Mechanisms of population establishment in insect invasions: Drosophilidae as a model system

by

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Declaration

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Summary

The mechanisms and traits influencing insect invasions are generally poorly understood. Drosophilids are an excellent model system for studying invasions and especially the adaptive processes occurring during invasions since the family has short generation times, diverse functional traits and variation in geographic distributions while possessing several notable invasive species. While there are many studies of environmental stress resistance or life-history traits and how these might influence population dynamics or geographic range limits in Drosophilidae, these studies have several potential shortcomings. Chief among these are perhaps concerns surrounding their use of Stock Centers (laboratory cultures) of varying time in culture and sometimes unknown geographic origins to infer trait-environment associations, niche requirements or evolutionary adaptive capacity. Traits can respond rapidly to laboratory rearing with laboratory cultures typically losing stress resistance and increasing fecundity and/or development rates. In this study, I sought to determine whether there is a significant and systematic effect of time spent in culture on estimates of environmental stress resistance and its thermal acclimation (i.e. phenotypic plasticity) of two wild-caught *Drosophila* species (*Drosophila melanogaster* and *Zaprionus vittiger*) between newly established lines (in the F_2 generation) and a later timepoint (F_8-F_{10} generation) in the laboratory under standard, controlled rearing conditions. A further objective was to identify the nature and magnitude of basal and plastic estimates of environmental stress resistance traits among four populations of *D. melanogaster* collected from different areas within South Africa to assess if geographic origin influences trait and plasticity estimates substantially within a single species. This was done by measuring traits of upper and lower thermal activity limits (CT_{MAX} and CT_{MIN}, respectively), the proportion of individuals surviving after 24 hours after exposure to a potentially lethal temperature (heat and cold survival survival), desiccation resistance, starvation resistance and the plasticity thereof in response to thermal acclimation at three temperatures (18, 23, 28 ˚C). There was significant variation in resistance to environmental stressors between earlier and later generations for *D. melanogaster* and *Z. vittiger*. *Drosophila melanogaster* generally increased resistance to environmental stressors after spending ten generations in the laboratory whilst *Z. vittiger* had decreased resistance. There was also significant variation in both thermal and

survival traits and the plasticity thereof between the four populations of *D. melanogaster*. Thus, it is clear that conditions at time of sampling and the species or population's geographic source can strongly mediate trait and plasticity assessments in laboratory cultures. Consequently, environmental stress resistance measured from Stock Centers lines or species may give a biased view which could influence tests of climate or niche matching and risk assessments. The divergent, idiosyncratic responses noted between my study species' means that more species would need to be assessed to understand the generality of the outcomes described here.

Opsomming

Meganismes en eienskappe wat suksesvolle indringings te weeg bring word nie goed verstaan nie. Die Drosofiliede verteenwoordig ‘n belangrike model sisteem vir die studie van indringerspesis en die adaptiewe prosesse wat gepaard gaan met suksesvolle indringings, omdat die familie diverse funksionele eienskappe besit, ‘n wye geografiese verspreding het en terselfdetyd uit verskeie kenmerkende indringerspesies bestaan. Daar bestaan verskeie studies wat die omgewings stres weerstands eienskappe van die Drosofiliede gemeet het, maar hierdie studies het verskeie tekortkominge. Een van die belangrikste tekortkominge is moontlik bekommernisse rondom die gebruik van ‘Stock Centers’ (laboratorium kulture) van verkeie ouderdomme en geografiese afkoms om omgewings assosiasies te maak of om evolutionêre adaptiewe kapasiteit te bepaal. Spesies eienskappe reageer vinnig op laboratorium kondisies en verloor tipies hulle weerstand tot omgewings kondisies met ‘n gepaardgaande toename in voortplanting. Die doel van hierdie studie was om te bepaal of die aantal tyd wat spandeer word in kultuur ‘n beduidende effek het op beramings van omgewings stress weerstand en termiese akklimasie (fenotiepiese plastisiteit) tussen nuutgestigte (F_2 generasie) Drosofilied spesies (*Drosophila melanogaster* en *Zaprionus vittiger*) en na die spesies tien generasies (F_{10} generasie) in kultuur onder standaard grootmaak praktyke spandeer het. ‘n Verdere doel van die studie was om die natuur en omvang van basale en plastiese beramings van omgewings stress weerstand eienskappe te identifiseer binne vier populasies van *D. melanogaster* wat gevang was in verskeie dele van Suid Afrika om sodoende te bepaal of geografiese afkoms ‘n beduidende impak kan hê op beramings van omgewingseinskappe en plastisiteit binne ‘n spesifieke spesies. Hierdie was gedoen deur die boonste en onderste termiese limiete (CT_{MAX} en CT_{MIN}), die proporsie van individue wat oorleef het 24 uur na blootstelling aan ‘n potensiële dodelike temperatuur (hitte en koue skok) sowel as oorlewing na uitdroging en uithongering, en hulle plastisiteit in reaksie tot termiese akklimasie by drie temperature (18°C, 23°C en 28°C), te bepaal. Daar was betekenisvolle en teenstrydige weerstand tot omgewing stressors tussen vroeër en latere generasies van *D. melanogaster* en *Z. vittiger*. *Drosophila melanogaster* het ‘n algemene toename in weerstand tot omgewing stressors gehad na tien generasies in die laboratorium, terwyl *Z. vittiger* ‘n afname ondergaan het. Daar was ook betekenisvolle verskille in beide termiese en oorlewingseienskappe sowel as hulle plastiese reaksie tussen die vier populasies van

D. melanogaster, en dus is dit duidelik dat die geografiese oorsprong van ‘n spesies of populasie ‘n effek kan hê op die assessering van eienskappe en hulle plastisiteit in laboratorium kulture. Dus is dit duidelik dat die kondisies tydens steekproefneming sowel as die spesies of populasie se geografiese oorsprong ‘n verdere invloed het op omgewingseienskap en platisiteit assesserings in laboratorium culture. As ‘n gevolg sal omgewings stress eienskappe wat gemeet is vanaf ‘Stock Centers’ ‘n bevoordeelde uitkyk gee en hierdie vooroordele kan ondersoeke van klimaat en nis ooreenstemmings beïnvloed en sodoende risiko assessering beïnvloed. Die uiteenlopende eienaardige reaksies opgemerk tussen my studie spesies beteken dat meer spesies geasseseer sal moet word om die algemeenheid van die uitkomste wat hier beskryf word te verstaan.

This thesis is dedicated to

The memory of my father, Sarel Opperman

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Preface

This thesis is presented as one chapter

Contents

Introduction

Invasive species

Species introduced and established intentionally or unintentionally beyond their native range are referred to as alien invasive species (Jeschke *et al.*, 2014). Alien invasive species can have numerous detrimental impacts on the natural world, and act as agents of ecological change by causing changes in ecosystem structures and the extinction of threatened species, by altering the structure of communities and by disrupting successional pathways (Clout and Williams, 2009; Jeschke *et al*., 2014; Simberloff *et al*., 2013). Invasive species can enter a country through several pathways, either intentionally or unintentionally, and in recent decades there has been an increase in the spread of invasive species through human vectors correlated with an increase in human movement across the world (Roderick and Navajas, 2015; Sacaggi *et al*., 2016). Invasive species also cause losses of important ecosystem services with accompanying impacts on the health of humans, livestock and wild animals (Bertelsmeier *et al*., 2016; Clout and Williams, 2009). For a species to become invasive, it needs to overcome several barriers to survive through the invasive stages of transport and introduction, and once introduced, population establishment and eventual spread (Blackburn *et al*., 2011). A suite of population demographic factors and traits likely influence the invasion process. Upon introduction to a new environment, high propagule pressure (the number of individuals introduced and frequency thereof) increases the chances of successful invasion (Duncan *et al.*, 2014). However, both demographic and genetic characteristics of the introduced species are chief in allowing spread of the species after the initial introduction (Szűcs *et al.*, 2014). A match between the introduced areas' climate and the environmental stress traits of the introduced species allows for niche occupation and increased population growth and spread of the invasive species post-introduction (Dixon *et al.*, 2009; Gilchrist *et al*., 2008; Rey *et al*., 2012). Thus, environmental stress resistance or thermal requirements of a species represent an important proxy for determining which species have the potential to become invasive and are frequently used in risk assessments and climate or niche models (e.g. Jarošík *et al*., 2015; Kumschick *et al.*, 2015).

Invasive insects have detrimental ecological and social impacts worldwide, however, Pysêk *et al.* (2008) found that studies on insects make up only 18% of invasive studies. Relative to insect

species diversity (c. 5.5 million species) this is a potentially huge literature bias. Well-known insect invaders include the Argentine ant (*Linepithema humile*), the big-headed ant (*Pheidole megacephala*), the gypsy moth (*Lymantria dispar*), the codling moth (*Cydia pomonella*), the Mediterranean fruit fly (*Ceratitis capitata*) and *Drosophila suzukii* among others (Huang *et al*. 2011). The Argentine ant, for example, has spread globally through the increased movement of people and trade goods (Suarez *et al*., 2001) and has reduced native ant populations by causing the collapse of various mutualisms between plants and native ant communities (Griffiths and Picker, 2011). In Fynbos vegetation in South Africa, the Argentine ants interfere with the burial of large proteaceous seeds by native ants, leading to changes in the plant community structure (Griffiths and Picker, 2011; Mothapo and Wossler, 2013).

In addition, the true fruit flies (Tephritidae), such as members of *Ceratitis* and *Bactrocera*, have led to substantial economic losses in the fruit industry as well as quarantine restrictions and phytosanitary measures in affected areas that lead to increased costs of fruit production and restricted market access (Sarwar, 2015). The Mediterranean fruit fly (Tephritidae) represents a major fruit fly pest which has readily invaded most of the world (Malacrida *et al.*, 2007) and which has become an important agricultural pest with associated economic losses for the fruit industry worldwide (De Meyer *et al.*, 2008; White and Elson-Harris, 1994). Their fast spread is due to the increased implementation of agriculture as well as the increased mobility of humans worldwide (Hill *et al*., 2016; Malacrida *et al.*, 2007).

Drosophilidae as invasive species

Several invaders from the *Drosophila* genus have also become important pests in the fruit industry. The most important include *Drosophila subobscura*, *Zaprionus indianus* and *D.suzukii* (the spotted wing *Drosophila*). *Drosophila subobscura* (native to Europe) has invaded both South and North America leading to severe economic and ecological impacts (Foucaud *et al*., 2016). *Zaprionus indianus* (native to East Africa, the Middle East and Southern Eurasia) has invaded India, Mexico and Canada among others and has also had detrimental impacts on the fruit industry in these countries (Alawamleh *et al*., 2016; Van der Linde *et al.*, 2006). *Drosophila subobscura* and *Z. indianus* are considered secondary pests as they lay their eggs in already-

damaged fruit (Lasa and Tadeo, 2015). *Drosophila suzukii* is originally from Japan but has subsequently invaded China, Myanmar, India, Italy, Thailand, Spain, Russia and North America where it causes severe damage in the fruit industry (Calabria *et al*., 2012; Walsh *et al*., 2011). It was first identified in 2008 from raspberries in California (USA) but an accurate identification was only made in 2009 after it had spread to many countries worldwide. It is unique in that it is a primary pest, unlike other *Drosophila* species, with a serrated ovipositor that allows it to lay its eggs in undamaged fruit and, consequently, leads to substantial fruit losses in the invaded areas (Calabria *et al*., 2012). It is a major pest on cherries, strawberries, pears and wine grapes, among others (Calabria *et al*., 2012; Walsh *et al*., 2011).

In order to become invasive, species are thought to go through four stages, namely transport, introduction, establishment and spread (Blackburn *et al*., 2011). The species will only become invasive if it survives all these stages overcoming the unique barriers posed in each instance (Blackburn *et al*., 2011). Successful invasions require an organism to have the ability to respond to diverse environmental conditions through either phenotypic plasticity or rapid genetic adaptive shifts (Perkins *et al*., 2011). As a result, *Drosophila* species act as an important model for investigating the underlying mechanisms of adaptive processes involved in successful invasions (Gibert *et al*., 2016) due to their wide geographical distribution as well as the presence of latitudinal clines for many morphological and physiological traits. In addition, they are easy to rear, have short generation times (leading to rapid evolutionary shifts) and there is a large amount of genomic data available for the genus (Gibert *et al*., 2016; Hoffmann, 2010).

Risk assessments determine the likelihood of a species becoming invasive together with the potential impact should it become invasive (Kumschick and Richardson, 2013). Except for risk assessments done on the widespread pest species *D. suzukii*, little risk assessments have been done on other potential Drosophilidae invaders (Berry, 2012). This is due to a lack of prioritization of members of the family, because of limited data availability and that the particular species' invasion may have little economic or ecological consequence (Kumschick *et al*., 2015). Data required for risk assessments typically includes propagule pressure as well as inherent biological variables (suitability of the climate, availability of resources and habitat, etc.) among others (Kumschick *et al*., 2015; see also Jarošík *et al*., 2015). These data can be used to

conduct a risk assessment for *Drosophila* species using a scoring system, like the newly developed generic impact scoring system (GISS) that will aid in elucidating potential future invaders (Nentwig *et al.*, 2016).

Estimating environmental tolerances and inferring population dynamics

Several theories have been put forward on what characteristics or traits makes a species a successful invader. These include propagule pressure, genetic similarity (species from the same families and/or genus), the ability of the species to disperse long distances and climate matching among others (Richardson and Pysêk, 2006). In weeds for example, traits of high reproductive potential such as autonomous seed production (uniparental reproduction) and higher germination rates increases invasive potential (Hao *et al*., 2011; van Kleunen *et al*., 2007), together with performance traits such as higher shoot- and leaf area allocation and increased growth rate (van Kleunen *et al*., 2010; van Kleunen *et al*., 2011) all increase invasive potential. Several traits make invertebrates successful invaders, including propagule pressure (Hee *et al*., 1999), traits of high reproductive potential such as a high number of offspring and a wide host range (Duncan *et al*., 2014) and long distance anthropogenic dispersal as a result of trade (fruit, flowers and the like) (Brown *et al*., 2011) among others. Climate matching puts forward the idea that the climate in an organism's native range can be compared to climates across the world in order to determine possible areas in which the organism could become invasive (Baker *et al.*, 2000; Bomford *et al*., 2009; Petersen, 2003; Thorn *et al.*, 2009). As a consequence, information on various aspects of the environmental physiology of *Drosophila* species are critical to ensure accurate risk assessment of potential invaders which will also allow for more accurate climate modelling to determine important risk areas for invasion.

Critical thermal limits are thought to represent the functional, though not necessarily lethal, limits to performance of insects. Estimates of critical thermal limits are frequently estimated and used in database compilations of upper thermal tolerance or geographic distribution (reviewed in Terblanche *et al*., 2011) and are defined as the temperature at which the insect's movements becomes irregular or loses function (e.g. righting response), causing it to be unable to escape the conditions that will ultimately lead to its death (Lutterschmidt and Hutchison, 1997).

Internationally, many physiological studies have used *Drosophila* as a model group to understand their trait-environment relationships, geographic range limits, range shifts or evolutionary adaptations (Bush *et al*., 2016; Hoffmann *et al*., 2002; van Heerwaarden *et al*., 2009; van Heerwaarden *et al*., 2016). Recent examples include Kellerman *et al*. (2012a) which assessed patterns of cold tolerance and desiccation resistance of 95 *Drosophila* species and found that desiccation and cold resistance are indeed linked to species distributions. Using the same species, they then compiled critical thermal maximum (CT_{MAX}) and critical thermal minimum (CT_{MIN}) measurements (Kellerman *et al*., 2012b) and found variation in upper thermal limits between the species and showed that species from drier regions had increased resistance to heat stress. In terms of thermal traits for *Drosophila*, several mechanisms have been studied including high and low temperature pre-treatments (acclimation), that lower thermal limits are generally more plastic than upper thermal limits (Kellett *et al.,* 2005), a linear reaction norm for both CT_{MAX} and CT_{MIN} across acclimation temperatures (Schou *et al.*, 2017) and significant differences in basal thermotolerance and their plastic responses (Nyamukondiwa *et al.*, 2011). Other studies assessing CT_{MAX} and CT_{MIN} and their environmental trait interactions found clinal patterns in *D. melanogaster* thermal traits in Australia and showed that simulating temperate conditions in five widespread and five restricted *Drosophila* species increased CT_{MIN} by 2-4°C whilst simulating tropical conditions increased CT_{MAX} by less than 1°C (Hoffmann *et al.*, 2005b; Overgaard *et al.*, 2011). Furthermore, Matzkin *et al.* (2011) showed that phylogenetic relatedness has a large impact on both desiccation and starvation resistance. *Drosophila melanogaster* was also found to exhibit clinal patterns in desiccation resistance as well as cuticular permeability and levels of melanisation (darker versus lighter flies) (Bazinet *et al.*, 2010; Hoffmann *et al.*, 2003; Parkash *et al*., 2008).

While there are many physiological studies available, little is known about *Drosophila* species and their stress resistance in South Africa. Nyamukondiwa and Terblanche (2010) collected *Zaprionus vitti*ger individuals from Stellenbosch and showed that maximum survival was achieved following cold-hardening at 7°C and 10°C. Klepsatel *et al.* (2013) investigated the thermal reaction norms between different populations of *D. melanogaster* sampled from South Africa, Ethiopia, Zambia, Switzerland and Austria and found no significant differences between the thermal reaction norms of the different populations for the morphological and reproductive

traits (fecundity, thorax length, wing area and ovariole number) they examined. To my knowledge, no other physiological studies have been done on Drosophilid species caught in South Africa. Other studies of African *Drosophila* species are mostly genetic or for compilation in large-scale population genetics or genomics. For example, Singh *et al.* (1982) investigated the genetic differences between *D. melanogaster* populations from nine continents which included a West African (Benin) population. They found significant genetic differentiation between the different populations, especially between the Northern and Southern hemispheres. Capy *et al.* (1993) examined morphometric differences between *D. melanogaster* and *Drosophila simulans* populations and included populations from South Africa (Johannesburg and Cape Town) as well as Egypt. This study showed morphometric differences between populations of both *D. melanogaster* and *D. simulans*, as well as between the Northern and Southern hemispheres for both species.

Variation in trait estimates

There is a long history of comparative studies of environmental stress responses within and among Drosophilid species (e.g. Castaneda *et al*.,2015; Hoffmann and Harshman, 1999; Hoffmann and Parsons, 1993; King *et al*., 1956; Lockwood *et al*., 2017; Smith and Smith, 1954). In nature, variation among seasons at specific geographic locations can elicit significant trait variation. Resistance to environmental stressors has been found to differ seasonally as well as geographically between populations of the same species (Hoffmann and Harshman, 1999; Sinclair *et al.*, 2012). In Australia, desiccation resistance of *D. melanogaster* was found to be higher under summer versus winter conditions (Hoffmann *et al*., 2005b). Similarly, in India, seasonal changes in moisture availability led to changes in desiccation resistance as well as body colour in *Drosophila jambulina* (Parkash *et al.*, 2009). Moreover, Behrman *et al.* (2015) found that *D. melanogaster* isofemale lines started from the generation that survived through the winter (females collected in Spring) had a higher tolerance to environmental stressors in comparison to isofemale lines started from females collected in the other seasons.

Since there is marked genetic variation in several traits from the cosmopolitan *D. melanogaster*, differences in environmental stress responses are largely expected between different populations

(Ayrinhac *et al*., 2004; David and Capy, 1988). There are several studies showing geographic variation in trait estimates between different populations of diverse Drosophilid species (Sinclair *et al*., 2012). Recently, Sgrò *et al.* (2010) showed latitudinal clines in heat tolerance among populations of *D. melanogaster* with populations at the tropics being more resistant to heat than those at higher latitudes. Earlier clinal studies documented increased heat resistance and decreased cold resistance in lower latitude populations compared to higher latitude populations (Hoffmann *et al*., 2002; Hoffmann *et al.*, 2005b). In *Drosophila buzzatii* clinal variation was found in thermal traits between populations along an altitudinal gradient in North-Western Argentina (Sørensen *et al*., 2005). Knockdown time following a heat shock treatment is higher in Australian *D. melanogaster* populations closer to the tropics in contrast with populations in temperate habitats. In addition, populations from warm regions are more heat tolerant and populations from cold areas are more cold tolerant in *D. melanogaster* from temperate (Denmark and Italy) and subtropical areas (Canary Islands and Mali) (Guerra *et al*., 1997; Hoffmann *et al.*, 2002). With regards to other physiological traits of stress resistance, significant differences in desiccation and starvation resistance have been widely reported between populations of the same species in *D. melanogaster*, *D. ananassae* and *Zaprionus indianus* in India (Karan and Parkash, 1998), *D. birchii* in Australia (Hoffmann *et al*., 2003) and in *D. melanogaster*, *D. pseudoobscura*, *D. nigrospiracula* and *D. mojavensis* from several countries (Matzkin *et al.*, 2007).

Inter-specific variation in environmental stress resistance traits are also well-documented. For thermal traits, precipitation and temperature are thought to influence CT_{MAX} values in several *Drosophila* species (Kellermann *et al.*, 2012b). A study by Anderson *et al*. (2014) also found that for 14 *Drosophila* species, the thermal traits of CT_{MIN}, lower lethal temperature and lethal time at low temperature were the best predictors of latitudinal distributions in *Drosophila* species worldwide. Additionally, widespread species were found to have higher cold resistance in Australian *Drosophila* compared to narrowly distributed *Drosophila* species (Overgaard *et al.*, 2011). Substantial variation in the heat acclimation response of several *Drosophila* species has also been found (Schou *et al.*, 2017). Differences between *Drosophila* species are also documented for desiccation and starvation resistance (Matzkin *et al*., 2009) with desiccation resistance correlated with distribution in some *Drosophila* species (Kellermann *et al*. 2012a).

These studies indicate the importance of taking both seasonal and geographic differences between populations of the same species into account when assessing stress resistance traits for a species.

Stock Centers and laboratory adaptation

Although physiological studies on Drosophilidae are abundant there are several potential shortcomings in these studies (Hodkinson, 2003) that are typically ignored or argued to be of little consequence to the major outcomes or conclusions reached (Chown *et al.*, 2003). For example, many inter-specific comparison studies make use of Stock Center lines, instead of newly established lines of flies. This is much less of an issue in inter-population studies which more typically would establish new lines from field collections (Hoffmann *et al*., 2001a; Kellermann *et al*., 2017; Sgró *et al*., 2010). Using species obtained from Stock Centers for experiments, instead of species collected from the wild, has the drawback of the laboratory colony potentially being inbred with a resultant decrease in genetic diversity (Ærsgaard *et al*., 2015) and possibly trait diversity. Additionally, the species could have become laboratory adapted potentially leading to a decrease in resistance to environmental stressors. Studies investigating these effects show that in laboratory stocks resistance to environmental conditions is lost and is instead replaced by selection on other traits such as increased fertility (Hoffmann *et al*., 2001b; Sgró and Partridge, 2000). Species kept in the laboratory for an extensive period are not subjected to natural selection and thus the results of stress resistance might not be a true representation of what may be happening in the wild if strong directional selection maintains the trait. Yet different species of *Drosophila* have vastly different responses to selection depending on the underlying adaptive capacity of the trait in question (Kellermann *et al.*, 2009; van Heerwaarden and Sgrò, 2014), thus it is unlikely that all the species and diverse traits used in comparisons from Stock Center lines have responded equally to these laboratory rearing environments. To improve the validity of data collected for risk assessment, wild collected species, kept under laboratory conditions for a short period of time would better represent the wild population as they will more closely match the wild populations (Najarro *et al.*, 2015).

Study objectives

This study aimed to investigate whether there is significant variation in environmental stress resistance traits in newly established laboratory lines (i.e. at the 2nd generation) compared to lines that have been in laboratory culture for a longer period (i.e. several generations). I chose the F_2 generation to represent the wild population and F_8-F_{10} to represent a time-point after laboratory stay. I assumed that the results between F_8-F_{10} would not significantly differ but that most variation that would be a consequence of lab culture or lab adaptation would likely occur within the first 3-6 generations under standard rearing conditions (see e.g. Bertoli *et al*., 2009; Sambucetti *et al*., 2010). To achieve this, I compared the same sets of traits scored recently after establishment and again later on in two wild-caught South African *Drosophila* species (*D. melanogaster* and *Z. vittiger*) reared under standard, controlled conditions. Further, I sought to determine whether estimates of the phenotypic plasticity of these traits remain constant over time in culture or diverge significantly under laboratory rearing conditions when acclimated at different temperatures. Thus, I measured in both the F_2 and F_8-F_{10} generation of two species their CT_{MAX}, CT_{MIN}, acute heat and chill survival after exposure to an extreme temperature, desiccation resistance and starvation resistance and the plasticity of each trait in response to thermal acclimation at three temperatures (18, 23, 28 ˚C). I made two general predictions for the outcome of my study: I expected a decrease in both basal resistance and their plastic responses due to laboratory adaptation, possibly driven by small population sizes, as previously shown (Hoffmann *et al.*, 2001; Sgró and Partridge, 2000). In addition, I expected there to be a decline in the plasticity of traits between the F_2 and F_{10} generations if plasticity is costly to maintain. Alternatively, if plasticity and basal stress resistance are traded-off directly, that basal stress resistance may decline in culture while plasticity could increase (or vice versa).

An additional objective of this work was to determine if population comparisons are subject to similar kinds of laboratory adaptation problems by identifying the nature and magnitude of trait variation and acclimation-induced phenotypic plasticity in four populations of *D. melanogaster* collected from different areas within South Africa. The F_2 generation of each *D. melanogaster* population was used to measure the same traits as for the species mentioned in the first objective. It is expected that there will be differences between environmental stress resistance of the

different *D. melanogaster* populations as there is known to be considerable variation between populations of the same species, especially if populations are sourced from climatically diverse habitats (David and Capy, 1988).

Aims:

1. To determine whether there is a large variation in environmental stress resistance traits and their plastic responses between newly established laboratory lines of *D. melanogaster* and *Z. vittiger* (i.e. at the 2nd generation) compared to lines that have been in laboratory culture for an extended period.

2. To determine the magnitude of trait variation and acclimation-induced phenotypic plasticity between four populations of *D. melanogaster* collected from geographically distinct areas within South Africa

Materials and Methods

Origin and maintenance of experimental flies

The *Drosophila* species used in this study were freshly collected from the field in Stellenbosch (STB), Brackenfell (BRAC), Durban (DB), the Cederberg (CD) and Polokwane (POL). For the species comparisons *Drosophila melanogaster* (STB) and *Zaprionus vittiger* (BRAC) were caught and compared and for the population comparisons *Drosophila melanogaster* from four different locations were sampled (STB, DB, CD and POL) (Figure 1).

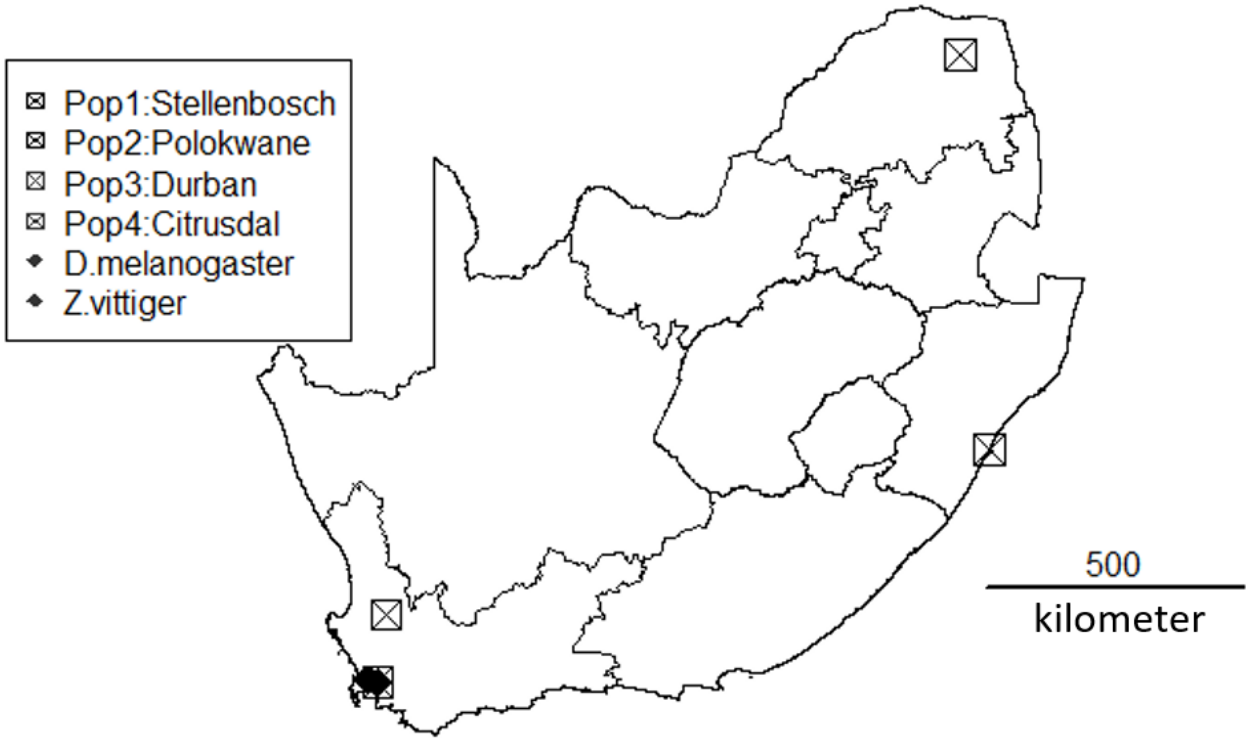

Figure 1: Sampling locations for the *Drosophila* populations and species within South Africa used in this study. Species (*D. melanogaster* and *Z. vittiger*) are represented by black diamondss and populations of *D. melanogaster* (Pop1: Stellenbosch, Pop2: Polokwane, Pop3: Durban and Pop4: Citrusdal) by the crossed squares.

Flies were caught by placing traps consisting of buckets filled with mixed fruits (oranges, bananas, apples and lemons) in suitable locations and then collected on the rotting fruit with a hand net or a plastic bag. After collection, flies were placed individually in 325 ml plastic bottles containing Bloomington's standard cornmeal medium (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/bloomfood.htm). Mated wild- caught females were used to start eighteen isofemale lines for each of the two species and for each of the four populations (David *et al*., 2005). Flies were then placed in an incubator (MRC LE-509, Holon, Israel) pre-set at 23°C and <10% relative humidity on a 12h day/night cycle and tipped into fresh medium until the F_2 generation when they were used for trait assays (Figure 2). Flies were tipped into new food at regular intervals to avoid overcrowding the bottles and the density of flies in each bottle were kept constant. Within the first 24 hours after eclosion of the F_2 generation the flies were put into new medium and three of the lines (chosen at random) were acclimated at 18°C, three at 23°C and three at 28°C for 48 hours. Acclimation temperatures were modified from constant temperate (19°C) and constant tropical (27°C) as used by Overgaard *et al*., 2011. After the 48 hours acclimation, the flies were placed back into the incubator set at 23°C until they were between 5-7 days old (about 24 to 48 hours), which is standard practice in *Drosophila* stress resistance studies (Kellermann *et al*., 2012a; Sgrò *et al*., 2010), and then environmental stress resistance traits were scored. It was assumed that the effects induced in the 48 hour acclimation period will last several days (as shown by Loeschcke *et al*. (1997) in which pretreated flies remained more resistant for several days in *Drosophila hydei* and *D. melanogaster*) since we were not particularly interested in any highly transient trait variation. For each species, nine of the eighteen lines were used for trials when the lines reached the F_2 generation and the remaining nine lines were kept in the laboratory until the tenth generation and then the same traits as for the F_2 generation were assayed.

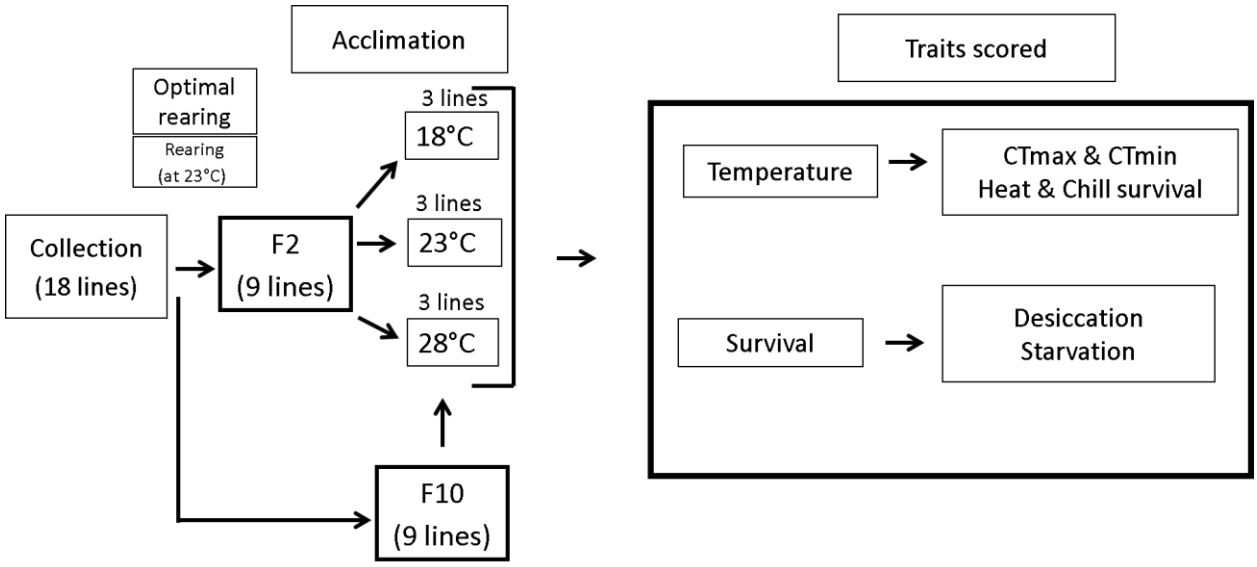

Figure 2: Experimental schematic diagram. For each species and population 18 isofemale lines were established and reared at 23°C until the F_2 generation after which lines were acclimated at 18°C, 23°C and 28°C (3 lines per acclimation) and returned to 23°C after 48 hours acclimation. After being acclimated for 48h, life history, temperature and survival traits were scored. The same procedure of acclimation followed by trait scoring took place at the F_{10} generation.

Environmental stress resistance

Temperature traits

CT_{MAX} and CT_{MIN} were determined by taking fifteen male and fifteen female 5-7 old flies (from each line) from the F_2 generation and placing them in eppendorf tubes (1.5ml). The eppendorf tubes were placed in a foam "boat" which was then placed in a circulating programmable refrigeration bath (Huber CC-410wl, Huber, Offenburg, Germany) filled with water for CT_{MAX} and with ethanol for CT_{MIN} (to prevent the bath liquid freezing at low temperatures). Fine-gauge (36-SWG) Type T thermocouples connected to a hand held two channel digital thermometer (Fluke 54 series II, Fluke Cooperation, China) were placed inside one of the eppendorf tubes and

on the "boat" to measure the representative temperature inside the tubes (eppendorf temperature) and the temperature on the boat (surface temperature). For CT_{MAX} the water bath was pre-set to 25°C (23°C in the eppendorf) and heated at 0.15°C/min (0.1°C/min ramping experience in the eppendorf). Flies were acclimated to the bath for 15min before ramping started. During ramping, flies were checked intermittently for coordinated movement and CT_{MAX} were scored as the temperature at which the fly lost all mobility (after all spasms has ceased and death ensues) (Lutterschmidt and Huthison, 1997). Flies that stopped all spontaneous movement were poked with a piece of fishing line to ensure that CT_{MAX} was reached. For CT_{MIN} the water bath was also pre-set to 25°C (23°C in the eppendorf) and then cooled at 0.15°C/min (0.1°C/min in the boat). The ramping temperature of 0.1°C/min were chosen in order to replicate the rate used in studies determining lethal temperatures of Stock Center derived *Drosophila* species and thus allowing comparisons to be more readily made with some of the existing literature (Kellermann *et al*., 2012b, Overgaard *et al*., 2011). CT_{MIN} were then scored as the temperature at which the fly lost all mobility. As with CT_{MAX} the flies were poked with a piece of fishing line to ensure that CT_{MIN} were reached.

Heat and Chill survival

Heat and cold survival were determined by placing fifteen male and fifteen female flies (5-7 day old) from each line individually into eppendorf tubes and placing them on the same foam boat setup described above in a pre-set programmable bath as for the thermal traits. Flies were placed at 38°C for 1h for the heat survival treatment and at 0°C for 2h for the cold survival treatment (as per Bechsgaard *et al*., 2013). After the treatments flies were placed into the incubator at 23°C for 24h after which survival was scored.

Survival traits

For desiccation resistance traits, 5-7 day old post-eclosion flies (15 males and 15 females) were placed individually into empty glass vials sealed with gauze. The vials were placed in an airtight desiccator (Duran 250mm, DIN 12491, Germany) and 80-90% of its volume filled with silica gel (Merck). The desiccator was placed in a dark incubator to suppress activity, at 23°C and <10% relative humidity (modified from Kellermann *et al*., 2012a). A hygrochron iButton (Maxim ibutton, Hygrochon Hi-Res (-20°C to +85°C) Acc 0.5°C, USA) was placed inside the desiccator

to confirm the temperature and relative humidity during the experiment. Survival was scored four to five times a day until the first fly died and was then scored hourly until all flies had died.

For starvation resistance 15 male and 15 female 5-7 day old flies per acclimation were placed individually into glass vials containing 5ml 0.5% agar solution (Matzkin *et al*., 2009). The vials were sealed with moist cotton wool, to achieve relatively high level of humidity and placed in an incubator set at 23°C. Separate measurements have confirmed that this will typically achieve constant >95% humidity for several days. Mortality was scored at the same time each day until all flies had died.

As a control for desiccation and starvation resistance 15 male and 15 female five to seven-day old flies were placed in glass vials containing a standard cornmeal medium covered with gauze and placed in a desiccator at 23°C. Deaths were scored twice a week until the first death and then daily until all flies had died.

Statistical analyses

The effect of the acclimation regimes and the number of generations spent in the laboratory on environmental stress resistance of the *Drosophila* species and populations were determined in Rstudio version 1.0.136 (R core team, 2013) and Statistica version 13 (Statsoft, Tulsa, Oklahoma, USA). All data were tested for normality by doing a Shapiro-Wilk normality test. All significance levels for tests were set at $p<0.05$.

Normality was tested by running a Kruskal-Wallis test and homogeneity of variances were determined by plotting the raw residuals over the predicted values. Variances were considered homogeneous if the residuals and the fitted values were uncorrelated. Depending on the outcome of these tests either a factorial ANOVA or generalized linear model (GLM) was run. If the data was non-parametric a generalized linear model factorial ANOVA was run with a poisson distribution and a log-link function. Overdispersion was checked and corrected for, if present.

For the thermal limits of the species data, CT_{MAX} or CT_{MIN} was used as dependent variables in the model and the independent variables were the generation (F_2, $F_{8\text{-}10}$), acclimation (18°C, 23°C, 28°C) and sex (male or female). For the populations of *D. melanogaster* CT_{MAX} and CT_{MIN} were

used as dependent variables and the independent variables were population (Stellenbosch, Citrusdal, Durban or Polokwane), acclimation (18°C, 23°C, 28°C) and sex (male or female).

For heat and cold survival of the species data, a generalized linear model (GLM) with a logit link function and a binomial distribution was run using the "*MASS*" package in R (Venables *et al.*, 2002) to assess the main effects and interaction of generation, acclimation and sex on the proportion survival 24 hours after exposure to a potentially lethal temperature. The same was done for the populations of *D. melanogaster* but the effects of population source, acclimation and sex on the proportion of survival were tested. The assumptions of normally distributed data and independent errors were met for all data. A post hoc ANOVA of the GLM was run to examine significant effects and the main interactions.

For the desiccation and starvation survival experiments, Kaplan-Meier survival curves were drawn using the '*survival*' package in R (Therneau and Grambsch, 2000) and showed the proportion survival over time for the different acclimation regimes (18°C, 23°C and 28°C). The Cox-proportional hazards model, also in the '*survival*' package, was used to determine the dependency of survival time on the predictor variables of desiccation and starvation. For the species, the main effects and interactions of generation, treatment, acclimation and sex on survival over time were determined using the coxph function in the '*survival*' package. For the populations of *D. melanogaster*, the main effects and interactions of origin of population, treatments, acclimation and sex on survival time were also determined using the coxph function. The proportionality of hazards assumption for the cox regression was met in all analyses (cox.zph function R). A post hoc ANOVA of the coxph model was run to examine significant effects and main interactions.

Results

Temperature treatments

Drosophila melanogaster

For CT_{MAX}, the data were normally distributed ($W=0.99$, $p>0.05$) and the variances homogeneous. There was a significant increase in basal resistance as well as plastic responses between the F_2 and F_{10} generations ($F=414.1$, *d.f.*$=1$, $p<0.0001$). There was also a significant

difference between acclimation responses (F=56.1, *d.f.*=2, p<0.0001) with 18°C and 28°C having significantly higher resistance to thermal stressors in comparison to individuals reared at the optimum temperature of 23°C. CT_{MAX} values for males and females also differed significantly with females having a higher CT_{MAX} (F=63.2, *d.f.*=1, p<0.0001). Both generation and acclimation had a significant impact on CT_{MAX} (F=4.2, *d.f.*=2, p<0.05) (Table 1; Figure 3).

For CT_{MIN}, the data was not normally distributed (W=0.98, p<0.05) but the residuals were homogeneous. The factorial GLM indicated a significant increase in CT_{MIN} values between the F_2 and F_{10} generation of *D. melanogaster*, (Wald's χ^2=166.42, *d.f.*=1, p<0.0001). Significant differences were also found between acclimations (Wald's χ^2= 56.1, *d.f.*=2, p<0.008) and the sexes (Wald's χ^2=9.64, *d.f.*=2, p<0.05). A significant interaction effect was found between generation and acclimation (Wald's χ^2=48.6, *d.f.*=2, p<0.05) of CT_{MIN} in *D. melanogaster* (Table 1; Figure 3).

Zaprionus vittiger

For $CT_{MAX,}$ data were non-normal (Shapiro-Wilks: W=0.95, p<0.05) and the variances were heteroscedastic. There was a pronounced decrease in both basal resistance and plastic responses between the F_2 and F_{10} generations (Wald's χ^2=190.3, *d.f.* =1, p<0.0001). There was a significant effect of acclimation (Wald's χ^2=72.46, *d.f.* =2, p<0.0001) but not sex (Wald's χ^2=1.40, *d.f.*=1, p=0.24). Additionally, there was a significant interaction effect between generation and acclimation (Wald's χ^2=20.68, *d.f.*=2, P<0.0001) and between acclimation and sex (Wald's χ^2=17.59, *d.f.*=2, p<0.0002). The interaction between generation, acclimation and sex was significant for CT_{MAX} (Table 1; Figure 3). There was a significant decrease in CT_{MIN} between the F_2 and F_{10} generations (Wald's χ^2=59.18, p<0.05). A significant effect of acclimation (Wald's χ^2=50.58, p<0.05) and sex was detected (Wald's χ^2=32.29, p<0.05). There were significant interaction effects between generation and acclimation (Wald's χ^2=39.82, p<0.05), generation and sex (Wald's χ^2=33.60, p<0.05), acclimation and sex (Wald's χ^2=88.86, p<0.05) as well as generation, acclimation and sex (Wald's χ^2=39.49, p<0.05) (Table 1, Figure 3).

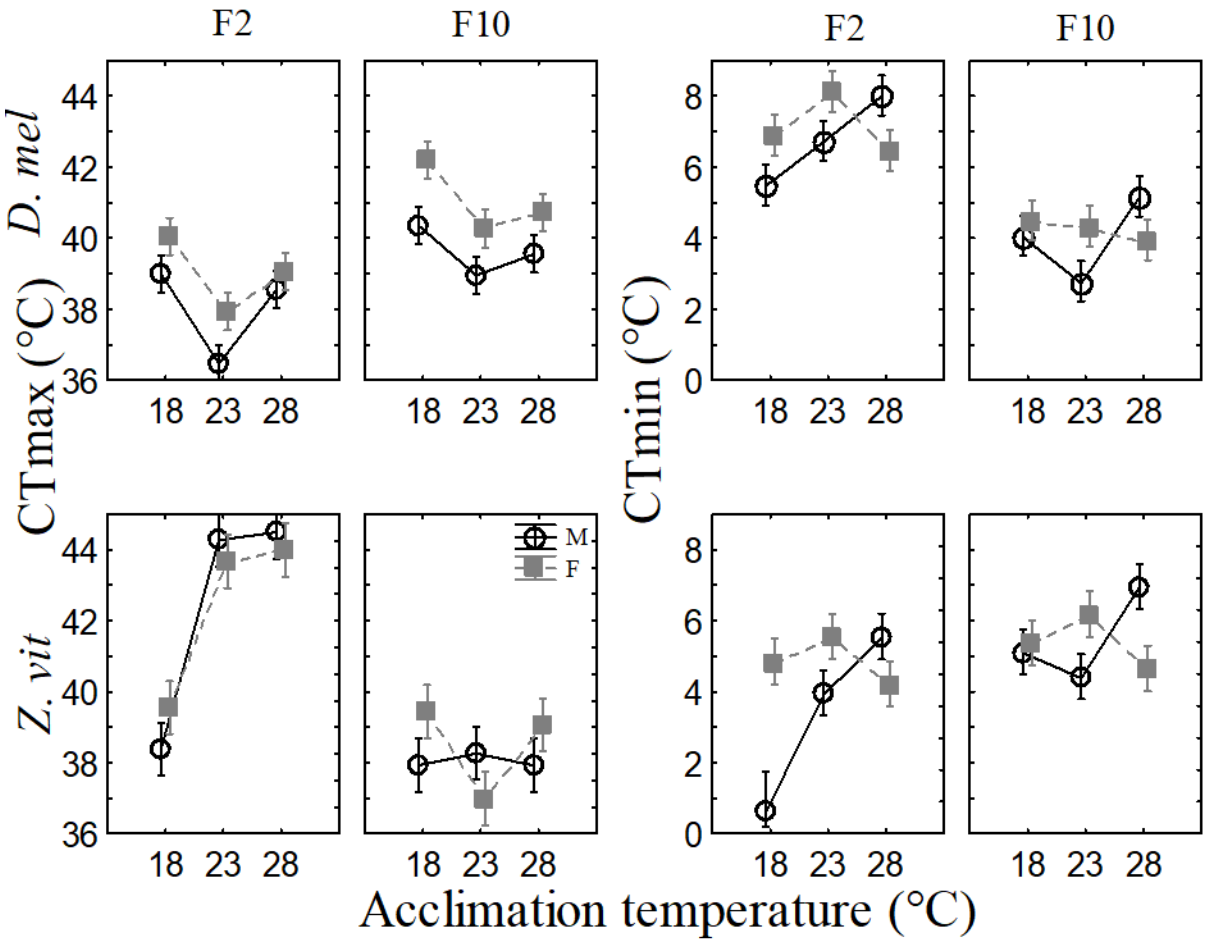

Figure 3: Box and whisker plot (mean ± SD) indicating the results of the temperature treatments performed on the two species *D. melanogaster* (*D. mel*) and *Z. vittiger (Z. vit)*. The figure shows the Critical thermal maximum (CT_{MAX}) and Critical thermal minimum (CT_{MIN}) results of *D. melanogaster* (first row) and *Z. vittiger* (second row) of the F_2 and F_{10} generations for the three acclimations of 18°C, 23°C and 28°C. Males are indicated by the black solid lines and unfilled circles; females are indicated by the grey stippled lines and filled squares.

Table 1: Temperature treatment results (CT_{MAX} and CT_{MIN}) from separate Factorial ANOVA and Generalized linear models (GLM) for species (*D. melanogaster* and *Z. vittiger*) and populations of *D. melanogaster* (significant effects indicated in bold).

Temperature treatments	Effect	F/ χ^2	*d.f*	p
D. melanogaster CT_{MAX}	**Generation**	**141.1**	**1**	**<0.0001**
	Acclimation	**56.1**	**2**	**<0.0001**
	Sex	**63.2**	**1**	**<0.0001**
	Generation * Acclimation	**4.2**	**2**	**<0.05**
	Generation * Sex	2.0	1	0.16
	Acclimation * Sex	1.6	2	0.20
	Generation*Acclimation*Sex	0.9	2	0.40
D. melanogaster CT_{MIN}	**Generation**	**166.42**	**1**	**<0.0001**
	Acclimation	**9.64**	**2**	**0.008**
	Sex	**9.64**	**2**	**<0.05**
	Generation * Acclimation	**20.68**	**2**	**<0.0001**
	Generation * Sex	0.16	1	0.69
	Acclimation * Sex	**45.28**	**2**	**<0.0001**
	Generation*Acclimation*Sex	5.36	2	0.069
Z. vittiger CT_{MAX}	**Generation**	**190.34**	**1**	**<0.0001**
	Acclimation	**72.46**	**2**	**<0.0001**
	Sex	1.40	1	0.24
	Generation * Acclimation	**104.42**	**2**	**<0.0001**
	Generation * Sex	0.81	1	0.37
	Acclimation * Sex	**17.59**	**2**	**<0.0002**
	Generation*Acclimation*Sex	5.24	2	0.072
Z. vittiger CT_{MIN}	**Generation**	**59.18705**	**1**	**<0.0001**
	Acclimation	**50.58265**	**2**	**<0.0001**
	Sex	**32.28649**	**1**	**<0.0001**
	Generation * Acclimation	**39.82445**	**2**	**<0.0001**
	Generation * Sex	**33.59683**	**1**	**<0.0001**
	Acclimation * Sex	**88.85645**	**2**	**<0.0001**
	Generation*Acclimation*Sex	**39.49350**	**2**	**<0.0001**
Populations CT_{MAX}	**Population**	**114.30**	**3**	**<0.0001**
	Acclimation	**12.65**	**2**	**<0.002**
	Sex	**4.06**	**1**	**<0.05**
	Population*Acclimation	**70.18**	**6**	**<0.0001**

	Population*Sex	**33.91**	**3**	**<0.0001**
	Acclimation*Sex	1.85	2	0.40
	Population*Acclimation*Sex	**51.05**	**6**	**<0.0001**
Populations CT_{MIN}	**Population**	**220.72**	**3**	**<0.0001**
	Acclimation	**27.7046**	**2**	**<0.0001**
	Sex	0.0057	1	0.94
	Population*Acclimation	**18.2168**	**6**	**0.006**
	Population*Sex	**144.4887**	**3**	**<0.0001**
	Acclimation*Sex	**10.3262**	**2**	**0.006**
	Population*Acclimation*Sex	**49.7351**	**6**	**<0.0001**

Heat and Chill survival

Drosophila melanogaster

The GLM indicated acclimation to have a significant effect on heat survival (F=17.37, *d.f.*=2, p<0.0001). Significant interactions were found between the generations and acclimation (F=12.43, *d.f.*=2, P<0.0001), acclimation and sex (F=4.08, *d.f.*=2, p<0.02) as well as generation, acclimation and sex (F=10.28, *d.f.*=2, p<0.0001). Flies acclimated to 23°C and 28°C had the highest percentage of individuals alive after 24h with the F_2 males from the 23°C acclimation having the highest percentage of individuals still alive (80%). For F_{10} flies, the highest survival occurred in the 28°C acclimation group with 18°C having no survival and 23°C having some survival but only in the females. For F_2 flies, highest survival was observed in the 23°C and 28°C groups with the 18°C group having the lowest survival (Table 2; Figure 4).

For cold survival, acclimation also had a significant effect (F=7.22, *d.f.*=2, p<0.001). Significant interactions were found between generations and acclimation (F=33.28, *d.f.*=2, P<0.0001), generation and sex (F=11.63, *d.f.*=1, p<0.0009), acclimation and sex (F=8.51, *d.f.*=2, p<0.0004) as well as generation, acclimation and sex (F=5.28, *d.f.*=2, p<0.006) (Table 2). In F_2 flies, highest survival was observed in the 23°C and 28°C acclimation groups with the 18°C group having the least survival (as seen in the heat survival results). In the F_{10} generation however there was higher survival in the 18°C group than in the other two acclimation groups (Figure 4).

Zaprionus vittiger

Generation (F=21.54, *d.f.*=1, p<0.0001), acclimation (F=19.55, *d.f.*=2, p<0.0001) and sex (F=21.54, *d.f.*=1, p<0.0001) all significantly influenced heat survival in *Z. vittiger*. Significant interactions were also found between generation and acclimation (F=12.01, *d.f.*=2, p<0.0001), generation and sex (F=7.75, *d.f.*=1, p<0.006), acclimation and sex (F=19.76, *d.f.*=2, p<0.0001) and generation, acclimation and sex (F=9.85, *d.f.*=2, p<0.0001) (Table 2). F_2 flies had a higher survival in the 18°C and 23°C acclimation groups compared to $F_{8\text{-}10}$ flies. In the 28°C acclimation group $F_{8\text{-}10}$ females had the highest survival and $F_{8\text{-}10}$ males the lowest survival. There were also significant differences between the sexes in the $F_{8\text{-}10}$ generation with females being more tolerant in the 18°C and 28°C acclimations and males being more tolerant for the 23°C acclimation (Figure 4).

For chill survival, acclimation (F=6.54, *d.f.*=2, p<0.002) and sex (F=6.46, *d.f.*=1, p<0.02) had a significant effect on recovery in *Z.vittiger*. In addition, there were significant interactions between all groups except for acclimation and sex (F=1.19, *d.f.*=2, p=0.31) (Table 2). In the F_2 generation, 18°C had the highest survival followed by 28°C and lastly 23°C. In the $F_{8\text{-}10}$ generation, however the highest survival was in the 28°C group followed by 23°C and lastly 18°C. In the F_2 generation, males had higher survival than females in the 18°C and 28°C group; and in the 23°C survival were similar. For the $F_{8\text{-}10}$ generation males and females had similar survival at 18°C acclimation, males had the highest survival at 23°C and females had the highest survival at 28°C (Figure 4).

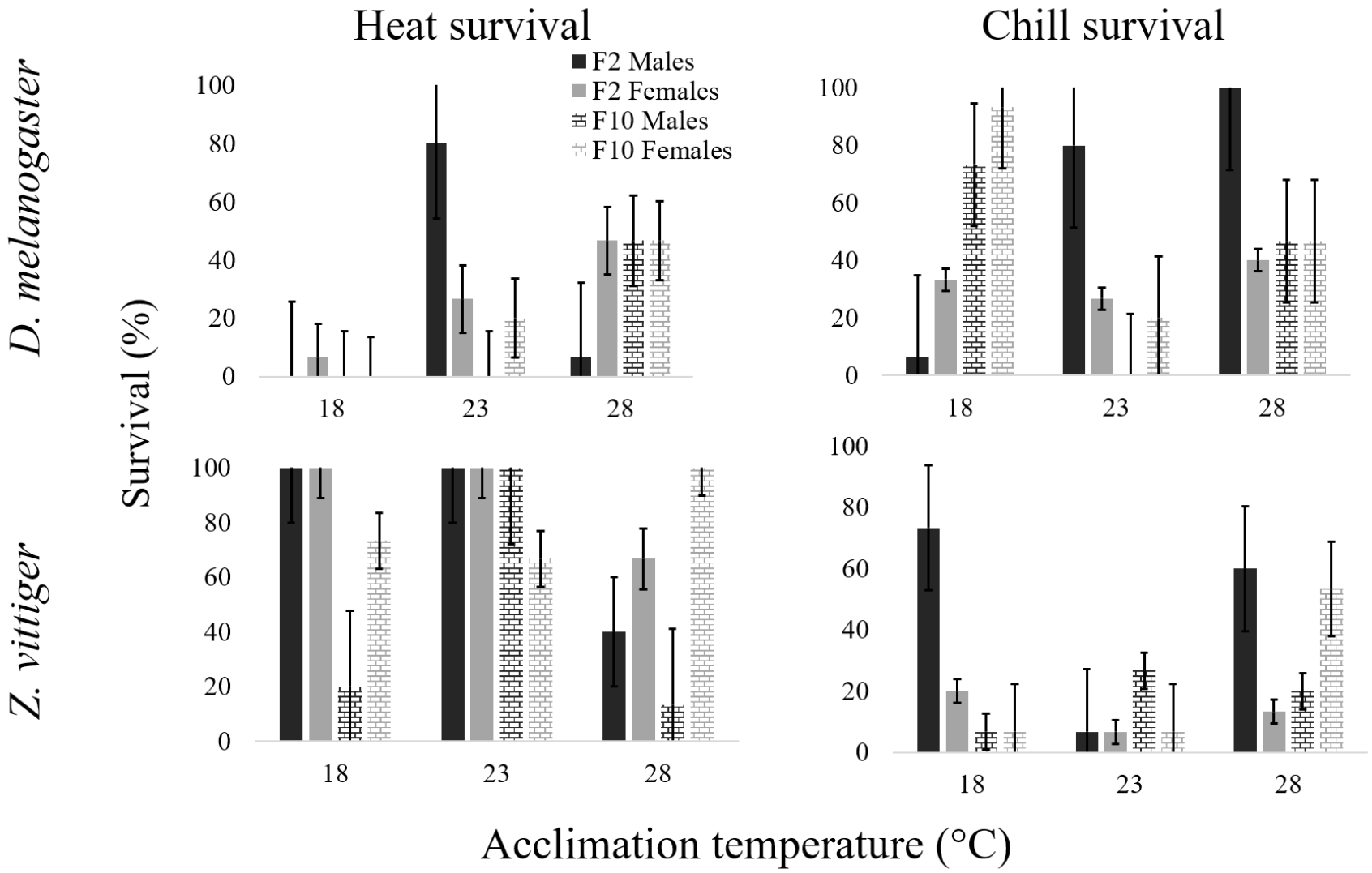

Figure 4: Column graph indicating the mean survival (%) and standard error following heat (38°C for 1hour) and cold survival (0°C for 2hours) treatments for *Drosophila melanogaster* and *Zaprionus vittiger*. Mean survival by Generation (F_2=second generation and F_{10}= tenth generation), acclimation (18°C, 23°C and 28°C) and sex (Males and Females) are shown

Table 2: Heat and cold survival results from generalized linear models for species (*D. melanogaster* and *Z. vittiger*) and populations of *D. melanogaster* (Significant effects indicated in bold).

Heat survival	Effect	F	*d.f.*	p
D. melanogaster	Generation	2.87	1	0.09
	Acclimation	**17.37**	**2**	**<0.0001**
	Sex	0.18	1	0.68
	Generation*Acclimation	**12.43**	**2**	**<0.0001**
	Generation*Sex	0.71	1	0.40
	Acclimation*Sex	**4.08**	**2**	**<0.02**
	Generation*Acclimation*Sex	**10.28**	**2**	**<0.0001**
Z. vittiger	**Generation**	**21.54**	**1**	**<0.0001**
	Acclimation	**19.55**	**2**	**<0.0001**
	Sex	**21.54**	**1**	**<0.0001**
	Generation*Acclimation	**12.01**	**2**	**<0.0001**
	Generation*Sex	**7.75**	**1**	**<0.006**
	Acclimation*Sex	**19.76**	**2**	**<0.0001**
	Generation*Acclimation*Sex	**9.85**	**2**	**<0.0001**
Populations	**Population**	**7.00**	**3**	**<0.0002**
	Acclimation	**4.041**	**2**	**<0.02**
	Sex	**18.33**	**1**	**<0.0001**
	Population*Acclimation	**16.26**	**6**	**<0.0001**
	Population*Sex	**6.53**	**3**	**<0.0003**
	Acclimation*Sex	**18.99**	**2**	**<0.0001**
	Population*Acclimation*Sex	**8.22**	**6**	**<0.0001**
Cold survival				
D. melanogaster	Generation	0.00	1	1.00
	Acclimation	**7.22**	**2**	**<0.001**
	Sex	1.30	1	0.26
	Generation*Acclimation	**33.28**	**2**	**<0.0001**
	Generation*Sex	**11.63**	**1**	**<0.0009**
	Acclimation*Sex	**8.51**	**2**	**<0.0004**
	Generation*Acclimation*Sex	**5.28**	**2**	**<0.006**
Z. vittiger	Generation	3.09	1	0.08
	Acclimation	**6.54**	**2**	**<0.002**
	Sex	**6.46**	**1**	**<0.02**
	Generation*Acclimation	**7.23**	**2**	**<0.001**
	Generation*Sex	**11.05**	**1**	**<0.002**
	Acclimation*Sex	1.19	2	0.31

	Generation*Acclimation*Sex	**6.92**	**2**	**<0.002**
Populations	**Population**	**18.43**	**3**	**<0.0001**
	Acclimation	**6.87**	**2**	**<0.002**
	Sex	1.40	1	0.24
	Population*Acclimation	**15.56**	**6**	**<0.0001**
	Population*Sex	**20.07**	**3**	**<0.0001**
	Acclimation*Sex	0.42	2	0.66
	Population*Acclimation*Sex	**13.55**	**6**	**<0.0001**

Survival treatments

Drosophila melanogaster

The Cox-proportional hazards model showed no difference in survival between the generations (χ^2=0.018, *d.f.*= 1, p>0.89). Although there were no differences between the generations overall, differences were evident in the starvation treatments where the 18°C individuals in the F_2 had a LT_{50} of 160.45 versus $F_{8\text{-}10}$ at 67.17, 23°C individuals at F_2 had a LT_{50} of 230.08 versus $F_{8\text{-}10}$ at 53.85 and 28°C F_2 survived 156.84 versus $F_{8\text{-}10}$ at 51.18 and thus there was a significant decrease between the generations for starvation resistance (χ^2=137.11, *d.f.*=1, p<0.0001). The model indicated significant differences between the treatments (χ^2=830.64, *d.f.*=2, p<0.0001). The desiccation treatment survived the shortest amount of time (median=14.5 h), followed by starvation (median=105.5 h) and then the control (median=519.5) (χ^2=830.64, *d.f.*=2, p<0.0001). There was no difference between the acclimation groups (χ2=1.4052, *d.f.*=2, p=0.50), but there were differences between the sexes (χ^2=28.38, *d.f.*=28.381 p<0.0001) for the starvation treatment. In both generations females survived significantly longer than males except in the 28°C acclimation of the F_2 generation where males performed better than females (Figure 5). Generation, treatment, acclimation and sex had an effect on the survival rate of *D. melanogaster* (Table 3; Figure 6a; Figure 7).

Zaprionus vittiger

The Cox-proportional hazards model indicated a significant difference between the generations (χ^2=56.21, *d.f.*=1, p<0.0001), treatments (χ^2=586.11, *d.f.*=1 p<0.0001) and acclimations (χ^2=17.56, *d.f.*=2, p<0.0002) in *Z. vittiger*. Significant interaction effects were detected between generation and acclimation (χ^2=43.15, *d.f.*=2, p<0.0001) as well as generation, treatment, and acclimation (χ^2=6.09, *d.f.*=2, p<0.05) (Table 3). The basal resistance for the desiccation, starvation and the control of the F_2 generation was significantly higher than that of the F_{10} generation in *Z. vittiger*. The acclimation responses of the F_2 generation were also higher than that of the $F_{8\text{-}10}$ generation. *Zaprionus vittiger* flies that underwent desiccation trials had an LT_{50} basal survival at the F_2 generation of 37 hours versus 16 hours at the $F_{8\text{-}10}$ generation, those undergoing starvation trials had an LT_{50} basal survival value of 140 hours at the F_2 versus 73 hours at $F_{8\text{-}10}$. The control flies had a basal LT_{50} survival of 452 hours at F_2 versus 431 hours at $F_{8\text{-}10}$. Plastic responses were lower than the basal resistance in the F_2 generation for desiccation and starvation resistance. However, for the control in the F_2 generation the 18°C acclimation and the basal (23°C) were similar and the 28°C acclimation flies survived the longest. For the F_{10} generation the 18°C acclimation had the highest survival rate for both desiccation and starvation resistance. The control group of the F_{10} also has the highest survival at the 28°C acclimation followed by 23°C and 18°C (Figure 6b; Figure 7)

Table 3: Survival treatment results from a Cox-proportional hazards model, indicating χ^2, degrees of freedom and p-value for *D. melanogaster*, *Z. vittiger* and the comparison between the four *D. melanogaster* populations (Significant effects indicated in bold).

Species	Effect	χ^2	*d.f.*	p
D. melanogaster	Generation	0.0181	1	0.89
	Treatment	**830.64**	**2**	**<0.0001**
	Acclimation	1.4052	2	0.4952
	Sex	**28.381**	**1**	**<0.0001**
	Generation*Treatment	**121.898**	**2**	**<0.0001**
	Generation*Acclimation	**20.836**	**2**	**<0.0001**
	Treatment*Acclimation	**27.8008**	**4**	**<0.0001**
	Generation*Sex	0.7468	1	0.39
	Treatment*Sex	**7.2803**	**2**	**<0.05**
	Acclimation*Sex	**9.2319**	**2**	**<0.01**
	Treatment*Acclimation*Sex	**24.76**	**4**	**<0.0001**
	Generation*Treatment*Acclimation	**33.91**	**4**	**<0.0001**
	Generation*Treatment*Sex	**27.7158**	**2**	**<0.0001**
	Generation*Acclimation*Sex	0.41	2	0.814
	Generation*Treatment*Acclimation*Sex	**10.50**	**4**	**<0.05**
Z. vittiger	**Generation**	**56.21**	**1**	**<0.0001**
	Treatment	**586.11**	**2**	**<0.0001**
	Acclimation	**17.5567**	**2**	**<0.0002**
	Sex	2.9741	1	0.085
	Generation*Treatment	0.7727	1	0.38
	Generation*Acclimation	**43.1480**	**2**	**<0.0001**
	Generation*Sex	1.4025	1	0.24
	Treatment*Acclimation	4.1159	2	0.13
	Treatment*Sex	0.2645	1	0.61
	Acclimation*Sex	0.3790	2	0.83
	Treatment*Acclimation*Sex	1.0746	2	0.58
	Generation*Treatment*Acclimation	**6.0932**	**2**	**<0.05**
	Generation*Treatment*Sex	0.0111	1	0.92
	Generation*Acclimation*Sex	0.7922	2	0.67
	Generation*Treatment*Acclimation*Sex	3.1029	2	0.21
Populations	**Population**	**23.02**	**4**	**<0.0002**
	Treatment	**1990.76**	**2**	**<0.0001**
	Acclimation	3.44	2	0.18
	Sex	**25.87**	**1**	**<0.0001**
	Population*Treatment	**305.46**	**6**	**<0.0001**
	Population*Acclimation	**23.25**	**6**	**<0.001**

Treatment*Acclimation	**31.92**	**4**	**<0.0001**
Population*Sex	5.62	3	0.13
Treatment*Sex	**9.20**	**2**	**<0.05**
Acclimation*Sex	**8.45**	**2**	**<0.05**
Population*Treatment*Acclimation	**65.38**	**12**	**<0.0001**
Population*Treatment*Sex	**29.37**	**6**	**<0.0001**
Population*Acclimation*Sex	**14.00**	**6**	**<0.05**
Treatment*Acclimation*Sex	5.66	4	0.23
Population*Treatment*Acclimation*Sex	**22.24**	**12**	**<0.05**

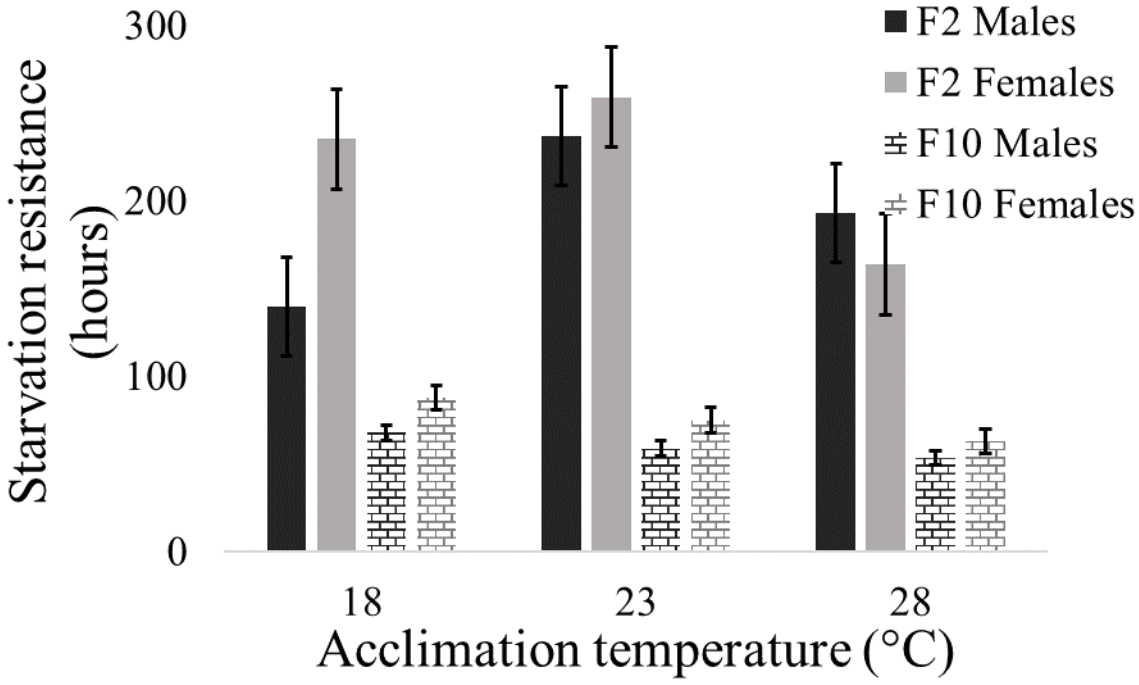

Figure 5: Column graph indicating the mean starvation resistance survival (hours) and its standard error in *D. melanogaster* at the three acclimations (18°C; 23°C; 28°C), for the two generations (F_2; F_{10}) indicating male and female responses

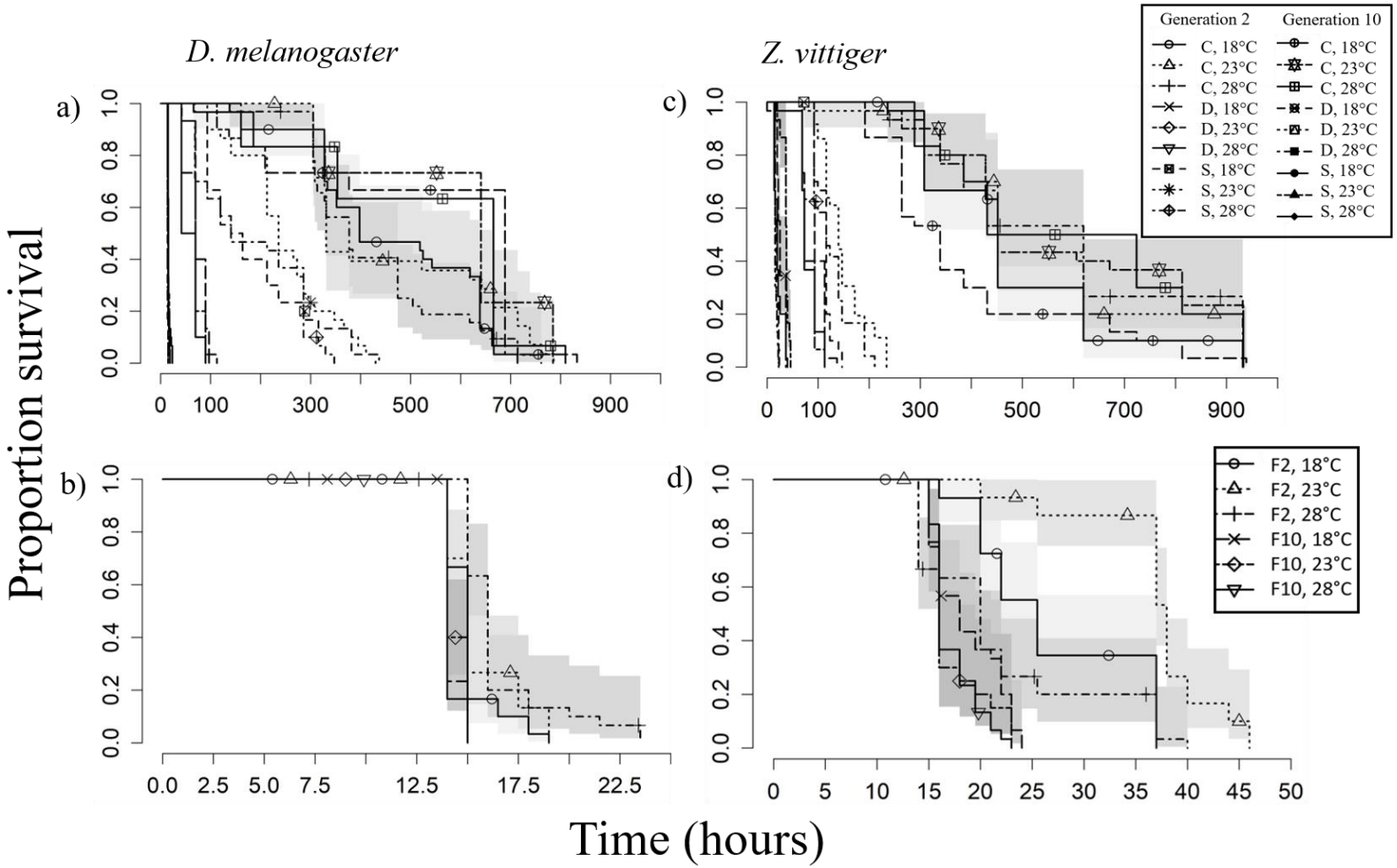

Figure 6: Kaplan-Meier Survival curves indicating a) the survival curves with standard error (grey area) for *D. melanogaster* depicting both generations (F_2 and F_{10}), all treatments (C=Control, D=Desiccation, S=Starvation) and the three acclimations (18°C, 23°C, 28°C), b) the survival curve with standard error for the Desiccation treatment of *D. melanogaster* depicting both generations (F_2 and F_{10} and acclimations (18°C, 23°C, 28°C)), c) the survival curve with standard error for *Z. vittiger* depicting both generations (F2 and F10), all treatments (C=Control, D=Desiccation, S=Starvation) and the three acclimations (18°C, 23°C and 28°C) and d) the survival curve with standard error for the Desiccation treatment of *Z. vittiger* depicting both generations (F_2 and F_{10} and acclimations (18°C, 23°C and 28°C)).

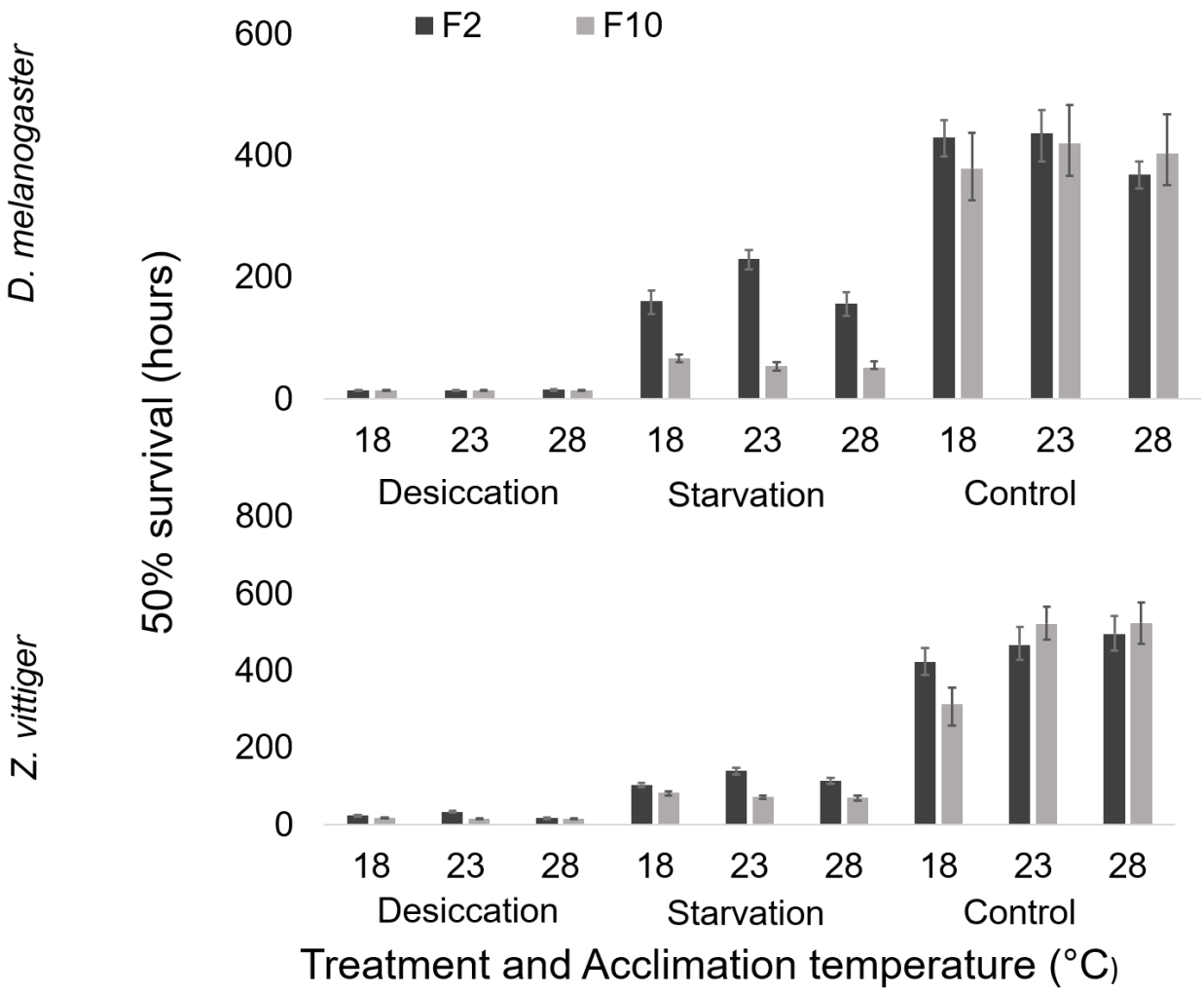

Figure 7: Column graph showing 50% survival (in hours) and 95% confidence limits for desiccation and starvation resistance as well as a control at 18°C, 23°C and 28°C for the second and tenth generation (F_2 and F_{10}) in *D. melanogaster* and Z. *vittiger*.

Populations of *D. melanogaster*

Temperature treatments

For CT_{MAX}, a Shapiro-Wilk normality test was performed, the data were normally distributed (W=0.028, p>0.05) and variances were homogeneous. There were significant differences between the populations (F=114.30, *d.f.*=3, p<0.0001), acclimation (F=12.65, *d.f.*=2, p<0.002) and sex (F=4.06, p<0.05). There were also significant interactions between population and acclimation (F=70.18, *d.f.*=6, p<0.0001), population and sex (F=33.91, *d.f.*=3, p<0.0001) and population, acclimation, and sex (F=51.05, *d.f.*=6, p<0.0001) (Table 1). For CT_{MAX}, the basal resistance (23°C) of the Stellenbosch population was the highest, followed by Citrusdal, Durban and Polokwane. There were also diverse plastic responses (acclimations 18°C and 28°C) between the populations. The 18°C acclimation in the Citrusdal population was similar to the basal resistance (23°C) but for the 28°C acclimation group females had a very low resistance (mean CT_{MAX} of 35°C) whilst the males had a higher CT_{MAX} of 38°C. For the Polokwane population, the plastic responses were very similar to the basal plastic response with a 1°C increase or decrease. Stellenbosch *D. melanogaster* had higher resistance in the 18°C and 28°C groups. Lastly, the Durban population looked similar to the Citrusdal population with the 18°C group being similar to the 23°C group and the 28°C group being very different, but here the females were more resistant with females having a CT_{MAX} of 39°C and males having a CT_{MAX} of 36°C (Figure 8a).

For CT_{MIN}, a generalized linear model was run after data were found to not be normally distributed (W=0.99, p<0.05) and the variances heterogeneous. Populations were significantly different from each other (χ^2=220.72, *d.f.*=3, p<0.0001) as well as the different acclimation groups (χ^2=27.70, *d.f.*=2, p<0.0001). There were also significant interactions between population and acclimation (F=18.2168, *d.f.*=6, p=0.006), population and sex (F=144.4887, *d.f.*=3, p<0.0001), acclimation and sex (F=10.3262, *d.f.*=2, p=0.006) and population, acclimation and sex (F=49.7351, *d.f.*=6, p<0.0001). Thus, population, acclimation and sex all had significant effects on CT_{MIN} (Table 1). Durban had the lowest CT_{MIN} and Stellenbosch the highest. Citrusdal and Polokwane averaged between 3°C and 7°C. Resistance of the plastic responses (18°C and 28°C) was higher than the basal resistance in the Citrusdal *D. melanogaster* population. In addition, resistance of the female flies was lower than the males in the Citrusdal population

(higher CT_{MIN}). Durban had the opposite response to Citrusdal with males having lower resistance (higher CT_{MIN}) (Figure 8). In the Durban population plastic responses also had higher resistance than the 23°C group, except for the 23°C females which had a lower resistance than the 23°C group. The Stellenbosch *D. melanogaster* had a lower basal resistance in both sexes except for 28°C acclimated males which had a higher basal resistance than the 23°C group. Lastly for the Polokwane population, the males had higher resistance in the acclimation groups 18°C and 28°C than in the 23°C group whilst the females had higher resistance in the 23°C group than in the plastic groups (Figure 8; Table 1).

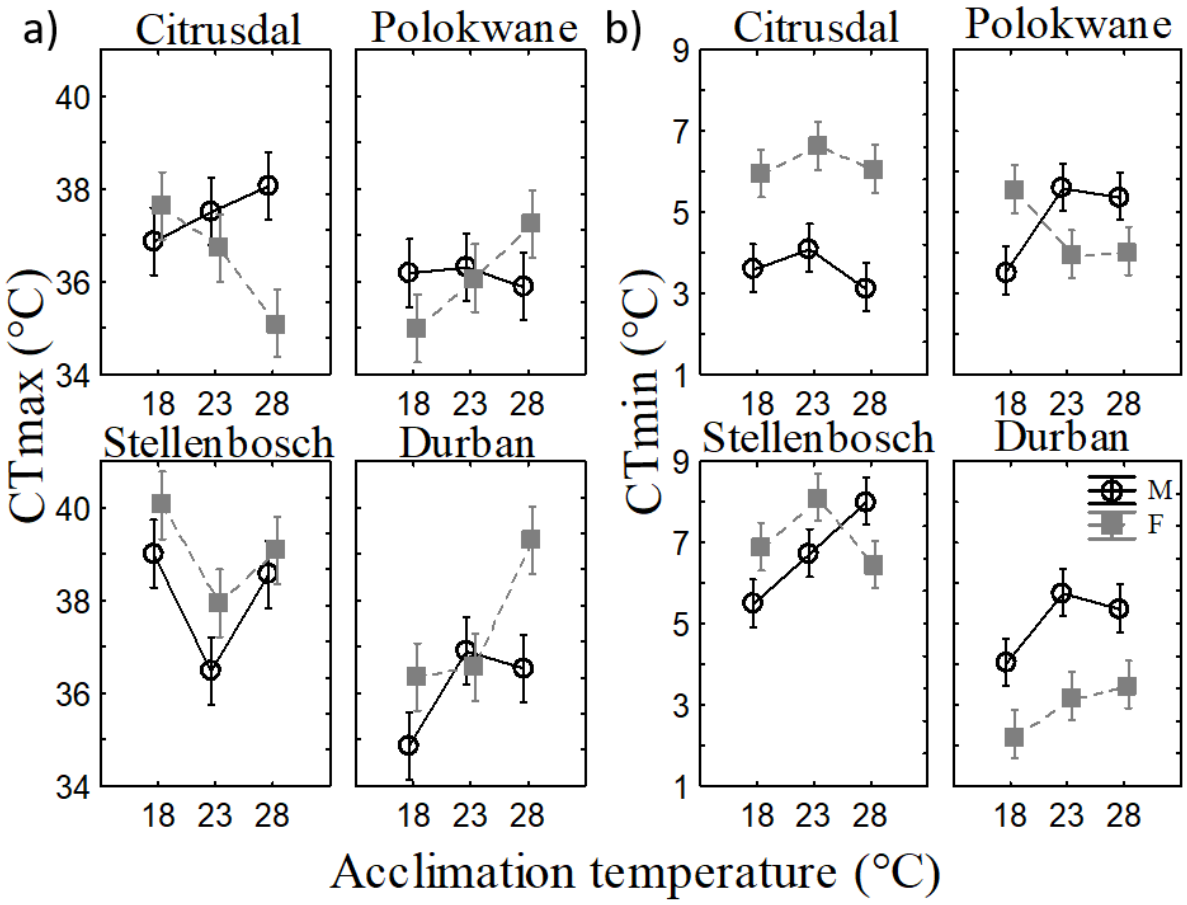

Figure 8: Box and whisker plots indicating mean and standard deviation of a) CT_{MAX} and b) CT_{MIN} of the four *D. melanogaster* populations (Citrusdal, Polokwane, Stellenbosch and Durban), indicating acclimations (18°C, 23°C and 28°C) and sex (Male and Female). The figure shows the Critical thermal maximum (CT_{MAX}) (first column) and Critical thermal minimum (CT_{MIN}) (second column) results of the four *D. melanogaster* of the F_2 generation at the three acclimations of 18°C, 23°C and 28°C. Males are indicated by the black solid lines and unfilled circles; females are indicated by the grey stippled lines and filled squares.

Heat and Chill survival

For heat survival, all effects and interactions were significant (Table 2). Significant differences were found between the populations, with Citrusdal having high survival in the 18°C acclimation group but no survival in the other two acclimation groups. In contrast, Polokwane had low survival over all acclimation groups and in both sexes. Durban females had high survival at 18°C but there was low survival in 18°C males and in 23°C and 28°C for both sexes. Lastly Stellenbosch had high survival at 23°C for males and 28°C for females but little to no survival in the rest of the groups. There were significant differences between the sexes with females having an increased survival percentage in the 18°C and 28°C groups but males having a higher percentage survival in the 23°C group overall (Figure 9).

In cold survival assays, populations ($F=18.43$, *d.f.*=3, $p<0.0001$) and acclimation ($F=6.87$, *d.f.*=2, $p<0.002$) had a significant effect on survival. There were also significant interactions between all the groups, except for acclimation and sex ($F=0.42$, *d.f.*=2, $p=0.66$) (Table 2). There were significant differences between the populations with Polokwane males having the highest survival at 18°C followed by Durban females. At 23°C, Stellenbosch male flies had the highest survival, followed by Durban females and at 28°C Stellenbosch males had the highest survival followed by Durban females (Figure 9).

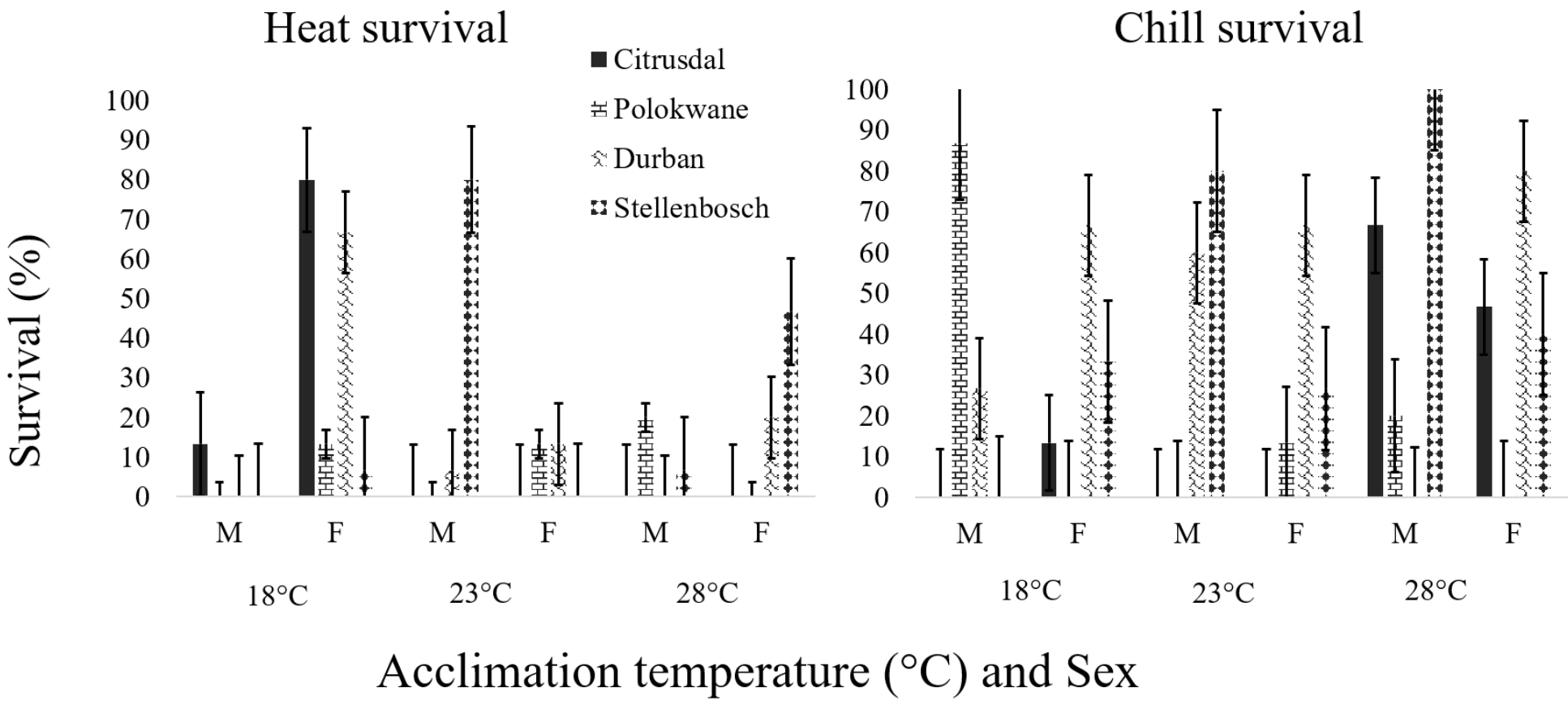

Figure 9: Column graph indicating the mean survival (%) and standard error 24 hours after heat (38°C for 1hour) and cold (0°C for 2 hrs) survival treatments for the four *Drosophila melanogaster* populations (Citrusdal, Polokwane, Durban and Stellenbosch). Mean survival for acclimation (18°C, 23°C and 28°C) and sex (Males and Females) are shown.

Survival treatments

The Cox-proportional hazards model showed a significant difference between the populations (χ^2=23.02, *d.f.*=4, p<0.0002), treatments (χ^2=1990.76, *d.f.*=2, p<0.0001) and sexes (χ^2=25.87, *d.f.*=1, p<0.0001). There were also significant interactions between all groups except populations and sex (χ^2=5.65, *d.f.*=3, p=0.13); and treatments, acclimations and sex (χ^2=5.66, *d.f.*= 4, p=0.23) (Table 3).

There was no clear difference between the desiccation treatments for the different populations, with all of them surviving at least 24h but with differing patterns of survival (Figure 10). There was a clear difference between the starvation trials for Stellenbosch and those for Citrusdal, Polokwane and Durban, with Stellenbosch populations surviving much longer (up to about 400 hours). The Citrusdal and Polokwane populations survived up to 140 hours and the Durban population up to ~100 hours (Figure 11; Figure 12). In addition, controls for Durban and Stellenbosch populations survived until about 800 hours whilst those of Citrusdal and Polokwane survived ~700 hours (Figure 11; Figure 12).

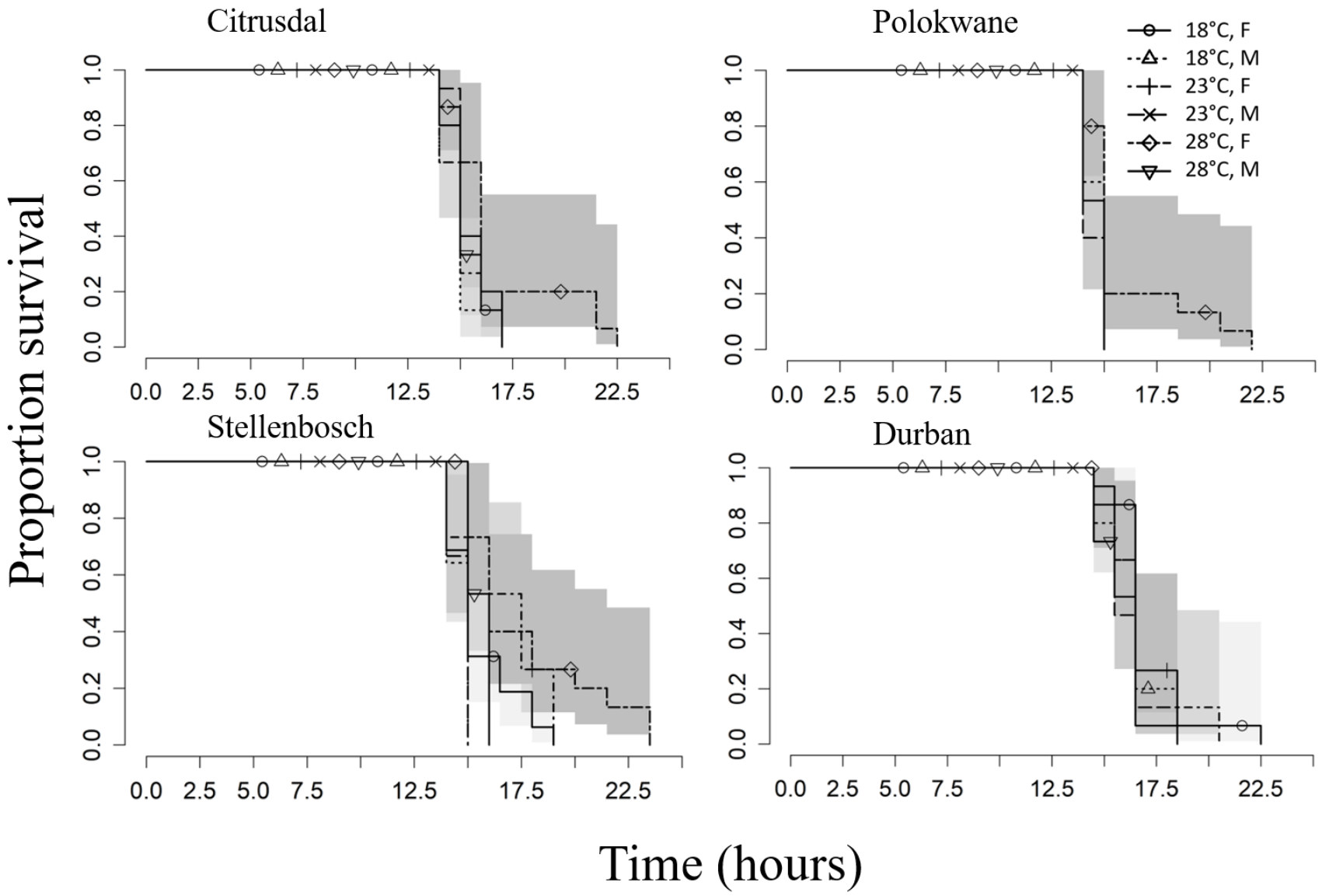

Figure 10: The survival curve with standard error for the Desiccation treatments of the four *D. melanogaster* populations (Citrusdal, Polokwane, Stellenbosch and Durban) depicting all acclimations (18°C, 23°C and 28°C) and both sexes (Male and Female).

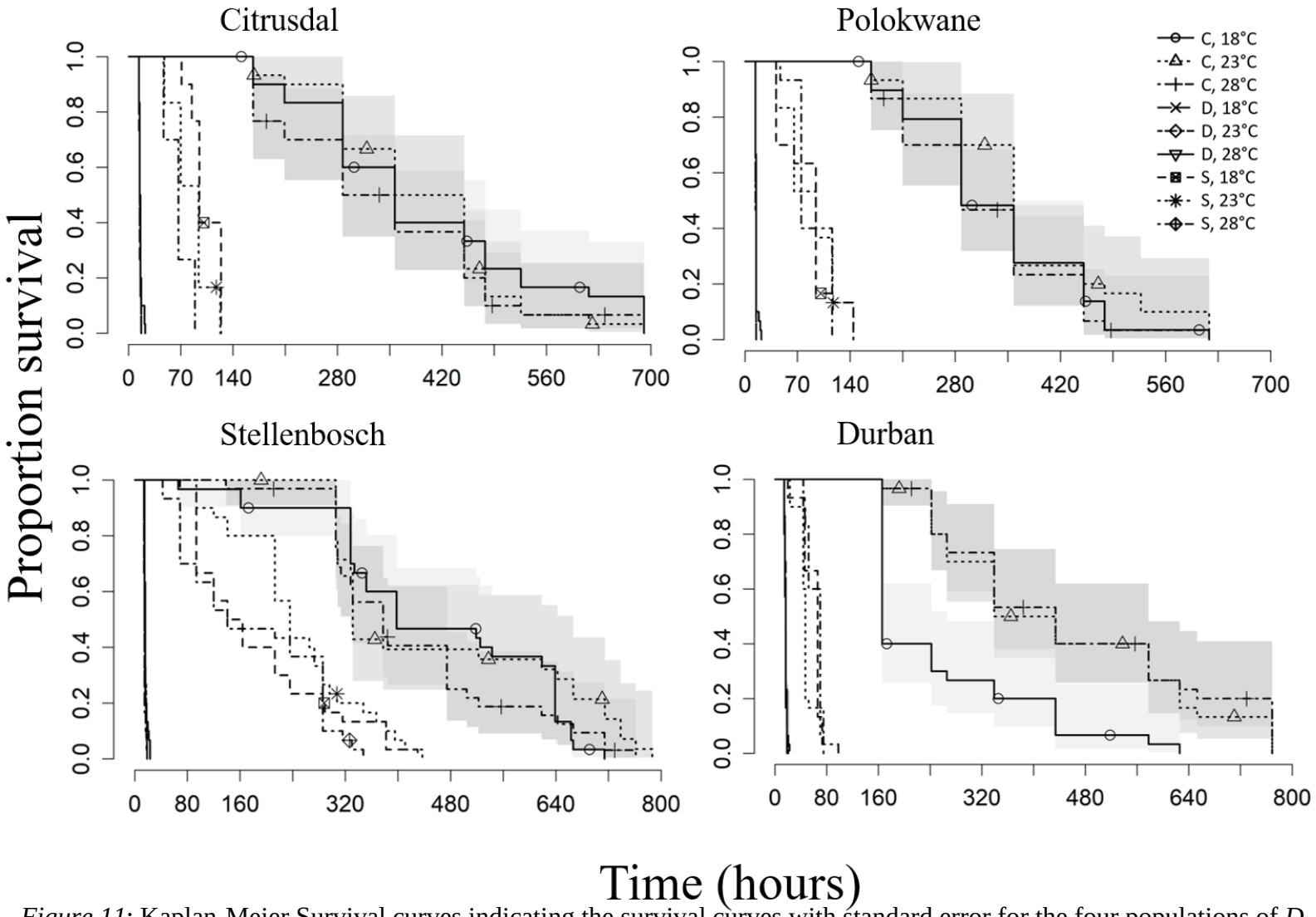

Figure 11: Kaplan-Meier Survival curves indicating the survival curves with standard error for the four populations of *D. melanogaster* namely Citrusdal, Polokwane, Stellenbosch and Durban, including all treatments (C=Control, D=Desiccation, S=Starvation) and all acclimations (18°C, 23°C and 28°C).

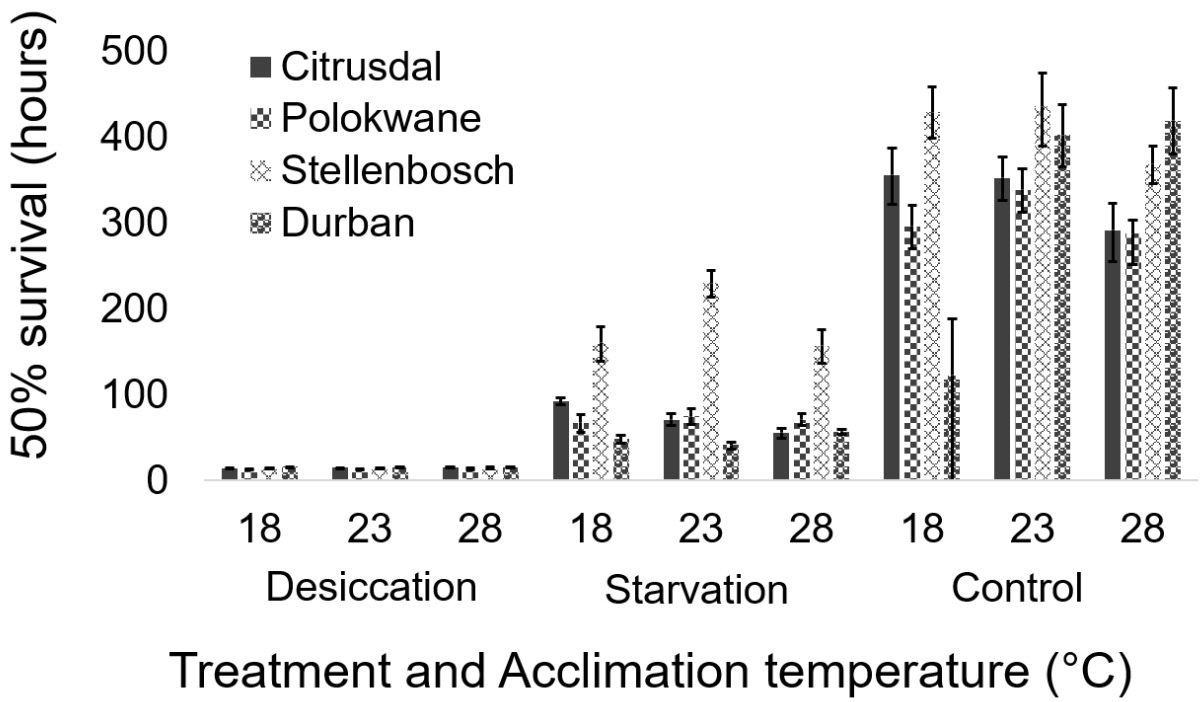

Figure 12: Column graph showing 50% survival (in hours) and 95% confidence limits for desiccation and starvation resistance as well as a control acclimated at 18°C, 23°C and 28°C for the four populations of *D. melanogaster* (Citrusdal, Polokwane, Stellenbosch and Durban).

Discussion

In this study I set out to determine whether there is any consistent variation in environmental stress resistance traits and their plastic responses between the F_2 and F_{8-10} generations of *D. melanogaster* and *Z. vittiger* in order to determine whether time spent in culture may have an impact on estimates of environmental stress resistance and the phenotypic plasticity of such traits. A further objective was to determine whether there was any significant variation in environmental stress resistance estimates of four geographically distinct populations of *D. melanogaster*. This was done to ascertain whether the geographic source of a particular species may have a further impact on environmental stress resistance estimates. My results show that there was indeed significant variation in environmental stress resistance early in culture compared to those same traits measured at a later time point in culture for the two species examined. Notably, however, the direction of these changes was different for the two species. *Drosophila melanogaster* had an unexpected and large increase in basal resistance over time in culture and *Z. vittiger* showed a pronounced but expected decrease in basal resistance. The results of *D. melanogaster* run counter to the expectation that increased time spent in culture will generally lead to decreased resistance to environmental stressors (Hoffmann *et al*., 2001b; Sgró and Partridge, 2000). The increased resistance in *D. melanogaster* could indicate that the laboratory culture is in fact a favorable environment due to a more nutritious diet leading to increased resistance to the stressors measured (Andersen *et al.*, 2010; Kristensen *et al.*, 2016; Trajkovic *et al*., 2017) or a release from various other field stressors (e.g. infection, predation).

Significant differences were found between the environmental stress resistance traits of the four *D. melanogaster* populations sampled across South Africa. This was not altogether surprising as clines of stress resistance or variation of traits among populations, correlating with local climate variation, are well documented (Hoffmann and Harshman, 1999; Hoffmann *et al.*, 2002; Hoffmann *et al.*, 2005b; Matzkin *et al.*, 2007; Sinclair *et al*., 2012; van Heerwaarden and Sgrò, 2011; van Heerwaarden *et al*., 2009). It is unclear precisely what is driving this inter-population variation in my sampling locations, but it is likely to be the outcome of a combination of population genetics (dispersal) factors and local climate factors selecting for fitter genotypes. The microclimate of an insect refers to the conditions that the particular insect experience and

can differ substantially from the local climate owing to biophysical factors (e.g. thermal inertia). Insects frequently display body temperatures that are substantially different from air temperature owing to a variety of behavioural and habitat specific interactions on thermal balance (Andrew & Terblanche, 2013; Woods *et al*., 2015). Nevertheless, macro- rather than micro-climate may be a significant driver of evolutionary variation in traits of stress resistance. Adaptation in thermotolerance for *D. melanogaster* along a microclimate gradient in 'Evolution Canyon' in Israel has been found (Hübner *et al*., 2013; Rashkovetsky *et al*., 2006) and thus the differences between my populations could be argued to be a result of climatic differences at the microclimate scale but as we do not yet have information on the microclimates of the *Drosophila* in our study this cannot be demonstrated (Duffy *et al*., 2015). Thus, I argue that environmental stress resistance estimates obtained from Stock Center lines (typically lines that have spent many and often unknown numbers of generations in culture) do not necessarily represent wild caught flies and that values obtained from one population likely do not represent the entire species. It is also clear from the acclimation treatments that there is considerable plasticity in most traits, sexes, populations, and species and, therefore, that season or environmental conditions prior to sampling may further influence the trait estimates. As a result, the use of trait estimates obtained through the use of Stock Center lines, even if these were relatively recently established in culture, may lead to biased results of trait-environment associations. This in turn will likely have consequences for the development of risk assessments or watch lists for invasive alien species and may affect understanding of traits and mechanisms underlying invasions. The challenge remains to understand the extent of the problem.

There was significant variation in CT_{MAX} and CT_{MIN} as well as their plastic responses to thermal acclimation between earlier and later generations of *D. melanogaster* and *Z. vittiger*. In *D. melanogaster* there was an increase in basal resistance between the second and tenth generation in CT_{MAX} and CT_{MIN} (Figure 3). This contrasted sharply with my expectation that there would be a general decrease in stress resistance with increased time in culture. The reaction to environmental stress by *D. melanogaster* was opposite to those shown in the literature previously in the same species albeit on different traits, namely desiccation and starvation resistance and life history traits (Hoffmann *et al*., 2001b; Sgrò and Partridge, 2000). This response could be unique to the traits of CT_{MAX} and CT_{MIN}, as to my knowledge no study has been done investigating the effect of prolonged culture on these specific traits for *D. melanogaster*. It could also be due to

laboratory adaptation. Although generally thought to reflect adaptation to more benign and stable thermal conditions, it is plausible to speculate that the increase in thermal tolerance I found could have been a result of a more nutritious and balanced diet in the laboratory setting, and/or a reduction in infections through the diet medium used (e.g. my fungicide application) or a reduction in secondary infections, which in turn may lead to more healthy or vigorous flies, and increased fitness or stress resistance in the laboratory. Trajkovic *et al*. (2017) indicated that fitness traits of *D. melanogaster* differed significantly in the long term when reared on different diets. Food composition in both the larvae and adults affects tolerance to environmental stressors in Drosophilidae flies (e.g. Andersen *et al.*, 2010; Kristensen *et al.*, 2016) although the mechanistic link between nutrition and thermal tolerance has been questioned in *Drosophila* (Overgaard *et al*., 2012) and other fly species (Mitchell *et al.*, 2017). Additionally, several studies on zoo animals (typically vertebrates) have indicated that many species are healthier, live longer and have higher breeding rates than their wild counterparts (Mason and Veasey, 2010; Müller *et al.*, 2010; Patton *et al.*, 2007). This is due to generally better conditions in captivity relative to the field such as enough food, safety from predators and veterinary care.

The decrease in stress resistance with generation time in *Z. vittiger* may be a result of relaxed selection on stress traits in the laboratory environment as a result of more benign and stable conditions (Hoffmann *et al.*, 2001b; Sgró and Partridge, 2000). To my knowledge, no previous studies have assessed the effect of time spent in culture on the environmental stress resistance of *Z. vittiger*. The responses of this species are in keeping with that predicted from other Drosophilids,a loss of resistance in laboratory culture. Several studies have shown that *Drosophila* species respond rapidly to heat and cold acclimation. Generally, heat pre-treatments will increase CT_{MAX} and cold hardening or acclimation will improve cold resistance by lowering CT_{MIN} or chill coma recovery times (Hoffmann *et al*., 1997; Levins, 1969; Sejerkilde *et al.*, 2003; Watson and Hoffmann, 1996). This was mostly shown to be the case in this study since, in both species, the warm acclimation (28°C) led to higher CT_{MAX} values in both generations tested. Cold acclimation also led to decreased CT_{MIN} except in the F_{10} males of both species (Figure 3). Plasticity was not apparently constrained by basal resistance for *D. melanogaster* since there was no significant correlation between plasticity and basal resistance for both CT_{MAX} and CT_{MIN} (Figure 13). However, plasticity and basal resistance appear coupled for CT_{MAX} of *Z. vittiger*, but not for their CT_{MIN}, since there was a strong positive correlation between plasticity

of CT_{MAX} and basal CT_{MAX} across lines and generations assayed (Figure 13). Additionally, the variation in average CT_{MAX} and CT_{MIN} values across treatments was substantial, with an average difference of up to 10°C in CT_{MAX} values of *Z. vittiger* (Figure 3). This suggests that CT_{MAX} and CT_{MIN} estimates vary depending on several factors such as time spent in culture, geographic origin, and thermal background. It also means any conclusion about the ecological or evolutionary relevance of these traits depends on which species was chosen and at what time point in culture, what the specific culture conditions were like, and perhaps even what population(s) were used to represent that species in culture. Lastly, with regards to heat and cold survival there was no generational effect in *D. melanogaster* but there was for heat survival in *Z. vittiger* which showed decreased survival between the F_2 and F_{10} generations. Once again, this decrease could be due to some form of laboratory adaptation although the exact mechanism remains unclear and could not be ascertained given my experimental design and time constraints. Given that different traits of heat or cold resistance can be regulated by different genes (Anderson *et al*., 2005), this is perhaps not surprising. For heat and cold survival, acclimation had a significant effect (Table 2; Figure 4). Hoffmann *et al.* (2002) found cold acclimation typically increased survival after cold survival treatments and warm acclimation increased survival after heat shock treatments. This was not always the case in my data with heat or cold survival showing diverse responses to acclimation and no clear consistent pattern.

In the survival treatments of *D. melanogaster,* no strong generational effect was found. However, for the starvation treatment in particular there was a significant decrease in survival between the F_2 and F_{10} generations (Figure 6). This could be due to laboratory adaptation which caused *D. melanogaster* to be unable to withstand adverse environmental conditions such as starvation. This counters the proposed notion of Hoffmann *et al.* (2001b) where they showed a rapid decrease in desiccation resistance between generations. This may be due to the difference in the amount of time spent in laboratory with mine spending ten generations (about ten weeks) in laboratory culture and those from the Hoffmann *et al*. (2001b) study spending three years in laboratory culture. A generation effect was found in *Z. vittiger* with starvation resistance showing a decrease between the generations as was expected but once again no difference in desiccation resistance. There was no significant difference in survival between the different acclimation regimes for *D. melanogaster* within desiccation resistance, starvation resistance and the control group flies. Similarly, Bubliy *et al.* (2012) showed a heat and cold pre-treatment had

no effect on desiccation and starvation resistance in *D. melanogaster*. In *Z. vittiger*, acclimation had a significant effect on desiccation resistance in the basal acclimation group (23°C) having an overall higher desiccation resistance which may indicate 23°C to be the optimal rearing temperature allowing optimal resistance to starvation. Plasticity was not apparently constrained by basal resistance for *Z. vittiger* however it appeared to be the case in desiccation resistance of *D. melanogaster* since plasticity and basal resistance were positively correlated (Figure 14). Generation assayed was important for both the basal resistance and for the magnitude of plasticity in *Z. vittiger*. It is therefore evident that species spending many generations in the laboratory do not respond to thermal acclimation in the same manner as more recently-collected species. The decrease in starvation resistance over time in culture were also found by Hoffmann *et al.* (2001b) for *D. melanogaster* and thus time spent in culture seems to cause a decrease in survival resistance.

For my second objective I hypothesized that there would be variation between the environmental stress resistance of four populations of *D. melanogaster* from geographically different areas within South Africa. My results indicate significant differences in thermal traits between the four populations of *D. melanogaster*. Basal resistance differed between 1°C and 2°C between populations for CT_{MAX} and between 1°C and up to 5°C for CT_{MIN}. There were also significant differences over all treatments with maximum variation of about 5°C in CT_{MAX} and 6°C in CT_{MIN}. In heat and cold survival, there were also large differences between the populations (Figure 9). There was also significant population variation between acclimation treatments in heat and cold survival, however, once again there were no clear patterns. For desiccation resistance the survival between the different populations was similar but for starvation resistance there were clear differences, with the Stellenbosch population surviving the longest, followed by Citrusdal, Polokwane and then Durban. This inter-population variation was largely to be expected as many studies have found significant differences in environmental stress resistance between populations of the same species from geographically or climatically different areas (Sinclair *et al.*, 2012). Among these, Overgaard *et al.* (2011) found that different populations of *D. melanogaster* in Australia had significantly different thermal resistance. Differences in environmental and life history traits has also been found in *D. buzzatii* populations of North-Western Argentina (Sørensen *et al*., 2005). Similarly, desiccation resistance, heat knockdown and chill coma recovery varied between *D. melanogaster* populations of Eastern Australia

(Hoffmann *et al.*, 2005b; Sørensen *et al*., 2005). Hoffmann and Harshmann (1999) also detailed significant inter-population variation in desiccation and starvation resistance in *D. melanogaster* as well as other species e.g. *D. simulans*. Diapause incidence, life history traits were also found to vary between geographically distinct populations of *D. melanogaster* in North America (Schmidt *et al.*, 2005). Plasticity was constrained by basal resistance in the starvation resistance treatment of the Durban population as there was a positive correlation between plasticity and basal resistance, but there was no significant correlation for any of the other populations and treatments (Figure 15). These results indicate that trait values obtained from studies using species of unknown origin (Stock Center species) do not represent all populations of that species. One therefore should refrain from assuming that environmental stress resistance estimates of a certain population are likely to reflect those of another one.

Stock Centers typically house species that are mass-bred and often derived from unknown or mixed locations (Kellermann *et al.*, 2012a). Thus, the species are susceptible to laboratory adaptation and sometimes also go through bottlenecks during laboratory establishment or suffer from inbreeding effects due to low population sizes. Many studies make use of environmental stress resistance of Drosophilid species derived from Stock Centers to draw inferences about various evolutionary or ecological processes (Kellerman *et al*., 2012a; Kellerman *et al*. 2012b; Matzkin *et al.*, 2011). These Stock Center-based trait comparisons are increasingly being used to model biogeographic responses to climate change. Araújo *et al.* (2013) mapped niche evolution to determine possible caveats with regards to the movement of species ranges under future climate change scenarios by using environmental stress resistance traits for different species from several sources, including thermal limits on insects from Kellermann *et al* (2012a; 2012b) which used *Drosophila* species derived from Stock Centers. Additionally, diverse sources of data were also used in species distribution modelling in a paper by Bush *et al.* (2016) in which the environmental stress resistance traits were once again derived from Kellermann *et al.* (2012b), as well as Hoffmann *et al.* (2003) whose *Drosophila* were sourced from a mass bred laboratory population which had spent many generations in culture and Blackburn *et al*. (2014) which used wild caught *Drosophila* which spent 5-7 generations in laboratory before trials. Overgaard *et al.* (2014) also mapped the distribution of *D. melanogaster* and its possible future distribution under climate change across the Australian continent by using wild caught *D. melanogaster* which were allowed to breed and assayed between the F_{10} and F_{25} generations depending on the traits

measured. These examples indicate that predictions of future climate change responses based on these models may be biased as time spent in culture (lab adaptation effects) and geographic origin is not accounted for in the data sources being used for the modelling. The results of my study indicate that using estimates of environmental stress resistance for species derived from Stock Centers might be problematic for drawing inferences about environmental niches, thermal specialization or trait-environment associations, especially if time in culture and laboratory adaptation is not accounted for. Stress trait and associated plasticity estimates might also not be a true representation of trait values from the natural populations. Additionally, as mentioned above, these species are often from unknown populations and as populations differ in their resistance to environmental stressors the trait values from mass bred stocks might not be reliable in representing the variety of environmental stress responses occurring naturally. Biased or skewed estimates of stress resistance are particularly problematic for climate matching and risk assessment studies. If the estimates of stress resistance are unreliable this could lead to unreliable risk assessments which in turn may result in species being assumed to have low climate similarity despite that climate matching would be quite high had these methodological effects been carefully accounted for. Thus, conclusions about which species make it onto high risk species' watchlists would be altered and could have major implications for detecting new invasions and being adequately prepared.

This study also represents one of the first comprehensive studies undertaken on wild caught South African *Drosophila* species and their environmental stress resistance. In addition it represents the first study on interpopulation variation in *Drosophila* species in South Africa, which should provide useful baseline data for future studies. Thus, a pressing need exists for more extensive studies on a larger variety of wild-caught South African *Drosophila* species. Globally, wild-caught *Drosophila* species could be used as extensively as possible in studies with lines spending a maximum of five generations in culture before traits are scored or with comparison between early and later time points in culture with a known number of generations. There exists a need to compare additional results from various traits on wild-caught *Drosophila* to those estimates made on Drosophilids derived from Stock Centers to ensure that results from risk assessment are robust and accurate. Additionally, more diverse studies need to be done on the effects of a prolonged laboratory stay on stress resistance traits as well as life history traits, especially fecundity, as it represents an important proxy for reproductive potential in risk

assessment studies (Kumshick *et al*., 2015). Other important factors that were omitted from this study but that could be incorporated into future studies include accounting for body mass in desiccation and starvation resistance trials, looking at the effect of starvation and desiccation hardening on desiccation and starvation resistance, using a larger sample size in trials and mass-rearing to account for possible inbreeding effects. Furthermore, the increase (or decrease) in stress resistance traits under laboratory adaptation in *D. melanogaster* that we observed may also come at a cost (or benefit) to other fitness-related traits such as fecundity, longevity, desiccation and starvation resistance (e.g. Hoffmann *et al*., 2005a), and future studies could consider measuring some of these traits simultaneously to determine any possible trade-offs. These results may have also been affected by biotic factors such as resource competition and crowding/density effects. For example, resource competition between species can change thermal ranges and abundance of a species within a particular range with a resulting shift in a species optimum range (Davis *et al*., 1998). Additionally, it has been found that larval overcrowding leads to the expression Hsp70 and increased longevity in adult *D. melanogaster* (Sørensen and Loeschke, 2001). Thus, addressing potential biotic factors on fitness traits in future studies will also have merit.

Invasive species represent one of the biggest ecological problems facing mankind currently and can lead to massive economic and biodiversity losses. Border control is critical to ensuring that invasive species as well as potentially invasive species do not enter the country (Saccaggi *et al.*, 2016). Decisions about which species have the potential to become invasive are based on risk assessment of that species and its underlying traits of physiology and life-history, including thermal requirements and reproductive rates. It is thus of utmost importance that the environmental stress resistance data used to infer results from risk assessments are accurate, or at least account for well-known methodological biases, as this could affect policy surrounding a particular species and its relative invasion potential.

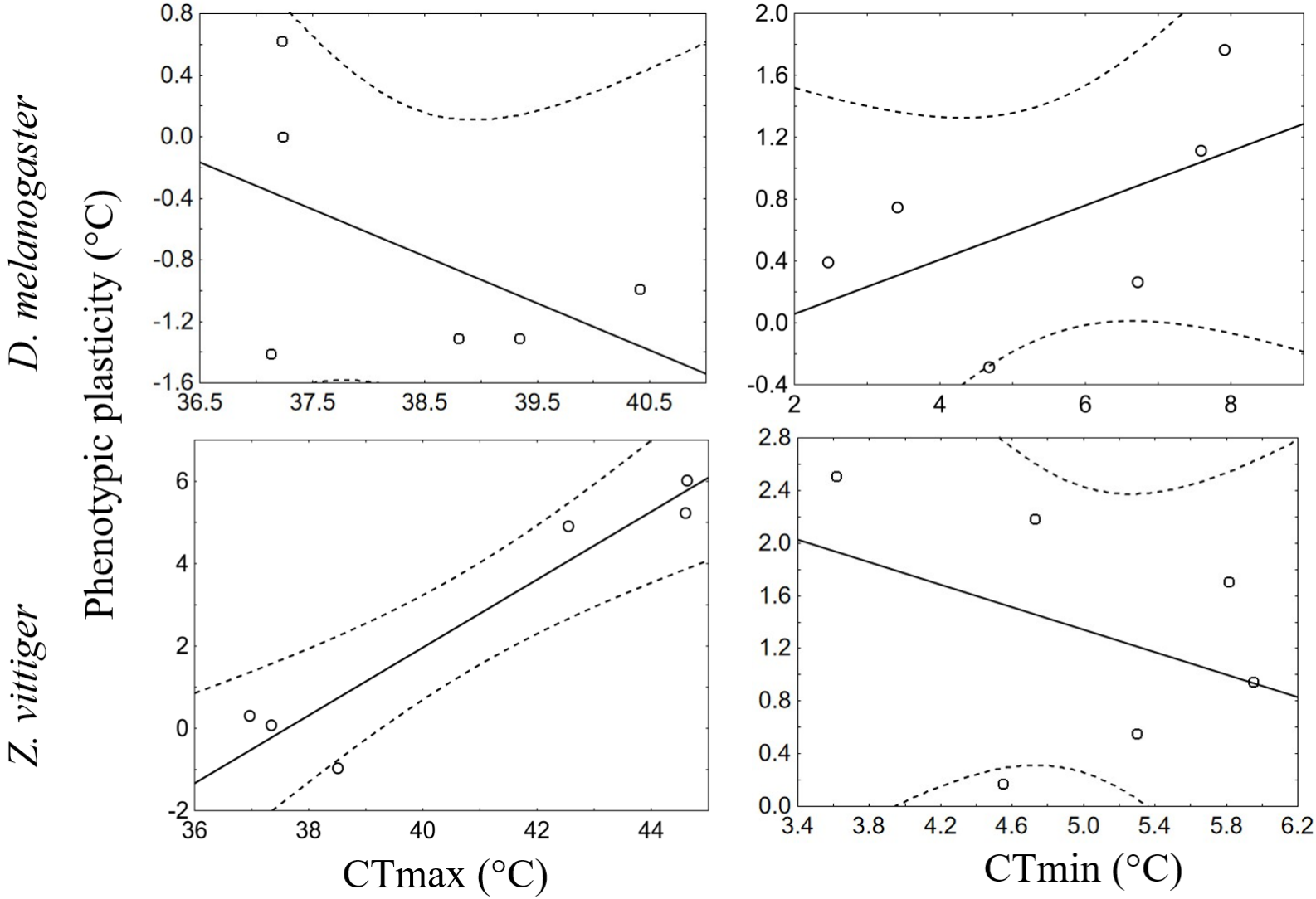

Figure 13: Scatterplots showing the correlation between plasticity, estimated as the difference between mean of the highest and lowest acclimation group's trait estimates (I.e. 28°C-18°C), against the basal resistance measured in the intermediate (control) group (23°C). Correlation statistics for the CT_{MAX} and CT_{MIN} of the two species (*Drosophila melanogaster* and *Zaprionus vittiger*) are shown in each plot and stippled lines show 95% confidence limits.

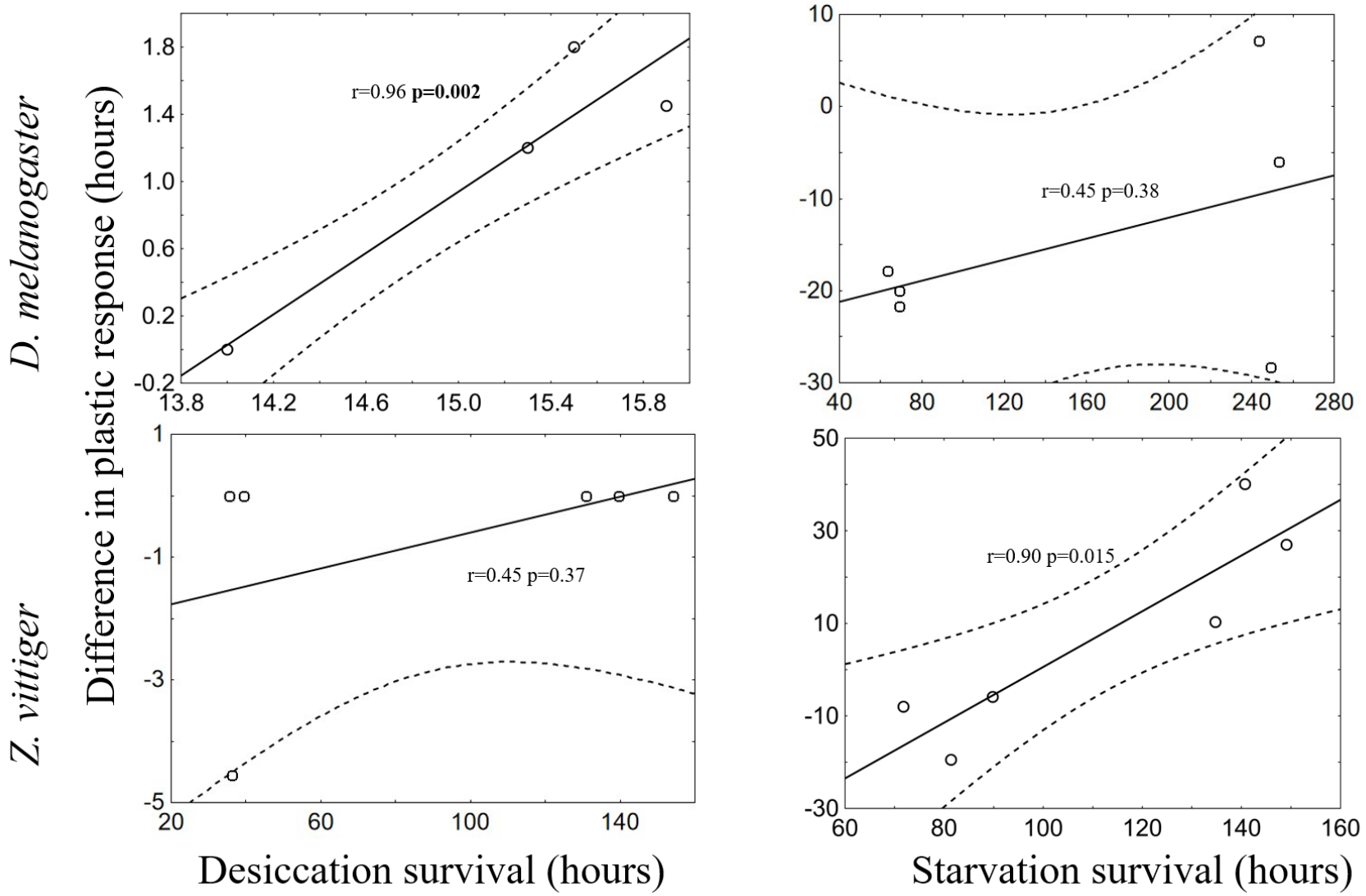

Figure 14: Scatterplot showing the correlation response and 95% confidence limits between basal resistance (23°C) and the difference of the plastic response (28°C-18°C) for the Desiccation and Starvation resistance of *D. melanogaster* and *Z.vittiger*.

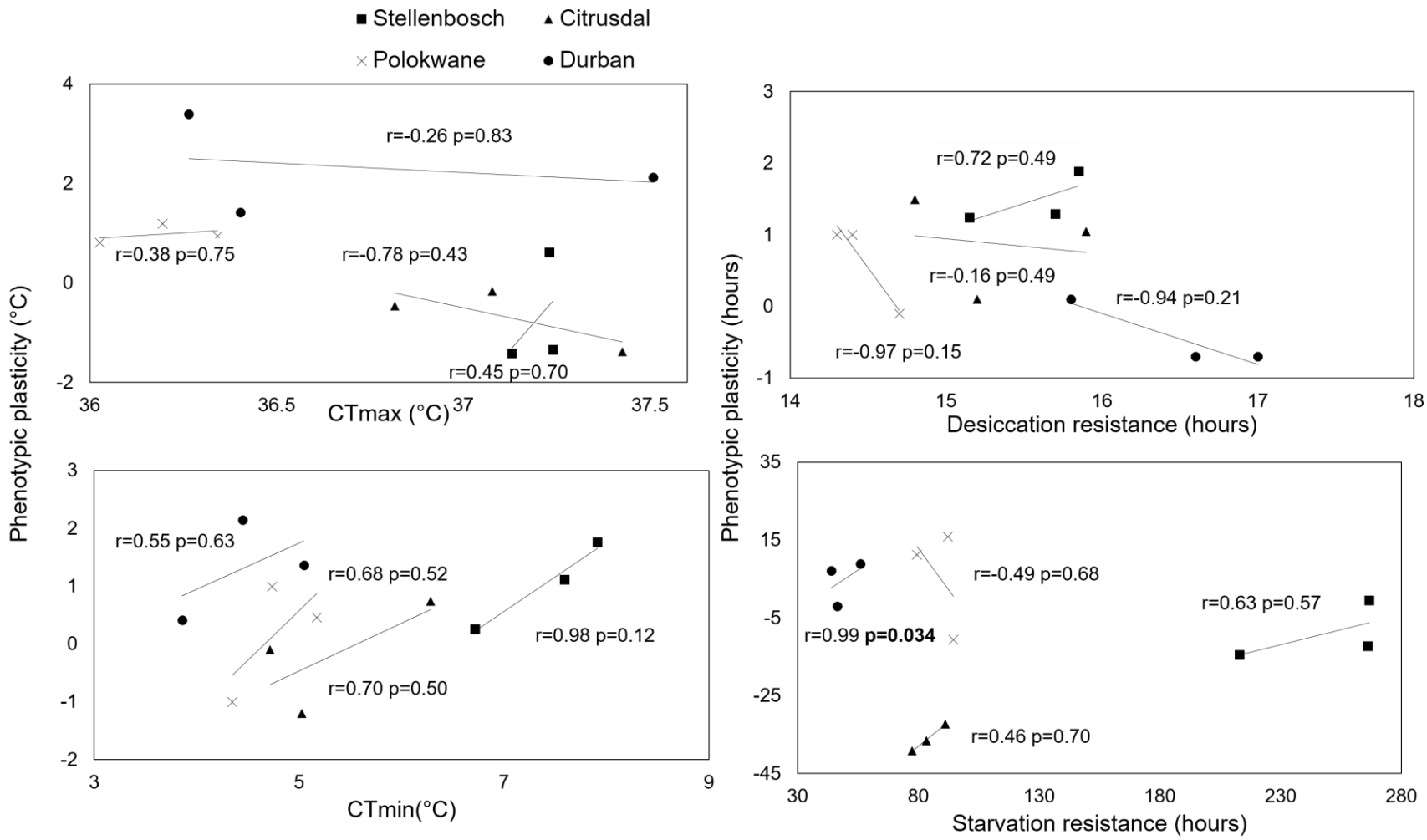

Figure 15: Scatterplot showing the correlation response between basal resistance (23°C) and the difference of the plastic response (28°C-18°C) for the CT_{MAX}, CT_{MIN}, Desiccation resistance and Starvation resistance of the four populations of *D. melanogaster* (Stellenbosch, Citrusdal, Polokwane and Durban).

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Supplementary material

Details of collection sites:

Species	Population	Specific site name	Coordinates
Drosophila melanogaster	Stellenbosch	J.S. Marais building quad	-33.9321, 18.860152
	Citrusdal	The Baths camping terrain	-32.7405063, 19.0349246
	Durban	SASRI (South African Sugar Research Institute)	-29.8586804, 31.0218404
	Polokwane	Thoyandou	-22.9761353, 30.4464797
Zaprionus vittiger	Brackenfell	Aroma Sands flats, Vredekloof	-33.8621563, 18.6799144