bisphosphate activity, preventing recycling of glycerol carbon back to glycogen during the winter months when polyols must be sustained for antifreeze protection. During the late aestivation period, the decrease in enolase and glyceraldehyde 3-phosphate dehydrogenase would have affected the glycolysis pathway function in *M. diplopterus*. Glyceraldehyde 3-phosphate dehydrogenase and enolase are functional in glycolysis for the breakdown of glucose for energy and carbon. The decrease in glyceraldehyde 3-phosphate dehydrogenase indicates higher activity of glucose breakdown compared to later in the aestivation cycle, where different mechanisms were used to create energy sources. Enolase is a key glycolytic enzyme which has a species-specific function in insects (Kikuchi *et al*., 2017). Fatty acid synthase is a multi-enzyme protein that catalyses fatty acid synthesis. During extended periods of nonfeeding, lipid metabolism is essential as an energy source (Gilbert, 1967).

The identification of proteins present at the different stages before and during aestivation, shows how *M. diplopterus* can become more tolerant to thermal stresses such as heat and cold treatments as the aestivation period progresses. The increase in heat shock proteins (HSP) such as smHsp20, Hsp60, 70, 83 and 90 during the mid and mid-late aestivation periods, compared to the early aestivation period is significant. The importance of Hsp70 and 90 in an organism's thermal response is well documented in literature (Lindquist and Craig, 1988; Guo and Feng, 2018). Heat shock proteins are identified and related to their molecular weight, for example, 70kDa HSP is referred to as Hsp70. These Hsp70s consist of HSC70 (heat shock cognate 70) and stress-inducible isoforms that are upregulated with long term cold exposure (Clark and Worland, 2008). These highly conserved proteins act as chaperones to stabilize and refold denatured proteins. In doing so, the insect can prevent the formation of cytotoxic aggregates (Parsell and Lindquist, 1993; Hartl, 1996; Fink, 1999). An increase in the expression of heat shock proteins is a reliable indicator of thermo-tolerance or thermal protection. In *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) the development of thermo-tolerance is linked to the production of Hsp70, which is virtually absent from non–stressed cells (Lurie and Jang, 2007).

The level of expression of Hsp70 will influence the outcome of thermo-tolerance; too little (induced by either a too short stress treatment or lowering the temperature) will restrict the level of thermo-tolerance that develops (Lurie and Jang, 2007). Over expression of Hsp70 has resulted in problems with *D. melanogaster* lines in that treated lines were unable to tolerate the stress, compared to control lines. Overproduction of Hsp70 can, however,

increase thermo-tolerance in some stages of development. Insects from warmer climates may have tighter control of HSP production in order to enable them to limit unnecessary production of HSPs, which could be an energy costly process. Thermo-tolerance has also been correlated with induction of HSP in insect pests after exposure to non-lethal temperatures (Neven, 2000). The presence of Hsp70 induced by heat conditioning treatment in codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae), is related to the survival rate of the insect after application of a quarantine heat treatment (Lurie and Jang, 2007). Accumulation of Hsp70 followed thermo-resistance to high temperatures in *D. melanogaster*. In studies conducted on *C. pomonella*, a decrease in Hsp70 was linked to disappearance of thermo-tolerance induced by a 35°C pre-treatment and the sensitivity of the insect to 50°C quarantine treatment (Yin *et al*., 2006). Research conducted by Kinene *et al*. (2019) indicated the potential of the Hsp90 gene to increase whiteflies, *Bemisia tabaci* (Hemiptera: Aleyrodidae), chances of survival against heat stress and other biotic factors. The increase, therefore, in Hsp70 and Hsp90 observed in *M. diplopterus*, is an indication that the insect has an increased ability to tolerate thermal stresses more effectively. The activity of various proteins and enzymes are involved in the defence mechanisms that protect *M. diplopterus* against thermal stresses.

Regulators through epigenetic mechanisms (e.g. DNA methylation and histone modification) of transcriptional suppression provides a mechanism for gene silencing during periods of hypometabolism for functions such as development and differentiation (Fraga *et al*., 2007; Storey and Storey, 2007; Storey and Storey, 2012). During a cell stress response mRNA's are reprogrammed and recruited to stress granules which are preserved and made available for rapid translation when organisms are exiting their hypometabolic state. These stress granules is another area for research in aestivating organisms (Kedersha and Anderson, 2009; Storey and Storey, 2012). Specific mRNA is preserved over winter in the fat cells of larvae of *Eurosta solidaginis* Fitch (Diptera: Tephritidae) and *Gynaephora groenlandica* (Lepidoptera: Lymantriidae) which allows for a rapid recovery to original levels when an increase in temperature occurs (Levin *et al*., 2003). Thus, indicating not one element or protein is solely responsible for the insects' survival and adaption to thermal and desiccation stresses, but an array of components working in synergy are able to achieve this.

Further research is required to better understand the environmental or genetic triggers that enable the insect to initialize the production of heat shock proteins etc. as defence mechanisms, allowing them to tolerate a variety of stresses. The large number of soluble proteins isolated during this study, but that could not be characterized, need to be identified,

as they may also shed some light on other mechanisms and elements involved in the physiology of aestivation in *M. diplopterus*.

Analysis of mechanisms involved in transcriptional and translational control will provide insight into the regulation of aestivation and the mechanisms insects use to control their metabolic arrest and extend their life span during unfavourable conditions (Storey and Storey, 2012). The research conducted in this study has indicated that multiple factors play a role in preparing and protecting *M. diplopterus* against thermal and desiccation stresses which it may encounter during its aestivation cycle. The changes observed in abundance or intensity of heat shock proteins is but one of the components that enable *M. diplopterus* to adapt for survival. The increased levels of Hsp70 and 90 observed here, as aestivation progressed, now provides concrete evidence for the mechanisms behind the possible increase in thermal tolerance in aestivating *M. diplopterus* reported by Okosun (2012). Increasing thermal tolerance in aestivating *M. diplopterus* makes development of a thermal phytosanitary treatment challenging, as a single temperature that will be effective throughout aestivation must be developed using insects at their most tolerant, which is difficult to ascertain. No blanket thermal treatment will be effective to control *M. diplopterus* throughout the aestivation period, which is when they affect fruit and can be found contaminating export fruit consignments.

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# Paper 4

# Macromolecular composition of aestivating grain chinch bug (*Macchiademus diplopterus)* in response to thermal stresses

## 3.2.1 Introduction

Through enzymatic reactions, physiological adaptions and behavioural changes, insects have developed the capacity to survive and flourish under a wide range of thermal environments (Lee and Denlinger, 1991). Within a single species, temperature tolerance may vary considerably according to developmental stage. The aestivation or diapause status could potentially also play a role in thermo-tolerance capabilities, as the low metabolic activity makes the diapausing insect more tolerant to environmental stresses such as heat (Denlinger and Yocum, 1998). One of the key phytosanitary pests of export fruit in South Africa, is the grain chinch bug, *Macchiademus diplopterus* (Distant) (Hemiptera: Lygaeidae). Since *M. diplopterus* is endemic to the southwestern Cape region of South Africa, countries importing fruit from South Africa impose phytosanitary or quarantine restrictions to prevent the introduction of a new pest on imported fruit. Pre- and postharvest measures to control the threat that *M. diplopterus* poses to growing export markets, are lacking. Developing thermal treatments that could be successfully used as phytosanitary postharvest mitigation treatments has been problematic in the past. *Macchiademus diplopterus* has displayed cold tolerance behaviour on various occasions surviving pre-cooling and cold storage treatments (Myburgh and Kriegler, 1967; Malumphy, 2011). Papers 1 and 2 in this dissertation report on the ability of *M. diplopterus* to also withstand high-temperature treatments during controlled atmosphere temperature treatment system (CATTS) studies, which resulted in unsatisfactory mortality levels. Paper 3 examined the physiological and molecular adaptions that take place as the insect moves into and through aestivation, and highlighted the significant biochemical changes that take place and contribute to changes in thermal tolerance.

Thermal stresses, which includes heat treatments, can alter the quantity and type of macromolecule present in the cell. The most conspicuous change occurs in protein synthesis, whereby a sudden increase in temperature could result in the present protein synthesis cycle ceasing, and the initiation of expression of a new set of proteins, such as heat shock proteins (Denlinger and Yocum, 1998). Biochemical

changes, and the expression of heat shock proteins in field-collected aestivating *M. diplopterus* was reported in Paper 3. However, the changes that can occur in response to a thermal stress, such as heat in a CATTS treatment or cold during long term cold storage, was not determined. Such external stresses can further influence thermal tolerance, beyond the natural tendency for improved thermal tolerance as the aestivation period progresses as seen in Paper 3.

Animal cells require energy, which is acquired through the ingestion of food (which contains complex carbohydrates, fats and proteins) subsequently broken down to simpler components which are essential for life processes. Insects have similar processes of food digestion, utilisation and storage, where energy not required immediately is stored as trehalose, glycogen or fat (Klowden, 2008). Glycogen is a polymeric form of glucose and can be depolymerized on demand as a fuel source (Steele, 1985). Glycogen and other cryo-protectants can be interconverted in response to temperature fluctuations (Storey and Storey, 1986). Glycogen is therefore not only an energy source, but can provide resources for defence mechanisms during thermal stresses as well.

Lipids are a key reserve used by insects during diapause or aestivation as an energy source, as they contain four times the energy per unit mass of sugars. Lipids and glycogen also have a crucial role as a fuel source for prolonged periods of flight for insects flying to overwintering sites (Amiri and Bandani, 2013). During cold acclimation, insects can restructure their membranes by increasing the unsaturated fatty acid to saturated fatty acid ratio to prevent freezing (Michaud and Denlinger, 2006; Kayukawa *et al*., 2007; Clark and Worland, 2008).

Carbohydrates are low in molecular weight and are involved in anti-freeze and cryo-protectant systems (Lee and Denlinger, 1991). It was found that cryo-protectant systems consist of glycerol and other low molecular weight sugars and polyhydric alcohols (e.g. sorbitol and mannitol) (Sømme, 1982; Clark and Worland, 2008). Research conducted on *Drosophila melanogaster* (Diptera: Drosophilidae) found when exposure to low temperatures occurred for 72 hours, elevated levels of glucose and trehalose were observed. The increase in glucose and trehalose which was observed was correlated with improved chill tolerance for survival following a cold shock at -5°C (Overgaard *et al*., 2007). Studies by Michaud and Denlinger (2006) on

*Sarcophaga crassipalpis* Macquart (Diptera: Sarcophagidae) indicated that increased levels of glycolytic metabolites occurred due to gradual cooling to low temperatures (0°C). The trehalose levels in *S. crassipalpis* were however reduced compared to above mentioned elevated levels in *Drosophila melanogaster*. This phenomenon illustrates how an organism's response in relation to the up or down-regulation of components, is unique.

To determine the mechanisms through which *M. diplopterus* can tolerate a variety of stresses, the insect's enzymatic reactions, physiological functions and behaviour (aestivation period), which are all influenced by temperature, need to be examined. The aim of the present study was to examine the physiology of aestivating *M. diplopterus* and its biochemical makeup in response to thermal stresses, and the potential influence of this biochemistry on survival under unfavourable conditions. Previous studies have indicated that *M. diplopterus* becomes more tolerant of thermal stresses deeper into its aestivation cycle. Investigating the effect of CATTS (heat) treatments and cold storage regimes (cold sterilisation and dual temperature regimes) on the biochemical composition (protein, glycogen, sugar and lipid content) of aestivating *M. diplopterus*, may provide insight into the insect's adaptation process when exposed to thermal phytosanitary treatments, and potential improved survival.

## 3.2.2 Materials and Methods

### Insects

Adult grain chinch bugs (*Macchiademus diplopterus*) were field collected in Ceres, (S 33:22'10"1, E 19:19'24"2), Western Cape, South Africa. Collections were done during the early phase of the aestivation cycle in December 2016 (week 48), and another was done midway through the aestivation cycle in March 2017 (week 11). This covered the period during which the highest volumes of fruit are exported. Aestivating *M. diplopterus* insects were collected from shelter sites under the bark of blue gum trees (*Eucalyptus globulus* Labill.), as well as from corrugated cardboard bands tied around the base of fruit trees. Collected insects were placed in perforated microtubes for treatment and storage. Insects were subjected to CATTS treatments in conjunction with different cold storage regimes (cold sterilisation and dual temperature) and mortality was determined at seven different evaluation time points. For each aestivation sampling period (early and mid) a total of 10 080 insects was used in

treatments and storage. This was made up of 6 replicates (40 insects per rep) per evaluation time point per cold storage regime per treatment.

**Treatments**

CATTS treatments were carried out in a laboratory-scale CATTS chamber manufactured by Techni-Systems (USA). The CATTS chamber is a flow through, airtight system with computerized temperature, dew point and atmospheric controls. The atmosphere inside the chamber is manipulated by injection of nitrogen, carbon dioxide and synthetic air (medical air in a compressed cylinder), humidified by micro-misting nozzles and passed over a heater element to increase air temperature. The chamber can accommodate 2 plastic trays (58.4cm x 38.1cm x 40.6cm). Probes allow data logging of temperature changes, gas levels (nitrogen, carbon dioxide and synthetic air) and relative humidity. Relative humidity of 80% was maintained, and a controlled atmosphere (1% $O_2$ and 15% $CO_2$) was applied. CATTS treatment conditions and the subsequent cold storage regimes used (standard cold sterilisation and a dual temperature) are described in detail in Table 3.2.1. The two CATTS treatments applied were 1) a treatment with a fast ramping heat rate to a high temperature, but with a short holding period; and 2) a slow ramping heat rate to a high temperature and with a long holding period. Control samples were not subjected to CATTS treatments, but only held at the two cold storage regimes. Control samples for comparison with cold stored samples were kept in the laboratory at room temperature. For cold storage, additional days were added to the two regimes to reach 45 days of total storage, as this represents the maximum shipping duration for stone fruit.

Table 3.2.1: CATTS and cold treatments applied to *Macchiademus diplopterus* during the 2016/2017 season. Relative humidity of 80% was maintained and controlled atmosphere set at 1% $O_2$ and 15% $CO_2$ (balance N2).

| Treatments | CATTS treatment conditions | Cold storage regime |
|---|---|---|
| **1** | 80°C.h$^{-1}$ ramp to air temperature of 56°C<br>Hold for 5 min | **Cold sterilisation:**<br>-0.5°C for 22 d plus 20 days at -0.5°C (standard regime)<br>plus another 3 days at -0.5°C to reach total storage of 45 days |
| **2** | 12°C.h$^{-1}$ ramp to air temperature of 35°C<br>Hold for 5 hours | |
| **3** | No CATTS treatment<br>(control) | |
| **4** | Same as Treatment 1 | **Dual temperature**:<br>10 days at -0.5°C + 7 days at 7.5°C followed by 25 days at -0.5°C (standard regime)<br>plus another 3 days at -0.5°C to reach total storage of 45 days |
| **5** | Same as Treatment 2 | |
| **6** | No CATTS treatment<br>(control) | |

Mortality, determined immediately after treatment and at different evaluation time points during cold storage (see Table 3.2.2), was assessed at each evaluation, as well as 8 h later to allow for metabolic adjustment of the insects to ambient temperature.

Table 3.2.2: Evaluation time point for mortality and macromolecule composition analysis of *Macchiademus diplopterus* during the 2016/2017 season. *Note: ST= standard cold sterilisation regime and DT = dual temperature.*

| | Evaluation time point |
|---|---|
| **1** | After CATTS (all samples) |
| **2** | After 10 days at -0.5°C (DT samples were moved to 7.5°C at this time) |
| **3** | After 17 days at -0.5°C (ST samples)<br>After 7 days stored at 7.5°C (DT samples - before being moved back to -0.5°C) |
| **4** | After 24 days of total storage (all samples) |
| **5** | After 31 days of total storage (all samples) |
| **6** | After 38 days of total storage (all samples) |
| **7** | After 45 days of total storage (all samples) |

Mortality was assessed by prodding the insects to check for any leg or antennal movement, indicating survival. Dead and alive insects were separated for determination of the macromolecule composition for each group. Mortality was calculated as the percentage of dead insects relative to total treated insects for each treatment.

After mortality assessment insects were placed in a -80°C freezer until the samples were freeze-dried to remove moisture and preserve content for analysis. 10 mg of sample was required for analyses, which was equivalent to 10 insects, therefore 10 grain chinch bugs were used per replicate for determination of macromolecules (6 replicates per aestivation period, evaluation time point, treatment and cold storage regime (N = 5040) and the excess stored for future use. Extractions from the freeze-dried samples were used to determine the levels of macromolecules (proteins, lipids, sugars and glycogen) present during the early and mid-aestivation sampling periods.

**Determination of macromolecules (total proteins, lipids, sugars, and glycogen)**

Extraction and analysis methods for the determination of total protein, lipid, sugar and glycogen content of freeze-dried samples of *M. diplopterus* were modified from Yuval *et al*. (1998), Olson *et al*. (2000), Lee *et al*. (2004) and Yi and Jean (2011).

***Extraction procedure:*** A 2% $Na_2SO_4$ solution was added to a microtube containing the ten insects per replicate, an electric grinding pestle was then used to grind samples for 30 s to 1 min. Ground samples were centrifuged for 1 min at 15 000 rpm and the supernatant was used in further analyses.

***Quantification:***

*Total protein measurement:* A Bradford protein assay protocol was followed to determine the total protein content of an aliquot taken from the supernatant produced in the extraction described above. Absorbance was measured at 595 nm using a microplate reader (Varioskan, Thermo Electron Corporation).

For the determination of lipid, sugar and glycogen content, a solution of chloroform and methanol (2:1) was added to the remaining supernatant. Samples were vortexed and centrifuged at 15 000 rpm for 4 min. This separated the supernatant into three layers: sugars in the top layer (~300 µl); glycogen as a viscous layer in the middle; and lipids in the bottom layer (~700 µl). Each layer per sample was transferred to a

separate marked microtube. The lipids and sugars were analysed immediately, and the glycogen layer was washed with methanol and stored at -20°C for later analysis.

*Total lipids measurement:* The lipid layer was evaporated using nitrogen to create a pellet. Sulphuric acid was added, and samples were heated at 90°C for 10 min. After cooling to room temperature, phosphoric acid with vanillin was added, and the sample was placed on a shaker for 20 min at room temperature. Absorbance was measured at 530 nm using the microplate reader.

*Sugar measurement:* Anthrone was added to the supernatant containing the sugar layer and inverted several times, after which it was boiled for 7.5 min. After heating, the samples were placed on ice and absorbance readings were taken at 620nm on the microplate reader.

*Glycogen measurement:* Water was added to the glycogen pellet and heated at 70°C for 20 min. Anthrone was then added to the sample and boiled for 7.5 min. After the samples had cooled to room temperature, absorbance readings were taken at 630nm on the microplate reader.

**Statistical analysis of data**

Insect mortality data for all treatments and evaluation periods were analysed using a four-way (factorial) ANOVA with STATISTICA version 13 (Statsoft, Inc., 2017). AVONA-generated P-values and the significant differences between means were determined using Fisher's least significant differences (LSD) test with a 95% confidence interval.

## 3.2.3 Results

### Insect mortality after treatment and during cold storage

Aestivation period, treatments (CATTS and cold storage regime) and evaluation time point (storage duration) all had a significant effect on the mortality of *M. diplopterus* ($F_{(30,168)} = 17.29$, $\rho < 0.0001$). The percentage mortality of all samples is presented in Table 3.2.3 See Appendix D for an outline of the interactions between all the factors in the analysis of mortality.

Trt 1 and 4, which represent the fast ramping heat rate and short holding period, yielded a 100% mortality immediately after treatment. Samples checked 8 h later confirmed that the treatment alone resulted in zero survival of treated insects, and

subsequent cold storage would not be required to control insects, if these treatments were applied. Therefore, no further analysis of samples in Trt 1 and 4 were done.

Trt 2 and 5, with the slow ramping heat rates and long holding period, did result in some mortality immediately after treatment (Trt 2 early samples – 40%, mid samples – 56%; Trt 5 early – 35%, mid – 33%) (Table 3). With subsequent cold storage, these levels of mortality increased over time. In the early samples, for both Trt 2 and Trt 5, only 10 days at -0.5 °C (evaluation time point 2) increased mortality to 100%. The ST regime samples remained at -0.5 °C for the duration of evaluation, and 100% mortality was maintained in these insects. However, the removal of DT regime samples after 10 days at -0.5 °C to 7.5 °C for 7 days, reduced mortality for those samples to 72% at that point (evaluation time point 3). After the 7 days, DT regime samples were returned to -0.5 °C, and consequently mortality increased to 100% again.

Trt 2 and Trt 5 also highlight the difference between the responses of early and mid aestivation samples to the thermal stresses of CATTS followed by cold treatment. This difference is also seen in Trt 3 and Trt 6, samples that underwent cold storage without CATTS treatment. In all treatments, the early samples were more susceptible, displaying higher levels of mortality than mid aestivation samples. For example, at evaluation time point 4 (24 days of total cold storage), 100% mortality was achieved in Trt 2 early samples, compared to 80% in Trt 2 mid samples (Table 3.2.3). This was similar for the Trt 3 samples stored under the ST regime, where 100% mortality was observed for early samples and 84% for mid samples. Under the DT regime, this difference between early and mid was also observed in Trt 5 and Trt 6, but it was highly pronounced in Trt 6 samples (not subjected to CATTS). Here, mortality at evaluation time point 4, was 86% in early samples, compared to only 7% in mid aestivation samples.

Although, overall, mid aestivation insects were more thermal tolerant than early aestivation insects, the DT regime stored mid aestivation insects had the highest thermal tolerance of all insects. Figure 3.2.1 illustrates how, after 45 days of total storage, mid aestivation insects stored under the DT regime, still exhibit some survival, particularly in Trt 6 where mortality was only 74%.

Table 3.2.3: Mean percentage mortality of *Macchiademus diplopterus* due to thermal stress (CATTS and cold treatment) during different aestivation (early and mid) at each evaluation time point. See Table 3.2.1 and 3.2.2 for descriptions for treatments and evaluation time points. *Values that do not have letters (a, b, c….) in common are significantly different from each other. Factorial ANOVA table for Factor A (Evaluation time point), Factor B (Aestivation period: Early or Mid).and Factor C (Treatments): F (30,168) = 17.29, ρ = <0.0001*

| | | | | Mortality (%) for each evaluation time point (A) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | (B) | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Treatments (C) | Cold sterilisation | Trt1 | Early | 100 ± 0.00a | - | - | - | - | - | - |
| | | | Mid | 100 ± 0.00a | - | - | - | - | - | - |
| | | Trt2 | Early | 40 ± 4.54mn | 100 ± 0.00a | 100 ± 0.00a | 100 ± 0.00a | 100 ± 0.00a | 100 ± 0.00a | 100 ± 0.00a |
| | | | Mid | 56 ± 9.62kl | 46 ± 4.74m | 63 ± 5.52jk | 80 ± 6.54defgh | 94 ± 3.33ab | 93 ± 2.25abc | 100 ± 0.00a |
| | | Trt3 control | Early | 0 ± 0.00p | 33 ± 7.07n | 64 ± 9.36ijk | 100 ± 0.00a | 100 ± 0.00a | 100 ± 0.00a | 100 ± 0.00a |
| | | | Mid | 0 ± .00p | 32 ± 1.65n | 49 ± 0.71lm | 84 ± 2.28cdefg | 100 ± 0.00a | 100 ± 0.00a | 100 ± 0.00a |
| | Dual temperature | Trt4 | Early | 100 ± 0.00a | - | - | - | - | - | - |
| | | | Mid | 100 ± 0.00a | - | - | - | - | - | - |
| | | Trt5 | Early | 35 ± 11.67n | 100 ± 0.00a | 72 ± 6.76hij | 99 ± 1.08a | 95 ± 2.00ab | 100 ± 0.00a | 100 ± 0.00a |
| | | | Mid | 33 ± 1.67n | 73 ± 7.66hi | 83 ± 7.61defg | 78 ± 5.00efgh | 89 ± 10.06bcd | 87 ± 6.56bcde | 94 ± 6.07abc |
| | | Trt6 control | Early | 0 ± 0.00p | 35 ± 3.00n o | 89 ± 1.96bcd | 86 ± 5.27bcdef | 94 ± 0.83ab | 100 ± 0.00a | 100 ± 0.00a |
| | | | Mid | 0 ± 0.00p | 14 ± 2.89 | 14 ± 7.73o | 7 ± 12.37op | 1 ± 2.65p | 77± 8.87fgh | 74 ± 7.77gh |

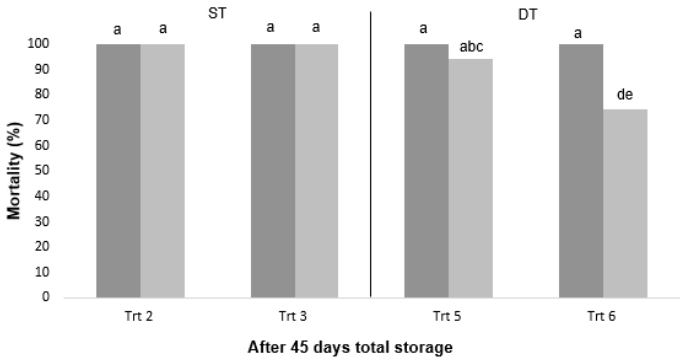


Figure 3.2.1. Mortality of *Macchiademus diplopterus* as a result of thermal stress (CATTS and cold treatment) during different aestivation (early and mid) after 45 days total cold storage, during the 2016/2017 season. *See Table 1 for descriptions for treatments, ($F_{(6,56)}$ = 6.1268, ρ < 0.0001). Bars with different letters (a,b,c…) are significantly different.*

Comparison of mortality during cold storage, with (Trt 2 and 5) and without (Trt 3 and 6) prior CATTS treatment, irrespective of the cold storage regime or aestivation period, highlights the additive effect of the CATTS treatment. This is evident at evaluation time point 2, that is 10 days after CATTS treatment (or not) and cold storage at -0.5 °C (Fig. 3.2.2). CATTS treatments applied for both early and mid-aestivating insects yielded a higher mortality than those only exposed to cold storage.

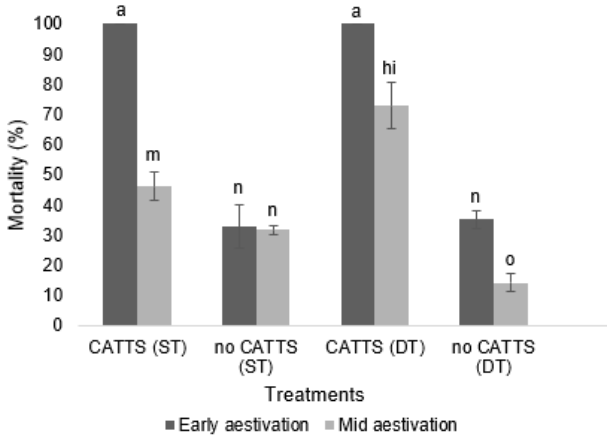


Figure 3.2.2. Mortality of *Macchiademus diplopterus* as a result of thermal stress (CATTS and cold treatment) during different aestivation (early and mid) after 10 days at -0.5 °C, during the 2016/2017 season. *See Table 3.2.1 and 3.2.2 for descriptions for treatments and evaluation periods. ($F_{(30,168)}$ = 17.29, ρ < 0.0001). Bars with different letters (a,b,c…) are significantly different.*

## Biochemical composition

The concentrations of the different macromolecules in early and mid aestivating *M. diplopterus* before application of treatment, therefore representing the natural state of the insect, are presented in Table 3.2.4. Insects collected during the mid aestivation period had

double the amount of total proteins (32 ± 5.08 µg/mg DW), and half the amount of sugars (38 ± 2.31 µg/mg DW), compared to early aestivation insects. The levels of total lipids and glycogen were similar for early and mid aestivation insects.

Table 3.2.4: Mean macromolecule concentration of *Macchiademus diplopterus* collected during the early and mid aestivation periods, before thermal treatments were administered. DW = dry weight

| | **Early aestivation** | **Mid aestivation** |
|---|---|---|
| **Total protein content (µg/mg DW)** | 16.05 ± 4.89 | 32.17 ± 5.08 |
| **Total lipid content (µg/mg DW)** | 122.3 ± 6.62 | 119.6 ± 3.27 |
| **Sugar content (µg/mg DW)** | 77.75 ± 4.16 | 37.66 ± 2.31 |
| **Glycogen content (µg/mg DW)** | 21.09 ± 4.62 | 28.3 ± 7.93 |

To more closely examine how macromolecule concentration may have changed due to thermal stress and influenced survival of insects during treatment, the macromolecule concentrations of alive and dead insects were compared. Since the mortality data (Table 3.2.3) showed that insects collected during the mid aestivation period are more thermotolerant than during early aestivation, this comparison is focussed on data relating to mid aestivating insects only. Table 3.2.5 summarises the macromolecule content of alive and dead insects, immediately after CATTS treatment and after subsequent 10 days cold storage at -0.5 °C (Trt 2 and Trt 5), as well as for insects not subjected to CATTS treatments and just cold stored (Trt 3 and Trt 6). After CATTS treatments, the insects that survived, had significantly lower levels of total protein and lipid content than those that died during treatment ($F_{(1,16)} = 9.29$, $\rho = 0.0077$; $F_{(1,16)} = 28.86$, $\rho = <0.0001$) respectively. The sugar content of insects that survived CATTS treatment was similar for Trt 2 and Trt 5, 65.56 ± 5.16 µg/mg DW and 65.84 ± 1.22 µg/mg DW, respectively, but was significantly lower in those that died during Trt 5 ($F_{(3,16)} = 28.84$, $\rho = <0.0001$), even though Trt 2 and Trt 5 were the same until evaluation time point 1. Glycogen content did not differ significantly in alive and dead insects after CATTS treatment. For all treatments, after 10 days cold storage at -0.5 °C, the insects that survived, had lower levels of total protein and lipid content than those that were dead. This was significantly so for Trt 6 for total protein content ($F_{(1,16)} = 9.29$, $\rho = 0.0077$) and Trt 3 and Trt 6 for total lipid content ($F_{(3,16)} = 28.84$, $\rho = <0.0001$). Also for all treatments, and after 10 days cold storage at -0.5 °C, surviving insects had significantly higher levels of sugar than those that died. Glycogen levels were also higher in surviving insects. Significantly so for Trt 2 and Trt 5 ($F_{(3,16)} = 112.0$, $\rho = <0.0001$).

Table 3.2.5: Mean macromolecule content of alive and dead *Macchiademus diplopterus* (collected during the mid aestivation period) at evaluation time point 1 (after CATTS treatment) and evaluation time point 2 (after 10 days cold storage at -0.5 °C). *See Table 3.2.1 for descriptions for treatments. Significant differences between the values for alive and dead insects at each evaluation are indicated in bold. DW = dry weight*

| | | **After CATTS** | | **After 10 days at -0.5°C** | |
|---|---|---|---|---|---|
| | | Alive | Dead | Alive | Dead |
| **Total protein content (µg/mg DW)** | Trt 2 | **22.46 ± 3.96** | **30.13 ± 8.14** | 23.47 ± 5.24 | 24.88 ± 5.48 |
| | Trt 3 | | | 26.10 ± 7.17 | 31.84 ± 9.56 |
| | Trt5 | **25.46 ± 2.82** | **32.50 ± 2.63** | 31.70 ± 5.05 | 40.25 ± 0.80 |
| | Trt 6 | | | **26.48 ± 2.67** | **40.27 ± 8.16** |
| **Total lipid content (µg/mg DW)** | Trt 2 | **108.57 ± 9.41** | **148.31 ± 4.55** | **59.54 ± 6.16** | **133.90 ± 9.41** |
| | Trt3 | | | **98.30 ± 10.50** | **121.29 ± 2.75** |
| | Trt 5 | **105.63 ± 4.90** | **135.57 ± 4.90** | 103.10 ± 2.16 | 102.50 ± 8.63 |
| | Trt 6 | | | **80.45 ± 0.59** | **102.55 ± 5.18** |
| **Sugar content (µg/mg DW)** | Trt 2 | 65.56 ± 5.16 | 70.95 ± 1.66 | **40.05 ± 5.75** | **23.53 ± 4.32** |
| | Trt 3 | | | **58.17 ± 9.51** | **39.25 ± 2.17** |
| | Trt 5 | **65.84 ± 1.22** | **40.65 ± 4.80** | **42.73 ± 2.53** | **32.97 ± 1.76** |
| | Trt 6 | | | **46.13 ± 10.40** | **29.99 ± 3.43** |
| **Glycogen content (µg/mg DW)** | Trt 2 | 27.54 ± 1.16 | 24.22 ± 3.77 | **38.00 ± 4.32** | **21.46 ± 4.58** |
| | Trt 3 | | | 21.73 ± 0.38 | 19.20 ± 1.62 |
| | Trt 5 | 23.93 ± 1.41 | 21.95 ± 0.32 | **38.54 ± 0.16** | **16.99 ± 1.07** |
| | Trt 6 | | | 16.82 ± 2.77 | 18.66 ± 0.91 |

In addition to mid aestivation insects being more thermotolerant, mortality data also showed that the DT cold storage regime also improved tolerance (Table 3.2.3 and Fig 3.2.1). Insects subjected to the DT regime survived for longer during cold storage in comparison to ST cold stored insects. To determine if these two groups of insects differed in macromolecule content, the levels of total protein, lipids, sugars and glycogen in alive and dead insects from

Trt 3 and Trt 6 (both no CATTS treatment), at evaluation time point 3 were compared (Table 3.2.6). At this time point ST stored insects had undergone a total of 17 days at -0.5 °C, and DT stored insects had undergone 10 days at -0.5 °C followed by 7 days at 7.5 °C (before being returned to -0.5 °C for the rest of the storage time). No significance in total proteins was observed between dead and alive insects or the two storage regimes. The total lipid content was significantly higher in ST stored insects compared to DT, and for ST stored insects, dead insects had a significantly higher total lipid content than those that survived ($F_{(1,8)}$ = 464.08, ρ = <0.0001). No difference in lipid content between alive and dead was observed for DT stored insects. For both storage regimes a significant difference between dead and alive insects was observed for the sugar content ($F_{(1,8)}$ = 174.34, ρ = <0.0001). In ST stored insects, the sugar content was lower in dead insects, however, in DT stored insects, it was higher. Significant difference in glycogen content was observed between dead and alive insects stored using the DT regime ($F_{(1,8)}$ = 15.87, ρ = 0.004). The glycogen content of alive insects was ~13 µg/mg DW higher than that of dead insects. No significant difference between alive and dead ST stored insects was observed.

Table 3.2.6: Mean macromolecule content of alive and dead *Macchiademus diplopterus* (collected during the mid aestivation period) at evaluation time point 3. For the standard cold sterilisation regime (ST) - after 17 days at -0.5°C and the dual temperature regime (DT) - after 10 days at -0.5°C followed by 7 days stored at 7.5°C. *For each macromolecule, different letters (a, b, c…j) indicate significant differences between values for alive and dead insects in both storage regimes. DW = dry weight*

| | | Evaluation time point 3 | |
|---|---|---|---|
| | | Alive | Dead |
| **Total protein content (µg/mg DW)** | ST | 35.89 ± 2.15a | 35.97 ± 7.55a |
| | DT | 30.81 ± 6.47a | 36.15 ± 4.40a |
| **Total lipid content (µg/mg DW)** | ST | 147.08 ± 8.31c | 212.11 ± 12.71b |
| | DT | 53.00 ± 6.32d | 41.65 ± 2.27d |
| **Sugar content (µg/mg DW)** | ST | 278.98 ± 0.61e | 248.50 ± 1.11f |
| | DT | 233.88 ± 7.41g | 247.09 ± 0.69f |
| **Glycogen content (µg/mg DW)** | ST | 23.39 ± 3.05j | 25.22 ± 2.45ij |
| | DT | 35.01 ± 0.60h | 28.47 ± 2.26i |

Taking into consideration the improved survival of DT stored mid aestivation insects, Table 3.2.7 shows the comparison between macromolecule content of early and mid-non-CATTS DT stored insects, where mortality for the early aestivation insects was 100%, and mid aestivation insects had reached 77 ± 8.87% mortality. This comparison is made at evaluation time point 6 (38 days total storage). All early aestivation insects had succumbed to the cold storage treatment at this point, and had significantly lower concentrations of all the macromolecules compared to mid aestivation insects, which still exhibited 23% survival.

Table 3.2.7: Mean macromolecule content of dead *Macchiademus diplopterus* (collected during the mid aestivation period) at evaluation time point 6 (38 days total storage period) for non-CATTS dual temperature regime treatment. *For each macromolecule, different letters (a, b) indicate significant differences between vaules for dead insects in early and mid aestivation.*

| | **Evaluation time point 6** | |
|---|---|---|
| | Early<br>100% mortality | Mid<br>77% mortality |
| **Total protein content (µg/mg DW)** | 28.82 ± 5.00b | 40.84 ± 4.58a |
| **Total lipid content (µg/mg DW)** | 44.90 ± 4.90b | 55.70 ± 2.05a |
| **Sugar content (µg/mg DW)** | 38.88 ± 617b | 100.43 ± 9.99a |
| **Glycogen content (µg/mg DW)** | 9.48 ± 136b | 23.85 ± 7.20a |

## Discussion

Insects have an array of physiological and biochemical adaptions which they can initiate to minimise potential injury due to thermal stresses. High or low-temperature survival is enhanced through the production of stress proteins such as heat shock proteins and other critical metabolites involved in cryo-protection (Denlinger and Yocum, 1998). The insects overall physiological state and health contribute significantly to its ability to tolerate thermal stresses (Denlinger and Yocum, 1998). Previous work on *M. diplopterus* indicated that thermo-tolerance may increase as insects move into and through the aestivation phase of their life cycle (Okosun, 2012). Paper 3 of this dissertation showed that *M. diplopterus* displays the means for mechanisms that increase thermo-tolerance, and that these are prevalent later in aestivation. Here, mortality results of insects subjected to thermal stresses clearly show that mid aestivation insects are more thermo-tolerant than early aestivation insects.

In *M. diplopterus* in its natural state, the macromolecule that differs most in concentration between early and mid aestivation insects is the total protein and sugar content. A higher concentration of total protein in mid aestivation insects compared to early aestivation insects, was also seen in Paper 3. In that study, the increase in individual heat shock proteins, Hsp70 and Hsp90, which occurred during the mid-aestivation period was illustrated and the importance of these and other proteins as defence mechanisms during aestivation was discussed. In the present study, although the total protein content did not significantly

change in response to thermal stresses, individual proteins could have increased as defence mechanisms, but these were not identified.

Survival of aestivating *M. diplopterus* after exposure to thermal stresses in the form of CATTS treatment and cold storage was most dependent on a higher sugar and glycogen content, as well the reserve of lipids available. Massive conversion of glycogen to polyols or sugars is required for cryoprotectant synthesis (Worland *et al*., 1998; Storey and Storey, 2012). Increased sugar levels indicate a potentially higher capacity for freeze tolerance, and a higher concentration of cryoprotectant available for the insect to utilise during stress. The accumulation of low molecular weight polyhydric alcohols (e.g. sorbitol and mannitol) enables antifreeze protection of the insect through freeze tolerance and freeze avoidance strategies (Storey and Storey, 1986). Glycogen and other cryoprotectant (low molecular weight sugars) can be interconverted in response to changing temperatures (Storey and Storey, 1986). Glycogen as a polymeric form of glucose is and energy reserve in animal cells, and can be depolymerised on demand to be used as a fuel source (Steele, 1985). Dual temperature (DT) cold-stored insects experienced an intermittent increase in temperature, as opposed to cold sterilisation (ST) stored insects, which experienced uninterrupted temperature conditions at -0.5 °C for the duration of storage. The physiological response to this intermittent increase in temperature is also reflected in the glycogen and sugar content of aestivating insects, in that the levels of these macromolecules were elevated in ST stored insects. This would enable DT stored insects to use their reserves for longer periods and allow extended survival. Further research is required to examine the rate at which resources are converted or depleted, and the effect this has on the insect's ability to survive prolonged exposure to low temperatures.

The effect of the intermittent increase in temperature in the DT regime is also reflected in the use of lipids. In starving insects, large reserves of lipids will delay the critical threshold which results in death. Lipid reserves in some starved insect species can be increased with conversion of amino acids into carbohydrates (Sonmez and Gulel, 2008). The conversion of amino acids may explain the high lipid content observed in ST stored insects compared to DT stored after 17 days total storage. This stored lipid composition adjustability could have been utilized during the temperature changes during DT storage. This may be play a role in the capabilities of temperature adaption, as the fluidity of storage lipids enables the accessibility of enzymes to energy reserves (van Dooremalen and Ellers, 2010). Lipid functions include freeze-tolerance, fuel source or as a precursor for the production of low molecular weight cryoprotectant molecules (Sinclair and Marshall, 2018). During cold

acclimation restructuring of the membranes takes place, where an increase in the unsaturated fatty acids to saturated fatty acid ratio occurs (Michaud and Denlinger, 2006). An increase in fatty acids as the insect transitions to diapause has been observed in insects such as *Chymomyza costata* Zetterstedt (Diptera: Drosophilidae), accompanied by enhanced cold hardiness (Clark and Worland, 2008). *Macchiademus diplopterus* had increases in total lipids for both cold storage regimes. This may have occurred to prevent ice formation as a freeze avoidance mechanism when exposed to prolonged periods at low temperatures. Further research however is required to fully understand the mechanism of lipid mobilisation and consumption by *M. diplopterus* as potential fuel source or defence mechanism.

The physiological state of *M. diplopterus*, whether in early or mid aestivation, directly affects the insects' ability to initialize successful adaptive strategies to survive. The resource level within mid aestivating insects is much larger than that of early aestivating insects, indicating a possible threshold concentration of components required to ensure survival. The physiological and biochemical state, as well as the aestivation period, will therefore determine the success of any thermal treatments applied for phytosanitary control. The mid aestivation period, in which the insect is at its most tolerant to thermal stresses aligns with the optimum export window for pome and stone fruit from South Africa. The high level of thermal tolerance of aestivating *M. diplopterus* renders CATTS and cold storage treatments insufficient for effective control of this key phytosanitary pest. Alternative postharvest mitigation treatments for *M. diplopterus* need to be investigated and developed. Fumigation treatment may be a feasible alternative that is not affected by or dependant on the physiological state of aestivation of *M. diplopterus* on export fruit requiring disinfestation.

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### 3.3.1 Conclusions

### Paper 3 and 4

Paper 3 and 4 constitute an in-depth study into the physiology of *Macchiademus diplopterus*, the grain chinch bug, to better understand its thermal tolerance level during aestivation and how this affects the potential of thermal postharvest treatments for this pest.

Biochemical data presented in Paper 3 clearly shows how the biochemical make-up, in terms of carbohydrates, fats and proteins, changes as *M. diplopterus* adults enter into aestivation and progress through this dormant period of their life cycle. The accumulation of energy reserves and protective mechanisms to maintain the integrity of cell structures through unfavourable conditions is evident. The identification of different functional groups of soluble proteins in insects at different times during the aestivation period highlighted how the emphasis on different functions changes as aestivation progresses. Thermo-tolerance is a physiological adaption, and has been correlated with the synthesis of certain proteins. Heat shock proteins are important in the protection of cells and cellular functions against thermal and desiccation stresses, and are found in the cytosol, mitochondria, chloroplast or nucleus within cells (Lurie and Jang, 2007). The high intensity in expression of heat shock proteins, in particular Hsp70 and Hsp90, observed in aestivating *M. diplopterus,* which increased later in aestivation, indicates the important role heat shock proteins play in the mechanisms employed by *M. diplopterus* to initiate its thermo-tolerant behaviour. Many other factors are also likely to play a role in temperature stress tolerance in *M. diplopterus*. It is not yet clear to what degree other identified elements, including some proteins that could not be characterised here, influence the mechanisms of protection against stress responses in *M. diplopterus*.

In Paper 4, mortality of *M. diplopterus* due to thermal stress (in the form of CATTS treatments and cold storage) clearly illustrates how thermal tolerance increases during aestivation. Quantification of macromolecules, in insects in their natural state, and after exposure to thermal stresses, shows how insects further in the aestivation cycle have a greater pool of relevant resources to draw from, which contributes to their survival. Higher levels of proteins suggest improved ability for protection of cells, and reserves of carbohydrates and fats improve cryoprotection during cold storage. Consequently, thermal treatment is not an option for phytosanitary control of *M. diplopterus.* Creating a blanket thermal treatment which could be applied throughout its aestivation cycle and provide efficient control without pre-conditioning the insect to tolerate those same stresses, will be challenging. Alternative

postharvest treatments are required, and this is the focus of the next chapter in this dissertation (Paper 5 and Paper 6). However, the physiology of *M. diplopterus*, particularly during aestivation, is interesting and complex and should be investigated in future to better understand all its mechanisms that improve survival in unfavourable conditions.

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# CHAPTER 4

# Paper 5

# Ethyl formate fumigation: its effect on stone and pome fruit quality, and grain chinch bug (*Macchiademus diplopterus*) mortality

## 4.1.1 Introduction

South Africa has a growing agricultural export industry with 44% of deciduous fruit produced in 2017 exported to international markets (Hortgro, 2018). One of the key phytosanitary pests that the export industry has to contend with is the grain chinch bug, *Macchiademus diplopterus* (Distant) (Hemiptera: Lygaeidae). The grain chinch bug was the reason for rejection of consignments of stone fruit from South Africa to the United Kingdom as far back as the 1920's (Malumphy *et al.*, 2012), and to date, this phytosanitary pest still impacts fruit exports from the Western Cape region of South Africa. During the 2017/2018 season approx. 1800 cartons of pears earmarked for the USA were rejected due to contamination with grain chinch bug. Another incident was reported during the 2017/2018 season with a consignment of peaches being placed on hold due to grain chinch bug interception (Horticulture Week, 2018).

The grain chinch bug is an indigenous pest of cultivated grain crops and wild grasses in the South Western Cape of South Africa and is considered a contaminant or hitchhiker pest of export fruit due to its shelter-seeking behaviour as it moves into aestivation (Myburgh and Kriegler, 1967; Slater and Wilcox, 1973; Annecke and Moran, 1982; Addison, 2005; Johnson and Addison, 2008). Aestivation is defined as a prolonged period of dormancy or quiescence, where the insect may be in a cryptobiotic state and highly tolerant to thermal stress and drought (Masaki, 2009). Insects in this state are able to change their metabolic functions enabling them to survive thermal stresses and even water scarcity for prolonged periods of time (Dingle, 1972; Dingle *et al*., 1980). During this period the insect only seeks shelter until more favourable conditions ends the dormancy cycle (Annecke and Moran, 1982; Mayer, 2016). During the summer months, when their host plants dry out, adult grain chinch bugs seek nearby shelter sites in large numbers where they enter aestivation. The aestivation period lasts from November until May and this coincides with the harvesting of various deciduous fruit cultivars grown in the region. Shelter-seeking grain chinch bugs may settle in the stylar or calyx ends of the fruit, or within bunches of grapes and can remain there throughout the harvesting and packing period.

Postharvest mitigation treatment for control of the grain chinch bug is challenging. Low temperature disinfestation treatments and hypobaric treatments at low temperatures have been shown to be ineffective (Addison, 2005; Grout and Tate, 2007). The cold hardiness ability of aestivating grain chinch bugs is evident from reports of survivors after a cold storage treatment of 8 weeks at -0.5°C on stone fruit, which was intercepted in the UK (Malumphy, 2011). Heat treatments combined with a controlled atmosphere also will not successfully control grain chinch bug without causing fruit damage (Johnson and Neven, 2011). With the use of methyl bromide being banned, the potential of alternative fumigants is being considered. These include phosphine, sulfuryl fluoride, carbonyl sulfide and ethyl formate (Ducom and Banks, 2006). Ethyl formate is considered to be the most promising fumigant to replace methyl bromide due to its insecticidal and fungicidal properties (Simpson *et al*., 2004; Ryan and De Lima, 2014). It has been recognized as a GRAS (Generally Recognized As Safe) chemical and is used in commercial manufacturing of artificial rum, as an organic solvent and as a flavouring for essences and lemonade (Budavari *et al*., 1989; FDA, 2014). As a disinfestation treatment ethyl formate has been used to control dried fruit pests since the 1920s (Simmons and Gertler, 1945). It kills insects rapidly and breaks down into natural occurring formic acid and ethanol (Desmarchelier *et al.*, 1998). Ethyl formate fumigation treatments are more effective against external surface pests than pests that are borne internally, such as fruit fly larvae (De Lima, 2010).

Liquid ethyl formate (EF) is highly flammable and application as a fumigant has been made possible by mixing with other compounds (Ryan and De Lima, 2014). A commercial formulation of EF + $CO_2$, known as Vapormate$^{TM}$, which can control a variety of pests, such as aphids, thrips, mites and mealybugs, has been applied to fresh commodities (such as lettuce, banana, pineapple, table grapes and citrus fruits). Approved concentrations are between 30 and 420 g/m$^3$ and exposure times range from 1 to 6 h (Linde, 2008). The ability of Vapormate$^{TM}$ to control grain chinch bug was previously investigated by Grout and Stoltz (2016) with promising results. The use of liquid ethyl formate and the commercial application thereof needs further research as Vapormate$^{TM}$ is very costly and may be a limiting factor for Vapormate$^{TM}$ fumigations (Yang *et al.*, 2017).

The primary purpose of this research was to determine the effective dose (concentration and duration) of liquid ethyl formate required to control grain chinch bug and the potential effect it could have on the quality of various fruit commodities.

## 4.1.2 Materials and Methods

***Insects***

Aestivating adult grain chinch bug (GCB) were field-collected in Ceres (S 33:22'10"1, E 19:19'24"2) in the Western Cape province of South Africa, as needed, during two deciduous fruit production seasons (2015/2016 and 2016/2017). Preliminary grain chinch bug mortality trials, phytotoxicity assessment, tasting trials, and packaging trials were conducted. The latter phytotoxic assessment and packaging trials also included GCB mortality trials.

***Fruit***

**Phytotoxicity assessment and tasting trials (2015/2016 season)**

At harvest, the Japanese plum (*Prunus salicina* Lindl.) cultivar, 'Songold', was sourced from a commercial packhouse in Franschhoek (33°54'33.2"S, 19°06'58.4"E), Western Cape, South Africa. The nectarine (*Prunus persica var. nucipersica)* (Suckow) C. K. Schneid cultivar, 'August Red', and two pear *(Pyrus communis* L.*)* cultivars, 'Golden Russet Bosc' and 'Forelle' were sourced from a commercial packhouse in Ceres (33°22'S, 19°19'E), Western Cape, South Africa.

**Packaging trial (2016/2017 season)**

The nectarine (*Prunus persica var. nucipersica)* cultivar, 'September Bright', and the pear *(Pyrus communis)* cultivar, 'Beurrè Bosc' were sourced at harvest from a commercial packhouse in Ceres.

The maturity at harvest for all the fruit types was within the picking window used for export of each fruit type, see Appendix C (DAFF 2015a, 2015b).

***Treatments***

**Dose - response trials (2015/2016)**

To determine the minimum concentration of ethyl formate (EF) required to obtain a 100% mortality of aestivating adult grain chinch bug, 500 ml Erlenmeyer flasks with rubber stoppers were used as small fumigation containers. Insects where placed into perforated microcentrifuge tubes (1ml, Eppendorf) which were placed inside the Erlenmeyer flask and sealed with the stopper. The free volume of the flask was determined to calculate the specific dose of liquid ethyl formate (µL) (reagent grade, 97%, Sigma-Aldrich) required for a specific concentration to be achieved.

Ethyl formate concentrations of 0 (control), 15, 20, 25, 30, 35, 37, 39, 42, 45 and 50 g/m$^3$ were used to determine the insects' response curve. Liquid ethyl formate was pipetted

through an opening in the stopper which was sealed after each dosage was applied. The flask remained sealed and at ambient for the duration of the treatment, which was 1 h. For each concentration the fumigation treatment was replicated three times with 40 grain chinch bug adults per replication (n = 120 per concentration). Insect mortality was assessed immediately after fumigation, and again 8 h after treatment (to allow for metabolic adjustment of any survivors). Mortality was assessed by prodding of the insects to check for any leg or antennal movement, indicating survival. Mortality was calculated as the percentage of dead insects relative to total treated insects for each treatment.

**Phytotoxicity assessment and tasting trials (2015/2016)**

Based on the findings of the dose-response trials, ethyl formate concentrations of 0 (control), 50, 100 and 150 g/m$^3$, were applied to test the effect of ethyl formate fumigation on fruit quality, and to determine the threshold for phytotoxic damage in each fruit type and cultivar. Three replicates for each concentration and cultivar were performed. Fruit was placed inside a 14 L glass desiccator. Based on individual fruit size, the number of fruit in a desiccator varied with each cultivar treated, and the free volume of the desiccator was, therefore, determined for each replicate per treatment. The free volume was used to calculate the amount of ethyl formate (µL) required to achieve the desired concentration inside the desiccator. Liquid ethyl formate was pipetted into an open glass petri dish placed on top of the fruit in each desiccator, which was closed immediately after the ethyl formate was applied and left for 1h at ambient temperature (Fig. 4.1.1).

Figure 4.1.1. Illustration of ‘August Red’ nectarines placed in 14 L desiccators for ethyl formate fumigation and phytotoxic assessment trials during the 2015/2016 season.

After treatment the fruit was removed from the desiccators and packed according to export standards per fruit type and subjected to commercial cold storage regimes. ‘Songold’ plums were packed in a two-layer 4.5 kg carton and ‘August Red’ nectarines were packed in two layers in 8 kg cartons, both with perforated high-density polyethylene shrivel sheets to retard moisture loss during cold-storage. The two pear cultivars, ‘Golden Russet Bosc’ and

'Forelle', were packed in MK4 (KaapAgri, Paarl) cartons with a green 37.5-micron inner bag which was folded closed. For above mentioned cultivars the layers within cartons where separated using pulp trays and 25 fruit were allocated for each evaluation per replicate and per treatment. Commercial cold storage regimes per cultivar were an intermittent warming regime for 'Songold' plums (Fig. 4.1.2), 4 weeks at -0.5 °C for 'August Red' nectarines, 12 weeks at -0.6 °C for 'Golden Russet Bosc' and 13 weeks at -0.6 °C for 'Forelle' pears. After cold storage fruit was also subjected to a simulated shelf life period of 7 days at 10 °C.

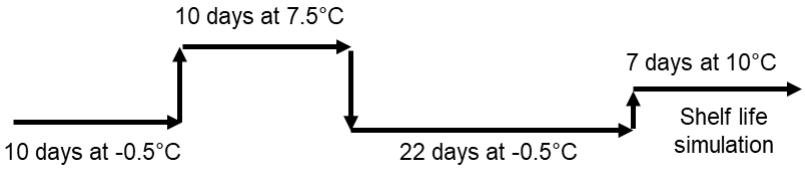



Figure 4.1.2. Illustration of intermittent warming regime used for cold storage of 'Songold' plums during the 2015/2016 season.

The effect of ethyl formate on fruit quality and assessment of the phytotoxic effects of ethyl formate fumigation was conducted at three evaluation points: immediately after treatment, after cold storage, and after cold storage plus shelf life simulation.

In addition, to determine the effect that ethyl formate fumigation could potentially have on the flavour profile of treated fruit, an informal tasting panel of five participants was compiled to complete a blind tasting and questionnaire for each cultivar at each evaluation, see Appendix C.

During the fumigation trials, the efficacy of the chosen ethyl formate concentrations to achieve mortality was also verified. To monitor insect mortality each replicate within a treatment contained 20 GCB adults placed at the bottom of the desiccator, and 20 placed on the top layer of the fruit inside perforated micro centrifuge tubes (1ml, Eppendorf). Insect mortality was assessed immediately after fumigation, immediately after the fruit came out of cold storage and again 8h after removal from cold-storage, to check for revival after metabolic adjustment of the insects to ambient temperature. Mortality was assessed by prodding of the insects to check for any leg or antennal movement. Mortality was calculated as the percentage of dead insects relative to total treated insects for each treatment.

**Packaging trial (2016/2017)**

To assess the efficacy of EF fumigation and to determine the minimum dosage required to control grain chinch bug adults on unpackaged fruit (fruit in open crates/ lugs) versus packaged fruit (fruit in cartons with inner bags or liners as per commercial use) fumigations

were conducted in an airtight chamber (1m x 0.5m x 0.8m). A small (15cm diameter) fan was placed at the bottom of the container. The chamber capacity allowed for three lugs or three cartons (commercially used for each cultivar) to be stacked one on top of the other for fumigation (Fig 4.1.3A). Each replicate run consisted of three lugs or cartons. Each lug or carton contained 30 fruit (90 fruit per replicated treatment). Treated insects were contained in perforated microcentrifuge tubes (1ml, Eppendorf) placed in fixed positions within the fumigation chamber. For each chamber side panel, tubes were placed in each of the top (position 1) and bottom (position 3) corners of the panel, and fixed to the middle (position 2) of each chamber wall (Fig 4.1.3B). This was done for each of the four panels of the fumigation chamber. Each tube contained 40 insects (480 insects in the chamber positions per replicated treatment). In addition to tubes with insects positioned inside the chamber, insects were included with the unpackaged and packaged fruit. In the unpackaged 'September Bright' nectarines, each of the three lugs in the chamber contained three tubes – one in a top corner (position 1), one in a bottom corner (position 2) and one in the middle amongst the fruit (position 3) (Fig 4.1.3C), totalling nine tubes and 360 insects in lugs per replicated treatment). The cartons of packaged 'September Bright' nectarines and 'Beurrè Bosc' pears also contained three tubes of insects per carton, as well as a piece of corrugated cardboard infested with sheltering grain chinch bugs contained inside a breathable material bag (to prevent bugs from escaping during treatment). In each carton, one tube was placed outside the shrivel sheet or bag (position 1) containing nectarines or pears, one was placed insider the liner amongst the top layer of fruit (position 2) and the other amongst the bottom layer of fruit (position 3) (Fig 4.1.3D).

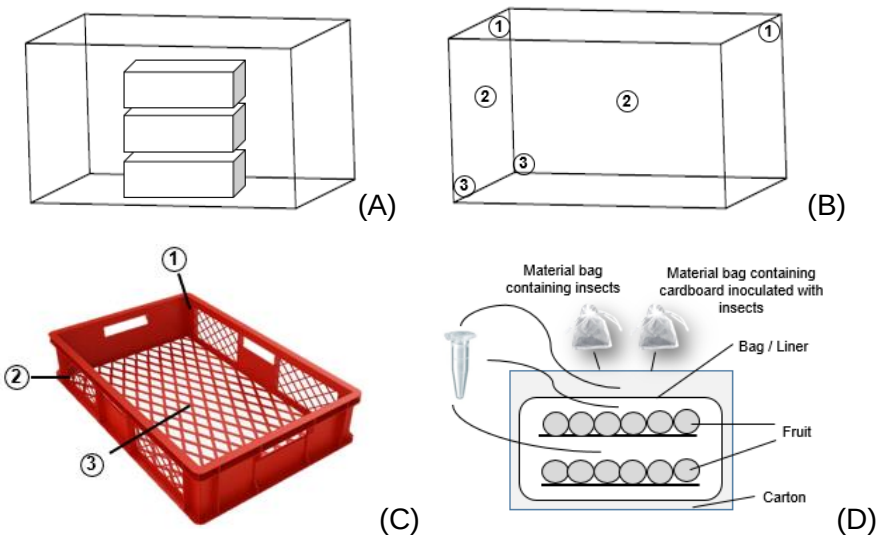



Figure 4.1.3. Diagrams to illustrate the placement of grain chinch bugs in perforated tubes within the fumigation chamber, lugs and packaging during the 2016/2017 season. A) Illustration of stacking of lugs or cartons for each replicate during treatment. B) Tubes placed on upper (1), middle (2) and lower positions (3) this distribution repeated for each of the four panels. C) Tubes placed on upper (1), middle (2) (between fruit) and lower (3) positions repeated for each lug. (D) Position of tubes and bags with inoculated cardboard sheets in nectarine and pear cartons.

A total of 360 insects in tubes were treated per carton per replicated treatment. The number of insects contained in the cardboard sheets was determined after treatment, since infestation of cardboard sheets was achieved by placing the pieces of cardboard inside laboratory containers with thousands of collected grain chinch bugs and allowing insects to move into the cardboard for shelter overnight. Insects sheltering inside corrugated cardboard sheets were included to evaluate the effectiveness of ethyl formate to penetrate fluted packaging. Fumigation treatments were conducted at ambient temperature (approx. 23°C) containing 6 replicates per treatment. Once fruit and insects were placed inside the chamber and free volume was calculated, the treatment was started by injecting the required amount of liquid ethyl formate through a resealable port onto a glass plate inside the chamber and the unit was kept closed for 1h.

Unpackaged ‘September Bright’ nectarines in lugs were fumigated at a concentration of 50 g/m$^3$ for 1h. To consider the effect of packaging, and to take the potential dilution effect of the packing into account, the ‘September Bright’ nectarines packed in commercial packaging (cartons with high density polyethylene shrivel sheets) were fumigated with ethyl formate concentrations of 50 g/m$^3$ and 100 g/m$^3$ for 1h. Based on the results for the packaged nectarines and due to the availability of fruit, packaged ‘Beurrè Bosc’ pears (fruit packed in a green 37.5 micron bag and MK4 carton) were used to determine if a concentration lower

than 100 g/m$^3$ (85 g/m$^3$ ) would be effective to control GCB within packaged fruit. After treatment fruit was stored using commercial cold storage regimes: 4 weeks at -0.5 °C for 'September Bright' nectarines and 12 weeks at -0.6 °C for 'Beurre Bosc' pears.

As for the previous season's trials, assessment of the phytotoxic effects of ethyl formate fumigation on unpackaged and packaged fruit was conducted at three evaluation points: immediately after treatment; after cold storage, and after cold storage plus shelf life simulation (7 days at 10 °C).

Insect mortalities for all treatments (controls and fumigated) were assessed immediately after fumigation, 8h after fumigation at ambient air (to allow for metabolic adjustment), immediately after cold-storage, as well as 8h after removal from cold storage (to allow for metabolic adjustment of the insects to ambient temperature). Mortality was again assessed by prodding. Mortality was calculated as the percentage of dead insects relative to total treated insects for each treatment.

***Fruit evaluation***

Maturity parameters of fruit for all trials were evaluated at harvest, after treatment, after cold storage and after cold storage plus shelf life simulation. Hue angle (°) was measured using a calibrated colorimeter (Minolta chroma meter CR-400, Japan) to quantify the change in ground colour of the fruit. Flesh firmness (N) was measured using a Fruit Texture Analyzer (Güss Manufacturing, Strand, South Africa). Total soluble solids (TSS), expressed as % Brix, was determined using a temperature controlled, digital refractometer (Atago digital refractometer PR-32a, Japan). Titratable acid (TA) (%) expressed as malic acid was determined through titration of 10 g of juice with 0.1 N NaOH to a pH end point of 8.2 using an automated titrator (Metrohm 719 S Titrino, Herisau, Switzerland). Both TSS and TA were measured using pooled juiced samples of 20 fruit per replicate per treatment.

After treatment, after cold storage and after cold storage plus shelf life simulation, external fruit quality parameters (shrivel, decay and external phytotoxic damage) and internal fruit quality parameters (such as internal browning, gel breakdown, pulpiness, mealiness, astringency and internal phytotoxic damage) were determined for all cultivars. External fruit quality was subjectively evaluated on 20 fruit and internal fruit quality was subjectively evaluated on 10 fruit per replicate per treatment and expressed as a percentage of the total fruit examined

Internal quality defects specific to the cultivars were internal browning and gel breakdown for 'Songold' plums, mealiness and pulpiness for both nectarine cultivars and incidence of

astringency and mealiness for all pear cultivars. Internal disorders were evaluated by cutting the fruit around the equatorial axis and separating the two halves of the fruit. Gel breakdown in plums is defined as the gelatinous breakdown of the inner mesocarp tissue surrounding the stone, while the outer mesocarp tissue displays no damage (Jooste, 2012). Fruit wedges were used from both nectarine and pear cultivars to assess for mealiness and pulpiness by squeezing the sample to examine the presence of free juice. Mealiness and pulpiness can be defined as fruit that appears dry with a coarse, floury texture; therefore the presence of free juice will be limited. Astringency in pears can be defined as the sensation of drying of the mouth which was assessed by tasting of fruit wedges. For this purpose, fruit were removed from cold storage the previous day to allow for acclimatisation to room temperature before tasting. Phytotoxicity is defined as any adverse effect that results in damage to the cuticle (formation of spot like necrosis) and penetration of the tissue resulting in cork like tissue.

***Statistical analysis of data***

Fruit quality and insect data for all trials were analysed using factorial ANOVA with STATISTICA version 13 (Statsoft, Inc., 2017). AVONA-generated P-values and the significant differences between means were determined using Fisher's least significant differences (LSD) test with a 95% confidence interval.

## 4.1.3 Results

***Preliminary GCB mortality trial (2015/2016)***

The preliminary mortality trial indicated that a minimum concentration of 50 g/m$^3$ for an hour is required to obtain a 100% mortality for grain chinch bug when fumigating with liquid ethyl formate in 500 ml Erlenmeyer flasks (Fig 4.1.4).

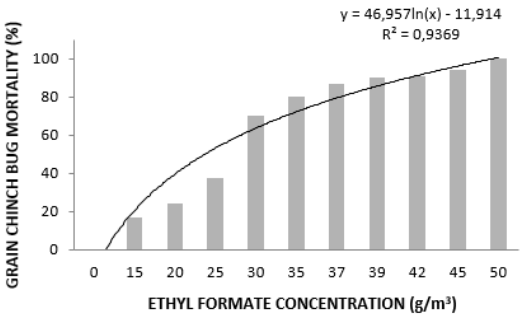
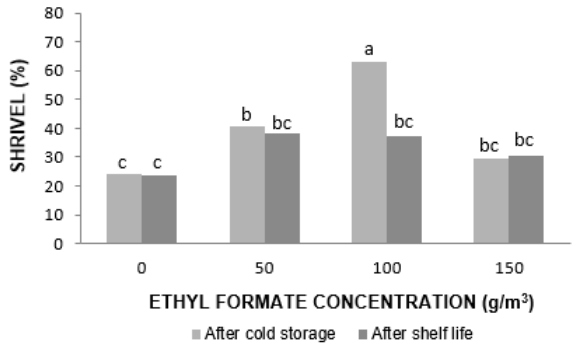



Figure 4.1.4. Grain chinch bug mortality (%) after exposure to different ethyl formate concentrations (g/m$^2$) per hour during the 2015/2016 season.

***Phytotoxic assessment and tasting trials (2015/2016)***

The various ethyl formate concentrations did not affect the hue angle, flesh firmness or total soluble solids of the fruit during treatment, as measurements in fruit fumigated at 50, 100 or 150 g/m$^3$ for 1 h did not differ significantly from each other or from those measured in the control fruit (Table 4.1.1). In 'Songold' plums and 'Golden Russet Bosc' pears, hue angle and flesh firmness changed significantly over cold-storage time. 'August Red' nectarines and 'Forelle' pears also had a significant decrease in flesh firmness during cold-storage. 'Golden Russet Bosc' and 'Forelle' pears were the only products in which a significant difference was observed for total soluble solids across evaluation periods, from after treatment to after shelf life. The only external quality parameter that was significantly affected by ethyl formate fumigation across all cultivars tested, was the incidence of shrivel in 'Songold' plums (Fig. 4.1.5).




| Effect | Df | MS | F | p |
|---|---|---|---|---|
| Evaluation | 3 | 5240 | 56.42 | <0.0001 |
| Treatment | 3 | 385.3 | 4.149 | 0.0136 |
| **Eval*Treatment** | **9** | **207.4** | **2.233** | **0.0458** |
| Error | 32 | 92.88 | | |
| $LSD_{5\%}$ | | | | 15.95 |

Figure 4.1.5. The effect of ethyl formate fumigation after 1 h on the incidence of shrivel in 'Songold' plums during the 2015/2016 season.

There was a significant interaction between cold-storage duration and ethyl formate concentration for shrivel. After cold storage, plums treated at 100 g/m$^3$ during fumigation had significantly higher levels of shrivel (63%) compared to the control (24%) and the other fumigation concentrations. After shelf life simulation the incidence of shrivel for the above-mentioned treatment decreased to 37%, resulting in no significant differences between the control (23%) and the other concentrations. Fruit fumigated at 50 g/m$^3$ also had significantly higher levels of shrivel after cold-storage compared to the control and fruit fumigated at 150 g/m$^3$. However, after shelf-life all the treatments had comparable levels of shrivel.

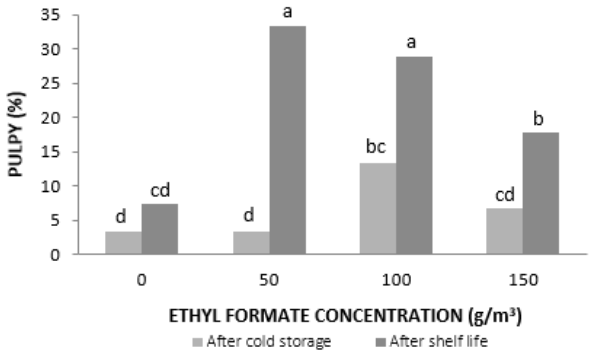
There was also a significant interaction between ethyl formate concentration and storage duration for the incidence of pulpiness in 'August Red' nectarines (Fig. 4.1.6). This was the only internal quality parameter affected across all cultivars tested. After shelf life simulation

significantly higher levels of pulpiness were observed for all ethyl formate treated fruit compared to the control.

Table 4.1.1. Effect of different ethyl formate concentrations and evaluation intervals on fruit maturity parameters of 'Songold' plums, 'August Red' nectarines, 'Golden Russet Bosc' and 'Forelle' pears during the 2015/2016 season.

| Commodity | Maturity parameters | [1] Ethyl formate [ ] g/m$^3$ | Ethyl formate concentration g/m$^3$ (A) | | | | [2] Evaluations (B) | | | *Prob > F* [3] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 50 | 100 | 150 | After treatment | After storage | After shelf life | *A* | *B* | *A x B* |
| 'Songold' plums | Hue Angle (°) | 0+50+100+150 | 101.35 | 99.94 | 101.29 | 100.82 | 107.60a | 97.45b | 90.71c | 0.2878 | ***<0.0001*** | 0.7575 |
| | Flesh firmness (N)[4] | 0+50+100+150 | 48.51 | 49.63 | 52.93 | 51.02 | 55.02a | 46.05b | 34.56c | 0.1929 | ***<0.0001*** | 0.3268 |
| | Total soluble solids (% Brix) | 0+50+100+150 | 13.71 | 13.06 | 13.64 | 13.25 | 13.52 | 13.40 | 13.67 | 0.1293 | 0.2854 | 0.0848 |
| 'August Red' nectarines | Hue Angle (°) | 0+50+100+150 | 51.58 | 48.42 | 48.11 | 47.60 | 47.88 | 47.57 | 47.17 | 0.2170 | 0.2119 | 0.5249 |
| | Flesh firmness (N)[4] | 0+50+100+150 | 75.24 | 78.63 | 80.03 | 80.63 | 84.48a | 76.22a | 41.59b | 0.7136 | ***<0.0001*** | 0.1420 |
| | Total soluble solids (% Brix) | 0+50+100+150 | 13.05 | 12.71 | 12.81 | 13.16 | 12.78 | 12.82 | 12.82 | 0.2614 | 0.1153 | 0.2592 |
| 'Golden Russet Bosc' pears | Hue Angle (°) | 0+50+100+150 | 76.78 | 76.20 | 76.56 | 76.44 | 82.51a | 71.54b | 64.41c | 0.7710 | ***<0.0001*** | 0.9917 |
| | Flesh firmness (N)[4] | 0+50+100+150 | 48.99 | 47.08 | 49.76 | 47.64 | 61.14a | 54.83b | 18.75c | 0.7096 | ***<0.0001*** | 0.5236 |
| | Total soluble solids (% Brix) | 0+50+100+150 | 16.92 | 16.64 | 16.43 | 16.43 | 16.19b | 17.30a | 17.23a | 0.2210 | ***<0.0001*** | 0.4573 |
| 'Forelle' pears | Hue Angle (°) | 0+50+100+150 | 90.11 | 90.61 | 87.63 | 87.04 | 92.93 | 85.37 | 82.50 | 0.0571 | ***<0.0001*** | 0.0680 |
| | Flesh firmness (N)[4] | 0+50+100+150 | 45.75 | 45.75 | 45.95 | 46.44 | 62.97a | 59.52b | 15.24c | 0.6215 | ***<0.0001*** | 0.4676 |
| | Total soluble solids (% Brix) | 0+50+100+150 | 16.23 | 16.43 | 16.43 | 16.54 | 15.84c | 16.72b | 17.23a | 0.2320 | ***<0.0001*** | 0.3689 |

[1] Data pooled for non-significant interactions across evaluation duration and ethyl formate concentrations, with 0, 50, 100 and 150 indicating concentration (g/m$^3$) of ethyl formate.
[2]Significant differences indicated by different letters next to values (P<0.05) according to the LSD test, for evaluation periods
[3] Two-way ANOVA table with for Factor A (Ethyl formate concentrations) and Factor B (Evaluation period).
[4] Conversion of Newton to kilogram, divide by 9.80




| Effect | Df | MS | F | p |
|---|---|---|---|---|
| Evaluation | 3 | 1276. | 38.28 | <0.0001 |
| Treatment | 3 | 145.9 | 4.377 | 0.0109 |
| **Eval*Treatment** | **9** | **108.8** | **3.264** | **0.0063** |
| Error | 32 | 33.34 | | |
| $LSD_{5\%}$ | | | | 9.554 |

Figure 4.1.6. The effect of ethyl formate fumigation for 1 h on the incidence of pulpiness in 'August Red' nectarines in the 2015/2016 season.

No internal or external phytotoxic damage was observed for all cultivars tested. Also, the informal tasting panel could not distinguish between the treated fruit and the control, which indicated that no sensory differences or off taste was detected (see Appendix C).

All three ethyl formate concentrations applied achieved 100% insect mortality for each of the four cultivars treated.

**Packaging trial (2016/2017)**

The evaluation conducted after treatment which included the control and treated fruit yielded a lower flesh firmness than the 52 N observed at harvest (Appendix C and Table 4.1.2). No significant differences were observed due to ethyl formate treatment for hue angle, flesh firmness or total soluble solids after treatment, after cold-storage or after shelf-life for 'September Bright' nectarines and 'Beurrè Bosc' pears (Table 4.1.2).

No external or internal fruit quality parameters were significantly affected by ethyl formate fumigation for 'September Bright' nectarines and 'Beurrè Bosc' pears for all evaluation periods during the packaging trial (see Appendix C).

'September Bright' nectarines placed in lugs and fumigated with 50 g/m$^3$ for 1 h yielded a 100% mortality of grain chinch bug, as was expected from the phytotoxic assessment and mortality trials (Table 4.1.3). The application of 50 g/m$^3$ to packaged fruit, however, did not yield a 100% mortality in insect-inoculated cardboard sections and cartons. The inoculated cardboard sections resulted in mortality of only 77%, and a 72% mortality was observed when insects were placed within the packaged cartons. This illustrated the poor penetration of fumigant or absorption effect that takes place as the fumigant moves through the

cardboard and packaging. Inoculated cardboard showed live insects in the areas with denser / thicker corrugated cardboard due to more cardboard layers. All the insects found in the thinner / less dense corrugated cardboard sections were dead.

Increasing the applied concentration to 100 g/m$^3$ allowed the fumigant to penetrate and disperse within the packaging, achieving a 100% mortality of insects placed with packaged fruit (Table 4.1.3). These results indicate that a higher concentration of ethyl formate was required to obtain 100% mortality of grain chinch bug.

'Beurrè Bosc' pears fumigated at 85 g/m$^3$ also yielded a 100% mortality of insects imbedded within the packaged fruit and corrugated cardboard sections (Table 4.1.4), indicating that a concentration below 100 g/m$^3$ was still effective in penetrating the packaging material. A concentration of 50 g/m$^3$ could, therefore, be applied to control grain chinch bug in unpackaged fruit, and 85 g/m$^3$ for packaged fruit.

Table 4.1.2. Effect of different ethyl formate concentrations and evaluation intervals on fruit maturity parameters for 'September Bright' nectarines and 'Beurrè Bosc' pears during the 2016/2017 season

| Commodity | Maturity parameters | [1] Ethyl formate [ ] g/m$^3$ | Ethyl formate concentration g/m$^3$ (A) | | | | | [2] Evaluations (B) | | | *Prob > F* [3] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 50 | 50[4] | 85[4] | 100[4] | After treatment | After storage | After shelf life | *A* | *B* | *A x B* |
| 'September Bright' nectarines | Hue Angle (°) | 0+50+50[5]+100[5] | 46.62 | 47.54 | 47.38 | NA | 46.09 | 47.80 | 47.54 | 45.37 | 0.8722 | 0.3025 | 0.1200 |
| | Flesh firmness (N)[4] | 0+50+50[5]+100[5] | 28.27 | 28.70 | 30.88 | NA | 27.67 | 32.72a | 29.44b | 24.47c | 0.1378 | ***<0.0001*** | 0.2202 |
| | Total soluble solids (% Brix) | 0+50+50[5]+100[5] | 14.43 | 14.49 | 14.07 | NA | 14.02 | 14.55a | 14.51a | 13.70b | 0.1311 | ***<0.0001*** | 0.9463 |
| 'Beurrè Bosc' pears | Hue Angle (°) | 0+85[5] | 77.11 | NA | NA | 77.40 | NA | 85.81a | 74.58b | 70.83c | 0.8985 | ***<0.0001*** | 0.9972 |
| | Flesh firmness (N)[4] | 0+85[5] | 50.68 | NA | NA | 53.69 | NA | 68.11a | 68.11a | 19.08b | 0.2289 | ***<0.0001*** | 0.1896 |
| | Total soluble solids (% Brix) | 0+85[5] | 16.26 | NA | NA | 16.47 | NA | 15.53b | 16.80a | 16.80a | 0.4733 | ***<0.0001*** | 0.5201 |

[1] Data pooled for non significant interactions across evaluation duration and ethyl formate concentrations, with (0, 50, 100 and 150) and (0 and 85) indicating concentration (g/m$^3$) of ethyl formate.
[2]Significant differences indicated by different letters next to values (P<0.05) according to the LSD test, for evaluation periods
[3]Two way ANOVA table for Factor A (Ethyl formate concentrations) and Factor B (Evaluation period).
[4] Indicates concentrations applied to packaged fruit
[5] Conversion of Newton to kilogram, divide by 9.80

Table 4.1.3. Insect mortality of *Macchiademus diplopterus* after 1 h of ethyl formate fumigation with and without packaging for 'September Bright' nectarines during the 2016/2017 season.

| Position | Ethyl formate treatment (g/m³) | | | |
|---|---|---|---|---|
| | **0 g/m³ (control)** | **50 g/m³ (fruit in lugs)** | **50 g/m³ (applied to packed fruit)** | **100 g/m³ (applied to packed fruit)** |
| Top section of fumigation chamber (*n= 160)* | 0% | 100% | 100% | 100% |
| Middle section of fumigation chamber (*n=160)* | 0% | 100% | 100% | 100% |
| Lower section of fumigation chamber (*n= 160)* | 0% | 100% | 100% | 100% |
| Top section in lugs (*n= 120)* | 0% | 100% | N/A | N/A |
| Middle section in lugs (*n= 120)* | 0% | 100% | N/A | N/A |
| Lower section in lugs (*n= 120)* | 0% | 100% | N/A | N/A |
| Inoculated cardboard (*n=120)* | 0% | N/A | 77% | 100% |
| Top layer in carton (*n=120)* | 0% | N/A | 100% | 100% |
| Middle section of carton (*n=120)* | 0% | N/A | 72% | 100% |
| Lower layer in carton (*n=120)* | 0% | N/A | 98.5% | 100% |

Table 4.1.4. Insect mortality of *Macchiademus diplopterus* after 1 h ethyl formate fumigation of packaged 'Beurrè Bosc' pears during the 2016/2017 season.

| Position | Ethyl formate treatment (g/m³) | |
|---|---|---|
| | **0 g/m³ (control)** | **85 g/m³** |
| Top section of fumigation chamber (*n=720)* | 0% | 100% |
| Middle section of fumigation chamber (*n=960)* | 0% | 100% |
| Lower section of fumigation chamber (*n=720)* | 0% | 100% |
| Inoculated cardboard (*n=720)* | 0% | 100% |
| Top layer in carton (*n=720)* | 0% | 100% |
| Middle section of carton (*n=720)* | 0% | 100% |
| Lower layer in carton (*n=720)* | 0% | 100% |

### 4.1.4 Discussion

With the application of any chemical for phytosanitary treatments there is always the potential risk of causing phytotoxic damage to the commodity, which would result in economic losses. Ethyl formate produces less phytotoxicity compared to other fumigants such as methyl bromide (Yang *et al*., 2016). In this study, no phytotoxic damage was observed, internally or externally, for all stone and pome fruit cultivars treated with ethyl formate. A study by Zoffoli *et al*. (2013), also found no visible phytotoxic damage when fumigating peaches and plums with 1% (~33.05 mg/L) and 3.5% (~115.68 mg/L) ethyl formate for 2h, respectively.

The significant differences observed in hue angle, flesh firmness and total soluble solids, in all the cultivars treated in this study, were due to ripening over time, and not the fumigation treatment, as controls ripening similarly. The ripening process of stone and pome fruit for example involves many biochemical changes such as chlorophyll and starch degradation, the production of volatile compounds and the accumulation of sugars and acids (Mkhathin, 2014). The decrease observed in hue angle and flesh firmness as a result of storage period and simulated shelf life was expected and can be correlated with other plum studies (Abdi *et al*., 1997; Jooste, 2012). The phenomenon observed in many fruit types during cold storage that results in the change in skin/peel colour in most fruit is due to chlorophyll degradation and the biosythesis of carotenoids and anthocyanins (Pentzer and Allen, 1944; Seymour *et al*., 1993; Monreal *et al*., 1999; Varasteh *et al.*, 2012). During ripening changes in the cell wall structure have been observed in many fruit. Cold storage affects the activity of numerous cell wall modifying enzymes influencing pectin metabolism (Brummell *et al.*, 2004). Pectin breakdown is hindered at -0.5°C, although cellulose degradation is not inhibited during cold storage, which could result in the observed decrease in flesh firmness of the pome and stone fruit (Taylor, 1996).

Focusing on export criteria for marketability and storage capabilities, the flesh firmness of all the cultivars tested during these trials, except ‘September Bright’ nectarines, was within the export requirements for South African export fruit (Pawley, 2007; DAFF, 2015a; 2015b). Since the decrease in flesh firmness in ‘September Bright’ nectarines, after treatment, was also observed in the control fruit, this was not due to the application of a fumigant. Both treated and control fruit had flesh firmness that measured below the 44N required for export. The fumigation treatments, done at ambient temperature, coincided with an unexpected heatwave at the time the trials were run. Inadequate temperature control in the room and the high environmental temperatures might have caused the decrease in flesh firmness seen

in the ‘September Bright’ nectarines. Temperature management in the postharvest handling chain for nectarines is, therefore, crucial to prevent the observed decrease in flesh firmness from occurring.

‘August Red’ nectarines treated with ethyl formate showed a higher incidence of pulpiness after shelf life simulation than the control samples. Pulpiness is a form of chilling injury or internal disorder. It has been shown to develop in peaches due to prolonged storage at low temperatures (Brovelli *et al.*, 1998). Harvest maturity or the influence of heat waves may have played a role in the product’s susceptibility to chilling injury, as the other nectarine cultivar, ‘September Bright’, presented no significant differences in pulpiness due to ethyl formate treatment. Heat treatments in various fruits and vegetables have been shown to reduce chilling injury by enhancing membrane integrity, increasing antioxidant activity and heat shock protein gene expression (Aghdam and Bodbodak, 2014). The unexpected heatwave experienced by ‘September Bright’ could potentially have influenced susceptibility to chilling injury compared to ‘August Red’. Research has shown that in some stone fruit, both the extremes of immature and more mature fruit have the potential to yield higher incidences of pulpiness (Lurie and Crisosto, 2005). A flesh firmness after harvest of 112.37N was observed for ‘August Red’ nectarines, which exceeds the maximum of 110.74N recommended for export (DAFF, 2015a). Flesh firmness, observed for ‘August Red’ nectarines was in some cases as high as 123N, which indicates that maturity could have influenced the incidence of pulpiness of treated ‘August Red’ nectarines, and not necessarily the fumigation. The control yielded a lower incidence of pulpiness only after the shelf life simulation, indicating that multiple factors which include the effect of ethyl formate fumigation, could have influenced the susceptibility of ‘August Red’ to chilling injury. To determine if the effect of ethyl formate on the incidence of pulpiness was an isolated incident or cultivar related, the effect of ethyl formate fumigation on ‘August Red’ nectarines needs to be investigated further.

The decrease in the incidence of shrivel levels that was observed for ‘Songold plums after shelf life simulation has been observed in other stone fruit cultivars as well (Jooste, 2012; Theron, 2015). Theron (2015) observed a 5 – 20% decrease in shrivel after shelf-life simulation compared to levels after cold storage during the 2013/2014 season. The observed decrease in shrivel levels during the shelf-life period could be due to cell wall disassembly, in conjunction with loss of turgidity of the mesocarp cells during fruit ripening at higher temperatures. The loss of cell wall integrity and turgor of the mesocarp cells as a result of fruit softening could possibly have resulted in the skin surface relaxing, making

shrivel less evident (Jooste, 2012). Brüggenwirth and Knoche (2016) also stated that decrease in skin stiffness or elasticity in sweet cherries is probably the result of enzymatic softening of the cell walls of the skin in the ripening fruit. Relaxation of the cell walls leads to a change in turgor by transpiration and decreasing pectin middle lamellae viscosity at higher temperatures. Commercial export fruit with shrivel levels exceeding 10% are usually rejected. In this trial the control 'Songold' plums also exceeded this requirement.

The effect of ethyl formate fumigation on the flavour of treated fresh horticultural products, such as grapes, citrus, strawberries, pineapples and bananas has been evaluated, with results indicating no adverse effects on taste (Misumi *et al*., 2013; Ryan and De Lima, 2014). The stone and pome fruit cultivars subjected to a taste assessment in the present study also showed no alteration to the flavour profile. Pupin *et al*. (2013) treated navel oranges and lemons with ethyl formate and measured the levels of its breakdown products, ethanol and formic acid, in treated and untreated fruit, and found no significant differences, providing more evidence that ethyl formate fumigation does not affect fruit flavour, nor does it leave residues on the fruit. Bessi *et al*. (2016) investigated the effect of ethyl formate fumigation on the sugar content (fructose, glucose etc.) in dates. They found that it did not affect the activity responsible for sucrose reduction, unlike other disinfestation techniques, like heating or freezing. The trials conducted during this study also found no significant differences in the total soluble solids due to ethyl formate treatment for all cultivars investigated.

The only other study done on the effect of ethyl formate fumigation on grain chinch bug survival is based on fumigation with Vapormate$^{TM}$, a formulation in which ethyl formate is mixed with $CO_2$ for stabilization of the active ingredient. In their study, Grout and Stoltz (2016) found that a Vapormate$^{TM}$ concentration of 170 g/m$^3$ for 6h at ambient was effective to control the grain chinch bug in the absence of fruit. With the addition of fruit, a higher concentration of 250 g/m$^3$ for 6 h was required to control the insects. Learmonth and Ren (2012) also found that an increased concentration of ethyl formate was required for effective control of adult eucalyptus snout weevil Gyllenhal 1833 (*Gonipterus scutellatus*) when apples were included in the efficacy trials. Absorption by commodities is an important consideration in fumigation treatment, as it can affect the availability of the fumigant and reduce effectivity to control the target pest(s). It has been shown that ethyl formate does not penetrate the host commodity sufficiently to kill internal pests, such as fruit fly larvae and eggs (Armstrong, 1992; Armstrong *et al*., 2014), and is therefore more suitable to control external pests, such as the grain chinch bug. However, some absorption by the commodity does occur and affects control of targeted external pests. A product such as grain is highly

absorptive to ethyl formate (Haritos *et al*., 2003). To reduce the absorption of ethyl formate by grain, a forced flow system was used to reduce the duration and interaction of ethyl formate near grain, successfully reducing the effect of absorption and improving efficacy of treatment. Grout and Stoltz (2016) did propose that the higher concentrations required to control grain chinch bug in the presence of fruit was due to the potential absorption effect of the commodities treated. However, in the present study, the effective concentration and duration of treatment did not differ when the grain chinch bugs where fumigated with fruit (in the desiccators and lugs). There was, however, an effect when packaging was included. During these trials a dilution effect was observed where insects survived in the areas with denser / thicker corrugated cardboard due to more cardboard layers. More packaging material can absorb more of the ethyl formate, while the type of packaging can also affect permeability and the distribution of the fumigant in the product. Sigmund (2005) examined the distribution and effectiveness of liquid ethyl formate when injected into small rice bags when it was applied as a fumigant. They compared polythene and nylon rice bags (750 g capacity) for permeability, and found that the nylon bags appeared to be less permeable to ethyl formate compared to the polythene, resulting in higher insect mortality in the nylon bags. It was recommended that a higher concentration be used with the polythene bags to increase mortality. Therefore, the absorptive capacity of the commodity, as well as the absorption and permeability of the packaging, are crucial considerations in fumigation treatment development.

Haritos *et al*. (2006) proposed that the $CO_2$ in Vapormate™ not only stabilises the product, but also enhances the efficacy of the fumigant through its synergistic effect with ethyl formate, and reduces the time required to kill the insects. To further address the challenges regarding the penetration of ethyl formate through grain and significant losses to grain sorption, Haritos *et al.* (2006) found the added impact of $CO_2$ beneficial. By adding $CO_2$, the efficacy was enhanced to control rice weevil (*Sitophilus oryzae* Linnaeus), the lesser grain borer (*Rhyzopertha dominica* F) and the flour beetle (*Tribolium castaneum*). However, Grout and Stoltz (2016) found that $CO_2$ did not contribute to increasing the mortality of the grain chinch bug when fumigated with Vapormate™. They concluded that the active ingredient, ethyl formate, was the driving force in controlling the grain chinch bug, and that the $CO_2$ provided no benefit other than stabilising the fumigant. A recent study on the effect of carrier gases, such as $CO_2$ and $N_2$, for the application of ethyl formate as a fumigant does, however, indicate that $CO_2$ contributes to the efficacy of the fumigation treatment. Brown *et al*. (2018) treated two postharvest pests of kiwifruit, namely greedy scale (*Hemiberlesia rapax*

Comstock) and obscure mealybug (*Pseudococcus viburni* Signoret), with ethyl formate in combination with either $CO_2$ or $N_2$. They found that higher mortality of both pest species was achieved when $CO_2$ was used. The loss of efficacy in the treatment when $N_2$ was used as the carrier gas was evident at both treatment temperatures (5°C and 15°C) used in their trials, but more significantly so at the lower temperature. Comparatively, $CO_2$ is more expensive and slower to deliver into a fumigation space than $N_2$, but given the results of their study, Brown *et al*. (2018) maintain that $CO_2$ is the preferred carrier gas for ethyl formate and technological solutions for more rapid $CO_2$ delivery are needed to develop ethyl formate fumigation for postharvest purposes. Simpson *et al*. (2007) demonstrated the mortality response of the Western flower thrip (*Frankliniella occidentalis* Pergande) to a range of ethyl formate concentrations in combination with $CO_2$. The addition of 10% $CO_2$ to ethyl formate had varying effects on the efficacy and this was influenced by the insect life stage. This same phenomenon was seen by Armstrong *et al*. (2014) where the effect of adding $CO_2$ to ethyl formate to increase efficacy was shown to vary between target pests. It appears, from the literature, that the response to the addition of $CO_2$ to the process of ethyl formate fumigation varies according to the target pest and the life stage treated.

The concentration of ethyl formate required to control grain chinch bug in the Grout and Stoltz (2016) study, in the presence of fruit – 250 g/m$^3$ Vapormate$^{TM}$ (equivalent to 42 g/m$^3$ ethyl formate) was close to that found to be effective in the present study, namely 50 g/m$^3$. However, the duration of effective treatments differed significantly – 6 h (Grout and Stoltz, 2016) versus 1 h in this study. Further research is required to clarify this difference if commercial application of ethyl formate is to be developed. Treatment duration in commercial fumigations impacts the feasibility of application and logistics surrounding fruit flow and quality. High volumes of fruit that require fumigation may only have a short window of time during the season for treatments to be applied.

In conclusion, fumigation with ethyl formate successfully controls a key phytosanitary pest of South African export fruit within a dose range that does not have adverse effects on fruit quality, at least for the stone and pome fruit cultivars treated in this study. In addition to the insecticidal properties of ethyl formate, which need to be tested on other insect pest species also affecting fruit exports, the fungicidal properties of this fumigant may also be beneficial in postharvest management. Research conducted by Utama *et al*. (2002) found that ethyl formate was germicidal against the plant pathogens, *Rhizopus. stolonifer, Colletotrichum musae* and *Pseudomonas aeruginosa*, and according to Palou *et al*. (2008) ethyl formate negatively affects, but does not completely inhibit the growth of *Penicillium digitatum* in

citrus. The potential for the use of ethyl formate fumigation to control the grain chinch bug, as well as certain other phytosanitary pests and pathogens, and maintain and expand market access for fresh agricultural export products from South Africa is crucial to ensure long term market sustainability.

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# Paper 6

# Central Composite Designs (CCD) as a predictive tool when fumigating pome and stone fruit with ethyl formate

## 4.2.1 Introduction

Currently, worldwide, postharvest research is focused on developing effective non-chemical disinfestation methods for phytosanitary pest control. This is due to a ban on the use of methyl bromide since its ozone depleting properties were identified (UNEP, 2000). Historically, methyl bromide fumigation was the most widely used fumigant due to its efficacy against a wide range of pests at concentrations that did not negatively affect commodity quality or marketability (Fields and White, 2002). Several fumigants exist that are alternatives to methyl bromide. These include phosphine, carbonyl sulfide and ethyl formate (Ducom and Banks, 2006). Phosphine fumigation has been key in controlling phytosanitary pests, but heavy reliance thereof has resulted in the development of resistance in several pest species (Shi *et al*., 2013; Bessi *et al*., 2016). Ethyl formate is recognized as a GRAS (Generally Recognized As Safe) chemical and is used as a flavouring agent in a variety of products (FDA, 2014). It is a volatile compound with low molecular weight, and is considered to be the most promising fumigant to replace methyl bromide due to its insecticidal and fungicidal properties (Nursten, 1970; Simpson *et al*., 2004).

In the development and application of any disinfestation treatment the balance between commodity and pest tolerances is an important concern in order to ensure maintaining fruit quality and ultimately, the market. Crucial factors such as fumigant concentration, fumigation duration and temperature should be considered when developing a successful fumigation treatment regime targeting a specific insect pest. The balance of all these factors will ensure that fruit quality is maintained while controlling the target pest.

A central composite design is an experimental design in which a minimal number of experiments yield maximum information about a process (Yang *et al*., 2009). Data generated using a central composite design is used to generate a regression model which is visualised through surface area plots. The use of regression analysis is a predictive modelling technique which examines the relationship between a dependent and independent variable(s). The generated regression model can be used as a predictive tool to determine either the optimal combination of factors (for example, fumigant concentration and fumigation duration) to yield a required result, or to provide predicted guidelines for prevention of undesirable outcomes (for example, phytotoxic damage). The illustration of

the regression model using surface area plots provides a visual response of factors (such as, ethyl formate concentration, fumigation duration and treatment temperature) for a specific parameter. The regression models for a specific commodity could, therefore, be used to assess maturity and quality parameters such as hue angle, flesh firmness and the product's susceptibility to phytotoxic damage.

Compiling a model for stone and pome fruit will present researchers with 'safe zones', or limits, when fumigating with liquid ethyl formate. This will enable researchers to determine if any phytotoxic or undesirable effect will be observed on the tested commodities when applying a critical dose and duration to control the targeted pest. The potential undesirable effect of liquid ethyl formate on other quality parameters such as hue angle, flesh firmness and sugars, which could potentially influence the export and marketability of the product, could also be predicted.

### 4.2.2 Materials and Methods

#### Fruit

'Golden Russet Bosc' pears *(Pyrus communis* L.*)* and nectarine (*Prunus persica var. nucipersica* (Suckow) C. K. Schneid) cultivars ('Alpine', 'August Bright' and 'August Red') were sourced from a commercial packhouse in Ceres (33°22'S, 19°19'E), Western Cape, South Africa on their respective optimum harvest dates. The Japanese plum (*Prunus salicina* Lindl.) cultivar, 'Songold', was sourced from a commercial packhouse on its optimum harvest date in Franschhoek (33°54'33.2"S, 19°06'58.4"E), Western Cape, South Africa. The average flesh firmness at harvest for all fruit was within the picking window used for export (DAFF 2015a, 2015b, data not shown). Pears were sourced and treated during the 2015/2016 and 2016/2017 season, and stone fruit was treated only during the 2016/2017 season.

#### Central composite design and fumigation treatments

A central composite design (CCD) experiment was created to run fumigation treatments on the selected fruit cultivars. See Table 4.2.1 for the different treatment durations and corresponding ethyl formate concentrations applied to test the effect of ethyl formate fumigation on fruit quality. The data obtained was used to generate regression models, and ultimately determine the threshold for phytotoxic damage in each fruit type and cultivar. The adequacy model was determined through diagnostic checking tests using analysis of variance (ANOVA) (Statsoft Inc. 2017).

Example of the regression equation used:

$y = b_0 + b_1C + b_2D + b_3C^2 + b_4D^2 + b_5C.D$

*Note: b = regression coefficient and $b_0$ = mean / intercept*

*C = ethyl formate concentration ($g/m^3$) and D = treatment duration (hours)*

Table 4.2.1: Central composite design experimental runs for ethyl formate fumigation of stone and pear cultivars treated during the 2015/2016 and 2016/2017 season.

| Treatment duration (hours) | Ethyl formate concentration ($g/m^3$) |
|---|---|
| 1 | 90 |
| 2 | 48 |
| 2 | 132 |
| 3.5 | 31 |
| 3.5 | 90 |
| 3.5 | 149 |
| 5 | 48 |
| 5 | 132 |
| 6 | 90 |

For each cultivar additional factors were introduced to determine if an interaction with ethyl formate would take place, that could potentially have an undesirable effect on fruit quality or induce phytotoxicity. Factors (temperature and harvest maturity) used to compile the regression models for ‘Golden Russet Bosc’ pears during the 2015/2016 and 2016/2017 seasons are outlined in Table 4.2.2.

Table 4.2.2: Factors included in central composite design experimental runs for compiling a regression model for ethyl formate fumigation of ‘Golden Russet Bosc’ pears during the 2015/2016 and 2016/2017 seasons.

| Season | Pulp temp. (°C) when fumigated | Harvest Maturity [1] | Experiment repeated[2] |
|---|---|---|---|
| 2015/2016 | 0 | ±69N | no |
| | 10 | ±69N | no |
| | 23 | ±69N | no |
| 2016/2017 | 0 | ±69N | yes |
| | 23 | ±69N | yes |

*Note:*
*[1]Conversion of Newton to kilogram, divide by 9.80. Firmness of ‘Golden Russet Bosc’ pears was measured using 8 mm tip.*
*[2] Repetition of CCD experiment taking all parameters into account to confirm results generated.*

During the 2016/2017 season 10°C was excluded from the experimental design as no differences between 0 and 10 were observed during the 2015/2016 season. Factors included in the experimental design for stone fruit, namely temperature, harvest maturity and pre-ripening (in 'August Red' nectarines) are outlined in Table 4.2.3.

Table 4.2.3: Factors included in central composite design experimental runs for compiling a regression model for ethyl formate fumigation of stone fruit during the 2016/2017 season.

| Commodity | Pulp temp. (°C) when fumigated | Harvest Maturity[1] | Pre-ripened[2] | Experiment repeated[3] |
|---|---|---|---|---|
| 'Songold' plums | 0 | ±69N | n/a | yes |
| | 23 | ±69N | n/a | yes |
| 'Alpine' nectarines (Early cultivar) | 0 | ±88N | n/a | yes |
| | 0 | ±69N | n/a | yes |
| | 23 | ±88N | n/a | yes |
| | 23 | ±69N | n/a | yes |
| 'August Bright' nectarines (Mid/Late cultivar) | 0 | ±88N | n/a | No[4] |
| | 0 | ±69N | n/a | |
| | 23 | ±88N | n/a | |
| | 23 | ±69N | n/a | |
| 'August Red' nectarines (Late cultivar) | 0 | ±69N | yes | yes |
| | 0 | ±69N | no | yes |
| | 23 | ±69N | yes | yes |
| | 23 | ±69N | no | yes |

*Note:*
*[1,]Conversion of Newton to kilogram, divide by 9.80. Firmness of stone fruit cultivars ('Songold' plums, 'Alpine', 'August Bright' and 'August Red' nectarines) determined using a 11.1 mm tip.*
*[2] Pre-ripening of nectarines involves a delay in cooling of fruit whereby fruit is kept 20 °C for approximately 48 hours after harvest to speed ripening.*
*[3] Repetition of CCD experiment taking all parameters into account to confirm results generated.*
*[4] Due to time constraints experiments were not repeated*

Fruit was placed inside 14 L glass desiccators for fumigation (Fig 4.2.1). Based on individual fruit size, the number of fruit in a desiccator varied with each cultivar treated, and the free volume of the desiccator was, therefore, determined for each treatment. The free volume was used to calculate the amount of ethyl formate (µL) required to achieve the desired

concentration inside the desiccator. Liquid ethyl formate was pipetted into an open glass petri dish placed on top of the fruit in each desiccator, which was closed immediately after the ethyl formate was applied, and left to fumigate for the required duration at the required temperature.

Figure 4.2.1. Illustration of ‘Songold’ plums in 14L desiccators for fumigation with liquid ethyl formate.

After treatment, fruits were packed according to export standards per fruit type and subjected to commercial cold storage regimes. ‘Golden Russet Bosc’ pears were packed in MK4 cartons with a green 37.5-micron inner bag. ‘Songold’ plums were packed in two layer 4.5 kg cartons, and the three nectarine cultivars (‘Alpine’, ‘August Bright’ and ‘August Red’) were packed in two layers in 8 kg cartons. The plums and nectarines were packed with perforated high density polyethylene shrivel sheets to retard moisture loss during cold-storage. Commercial cold storage regimes per cultivar were used. ‘Golden Russet Bosc’ pears were stored for 12 weeks at -0.6°C. An intermittent warming regime was used for ‘Songold’ plums, namely 10 days at -0.5°C, 7 days at 7.5°C and 25 days at -0.5°C. The three nectarine cultivars were stored for 4 weeks at -0.5°C, respectively. After cold storage fruit was also subjected to a simulated shelf life period of 7 days at 10 °C.

The effect of ethyl formate on fruit quality and assessment of the phytotoxic effects of ethyl formate fumigation was conducted at three evaluation points: immediately after treatment, after cold storage, and after cold storage plus shelf life simulation.

**Fruit evaluations**

Maturity parameters of fruit for all trials were evaluated at harvest, after treatment, after cold storage and after cold storage plus shelf life simulation. Hue angle (°) was measured using a calibrated colorimeter (Minolta chroma meter CR-400, Japan) to quantify the change in ground colour of the fruit. Flesh firmness (N) was measured using a Fruit Texture Analyzer (Güss Manufacturing, Strand, South Africa). Stone fruit flesh firmness was measured with an 11.1 mm tip, on two peeled cheeks per fruit. Pear cultivar flesh firmness was measured

using an 8 mm tip on two peeled cheeks per fruit. Total soluble solids (TSS), expressed as % Brix, were determined using a temperature controlled, digital refractometer (Atago digital refractometer PR-32a, Japan). Titratable acid (TA) (%) expressed as malic acid was determined through titration of 10 g of juice with 0.1 N NaOH to a pH end point of 8.2 using an automated titrator (Metrohm 719 S Titrino, Herisau, Switzerland). Both TSS and TA were measured using pooled juiced samples of 20 fruit per replicate per treatment.

After treatment, after cold storage and after cold storage plus shelf life simulation external fruit quality parameters (shrivel, decay and external phytotoxic damage) and internal fruit quality parameters were determined for all cultivars. External fruit quality was subjectively evaluated on 20 fruit and internal fruit quality was subjectively evaluated on 10 fruit per replicate per treatment and expressed as a percentage of the total fruit examined.

Internal quality defects examined specific to the cultivars were internal browning and gel breakdown for ‘Songold’ plums, mealiness and pulpiness for the nectarine cultivars and incidence of astringency and mealiness for the pear cultivar. Internal disorders were evaluated by cutting the fruit around the equatorial axis and separating the two halves of the fruit. Internal browning was observed as a brown discolouration of the mesocarp tissue directly beneath the skin of the fruit that spreads throughout the entire mesocarp tissue in severe cases. Gel breakdown in plums is defined as the gelatinous breakdown of the inner mesocarp tissue surrounding the stone, while the outer mesocarp tissue displays no damage (Jooste, 2012). Fruit wedges were used from both nectarine and pear cultivars to assess for mealiness by squeezing the sample to examine the presence of free juice. Mealiness can be defined as fruit that appears dry with a coarse, floury texture; therefore the presence of free juice will be limited. Astringency in pears can be defined as the sensation of drying of the mouth, which was assessed by the tasting of fruit wedges. Phytotoxicity is defined as any adverse effect that results in damage to the cuticle (formation of spot like necrosis) and penetration of the tissue resulting in cork like tissue.

**Statistical analysis**

The statistical analysis was performed using STATISTICA version 13 (Statsoft, Inc., 2017). The experimental data were analyzed by multiple regression analysis through the least square method. Two different tests, namely sequential sum of squares and model summary statistics, were carried out on the experimental data in order to determine the adequacy of the various models. The regression coefficients of all the terms (linear, quadratic, and interaction) involved in the model, and their effect were analyzed by analysis of variance

(ANOVA) and ANOVA tables were generated. All the terms of the model were tested and verified statistically by F-test at probability levels ($P < 0.05$). Adequacy of the developed models was tested by performing coefficient of determination ($R^2$), adjusted coefficient of determination ($R^2$ adj) and predicted coefficient of determination ($R^2$ pre). After fitting the models, surface and contour plots were constructed to predict the relationship between the independent variables and responses.

### 4.2.3 Results

***'Golden Russet Bosc' pears (season 2015/2016 and 2016/2017)***

No significant differences were observed in parameters such as hue angle, flesh firmness and total soluble solids for all models generated over two seasons (Appendix D). The different ethyl formate concentrations and fumigation durations applied during fumigating did not affect the products' ability to meet the criteria used for export as outlined by DAFF (DAFF, 2015b). s

Phytotoxic damage was only observed after shelf life simulation. Blackening around the stem and calyx ends (Fig 4.2.2A and B) and wound blackening of bruised tissue (Fig 4.2.2C) occurred during both the 2015/2016 and 2016/2017 seasons. As no phytotoxic damage was observed immediately after treatment and cold storage the data of these two evaluations were excluded from the model. Models for phytotoxic damage were, therefore, compiled using only data generated after the shelf life simulation.

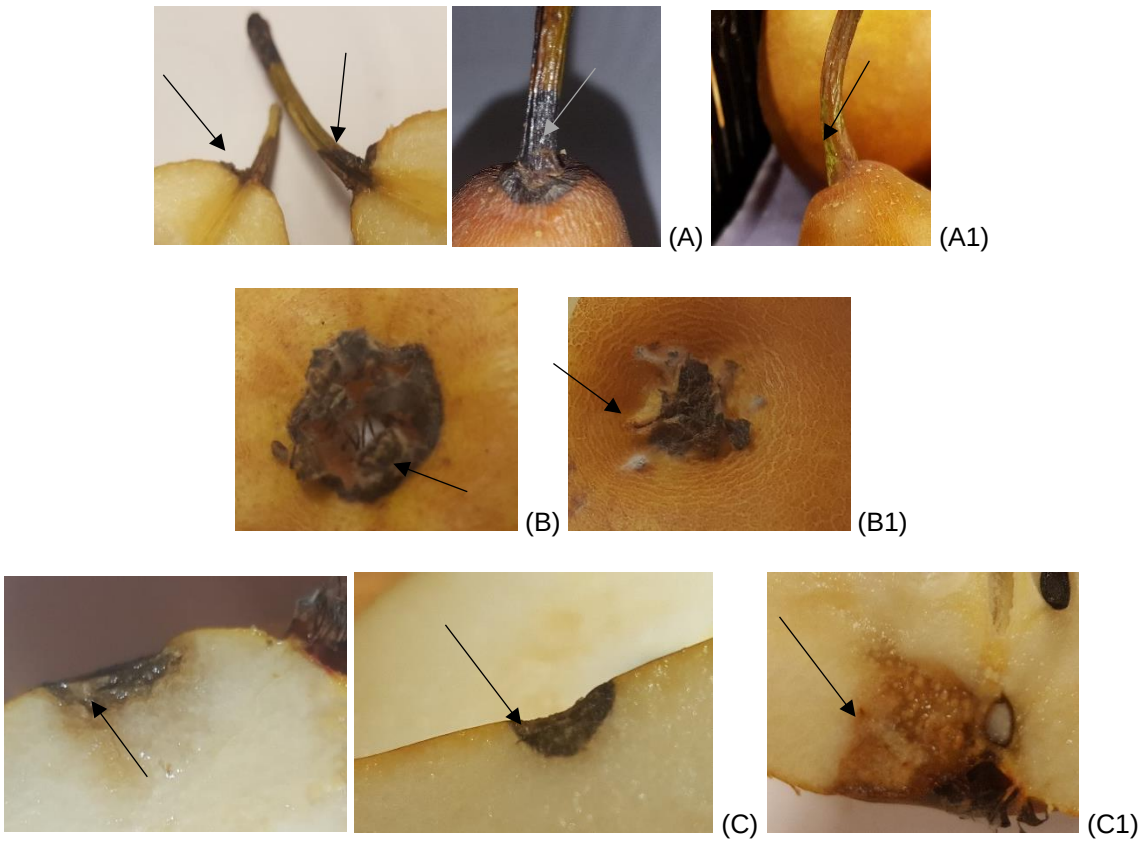



Figure 4.2.2. Photographs of the (A) stem, (B) calyx and (C) wound blackening that occurred due to high concentrations of ethyl formate (132g/m$^3$) with prolonged exposure times (5 hours). Control fruit images (A1, B1 and C1) are given on the right for comparison.

There was a significant interaction between concentration and treatment duration for total phytotoxic damage observed after shelf-life simulation during the 2015/2016 season (Table 4.2.4). During the 2016/2017 season, however, no interaction was observed for total phytotoxicity. This could be due to the differences not being as pronounced as those observed during the 2015/2016 season.

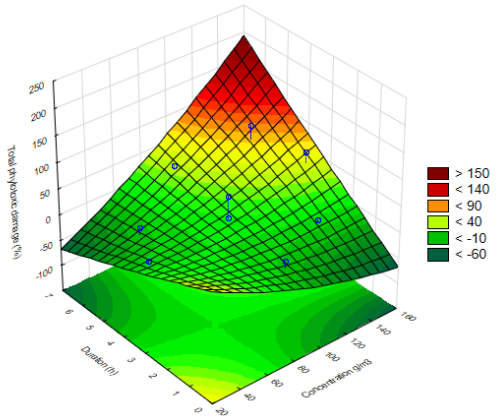
Table 4.2.4. ANOVA results illustrating influence of concentration and duration on the incidence of total phytotoxic damage on 'Golden Russet Bosc' pears ($R^2$ = 0.79) during the 2015/2016 season.

| Factor | p |
|---|---|
| **(1) Concentration (g/m$^3$) (L)** | **0.0108** |
| Concentration (g/m$^3$) (Q) | 0.2653 |
| **(2) Duration (hours) (L)** | **0.0207** |
| Duration (hours) (Q) | 0.8667 |
| (3) Temperature (°C) (L) | 0.1415 |
| Temperature (°C) (L) | 0.4593 |
| **(1) by (2)** | **0.0142** |
| (1) by (3) | 0.4073 |
| (2) by (3) | 0.4195 |

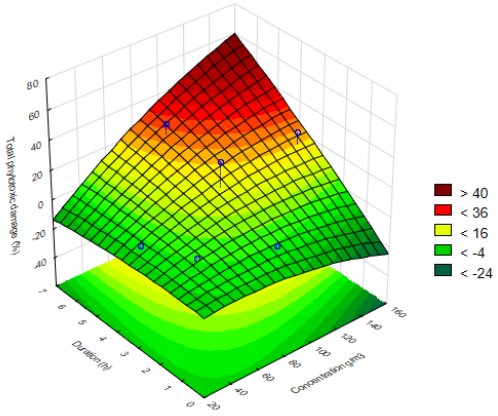
*Note: L: linear fit of the data; Q: quadratic fit of the data.*

The $R^2$ value of 0.79 which was observed during 2015/2016 season indicated that there was a good fit of the model (Fig 4.2.3 A1). The surface area plots indicated that total phytotoxic damage increased with higher concentrations of ethyl formate (149 g/m$^3$) for extended treatment durations (> 3.5 h). Although no significant interaction was observed during the 2016/2017 season the same pattern of damage was observed with an $R^2$ value of 0.64 (Fig 4.2.3 A2). During the 2015/2016 season pulp temperature of the fumigated pears appeared to play a role in their susceptibility to phytotoxic damage.

Fruit at room temperature (~23°C) yielded higher levels of total phytotoxic damage when fumigated with ethyl formate at concentrations above 120 g/m$^3$ (Fig 4.2.3 B1) compared to 0°C and 10°C (data not shown). Although the differences were not statistically significant, a similar pattern was observed during the second season (Fig 4.2.3 B2). The products' susceptibility to phytotoxic damage due to the pulp temperature of the fruit during fumigation is illustrated by Figure 4.2.4. Figure 4.2.5 illustrates an example of the predicted percentage of phytotoxic damage that could occur after cold storage plus shelf life for the different ethyl formate concentrations and treatment durations when fumigated at 23°C.



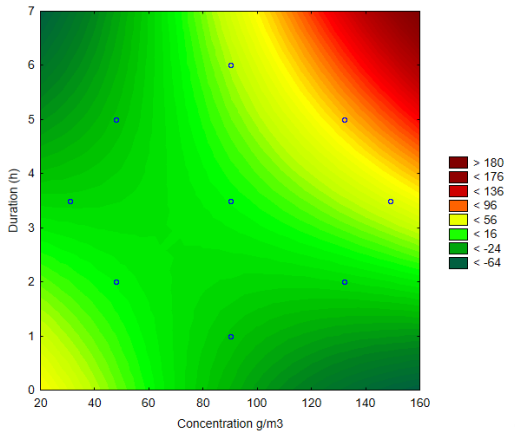

(A1)



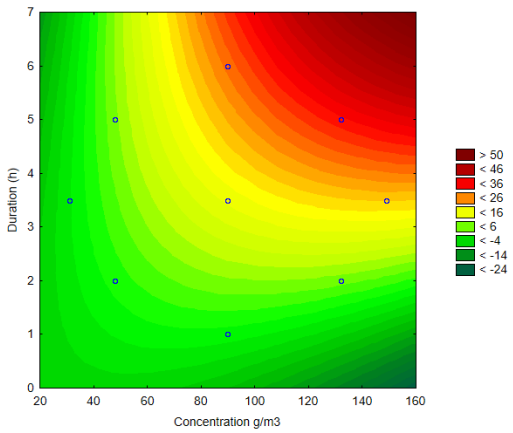

(A2)

| Factor | p |
|---|---|
| (1) **Concentration (g/m³) (L)** | 0.0108 |
| **Concentration (g/m³) (Q)** | 0.2653 |
| (2) **Duration (hours) (L)** | 0.0207 |
| **Duration (hours) (Q)** | **0.8667** |
| ***(1) by (2)*** | ***0.0142*** |

| Factor | p |
|---|---|
| (1) **Concentration (g/m³) (L)** | 0.4974 |
| **Concentration (g/m³) (Q)** | 0.8411 |
| (2) **Duration (hours) (L)** | 0.4581 |
| **Duration (hours) (Q)** | 0.9541 |
| (1) **by (2)** | 0.6902 |




(B1)




(B2)

Figure 4.2.3. Surface area plots illustrating 'Golden Russet Bosc' pears susceptibility to phytotoxic damage due to the interaction between fumigation duration and concentration. The colour bar on the right indicates the percentage of phytotoxic damage that occurred.

*Note:*

*A1 and B1: Total phytotoxic damage (stem, calyx and wound blackening) that occurred in pears fumigated at 23°C during the 2015/2016 season ($R^2$ = 0.79).*

*A2 and B2: Total phytotoxic damage (stem, calyx and wound blackening) that occurred in pears fumigated at 23°C during the during the 2016/2017 season ($R^2$ =0.64).*

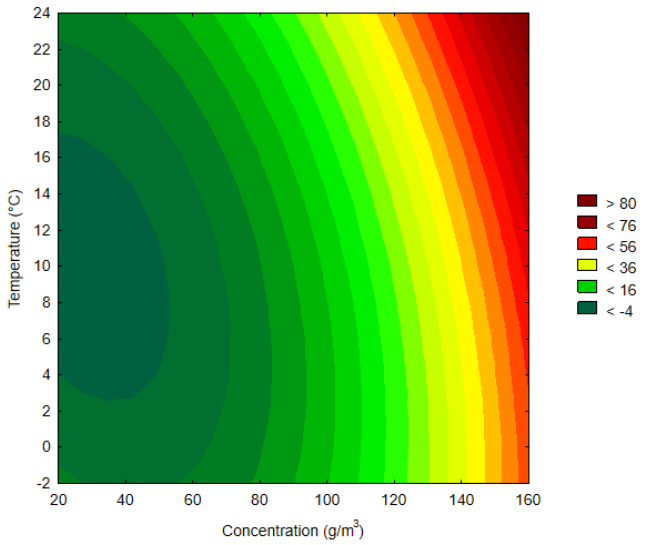



Figure 4.2.4. Illustration of the product's susceptibility to phytotoxic damage due to the pulp temperature of the fruit. Colour bar on the right indicates the percentage of total blackening (stem, calyx and wound) that occurred.

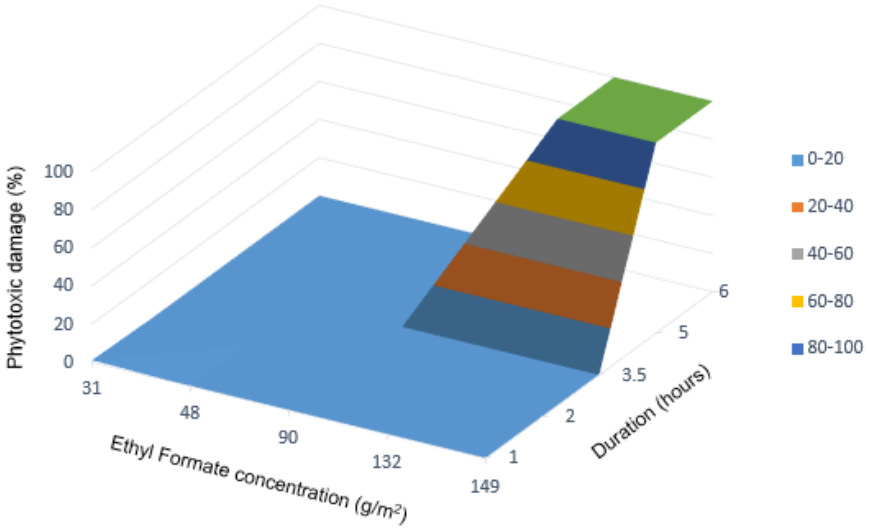



Figure 4.2.5. Illustration of total phytotoxic damage on 'Golden Russet Bosc' pears that is projected by regression model after cold storage plus shelf life simulation due to ethyl formate fumigation at 23°C.

### *'Songold' plums*

No phytotoxic damage was observed for the various ethyl formate treatments using different fumigation durations and temperatures. Repetition of the model yielded the same results. No significant differences were observed for maturity parameters for all the evaluations between treatments (Appendix D). No significant differences were observed after shelf life simulation due to treatment for internal quality parameters, such as total chilling injury

(combination of gel breakdown and internal browning), or external parameters, such as shrivel (Appendix D).

***'Alpine', 'August Bright' and 'August Red' nectarines***

None of the cultivars yielded any phytotoxic damage after cold storage or shelf life simulation, regardless of the cultivar being an early, mid-, or late season cultivar, harvested at the upper or lower end of the picking window, or having undergone pre-ripening.

For all three nectarine cultivars no significant differences were observed for any of the parameters evaluated across the different evaluation periods, taking all additional factors into account (Appendix D). The different ethyl formate concentrations and fumigation durations applied during fumigating did not affect the products' ability to meet the criteria used for export as outlined by DAFF (DAFF, 2015b).

### 4.2.4 Discussion

Ethyl formate fumigation of the pear and stone fruit cultivars included in this study is a promising potential alternative postharvest mitigation treatment, as the only phytotoxic damage observed was observed in the pear cultivar. Also, this phytotoxic damage only occurred at the upper end of the concentration range and fumigation times tested. The fumigation limits, or 'safe zones', identified here allow for further development of ethyl formate fumigation treatments that will maintain fruit quality of 'Golden Russet Bosc' pears, as well as the stone fruit cultivars treated here, as no phytotoxic damage was observed for any of the stone fruit subjected to the same treatments. The pulp temperature of pears during fumigation also had an effect on the products' susceptibility to phytotoxic damage. Higher ethyl formate concentrations in combination with higher pulp temperature could increase the products' susceptibility to phytotoxic damage. This should be considered when developing a fumigation treatment for fruit that will to be fumigated immediately after harvest, which could potentially be at temperatures higher than those evaluated during these trials.

'Golden Russet Bosc' pears are russetted, brown skinned, elongated pears, which, in the present study, were more sensitive to ethyl formate fumigation than the stone fruit cultivars treated here. 'Bosc' pears have been shown to be more susceptible to decay than other smooth-skinned pear cultivars, such as Anjou (Kupferman, 1998). Kupferman (1998) also showed that pome fruit (apples and pears) with thick skins and waxy coatings are less susceptible to phytotoxicity and decay than thin-skinned varieties, implying that the thicker skin and waxy coating provides a level of protection for the fruit. Also, skin thickness may not be uniform for an individual fruit. For example, in apple cultivars there can be

considerable difference in thickness between cultivars and seasons within the same fruit (Homutová and Blažek, 2006). This could be one of the reasons that 'Golden Russet Bosc' pears were more susceptible to damage in the first season compared to the next. It has also been observed that the sun exposed side of apples have a thicker cuticle than the shaded side, and late ripening apple cultivars also have thicker cuticles and hypodermises than earlier ripening fruit (Homutová and Blažek, 2006). Previous research has shown that the surface area and type of commodity influences the products ability to absorb ethyl formate. Paper 6 discussed the importance of the potential absorption effect that could occur, influencing the concentration of ethyl formate which has to be applied to remain effective to control target pests. All these factors potentially influence a cultivar's susceptibility to phytotoxic damage.

Research conducted by Zoffoli *et al*. (2013) using liquid ethyl formate to fumigate various commodities found similar results to those seen here, for plum cultivars. They found when applying a maximum concentration of 3.5% ethyl formate (~ 116 g/m$^3$) for a maximum duration of 2 h (at 20°C) to 'Angeleno' plums no phytotoxic damage was observed. They did however observe sunken areas on 'Sweet September' peaches when exposed to 2% ethyl formate (~ 65 g/m$^3$) for exposure after an hour. This result indicates that peaches could potentially be more sensitive to ethyl formate fumigation compared to plum and nectarine cultivars.

In the last 15 years research has shown that a wide range of ethyl formate concentrations (24-160 g/m$^3$) are effective in controlling target pests on a wide range of fresh agricultural products, without any phytotoxic effect (Simpson *et al*., 2004; Simpson *et al.*, 2007; Van Epenhuijsen *et al.*, 2007; Misumi *et al.*, 2013; Pupin *et al.*, 2013; Zoffoli, *et al*., 2013). However, phytotoxic damage can occur, and knowing the conditions that are conducive to damage prior to testing efficacy against insect pests can accelerate the development of phytosanitary treatments. Using central composite designs, the phytotoxic limits determined here for 'Golden Russet Bosc' pears serves to provide a predictive tool when assessing the potential of ethyl formate fumigation for a variety of insects with different critical dose criteria. In addition, since no phytotoxic damage was observed in the stone fruit cultivars treated here, these limits are applicable to those cultivars as well. The critical concentration/duration requirements to control target pests ultimately determine the feasibility of using EF for postharvest fumigation. The 'safe zones' allow researchers to work within ranges that avoid any undesired effect on fruit quality while screening the variety of insects that require phytosanitary control. These limits could also be used to guide the development of

fumigation treatments using Vapormate$^{TM}$, a commercially available formulation of ethyl formate and $CO_2$, where $CO_2$ is added to reduce flammability and potentially enhance treatment efficacy. Although challenges exist with the application of ethyl formate fumigation, whether formulated as Vapormate$^{TM}$, or not, these are outweighed by the benefits of this alternative phytosanitary risk mitigation option, to the environment as well as fresh produce export industries.

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# CHAPTER 5

## 5.1 Concluding comments

International trade of agricultural products has become an essential part of the global economy, but not without significant challenges. One area with many challenges is phytosanitation and the postharvest control of phytosanitary insect pests on fresh agricultural product. This dissertation addresses a variety of issues related to phytosanitary control and fresh fruit exports from South Africa, provides solutions to some of the associated challenges, presents new information on a difficult-to-control pest species, *Macchiademus diplopterus*, as well as a new effective method for its control in future.

The viability of CATTS technology as alternative phytosanitary treatment was focused on controlling three key South African pests. The banded fruit weevil, *Phlyctinus callosus*, was the easiest to control with CATTS treatments. The false codling moth, *Thaumatotibia leucotreta*, was challenging to control without detrimental side effects on fruit quality. Internal pests such as this are difficult to control with CATTS, as the heat and atmospheric changes must penetrate the fruit sufficiently to increase stress conditions for the insect. This study has shown, however, that certain heat conditions can be used to pre-condition chill sensitive stone fruit, which are affected by cold sterilisation required to control *T. leucotreta.* This requires further research but is a feasible option to control other pests such as *P. callosus*, while pre-conditioning the fruit for subsequent cold treatment against *T. leucotreta.* The grain chinch bug, *M. diplopterus*, was the most tolerant to CATTS treatments, and highly thermal (heat and cold) tolerant behaviour was observed. The physiological study provided insight into some the mechanisms involved, indicating the ineffectiveness of control using thermal treatments. Ethyl formate fumigation was, however, highly effective in controlling *M. diplopterus*. This technology could not only be used for controlling *M. diplopterus* but various other external insects not sufficiently controlled with thermal treatments. More research is required to further develop this technology, but it provides a solution for a long-standing phytosanitary pest problem.

Commercial application of alternative phytosanitary treatments, such as those examined during this study, will enable the South African export industry to maintain existing markets by offering improved measures for phytosanitation, as well as expand and access new markets and continue to participate and grow in international trade.

# Appendix A

## Chapter 2

*Paper 2*: Chill sensitive 'Songold' Japanese plums (*Prunus salicina* Lindl.) and heat treatment for phytosanitary purposes

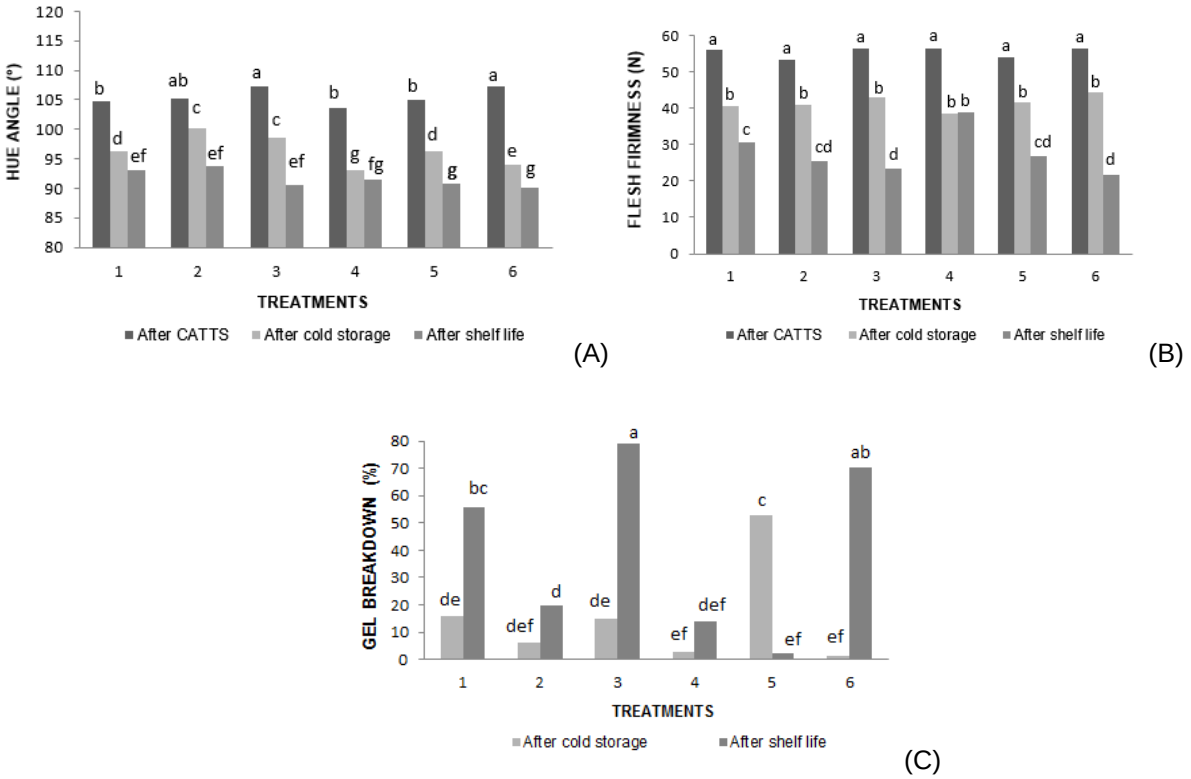
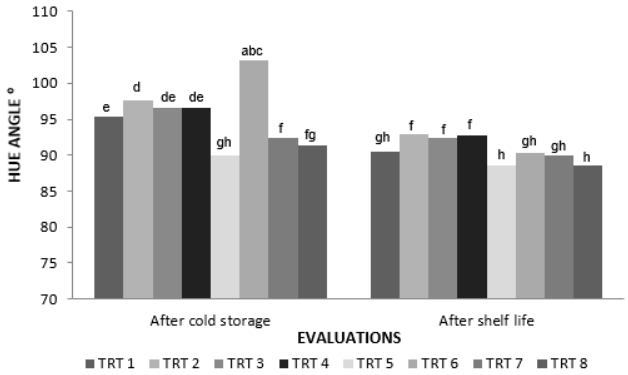
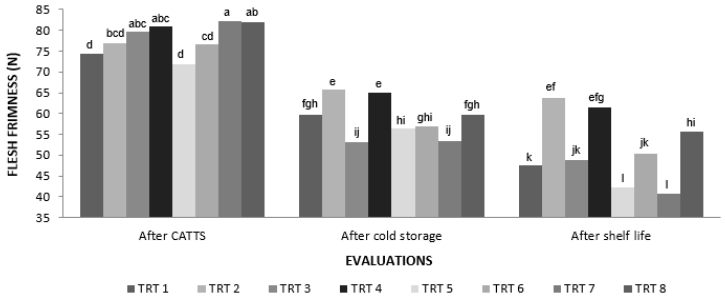



Figure A.1. The effect of CATTS treatments on the hue angle (A), flesh firmness (B) and gel breakdown (C) in 'Songold' plums during the 2015/2016 season. *Note: Trt 1 = 80°C* $h^{-1}$ *ramp until air temp 56°C, when pulp temp reached 42°C hold 5 min (stored using cold sterilisation regime); Trt 2 = 80°C* $h^{-1}$ *ramp until air temp 56°C, when pulp temp reached 42°C hold 5 min then bring air temp down to 40°C and hold for 90min (stored using cold sterilisation regime); Trt 3 = No CATTS treatment (control (stored using cold sterilisation regime); Trt 4 = same as Treatment 1 (stored using intermittent warming regime); Trt 5 = same as treatment 2 (stored using intermittent warming regime); Trt 6 = No CATTS treatment (control stored using intermittent warming regime)*




(A)




(B)

Figure A.2. The effect of CATTS treatments on the hue angle (A) and flesh firmness (B) in 'Songold' plums during the 2016/2017 season. *Note: Stored using cold sterilisation regime: Trt 1 = Ramp 12°C.* $h^{-1}$ *until pulp temperature is 35°C. Hold for 5 hours (no Smartfresh™); Trt 2: Ramp 12°C.* $h^{-1}$ *until pulp temperature is 35°C. Hold for 5 hours (Smartfresh™); Trt 3 = control (no Smartfresh™); Trt 4 = control (Smartfresh™). Stored using duel temperature regime: Trt 5 = same as Treatment 1; Trt 6 = same as treatment 2; Trt 7 = same as treatment 3 (Control = no Smartfresh™); Trt 8 = same as treatment 4 (Control = Smartfresh™)*

# Appendix B

## Chapter 3

*Paper 4:* Macromolecules composition of aestivating grain chinch bug (*Macchiademus diplopterus*) in response to thermal stresses

Table B1: Mortality of *Macchiademus diplopterus* after treatment (heat and cold storage) during different aestivation (early and mid) and evaluation time points, during the 2016/2017 season. *See Table 3.2.1 and 3.2.2 for descriptions for treatments and evaluation time points.*

| Effect | Df | MS | F | ρ |
|---|---|---|---|---|
| Aestivation Period | 1 | 8205 | 214.27 | <0.0001 |
| Evaluation Period | 6 | 11423 | 298.32 | <0.0001 |
| Treatment | 5 | 15782 | 412.16 | <0.0001 |
| Aestivation Period x Evaluation Period | 6 | 721 | 18.82 | <0.0001 |
| Aestivation Period x Treatment | 5 | 2322 | 60.64 | <0.0001 |
| Evaluation Period x Treatment | 30 | 1832 | 47.86 | <0.0001 |
| ***Aestivation Period x Evaluation Period x Treatment*** | ***30*** | ***662*** | ***17.29*** | ***<0.0001*** |
| Error | 168 | 38 | | |

Table B2: Total protein content of dead and alive *Macchiademus diplopterus* after treatment (heat and cold storage) during different aestivation (early and mid) and evaluation time points, during the 2016/2017 season. *See Table 3.2.1 and 3.2.2 for descriptions for treatments and evaluation time points.*

| Effect | Df | F | ρ |
|---|---|---|---|
| Aestivation Period | 1 | 220,865 | <0.0001 |
| Evaluation time point | 6 | 10,779 | <0.0001 |
| CATTS treatment | 1 | 0,648 | 0,4215 |
| Cold storage | 1 | 8,230 | 0,0045 |
| **Insects (dead or alive)** | **1** | **31,949** | **<0.0001** |
| CATTS x Cold storage | 1 | 6,007 | 0,0150 |
| CATTS x Insects | 1 | 3,497 | 0,0628 |
| Cold storage x Insects | 1 | 0,233 | 0,6297 |
| CATTS x Cold storage x Insects | 1 | 0,499 | 0,4809 |
| Residuals | 222 | | |

Table B3: Total lipid content of dead and alive *Macchiademus diplopterus* after treatment (heat and cold storage) during different aestivation (early and mid) and evaluation time points, during the 2016/2017 season. *See Table 3.2.1 and 3.2.2 for descriptions for treatments and evaluation time points.*

| Effect | Df | F | ρ |
|---|---|---|---|
| Aestivation Period | 1 | 20,8972 | <0.0001 |
| Evaluation time point | 6 | 9,5732 | <0.0001 |
| CATTS treatment | 1 | 1,6268 | 0,2035 |
| Cold storage | 1 | 0,1553 | 0,6939 |
| Insects (dead or alive) | 1 | 3,0595 | 0,0817 |
| CATTS x Cold storage | 1 | 1,0443 | 0,3079 |
| CATTS x Insects | 1 | 3,1355 | 0,0780 |
| Cold storage x Insects | 1 | 0,1330 | 0,7157 |
| CATTS x Cold storage x Insects | 1 | 2,5942 | 0,1087 |
| Residuals | 222 | | |

Table B4: Sugar content of dead and alive *Macchiademus diplopterus* after treatment (heat and cold storage) during different aestivation (early and mid) and evaluation time points, during the 2016/2017 season. *See Table 3.2.1 and 3.2.2 for descriptions for treatments and evaluation time points.*

| Effect | Df | F | ρ |
|---|---|---|---|
| Aestivation Period | 1 | 149,1804 | <0.0001 |
| Evaluation time point | 6 | 14,3753 | <0.0001 |
| CATTS treatment | 1 | 0,7110 | 0,4000 |
| Cold storage | 1 | 7,7226 | 0,0059 |
| Insects (dead or alive) | 1 | 0,7839 | 0,3769 |
| CATTS x Cold storage | 1 | 0,1225 | 0,7267 |
| CATTS x Insects | 1 | 3,9516 | 0,0481 |
| Cold storage x Insects | 1 | 1,6471 | 0,2007 |
| CATTS x Cold storage x Insects | 1 | 0,5299 | 0,4674 |
| Residuals | 222 | | |

Table B4: Glycogen content of dead and alive *Macchiademus diplopterus* after treatment (heat and cold storage) during different aestivation (early and mid) and evaluation time points, during the 2016/2017 season. *See Table 3.2.1 and 3.2.2 for descriptions for treatments and evaluation time points.*

| Effect | Df | F | ρ |
|---|---|---|---|
| Aestivation Period | 1 | 196,692 | <0.0001 |
| Evaluation time point | 6 | 6,732 | <0.0001 |
| CATTS treatment | 1 | 0,089 | 0,7657 |
| Cold storage | 1 | 5,596 | 0,0189 |
| Insects (dead or alive) | 1 | 5,067 | 0,0254 |
| CATTS x Cold storage | 1 | 0,210 | 0,6470 |
| CATTS x Insects | 1 | 16,765 | <0.0001 |
| Cold storage x Insects | 1 | 2,211 | 0,1385 |
| CATTS x Cold storage x Insects | 1 | 2,064 | 0,1523 |
| Residuals | 218 | | |

Table B5: Total protein content of dead *Macchiademus diplopterus* after treatment (heat and cold storage) during different aestivation (early and mid) and evaluation time points, during the 2016/2017 season. *See Table 3.2.1 and 3.2.2 for descriptions for treatments and evaluation time points.*

| Effect | Df | F | ρ |
|---|---|---|---|
| Aestivation Period | 1 | 144,48 | 0,00 |
| CATTS treatment | 1 | 5,97 | 0,02 |
| Cold storage | 1 | 3,57 | 0,06 |
| Evaluation time point | 4 | 1,63 | 0,18 |
| Aestivation Period x CATTS | 1 | 0,41 | 0,52 |
| Aestivation Period x Cold storage | 1 | 0,00 | 0,98 |
| Aestivation Period x Evaluation time point | 4 | 1,75 | 0,15 |
| CATTS x Cold storage | 1 | 1,29 | 0,26 |
| CATTS x Evaluation time point | 4 | 0,67 | 0,61 |
| ***Cold storage x Evaluation time point*** | ***4*** | ***5,97*** | ***0,00*** |
| Aestivation Period x CATTS x Cold storage | 1 | 0,18 | 0,68 |
| Aestivation Period x CATTS x Evaluation time point | 4 | 0,15 | 0,96 |
| Aestivation Period x Cold storage x Evaluation time point | 4 | 1,66 | 0,17 |
| CATTS x Cold storage x Evaluation time point | 4 | 1,93 | 0,11 |
| Aestivation Period x CATTS x Cold storage x Evaluation time point | 4 | 3,86 | 0,01 |
| Residuals | 80 | | |

Table B6: Total lipid content of dead *Macchiademus diplopterus* after treatment (heat and cold storage) during different aestivation (early and mid) and evaluation time points, during the 2016/2017 season. *See Table 3.2.1 and 3.2.2 for descriptions for treatments and evaluation time points.*

| Effect | Df | F | ρ |
|---|---|---|---|
| Aestivation Period | 1 | 407,06 | 0,00 |
| CATTS treatment | 1 | 6,34 | 0,01 |
| Cold storage | 1 | 13,80 | 0,00 |
| Evaluation time point | 4 | 137,36 | 0,00 |
| Aestivation Period x CATTS | 1 | 149,56 | 0,00 |
| Aestivation Period x Cold storage | 1 | 7,26 | 0,01 |
| Aestivation Period x Evaluation time point | 4 | 33,64 | 0,00 |
| CATTS x Cold storage | 1 | 52,80 | 0,00 |
| CATTS x Evaluation time point | 4 | 71,06 | 0,00 |
| Cold storage x Evaluation time point | 4 | 97,21 | 0,00 |
| Aestivation Period x CATTS x Cold storage | 1 | 0,35 | 0,56 |
| Aestivation Period x CATTS x Evaluation time point | 4 | 14,46 | 0,00 |
| Aestivation Period x Cold storage x Evaluation time point | 4 | 119,16 | 0,00 |
| CATTS x Cold storage x Evaluation time point | 4 | 7,70 | 0,00 |
| ***Aestivation Period x CATTS x Cold storage x Evaluation time point*** | ***4*** | ***34,18*** | ***0,00*** |
| Residuals | 80 | | |

Table B7: Sugar content of dead *Macchiademus diplopterus* after treatment (heat and cold storage) during different aestivation (early and mid) and evaluation time points, during the 2016/2017 season. *See Table 3.2.1 and 3.2.2 for descriptions for treatments and evaluation time points.*

| Effect | Df | F | ρ |
|---|---|---|---|
| Aestivation Period | 1 | 3911,83 | 0,00 |
| CATTS treatment | 1 | 40,92 | 0,00 |
| Cold storage | 1 | 47,80 | 0,00 |
| Evaluation time point | 4 | 302,35 | 0,00 |
| Aestivation Period x CATTS | 1 | 0,02 | 0,88 |
| Aestivation Period x Cold storage | 1 | 8,21 | 0,01 |
| Aestivation Period x Evaluation time point | 4 | 530,88 | 0,00 |
| CATTS x Cold storage | 1 | 11,57 | 0,00 |
| CATTS x Evaluation time point | 4 | 15,03 | 0,00 |
| Cold storage x Evaluation time point | 4 | 25,09 | 0,00 |
| Aestivation Period x CATTS x Cold storage | 1 | 18,31 | 0,00 |
| Aestivation Period x CATTS x Evaluation time point | 4 | 9,01 | 0,00 |
| Aestivation Period x Cold storage x Evaluation time point | 4 | 25,59 | 0,00 |
| CATTS x Cold storage x Evaluation time point | 4 | 10,54 | 0,00 |
| ***Aestivation Period x CATTS x Cold storage x Evaluation time point*** | ***4*** | ***24,47*** | ***0,00*** |
| Residuals | 80 | | |

Table B8: Glycogen content of dead *Macchiademus diplopterus* after treatment (heat and cold storage) during different aestivation (early and mid) and evaluation time points, during the 2016/2017 season. *See Table 3.2.1 and 3.2.2 for descriptions for treatments and evaluation time points.*

| Effect | Df | F | ρ |
|---|---|---|---|
| Aestivation Period | 1 | 376,20 | 0,00 |
| CATTS treatment | 1 | 21,97 | 0,00 |
| Cold storage | 1 | 18,86 | 0,00 |
| Evaluation time point | 4 | 15,58 | 0,00 |
| Aestivation Period x CATTS | 1 | 23,67 | 0,00 |
| Aestivation Period x Cold storage | 1 | 2,26 | 0,14 |
| Aestivation Period x Evaluation time point | 4 | 5,97 | 0,00 |
| CATTS x Cold storage | 1 | 1,80 | 0,18 |
| CATTS x Evaluation time point | 4 | 2,89 | 0,03 |
| Cold storage x Evaluation time point | 4 | 8,70 | 0,00 |
| Aestivation Period x CATTS x Cold storage | 1 | 3,35 | 0,07 |
| Aestivation Period x CATTS x Evaluation time point | 4 | 4,49 | 0,00 |
| Aestivation Period x Cold storage x Evaluation time point | 4 | 6,10 | 0,00 |
| CATTS x Cold storage x Evaluation time point | 4 | 14,57 | 0,00 |
| ***Aestivation Period x CATTS x Cold storage x Evaluation time point*** | ***4*** | ***10,26*** | ***0,00*** |
| Residuals | 82 | | |

# Appendix C

## Chapter 4

*Paper 5:* Ethyl formate fumigation: its effect on stone and pome fruit quality, and grain chinch bug (*Macchiademus diplopterus*) mortality

## Figures

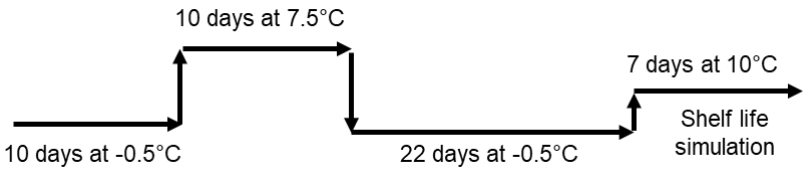
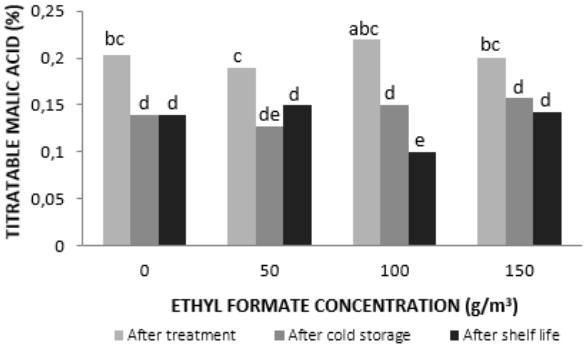
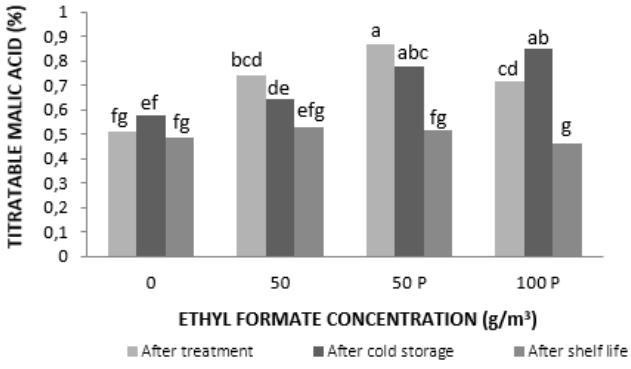



Figure C1. Intermittent warming regime used for cold storage of 'Songold' plums during the 2015/2016 season




| Effect | Df | MS | F | P |
|---|---|---|---|---|
| Evaluation | 3 | 0.0248 | 72.585 | <0.0001 |
| Treatment | 3 | 0.0001 | 0.3410 | 0.7955 |
| **Eval*Treatment** | **9** | **0.0008** | **2.466** | **0.0291** |
| Error | 32 | | | |
| $LSD_{5\%}$ | | | | 0.0 |

Figure C2. The effect of ethyl formate fumigation on the percentage of titratable malic acid present in 'Russet Gold Bosc' pears during the 2015/2016 season.




| Effect | Df | MS | F | P |
|---|---|---|---|---|
| Evaluation | 2 | 0.3582 | 32.75 | <0.0001 |
| Treatment | **3** | 0.1216 | 11.12 | <0.0001 |
| **Eval*Treatment** | **6** | **0.0520** | **4.75** | **<0.0001** |
| Error | 60 | 0.0109 | | |
| $LSD_{5\%}$ | | | | 0.0 |

Figure C3. The effect of ethyl formate fumigation on titratable malic acid in 'September Bright' nectarines during the 2016/2017 season. *The 'P' annotation in the graph indicates concentrations applied to packaged fruit.*

# Tables

Table C1. Harvest maturity parameters of ‘Songold’ plums, ‘August Red’ nectarines, ‘Russet Gold Bosc’ and ‘Forelle’ pears (during the 2015/2016 season) and ‘September Bright’ nectarines and ‘Beurre Bosc’ pears (during the 2016/2017 season).

| | ‘Songold’ plums | ‘August Red’ nectarines | ‘Russet Gold Bosc’ pears | ‘Forelle’ pears | ‘September Bright’ nectarines | ‘Beurre Bosc’ pears |
|---|---|---|---|---|---|---|
| Hue Angle (°) | 107.66 | 50.61 | 86.59 | 93.24 | 53.62 | 85.82 |
| Flesh firmness (N)[1] | 66.74 | 112.37 | 58.74 | 62.75 | 52.06 | 67.91 |
| Total soluble solids (% Brix) | 13.08 | 13.32 | 15.69 | 15.75 | 15.15 | 15.48 |
| Titratable malic acid (%) | 1.15 | 1.00 | 0.23 | 0.15 | 0.58 | 0.23 |

[1] Conversion of Newton to kilogram, divide by 9.80

Table C2: Effect of different ethyl formate concentrations and evaluation intervals on the quality of 'Songold' plums (2015/2016 season).

| Examination parameters | [1] Ethyl formate [ ] g/m$^3$ | [2] Evaluations (A) | | | | [3] Ethyl formate [ ] g/m$^3$ (B) | | | | *Prob > F* [4] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | At harvest | After treatment | After storage | After shelf life | 0 | 50 | 100 | 150 | *A* | *B* | *A x B* |
| Hue Angle (°) | 0+50+100+150 | 101.35a | 107.68a | 97.45b | 90.71c | 101.35 | 99.94 | 101.29 | 100.82 | ***<0.0001*** | *0.2878* | *0.7575* |
| Total soluble solids (% Brix) | 0+50+100+150 | 13.08 | 13.52 | 13.40 | 13.67 | 13.71 | 13.06 | 13.64 | 13.25 | *0.2854* | *0.1293* | *0.0848* |
| Titratable malic acid (%) | 0+50+100+150 | 1.15c | 1.03b | 1.33a | 1.12c | 1.13 | 1.15 | 1.16 | 1.16 | ***<0.0001*** | *0.7105* | *0.1477* |
| Flesh firmness (N) [5] | 0+50+100+150 | 66.75a | 55.02b | 46.05c | 34.56d | 48.51 | 49.63 | 52.93 | 51.02 | ***<0.0001*** | *0.1929* | *0.3268* |
| External phytotoxic damage (%) | 0+50+100+150 | 0 | 1.67 | 0 | 0 | 0 | 1.11 | 0 | 0 | *0.1670* | *0.5398* | *0.6756* |
| Gel breakdown (%) | 0+50+100+150 | 0b | 0b | 1.39b | 10.94a | 8.33 | 12.80 | 11.59 | 12.41 | ***<0.0001*** | *0.8284* | *0.9825* |
| Internal browning (%) | 0+50+100+150 | 0b | 0b | 4.65a | 2.08b | 2.08 | 2.08 | 2.57 | 5.77 | ***0.0049*** | *0.5777* | *0.1328* |
| Total chilling injury (%) | 0+50+100+150 | 0b | 0b | 11.81b | 45.82a | 10.42 | 14.88 | 14.16 | 18.18 | ***<0.0001*** | *0.7031* | *0.9859* |

[1] Data pooled for non-significant interactions across evaluation duration and ethyl formate concentrations, with 0, 50, 100 and 150 indicating concentration g/m$^3$) of ethyl formate.

[2] Values with different superscrips in the same row followed indicate significant differences (P<0.05) according to the LSD test, for evaluation periods

[3] Values with different superscrips in the same row followed indicate significant differences (P<0.05) according to the LSD test, for different ethyl formate concentrations

[4] Two way ANOVA table with complete randomised design for Factor A (Evaluation period) and Factor B (Ethyl formate concentrations).

[5] Conversion of Newton to kilogram, divide by 9.80

Table C3: Tasting panel summary of ethyl formate fumigation sensory effect on ‘Songold’ plums (Season 2015/2016)

| Evaluation | Ethyl formate concentrations ($g/m^3$) | Does the fruit taste similar to control? | Flesh Firmness | Acidity | Off taste present |
|---|---|---|---|---|---|
| After cold storage | 0 | Undecided | Moderate | Moderate | No |
| | 50 | No | Moderate | Not acidic | No |
| | 100 | Yes | Moderate | Not acidic | No |
| | 150 | Undecided | Moderate | Not acidic | No |
| After shelf life simulation | 0 | No | Moderate | Moderate | No |
| | 50 | No | Firm | Moderate | No |
| | 100 | No | Moderate | Mildly Acidic | No |
| | 150 | Undecided | Moderate | Moderate | No |

Table C4: Effect of different ethyl formate concentrations and evaluation intervals on the quality of 'August Red' nectarines (2015/2016 season).

| Examination parameters | [1] Ethyl formate [ ] g/m$^3$ | [2] Evaluations (A) | | | | [3] Ethyl formate [ ] g/m$^3$ (B) | | | | *Prob > F* [4] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | At harvest | After treatment | After storage | After shelf life | 0 | 50 | 100 | 150 | *A* | *B* | *A x B* |
| Hue Angle (°) | 0+50+100+150 | 50.61 | 47.88 | 47.57 | 47.17 | 51.58 | 48.42 | 48.11 | 47.60 | *0.2119* | *0.2170* | *0.5249* |
| Flesh firmness (N)[5] | 0+50+100+150 | 112.37a | 84.48b | 76.22b | 41.59c | 75.24 | 78.63 | 80.03 | 80.63 | ***<0.0001*** | *0.7136* | *0.1420* |
| Total soluble solids (% Brix) | 0+50+100+150 | 13.32 | 12.78 | 12.82 | 12.82 | 13.05 | 12.71 | 12.81 | 1316 | *0.1153* | *0.2614* | *0.2592* |
| Titratable malic acid (%) | 0+50+100+150 | 1.0a | 0.96b | 0.73c | 0.54d | 0.82 | 0.79 | 0.82 | 0.80 | ***<0.0001*** | *0.2268* | *0.2563* |
| Wooliness/ Mealiness (%) | 0+50+100+150 | 0b | 0b | 0b | 63.23a | 13.22 | 13.86 | 18.57 | 17.59 | ***<0.0001*** | *0.3270* | *0.3310* |

[1] Data pooled for non-significant interactions across evaluation duration and ethyl formate concentrations, with 0, 50, 100 and 150 indicating concentration g/m$^3$) of ethyl formate.

[2] Values with different superscrips in the same row followed indicate significant differences (P<0.05) according to the LSD test, for evaluation periods

[3] Values with different superscrips in the same row followed indicate significant differences (P<0.05) according to the LSD test, for different ethyl formate concentrations

[4] Two way ANOVA table with complete randomised design for Factor A (Evaluation period) and Factor B (Ethyl formate concentrations).

[5] Conversion of Newton to kilogram, divide by 9.80

Table C4. Tasting panel summary of ethyl formate fumigation sensory effect on 'August Red' nectarines (Season 2015/2016)

| Evaluation | Ethyl formate concentrations ($g/m^3$) | Does the fruit taste similar to control? | Flesh Firmness | Acidity | Off taste present |
|---|---|---|---|---|---|
| After cold storage | 0 | No | Very firm | Moderate | No |
| | 50 | Yes | Moderate | Moderate | No |
| | 100 | Undecided | Moderate | Moderate | No |
| | 150 | No | Undecided | Undecided | No |
| After shelf life simulation | 0 | Yes | Undecided | Undecided | No |
| | 50 | Undecided | Moderate | Moderate | No |
| | 100 | Yes | Very soft | Not acidic | No |
| | 150 | Yes | Moderate | Not acidic | No |

Table C6. Tasting panel summary of ethyl formate fumigation sensory effect on 'Russet Gold Bosc' pears. Season 2015/2016

| Evaluation | Ethyl formate concentrations ($g/m^3$) | Does the fruit taste similar to control? | Flesh Firmness | Acidity | Off taste present |
|---|---|---|---|---|---|
| After cold storage | 0 | Yes | Very firm | Not acidic | No |
| | 50 | Yes | Very firm | Not acidic | No |
| | 100 | Yes | Very firm | Not acidic | No |
| | 150 | Yes | Very firm | Not acidic | No |
| After shelf life simulation | 0 | Yes | Moderate | Not acidic | No |
| | 50 | Yes | Very soft | Not acidic | No |
| | 100 | Yes | Very soft | Not acidic | No |
| | 150 | Yes | Very soft | Not acidic | No |

Table C7. Effect of different ethyl formate concentrations and evaluation intervals on the quality of 'Russet Gold Bosc' pears (2015/2016 season)

| Examination parameters | [1] Ethyl formate [ ] g/m$^3$ | [2] Evaluations (A) | | | | [3] Ethyl formate [ ] g/m$^3$ (B) | | | | *Prob > F* [4] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | At harvest | After treatment | After storage | After shelf life | 0 | 50 | 100 | 150 | *A* | *B* | *A x B* |
| Hue Angle (°) | 0+50+100+150 | 86.59a | 82.51b | 71.54c | 64.41d | 76.78 | 76.20 | 76.56 | 76.44 | ***<0.0001*** | *0.7710* | *0.9917* |
| Flesh firmness (N)[5] | 0+50+100+150 | 58.74ab | 61.14a | 54.83b | 18.75c | 48.99 | 47.08 | 49.76 | 47.64 | ***<0.0001*** | *0.7096* | *0.5236* |
| Total soluble solids (% Brix) | 0+50+100+150 | 15.69b | 16.19b | 17.30a | 17.23a | 16.92 | 16.64 | 16.43 | 16.43 | ***<0.0001*** | *0.2210* | *0.4573* |
| Shrivel (%) | 0+50+100+150 | 0 | 0 | 0 | 31.49a | 6.55 | 8.61 | 6.19 | 10.14 | ***<0.0001*** | *0.8228* | *0.9683* |

[1] Data pooled for non-significant interactions across evaluation duration and ethyl formate concentrations, with 0, 50, 100 and 150 indicating concentration g/m$^3$) of ethyl formate.

[2] Values with different superscrips in the same row followed indicate significant differences (P<0.05) according to the LSD test, for evaluation periods

[3] Values with different superscrips in the same row followed indicate significant differences (P<0.05) according to the LSD test, for different ethyl formate concentrations

[4] Two way ANOVA table with complete randomised design for Factor A (Evaluation period) and Factor B (Ethyl formate concentrations).

[5] Conversion of Newton to kilogram, divide by 9.80

Table C8. Effect of different ethyl formate concentrations and evaluation intervals on the quality of 'Forelle' pears (2015/2016 season)

| Examination parameters | [1] Ethyl formate [ ] g/m$^3$ | [2] Evaluations (A) | | | | [3] Ethyl formate [ ] g/m$^3$ (B) | | | | *Prob > F* [4] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | At harvest | After treatment | After storage | After shelf life | 0 | 50 | 100 | 150 | *A* | *B* | *A x B* |
| Hue Angle (°) | 0+50+100+150 | 93.24 | 92.93 | 85.37 | 82.50 | 90.11 | 90.61 | 87.63 | 87.04 | ***<0.0001*** | *0.0571* | *0.0680* |
| Flesh firmness (N)[5] | 0+50+100+150 | 6.40a | 6.43a | 6.08b | 1.56c | 5.08 | 5.11 | 5.10 | 5.16 | ***<0.0001*** | *0.6215* | *0.4676* |
| Total soluble solids (% Brix) | 0+50+100+150 | 15.75c | 15.84c | 16.72b | 17.22a | 16.23 | 16.43 | 16.43 | 16.54 | ***<0.0001*** | *0.2320* | *0.3689* |
| Titratable malic acid (%) | 0+50+100+150 | 0.15b | 0.18a | 0.14c | 0.15b | 0.15 | 0.15 | 0.16 | 0.15 | ***<0.0001*** | *0.9768* | *0.0804* |
| Shrivel (%) | 0+50+100+150 | 0b | 0b | 3.47b | 12.73a | 3.00 | 1.39 | 5.56 | 6.25 | ***<0.0001*** | *0.0524* | *0.0925* |
| Mealiness (%) | 0+50+100+150 | 0b | 0b | 0b | 51.34a | 10.71 | 12.50 | 13.54 | 14.58 | ***<0.0001*** | *0.6636* | *0.8402* |
| Astrigency (%) | 0+50+100+150 | 100a | 100a | 77.5b | 27.86c | 80.77 | 79.54 | 73.13 | 72.92 | ***<0.0001*** | *0.4266* | *0.7575* |

[1] Data pooled for non-significant interactions across evaluation duration and ethyl formate concentrations, with 0, 50, 100 and 150 indicating concentration g/m$^3$) of ethyl formate.

[2] Values with different superscrips in the same row followed indicate significant differences (P<0.05) according to the LSD test, for evaluation periods

[3] Values with different superscrips in the same row followed indicate significant differences (P<0.05) according to the LSD test, for different ethyl formate concentrations

[4] Two way ANOVA table with complete randomised design for Factor A (Evaluation period) and Factor B (Ethyl formate concentrations).

[5] Conversion of Newton to kilogram, divide by 9.80

Table C9. Tasting panel summary of ethyl formate fumigation sensory effect on 'Forelle' pears. Season 2015/2016

| Evaluation | Ethyl formate concentrations ($g/m^3$) | Does the fruit taste similar to control? | Flesh Firmness | Acidity | Off taste present |
|---|---|---|---|---|---|
| After cold storage | 0 | Undecided | Very firm | Not acidic | No |
| | 50 | Yes | Very firm | Not acidic | No |
| | 100 | Undecided | Very firm | Not acidic | No |
| | 150 | Yes | Very firm | Not acidic | No |
| After shelf life simulation | 0 | Yes | Moderate | Not acidic | No |
| | 50 | Yes | Moderate | Not acidic | No |
| | 100 | Yes | Moderate | Not acidic | No |
| | 150 | Yes | Moderate | Not acidic | No |

Table C10. Insect mortality of grain chinch bug after ethyl formate fumigation with and without packaging for 'September Bright' nectarines, season 2016/2017.

| | Ethyl formate treatment ($g/m^3$) | | | |
|---|---|---|---|---|
| Position | 0 $g/m^3$ | 50 $g/m^3$ (lugs) | 50 $g/m^3$ (packaging) | 100 $g/m^3$ (packaging) |
| Inoculated cardboard | 0% | N/A | 77% | 100% |
| Top section of fumigation chamber | 0% | 100% | 100% | 100% |
| Middle section of fumigation chamber | 0% | 100% | 100% | 100% |
| Lower section of fumigation chamber | 0% | 100% | 100% | 100% |
| Top section in luggs | 0% | 100% | N/A | N/A |
| Middle section in luggs | 0% | 100% | N/A | N/A |
| Lower section in luggs | 0% | 100% | N/A | N/A |
| Top layer in carton | 0% | N/A | 100% | 100% |
| Middle section of carton | 0% | N/A | 72% | 100% |
| Lower layer in carton | 0% | N/A | 98.5% | 100% |

Table C11. Effect of different ethyl formate concentrations and evaluation intervals on the quality of 'September Bright' nectarines (2016/2017 season)

| Examination parameters | [1] Ethyl formate [ ] g/m$^3$ | [2] Evaluations (A) | | | | [3] Ethyl formate [ ] g/m$^3$ (B) | | | | *Prob > F* [4] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | At harvest | After treatment | After storage | After shelf life | 0 | 50 | 100 | 150 | *A* | *B* | *A x B* |
| Hue Angle (°) | 0+50+100+150 | 53.62 | 45.37 | 47.54 | 47.80 | 47.38 | 46.62 | 47.54 | 46.09 | *0.3025* | 0.8722 | 0.1198 |
| Flesh firmness (N)[5] | 0+50+100+150 | 52.06a | 36.17b | 32.89c | 27.92d | 30.880 | 32.87 | 33.30 | 32.27 | ***<0.0001*** | 0.3560 | 0.2202 |
| Total soluble solids (% Brix) | 0+50+100+150 | 15.15a | 14.55b | 14.51b | 13.70c | 14.07 | 14.43 | 14.49 | 14.02 | ***<0.0001*** | 0.1311 | 0.9463 |
| Shrivel (%) | 0+50+100+150 | 0 | 0 | 11.88 | 16.60 | 4.54b | 15.1a | 9.44b | 8.89b | ***<0.0001*** | 0.0039 | 0.2385 |
| Decay (%) | 0+50+100+150 | 0b | 0b | 0b | 0.6a | 0 | 0 | 0.28 | 0.56 | ***0.0378*** | 0.2486 | 0.2256 |
| Pulpiness (%) | 0+50+100+150 | 0b | 0b | 0b | 35a | 5.6 | 10 | 13.3 | 17.78 | ***<0.0001*** | 0.2361 | 0.2095 |
| Wooliness (%) | 0+50+100+150 | 0b | 0b | 0b | 29.17a | 4.44 | 6.67 | 12.2 | 15.56 | ***<0.0001*** | 0.2154 | 0.1832 |

[1] Data pooled for non-significant interactions across evaluation duration and ethyl formate concentrations, with 0, 50, 100 and 150 indicating concentration g/m$^3$) of ethyl formate.

[2] Values with different superscrips in the same row followed indicate significant differences (P<0.05) according to the LSD test, for evaluation periods

[3] Values with different superscrips in the same row followed indicate significant differences (P<0.05) according to the LSD test, for different ethyl formate concentrations

[4] Two way ANOVA table with complete randomised design for Factor A (Evaluation period) and Factor B (Ethyl formate concentrations).

[5] Conversion of Newton to kilogram, divide by 9.80

Table C12. Effect of different ethyl formate concentrations and evaluation intervals on the quality of 'Beurrè Bosc' pears (2016/2017 season)

| Examination parameters | [1] Ethyl formate [ ] g/m$^3$ | [2] Evaluations (A) | | | | [3] Ethyl formate [ ] g/m$^3$ (B) | | *Prob > F* [4] | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | At harvest | After treatment | After storage | After shelf life | 0 | 85 | *A* | *B* | *A x B* |
| Hue Angle (°) | 0+50+100+150 | 85.82a | 85.81a | 74.58b | 70.83c | 77.11 | 77.40 | ***<0.0001*** | 0.8985 | 0.9972 |
| Flesh firmness (N)[5] | 0+50+100+150 | 67.91a | 68.11a | 66.47a | 19.08b | 50.68 | 53.69 | ***<0.0001*** | 0.2289 | 0.1896 |
| Total soluble solids (% Brix) | 0+50+100+150 | 15.48b | 15.53b | 16.80a | 16.80a | 16.26 | 16.47 | ***<0.0001*** | 0.4733 | 0.5201 |
| Titratable malic acid (%) | 0+50+100+150 | 0.227 | 0.222 | 0.2033 | 0.1753 | 0.21a | 0.19b | ***<0.0001*** | 0.0055 | 0.4672 |
| Shrivel (%) | 0+50+100+150 | 0b | 0b | 0b | 27.2a | 6.73 | 10.3 | ***<0.0001*** | 0.4138 | 0.5214 |
| Decay (%) | 0+50+100+150 | 0c | 0c | 10.9b | 18.7a | 7.93 | 11.2 | ***<0.0001*** | 0.3498 | 0.7691 |

[1] Data pooled for non-significant interactions across evaluation duration and ethyl formate concentrations, with 0, 50, 100 and 150 indicating concentration g/m$^3$) of ethyl formate.
[2] Values with different superscrips in the same row followed indicate significant differences (P<0.05) according to the LSD test, for evaluation periods
[3] Values with different superscrips in the same row followed indicate significant differences (P<0.05) according to the LSD test, for different ethyl formate concentrations
[4] Two way ANOVA table with complete randomised design for Factor A (Evaluation period) and Factor B (Ethyl formate concentrations).
[5] Conversion of Newton to kilogram, divide by 9.80

# Appendix D

## Chapter 4

*Paper 6:* Central Composite Designs (CCD) as a predictive tool when fumigating pome and stone fruit with ethyl formate

Table D1. ANOVA results illustrating influence of concentration and duration on hue angle (°), flesh firmness (N) and total soluble solid levels (% Brix) of 'Russet Gold Bosc' pears after shelf life simulation during the 2015/2016 and 2016/2017 season. *The (L) indicates a linear fit and (Q) a quadratic fit of the data*

| | **2015/2016** | | | **2016/2017** | | |
|---|---|---|---|---|---|---|
| **Factors** | **Hue angle (°)** ρ = | **Flesh firmness (N)** ρ = | **Total soluble solid levels (% Brix)** ρ = | **Hue angle (°)** ρ = | **Flesh firmness (N)** ρ = | **Total soluble solid levels (% Brix)** ρ = |
| **(1)** Concentration (g/m$^3$) (L) | 0.4005 | 0.9996 | 0.3616 | 0.9204 | 0.9972 | 0.5825 |
| Concentration (g/m$^3$) (Q) | 0.2076 | 0.0639 | 0.8550 | 0.1666 | 0.5439 | 0.4692 |
| **(2)** Duration (hours) (L) | 0.7572 | 0.2145 | 0.4007 | 0.3786 | 0.5409 | 0.2642 |
| Duration (hours) (Q) | **0.0365** | **0.0354** | 0.9763 | 0.5802 | 0.3151 | 0.3855 |
| **(3)** Temperature (°C) (L) | 0.0884 | 0.7839 | 0.9890 | - | - | |
| Temperature (°C) (L) | 0.1751 | 0.0814 | 0.8300 | - | - | |
| **(1)** by (2) | 0.0621 | 0.0759 | 0.8041 | 0.3639 | 0.7932 | 0.8695 |
| **(1)** by (3) | 0.7455 | 0.3488 | 0.7210 | - | - | |
| **(2)** by (3) | 0.0574 | 0.5685 | 0.7986 | - | - | |

Table D2. ANOVA results illustrating influence of concentration and duration on hue angle (°), flesh firmness (N), shrivel (%), gel breakdown (%) and internal browning (%) of 'Songold' plums after shelf life simulation fumigated at ambient temperature during the 2016/2017 season. *The (L) indicates a linear fit and (Q) a quadratic fit of the data*

| | ***Hue angle (°)*** | ***Flesh firmness (N)*** | ***Shrivel (%)*** | ***Gel breakdown (%)*** | ***Internal browning (%)*** |
|---|---|---|---|---|---|
| **Factors** | ρ = | ρ = | ρ = | ρ = | ρ = |
| (1) Concentration ($g/m^3$) (L) | 0,6155 | 0,6277 | 0,6319 | 0,3079 | 0,1399 |
| Concentration ($g/m^3$) (Q) | 0,5368 | 0,7162 | 0,8137 | 0,6732 | 0,4863 |
| (2) Duration (hours) (L) | 0,1142 | 0,7081 | 0,4010 | 0,5915 | 0,6180 |
| Duration (hours) (Q) | 0,4026 | 0,4808 | 0,6796 | 0,4481 | 0,4668 |
| (1) by (2) | 0,3801 | 0,5212 | 0,8649 | 0,6723 | 0,4510 |
| Lack of fit | 0,5172 | 0,6891 | 0,6319 | 0,3586 | 0,1399 |

Table D3. ANOVA results illustrating influence of concentration and duration on hue angle (°), flesh firmness (N), total soluble solids (%Brix) and pulpiness (%) of 'Alpine' nectarines (harvest 2) after shelf life simulation fumigated at ambient temperature during the 2016/2017 season. *The (L) indicates a linear fit and (Q) a quadratic fit of the data*

| | ***Hue angle (°)*** | ***Flesh firmness (N)*** | ***Total soluble solids (% Brix)*** | ***Pulpiness (%)*** |
|---|---|---|---|---|
| **Factors** | ρ = | ρ = | ρ = | ρ = |
| (1) Concentration ($g/m^3$) (L) | 0,5386 | 0,5424 | 0,4460 | 0,5003 |
| Concentration ($g/m^3$) (Q) | 0,1174 | 0,4890 | 0,8106 | 0,6889 |
| (2) Duration (hours) (L) | 0,1446 | 0,1873 | 0,5438 | 0,1284 |
| Duration (hours) (Q) | 0,6238 | 0,9006 | 0,0408 | 0,1392 |
| (1) by (2) | 0,1695 | 0,8284 | 0,0562 | 0,1052 |
| Lack of fit | 0,5386 | 0,6475 | 0,4460 | 0,5003 |

Table D4. ANOVA results illustrating influence of concentration and duration on hue angle (°), flesh firmness (N), total soluble solids (%Brix) of 'August Bright' nectarines (harvest 2) after shelf life simulation fumigated at ambient temperature during the 2016/2017 season. *The (L) indicates a linear fit and (Q) a quadratic fit of the data*

| | ***Hue angle (°)*** | ***Flesh firmness (N)*** | ***Total soluble solids (% Brix)*** |
|---|---|---|---|
| **Factors** | ρ = | ρ = | ρ = |
| (1) Concentration (g/m$^3$) (L) | 0,3909 | 0,7471 | 0,2482 |
| Concentration (g/m$^3$) (Q) | 0,7981 | 0,7583 | 0,9393 |
| (2) Duration (hours) (L) | 0,6179 | 0,1692 | 0,8869 |
| Duration (hours) (Q) | 0,2876 | 0,9361 | 0,1100 |
| (1) by (2) | 0,6028 | 0,2792 | 0,1254 |
| Lack of fit | 0,3389 | 0,9442 | 0,2482 |

Table D5. ANOVA results illustrating influence of concentration and duration on hue angle (°), flesh firmness (N), total soluble solids (%Brix), shrivel (%) and overripe (%) of 'August red' nectarines (pre-ripened) after shelf life simulation fumigated during the 2016/2017 season. *The (L) indicates a linear fit and (Q) a quadratic fit of the data*

| | ***Hue angle (°)*** | ***Flesh firmness (N)*** | ***Total soluble solids (% Brix)*** | ***Shrivel (%)*** | ***Overripe (%)*** |
|---|---|---|---|---|---|
| **Factors** | ρ = | ρ = | ρ = | ρ = | ρ = |
| (1) Concentration (g/m$^3$) (L) | 0,7282 | 0,3233 | 0,7329 | 0,3098 | 0,1137 |
| Concentration (g/m$^3$) (Q) | 0,9445 | 0,7635 | 0,8924 | 0,3537 | 0,2274 |
| (2) Duration (hours) (L) | 0,8887 | 0,6028 | 0,5853 | 0,9007 | 0,2348 |
| Duration (hours) (Q) | 0,9950 | 0,7540 | 0,2412 | 0,2619 | 0,5705 |
| (1) by (2) | 0,8168 | 0,3689 | 0,9360 | 0,4297 | 0,4778 |
| Lack of fit | 0,8257 | 0,9210 | 0,7329 | 0,9438 | 0,4036 |