



Cross resistance to environmental stressors in Natal fruit fly, *Ceratitis rosa* Karsch (Diptera: Tephritidae) and its underlying physiological mechanisms

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A Dissertation/Thesis Submitted to the College of Science in Partial Fulfilment of the Requirements for the Award of the Degree of Master of Science in Biological Sciences (Applied Entomology) of BIUST

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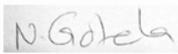
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Dr C. Nyamukondiwa (Supervisor)

Signature: 

Date: 26 June 2017

DEDICATION

I dedicate this thesis to my family and friends.

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ABSTRACT

Plastic adjustments of physiological tolerance to a particular stressor can result in fitness benefits for resistance that might manifest not only in that same environment, but might also be advantageous when faced with alternative environmental stressors, a phenomenon termed cross-tolerance. The nature and magnitude of cross-tolerance responses can provide important insights into the underlying genetic architecture, potential constraints on, or versatility of, an organism's stress responses. The current study tested for cross-tolerance to a suite of abiotic factors that likely contribute to setting insect population dynamics and geographic range limits: heat, cold, desiccation and starvation resistance in adult mixed sex *Ceratitis rosa* following acclimation to all of these isolated individual conditions prior to stress assay. Traits of stress resistance measure included critical thermal (activity) limits, chill coma recovery time (CCRT), heat knockdown time (HKDT), desiccation and starvation resistance. In agreement with a range of other studies, we found that acclimation to one stress typically increased resistance for that same stress experienced later in life. A more novel result is that substantial evidence for cross-tolerance was found. For example, we found an improvement in heat tolerance (critical thermal maxima, CT_{max}) following starvation or desiccation hardening; improved desiccation resistance following cold acclimation, and enhanced starvation resistance following desiccation hardening, indicating pronounced cross tolerance to these environmental stressors for the traits examined. The study further investigated underlying physiological mechanisms to the observed cross tolerance by measuring water balance traits, carbohydrates and lipid content of acclimated and control adult *C. rosa*. Overlaps in physiological mechanisms such as reduced water loss rates, and high lipid content observed in both heat and starvation treatment *C. rosa* flies were determined to be the likely mechanisms behind the observed increased heat tolerance following desiccation hardening, and improved heat tolerance following starvation acclimation respectively. However physiological mechanisms were not exhaustive hence there is need to conduct further research to explain all observed cross tolerance. The major implications of the results are: 1) the existence of cross tolerance could explain how species are able to survive multiple stress environments, 2) that a set of common underlying physiological mechanisms might exist between apparently divergent stress responses in species, and (3) suggest that stress resistant traits likely coevolved to cope with diverse or simultaneous stressors.

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CHAPTER 1

GENERAL INTRODUCTION

1.0 Background

The interaction between species environmental trait resistance, plasticity of that trait and climate change, determine its success or failure in a changing world (Foden et al., 2013). Global climate change has brought increased magnitude of environmental stressors such as increase in global average temperatures (William et al., 2015), changes in rainfall patterns, and extreme climatic events vis-a-vis frequent occurrences of cold snaps, heat waves and several cases of drought (Trenberth 2011) and wildfires (IPCC 2014). As such, organisms faced with these overlapping and multiples stressors simultaneously must adapt morphologically, behaviourally or physiologically, in time and space or otherwise face extinction (Chevin et al. 2010; reviewed in Chown & Nicolson 2004; Angilletta 2009). Recently increases in global temperatures have been associated with shifts in geographic distribution of both terrestrial and aquatic organisms (Hickling et al., 2006; Stange & Ayres, 2010), with great concern being on the population dynamics and biogeography of insects (Kiritani, 2013), especially economically important insect pests like the Natal fruit fly, *Ceratitits rosa* (De Meyer et al., 2008). This has brought several biosecurity concerns for so many nations, as countries battle increased reports of invasive alien insect species, with direct consequences on health, agricultures and overall human wellbeing. The survival abilities (resistance mechanisms) of these economic insect pests in the face of multiple stressors under current global climate change are currently not well elucidated. Nevertheless, it remains critical due to insects' economic significance in agriculture, food security and health, coupled with costs associated with eradication and control and associated consequences, for example, barriers to international trade (Benjamin et al., 2012).

Literature review

1.1. Effects of environmental stress on ectotherms

The interaction between an insect species traits, plasticity of those traits and climate change, determine its survival in a changing world (Foden et al., 2013). The ability of insects to successfully inhabit most environmental niches depends on their plasticity and ability to survive under these changing environments (Davies et al., 2014). There are various environmental stressors including; heat, drought, cold, starvation, ultraviolet radiation, salinity and others (Chown & Nicolson 2004) with starvation, desiccation and exposure to extreme heat and cold being the most stressful environmental factors to insects (Sisodia et al.,

2015). Extensive research has been done to determine the effects of environmental factors on insects' growth, development, survival, reproduction and longevity (Ouedraogo et al., 1996; Thomas et al., 2004; Deutsch et al., 2008; Norhisham et al., 2013; Bahar et al., 2012). Nevertheless, similar work looking into physiological mechanisms underlying tolerance to these stressors have not received much attention.

Being ectotherms, most insects have body temperatures similar to that of the environment, therefore, as environmental temperature fluctuate, so do their body temperatures (Chown & Nicolson 2004; Angilletta 2009). This is mostly true for almost all small insects whose body weight is <1 g (Stevenson 1985; Heinrich 1993; Angilletta 2009). The effect of temperature on insects is best described by the thermobiological scale as proposed by (Vannier 1994) (see Fig. 1.1). At the centre of the scale is the optimum temperature, which is the temperature that allows optimum performance or activity of the insect. Each insect species has an optimal temperature range for development (Ricalde et al., 2012), for example the optimum temperature for normal growth of silkworms is between 20°C and 28°C (Rahmathulla 2012). It is at this optimum temperature that several physiological functions such as growth, metabolism, and reproduction reach a maximum (Vannier 1994; Reznik et al., 2009).

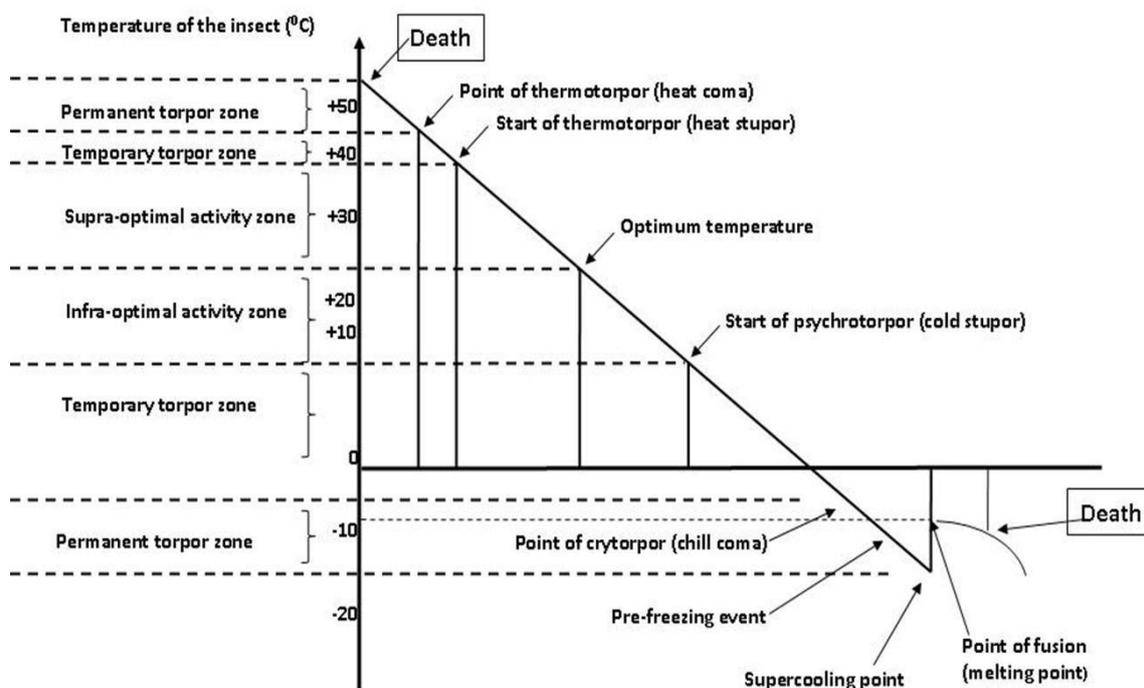


Figure 1.1: The Thermobiological scale (redrawn from Vannier 1994)

If temperatures increase or decrease beyond the optimum temperature, this will not result immediately in death of insects, but first an insect will enter a physiological state called heat stupor and cold stupor respectively (Fig. 1.1). These “Vannier stupors” are known as the critical thermal limits (CTLs), categorised as critical thermal minima (CT_{\min} for low temperature) and critical thermal maxima (CT_{\max} for high temperature). CTLs have both been defined as the temperature at which each individual insect loses co-ordinated muscle function, consequently losing the ability to respond to mild stimuli e.g. probing (Nyamukondiwa & Terblanche 2009). These parameter have been used in several studies as a measure of insects heat tolerance (Nyamukondiwa et al., 2013; Chidawanyika et al., 2017) and cold tolerance (Nyamukondiwa & Terblanche 2009; Kelty & Lee Jr 2001) respectively. However changes in thermal limits occurs in many terrestrial ectotherms due to plastic changes (Hoffman et al., 2013). And often than not these plastic changes tend to have less effects on upper thermal limits than on lower limits (Hoffmann et al., 2013). Below and above CTLs, insect development does not occur, as such an insect critical thermal limit temperature range can therefore be a useful indicator of an insect’s potential distribution and abundance. Further increasing or decreasing beyond either CT_{\max} or CT_{\min} , an insects will enter into heat or cold coma respectively. Several studies have used heat coma as measure of insects’ heat tolerance, by assessing the time taken for an insect to go into heat coma (Sørensen & Loeschcke 2001; Weldon et al., 2011; Bublly et al., 2012, 2013; Parkash et al., 2014b; Bauerfeind et al., 2014). Cold coma on the other hand has been used in studies which used chill coma recovery tome (CCRT) as an adaptation of insects to cold tolerance (Sinclair et al., 2007; Weldon et al., 2011; Bublly et al., 2012; Karl et al., 2014; Scharf et al., 2015). Beyond coma an insect will enter a stage of irreversible trauma and death (Chown & Nicolson 2004).

Relative humidity refers to the amount of water vapor in the atmosphere. Both low and high humidity levels affect the physiology of many insects’ species (Norhisham et al., 2013), because they are generally vulnerable owing to their high surface area to volume ratio. The ability of an insect to survive low humidity environments depend on both its body water content and the rate of water loss (Sisodia et al., 2015) which depend on the interaction between humidity and temperature (Han et al., 2008). Low relative humidity prolong developmental time (Han et al., 2008), by preventing egg hatching and embryo development. Low relative humidity has also been shown to reduce egg laying capacity in mosquitoes

Culex pipiens (Diptera; Culicidae) (Benoit et al., 2010) as well as increased egg mortality in the bamboo borer, *Dinoderus minutus* Fabricius (Coleoptera; Bostrichidae) (Norhisham et al., 2013). High humidity on the other hand contributes to population increase in stored product pests, as shown in *Callosobruchus maculatus* (Fabricius) (Coleoptera: Bruchidae), where the females showed higher fecundity and longer adult lifespan at high humidity (Ouedraogo et al. 1996).

Precipitation and extreme events such as flooding and hurricanes have been predicted to increase due to global climatic changes (Cornelissen 2011). These may affect insects feeding, leading to most insects species encountering periods of food shortages and hence starvation stress (Marron et al., 2003; Chahal et al., 2013). Due to this changing climate, there are less rains, less plants and deforestation in other parts of the world. Starvation is when the food an organism consume does not meet its minimum energy requirement (Scharf et al., 2016). Insects require food to supply enough energy requirements for growth, maintenance and reproduction. Starvation has been shown to result in undesirable consequences such as reduced egg laying (Wayne et al., 2006) and decreased lifespan (Rion & Kawecki 2007).

Although much has been done on how single abiotic stressors affect insects (Wayne et al., 2006; Rion & Kawecki 2007; Benoit et al., 2010; Norhisham et al., 2013), environmental stressors do not occur in isolation, multiple environmental stressors are often experienced simultaneously in nature (Sinclair et al., 2013). For example; insects which overwinter in temperate and polar environments often experience both low temperatures and low water availability (Danks 2000). Periods of heat stress also often coincides with desiccation stress (Bubliy et al., 2012). Several studies however have provided evidence which suggest that, the impacts of multiple stressors may synergistically exceed the effects of individual stressors (Liess et al., 2016; Bubliy et al., 2013). Wild bee populations and honey bee colonies have declined due to combined effects of pesticides and parasites (Pettis et al., 2012), and reduced floral abundance (Holmstrup et al., 2010; Liess et al., 2016) and/or weather (Henry et al., 2014). In a related study, hardening at combined heat and desiccation treatments increased resistance in *D. melanogaster* in both heat and desiccation resistance (Bubliy et al., 2013). These studies are not exhaustive of all possible combination of stress factors and have not been done in all insects' taxa. Information of the effects of combined stressors is necessary due to climate change which has brought with it increased climate variability. Due to these

increased climate fluctuations, insects find themselves in different multiple environmental factors. Their ability to survive remains unknown. Therefore, there is need to know how the effects of one environmental factor will affect insects species upon exposure to another environmental factor.

1.2. Adaptation of ectotherms to environmental stress

Insects are found in a wide range of environments, experiencing changing biotic and abiotic factors. Since a changing environment demands organisms to adapt in order to survive (reviewed in Chown & Nicolson 2004; Angilletta 2009; Chevin et al. 2010), insect species have evolved a number of behavioural, morphological and physiological strategies (Lencioni 2004; Sheikh et al., 2017) in order to cope with changing environmental circumstances. Generally, most species develop a combination or several of these survival strategies (Irons et al., 1993) and are used interchangeably or overlapping in surviving stressful environments. Adaptation of insects to different environmental conditions is very important especially for invasive species such as *C. capitata*, *Bactrocera dorsalis* (Diptera: Tephritidae) (Pieterse et al., 2017) and potentially invasive species such as *C. rosa* and *C. fasciventris* (Diptera: Tephritidae) (Baliraine et al., 2004). This ability play a great role in their ability to invade new areas, establishment and dispersal into novel environments (Weldon et al., 2016).

1.2.1. Behavioural adaptation

Behavioural adaptations deal with animal actions and are the immediate responses to stress (Even et al., 2012). There are two types of behaviour; innate and learned behaviour (Sheikh et al., 2017). Innate behaviour is usually heritable; present in animals raised in isolation from others, performed in the same way each time by each individual, not modified by development or experience and is fully developed or expressed at first performance, while on the other hand learned behaviour is not heritable, is absent in animals raised in isolation from others, adaptable and progressive (Sheikh et al., 2017). Some of the behavioural responses insects use to adapt to various stressors include: shade seeking, hibernating during winter, summer diapause (aestivation) and migration.

1.2.1.1. Shelter seeking

An appropriate shelter is essential for insects' survival, as such depending on the environmental condition experienced, insects tend to seek certain shelter to avoid deleterious

environmental conditions. For example in order to avoid water loss some insects move down the soil profile (Clark 2008), while during starvation most ectotherms seek cooler habitats such that energy expenditure is passively suppressed by temperature and during extreme heat they will seek shades.

1.2.1.2. Hibernation (winter diapause)

Hibernation refers to the state in which insects survive the winter (Lee 2003). It involves entry into a dormant state called ‘diapause’, hence hibernation can be called winter diapause. (Lee 2003). Diapause is an alternative developmental pathway, which enhances survival by ensuring that development or reproduction coincide with favourable conditions (Danks 2001). It can occur at different life stages (Pullin 1996) in different insects, and in both space and time. There are two types of diapause; facultative and obligatory diapause. Facultative diapause occurs as a response to unfavourable environmental conditions and is optimal while obligatory diapause occurs each generation independently from environmental conditions to unfavourable conditions (Denlinger 2002; Kostal et al., 2008). Hibernation is therefore facultative diapause, because morphological development is arrested by low temperatures, absence of food and / photoperiod (Hodek 2012). Examples of insects that hibernate during winter include lady beetle, *Harmonia axyridis*; Coleoptera: Coccinellidae (Watanabe 2002; Sheikh et al., 2017), cabbage beetle, *C. bowringi*, and carrot weed, *Parthenium hysterophorus*, which during late spring when conditions become favourable they resume their development (Hodek 2012).

1.2.1.3. Aestivation (summer diapause)

Aestivation is a dormant state for insects to pass summer (Lee 2003), thus it is an adaptation to survive unfavourable high temperatures conditions (Nakai & Takeda 1995), or a response to survive food shortage due to summer drought (Nagasaka 1992; Braby 1995; Kostal & Hodek 1997). Examples of the insects’ taxa which pass summer temperatures in aestivation include the African Malaria mosquito, *Anopheles gambiae* (Lehmann et al., 2010; Dao et al., 2014) and the cabbage butterfly, *Pieris melete* (Xue et al., 1997) and the large white butterfly, *Pieris brassicae* (Spieth & Schwarzer 2001).

1.2.1.4. Migration

Migration of certain insects has been observed mainly for reasons of searching for food, searching for a summer or winter habitat, searching for breeding sites and/ escaping from predators (Lencioni 2004; Chapman et al., 2015). This migratory behaviour can be over a short or long distance (Dingle 2014). Examples of migratory insects include desert locust, *Schistocerca gregaria* (Homberg 2015) and butterflies (Dingle 2014). This mechanism allows insects to optimize growth and fitness through evading stressful environmental conditions in space rather than coping with them *in situ*.

1.2.2. Morphological adaptation

Morphological adaptations include adaptations via various physical features which enable an insect to live a stress free life in adverse conditions (Sheikh et al., 2017). Some of morphological adaptations insects use to adapt to various environmental stressors include melanism, reduction in insect body size, hairiness and reduction and / the absence of wings (Lencioni 2004).

1.2.2.1. Melanism

Colour in insects' species is extremely diverse between species, between populations of the same species, and between individuals within a population in space and time (Rajpurohit et al., 2016). Melanism has been linked to several stressors including thermal, ultraviolet radiation, infection and desiccation (Dombeck & Jaenike 2004; Rajpurohit et al., 2008; Johnson et al., 2011; Bastide et al., 2014). For example increased melanisation has been associated with higher fitness under thermal and desiccation stresses in *D. melanogaster* (Parkash et al., 2008: 2009). There has also been records of increased melanism and disease resistance, which has been attributed to phenomenon known as Density-independent prophylaxis (DDP) (Wilson et al., 2001). According to this phenomenon individual invest more in immune function when at high population density in order to reduce density pathogen transmission rates (Reeson 1998), and this increase in resistance is said to be accompanied by cuticular melanism (Wilson et al., 2001).

1.2.2.2. Reduction in body size

Terrestrial and aquatic insects from polar and alpine regions generally exhibit smaller body sizes than relative species at lower altitudes and latitudes (Lencioni 2004). The smaller body

size allow for a faster growth and development, hence allow these insects to survive food shortage (Lencioni 2004). Also a small size enable insects to find sheltered microhabitats for protection during winter.

1.2.2.3. Hairiness /pubescence

Some insects from Polar Regions develop big and hairy bodies in contrast to similar species from warmer environments (Lencioni 2004). For example bumble bees exhibit big size and hairiness to allow them to maintain the heat generated by the contraction of the flight muscles. Also cuticular permeability varies in insects depending on their habitat and lifestyle (Chapman et al., 1998). Thus the type and quantity of lipids on insect cuticle is one of the factors why insects are adapted to different environment (Nation 2001). For example larvae of *Sarcophaga bullata* which lives in wet environments has only small amount of cuticular hydrocarbons on their cuticle, while adults which live on dry environments have a larger quantity of surface lipids to offer protection from desiccation (Nation 2001).

1.2.2.4. Reduction or absence of wings

Wing reduction has been observed in several insect orders: Diptera, Plecoptera and Coleopteran (Brittain 1990), and also in many insects from extreme environments such as islands, polar region and mountains. Wing reduction can be an adaptation to periods of food shortage (Brittain 1990) or cold (Margesin 1999), where resources are redirected towards growth and reproduction and not towards wing construction which is highly energy consuming (Brittain 1990; Margesin 1999).

1.2.3. Physiological adaptation

If the environment changes in such a way that insects cannot avoid or cope using behavioural or morphological responses, they will be faced with either adapting to the new environment or otherwise face extinction (Thomas et al., 2004; Chevin et al., 2010). A form of phenotypic plasticity which involves changes in the physiological phenotype of an organism, enabling organisms to increase their resistance after pre- exposure to some form of environmental stress is referred to as acclimation/hardening (Bubliy et al., 2012).

1.2.3.1. Acclimation/ Hardening

Despite hardening/acclimation often used interchangeably, the terms are different, with acclimation used to describe long term (days to weeks) while hardening refers to short term (minutes to hours) exposure to sub lethal conditions (Cossins & Bowler 1987; Chown & Nicolson 2004) which confers improved survival to an otherwise lethal stress environment.

These treatments typically give reversible and/irreversible physiological changes in insects' physiology (Hoffmann et al., 2003; Weldon et al., 2011), and can be induced either during development (developmental acclimation), during adulthood (adult acclimation) (Parkash et al., 2014b) or may have a synergistic effect (Scharf et al., 2015).

Acclimation can be induced in the laboratory through processes known as rapid cold hardening (RCH) (for low temperatures) and rapid heat hardening (RHH) (for high temperatures) (see Rinehart et al., 2000b) with some studies documenting that exposing insects to moderate stress may improve survival to that particular stress later in life, a phenomenon termed beneficial acclimation (Leroi et al., 1994; Terblanche & Kleynhans 2009). According to the beneficial acclimation theory, exposing insects to moderate stress improves their survival to that particular stress later in life, compared to an organism that did not have the opportunity to acclimate to that particular environment. That is to say, the benefits of plasticity may only be possible if the exposed trait match the pre exposure trait. Studies have shown that insects with pre-exposure to less severe heat (Sørensen & Loeschcke 2001; Sejerkilde et al., 2003; Chidawanyika & Terblanche 2011; Karl et al., 2014; Bauerfeind et al., 2014), cold (Kelty & Lee 2001; Sinclair et al., 2007; Sinclair & Roberts 2005; Denlinger & Lee 2010; Fischer et al., 2010; Chidawanyika & Terblanche 2011; Karuppaiah & Sujayanad 2012; Karl et al., 2014), desiccation (Bayley et al., 2001; Sjørnsen et al., 2001; Bazinet et al., 2010; Bauerfeind et al., 2014; Parkash et al., 2014a) and starvation acclimation (Bubliy et al., 2012; Karl et al., 2014) have improved survival ability to the same lethal stress later in life.

Although beneficial acclimation is appealing and widely supported, there are several studies which have challenged its generality (Hoffmann 1999; Zamudio et al., 1995). Some of the challenges were that fitness benefits may or may not be as a result of acclimation alone, and also majority of the research was focused on the benefits of acclimation rather than the costs (Basson et al., 2011). For example, RCH increases cold tolerance, long term exposure may

impact on insects' performance by incurring damage (Le Bourg 2007) or cost to key life history traits (Basson et al., 2011). These challenges led to the conclusion that the beneficial acclimation hypothesis is not the only possible response of phenotypic plasticity. Other competing hypotheses included the optimal developmental temperature hypothesis (OTH), cooler is better hypothesis (CBH), and the warmer is better hypothesis (WBH) (Zamudio et al., 1995; Huey & Berrigan 1996; Huey et al., 1999). According to the OTH, organisms raised at intermediate temperatures, though small in size, will perform better across all temperatures than do organisms raised at high or low temperatures (Zamudio et al., 1995). The Cooler is Better Hypothesis (CBH) on the other hand states that organisms raised at cool temperatures have higher relative fitness across all temperatures than do organisms raised at optimum or high temperatures. Lastly organisms raised at high temperatures have higher relative fitness across all temperatures than do those raised at intermediate or cool temperatures in what is known as the WBH (Knies et al., 2009).

1.2.3.2. Cross tolerance

In addition to all these hypothesis, further studies have shown that acclimation to one stress not only improves resistance to the similar stress, but also to other stressors in what is known as the cross resistance (Bubliy et al., 2012). Cross tolerance or multiple-stress resistance, refers to a phenomenon in which one type of stress can confer a plastic response leading to increased resistance to a different kind of stress (MacMillan et al., 2009; Bubliy et al 2012; Sinclair et al., 2013; Ponnanna & Krishna 2014). For example although RCH improves low temperature survival, improved survival at low temperatures can also occur after mild high temperature treatments (Chidawanyika & Terblanche 2011), an indication of cross tolerance/resistance. Cross tolerance has been investigated in a number of insect's species; *D. melanogaster* (Bubliy et al., 2012; Parkash et al., 2014b; Bauerfeind et al., 2014), *D. simulans* (Bubliy et al., 2013), *Folsomia candida* (Bayley et al., 2001; Holmstrup et al., 2002), *Belgica Antarctica* (Benoit et al., 2009), *Cydia pomonella* (Chidawanyika & Terblanche 2011), *Thaumatotibia leucotreta* (Boardman et al., 2015), *Tribolium castaneum* (Scharf et al., 2015), goldenrod gall fly, *Eurosta solidaginis* (Levis et al., 2012), *Zaprionus indianus* (Kalra et al., 2017). In Diptera, most of the research to date has focused on *D. melanogaster*. Nevertheless, there is need to investigate cross tolerance in other insect species especially insects of economic importance such as *C. rosa*, before any generalisations are made on cross tolerance across insect taxa.

1.3. Physiological mechanisms underlying stress

1.3.1. Synthesis of heat shock proteins

Insects respond to various abiotic stresses by increasing the synthesis of heat shock proteins (HSPs) (Zhao & Jones, 2012). Heat shock proteins act as molecular chaperones which play a role in the transportation, folding and assembling of degraded or misfolded proteins (Kalosaka et al., 2009). In most cases, the genes encoding these proteins are rapidly upregulated at the onset/after stress (Hoffmann et al., 2003), hence protects cells during stress, and also help in the recovery. However when more favourable conditions return, the Hsps are again down-regulated, a feature that is essential because expression of Hsps during non-stress conditions can lead to deleterious effects, including retardation and cessation of development (Feder et al., 1992). The role of Hsp70 in heat tolerance has been well studied in *D. melanogaster* (Feder et al., 1999), Mediterranean fruit fly, *C. capitata* (Kalosaka et al., 2009), codling moth, *C. pomonella*, flesh fly; *Sarcophaga crassipalpis* and pea leaf miners, *Lyriomyza huidobrensis* (Huang et al., 2007). However, the presence and role of Hsp70 in *C. rosa* is unknown. Originally described from *D. melanogaster* as a response to high temperature, and hence their name, Hsps are up-regulated by diverse stresses with the majority of studies documenting increased expression due to heat (Kalosaka et al., 2009), cold shock, desiccation (Tammarielo et al., 1999; Hayward et al., 2004; Chen et al., 2005; Rinehart et al., 2007; Chowanski et al., 2015), anoxia, and heavy metals (Shu et al., 2010), but to our knowledge, no research to date has assessed the expression of hsp70 following starvation treatment, and especially for economic insects e.g. *C. rosa*.

1.3.2. Use of energy reserves

During starvation most animals including ectotherms exploit their body reserves and lose body mass (Scharf et al., 2016). Energy reserves in animal cells include glycogen and triglycerides (Arrese & Soulages 2010). The amount of energy reserves accumulated in insect fat body differs among insects' species, however lipids are the most abundant representing more than 50% of insect dry weight. Glycogen is stored in form which can be readily degraded on demand to be used as a glycolytic fuel while triglyceride can be used for energy production throughout β -oxidation (Athenstaedt & Daum 2006). Triglycerides however have a higher caloric content per unit of weight than glycogen, and provide a source of water upon oxidation, yielding almost two times more metabolic water than glycogen.

Extensive research has been done on the use of lipid reserves during starvation stress in various insects' species (Ballard et al., 2008; Arrese & Soulages 2010). However, the relationship between starvation and lipid content is not universal (Hofmann et al., 2001), as lipid reserve can also be induced by poor protein diet of adults (Piper et al., 2005). Also studies have recorded a link between the accumulations of lipids with other environmental stressors such as heat hardening (Kalra et al., 2017) and cold hardening (Chowanski et al., 2015). In a related study most insects accumulate energy reserves in the form of triacylglycerols prior to the beginning of winter (Lencioni 2004; Moghadam et al., 2011), symbolising energy investment and also the role of these lipids as mechanisms for low wither temperature survival.

1.3.3. Water balance strategies

Insects' small body size, which gives a large surface area to volume ratio makes them particularly susceptible to dehydration (Gibbs 2002). Insects use various physiological mechanism to survive desiccation stress including reduce water loss (Weldon et al., 2016), acquire water (Sjursen et al., 2001; Bazinet et al., 2010; Aggarwal et al., 2013), and/increase tolerance to water loss (Gibbs et al., 2003). While some insets need to use all the three mechanism in order to survive desiccation stress for example *D. immigrans* (Parkash et al., 2012a), others such as *D. nasuta* use only one of the three mechanisms (Parkash et al., 2012a). Although majority of investigations are from *Drosophila* species, water balance characteristics have also been studied in *C. capitata*, *C. rosa* and *C. cosyra* as strategies for surviving desiccation tolerance (Weldon et al., 2016). Since most insect body water is lost via the cuticle (Gibbs 2002; Gibbs et al., 2003; Chown & Nicolson 2004), the ability to limit water loss enables most insects' species to survive periods of desiccation stress. Studies have also shown that insects can use balance characteristics to adapt to other environmental stressors including high temperature (Kleynhans & Terblanche 2011), and low temperature environment (Hayward et al., 2007; Elnitsky et al., 2009; Holmstrup et al., 2010; Alvarado et al., 2015). This suggests the possibility of cross protection effects between desiccation stress and thermal stress. The involvement of water balance in starvation tolerance to our knowledge remain less investigated. However research has shown *D. melanogaster* reared at high humidity show increased starvation tolerance accompanied by increased levels of lipids (Parkash et al., 2014b).

1.3.4. Cold tolerance strategies

Insect cold tolerance can be classified based on how 'cold hardy' a particular species is, i.e. how an insect cope with extreme low temperature. Three categories emanate from this classification comprising chill susceptible, freeze avoiding and freeze tolerant animals. Chill susceptible insects refers to insects which can survive at low temperatures of 0 to °C, but die at relatively high subzero temperatures (Bale 1993; Andersen et al., 2015). The chill susceptibility of insects can be assessed from the critical thermal minimum (CT_{min}), which is defined as the temperature at which an insect loses neuromuscular coordination during a gradual decrease in temperature (Hazel & Bale 2011). Mechanisms underlying chill susceptibility are not well understood, but thought to be inability to maintain ion and water homeostasis, leading to muscular dysfunction and ultimately chill-injury and death (Overgaard & Macmillan 2017). Freeze-avoiding insects are those insects which cannot tolerate the formation of ice within their bodily fluids, as such they implement strategies to depress the temperature at which their bodily fluids will freeze (Andersen et al., 2015; Sheikh et al., 2017). This is achieved through the accumulation and synthesis of cryoprotectants and antifreezers, which reduce the lethal freezing temperature of the body (Duman 2002). Glycerol is by far the most common protectant used by insects and other terrestrial arthropods, however other carbohydrates including sorbitol, mannitol, ribitol, xylitol, erythritol, ethylene glycol, glucose (Storey 2004), trehalose, and sucrose are also used as cryoprotectant by several other animals (Lencioni 2004). The depressive effect of glycerol on the super cooling point (SCP) is thought to be due to the high viscosity of glycerol solutions at low temperatures, which inhibit ice nucleating agents activity hence depress the SCPs far below the environmental temperature (Lencioni 2004). However not all freeze avoidant insects produce cryoprotectants. Hibernating insects produce what are known as thermal hysteresis factors (THFs) (Sheikh et al., 2017), which unlike cryoprotectants act directly on the ice crystals by adsorbing to the developing crystals to inhibit their growth hence reduce the chance of lethal freezing.

On the other hand, freeze tolerance refers to controlled freezing of body water in extracellular fluid spaces while preserving the liquid state of the cytoplasm (Sheikh et al., 2017). Thus, unlike freeze avoiding, freeze tolerant insects can tolerate intracellular ice formation, and insects survive the winter season in a frozen state with much of the water converted to extracellular ice (Danks 2004). These insects initiate freezing at relatively high temperatures

through the production of ice nucleating proteins (Marchand 1996; Duman 2001; Sheikh et al., 2017), which moderate the rate of ice growth, hence adjust more slowly to the mechanical and osmotic pressures that are imposed by ice formation. An example of a freeze tolerant insect is the fly *Heleomyza borealis* which is able to survive temperatures as low as -60°C (Worland et al., 2000).

1.4. Biology and ecology of *Ceratitis rosa*

Tephritidae fruit flies are the most damaging insect pests of fruit crops (Duyck & Quilici 2002; Ekesi et al., 2007). Bulk of them are highly polyphagous, invasive and poses a significant biosecurity threat worldwide. *Ceratitis rosa* is a serious pest of cultivated fruit (De Meyer 2001; Copeland et al. 2006) infesting over 90 species of wild and cultivated crops (De Meyer et al. 2002). The female fly punctures soft and tender fruits with her ovipositor and lay eggs below the exocarp of the fruit. Maggots that hatch from the eggs bore into the fruits and feed on fruit structures hence fruit flies are a limiting factor to obtaining high yields and quality fruits. In addition to direct losses, fruit fly infestation can result in serious losses in trade value and export opportunities due to strict quarantine regulations imposed by most countries, and also enormous control and eradication costs (Nyamukondiwa et al., 2013). *Ceratitis rosa* has a wide range on the Africa continent (De Meyer et al., 2008; Nyamukondiwa et al., 2013) and has been suggested to prefer cooler and wetter environments (Duyck & Quilici 2002; Normand et al, 2000; Nyamukondiwa & Terblanche 2009), suggesting a greater potential in temperate regions (see Nyamukondiwa et al., 2010). Its potential for establishment in these areas is limited by its inability to survive periods of repeated low temperature treatments which are typical of temperate areas (Nyamukondiwa et al., 2010). However due to climate change, *C. rosa* is likely to be faced with multiple stressors and information on how it will respond when faced with such, is important for future phenology predictions, and can help in early warning systems and consequently efficacious control. This will be significant to climate change prediction models of insects of economic importance, such as *C. rosa* and other related taxa.

1.5. Justification of study

Global climate change has brought a lot of environmental stressors such as increase in average temperatures and variability (IPCC 2014; William et al., 2015), changes in rainfall patterns and extreme climatic events vis-a-vis frequent occurrences of cold snaps, heat waves

(Perkins et al., 2012) and cases of drought (Trenberth 2011) and wildfires. As such organisms must adapt morphologically, behaviourally or physiologically in time and space to prevent extinction (reviewed in Chown & Nicolson 2004; Angilletta 2009; Chevin et al. 2010). Recently increases in global temperatures have been associated with shifts in geographic distribution of both terrestrial and aquatic organisms (Hickling et al., 2006; Vanhanen et al., 2007; Stange & Ayres 2010). Great concern is on the population dynamics and biogeography of insects (Kiritani 2013), especially economically important insect pests like the Natal fruit fly, *Ceratitidis rosa* (De Meyer et al., 2008), which poses significant biosecurity threats upon introduction to novel environments. The survival abilities (resistance mechanisms) of these economic pests in the face of climate change is imperative, due to their extensive damage and economic losses of major fruits and vegetable crops worldwide, coupled with costs of their eradication and barriers to international trade (Benjamin et al., 2012).

1.6 Objectives

This research was therefore aimed at (1) investigating the ability of *C. rosa* to adapt to multiple environmental stressors of heat, relative humidity, starvation and cold, (2) to identify thereof, any overlaps in mechanisms underlying stress tolerance such as differences in energy reserves and water balance traits.

1.7 Hypotheses

The hypothesis of the study was that *C. rosa* flies possess some form of cross tolerance to environmental stressors, which enables it to survive multiple stress environments brought about by climate change. The other hypothesis was that the existence of cross tolerance may be due to overlap or sharing of physiological resistance mechanisms or regulatory pathways (also known as cross talk).

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CHAPTER 2

Plasticity and cross tolerance to heterogenous environments: divergent stress responses co-evolved in an African fruit fly, *Ceratitis rosa*.

2.1. Introduction

Understanding how climate and local weather influences animal population dynamics and geographic range limits has been a long-standing issue in ecology and evolution for several reasons. Chief among them perhaps are concerns surrounding the increased incidence of extreme weather events (e.g. cold snaps, heat waves) (Gunderson et al., 2016; Williams et al., 2016), or atypical seasons (warming winters) (Ambrosini et al., 2016; Shepherd 2016; Uelmen et al., 2016), the relative importance of changing means vs. extremes (Camacho *et al.*, 2015; Sheldon & Dillon 2016) and their implications for estimating population dynamics of insect pest species, disease vectors or those of conservation concern (Walther et al., 2002; Williams et al. 2015; Boggs 2016). Increased mean temperature and variability thereof affects insects' life history and demography (Khaliq et al., 2014; Colinet et al., 2015) and thus population dynamics and biogeography (Hoffmann et al, 2003; Lobo 2016; Torossian et al., 2016). Mechanistic models will play a major role in helping forecasting to become robust and determining causal influences in population responses to climate change is also increasingly emphasized yet any trait-based approaches requires consideration of a diverse suits of traits and their responses within and between generations to diverse climate variability (Chown 2012; Seebacher & Franklin 2012) yet temperature is frequently the focus of attention despite several calls to broaden the suite of environments and traits considered to better describe fundamental niches.

Adaptation may take two major forms: phenotypic plasticity at the individual level and within generation or changes in genetic composition through natural selection, a process that favours the survival of the 'fittest' genotypes (Frankham & Kingsolver 2004; Angilletta 2009; Sgro et al., 2016). As such, phenotypic plasticity of different fitness-related traits is an important mechanism enhancing survival of species when introduced to novel and often stressful environments (Ghalambor et al., 2007; Schilthuizen & Kellermann 2014; Hill et al., 2016).

Physiological responses that increase an organism's resistance after exposure to some form of non-lethal stress are referred to as plasticity, acclimation or hardening (West-Eberhard 2003; Chown & Terblanche 2007), depending on the time-scale and severity of the treatment (reviewed in e.g. Seebacher 2005; Sgrò et al., 2016). Although typically examined in isolation for a single trait and environment, pronounced fitness benefits have been documented across a range of species. For example, mild heat treatments frequently improve what would have been lethal heat survival in diverse insect taxa (Nyamukondiwa et al., 2010, 2011; Weldon et

al., 2011; Bublly et al., 2013) and similar types of responses are well documented for cold responses at a range of time-scales and under diverse conditions (e.g. Kelty & Lee 2001; Nyamukondiwa et al., 2010, 2011; Findsen et al., 2013). Similarly, preacclimation to mild drought stress has also been shown to enhance drought tolerance in soil dwelling springtails *Folsomia candida* (Bayley et al., 2001).

In some instances, acclimation to one stress can confer resistance to a distinctly different form of stresses in what is termed cross tolerance (Bublly et al., 2012, 2013). Multiple abiotic stressors are often experienced simultaneously by organisms in nature, and as such, responses to these stressors may share signal pathways (“cross talk”) or protective mechanisms (“cross tolerance”) (Sinclair et al., 2013). Such phenomena may symbolize shared physiological resistance mechanisms and the possibility of joint evolution of resistance traits. Evolution of stress resistance and relationships among resistance and life history traits has been investigated by comparing correlated responses to selection on these traits (see Harshman & Hoffmann 2000; Kelly et al., 2016). Positive correlations have been detected between ecologically significant stress resistance traits, e.g. desiccation and starvation (Hoffmann & Harshman, 1999) while some studies have indicated negative results for potentially related desiccation and temperature resistance (e.g. Hoffmann 1990; Watson & Hoffmann 1996). Moreover, Hoffmann et al., (2002) recorded sex specific trade-offs between starvation and low temperature tolerance and such negative plastic correlations and probably genetic tradeoffs between traits may affect insect population phenology and biogeography. Nevertheless, experimental evidence for cross-resistance following hardening and acclimation is limited (but see Coulson & Bale 1991; Bublly et al., 2012) and is not exhaustive of all combinations of environmental stress factors (Sinclair et al., 2013) (see Table 2.1). Since most cross tolerance data is based on *Drosophila* (Table 2.1), it remains unclear how different insect taxa may respond when faced with multiple stress environments. Consequently, it is highly critical to test responses of different species versus multiple stressor interactions, to check any generalities (e.g. do most or all species respond similarly to identical stress combinations), before we can make robust predictions on the effect of multiple stressors on insects under global change (see Kaunisto et al., 2016). Furthermore, results on studies looking at the correlations across different environmental stressors have been resistance to multiple stressors may be due to shared regulatory (‘cross talk’) or mechanistic (cross tolerance) pathways (see Sinclair et al., 2013). Nevertheless, organisms in

nature are likely to face multiple overlapping stress scenarios, hence cross plastic environmental stress responses may be significant in the face of climate change.

Ceratitis rosa is a multivoltine, highly polyphagous fruit fly insect pest of most commercially grown fruits. This fruit fly is considered a biosecurity threat and a burden to agriculture as it is a barrier to economic transformation through direct crop losses, costs of control practices, and reduces market access (Nyamukondiwa et al., 2013). Correlative ecological niche modelling suggest *C. rosa* may have a more restricted distribution relative to its congener *C. capitata*. While *C. rosa* has a potentially broad range (Africa and Southern Europe), it may not thrive in central and western regions of Southern Africa, as well as Sahelian zone, where conditions are predominantly dry (De Meyer et al., 2008; Hill et al., 2016). Moreso, with global change and shifts in ecological niches, Hill et al., (2016) predicted an overall decrease in *C. rosa* climate suitability and a consequent poleward shift in species distribution. Nevertheless, *C. rosa* remains invasive (Nyamukondiwa et al., 2010) and thus likely to establish in novel environments. The acquisition of cross tolerance is very crucial in fluctuating environments, and as such cross tolerance may be a very significant mechanism of surviving multiple stressors in fluctuating environments under global change, and may likely aid *C. rosa* invasion potential and its ability to thrive upon introduction to a novel multiple stress environment. This research therefore aims to test adult cross-resistance following acclimation treatments to several different environmental stressors using a laboratory population of *C. rosa* as model organism. While most cross tolerance work has been exclusively done on *Drosophila* (see Bublly et al., 2012, 2013) (Table 2.1), it remains unclear how common coevolution of plastic stress responses is, especially for non *Drosophilids*. Moreover, for *Drosophila*, Kellermann et al. (2012a) showed these species have low evolutionary potential e.g for upper thermal limits. Indeed, Kellermann et al. (2012a) found that precipitation played a more significant role in driving *Drosophila* ranges and that the interaction between temperature and precipitation drives high temperature tolerance. Similarly, Kellermann et al. (2012b) evolutionary responses to cold resistance is likely slow, suggesting *Drosophila* species distributions are shaped by evolutionarily conservative climate responses, and a constrained potential for rapid adaptation to climate change. In consequence, cross tolerance and co-evolution to heterogenous environments ought to be investigated in a diversity of insect taxa before any generalities can be made on the effects of multiple stress

environments on insect population dynamics. The objective of this study was therefore to investigate cross tolerance in insect species, using adult *C. rosa* as a model organism.

Table 2.1: Summary survey table of species that have been examined for cross resistance (and whose traits have been shown to interact positively, i.e. cross tolerance), the pre-treatment stress factors and the cross stress factor tested. CCRT= Chill coma recovery time, HKDT= Heat knockdown time, SCP= Supercooling points, ULT= upper lethal temperature.

Insect Species	Trait 1 (Treatment factor)	Trait 2 (Stress factor tested)	Reference
<i>D. melanogaster</i>	Desiccation	Low temperature (CCRT)	Bubliy <i>et al.</i> , 2012
	Desiccation	High temperature (HKDT)	Bubliy <i>et al.</i> , 2012
	Starvation	Desiccation	Bubliy <i>et al.</i> , 2012
	High humidity	High temperature (HKDT)	Parkash <i>et al.</i> , 2014
	High humidity	Starvation resistance	Parkash <i>et al.</i> , 2014
	Low temperature	Desiccation resistance	Bauerfeind, <i>et al.</i> , 2014
	Low temperature	Starvation resistance	Bauerfeind, <i>et al.</i> , 2014
<i>D. simulans</i>	Relative humidity	High temperature (HKDT)	Bubliy <i>et al.</i> , 2013
<i>Acheta domesticus</i>	High temperature	Low temperature (CCRT)	Lachenicht <i>et al.</i> , 2010
<i>Folsomia candida</i>	Desiccation	Low temperature (CCRT)	Holmstrup <i>et al.</i> , 2002
	Starvation	Low temperature (CCRT)	Bayley <i>et al.</i> , 2001
	Mercury	Low temperature (LLT)	Holmstrup <i>et al.</i> , 2008
<i>Paractora dreuxi</i>	Low temperature	High temperature survival (ULT)	Marais <i>et al.</i> , 2009
<i>Tribolium castaneum</i>	Low temperature	Starvation tolerance	Scharf <i>et al.</i> , 2015
<i>Belgica antarctica</i>	Dehydration	High temperature (ULT)	Benoit <i>et al.</i> , 2009
	Dehydration	Low temperature (LLT)	Benoit <i>et al.</i> , 2009
<i>Thaumatotibia leucotreta</i>	Hypoxia	Low temperature (LLT)	Boardman <i>et al.</i> , 2015
<i>Cydia pomonella</i>	High temperature	Low temperature survival (LLT)	Chidawanyika & Terblanche, 2011

2.2. Materials and Methods

2.2.1. Fly rearing and maintenance

Colony of *C. rosa* flies were obtained as live pupae from Citrus Research International, Nelspruit, South Africa. The colony has been reared in the laboratory for ~300 generations under mass and outbred conditions, maintained in high numbers, and is regularly supplemented with wild flies to minimise inbreeding depression and genetic drift. Upon arrival at Stellenbosch University, pupae were distributed evenly into several petri dishes and placed into a rearing cage (Bugdorm-BD43030F, Megaview Science Co., Ltd, Taiwan) for eclosion. The flies were provided with dry sugar, water-soaked cotton wool and yeast powder (Biolab, Merck, Wadeville, Gauteng, South Africa). The cage was then placed in an incubator set to 25 °C, 75±5% RH with a 12:12 h photoperiod until adult eclosion. Within 48h of eclosion, adult *C. rosa* flies were separated according to sex using gross morphology into 5L plastic containers furnished with sugar, water and yeast. Each 5 L container contained ±150 adult flies of the same sex. A mesh-covered jar containing saturated NaCl solution was inserted into each of the 5 L plastic container to maintain relative humidity of 75 ±5 % (Weldon *et al.*, 2011) before sealing the container with a lid.

2.2.2. Acclimation treatments

2.2.2.1. Heat pretreatment

At 7 days after adult eclosion, virgin female and male *C. rosa* flies were separately placed into 20mm X 90mm glass vials with plastic lids in groups of 10 flies of the same sex. The glass tubes were immersed in a circulating programmable refrigeration bath (Huber CC410 WL, Offenburg, Germany) containing 1:1 water: propylene glycol set at 36 °C for 1 h. Pretreatment of flies at 36 °C (1 h) has been shown to significantly improve survival during an otherwise lethal 2 h exposure at 41°C in both *C. rosa* and *C. capitata* (Nyamukondiwa *et al.*, 2010). Since the heat shock response requires the de novo synthesis of heat shock proteins, a recovery period at mild temperature may be required to elicit responses (reviewed in e.g. Denlinger & Lee 2010). Therefore a 30 minutes recovery period under standard benign conditions in a constant environmental chamber 25 °C, 75% RH was therefore given to elicit the heat shock response. Control flies were sorted in the same way and placed in similar glass vials but returned to the optimal environmental rearing conditions (25°C; 75% RH) for subsequent stress survival or trait scoring.

2.2.2.2. *Cold treatment*

As in the heat treatments described above, virgin female and male flies were placed separately in 5L plastic containers 2 days post eclosion. Each 5L plastic container had access to food (sugar and yeast) and water plus a vial filled with saturated salt solution and covered by insect mesh to maintain the relative humidity at ~75%. The containers were then placed in an incubator set at 20 °C for 5 days. These conditions are sufficient to elicit cold acclamatory responses in *Ceratitis* species (see Nyamukondiwa et al., 2010; Weldon et al., 2011). Control flies were sorted in the same way as treatment flies, and placed in similar glass vials but returned to the optimal environmental rearing conditions (25°C; 75% RH) for subsequent stress survival or trait scoring.

2.2.2.3. *Desiccation treatment*

At 7 days after adult eclosion, virgin females and male *C. rosa* were individually placed in ventilated 0.65ml micro-centrifuge tubes. Six tubes were then placed in a sponge rack and put in a 250ml plastic container containing about 50g of silica gel at the bottom and sealed with an air-tight lid (Gibbs et al., 1997; Terblanche & Kleynhans 2009). These desiccators were then placed in an incubator set at 25 °C for a period of 15 h. This duration is sufficient to elicit ~35% body water loss to like invertebrates (e.g. Weldon et al., 2016). After 15 h of desiccation stress, flies of each sex were removed from the desiccators and given a recovery period of 6 h with food and water at 25°C; 75% RH.

2.2.2.4. *Food deprivation/fasting treatment*

At 5 days following emergence, virgin female and male *C. rosa* flies were placed separately in 5L plastic containers which were then placed in an incubator set at 25°C (48 h) with no food. However, a wet cotton wool was placed inside each container to prevent mortality associated with desiccation (Parkash et al., 2014). A dish filled with saturated NaCl solution and covered by insect mesh to maintain the relative humidity at 75% was also placed in each of the containers. After 2 days of food deprivation the flies were given a 12h recovery period (provided with water and food, at 25 °C; 75%RH). At the same time, control flies were sorted in the same way as treatment flies, and placed in 5L plastic containers but with access to food *ad libitum* at benign rearing conditions (25°C; 75% RH) for subsequent stress survival or trait scoring. In all food deprivation pre-treatments, the actual temperature and RH experienced by the flies during acclimation in the incubators was recorded at hourly interval using

thermochron temperature and humidity loggers attached to the inside of the sealed container containing the flies (DS1923 ibutton, Maxim Integrated Products, Sunnyvale CA, USA). After subjecting the flies to the respective pre-treatment conditions, the flies were tested for different environmental stressors (Table 2.2). Stress resistance tests were all done using 7 day old adult flies to control for any potential age related differences in environmental stress resistance (see e.g. Nyamukondiwa & Terblanche 2009).

2.2.3. Stress resistance tests

2.2.3.1. Temperature stress resistance

We scored two different traits of high temperature tolerance and two traits of low temperature tolerance. First, we investigated tolerance to high temperature stress, measured as upper critical temperatures to activity (CT_{max}) and heat knockdown time (HKDT). For CT_{max} experiments, *Ceratitis rosa* adult flies were individually weighed to 0.1 mg using a calibrated electronic microbalance (Model MS 104S /01, Mettler Toledo equipment, Switzerland) before and after performing CTLs. Individual *C. rosa* were placed into a double jacketed chamber ('organ pipes') connected to the programmable bath (Grant GP200-R4, Grant Instruments, UK) (as in e.g. Nyamukondiwa & Terblanche 2009). This was repeated twice for each experiment to get N= 20 flies. A thermocouple (type K, 36 SWG) connected to a digital thermometer (Fluke 54 series II, Fluke Cooperation, China; accuracy: 0.05°C) was inserted into the control chamber to record chamber temperature. CT_{max} experiments started at a set temperature of 25 °C from which temperature increased at a rate of 0.25 °C/min until the flies reached upper temperature limit of activity (Table 2.2) (Nyamukondiwa & Terblanche 2009). CT_{max} was defined as the temperature at which each individual insect lost co-ordinated muscle function, consequently losing the ability to respond to mild stimulus (e.g. gentle prodding). In the case of CT_{max} , this loss of muscle function coincided with death such that recovery was not possible upon removal from the assay. Immediately after CT_{max} assays, the flies were weighed once again individually on the electronic microbalance to determine water loss during experiment. For HKDT experiments, both treatment and control virgin *C. rosa* adults were weighed in ventilated 7ml plastic vials of known weight before acute exposure to a fixed temperature of 43.0±0.3 °C on the thermal stage following Weldon et al., (2011). HKDT was defined as the time (in minutes) that it takes to knockdown the insects, corresponding to the time it takes to lose locomotor function (as in Weldon et al., 2011).

Second, we investigated tolerance to low temperatures stress, measured as lower critical temperatures to activity (CT_{min}) and chill coma recovery time (CCRT), both of which are related to insect geographic distributions and are widely employed in assaying chilling stress resistance (Andersen *et al.* 2015). For CT_{min} experiments, the same methodology was followed as in CT_{max} , with necessary modifications in the ramping protocol. Briefly, ten replicate individual *C. rosa* flies were placed into a double jacketed chamber connected to a programmable bath. A thermocouple (type K, 36 SWG) connected to a digital thermometer, was inserted into the control chamber to record chamber temperature. CT_{min} experiments started at a set point temperature of 25 °C, ramping down at 0.25 °C/min until the flies reached their lower temperature limit to activity (Table 2.2). This was repeated twice for each experiment to get N= 20 flies. CT_{min} was defined as the temperature at which each individual lost co-ordinated muscle function. For CCRT, both treated and control flies of *C. rosa* (n =20) were tested for CCRT after exposure to the different environmental stressors (Table 2) following Weldon *et al.*, (2011). Briefly, flies were weighed in individual 7ml screw-cap plastic vials of known weight with two 1mm diameter holes pierced through the caps for ventilation. The vials were then placed into a large zip-lock bag which was then plunged into a water bath set at 0 °C for 1 hour. These assay conditions are sufficient to induce chill coma (see Weldon *et al.*, 2011). Following chill coma, the plastic vials were then placed on the thermal stage held at 25 °C and CCRT was recorded. CCRT was defined as the time required for each fly to stand on its legs, without any interference or stimulation from the observer, following chill coma.

2.2.3.2. Starvation Resistance

Five replicates of five insects for each sex were used for starvation resistance. This was done to account for the potential confounding effects of mating status on starvation resistance (see Rush *et al.*, 2007). Both treated and control adult flies were placed separately into 100ml plastic containers covered with mesh. The 100ml plastic containers were then placed in a 5L container held in an incubator (25 ±1 °C, 75±5% RH), until the last fly died (as in e.g. Parkash *et al.*, 2014). All the flies were provided with water (supplied as a wet cotton wool placed in each plastic container) during the whole experiment in order to avoid mortality associated with desiccation while ‘starving’ (Bubliy *et al.*, 2012; Parkash *et al.*, 2014). Mortality was assessed daily at 8am, 2pm and at 8pm until all the flies were dead (Goenaga *et al.*, 2011). Mortality data were then used to estimate the time taken for 50% of flies to die

(LT₅₀) in hours.

2.2.3.3. Desiccation Resistance

Desiccation resistance was assessed using 6 replicates of 6 adult flies per replicate for each sex (Table 2.2). Flies were placed in 0.65ml ventilated centrifuge tubes which were then put in small desiccators containing 50g of silica gel (Terblanche & Kleynhans 2009; Parkash & Ranga 2014). The desiccators were then placed in an incubator set at 25°C for a period of 36 h. Flies were provided with neither food nor water during the desiccation period. The survival rate (defined as the proportion of live to dead flies) under desiccation conditions was then recorded 24 h post the 36h desiccation treatment period. Mortality in this case was defined as the inability to respond to mild stimuli (e.g. prodding). During the recovery period flies were provided with water and food.

Table 2. 2: Summary of the conditions that were used to test abiotic stress resistance in *Ceratitis rosa* adult flies

Stress assay	Cold resistance	Heat resistance	Desiccation resistance	Starvation resistance
Number of vials or flies per treatment	(20 replicate individual flies) CT _{min}	(20 replicate individual flies) CT _{max}	6 replicates/6 flies each/sex/	2 replicates, 25 flies each/sex
Period of exposure to stress	Down to CT _{min}	Up to CT _{max}	36h	up to 10 days
Temperature (°C)	Ramped down (0.25°C/min) starting at 25°C	Ramped up(0.25°C/min) starting at 25 °C	25°C	25°C
Relative humidity	75%	75%	15-30%	75%
Culture medium	No medium	No medium	No medium	Water only (moist cotton wool)

2.2.4. Data analysis

To examine the effects of acclimation conditions on diverse trait responses treatment groups were analysed using generalized linear model (GLM) in R v. 3.1.2. (R Development Core Team). Minimal adequate models were determined based on Akaike’s information criterion (AIC) using the ‘step’ function in R, involving stepwise omission of the least influential

parameters from the model. Overlap in 95% confidence limits (CLs) was used to identify statistically homogeneous median groups.

For starvation and desiccation assays, analysis was firstly done considering each sex separately. However, as sex had no significant effect on desiccation resistance this factor was removed from subsequent analysis, but in the case of starvation resistance sex was significant and retained in the subsequent models. Effects of pre-treatment on desiccation resistance were analysed as the proportion of live to dead flies following 36h of desiccation stress. Starvation resistance was analysed as the time taken for 50% of flies to die (LT₅₀) in hours. Sexes were analysed separately, since it had a significant effect on starvation resistance (P<0.0001). Kruskal-Wallis *post hoc* tests were used to separate statistically homogeneous groups.

2.3. Results

2.3.1. Temperature stress resistance

Acclimation had significant effects on the *C. rosa* CT_{max} (Table 2.3). Heat hardening, desiccation and fasting conditions significantly improved CT_{max} (Fig. 2.1.A). Cold acclimated flies did not significantly differ from the heat exposed, desiccation and fasted treatment flies. However, these cold acclimated flies did not significantly differ from the untreated control group in terms of CT_{max} (Fig. 2.1.A). Pre-treatments did not have any significant effects on heat tolerance, measured as heat knockdown time (HKDT) (Table 2.3; Fig. 2.1.B). Generally HKDT did not differ between each treatment groups nor from the control group (Table 2.3).

Cold acclimation significantly enhanced *C. rosa*'s low temperature tolerance scored as CT_{min} (Table 2.3). Moreover, cold acclimated flies were significantly different from all other treatments (except for desiccation treated flies), which were not significantly different from each other (Fig. 2.1.C). There was no evidence of cross tolerance across all treatments for CT_{min} (Table 2.3; Fig 2.1.C). When cold tolerance was scored as CCRT, no effects of any of the treatments were detected (Table 2.3). Treatment groups were not significantly different from the control group (Fig. 2.1.D). However, for CCRT, heat exposed and cold acclimated flies differed significantly from desiccation and starvation treated flies.

2.3.2. Desiccation resistance

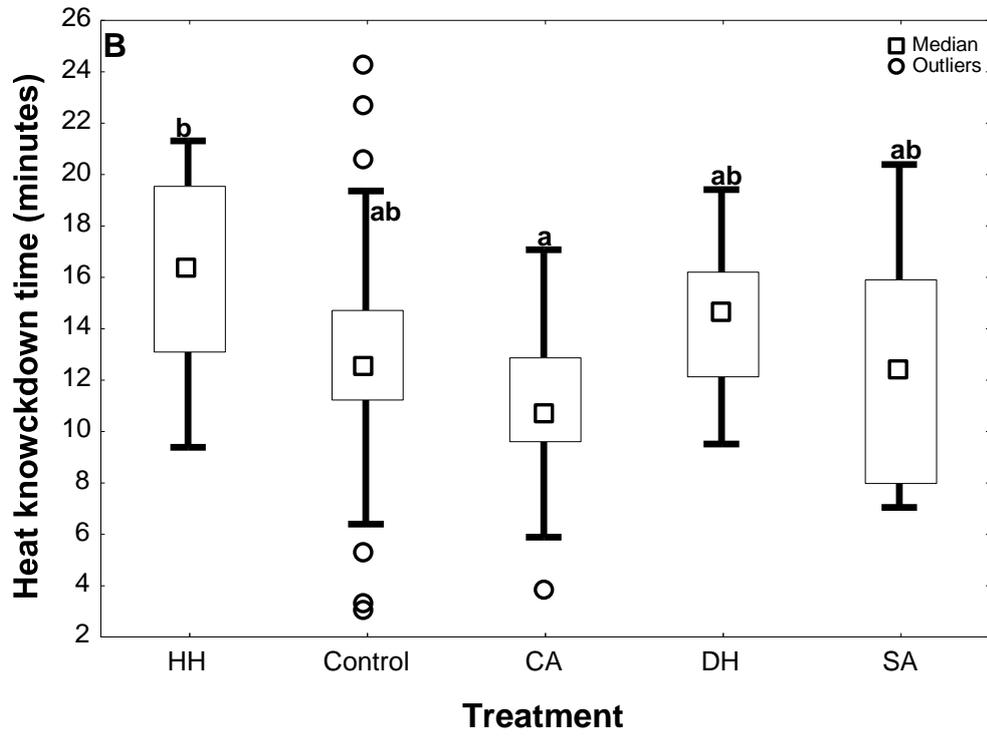
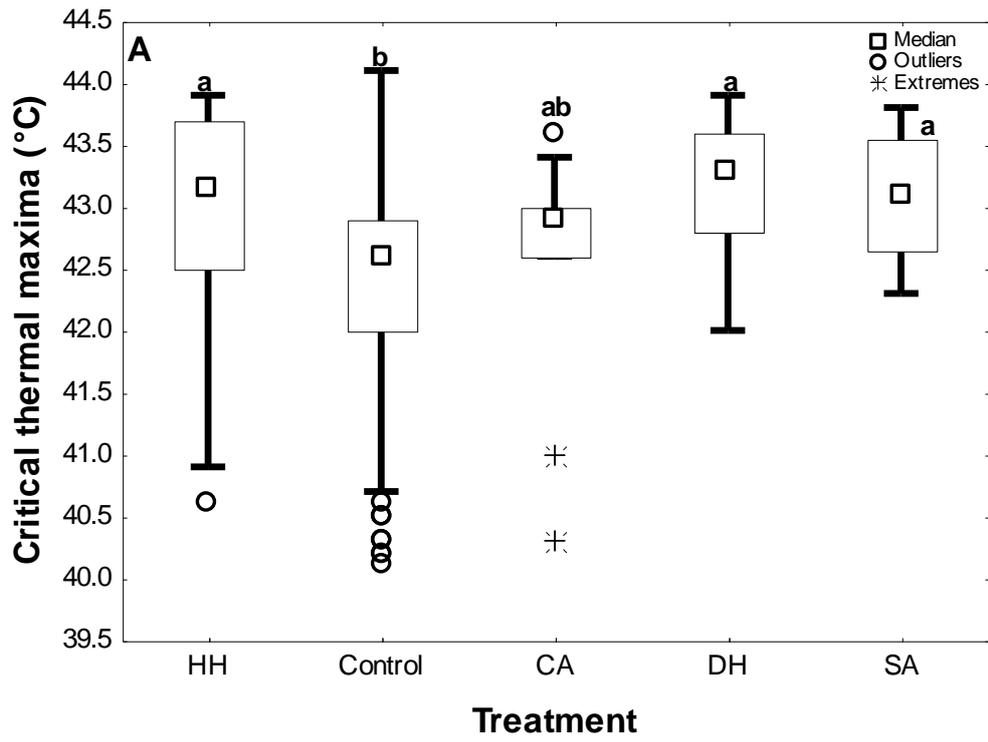
Desiccation hardening significantly improved desiccation resistance (Table 2.3; Fig. 2.2.A). Similarly, cold treatment significantly improved desiccation resistance relative to the control (Fig. 2.2.A) Heat and starvation pre-treatments had significantly lower desiccation resistance relative to desiccation hardened, cold acclimated and the control flies (Fig. 2.2.A).

2.3.3. Starvation resistance

There was a sex effect on starvation resistance ($p < 0.0001$) hence male and female *C. rosa* were analysed separately. Generally, females had an enhanced starvation resistance relative to the male flies (Fig. 2.2.B; C), measured as LT_{50} . For the female flies, starvation acclimation significantly improved starvation resistance (Fig. 2.2.B). However, heat and desiccation treated female flies did not significantly differ from the control (Fig. 2.2.B). Nevertheless, cold acclimation had significant negative effects on female *C. rosa* starvation resistance (Fig. 2.2.B). Starvation, heat and cold pre-treatments had no significant effects on male *C. rosa* starvation tolerance (Fig. 2.2.C). However, desiccation pre-treatments significantly improved starvation resistance in male flies.

Table 2.3: Summary results of the minimum adequate generalized linear models (glm) explaining the effects of acclimation [heat hardening (HH), cold acclimation (CA), desiccation hardening (DH) and starvation acclimation (SA)] on the various stress resistance traits in *C. rosa*.

Variable	Estimate	Standard Error	t value	Pr(> t)
<i>Critical thermal maxima</i>				
Intercept	22.182	1.475	15.041	<0.001
CA	-2.515	2.316	-1.086	0.280
DH	2.618	2.638	0.992	0.323
SA	1.318	2.638	0.500	0.619
Control	-6.635	1.754	-3.782	0.001
<i>Critical thermal minima</i>				
Intercept	19.273	1.395	13.818	<0.001
CA	-10.806	2.191	-4.933	<0.001
DH	-3.273	2.495	-1.312	0.192
SA	-0.373	2.495	-0.149	0.882
Control	-0.877	1.659	-0.528	0.598
<i>Heat knockdown time</i>				
Intercept	63.917	9.587	6.667	<0.001
CA	-3.877	11.663	-0.332	0.740
DH	0.583	14.220	0.041	0.967
SA	2.083	12.862	0.162	0.872
Control	-11.567	10.502	-1.101	0.273
<i>Chill coma recovery time</i>				
Intercept	50.600	6.808	7.433	<0.001
CA	-8.733	9.627	-0.907	0.367
DH	8.600	10.764	0.799	0.426
SA	7.300	10.764	0.678	0.499
Control	-5.980	7.762	-0.770	0.443
<i>Desiccation resistance</i>				
Intercept	-1.105	0.438	-2.525	0.012
CA	1.807	0.548	3.300	0.001
DH	2.374	0.625	3.801	0.001
SA	0.614	0.562	1.094	0.274
Control	1.554	0.462	3.366	0.001
<i>Starvation resistance</i>				
Intercept	36.220	2.992	12.107	<0.001
CA	-2.760	4.231	-0.652	0.515
DH	-6.856	4.373	-1.568	0.118
SA	-3.487	4.347	-0.802	0.423
Control	-7.033	3.348	-2.101	0.036



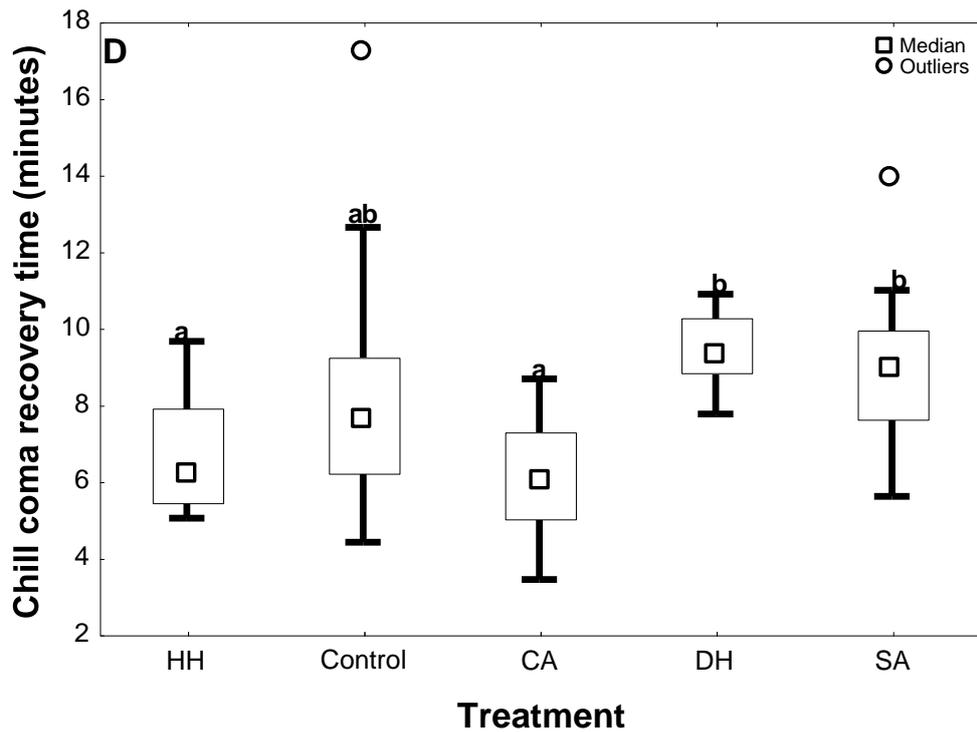
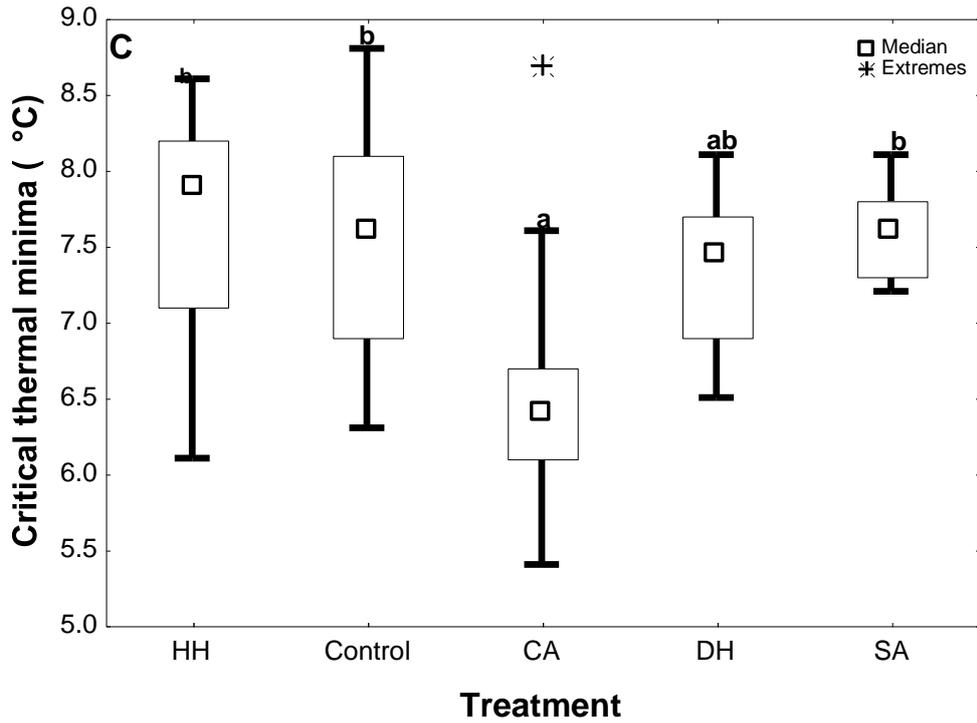
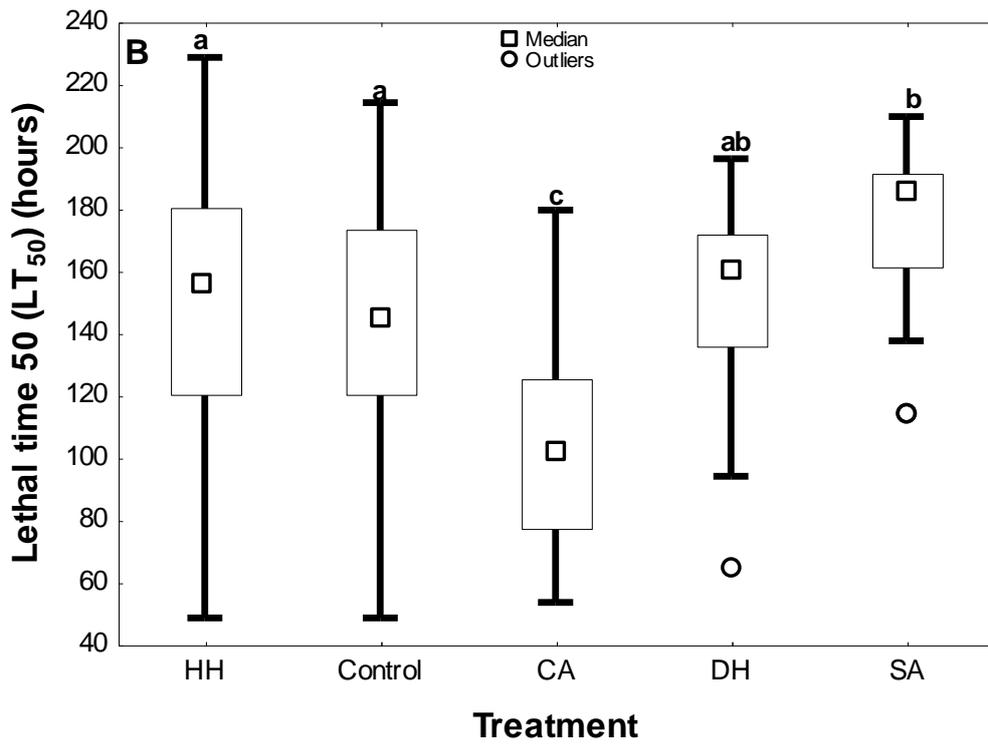
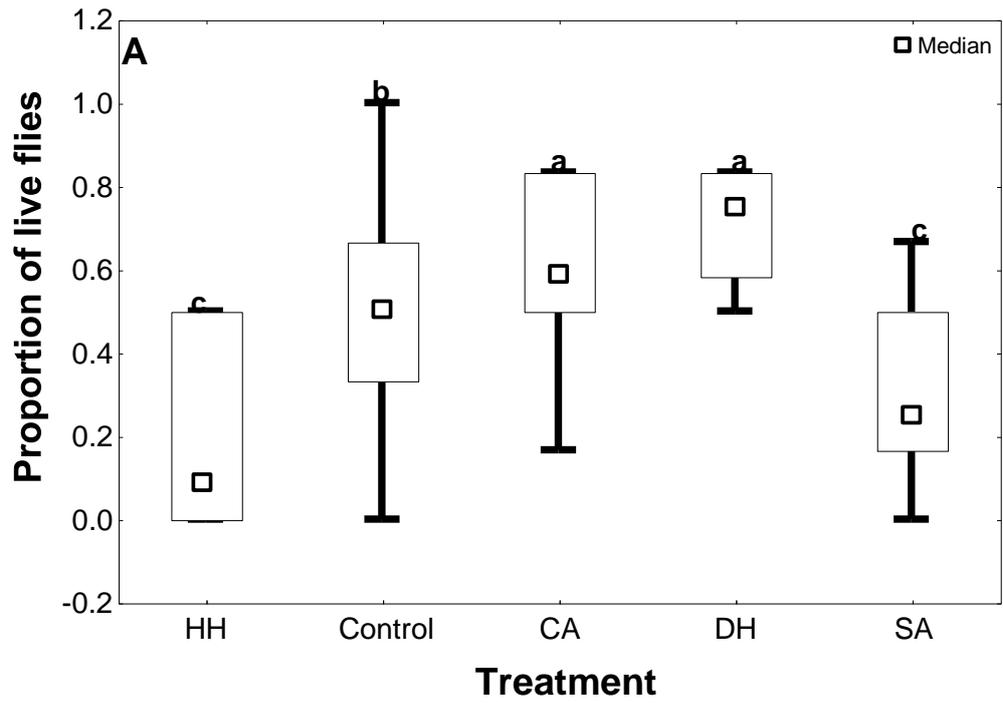


Figure 2.1. Effects of pretreatment on (A) critical thermal maxima, (B) heat knockdown time (C) critical thermal minima and (D) chill coma recovery time in adult *C. rosa*. Values represent medians \pm 95% CLs. Kruskal Wallis *post hoc* tests were used to separate statistically homogeneous groups at $P = 0.05$. Group medians with the same letter are not significantly different. (CA=cold acclimation; HH=heat hardening; DH=desiccation hardening; SA= starvation acclimation).



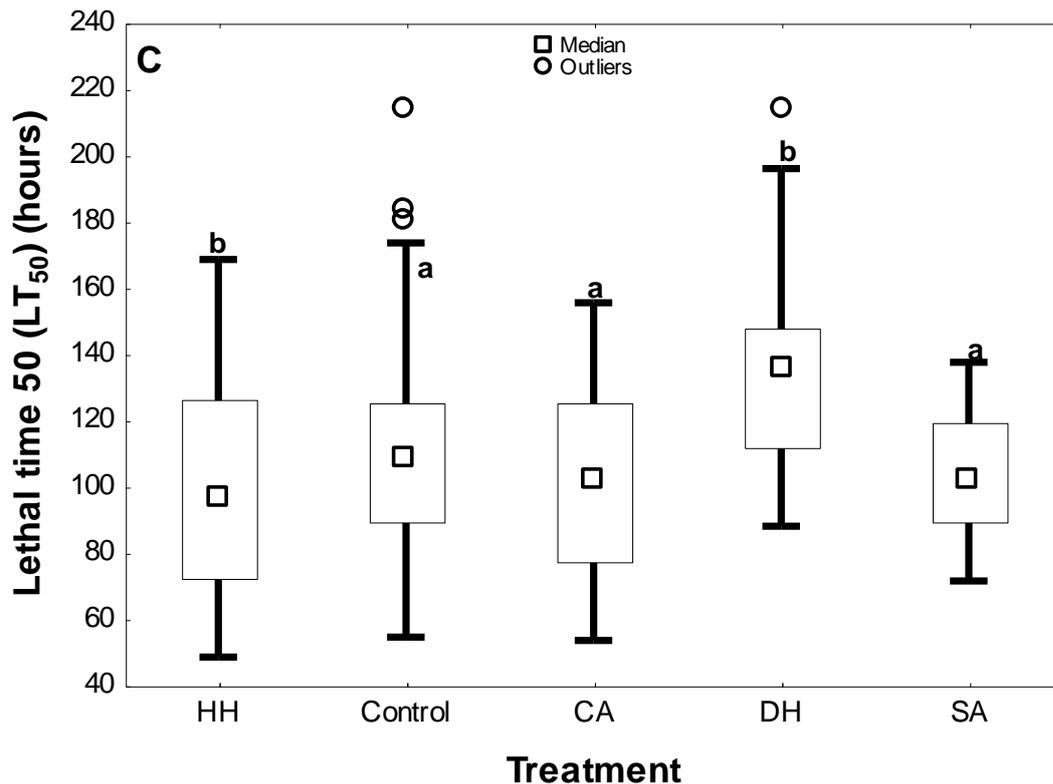


Figure 2.2. Effects of pretreatment on *C. rosa* (A) desiccation resistance, measured as survival of adult *C. rosa* under desiccation stress; (B) adult female starvation resistance measured as the time it takes to ‘kill’ half of the test organisms (LT_{50}) and (C) adult male starvation resistance. Values represent medians \pm 95% CLs. Kruskal Wallis *post hoc* tests were used to separate statistically homogeneous groups at $P = 0.05$. Group medians with the same letter are not significantly different. (CA=cold acclimation; HH=heat hardening; DH=desiccation hardening; SA= starvation acclimation).

2.4. Discussion

Our results show that acclimation to a particular stress, significantly improves survival upon exposure to that particular stress later in the organism’s lifetime, an indication of beneficial acclimation hypothesis (BAH) (Leroi et al., 1994). Furthermore, we report novel improvements in heat tolerance (CT_{max}) following starvation or desiccation acclimation; improved desiccation resistance following cold acclimation, and enhanced starvation resistance following desiccation hardening, indicative of significant cross tolerance to these environmental stressors for the traits examined (as in Scharf et al., 2015; Sinclair et al., 2013; Bublly et al., 2012, 2013). Another novel result of the present study is that, some traits of the same stress resistance differed in their responsiveness to the same conditions (e.g. heat knockdown time was less cross-resistant than CT_{max}) (see Fig. 2.1A; B). While there has been

several reports on beneficial acclimation (Leroi et al., 1994; Karl et al., 2014; Bahrndorff et al., 2016), investigations on cross resistance have been limited (Bublily et al., 2012, 2013). To our knowledge, this is the first report on cross resistance, involving a nearly exhaustive combination of environmental stressors likely to be faced by an organism in a changing climate. Such cross tolerance work has been limited to *Drosophila* (Bublily et al., 2012), with none on other insect taxa. However, for non-*Drosophilid* insects' cross-tolerance responses remain unclear regardless of its critical importance to understanding any generalities of how ectotherms respond to overlapping stressful environments. Such data are important in making robust predictions on insects faced with novel climates and upon introduction or dispersal into non-native environments.

Previous work on the relationship between high temperature and desiccation resistance has been equivocal (Sgro et al., 2010). We observed significant cross tolerance between traits of desiccation and high temperature tolerance (CT_{max}), in agreement with related studies on *Belgica Antarctica* (Benoit et al., 2009) and *D. melanogaster* (Bublily et al., 2012). This cross tolerance may be a result of Hsp70 expression, which are likely upregulated during desiccation stress (Bayley et al., 2001). Indeed, similar results have been reported in like Dipteran species, e.g. *Sarcophaga crassipalpis* (Tammariello et al., 1999; Hayward et al., 2004 but see Sinclair et al., 2007) and other distant insect taxa (Benoit et al., 2010; Mizrahi et al., 2010; Gusev et al., 2011), indicating the significant role of Hsps in desiccation related stress. Nevertheless, Sinclair et al., (2007) found no correlation between desiccation stress and Hsp expression in *D. melanogaster*. While desiccation hardening may be linked with Hsp expression, likely improving heat tolerance, desiccation hardening did not improve high temperature tolerance in *Sarcophaga crassipalpis* (Tammariello et al., 1999). This may reflect taxa related differential Hsp expression following desiccation. However, Hsp expression following desiccation has not been reported in *C. rosa* and thus warrants further investigation. Current results also showed that starvation acclimation improved CT_{max} for adult *C. rosa* (see Table 2.3). A starvation induced cross protection against heat has been recorded in bacteria (Jenkins et al., 1988; Preyer & Oliver 1993; Raheb et al., 2008). The mechanism behind this cross protection has been linked to certain proteins synthesised during starvation called starvation proteins. A subset of these starvation related proteins, known as pex proteins can be induced during heat and oxidation stress (Renyé et al., 2004). Nevertheless a negative relationship between starvation and thermal tolerance has been

observed in *D. melanogaster* (Bubliy et al., 2012), *Tribolium castaneum* (Scharf et al., 2015) and *Ceratitis* species (Nyamukondiwa et al., 2009). Failure of cold acclimation to improve heat tolerance is inconsistent with Rajamohan & Sinclair (2008) who reported the likelihood of an overlap between high and low temperature tolerance mechanisms through e.g. through Hsps. This inconsistency between these results could propably be due to the difference in insect species, the differences in duration of the cold pretreatment between the two studies or differences associated with the developmental stages tested. Nevertheless, the result is consistent with like work which documented some degree of independence between low and high temperature tolerance and mechanisms thereof (Hoffman et al., 2003). Several studies have shown that cold acclimation may impair heat tolerance in related taxa (Bauerfeind et al., 2014; Karl et al., 2014; Scharf et al., 2015), likely suggesting that the mechanisms for resistance to the stressors involved are decoupled. We also recorded that some traits of similar stress resistance differed in their responsiveness to the same conditions (e.g. HKDT time was less cross-resistant than CT_{max}). The lack of acclimation induced cross resistance for HKDT relative to CT_{max} may suggest that under overlapping stress environments, the evolution of shared physiological tolerance mechanisms may be constrained and varies across different traits.

In the present study, we also report on novel improved desiccation resistance following cold acclimation, an indication of substantial cross tolerance. To our knowledge, this is the first report indication a cross tolerance in that direction. Previous research has reported increase in cold tolerance following desiccation hardening for different insect species, an indication of probable overlap in mechanisms between the two environmental stressors (Bayley et al., 2001; Sinclair et al., 2007; Benoit et al., 2009), while Bubliy et al., (2012) showed no effect of cold acclimation on desiccation resistance in *D. melanogaster*. The reason why cold acclimation improved desiccation resistance in *C. rosa* is likely unknown. However a possible explanation to these results could be due to reduced water loss during cold acclimation. This conservation of water allowed *C. rosa* adult to be tolerant to desiccation .furthermore cold acclimation might have led to reduced developmental time, making cold acclimated flies younger and more tolerant to desiccation than older (control) flies, since desiccation resistance is said to decrease with age (Gibbs & Markow 2001)Our results also showed that desiccation hardening may likely improve survival under starvation stress, an indication of cross tolerance for the environmental stressors. This result is in tangent with like

research to date. For example, Karl et al., (2014) revealed no desiccation acclimation effect on starvation resistance of the tropical butterfly *Bicyclus anynana*. Similarly, in *D. melanogaster*, both heat and desiccation hardening had significantly negative effects on starvation resistance (Bubliy et al., 2012). Thus, although there has been no cross tolerance effects reported between desiccation pre-treatment effects for starvation resistance, the results of the present study suggest otherwise. This may suggest species related differences in cross tolerance and likely mechanisms thereof. Hence different species may need to be explored in isolation before any generalities can be concluded on cross tolerance, and how it facilitates environmental stress adaptation under climate change.

In addition to cross tolerance effects recorded above, heat hardening significantly improved high temperature tolerance, measured as CT_{max} (Table 2.3; Figure 2.1 A) in keeping with the BAH (Leroi et al., 1994; Terblanche & Kleynhans 2009; Karl et al., 2014; Bahrndorff et al., 2016), and the ‘hot is better’ hypothesis (Kingsolver & Huey 2008, Angilletta et al., 2010). Mechanisms linked to this improved CT_{max} following a mild high temperature pre-treatment may include the synthesis of heat shock proteins particularly Hsp70 (Feder et al., 1999; Hoffmann et al., 2003; Kristensen et al., 2005; Kalosaka et al., 2009). Heat shock proteins are thought to prevent protein denaturing caused by high temperatures and thus an essential component dictating fitness in insects inhabiting warm climates (Rinehart et al., 2006; Overgaard et al., 2010). The current study also found no significant acclimation effects on HKDT. Previous related research has shown a positive correlation between heat, cold, desiccation and starvation acclimation for traits of heat tolerance measured as HKDT in different insects’ taxa (Bubliy et al., 2012; Sinclair et al., 2013; Karl et al., 2014; Scharf et al., 2015). This increase in heat tolerance following acclimation may probably be a result of Hsps. The reason for lack of improvement in survival to high temperature (HKDT) following acclimation in the current study is unknown. The heat shock response may need a brief period at benign temperature to elicit a response, and lack of this recovery period thereof may have affected a response. Second, the lack of acclamatory effects for HKDT response may be taxa related. In a similar experiment, Weldon et al., (2011) found no effect of high temperature acclimation for HKDT in *C. rosa*. Nevertheless, flies acclimated optimally at benign temperatures (24.5°C) marginally improved heat tolerance (Weldon et al., 2011), indicative of optimal developmental temperature hypothesis (see Bahrndorff et al., 2016).

By contrast to traits CT_{max} where cross tolerance was recorded, only cold acclimation seemed to significantly improve cold tolerance measured as CT_{min} , a clear indication there may not be any cross tolerance for the factors tested here. Following pre-treatment at low temperatures, it is generally expected that organisms should do better at low temperatures, e.g. the BAH (see Leroi et al., 1994). The “cold is better” hypothesis also stipulates that individuals reared at lower temperatures may develop larger body size, conferring some fitness advantages in different environments (Huey & Berrigan 1996; Scharf et al., 2015, 2016a). Indeed, our results are consistent with related work on *Drosophila* (Kelty & Lee 2001; Nyamukondiwa et al., 2011) and *Ceratitis species* (Nyamukondiwa & Terblanche 2010) which confirmed the BAH. Mechanisms enhancing cold tolerance comprise carbohydrate cryoprotectants, depression of supercooling points, membrane remodelling, the inhibition of apoptosis, Hsp upregulation and ion homeostasis (Denlinger & Lee 2010). Low temperature pre-treatments enhanced Hsp expression with consequent low temperature survival improvement in *S. crassipalpis* (Rinehart et al., 2000, 2007) and *D. melanogaster* (Sejerkilde et al., 2003). Similarly, proline and trehalose accumulation has been shown to significantly improve cold tolerance in cold acclimated *D. melanogaster* larvae (Kostal et al., 2011). Although desiccation hardening seemed not to affect cold tolerance (CT_{min}), related studies documented cross tolerance between desiccation hardening and cold tolerance (Bayley et al., 2001; Sinclair et al., 2007; Benoit et al., 2009; Sinclair et al., 2013). This cross resistance may likely be explained through the synthesis of sugars and polyols in invertebrates under desiccation stress, and likely having roles in cold tolerance (Bayley et al., 2001; Denlinger & Lee 2010). In a related study, trehalose levels seemed to significantly increase during dehydration (Goyal et al., 2005; Benoit et al., 2009), likely explaining that these sugars, besides regulating low temperature tolerance, may also play a significant role in desiccation resistance. Heat hardening and starvation acclimation did not have any effect on cold tolerance (CT_{min}), in keeping with findings by Scharf et al., (2016a). However, there has been mixed results regarding the effects of starvation acclimation on cold tolerance. While our results show no effects (as in Scharf et al., 2016a), some related studies have indicated significant negative (Nyamukondiwa et al., 2009; Karl et al., 2014; Scharf et al., 2016b) and positive (Holmstrup et al., 2002) effects of starvation acclimation on cold tolerance. Similar to HKDT results, we recorded no pre-treatment cold tolerance (CCRT). In related studies, Weldon et al., (2011) showed a significant low temperature acclamatory effect on *C. rosa* CCRT while Bublly et al., (2012) showed cold acclimation improves CCRT in a closely related Dipteran *D.*

melanogaster. In some distant taxa, cold pre-treatment also improved CCRT in migratory locusts (Findsen et al., 2013) and crickets (Alvarado et al., 2015). The mechanism behind chill coma onset has been linked to the depolarization of cell resting potentials, leading to loss of neuromuscular excitability. As such, chill coma may lead to an increased K^+ concentration in the hemolymph due to the loss of an organism's ion and water balance. Hence for any organism to recover from chill coma, it requires reestablishment of ion homeostasis (MacMillan & Sinclair 2011; MacMillan et al., 2012, 2016). Recovery of hemolymph K^+ was faster in rapid cold hardened locusts, which were also had significantly higher cold tolerance than non-hardened ones (Findsen et al., 2013; Alvarado et al., 2015). Lack of improvement of CCRT following acclimation may be as a result of *C. rosa* failure in establishment of ion homeostasis following chill coma. Furthermore, lack of cross tolerance reported here may suggest that CCRT may likely be genetically independent from other measures of stress tolerance tested here (see discussions in Gerken et al., 2016).

Desiccation hardening significantly improved *C. rosa* desiccation resistance, and indication of beneficial acclimation and in keeping with previous results for *D. melanogaster* (Bazinet et al., 2010; Bublly et al., 2012; Stinziano et al., 2015) and springtails *Folsomia candida* (Sjursen et al., 2001). Similarly, flies selected for desiccation resistance showed significantly higher tolerance for desiccation compared to non-selected controls (Folk et al., 2001; Gibbs et al., 1997). Insects use various mechanisms to improve their desiccation resistance including a general increase body water content, increase in metabolic water, decreasing water loss rates (Bazinet et al., 2010; Parkash et al., 2014; Baurfeind et al., 2014) and increasing the amount of water able to be lost before death (Bazinet et al., 2010). *Drosophila melanogaster* selected for enhanced desiccation resistance have been found to contain about 34% more water than control flies (Folk et al., 2001). In a related study, desiccation selected flies had significantly higher carbohydrates, especially glycogen than control flies (Gibbs et al., 1997), which may serve as metabolic water during periods of desiccation stress. Our results also showed negative effects of starvation acclimation on desiccation resistance, contrary to a cross protection effect reported in *D. melanogaster* for the two environmental stressors (Bublly et al., 2012). The lack of desiccation resistance following starvation acclimation may be low energy reserves in the starved flies. Energy reserves may have been depleted during the acclimation hence compromising fitness, as has been shown in *Ceratitis* species (Nyamukondiwa & Terblanche 2009). This may also indicate that desiccation resistance may

be an energy consuming process that requires a significantly high energy investment. Heat hardening showed no significant effects on adult *C. rosa* desiccation resistance, in agreement with Bublik et al., (2012) studies on *D. melanogaster*. Nevertheless, some positive cross tolerance effects between traits of desiccation and heat resistance have been reported in the same species (Hoffmann 1990; Bublik & Loeschcke 2005). This may be because, in nature periods of desiccation stress often coincide with periods of heat stress, and as such, the mechanisms behind protection to both stressors may have coevolved. Starvation acclimation generally improved starvation resistance in female *C. rosa*, in keeping with the BAH. Moreover, females generally had significantly higher starvation resistance than the males. Enhanced starvation resistance may likely be linked with greater lipid reserves (Chahal et al., 2013), change in carbohydrates and lipid metabolism (Wang et al., 2016), and reduction in reproduction (Rion & Kawecki 2007; Hoffmann & Harshman 1999). Indeed, several studies have shown a positive correlation between starvation resistance and body lipid content (Ballard et al., 2008; Dussutour et al., 2016; Holmstrup et al., 2016), signifying the role of lipids in tolerance to environmental desiccation. In our experiment, we used virgin flies, which may have likely accumulated body lipid reserves, as a reproductive investment (see Golebiowski et al., 2016). During reproduction, many organisms use these lipid reserves, hence reduced reproduction can lead to increase in lipid storage (Corona et al., 2009; Judd et al., 2011), as may be the case for the current study. While there was no significant cross tolerance effect between heat and cold acclimation for starvation resistance, we recorded an improved starvation resistance following desiccation pre-treatment in males. This is consistent with previous studies to date showing no cold pre-treatment effects on starvation resistance in *D. melanogaster* (Gerken et al., 2016). However, contrastingly positive cross tolerance effects between cold acclimation and starvation resistance have been recorded in *D. melanogaster* (Bublik et al., (2012) and red flour beetle (Scharf et al., 2015). Improved starvation resistance following cold acclimation may be attributed to reduced activity and metabolic rate at lower relative to higher temperatures (De Vries & Appel 2013), increased storage reserves e.g. body lipids (Ballard et al., 2008; Dean et al., 2016), which are made available during starvation stress.

2.5. Conclusion

The current study reports cross tolerance in *C. rosa*, the first report investigating *Ceratitis* species. Generally in all cases, acclimation of organisms to a particular stress improved

survival to that particular stress as compared to flies which did not have a chance to be acclimated, in support the BAH. Moreover, we report on novel (1) improvement in CT_{max} following desiccation and starvation acclimation; (2) enhanced desiccation resistance following cold acclimation and (3) improved starvation resistance following desiccation acclimation in males indicating substantial cross tolerance to these environmental stressors. To our knowledge, this is the first report, of cross tolerance to environmental stressors, in a Tephritid fruit fly, *C. rosa*, and one that is nearly exhaustive of all environmental stressors likely to be faced by insects under global change. Our study supports the hypothesis that species may respond differently when faced with multiple stress environments, and thus cross tolerance in different insect taxa should be separately investigated before making robust predictions on cross tolerance and its effects on insect population dynamics. Overall, results of the current study has two critical implications that are of broad significance: 1) that a set of common underlying physiological mechanisms may exist between stress responses in *C. rosa*, and 2) that these traits likely co-evolved to cope with diverse multiple stressors. Such mechanisms may be particularly advantageous in the face of novel climates and upon introduction or dispersal into non-native climates.

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CHAPTER 3

Physiological mechanisms underlying cross tolerance to multiple environmental stressors in *Ceratitis rosa* Karsch (Diptera: Tephritidae)

3.1. Introduction

Climate change has brought with it increased average global temperatures (Weldon et al., 2016), increased temperature variability (William et al., 2015), changes in rainfall patterns and extreme climatic events such as the frequent occurrences of cold snaps, heat waves and severe cases of drought (Trenberth 2011) and wildfires (IPCC 2014). This suggests climate change, is often associated with multiple abiotic stressors. The ability of species to survive the existence of multiple environmental stressors is therefore of major interest in biology (Weldon et al., 2016), since species are expected to tolerate both environmental extremes simultaneously using single mechanisms for each different stress factor or show crosstolerance effects for these stressors. Cross tolerance is a phenomenon in which acclimation to one stress can confer resistance to other stresses (Bubliy et al., 2012, 2013; Sinclair et al., 2013). This has been investigated in a number of insect species *vis a vis* *D. melanogaster* (Bubliy et al., 2012; Parkash et al., 2014; Bauerfeind, et al., 2014), *D. simulans* (Bubliy et al., 2013), *Folsomia candida* (Bayley et al., 2001; Holmstrup et al., 2002), *Belgica Antarctica* (Hayward et al., 2007; (Elintsky et al., 2009; Benoit et al., 2009), *Cydia pomonella* (Chidawanyika & Terblanche 2011), *Thaumatotibia leucotreta* (Boardman et al., 2015), *Tribolium castaneum* (Scharf et al., 2015), goldenrod gall fly, *Eurosta solidaginis* (Williams & Lee 2011; Levis et al., 2012) and *Zaprionus indianus* (Kalra et al., 2017) and *C. rosa* (see Chapter 2). Currently no research has been exhaustive on all environmental stresses likely to be faced by an organism in nature.

Evidence of positive cross tolerance has been reported elsewhere, and includes increased heat resistance following desiccation acclimation, and increased desiccation resistance following starvation acclimation (Bubliy et al., 2012). In another study shorter photoperiods decreased cold tolerance, whereas longer photoperiods enhanced desiccation resistance in *D. melanogaster* (Bauerfeind et al., 2014). Furthermore, cross tolerance effects have been recorded between cold acclimation and starvation resistance in *D. melanogaster* (Bubliy et al., 2012) and red flour beetle (Scharf et al., 2015). Increased desiccation resistance after heat hardening, improved heat tolerance after starvation acclimation and desiccation hardening were also reported in *Z. indianus* (Kalra et al., 2017). Although there is quite some evidence pertaining to the existence of cross tolerance, mechanisms underlying cross tolerance are limited. To date extensive research has been dedicated to understanding physiological mechanisms underlying individual environmental stressors. Physiological mechanisms

underlying cold tolerance include cryoprotectants (Storey 2004; Lencioni 2004) antifreezes (Sheikh et al., 2017), aquaporins (Chowanski et al., 2015) and heat shock proteins (Kelty & Lee 2001). Similar, heat resistance has been associated with the synthesis of heat shock proteins (Kalosaka et al., 2009). Starvation resistance has been linked with upregulation of Hsps and accumulation of lipid reserves (Ballard et al., 2008; Arrese & Soulages 2010). Similarly, desiccation resistance mechanisms comprise reduced water loss (Weldon et al., 2016), water absorption (Bazinet et al., 2010; Aggarwal et al., 2013) and tolerance to increased dehydration (anhydrobiosis) (Gibbs et al., 2003; Watanabe 2006; Mitsumasu et al., 2010). However mechanisms underlying resistance to multiple environmental stressors in *C. rosa* remain limited (though reported in Chapter 2), except for dehydration tolerance (see Weldon et al., 2016).

This research was therefore aimed at investigating mechanisms underlying cross resistance to environmental stressors in *C. rosa* (following cross tolerance reports in chapter 2). Physiological mechanisms underlying cross-resistance in *C. rosa* were determined by measuring water balance traits, body lipid and carbohydrates composition following acclimation treatments to several major different environmental stressors such as cold, heat, starvation and desiccation. Overlaps in any of these physiological mechanisms, will therefore be used to explain the observed cross tolerance in *C. rosa* (Chapter 2). Similar overlapping physiological mechanisms has been reported in other studies, for example the accumulation of body lipids has been linked to other environmental stressors other than starvation such as heat hardening (Kalra et al., 2017) and cold acclimation (Moghadam et al., 2011; Chowanski et al., 2015). Similarly heat shock proteins have also been implicated in other stressors such as cold (Rinehart et al., 2007; Chowanski et al., 2015) and desiccation (Tammarielo et al., 1999; Hayward et al., 2004). Water balance characteristics are not only restricted to surviving desiccation stress, but also to adapting to other environmental stressors such as high temperatures (Kleynhans & Terblanche 2011) and low temperatures (Hayward et al., 2007; Elnitsky et al., 2009; Holmstrup et al., 2010; Alvarado et al., 2015). It is therefore important to experimentally determine these in *C. rosa* before making any generalization about cross tolerance mechanisms in insects. This is because mechanisms may vary within and across insect taxa and in space and time. The results of this study may help explain potential mechanisms likely aiding the ability of *C. rosa* to survive multiple stress environments, its invasion potential and ability to thrive upon introduction to a novel multiple stress

environments. The objective of this chapter was to identify cross tolerance mechanisms associated with chapter 2.

3.2. Materials and Methods

3.2.1. Fly rearing and maintenance

Colony of *C. rosa* flies were obtained as live pupae from Citrus Research International, Nelspruit, South Africa. The insect culture, which has been reared in the laboratory for ~300 generations is mass-bred and maintained in high numbers, with regular supplementation with wild flies to minimise inbreeding depression and genetic drift. Upon arrival at Botswana International University of Science and Technology pupae were distributed evenly into several petri dishes and placed in a Bugdorm rearing cage (32.5 x 32.5 32.5 cm) (Bug Dorm™, MegaView Science Co., Ltd, Taichung, Taiwan). The flies were provided with dry sugar, water-soaked cotton wool and yeast powder (Biolab, Merck, Wadeville, Gauteng, South Africa). The cage was then placed in an incubator (HPP 260, Memmert, GmbH + Co. KG, Germany) set to 25 °C, 75±5%RH with a 12:12 h photoperiod until adult eclosion. Within 48h following eclosion, adult *C. rosa* flies were separated according to sex into 5L plastic containers furnished with sugar, water and yeast. Each 5L container contained about 150 adult flies of the same sex. A petri dish containing saturated NaCl solution and covered by insect screen mesh was inserted into each of the 5L plastic container to maintain relative humidity of 75 ±5 % at to 25 °C (Weldon et al., 2011) before sealing the container with a lid.

3.2.2. Acclimation treatments

3.2.2.1. Heat Hardening (HH)

At 7 days after adult eclosion, virgin female and male *C. rosa* adults were separately placed into 20mm X 90mm glass vials with plastic lids in groups of 10 flies of the same sex. The glass tubes we then put into a zip lock bag which was then immersed in a water bath (Huber CC410 WL, Offenburg, Germany) containing 1:1 water: propylene glycol set at 36 °C for 1 h. Pre-treatment of flies at 36 °C (1h) has been shown to significantly improve survival during an otherwise lethal 2h exposure at 41°C in both *C. rosa* and *C. capitata* (Nyamukondiwa et al., 2010). Since the heat shock response require the de novo synthesis of heat shock proteins, a recovery period at mild temperature may be required to elicit responses (reviewed in Chown & Nicolson, 2004; Lee & Denlinger 2010). Therefore a 30 minutes recovery period under standard benign conditions in a constant environmental chamber 25

°C, 75% RH was therefore given to elicit the heat shock response. Control flies were left in their rearing containers at optimal environmental conditions (25°C; 75% RH).

3.2.2.2. *Cold Acclimation (CA)*

As in HH assays virgin female and male adult flies were placed separately in 5L plastic containers 2 days post eclosion. Each 5L plastic container had access to food (sugar and yeast) and water plus a vial filled with saturated salt solution and covered by insect mesh to maintain the relative humidity at ~75%. The containers were then placed in an incubator set at 20 °C for 5 days. This temperature time duration is suffice to elicit RCH responses in *Ceratitis* species (see Nyamukondiwa et al., 2010; Weldon et al., 2011). Control flies were kept in their rearing cages at optimal conditions.

3.2.2.3. *Desiccation Hardening (DH)*

At 7 days after adult eclosion, virgin females and male *C. rosa* adults were individually placed in ventilated 0.65ml micro-centrifuge tubes. About 6 tubes were then placed in a sponge rack and put in a 250ml plastic container containing about 50g of silica gel at the bottom and sealed with a lid (Gibbs et al., 1997; Terblanche & Kleynhans 2009). The mini desiccators were then placed in an incubator set at 25 °C for a period of 15h. After 15 hour of desiccation stress, flies of each sex were removed from the desiccators and given a recovery period of 6 h with food and water at 25 °C; 75% RH. Duration of desiccation hardening/stress (15h) was deduced following preliminary assays. Desiccation hardening time was defined as that duration that caused mortality in 50% of the organisms during trial experiments.

3.2.2.4. *Starvation Acclimation (SA)*

At 5 days following emergence, virgin female and male *C. rosa* flies were placed separately in 5L plastic containers which were then placed in an incubator set at 25°C (48 h) with no food. However, a wet cotton wool was placed inside each container to prevent mortality associated with desiccation (Parkash et al., 2014). A dish filled with saturated NaCl solution and covered by insect mesh to maintain the relative humidity at 75% was also placed in each of the containers. After 2 days of hardening the flies were given a 12h recovery period (provided with water and food, at 25 °C; 75%RH). At the same time, control flies were kept in their rearing cages at benign conditions. In all acclimation pre-treatments, the actual temperature and RH experienced by the flies during acclimation in the incubators was

recorded at hourly interval using thermocron temperature and humidity loggers attached to the inside of the sealed container containing the flies (DS1923 ibutton, Maxim Integrated Products, Sunnyvale CA, USA).

3.2.3. Environmental stress resistance assays

3.2.3.1. *Body water content (BWC)*

Body water content (BWC) of adult *C. rosa* flies was determined gravimetrically following methods by Gibbs & Marzkin (2001). Treatment and control *C. rosa* flies of each sex were weighed in pre-weighed 0.60ml Eppendorf tubes using a RADWAG microbalance (model AS 220.R2, Poland) to obtain wet body mass. Thereafter the flies were dried in an oven (UF160, Memmert, Germany) at 60 °C for 24 h to obtain the dry mass. BWC was thereafter calculated as the difference between wet mass and dry mass (Gibbs & Marzkin 2001).

3.2.3.2. *Water Loss Rates (WLR)*

WLR under desiccating conditions was determined gravimetrically for control and treatment organisms following method by Terblanche & Kleynhans (2009). Immediately after acclimation, *C. rosa* treatment and control flies were individually weighed in pre-weighed perforated 0.60ml ventilated Eppendorf tube. The tubes were individually placed in a plastic vial containing 10g of silica gel (Glassworld, South Africa). Thereafter the vials were placed in climate chamber (25 °C, 25 % RH, 12:12 h photoperiod) for 18h. WLR (expressed as g H₂O/h) was calculated as the difference in body mass divided by the duration of the desiccation period (Terblanche & Kleynhans 2009).

3.2.3.3. *Body Lipids Content (BLC)*

BLC of acclimated and control flies were determined following method by Parkash et al., (2014). Individual adult male and female acclimated and control *C. rosa* adult flies were weighed in pre-weighed 2ml Eppendorf tubes, then dried in an oven (UF160, Memmert, Germany) at 60 °C for 48 h. Immediately after drying the flies were weighed on a RADWAG microbalance (model AS 220.R2, Poland; with precision 0.001mg). Thereafter, 1.5ml of diethyl ether was added to each tube, and the tubes gently agitated at 250 rpm for 24h at 37 °C using orbital shaker. The diethyl ether was then removed from the tubes, and the flies dried again at 60 °C for 24h, then reweighed. The lipid content of each fly was calculated by subtracting the lipid free dry mass from the initial dry mass (Ballard et al., 2008).

3.2.3.4. Glucose assay

3.2.3.4.1. Preparation of reagents

Phosphate buffered saline

Phosphate buffered saline (PBS) was prepared by dissolving 5 phosphate buffered saline tablets (79382, Sigma-Aldrich, St. Louis, MO, USA) in 1000ml of distilled water, followed by autoclaving.

Glucose oxidase reagents

The glucose oxidase (GO) reagent was prepared following the manufacturer instructions (Sigma-Aldrich, St. Louis, MO, USA) by dissolving the contents of the capsules in 39.2mL of deionised water in an amber bottle. Next o-dianisidine reagent was then reconstituted with 1.0mL deionised water in an amber bottle (protects contents from light), and the vial inverted several times to dissolve the contents. Finally 0.8mL of o-Dianisidine reagent was added to the amber bottle containing 39.2mL of glucose oxidase reagent to make 40mL of glucose assay reagent. The assay reagent was then stored at 4°C until use.

Glucose standards

Glucose standards were prepared by diluting different amounts of glucose standard (Dglucose, 1.0mg/ml in 0.1% benzoic acid, Sigma) with cold Phosphate Buffered Saline (BPS) (Tennessee et al., 2014) up to a final volume of 1000µl of each standard. To prepare the standards, 160 µl of 1mg/ml glucose was diluted with 840 µl PBS (1000µl final volume) for 0.16 mg/ml standard. Then five 2-folds serial dilutions into PBS were prepared (i.e. 500µl of 0.16mg/ml + 500µl PBS for 0.08 mg/ml standard and so on).

Sample preparation and homogenisation

Free glucose was determined from whole insect homogenate following methods by Tennessee et al., (2014). Following respective acclamatory conditions plus control, flies were rinsed with 1ml cold PBS to remove any traces of food which might be stuck on the insect body (Morris et al., 2012). Thereafter acclimated flies in groups of 5 (Marshall & Sinclair, 2010 ; Morris et al., 2012) were transferred to 1.5mL microcentrifuge tubes, snap frozen in liquid nitrogen and stored at -80°C.

Frozen samples were removed from freezer and placed on ice until PBS treatment. Sample ice treatment prevents the endogenous enzymatic degradation of glycogen and trehalose into free glucose, which may have confounding effects of the results. Using a pestle motor and 1.5ml disposable pellet pestles, samples were thereafter homogenised, and the supernatant heated for 10 minutes at 70°C, followed by centrifugation for 3 minutes at maximum speed in a refrigerated table top centrifuge that has been pre-chilled to 4°C (Tennesen et al., 2014; Morries et al., 2012). The resulting supernatant was transferred to new pre-chilled 1.5ml microfuge tubes, and stored at -80 °C until analysis (Tennesen et al., 2014).

3.2.3.4.3. *Glucose determination*

30µl of each glucose standard solution each glucose standard solution measuring 30µl , 30µl of PBS (Blank) and 30µl of each sample (all in triplicate) were added into individual wells in a 96 well plate, followed by the addition of 100µl of the Glucose oxidase reagent (Tennesen et al., 2014). The plate was sealed with parafilm to prevent evaporation, incubated for 30 minutes at 37°C, after which the reaction was terminated by adding 100µl of 12N sulphuric acid. The 96 well plate was then put in a plate reader used to measure absorbance at 540nm. Glucose concentration in samples was determined from the glucose standard curve (as in Bazinet et al., 2010).

3.2.4. *Statistical analysis*

Analysis of variance (ANOVA) was used to compare body water, water loss rates, body lipid and glucose content of treatment and control adult *C. rosa* flies using STATISTICA version 13.2 (Statsoft Inc.). Tukey`s HSD was used for all *post hoc* comparisons.

3.3. Results

3.3.1. *Body water content of acclimated adult C. rosa*

There was a statistical significance ($p < 0.001$) on the effects of acclimation on body water content of adult *C. rosa* (Table 3.1). Our results show that at the end of each acclimation treatment, desiccated and starvation acclimated *C. rosa* flies had more body water content, while heat and cold acclimated flies had less but not significantly different body water content of the control (Fig. 3.1.A). However the interaction between treatment and sex was not statistically significant ($p > 0.05$) (Table 3.1, Fig. 3.2.A). Thus there was no statistical significance on the body water content between males and females of the same acclimation

treatment. The effects of sex on body water content was also statistically significant ($p < 0.01$), with females generally having more body water content than males (Table 3.1, Fig. 3.3.A).

3.3.2. *Body water loss rate of acclimated adult C. rosa*

There was a statistical significance ($p < 0.0001$) on the effects of acclimation on body water loss rates of adult *C. rosa* (Table 3.2). Desiccated and heat hardened flies exhibited lower water loss rates (measured in mg/h) than non-acclimated flies (Fig 3.1.B) though not significantly different. Water loss rates were however significantly higher in cold and starvation acclimated flies (Fig. 3.1.B). However the interaction between treatment and sex was not statistically significant ($p > 0.05$) (Table 3.2; Fig. 3.2.B). Thus there was no statistical significance on water loss rate between males and females of the same acclimation treatment. There effects of sex on water loss rate on the other hand was also statistically significant ($p < 0.01$) (Table 3.2, Fig. 3.3.B), with females having lower water loss rates than males.

3.3.3. *Body lipid content of acclimated adult C. rosa*

There was a statistical significance ($p < 0.0001$) on the effects of acclimation on the body lipid content of adult *C. rosa* (Table 3.3). Heat hardened flies had a significantly higher lipid content than non-acclimated flies. Similarly, starvation acclimated flies also generally had a higher but not significant body lipid content (Fig 3.1.C). Cold and desiccation acclimated flies on the other hand had generally lower body lipid content, but these did not significantly differ from the control (Fig 3.1.C). However the interaction between treatment and sex was not statistically significant ($p > 0.05$) (Table 3.3; Fig. 3.2.C), indicating the body lipid content of females and males from the same acclimation treatment did not significantly differ from each other. There effects of sex on body lipid content was also not statistically significant ($p > 0.05$) (Table 3.3, Fig 3.3.C).

3.3.4. *Glucose content of acclimated adult C. rosa*

There was a statistical significance ($p < 0.0001$) on the effects of acclimation on glucose content of adult *C. rosa* (Table 3.4). Heat, cold and desiccation hardened flies did not significantly differ from the control in their glucose content (Fig 3.1.D), indicating the acclimation treatments did not improve the amount of glucose content. However, starvation acclimated flies had significantly lower glucose content than all other treatments (Fig 3.1.D)

indicating a negative effect of starvation acclimation on glucose content. However the interaction between treatment and sex was not statistically significant ($p > 0.05$) (Table 3.4; Fig 3.2.D), indicating no statistically significant difference on the glucose content between males and females of the same acclimation treatment. The effects of sex on glucose content however was also statistically significant ($p < 0.01$) (Table 3.4, Fig. 3.3.D), with females having less glucose content than males.

Table 3.1: Summary table of the full factorial ANOVA on the effects of treatment, sex and treatment x sex on body water content of acclimated and control adult *C. rosa*. SS = sums of squares, DF =degrees of freedom.

Effect	SS	DF	MS	F	<i>P-value</i>
Intercept	8165.850	1	8165.850	10805.83	0.000000
	17.565	4	4.391	5.81	0.000279
Treatment	5.334	1	5.334	7.06	0.009062
Sex	6.795	4	1.699	2.25	0.068480
Treatment *Sex Error	83.126	110	0.756		

Table 3.2: Summary table of the full factorial ANOVA on the effects of treatment, sex and treatment x sex on water loss rates of acclimated and control adult *C. rosa*. SS = sums of squares, DF =degrees of freedom.

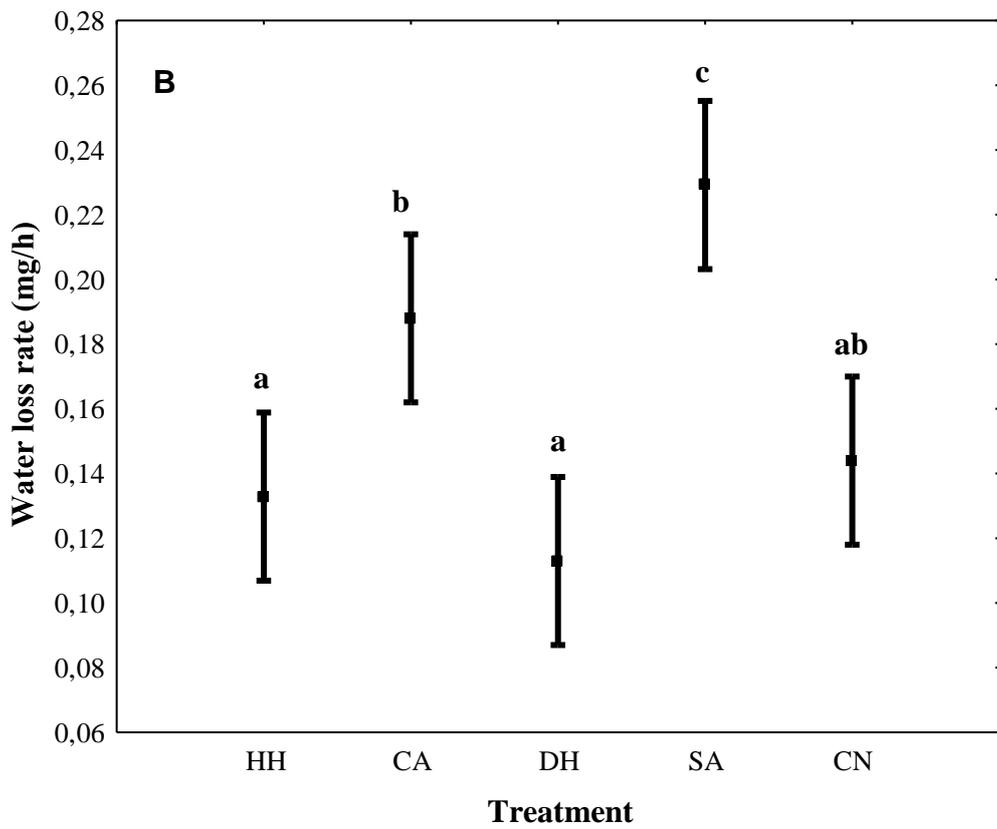
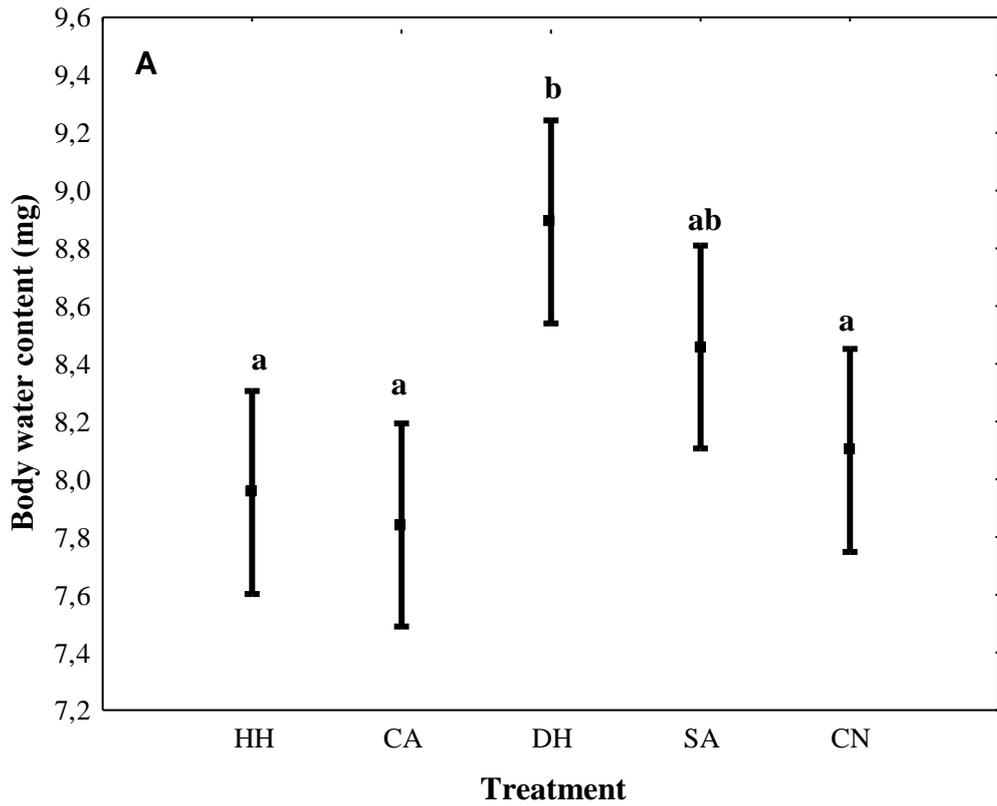
Effect	SS	DF	MS	F	<i>P-value</i>
Intercept	1.562782	1	1.562782	777.7505	0.000000
	0.105137	4	0.026284	13.0809	0.000000
Treatment	0.014346	1	0.014346	7.1397	0.010151
Sex	0.005137	4	0.001284	0.6391	0.637058
Treatment *Sex Error	0.100468	50	0.002009		

Table 3.3: Summary table of the full factorial ANOVA on the effects of treatment, sex and treatment x sex on body lipid content of acclimated and control adult *C. rosa*. SS = sums of squares, DF =degrees of freedom.

Effect	SS	DF	MS	F	<i>P-value</i>
Intercept	84,04834	1	84,04834	1539,769	0,000000
	1,80151	4	0,45038	8,251	0,000036
Treatment	0,02677	1	0,02677	0,490	0,487039
Sex	0,07984	4	0,01996	0,366	0,831888
Treatment *Sex					
Error	2,67467	49	0,05459		

Table 3.4: Summary table of the full factorial ANOVA on the effects of treatment, sex and treatment x sex on glucose content of acclimated and control adult *C. rosa*. SS = sums of squares, DF =degrees of freedom.

Effect	SS	DF	MS	F	<i>P-value</i>
Intercept	0,149549	1	0,149549	2843,122	0,000000
	0,010227	4	0,002557	48,608	0,000002
Treatment	0,000273	1	0,000273	5,196	0,045831
sex	0,000116	4	0,000029	0,551	0,702868
Treatment *sex					
Error	0,000526	10	0,000053		



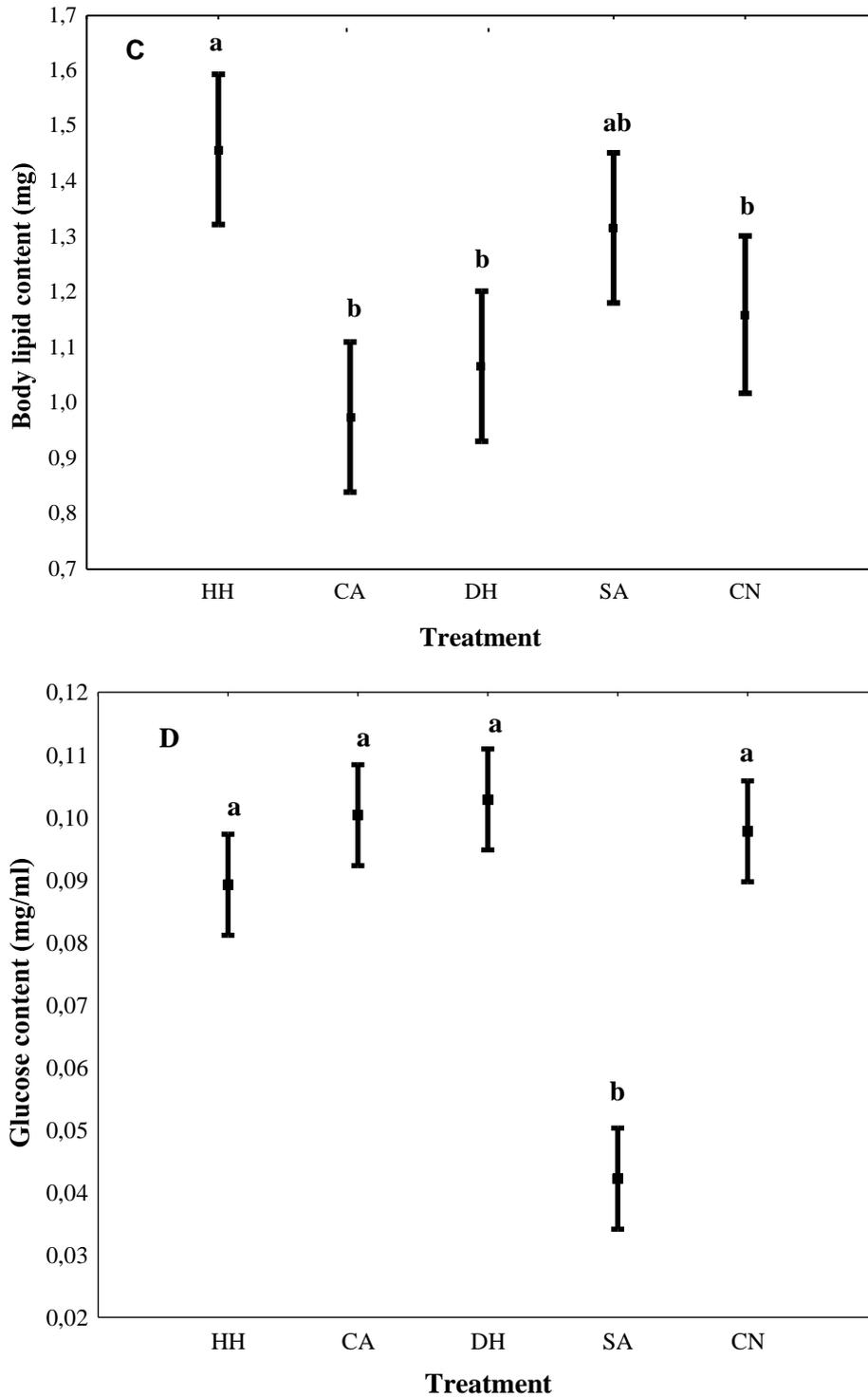
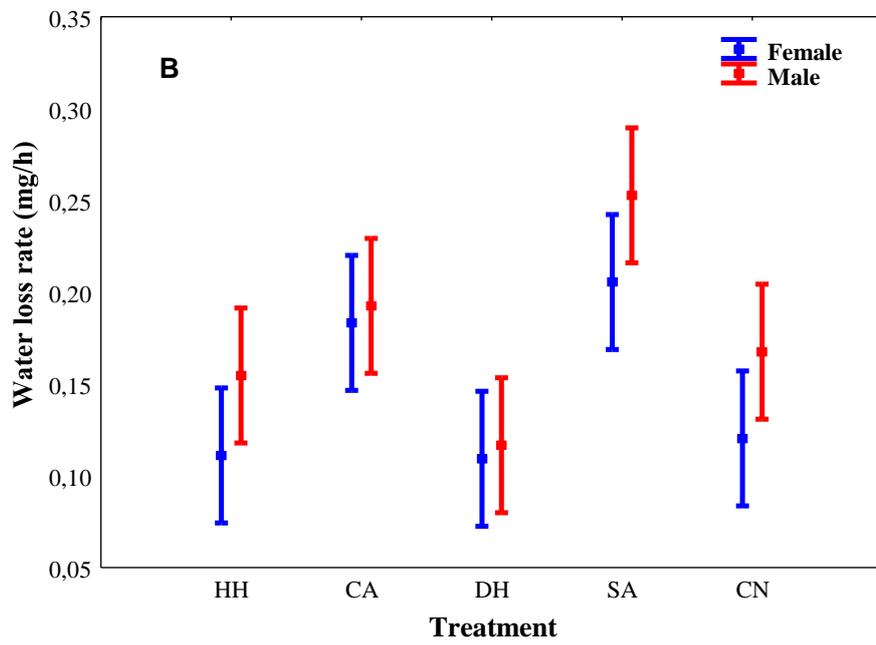
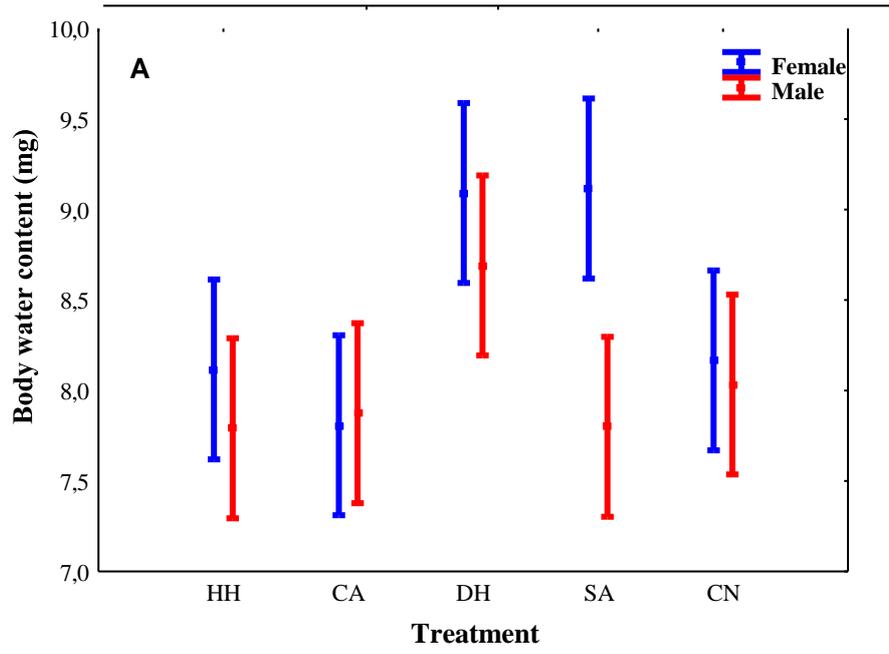


Figure 3.1: Summary effects of the effects of acclimation (HH, CA, DH, SA vs control) on (A) body water content (B) water loss rate (C) body lipid content (D) Glucose content of adult *C. rosa*. HH= heat hardening, CA= cold acclimation, DH= desiccation hardening, SA= starvation acclimation and CN= control. Errors bars represent 95% CLs (N = 50 per group). Means with the same letter are not significantly different from each other.



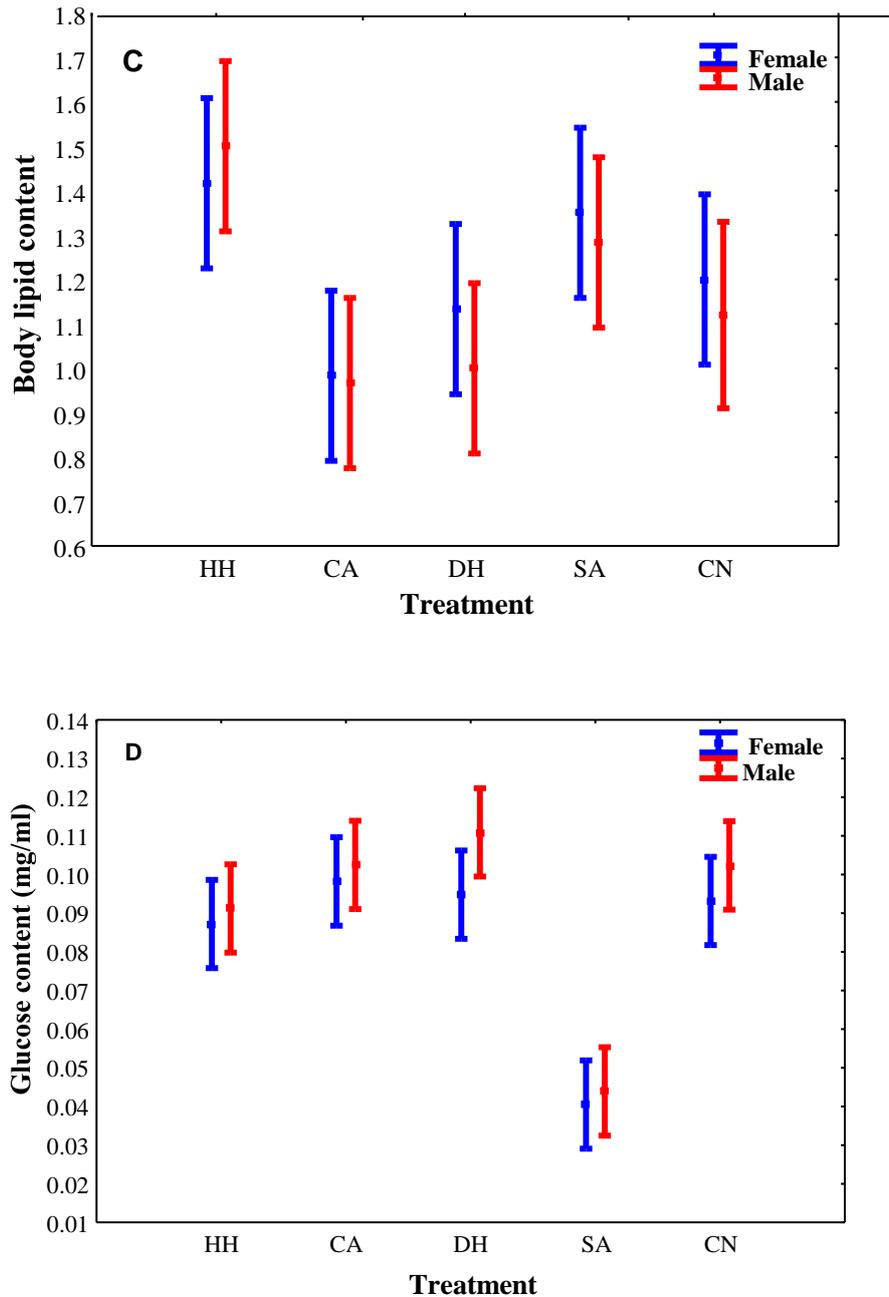
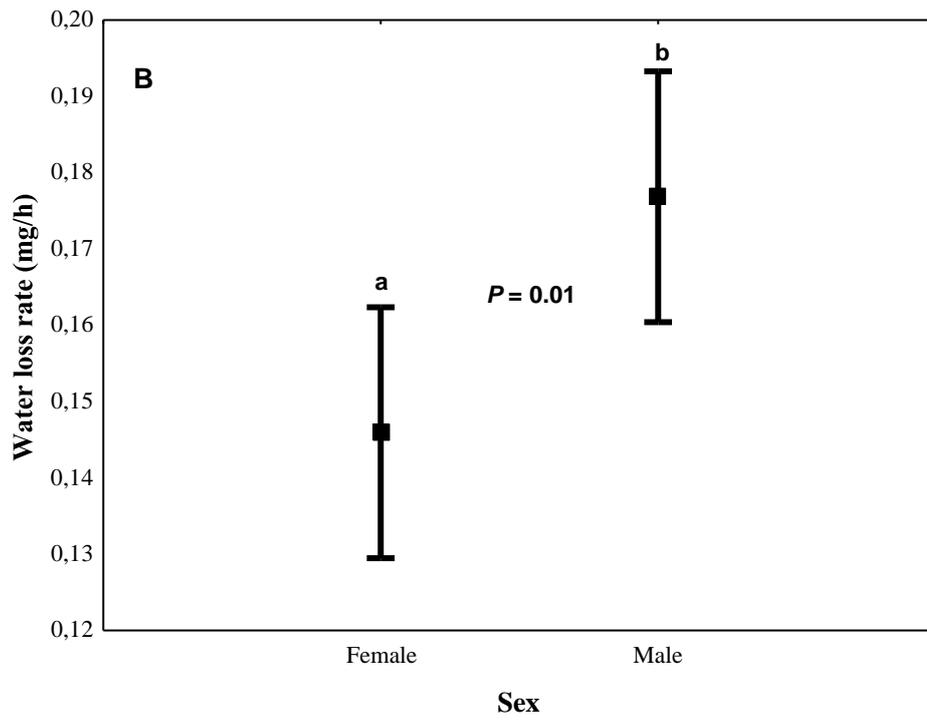
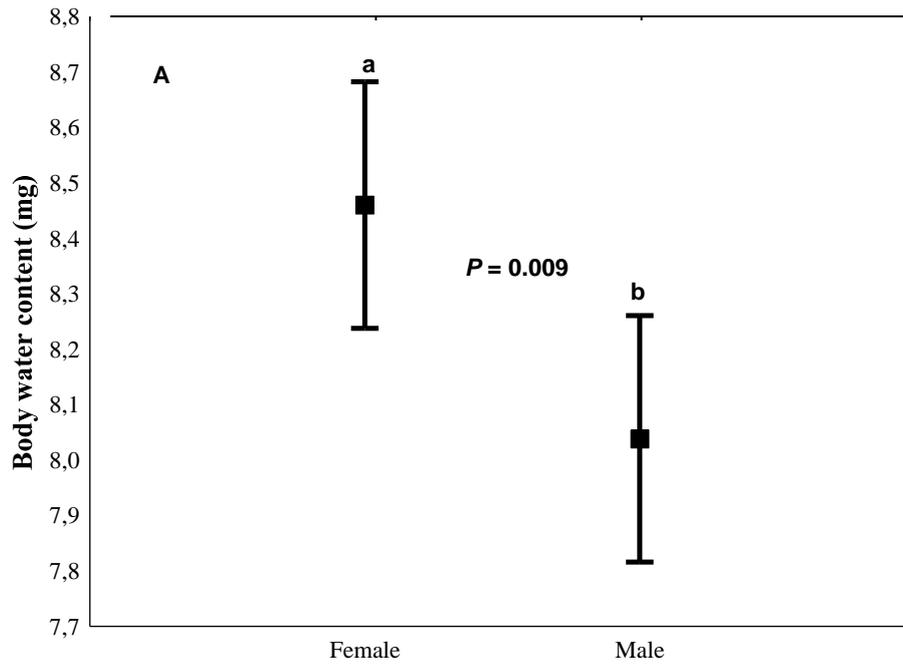


Figure 3.2: Summary interaction between treatment x sex following acclimation (HH, CA, DH, SA vs control) on (A) body water content (B) water loss rate (C) body lipid content (D) Glucose content of adult *C. rosa*. HH= heat hardening, CA= cold acclimation, DH= desiccation hardening, SA= starvation acclimation and CN= control. Errors bars represent 95% CLs (N = 50 per group). Means with the same letter are not significantly different from each other.



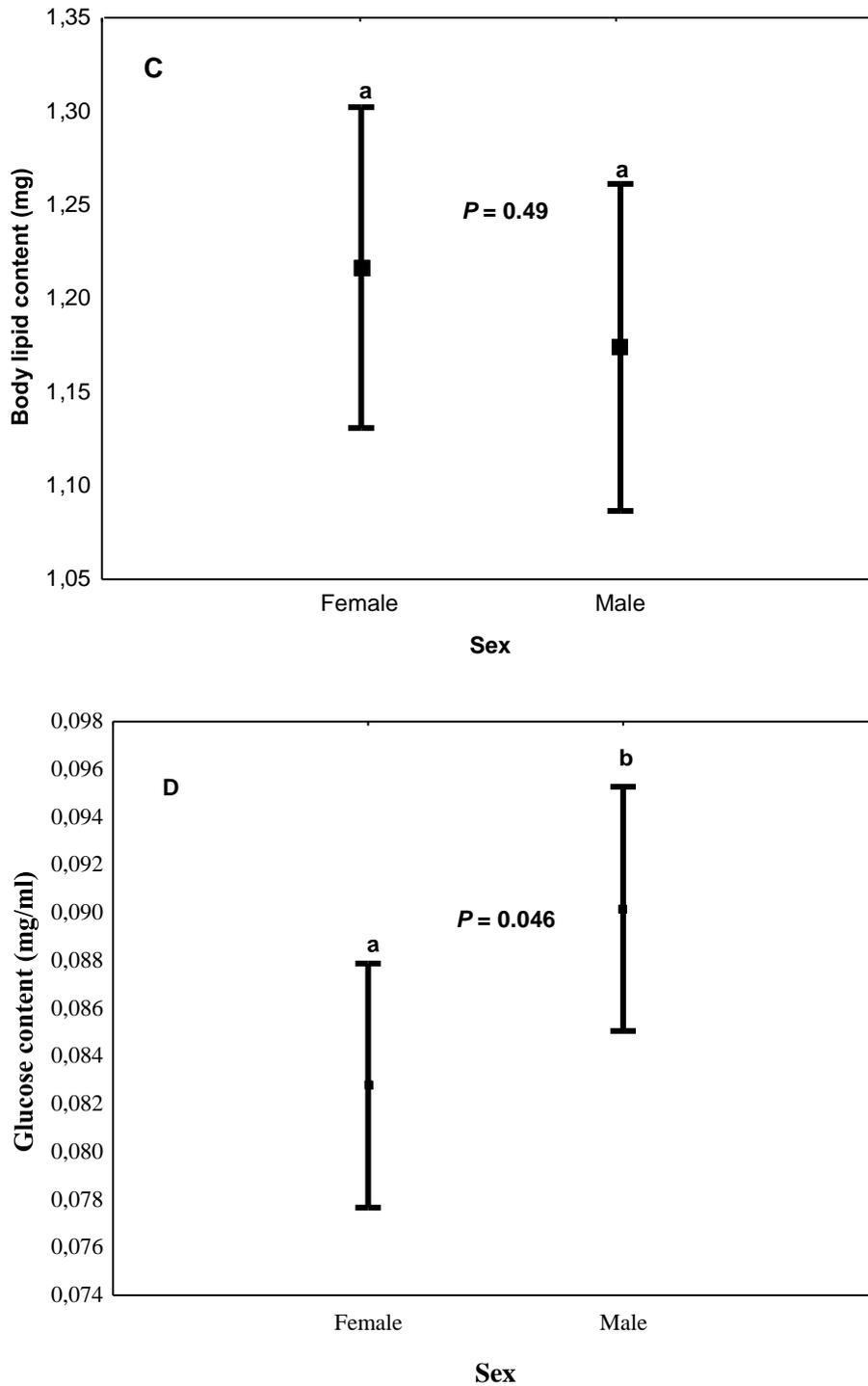


Figure 3.3: Summary effects of the effects of sex on (A) body water content (B) water loss rate (C) body lipid content (D) Glucose content of adult *C. rosa*. Errors bars represent 95% CLs (N = 50 per group). Means with the same letter are not significantly different from each other.

3.4. Discussion

Experimental evidence here found that acclimation to one stress (heat, cold, desiccation and starvation) typically increased resistance for that same stress later in life, a confirmation of the beneficial acclimation hypothesis. Furthermore we also found four cases of cross tolerance; increased heat tolerance following desiccation, improved heat tolerance following starvation hardening; improved desiccation tolerance following cold acclimation, and enhanced starvation tolerance following desiccation hardening, signifying significance cross tolerance to these environmental stress resistance traits. Nevertheless, mechanisms underlying such cross tolerance remains not elucidated.

3.4.1. Cross tolerance effects of desiccation hardening on heat tolerance

Desiccation hardening improved heat tolerance measured as critical thermal maxima (CT_{max}), as observed by (Bubliy & Loeschcke (2005) and Bubliy et al., (2012) (Chapter 2). This suggest that the two environmental stressors (heat and desiccation) may share similar mechanisms, especially since during summer, where higher ambient temperatures often leads to the reduction in environmental relative humidity (Kalra et al., 2017). Our result indicate that both heat hardening and desiccation hardened flies generally shared lower body water loss rates and reduced glucose content, but in both cases these results were not necessarily significantly different from non-acclimated *C. rosa* flies. Reduced water loss has also been observed in various insects following both desiccation hardening (Benoit et al., 2009) and heat hardening (Terblanche & Chown 2006). However heat hardening did not result in body water loss while desiccation hardened flies had significantly lower body water content than control flies. Several studies have assessed water balance characteristic in relation to desiccation tolerance (Gibbs et al., 1997; Parkash et al., 2008; Benoit et al., 2009; Weldon et al., 2016). However, to my knowledge, no study has assessed its relation to heat tolerance; as such further investigations are needed to determine whether water balance traits were behind the observed increased heat tolerance following desiccation hardening. Studies have shown that both desiccation hardening (Bayley et al., 2001; Benoit et al., 2009) and heat hardening (Tammarielo et al., 1999; Feder & Hofmann 1999; Kalosaka et al., 2009) elicit the synthesis of heat shock proteins which play a role in heat tolerance. Heat-shock proteins bind to unfolded proteins and either assist in their refolding, prevent their nonspecific aggregation, or target them for degradation (Feder & Hofmann 1999; Saibil 2013). Nevertheless, further

studies are needed to confirm upregulation of Hsps following desiccation hardening in *C. rosa* and its role in the current observed cross tolerance effects.

3.4.2. Cross tolerance effects of starvation hardening on heat tolerance

Starvation hardening led to improved heat tolerance measured as CT_{max} , while other treatments gave no cross tolerance (Chapter 2). Heat knockdown was however not affected by starvation acclimation as observed by Scharf et al., (2016). Our result indicate that both heat hardening and starvation acclimation recorded higher body lipid content than control flies, though the lipid content of starved *C. rosa* were not significantly higher. Since heat hardening flies were heat tolerant, this could probable suggest that body lipid content likely play a role in heat tolerance hence this could probably explain why starvation hardened flies were heat tolerant. Our assessment of glucose content indicated that starved *C. rosa* flies had significantly lower glucose content, suggesting that the accumulation of lipids, probably came from the conversion of glucose to lipids. Generally insects have a higher capacity to covert glucose to lipids, than to convert glucose to glycogen, which explains the tendency for insects to have high lipids than glycogen (Arresse & Souglases 2010). The accumulation of lipid at the onset of starvation has been observed in various insects (Djawdan et al., 1998), as a mechanism for starvation tolerance, however whether this increased lipid content during starvation hardening was behind the observed improved heat tolerance in *C. rosa* is unknown. In a recent study, elevated temperature stress has been shown to deplete fat body store in *Drosophila melanogaster* (Klepsatel et al., 2016). According to the concept of energy-limited tolerance to stress (Sokolova 2013), stressful conditions affect energy allocation by modulating energy demands for different processes (Klepsatel et al., 2016). Thus during stressful conditions energy reserves are reduced or depleted in order to redirect energy towards mechanisms of protection and damage repair. This suggest that, probably the lipid reserves that accumulated during starvation hardening, may have been redirected towards the energetically demanding production of Hsps (Tomanek 2010) hence allowing *C. rosa* flies to be more tolerant to heat stress. Also Hsps have been known to be synthesised following other stressors such as nutrient deficiency (Salvucci et al., 2000). Therefore this means there is need to investigate the synthesis of Hsps during starvation in *C. rosa* which could have led to the observed cross tolerance effect. A more fruitful study direction would be to also investigate trehalose and glycogen concentrations, since they are the primary source of energy for flight in dipteran insects (Arresse & Souglases 2010).

3.4.3. Cross tolerance effects of cold acclimation on desiccation tolerance

Cold acclimation increased desiccation tolerance in *C. rosa* (Chapter 2), as observed in other studies which have recorded this type of cross tolerance in other insects' species (Parkash et al., 2013; Bauerfeind et al., 2015). Mechanisms for increased desiccation tolerance after cold acclimation include reduction in water loss (Parkash et al., 2013), caused by reduced cuticular permeability. Since majority of insects' water loss occurs via the cuticle (Stinziano et al., 2015), reduced cuticular water loss have been associated with increased desiccation resistance (Parkash et al., 2008). Reduced water loss due to reduced cuticular permeability following rapid desiccation hardening improved desiccation stress in like Diptera e.g. *D. melanogaster* (Bazinet et al., 2010). However in this study, water loss rate of cold acclimated *C. rosa* flies was higher but not significantly different from control treatment. This suggests other mechanism could have been behind the observed cross tolerance. In many terrestrial arthropods, both cold and desiccation stimulates the production of low molecular weight compounds (Chown & Nicolson 2004). These low molecular weight compounds have been implicated in the atmospheric absorption of water in sprintails (Bayley & Holmstrup 1999) hence could lead to protection against desiccation stress. However our results did not show the possibility of water absorption during cold acclimation as the body water content of cold acclimated flies was lower but not significantly different from control flies, unlike desiccation hardened flies which had significantly higher BWC than control flies.

3.4.4. Cross tolerance effects of desiccation hardening on starvation tolerance

Results indicate that desiccation hardening increased starvation tolerance in *C. rosa* (Chapter 2). This result is in keeping with observations by Bublly et al., (2012), in which starvation acclimated *D. melanogaster* showed improved desiccation tolerance, signifying a cross tolerance effect between the two environmental stressors. Mechanisms underlying starvation tolerance usually include the accumulation of lipids. Nevertheless, current results showed no significant increase in lipid content following desiccation hardening. Desiccation hardened *C. rosa* flies exhibited lower but not significantly different body lipid content that control flies. This may mean other mechanisms other than lipids may be at play during the observed cross tolerance, and thus warrant further exploration, to determine exact causes of the observed cross tolerance.

3.4.5 Sex effects of life history traits

The interactions between treatment and sex were not significant in all results, except in starved *C. rosa* flies. A significantly higher body water content was observed in starved females than males. This may partly be because, in preparation for starvation stress, insects normally accumulate energy reserves e.g. lipids (Ballard et al., 2008; Arrese & Soulages 2010). Furthermore, females tested in these experiments were reproductively mature, and in most cases reproductive investment in insects may involve accumulation of lipid reserves (Hilary & Wolf 2011). These lipids may serve as metabolic water and hence the trend observed here. However generally irrespective of treatment, females showed a significantly higher body water than males. This may be because females were bigger in size than males, as also observed by Weldon et al., (2016) in which females of *Ceratitis* species including *C. rosa* were found to be bigger in size than males. Similarly, Gibbs et al. (2001) also showed that female *Drosophila* species were significantly larger in size than males. Females insects generally have larger body sizes compared with their male counterparts, especially following sexual maturity due to the accumulation of energy reserves for reproductive investment (in the female) (Hilary & Wolf 2011), while males are smaller due to paternal investment (Thornhill 1979). Insects paternal investments include the females receiving food collected or captured by males, and/ males themselves being eaten after mating (Thornhill 1979). The smaller size of males could have also contributed to the observed higher water loss rates, due to the surface area occupied versus the volume ratios, slightly favoring females relative to the males. Body lipid content on the other hand was not significantly different between males and females. However generally females insects and arachnids have higher lipids than males for biological activities such as locomotion, reproduction (Hilary & Wolf 2011), and as sources of higher metabolic water than glycogen (Gibbs et al., 1997; Arrese & Soulages 2010).

3.5. Conclusion

Mechanisms underlying cross resistance in *C. rosa* studied here suggest that some stress resistance mechanisms are shared among the different stressors, for example mechanisms against cold and desiccation, desiccation and heat tolerance, and starvation and heat tolerance. However before any generalisations can be made regarding cross resistance and its underlying mechanisms, extensive research need to be done on 1) costs associated with acclimation, reason why some insects do not acclimate, 2) species effect on plastic responses.

Furthermore, the role of Hsps and other carbohydrate protectants (e.g. trehalose, sorbitol) in the cross tolerance observed here ought to be investigated

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CHAPTER 4

GENERAL DISCUSSIONS

Ceratitis rosa has a wide range on the African continent (De Meyer et al., 2008; Nyamukondiwa et al., 2013) and has been suggested to prefer cooler and wetter environments (Duyck & Quilici 2002; Normand et al, 2000; Nyamukondiwa & Terblanche 2009), suggesting a greater potential in temperate regions. Its potential for establishment in these areas is limited by its inability to survive periods of repeated low temperature treatments which are typical of temperate areas (Nyamukondiwa et al., 2010). However due to climate change, *C. rosa* is likely to be faced with multiple stressors and information on how it will respond when faced with such simultaneous multiple stressors, is significant. This may help explain the invasion potential of this species in particular, and insects with general under increased magnitude of climate change. I therefore investigated the ability of *C. rosa* to tolerate stressful environmental factors such as heat, cold, desiccation and starvation following selected acclimation/hardening treatments (Chapter 2). Stress resistance traits measured included: critical thermal limits (CT_{max} and CT_{min}), chill coma recovery time (CCRT), heat knockdown time (HKDT), desiccation and starvation tolerance. This was followed by testing mechanisms underlying tolerance to these environmental stressors (Chapter 3), to look at possible overlap in mechanisms underlying tolerance to different stressors, symbolic of cross tolerance.

Our results showed that acclimation to one stress increased resistance for the same stress later in life, typifying beneficial acclimation hypothesis (Leroi et al., 1994). Previous studies have also reported that heat hardening improved heat tolerance (Kalosaka et al., 2009; Bublly et al., 2012), cold acclimation improves cold tolerance (Bublly et al., 2012) desiccation hardening improves desiccation tolerance (Sjursen et al., 2001; Bazinet et al., 2010; Bublly et al., 2012; Stinziano et al., 2015) and starvation acclimation improves starvation tolerance (Bublly et al., 2012), in support of what is known as the beneficial acclimation hypothesis. The current study also recorded four cases of cross tolerance. There was increased heat tolerance (measures as CT_{max}) following desiccation hardening (Fig. 2.1A, chapter 2). However no effect was recorded in heat tolerance measured as heat knockdown time, showing different results from those obtained by Bublly et al., (2012). Similarly cross tolerance has been observed in *Belgica antartica* (Benoit et al., 2009), where dehydration increased survival at high temperatures. Less attention has been paid to understanding the link between dehydration and heat tolerance (Benoit et al., 2009), with most of the focus on

studying the link between dehydration and cold tolerance. Several studies have recorded improved cold tolerance following desiccation hardening (Bayley et al., 2001; Sinclair et al., 2007; Benoit et al., 2009), contrary to our results in which we found improved desiccation tolerance following cold acclimation (Fig. 2.2A, chapter 2). The study also present improved heat tolerance (measured as CT_{max}) following starvation hardening (Fig. 2.1A, chapter 2), and enhanced starvation tolerance following desiccation hardening (Fig. 2.2C, chapter 2). Though our results indicate that only desiccation hardening improved starvation tolerance, positive cross tolerance effects between cold acclimation and starvation resistance have been recorded in other insects e.g. *D. melanogaster* (Bubliy et al., 2012) and red flour beetle (Scharf et al., 2015).

Our studies on mechanisms underlying tolerance to environmental stressors (and cross tolerance) (Chapter 3) showed low levels of glucose observed in starvation acclimated flies could be behind the observed improved heat tolerance following starvation acclimation. According to Salvucci et al., (2000), nutrient deficiency can be one of the triggers of heat shock protein synthesis, which has been reported to play a role in heat tolerance (Feder & Hofmann 1999; Saibil 2013). Starvation acclimated *C. rosa* flies were also found to have high lipid content. However, whether this increased lipid content played a role in the observed heat tolerance is unknown. However, according to the concept of energy- limited tolerance to stress (Sokolova 2013), stressful conditions affect energy allocation by modulating energy demands for different processes (Klepsatel et al., 2016). Thus during stressful conditions energy reserves are reduced or depleted in order to redirect energy towards mechanisms of protection and damage repair. This suggest that, probably the lipid reserves that accumulated during starvation hardening, may have been redirected towards the energetically demanding production of heat shock proteins (Tomanek 2010) hence allowing *C. rosa* flies to be more tolerant to heat stress. Heat shock proteins, though not analysed in this study could also explain the observed heat tolerance following desiccation hardening. This goes to show that temperature tolerance may be an energy demanding process (see Nyamukondiwa et al., 2010), and that lipid accumulation may play a critical role in modulating temperature tolerance. Studies have shown that both desiccation hardening (Bayley et al., 2001; Benoit et al., 2009) and heat hardening (Tammarielo et al., 1999; Feder & Hofmann 1999; Kalosaka et al., 2009) elicit the synthesis of heat shock proteins which play a role in heat tolerance. In addition, trehalose which was also not assayed in this study, has been shown to accumulate

during dehydration in *Belgica antarctica* (Benoit et al., 2009). This accumulated trehalose has been suggested to enable organisms to survive heat stress by playing a role in the stabilisation of heat sensitive proteins (Bowler 2005).

Our results of water loss rates however could not explain the observed improved desiccation tolerance following a cold acclimation treatment, suggesting other mechanisms may have accounted for our observed desiccation resistance. Mechanism for increased desiccation tolerance after cold acclimation include reduction in water loss (Parkash et al., 2013), caused by reduced cuticular permeability. However in our study water loss rates of cold acclimated flies were not significantly different from control flies, suggesting mechanisms accounting for resistance between the two stressors may be decoupled, at least in *C. rosa*. Our results also did not show the possibility of water absorption during cold acclimation as the body water content of cold acclimated flies were lower but not significantly different from control flies, unlike desiccated flies which had significantly higher body water content and were desiccation tolerant than control flies. Last, mechanisms studied here could not explain the observed increase in starvation tolerance after desiccation hardening (see chapter 2 and 3). The reason may be that mechanisms for starvation tolerance has been attributed to the accumulation of lipids reverses (Ballard et al., 2008; Dussutour et al., 2016; Holmstrup et al., 2016). In the present study, there was no indication of significant lipid accumulation following desiccation hardening. Desiccation hardened *C. rosa* flies exhibited lower but not significantly different body lipid content that control flies, suggesting other mechanisms may be at play. In consequence, other mechanisms ought to be explored to explain the cross tolerance observed here.

4.1. Conclusion

Information on the ability of *C. rosa* to confer resistance to environmental factors following acclimation/hardening to abiotic factors is the first step to understanding how *C. rosa* will deal with heterogeneous stressful environments, and may help explain its invasion potential in the face of climate change. Evidence of beneficial acclimation across environmental stressors tested here symbolise *C. rosa* may be able to adjust its thermal tolerance through plasticity, a key attribute that may enhance its invasion potential, and possibility of thriving upon introduction to novel environmental habitats. Furthermore, significant cross tolerance effects in *C. rosa* indicate it may be able to thrive under multiple heterogeneous

environmental stressors, typical under climate change and may have a competitive advantage over like species. This information is critical for future phenology predictions, and can also help in early warning systems and consequently efficacious control of the economic insect pest. Although mechanisms of cross resistance investigated here were not exhaustive, they provide significant insight that stress resistance mechanism may be shared across the different environmental stressors, hence enabling organisms to adapt to a changing climate. The information indicating the sharing of protective mechanisms across different stressor can also suggest the co-evolution of stress resistance mechanism. Nevertheless, a more fruitful area for further study will be looking at other mechanisms associated with cross tolerance, including but not limited to measurement of Hsp upregulation, carbohydrates (e.g. trehalose), glycogen, glycerol and the role of aquaporins.

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