Sources of variation for heat resistance in *Drosophila hydei*: developmental rearing and hardening acclimation, cross generational effects, (sex) and laboratory adaptation.

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Abstract

Temperature is often seen as being the most important intrinsic variable which shapes how successfully an ectotherm persists in its environment. How ectotherms respond to increases in temperature, a likely occurrence given current climate change trends, will therefore often shape their future survival and distribution patterns. *Drosophila* is a widely used model to study adaptation to changing thermal conditions, with studies focussing on both genetic and non-genetic factors associated with adaptation. Plastic effects on thermal resistance have been studied in *Drosophila* with short term (thermal shock hardening) and long term (acclimation) exposure to temperature eliciting non-genetic responses. The evolution of these plastic effects are not currently well understood, but the field remains important in determining short term responses in nature. Selection experiments measuring the underlying genetic potential of populations to modulate their upper and lower thermal limits have been carried out on several species, with both the thermal assay used as well as the environmental conditions being used for testing considered important in dictating the selection response.

In this thesis, the main theme which permeates throughout involves the sources of variation which influence heat resistance in *Drosophila hydei*. In the second Chapter of this thesis, the presence of cross generational effects was investigated for heat resistance in *D. hydei*. A thermal rearing regime was developed to investigate whether these short term effects were detectable at different stages of introduction in the laboratory environment and for how long they persist. Weak evidence for cross generational effects were detected in populations newly introduced into the laboratory, however these effects were not consistent in direction. When populations were re-tested, no evidence for cross generational effects was found. As a by-product of the re-testing process, a uniform increase for both populations and experimental condition was detected for increased heat resistance. The possibility for laboratory adaptation was posed and laid the groundwork for the third Chapter of this thesis.

To investigate potential laboratory adaptation, an experimental protocol involving multiple populations of *D. hydei* with different times spent in the laboratory but tested for their heat resistance at the same time was used. As well as using two developmental rearing temperatures, the effects of sex, and of heat hardening were incorporated into the experiment. Two experimental timepoints were used with six months separating them. Most populations were re-tested at the second timepoint which allowed direct comparison for the evolution of those populations. Evidence
for laboratory adaptation was found, with the oldest population displaying significantly lower levels of heat resistance compared to all other populations. Unusually high heat resistance levels were recorded for populations newly introduced into the laboratory, while populations showed an increase in resistance between the two experimental timepoints. The effects of developmental rearing temperature were substantial, and were matched only by population differences. The influence of hardening was non-significant as a standalone variable, but it did interact significantly with the sex of an individual.

This thesis has added knowledge to the field of thermal biology and has supplemented other studies showing the ability of *Drosophila* to modulate its thermal resistance in response to differing environmental variables. The effects of laboratory adaptation on heat resistance have rarely been considered in the literature, and this study, whilst not definitive, suggests the importance of this factor in adaptation.
Declaration

This is to certify that:

i. the thesis comprises only my original work towards the Masters degree

ii. due acknowledgement has been made in the text to all other material used,

iii. the thesis is less than 50,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Michele Johnstone
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Chapter One: Introduction

1.1 The importance of temperature

Temperature is one of the most important factors which can limit and shape the distribution and abundance of animals, particularly ectotherms (Mayr 1963; MacArthur 1972; Cossins & Bowler, 1987). The threat of human induced climate change will lead to both an increase in average mean temperature, and an increase in frequency of extreme weather events (Collins et al 2013). Due to the ever increasing rising global temperatures due to climate change (IPCC 2013), it is likely that species' distributions will shift in response (Parmesan & Yohe, 2003; Thomas et al., 2004; Perry et al, 2005; Chown & Gaston, 2008; Portner & Farrell, 2008; Hofmann & Todgham, 2010). The risk of local and widespread extinction will also increase (Deutsch et al, 2008; Sinervo et al, 2010; Overgaard et al, 2014).

Three mechanisms will shape the response of individual organisms to climate change; these include the ability to migrate to new favourable habitats, phenotypic plasticity, and adaptive evolution (Holt 1990; Davis et al, 2005; Williams et al, 2008; Dam 2013; Chirgwin et al, 2015). A combination of the three will be key in the early stages of adapting to climate change (Williams et al, 2008), but only organisms with both the capacity to locate suitable habitats and sufficient dispersal ability will be able to utilise migratory mechanisms (Hughes 2000). For those unable to disperse, phenotypic plasticity will be needed, especially in the short term, to tolerate changing conditions.

Temperature is also the most important factor which dictates the rates of physiological and biochemical processes for animals, with its impact on determining population growth rates, physiological performance, and most importantly, survival, well known (Cossins & Bowler, 1987; Angilletta, 2009, Overgaard et al, 2014). The fundamental niche of animals is shaped largely by environmental temperature. Insects are generally isothermal to ambient temperature and thus their overall fitness is mediated by the temperature in which they live (Johnson and Bennett 1996; Warren et al. 2001; Andrew & Hughes 2005). Insects must therefore have the capacity to respond to stressful thermal changes. Insects can choose to behaviourally move away from a stressful environment or to physiologically adapt (Addo-Bediako et al, 2002; Hoffmann et al, 2003; Chown & Nicolson, 2004; Angilletta et al, 2010).
Drosophila is widely used as a model organism in studies of thermal biology (Maynard-Smith 1956; Hollingsworth & Bowler 1966; David et al 1983; Hoffmann & Parsons 1991; Hoffmann et al, 2003) and its genome has been sequenced for several species (Adams et al, 2000; Clark et al, 2007). Due to its short generation time, Drosophila can be manipulated quite easily in order to study the effects of both and long term adaptation to temperature (Hoffmann et al, 2003). Drosophila species can have very different thermal niches and distributions and thus form a valuable model for investigating changing temperatures on both widespread and specialist species (Stanley 1980; Stratman & Markow 1998; Gibert et al, 2001; Hoffmann et al, 2003).

In the laboratory, various methods are known for measuring thermal resistance suitable for both genetic and non-genetic studies (Hoffmann et al, 2003). Short term exposure to a sublethal temperature prior to a thermal resistance test is known as hardening (Cossins & Bowler 1987). These changes are plastic and reversible, (but some may persist (Khazaeli et al, 1997)), and can be considered beneficial if the hardening treatment causes an increase in thermal resistance (Hoffmann et al, 2003; Terblanche et al, 2011). Longer term exposure to sublethal temperatures is called acclimation and can range from a few days to weeks and can even encompass the entire developmental cycle (Hoffmann et al, 2003; Terblanche et al, 2011). Both hardening and acclimation, including rearing temperatures, are considered non-genetic in nature. On the other hand, genetic changes can be elicited through the process of selection and epigenetic changes as well as other forms of cross generation effects.

1.2 Genetic changes in temperature responses

The process of adaptive evolution involves genetic factors and organisms will often need to utilise this mechanism to a certain extent to be able to persist in face of long term temperature increases (Gienapp et al, 2008). Evolutionary change has a notorious reputation for being very slow. However, when dealing with an increase in temperature or decrease in annual rainfall for example, evolutionary changes can be remarkably fast. This is especially true for species with short lifecycles (Huey et al, 2000; Franks et al, 2007; Lohbeck et al, 2012). This adaptive capacity is dependent on a population’s innate heritable genetic variation for trait which is under selection pressure (Lynch & Walsh, 1998; Conner & Hartl, 2004). To emphasize this point, a population which possesses limited heritable variation for trait/s under selection will have a decreased capacity to respond via
evolutionary processes and this has been shown in *Drosophila* selection experiments (Hoffmann et al, 2003a; Kellermann et al, 2006).

Evolutionary adaptation to environmental change may also be constrained by covariation between traits despite the presence of genetic variation within a population (Dam 2013). In this sense, more than one trait could be under selection in a population for countering a rise in temperature, and thus a combination of both genetic variation and covariation between traits under selection will dictate the extent to which one trait can respond (Lynch & Walsh 1998; Brooks 2000; Dam 2013; Blackburn et al, 2014). There is a risk that by measuring only one trait, an over- or underestimate of the actual adaptive capacity for that population to increase its heat tolerance may occur, if different traits are under selection due to warmer conditions. For heat tolerance, further study is needed to understand associated patterns of trait covariation, but this has been rarely tested in the laboratory or field (van Heerwaarden & Sgro 2013; Blackburn et al, 2014). Other traits, which will contribute to a response to changing environmental conditions such as desiccation and cold tolerance, must also be considered in this context.

Selection experiments are a good resource when needing to investigate the adaptive potential and heritable genetic variation of populations. Selection experiments can be used to select for increased fertility or survival upon exposure to extreme thermal conditions and levels of heritable variation can be quantified in traits such as cold and heat resistance (Gibbs 1999; Hoffmann et al, 2003). There are two main forms of selection experiments (Fuller et al, 2005). In the first, known as laboratory natural selection, replicate lines are divided into two or more experimental treatments and the response over time is examined (Rose et al, 1990; Travis & Reznick, 1998). Experimental treatments could include high or low temperature, as well as density and food manipulations. Evolution occurs through this process and the individuals which go on to form the next generation are not manipulated directly. The second form, known as artificial selection, involves a process whereby the individuals selected to propagate the next generation are chosen if they meet a certain standard or criteria (Rose et al, 1990; Fuller et al, 2005). A truncation process can be utilised by selecting the top x% or bottom x% of the individuals involved in the assay to form the next generation (Fuller et al, 2005). Selection experiments have been utilised to investigate stress resistance in *Drosophila*, as well as determining the capacity for increases in resistance (Hoffmann & Parsons 1989; Gibbs 1999; Hoffmann et al, 2003a). In *D. melanogaster*, the adaptive potential to rapidly increase heat tolerance has been shown in selection experiments (Huey et al, 1992; McColl et al, 1996; Bubliy & Loeschcke 2005), with a recent example showing a 0.5°C increase in upper thermal tolerance compared to control lines after 10 generations of selection in *D. melanogaster* (Hangartner & Hoffmann 2015).
Selection experiments involving both desiccation and cold resistance have shown that heritability is low in restricted tropical *Drosophila* species (Hoffmann et al, 2003a; Kellerman et al, 2009). Meanwhile selection for increased desiccation resistance has been shown in *Drosophila* to be possible in more widespread generalist species (Hoffmann & Parsons 1989; Blows & Hoffmann 1993; Hoffmann & Parsons 1993). This highlights issues facing many ectotherms in the tropics and the reality that they may be evolutionarily constrained in their ability to respond to future climate change. Heritable variation was found when selecting for increased heat resistance in both *D. melanogaster* (Huey et al, 1992; McColl et al, 1996; Gilchrist & Huey 1999) and *D. simulans* (Jenkins & Hoffmann 1994). Heritable variation has also been identified in the field (Jenkins & Hoffmann 1994). This further indicates the issues facing many ectotherms in the tropics and the reality that they may be evolutionarily constrained in their ability to respond to future climate change. In *Drosophila*, both cold tolerance and desiccation resistance, rather than heat resistance, has been shown to be a better indicator in predicting distribution limits although heat can be important in dry conditions (Gibbs & Matzkin, 2001; Gibert et al, 2001; Kimura 2004; Kellermann et al, 2012a + b).

Selection experiments have been criticised due to their inherent vulnerability to artefacts and also for not adequately representing the natural environments and their varying conditions (Harshman & Hoffmann 2000). An estimate of the adaptive potential of a population can be successfully determined using selection experiments and this can in turn lead to information relating to whether upper or lower thermal limits can be shifted. However, whether these laboratory simulations truly reflect what could hypothetically occur in nature remains to be seen, particularly as heritable variation can depend on environmental factors.

Many ectotherms are already persisting very close to their upper thermal limits, as measured by exposure to extreme heat experiments, and would be considered prime candidates for potential extinction risk due to climate change (Deutsch et al, 2008; Huey et al, 2009, 2012; Duarte et al, 2012; Hoffmann et al, 2013). Whether these upper thermal limit traits are subject to evolution has not been taken into consideration in the context of climate change and it remains to be seen if species are able to utilise evolutionary changes much (Kearney et al, 2009; Kingsolver et al, 2011; Hoffmann et al, 2013; Hangartner & Hoffmann 2015). An estimate of the adaptive capacity of upper thermal limits is important in predicting future organism distribution changes and extinction risk caused by an increase in temperature (Deutsch et al, 2008; Angilletta 2009; Huey et al, 2009; Sunday et al, 2011; Diamond et al, 2012; Hoffmann et al, 2013).

Most studies have however relied on indirect estimates of adaptive capacity. Studies that have directly tested the adaptive capacity of upper thermal limits remain sparse at the present (Hoffmann
et al, 2013; Hangartner & Hoffmann 2015). Low levels of genetic variation for heat tolerance were found to limit the increase in upper thermal limits in copepods (Kelly et al, 2012, 2013), but recent work on widespread Drosophila species suggests that the capacity for evolution of upper thermal limits might not be lacking and needs further investigation (van Heerwaarden & Sgro 2013; Blackburn et al, 2014; Hangartner & Hoffmann 2015). Van Heerwaarden (2015) showed that upper thermal limits were able to increase in two rainforest Drosophila species when subjected to a projected summer thermal regime. This same adaptive capacity was not found when the flies were subjected to winter or constant thermal regimes.

Such studies highlight the difficulty in estimating the approximate adaptive capacity of populations, particularly for restricted, tropical ectotherms, under conditions which will match future climatic changes. They may not otherwise provide much useful information in elucidating how populations will respond to climate change in an evolutionary sense.

Upper thermal limits are critical in the debate surrounding climate change, and the ability, or inability, of organisms to modify their upper thermal limits remains a vital question. As discussed above, in selection experiments, an evolutionary response is involved. But upper thermal limits and stress resistance in general can also be influenced by phenotypic plasticity, where non-genetic factors are involved. These non-genetic changes can be transmitted to the next generation through maternal environmental effects and epigenetic mechanisms and could later be selected through evolutionary processes. Phenotypic plasticity represents an adaptive mechanism in its own right. Plasticity may come to form the first intrinsic line of defence for many ectotherms under climate change.

1.3 Plastic changes in temperature responses

Phenotypic plasticity is the term used to describe the process in which an organism may modify their phenotype in response to environmental changes through non-genetic processes, leading to behavioural and physiological adjustments (Hoffmann et al, 2003; West-Eberhard 2003; Charmantier et al, 2008; Nicotra et al, 2010; Overgaard et al, 2011; Zizzari & Ellers 2014). Simply put, plasticity refers to the way in which the environment is able to modify the phenotype of the individual or the genotype of an individual (Auld et al, 2010). Plasticity has become important in the context of climate change as it will make up the first line of defence for many organisms, especially ectotherms, as plastic changes allow rapid responses to changing conditions. As discussed earlier, evolutionary
responses will take place, but often over a longer time span, and plastic changes in the short term may end up facilitating persistence under future environmental conditions if they confer a fitness advantage to populations. In the context of climate change, understanding how different traits can respond plastically to thermal changes is critical. It has been shown that stress resistance traits are indeed under the influence of plastic changes in many species (Hoffmann et al, 2003; Angilletta 2009). However, there is a challenge in linking laboratory assays to field conditions. For instance, Overgaard et al, (2011) conducted a comparative study of Drosophila and found that upper thermal limits exhibited little capacity for phenotypic plasticity. However, when an alternative assay was used, plastic responses were found (Hoffmann et al, 2003; Mitchell et al, 2011). This demonstrates the need to account for variation in assays and the way they are designed in order to tease out various plastic responses to thermal fluctuations.

Phenotypic plasticity can also help explain geographical variation in thermal tolerances in Drosophila (Ayrinhac et al, 2004; Hoffmann et al, 2005). It is thought that local adaptation and phenotypic plasticity may evolve together in animals (Calosi 2008; Jensen et al, 2008; Cottin et al, 2012), making it difficult to partition their relative contribution as well as accounting for local phenotypically plastic adaptation (Jensen et al, 2008; Bedulina et al, 2010). With the advent of climate change and predicted increases in global temperatures, any link and interplay between plastic responses and local adaptation needs to be understood. These interactions will likely be key in determining the fate of species and populations (Somero 2010).

It has been predicted that tropical species will exhibit lower potential for phenotypic plasticity compared to temperate species, which are subjected to a wider range of temperatures (Janzen, 1967; Levins, 1969; Ghalambor et al, 2006; Chown & Terblanche, 2007). Temperate environments tend to be more variable and therefore provide reliable environmental cues allowing plasticity to function optimally (Levins 1968; Ghalambor et al, 2006). Conversely, plasticity in more stable, tropical environments is quite low, and in some cases has been lost entirely, perhaps as it may incur costs (Levins 1968; Ghalambor et al, 2006). This lack of temperature variation has seen tropical species having very narrow thermal tolerance limits (Janzen 1967; Lips 1998; Ghalambor 2006). Any increase in temperature may severely affect tropical species, particularly ectotherms, who may already be persisting close to their upper thermal limit (Kimura 2004; Fischer 2010; Kellerman et al, 2012a). From a climate change perspective, tropical species are seen as being at most risk (Pounds et al, 2006; Deutsch et al, 2008; Raxworthy et al, 2008; Chen et al, 2009; Huey et al, 2009), but temperate species may also face an uncertain future (Kellerman et al, 2012b; Overgaard et al, 2014). Moreover, recent research has also called into question the notion that temperate, widespread Drosophila species have a larger capacity for exhibiting phenotypic plasticity for thermal tolerance.
limits, instead suggesting that species distributions are actually linked to innate thermal tolerance limits (Overgaard et al, 2011; Overgaard et al, 2014). Specifically, it appears the assay used to assess upper thermal limits may influence conclusions (Terblanche et al, 2007; Mitchell & Hoffmann, 2010; Sgrò et al, 2010; Rezende et al, 2011; Santos et al, 2011; Blackburn et al, 2014). Moreover, a multivariate approach across multiple traits and assays may be needed to assess upper thermal limits (van Heerwaarden & Sgro 2013; Blackburn et al, 2014).

Climate change will lead to an increase in global temperatures, and this provides a path for ectotherms to increase both their reproductive rate and decrease their development time, which would lead to both population increases and additional generations per year (Bentz & Powell 2014). Whilst insects will develop more rapidly in warmer conditions, the consequences of this perceived advantage in the warmer months may then become a disadvantage as individuals are not then well suited to winter conditions, which could lead to a population decrease (Coleman et al, 2014; Dworschak et al, 2014; Stuhldreher et al, 2014). There may be a balance between benefits gained in extreme periods and at other times of the year.

Recent acclimation and hardening studies in the laboratory have begun to replicate the types of environmental conditions ectotherms will encounter in the field, encompassing cyclic thermal regimes to better reflect seasonal changes during the year (van Asch et al, 2007; van Asch et al, 2010). In particular, both cold (Overgaard et al, 2011) and heat (Sarup & Loeschcke 2010; Bozinovic et al, 2011) resistance was increased through the fluctuation of temperatures (as opposed to constant temperatures) during developmental acclimation in Drosophila. Only cold tolerance has been shown to be influenced by fluctuating thermal regimes in adults in Drosophila (Kelty & Lee 2001). Thermal regimes involving fluctuating temperatures remain an area requiring further investigation.

The benefits of variable thermal regimes have been demonstrated in that they provide an increase in rapid heat hardening responses, but at the same time, can reduce rapid cold hardening responses in some species (Terblanche et al, 2010). Conversely, the opposite has also been found to be true (Hawes et al, 2008). Plastic changes therefore may need to be studied on a species by species basis when considering responses to changes in temperature brought on by climate change (Sgro et al, 2015). It is accepted that far more work is needed in this area to successfully ascertain the full range of net benefits of plasticity (Sgro et al, 2015).

The process of plasticity can be further complicated by the possibility of interactions between independent plastic responses such as hardening and acclimation. In D. melanogaster, acute cold survival was increased in response to cold acclimation, but no advantage was found when this cold
acclimation was paired with a cold hardening prior to testing (Rajamohan & Sinclair 2009). Similarly, plastic responses to differing food quality led to changes in thermal plasticity and induction of diapause (Stillwell et al, 2007; Anderson et al, 2010; Lu et al, 2013). The environmental changes which will be caused by climate change will remain important in determining which plastic responses will be triggered and the combination of different plastic responses and their potential interaction will shape how animals are able to respond.

1.4 Cross generational effects

The most common form of cross generational effect is a maternal effect, which is where the environment the mother experiences can influences the phenotype of the offspring in the next generation (Mousseau & Fox 1998). In insects, both diapause response and stress resistance has been shown to be maternally transmitted (Mousseau & Dingle 1991; Coleman et al, 2014), with grandmaternal effects lasting more than one generation also being recorded (Voinovich et al, 2013). When combined with paternal effects, such as fertility control (Avila et al, 2011, Crean et al, 2013), these effects constitute cross generational plasticity (Sgro et al, 2015). The mechanism behind these cross generational effects are often epigenetic in nature. The methylation of genes and nucleosome interactions are thought to be involved and have been proposed as a framework for explaining how DNA expression can be altered even in insects (Sgro et al, 2015). These epigenetic pathways can be seen to contribute to cross generational effects, and considering methylation of genes can last from one to five generations, this provides a suitable underlying mechanism of cross generation plasticity in general (Badyaev & Uller 2009; Bonduriansky et al, 2012; Glastad et al, 2014; Sgro et al, 2015).

The evolution of cross generational plasticity and its adaptive potential will require an understanding of environmental variation, maternal selection and the fitness of offspring of the individuals in question (Marshall & Uller 2007; Uller et al, 2013 Burgess & Marshall 2014). It has long been considered that for cross generational effects to be deemed beneficial, both the parental and offspring environments must be similar (Burgess & Marshall 2011; Uller et al, 2013; Burgess & Marshall 2014), but it is now accepted that parental effects are not always adaptive and the question of both parental and offspring fitness remains crucial (Burgess & Marshall 2014). In terms of traits which would relate to climate change, the literature is scarce in terms of examining the role cross generational effects. In widespread species of Drosophila, it was found that parental exposure to both heat (Sgro & Hoffmann 1998) and cold (Watson & Hoffmann 1996) stress brought about
reduced fecundity in offspring. Whilst in *D. serrata*, cold stress exposure to mothers resulted in increased fecundity and the opposite was true if the grandmother was exposed to this cold stress (Magiafoglou & Hoffmann 2003). Even so, cross generational plasticity has rarely been tested for stress resistance under field environmental conditions (Overgaard & Sorenson 2008). Jenkins and Hoffmann (1994) tested *D. simulans* for maternal effects in heat resistance directly in the field and Schiffer et al, (2013) tested many species of *Drosophila* from both temperate and tropical locations for cross generational effects and found they were present for heat resistance and only to a much lower extent for cold resistance. Nonetheless, these cross generational effects were still much smaller than those present for intragenerational plastic effects (Uller et al, 2013). Questions still remain on whether cross generational effects can persist for more than one generation as most studies have shown, but some studies have suggested they can persist for longer periods of time (Magiafoglou & Hoffmann 2003; Saastamoinen et al, 2013, Schiffer et al, 2013), and that the environment in which the progeny develops can alter these effects (Watson & Hoffmann 1996). The adaptive significance for cross generational effects is yet to be substantiated when considered from a multiple generations point of view. and this is important from a climate change point of view.

1.5 Plasticity costs and mechanisms

The mechanisms behind the evolutionary responses to plasticity remain complex and theoretical models have highlighted the need to balance both the benefits and costs involved determining the realised adaptive value of plasticity in the face of environmental variation (Sultan & Spencer 2002; Chevin et al, 2010; Sgro et al, 2015). By failing to account for fitness costs, which arise due to plastic responses, the perceived adaptive significance will be inflated (Chevin et al, 2010). DeWitt et al, (1998) contrasted two types of restrictions: costs, which lead to reduced fitness when a trait is produced via plasticity rather than constitutively, and limits, an inability to produce the optimal trait value.

Thermal stress causes almost all organisms to undergo a heat shock response, with heat shock proteins being the major driver of this process (Lindquist & Craig, 1988; Morimoto et al, 1994; Feder & Hofmann, 1999). Heat shock proteins (HSPs) behave as molecular chaperones, assisting in maintaining proper cellular function, which can often be thrown off dramatically in the onset of thermal stress. One of the most studied HSPs, HSP70, has been shown to diminish cellular damage during thermal stress in *Drosophila* (Georgopoulos & Welch, 1993; Parsell & Lindquist, 1993;
Sørensen et al., 2003). Consequently, evidence of HSP70 being produced in response to stressful temperatures has also been shown in *Drosophila* (Feder et al., 1996; Krebs & Feder 1997; Sørensen et al., 2003). Despite the apparent benefits of HSP70 induction for thermal stress, there have been many documented cases of deleterious effects involving improper development and reduced reproductive capacity in *Drosophila* (Feder et al., 1992; Krebs & Loeschcke, 1994; Krebs & Feder, 1997; Silbermann & Tatar, 2000). Interestingly, the induction of HSP70 in the absence of a stressful stimulus can prove deleterious (Krebs & Loeschcke, 1994; Krebs & Feder, 1997a,b).

Laboratory lines which are kept at different, but constant, temperatures have been shown to differ in both the expression of HSP70 and thus induction of thermotolerance (Stephanou et al., 1983; Huey et al., 1991; Bettencourt et al., 1999). The question of whether HSP70 expression can and is being selected during generations of no stress and constant temperature is something that remains unclear. There is a possibility that the first few generations in which lines are introduced into the laboratory could potentially cause HSP70 expression to change in response to laboratory conditions. Whether natural levels of HSP70 (and thermotolerance) will ever return to these lines remains a question worth pursuing. Krebs et al., (2001) has suggested that maintaining high numbers of individuals for laboratory lines will prevent loss of thermal tolerance regardless of the amount of time spent in a laboratory environment.

HSP70 is involved in the response to hardening treatments and is seen as being part of the process in increasing heat tolerance (Solomon et al., 1991; Feder & Krebs, 1997). At the same time, rapid increases in temperature do not seem to induce the expression of HSPs, and they seem to play a much more diminished role in both cold resistance and knockdown heat resistance assays (Sorensen et al., 2001).

For longer term exposure to thermal environments, HSPs become less of a player in the response to increased resistance. Physiological changes such as membrane lipid composition, sugar and polyol concentrations and metabolic rate have all been shown to play a larger role in shaping a response to acclimation, as opposed to the heat shock response involving HSPs (Hazel, 1995; Hendrix & Salvucci, 1998; Hoffmann & Parsons, 1997). Developmental rearing temperature has also been shown to influence changes in phospholipid composition in membranes in response to different rearing temperatures in *Drosophila* (Ohtsu et al., 1999).
1.6 Assays of heat resistance

In assessing the heat tolerance of populations, the type of assay used can strongly influence the results (Terblanche et al., 2007; Mitchell & Hoffmann 2010; Sgro et al., 2010; Overgaard et al., 2014). Heat tolerance can be measured in several ways; scoring fertility, longevity, and behaviour during a heat stress are popular methods in determining heat tolerance (Angilletta 2009). The most common form of measuring thermal tolerance in Drosophila and other small insects involves assessing their knockdown time. This measure involves recording the time until physical incapacitation occurs. The way in which knockdown time, and by extension, thermal tolerance, is measured experimentally in Drosophila depends on the experimental assay chosen. There are two main approaches to assess knockdown time and these involve static, or constant, temperature assays and those that are dynamic, or variable, which could involve a gradual increase or decrease in temperature during the experimental assay (Lutterschmidt & Hutchison 1997, Hoffmann et al., 2003, Terblanche et al., 2007). Static assays involve a constant stressful temperature maintained throughout the experiment (Levins 1969, Hoffmann et al., 2002, Hoffmann et al., 2003). For most Drosophila species, a temperature in the high 30's seems to be a popular choice when setting static temperature thresholds. A temperature of 39°C was chosen by Hoffmann et al., (2002) to measure heat tolerance in Drosophila melanogaster, whilst van Heerwaarden & Sgro (2013) selected a temperature of 38.5°C to measure upper thermal limits in Drosophila simulans.

Dynamic, or ramping, assays involve the steady increase in temperature during an experimental assay whereupon the knockdown time of an organism is measured against the corresponding current temperature during the assay (Terblanche et al., 2007; Mitchell & Hoffmann, 2010; Overgaard et al., 2012; Sgro et al., 2010; Terblanche et al., 2011, van Heerwaarden & Sgro 2013). A starting temperature of 28°C is often chosen, with many Drosophila species reaching their final knockdown time during the high 30's, not dissimilar to the static temperatures chosen mentioned above. Compared to a static heat assay, a ramping assay is seen as being more ecologically relevant, that is, something an organism is more likely to experience in the field (Terblanche et al., 2007; Terblanche et al., 2011). When trying to ascertain how an organism will respond to future climatic changes, it is essential to consider these real world changes in a laboratory setting rather than simply assuming behaviour and resistance displayed in the laboratory will be replicated. The starting temperature and temperature rate of increase are also factors which can influence knockdown time during ramping assays (Terblanche et al., 2007; Chown et al, 2009; Mitchell & Hoffmann 2010; Sgro et al, 2010; Sørensen et al, 2013; van Heerwaarden & Sgro 2013; Rezende et al, 2014).
A starting temperature of 28°C is often used for *Drosophila* for thermal ramping assays (Mitchell & Hoffmann 2010, Schiffer et al, 2013, van Heerwaarden & Sgro 2013) with two prevailing methods used to correctly reflect the rate of change of temperature in the field. This first ramping type was the '0.06' method which after commencing at 28°C, increased at a constant rate of 0.06°C for every ten minutes until reaching the final temperature of the assay (Mitchell & Hoffmann 2010). A final temperature of 38°C or higher (Mitchell & Hoffmann 2010, Sgro et al, 2010), or an increase until all flies have been knocked out during the assay have been used. The second ramping type involves the '0.2' method which is relatively faster when compared to the '0.06' method as it involves a rate of increase of 0.2°C for every ten minutes before finishing at 38°C (Schiffer et al, 2013). There are two main distinctions to be made for these two approaches to thermal ramping. The first obviously involves the speed at which the two methods increase in temperature throughout the assay. It would be presumed that short term acclimation responses would be involved during the '0.06' method when compared to the '0.2' method. However, the '0.2' method involves reaching the final temperature of 38°C at only the 65-minute mark, compared to a much later mark for the '0.06' assay if using a final temperature of 38°C. A longer time spent at the final experimental temperature poses interesting questions in terms of both the potential for acclimation and comparisons back to a static assay.

There has been disagreement among biologists about which assays are able to accurately reflect heat tolerance and how valid inferences of thermal adaptation in the laboratory really are (Rezende et al, 2011; Terblanche et al. 2011; Overgaard et al, 2012; van Heerwaarden et al, 2012; Hangartner & Hoffmann 2015). Different methodologies and the inferences they make in relation to climate change remain a bone of contention among biologists (Chown et al, 2009; Mitchell & Hoffmann 2010; Rezende et al, 2011; Hangartner & Hoffmann 2015).

Interestingly, heat tolerance heritability and additive genetic variance is reduced when slow ramping rates are utilised compared to static heat assays, meaning selection for heat tolerance may be constrained evolutionarily when there are more gradual increases in temperature (Hoffmann 2010; Mitchell & Hoffmann 2010; Blackburn et al, 2014). A multivariate approach has been used to show that ramping and static knockdown assays are not in fact genetically correlated with each other and as such should be treated as having an independent genetic pathway to illustrate heat tolerance (Blackburn et al, 2014).
Chapter Two - Cross generational effects and initial evidence for laboratory adaptation

2.1 Introduction

A relatively poorly investigated form of phenotypic plasticity is one that can last over two or more generations. These effects have been termed cross generational, or transgenerational, effects, and can involve epigenetic mechanisms. It is thought that cross generational effect mechanisms could be the first point of call for an ectotherm’s ability to respond to different and challenging thermal conditions (Bonduriansky & Day 2009; Bonduriansky et al, 2012; Donelson et al, 2012). Whether these short term effects become adaptive will depend on what thermal conditions the offspring of individuals encounter. The effect can be positive in that fitness is increased when a similar environment is encountered by offspring as was encountered by the parents, but it can also be negative, in that fitness is increased only if dissimilar conditions are encountered in the offspring generation (Crill et al, 1996; Watson & Hoffmann 1996; Mousseau & Fox 1998; Galloway & Etterson 2007, Burgess & Marshall 2011,). It is thought that positive cross generational effects could be more adaptive in Drosophila species living in warm conditions as they are more likely to experience successive generations of stable conditions. Schiffer et al, (2013), when searching for cross generational effects in Drosophila and their direction, instead found three of the species displaying positive cross generational effects were in fact widespread, temperate species.

Both maternally (Jenkins & Hoffmann 1994; Watson & Hoffmann 1995; Watson & Hoffmann 1996; Mousseau & Fox 1998; Hercus & Hoffmann 2000; Gilchrist & Huey 2001; Bacigalupe et al, 2007; Yanagi & Tuda 2010; Zizzari & Ellers 2014) and paternally (Magiafoglou & Hoffmann 2003) derived characteristics have been suggested as mechanisms for cross generational effect transfers to the next generation. These sex related effects are nonetheless nongenetic effects whose inheritance seems to be closely tied to maternally derived RNAs and DNA methylation (Badyaev & Uller 2009, Boyko et al, 2010, Lyko 2011; Bonduriansky et al, 2012; Bonasio 2014, Glastad et al, 2014).

Very few Drosophila papers have demonstrated the existence of cross generational effects with respect to heat resistance. In terms of heat resistance, Jenkins & Hoffmann (1994) showed that D. simulans tested directly from the field displayed cross generational effects with progeny being
influenced by maternal environmental conditions. Schiffer et al, (2013) found that F1 flies tended to be more heat resistant than the F2 individuals, suggesting a negative cross generational effect as both generations had been reared at 19°C. Moreover, individuals reared at 28°C for the F2 generation displayed a higher heat resistance than individuals reared at 19°C for the F1 generation. *Drosophila hydei* was one of the species which Schiffer et al, (2013) found to exhibit cross generational effects with respect to heat resistance.

Cross generation effects on cold resistance are also poorly studied for *Drosophila*. Watson and Hoffmann (1996) showed through cold selection experiments that after initial increases in cold resistance, further selection caused a decrease in cold resistance. However, after relaxing cold selection for one generation, there was a reversal of this decrease in cold resistance, suggesting cross generational effects and potential adaptive capacity. Schiffer et al, (2013) demonstrated that cold resistance may not be affected much by cross generational effect mechanisms. However life history traits have been shown to be affected by parental cold stress (Rako & Hoffmann 2006, Watson & Hoffmann 1995, 1996). In tropical *D. melanogaster* and *D. simulans* it was shown that maternal exposure to a cold stress led to increased progeny cold resistance, whilst paternal exposure led to a decrease in cold resistance in the progeny (Watson & Hoffmann 1995). Rako & Hoffmann (2006) showed that a low rearing temperature can also increase cold resistance and this would be an example of a positive cross generational effect. Interestingly, cold hardening in *D. melanogaster* can increase heat resistance in the offspring generation (Sejerkilde et al, 2003).

In this study I aim to assess both the presence and significance of short term cross generational effects in relation to heat resistance. In particular this study will aim to explore the many different ways heat resistance can be influenced by plasticity, and whether any benefits or negative effects to different conditions are then able to passed on to further generations. The environmental variable I chose to manipulate to investigate these effects was developmental rearing temperature. As discussed in the previous chapter, the environment in which an ectotherm develops can lead to significant changes in resistance. I have chosen to focus on heat resistance and whether developmental rearing temperature influences measures of adult heat resistance. In the context of climate change, the way heat resistance can be acquired or modified and then potentially passed on in the short term is rather topical. The evolutionary impact of these poorly understood cross generational effects is also unclear. I hope to understand whether ectotherms have the ability to utilise plastic effects in order to respond to changes in temperature. The species chosen for this study, *Drosophila hydei*, is a widespread (cosmopolitan) generalist species, similar in ecology to the main *Drosophila* model, *D. melanogaster*, which has been used extensively in thermal biology studies (Hoffmann et al, 2003).
I hypothesise that higher developmental rearing temperatures will lead to increased heat resistance when measured via adult heat knockdown time. Conversely, I predict that lower developmental rearing conditions will result in a decrease in heat resistance measured via adult heat knockdown time. To test the presence of cross generational effects, different rearing temperatures were chosen, as well as different combinations of these rearing temperatures over a three generation timespan, the scope of which could equate to the very early stages of adaptation to new environmental conditions. I formulated experimental treatment groups to reflect both consistent developmental rearing temperatures, as well as those which are inconsistent and change from F1 to their F2 generation. By comparing the treatment groups which switch temperatures to those which remain consistent, I can then work out the respective effect size of cross generational effects when comparing these two groups. This effect size can then be compared to normal rearing at a constant low or high temperature to indicate the strength of any potential cross generational effect.

The presence of cross generational effects was one aspect of this study. I also tested whether these effects would materialise regardless of time spent in a laboratory environment. By using consistent populations, the presence of cross generational effects shortly after introduction into the laboratory as well as many generations removed from the field can be assessed. Furthermore, to determine the effects of either parental or grandparental mechanisms on heat resistance, a generation of uniform temperature was used for all experimental treatments after their F1 and F2 generations were completed. It would be expected that a levelling off for heat resistance at the F3 stage would occur if only parental effects are involved. Conversely, grandparental effects could be considered relevant to this study if, after a uniform temperature generation, differences are still detected among experimental groups.

## 2.2 Methods

### 2.2.1 Field Flies and rearing

For this study flies were collected on two different occasions, one year apart. For the first collection, *Drosophila hydei* (Sturtevant) were collected from Kulnura, NSW on the 18 March 2013. A second collection occurred in early 2014, with two populations of *Drosophila hydei* being collected during March 2014 from Kulnura, NSW and Innisfail, QLD. All flies collected in the field were sexed and shipped. In referring to the populations used in this study I will reference both the location and year of collection in a shorthand code in order to maintain brevity and clarity. The initial population of *D.*
*Hydei* collected in 2013 will be referred to as '2013-NSW', whilst the 2014 NSW population will be '2014-NSW', with the 2014 QLD population being '2014-QLD'.

Field flies were set up in the laboratory using the offspring of 21 iso-female lines to create the mass bred generation. The parental generation was cultured at 25°C on a 12h:12h light:dark photoperiod to produce the F1 offspring. All flies were held and reared on a medium composed of dextrose (7.5% w/v), cornmeal (7.3% w/v), inactive yeast (3.5% w/v), soy flour (2% w/v), agar (0.6% w/v), nipagin (1.6% w/v), and acid mix (1.4% 10:1 proprionic acid:orthophosphoric acid). Prior to testing, all flies were sexed using CO₂ anaesthesia and allowed to recover on laboratory medium for a minimum of 24 hours.

### 2.2.2 Experimental procedure

Three environmental rearing temperatures were used over the three generations of the experiment; 19°C, 25°C, and 28°C. 19°C was chosen to represent the normal temperature in temperate areas; 28°C was chosen to represent the normal daily temperature in a hotter environment, for a tropical climate for example; 25°C was seen as the control temperature and in which most flies are reared in a laboratory.

Five treatments were formulated based on combinations of the rearing temperatures. At the F2 stage, the five treatment groups were: '1919' flies reared at 19°C for two generations; '1928' flies reared at 19°C for the first generation and at 28°C for the next generation; '2819' flies reared at 28°C for the first generation and at 19°C for the next generation; '2828' flies reared at 28°C for two generations; and, '2525' flies reared at 25°C for two generations as the control group. There were slight differences between how the 2013 and 2014 flies were treated after this initial two generations of thermal regimes. For the 2013 study, the initial population was subjected to the above thermal regime and then reared at 25°C for one extra generation (Figure 2.1). The same population was used at a later stage (derived from a sample which had been continuously reared at 25°C upon introduction into the laboratory environment), with the thermal regime commencing at generation F5 from the field, with testing occurring at F6 and at F7 (again with an extra generation at 25°C), as is shown in Figure 2.2. The 2014 study again followed the same rearing protocol as described above and shown in Figure 2.1, with the only divergence being the continuation of testing after each subsequent generation at 25°C. Testing continued each generation until the F8 stage, with the two 2014 populations being subjected to normal laboratory conditions (25°C), with these natural conditions being the only condition involved.
Flies were transferred to fresh media and allowed to lay for up to 2 days and then removed. Vials containing newly laid eggs were transferred to their respective temperatures for the commencement of the F1 generation. All treatments upon eclosion from the experimental thermal regime (at the F2 and F3 stage for the 2013 and 2014 flies, and at the F6 and F7 for the 2013 flies) were transferred to 25°C to allow for similar maturation times before being tested, as temperature has been shown to influence maturation times (Gilchrist & Huey 2001; Johnstone unpublished data). Flies from each sex and treatment were tested at the same time. To ensure that all treatments could be compared simultaneously, treatments were set up in a staggered manner to compensate for differences in developmental time.

Figure 2.1 - Experimental protocol detailing the rearing temperature regime for the cross generational effects thermal regime. This regime was used for both the 2013 and the 2014 studies. Further generations (F4, F5...etc.) were added to this design for the 2014 study and were not included in this diagram to avoid duplication of information.
2.2.3 Heat knockdown procedure

Heat knockdown testing was performed at both the F2 and F3 stage, with male and female flies being tested separately for both the 2013 and 2014 collected flies. Testing at the F4, F5, F6, and F8 stage was also carried out on the 2014 *D. hydei* flies. The 2013 *D. hydei* flies were also tested again at the F6 and F7 stage (from the field).

A minimum of ten flies per sex, population and treatment were used for each respective test and were placed individually into numbered glass specimen vials (50 mm height x 12 mm diameter). The vials were randomised and placed on a custom-built Perspex frame before being immersed into a 28°C custom-built water bath. Temperature was controlled using a Ratek SP599 thermoregulator with a REX-P24 controller.

A temperature ramping protocol was used to measure the heat resistance of the flies. The protocol I used was the '0.2' protocol (Schiffer et al. 2013) which involved having the water at 28°C for the first 15 minutes of the experiment followed by an incremental increase of 0.2°C per 10 minutes until reaching 38°C, after which the temperature remained at this level for the remainder of the experiment. Heat knockdown was defined as the point at which a fly was rendered unconscious, and unable to hold itself upright or be seen moving its mouthparts and did not respond to a light stimulus (a beam of light from a 12 LED hand torch) or gentle tapping of the vial. Heat knockdown time was recorded to the nearest second and then converted to minutes for data analysis. Flies were tested between the ages of 9 and 12 days post emergence to ensure flies were sexually mature upon testing (Markow 1985; unpublished data).

The heat assay was chosen to simulate environmental conditions experienced in the field, with the ramping assay best suited to this task. The ramping protocol chosen was the '0.2' protocol. It reached its final temperature of 38°C at the 65 minute mark, and was then maintained at this temperature until the end of the experiment. In a way this assay reflected an intermediate between a traditional direct heat assay and a slow, ramping heat assay (Mitchell & Hoffmann 2010; Terblanche et al. 2011). Furthermore, it is thought the '0.2' protocol simulates the maximum rate of temperature increase likely to be experienced in the field (Terblanche et al. 2011), which was important in my study of how *Drosophila* will respond in the short term to fluctuations in temperature.
Figure 2.2 - Experimental protocol detailing the rearing temperature regime for the repeat testing of the CDS *D. hydei* population. Testing occurred at both the F6 and F7 stage (relative to collection from the field). Generations 1-4 held at 25°C in a laboratory environment.

2.2.4 Analysis

Sex effects were large and sexes were therefore treated separately. I performed a one way ANOVA for each sex and population used during the study using R (R Core Team 2013) to determine whether developmental rearing temperature was significantly different among the experimental groups. Separate ANOVAs were used for each generation of testing. Where differences were detected, I then performed a post-hoc Tukey HSD test to determine which experimental treatments differed significantly from each other and whether I could find patterns between treatments to tease out cross generational effects. All heat knockdown data was analysed untransformed as it was mostly normally distributed.
### 2.3 Results

Table 2.1 - One way ANOVAs for the 2013-NSW *D. hydei* population indicating the significance of experimental treatment groups for heat resistance. Significant differences are indicated in bold. \( n \geq 10 \) for both sexes and all generations of testing.

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<td><strong>Male F2</strong></td>
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<tr>
<td>Treatment</td>
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<tr>
<td>Error</td>
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Table 2.2 - One way ANOVAs for the 2014-NSW (left) and 2014-QLD (right) *D. hydei* population indicating the significance of experimental treatment groups for heat resistance. Significant differences are indicated in bold. n ≥ 10 for both sexes and all generations of testing.

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Figure 2.3 - Mean (±SE) heat resistance of males, as measured by heat knockdown time (minutes), for experimental treatment groups for both the initial (F2 + F3) and repeat (F6 + F7) cross generational effects study with 2013-NSW D. hydei. a) Male F2 b) Male F3 c) Male F6 d) Male F7. n ≥ 10 for each of the treatment groups, and dissimilar letters indicate statistical significance between treatment comparisons.
Figure 2.4 - Mean (±SE) heat resistance of females, as measured by heat knockdown time (minutes), for experimental treatment groups for both the initial (F2 + F3) and repeat (F6 + F7) cross generational effects study with 2013-NSW D. hydei. a) Female F2 b) Female F3 c) Female F6 d) Female F7. n ≥ 10 for each of the treatment groups, and dissimilar letters indicate statistical significance between treatment comparisons.
Figure 2.5 - Mean (±SE) heat resistance of males, as measured by heat knockdown time (minutes), for experimental treatment groups for the 2014 cross generational effects study. Presented here are the *D. hydei* results for the male 2014-NSW population at the a) F2, b) F3, and c) F4 generations of testing. *n* ≥ 10 for each of the treatment groups, and dissimilar letters indicate statistical significance between treatment comparisons.
Figure 2.6 - Mean (±SE) heat resistance of females, as measured by heat knockdown time (minutes), for experimental treatment groups for the 2014 cross generational effects study. Presented here are the *D. hydei* results for the female 2014-NSW population at the a) F2, b) F3, and c) F4 generations of testing. n ≥ 10 for each of the treatment groups, and dissimilar letters indicate statistical significance between treatment comparisons.
Figure 2.7 - Mean (±SE) heat resistance of males, as measured by heat knockdown time (minutes), for experimental treatment groups for the 2014 cross generational effects study. Presented here are the *D. hydei* results for the male 2014-QLD population at the a) F2, b) F3, and c) F4 generations of testing. n ≥ 10 for each of the treatment groups, and dissimilar letters indicate statistical significance between treatment comparisons.
Figure 2.8 - Mean (±SE) heat resistance of females, as measured by heat knockdown time (minutes), for experimental treatment groups for the 2014 cross generational effects study. Presented here are the D. hydei results for the female 2014-QLD population at the a) F2, b) F3, and c) F4 generations of testing. n ≥ 10 for each of the treatment groups, and dissimilar letters indicate statistical significance between treatment comparisons.
Figure 2.9 - Side by side comparison displaying average heat resistance for the 25°C control group, as measured by heat knockdown time in minutes, for the 2013-NSW population when tested initially from the field (F2 + F3 generation) compared to when the same thermal regime was repeated (F6 + F7 generation). n ≥ 10 for each of the generations of testing.

Figure 2.10 - A sequential examination of heat resistance for the 25°C control group, as measured by heat knockdown time in minutes, for the 2014 cross generational effects study. Both the 2014-NSW and 2014-QLD March populations were used to illustrate this trend of an increase in heat resistance. n ≥ 20 for each of the generations of testing.
2.3.1 2013 Cross generational study

Initial tests F2/F3

Statistically significant differences between the rearing treatments are illustrated by dissimilar letters in the Figures above, whilst statistical significance is indicated by bold P values in the Tables above. For the 2013 cross generational study when tested at the F2 stage, a significant treatment difference was found for both males ($F_{4,45} = 7.15$, $P<0.0001$) and females ($F_{4,45} = 27.25$, $P<0.0001$). Flies reared at 28°C for two generations were more heat tolerant compared to flies reared at 19°C for two generations (Figure 2.3a). There was some evidence for cross generational effects, but more compelling were the immediate and combined environmental effects, with a lower rearing environmental temperature corresponding to decreased heat resistance and the opposite result for a higher rearing temperature. For male knockdown time, the 1928 treatment group was on average 6% lower than the 2828 treatment group, hinting at a potential negative consequence of a lowered F1 temperature resulting in a loss of heat resistance and the potential presence of a cross generational effect. On the other hand, heat knockdown time for the 2819 treatment group differed by only 2 minutes on average compared to the 1919 treatment group. There were larger differences when immediate environmental differences were considered, with the 2828 treatment group performing on average 14% better for knockdown time compared to the 2819 treatment group, and the 1919 treatment group performing on average 12% worse compared to the 1928 treatment group. Combining these effects, the 2828 treatment group performed 15% better for heat knockdown time compared to the 1919 treatment group.

The female data displayed a similar trend to the male data (Figure 2.4a). A similar cross generational trend was found, with the 1928 treatment group having 11% lower heat resistance compared to the 2828 treatment group. The 1919 and 2819 treatment groups showed resistance levels within one minute of each other, providing no evidence of a cross generational effect passing from the F1 to F2 generation. Immediate environmental effects were again large, with the 2828 group performing 27% better than the 2819 group, and the 1919 treatment group performing 23% worse than the 1928 treatment group. The largest difference was between the 1919 and 2828 treatment groups, with the 1919 group being 36% lower than the 2828 group for heat knockdown time.
After rearing at 25°C for the F3 generation, it was expected that all five treatments would have similar levels of resistance in the absence of effects lasting across multiple generations. A significant treatment difference was found for both males ($F_{4,45} = 2.88, P = 0.03$) and females ($F_{4,45} = 5.69, P < 0.001$) (Figures 2.3b + 2.4b). In contrast to the F2 results, the treatments which had been at 19°C at the F2 stage (191925 + 281925) performed the best on average for heat resistance, whilst the two treatments which were kept at 28°C for their F2 generation performed the worst on average. The increase in temperature (from 19°C to 25°C) led to higher resistance for treatments which were at 19°C for the F2 generation, whilst a decrease in temperature (from 28°C to 25°C) led to a decrease in heat resistance for treatments reared at 28°C for their F2 generation.

The cross generational effect detected in both sexes at the F2 generation between the '1928' and '2828' treatments (Figures 2.3b + 2.4b) was also present here at the F3, but reversed. After a generation at 25°C, the '1928(25)' treatment had a higher mean resistance than the '2828(25)' treatment. Despite both treatments having identical F2 and F3 rearing temperature regimes, a difference in their mean heat resistance is present. This suggests a potential grandparental effect, i.e., their F1 rearing temperature eliciting a response for more than one generation.

**Repeat at F6 generation**

The same procedure as used in the above experiments was applied here, but on stocks which had spent ~6 generations at 25°C in the laboratory, and the results are found in Figures 2.3c and 2.4c. When tested at the F6 stage, there were two key results. The first was the apparent absence of generational trends and the second was an apparent substantial increase in average heat resistance across all treatment groups when compared to the earlier experiment. For the males, there was no significant difference between the treatment groups ($F_{4,45} = 0.79, P = 0.54$), however the female data was marginally significant ($F_{4,45} = 2.77, P = 0.04$). Male treatments were all within 200 ± 10 minutes for heat knockdown. Female data was less consistent and mirrored the trends found in the original experiment, apart from the 1928 treatment group which had the highest heat resistance in the assay. On average across all five treatments, there was an increase of 47% for males and 44% for females in heat knockdown time when the population was re-tested, although it should be emphasized that these are comparisons of experiments undertaken at different times. For both sexes, the 1919 and 2819 treatments displayed the highest increase in heat resistance.
An extra generation at 25°C revealed similar results (Figures 2.1d + 2.2d). The male treatment groups did not differ significantly ($F_{4,45} = 1.82, P = 0.14$), whereas the female groups did marginally differ ($F_{4,45} = 2.91, P = 0.03$). Male treatments were all within 204 ± 6 minutes for heat knockdown, reflecting a very similar range to what was seen for the F6 stage. Female data was comparable to the male data, with all treatments being within 216 ± 8 minutes for heat knockdown. No female treatment groups differed significantly from each other in posthoc comparisons.

2.3.2 2014 Cross generational study

A repeat of the 2013 cross generational experiment was undertaken with two newly collected populations. The two 2014 populations, 2014-NSW, and 2014-QLD, were tested at each generation from the F2 stage onwards. It was again found that flies reared at 28°C for two consecutive generations produced flies with the highest heat resistance, whilst the opposite was true for flies reared at 19°C for two consecutive generations (Figures 2.5a, 2.6a, 2.7a, 2.8a). There was a significant treatment difference for both males ($F_{4,95} = 3.49, P = 0.01$) and females ($F_{4,95} = 3.27, P = 0.01$) for the 2014-NSW population. Furthermore, female treatments were significantly ($F_{4,45} = 3.12, P = 0.02$) different whilst males did not display treatment differences ($F_{4,45} = 2.17, P = 0.09$).

For the male data, relative to the 2828 treatment, the heat resistance of the 1928 treatment decreased 5.4% (2014-QLD) and 3% (2014-NSW), suggesting 19°C at the F1 stage reduced heat resistance at the F2 stage. Similarly, relative to the 1919 treatment, heat resistance of the 2819 treatment increased 7.8% (2014-QLD) and 5.3% (2014-NSW), suggesting a 28°C F1 temperature could increase heat resistance at the F2 stage. For the female data, relative to the 2828 treatment, the heat resistance of the 1928 treatment decreased 7% (2014-QLD) and 2.7% (2014-NSW), suggesting a 19°C F1 temperature could reduce resistance at the F2 stage. Similarly, relative to the 1919 treatment, the heat resistance of the 2819 treatment increased 9.2% (2014-QLD) and 5.5% (2014-NSW), suggesting a 28°C F1 temperature increased resistance at the F2 stage.

For the F3 generation, which involved testing the five treatments after they had all undergone a generation at 25°C, a general increase of 14.9% (or ~22 minutes) across all treatments was found (Figures 2.5b, 2.6b, 2.7b, 2.8b contrasted with Figures 2.5a, 2.6a, 2.7a, 2.8a). There was no longer any significant treatment differences for both sexes ($P>0.05$). Similar to what was found at the F3 stage for the 2013 cross generational experiment (Figures 2.3, 2.4), the '192825' treatment group
had a higher heat resistance (3.3% increase for Males, 7.6% increase for Females) compared to the '282825' treatment group. Due to the F2 and F3 rearing temperature being exactly the same for both treatment groups mentioned, this trend is not consistent unless seen in the context of grandparental effects being responsible. Instead, the '281925' treatment maintained a higher heat resistance (3.1% increase for Males, 7% increase for Females) compared to the '191925' treatment at the F3 stage.

The F4 stage of testing, which was undertaken after another generation at 25°C for development, again showed no statistical significance between the treatment groups (P>0.05). It is at this stage that any past developmental rearing condition differences have been lost, as all treatments displayed heat resistance that was within 5 minutes of each other for both sexes (Figures 2.3c, 2.4c, 2.5c, 2.6c). The one grandparental effect detected at the F3 stage of testing had now disappeared.

Any cross generational effects due to developmental rearing conditions appear to not be maintained after a minimum of two generations at 25°C.

25°C control group changes

To investigate the potential effects of laboratory conditions and how they were influencing the heat resistance of the *D. hydei* populations, I chose to graphically present the results of the control 25°C group, which was kept at this temperature throughout the experiment. As shown in both Figures 2.9 and 2.10, a substantial difference can be detected between newly introduced (and tested) populations of *D. hydei* into a laboratory environment compared to those same populations when tested a few generations afterwards. For 2013-NSW males there was an increase of 69 minutes for heat knockdown time at the F6/F7 stage compared to when initially tested at the F2/F3 stage; a significant difference between these two means was present (P<0.05). For 2013-NSW females an even greater increase of 86 minutes for heat knockdown time was found for the F6/F7 stage of testing compared to the F2/F3 stage; a significant difference was noted (P<0.05).

A continuous increase in heat knockdown time was noted for both the 2014-NSW and 2014-QLD populations as shown in Figure 2.10. A mean heat knockdown time, as taken as an average between both of the populations, was first measured at the F2 stage of testing as being ~148 minutes. This figure then increased significantly to a value of ~204 minutes by the time the F8 generation was tested. This equated to an increase of 56 minutes. This trend was consistent for both sexes.
2.4 Discussion

Weak evidence for cross generational effects related to heat resistance were found in this study, with stronger evidence for acclimation effects influencing heat resistance. When flies collected in 2013 were first collected from the field and exposed to specific rearing temperature regimes, weak evidence for cross generational effects were found. When the same population was re-tested four generations later, there was no evidence for any signs which could be characterised as being a cross generational effect. This was then repeated in 2014 with two different populations, and comparable results were found. Both populations of 2014 *D. hydei* behaved in a similar fashion, with some evidence for cross generational effects for heat resistance, but this trend disappearing when re-tested generations later. However the nature of the cross generational effects was not entirely consistent between the two experiments. With both the 2013 and 2014 populations, a negative cross generational effect was detected in regards to heat resistance with a low F1 rearing temperature lowering heat knockdown time when tested at the F2 stage. For the 2014 populations, a positive cross generational effect for heat resistance was detected, with a high F1 rearing temperature increasing heat resistance at the F2 stage. This trend was however not found for the 2013 population.

Whereas cross generational effects were relatively weak and not entirely consistent, the evidence for differences in heat resistance related to developmental acclimation was substantial. This finding was in line with others in showing the importance of developmental rearing temperature on influencing heat resistance. Other studies on similar species of *Drosophila* and other ectotherms have revealed results consistent to mine with thermal resistance traits being considered (Jenkins & Hoffmann 1994; Hoffmann et al, 2003; Rako & Hoffmann 2006; Schiffer et al, 2013), as well as life history traits (Watson & Hoffmann 1996; Magiafoglou & Hoffmann 2003; Rako & Hoffmann 2006; Bentz & Powell 2014). Fluctuating temperatures during development have also been shown in the literature to influence stress resistance traits (Sarup & Loeschcke 2010; Bozinovic et al, 2011; Overgaard et al, 2011) but was not covered in my study. The environment of the developing offspring has been shown to consistently influence stress resistance levels into adulthood, and my study reinforces this in *D. hydei*.

The literature for cross generational effects on stress resistance remains sparse for *Drosophila* (Jenkins & Hoffmann 1994; Watson & Hoffmann 1996; Schiffer et al, 2013), and evidence for beneficial effects even more so (Watson & Hoffmann 1996; Schiffer et al, 2013). Beneficial effects for stress resistance traits in the context of climate change could include an increase in heat
resistance, which would allow an individual to persist in the light of increased temperatures. Beneficial cross generational effects may also involve an increase in cold resistance, with Rako and Hoffmann (2006) finding a low rearing temperature increased adult cold resistance. For heat resistance, Schiffer et al, (2013) provided evidence for beneficial cross generational effects in three *Drosophila* species, with a high F1 temperature increasing resistance when tested at the F2 stage which had involved a low rearing temperature. For the cross generational effect detected at the F2 stage to become adaptive or persist, this high temperature would then constitute the new environment of the progeny. In this situation, an individual may benefit from an increase in temperature if it were within the parameters experienced by its parents. For the 2014 experiment in the current study using two populations of *D. hydei*, a consistent trend was found between the two populations to suggest a beneficial cross generational effect. The approach is similar to that used in Schiffer et al, (2013) in that an F1 rearing temperature of 28°C was followed by an F2 rearing temperature of 19°C and led to an increase in heat resistance compared to two generations at 19°C. Were the progeny to then experience this high temperature of 28°C during their lifetime, this cross generational effect may persist. In the context of climate change this represents a possible adaptive change, even if the effects appear to be relatively small.

Negative cross generational effects were also found for heat resistance by Schiffer et al, (2013) whereby F1 flies were more heat resistant compared to F2 flies. For both the 2013 and 2014 experiments in this study, I was also able to detect a potential negative cross generational effect for heat resistance. High developmental rearing temperature at the F1 stage lead to an increase in heat resistance when the F2 stage involved a low rearing temperature. Again, for this trend to have persisted the progeny would need to have experienced this low temperature post-eclosion.

My study differed to that conducted by Schiffer et al, (2013) in that flies were not directly tested from the field. Instead, my populations were given two generations within the laboratory environment prior to testing. In this design, I was particularly interested in the effects of F1 rearing temperature when tested later at the F2 stage, with Schiffer et al, (2013) testing at both the F1 and F2 stages. This was done to identify specific cross generational effects related directly to developmental rearing temperature, with consistent developmental rearing temperature providing a comparison group to test the strength of these effects.

The repeatability of the positive or negative cross generational effects remains an issue. The ability to detect the negative effect at different timepoints (2013 and 2014) as well as between three different populations (2013-NSW, 2014-NSW and 2014-QLD) is encouraging and remains a point of consistency throughout this study, even if positive effects were not consistently detected. As has
been shown in this study, and that conducted by Schiffer et al, (2013), cross generation effects can be detected but appear relatively small.

Signs of potential laboratory adaptation

There were two further findings which required further investigation. They both involved the time populations were spending in a laboratory environment. Firstly, the capacity to categorically detect cross generational effects related to heat resistance in this study may depend on the time stocks are in the laboratory. Trends seemed clear when newly collected populations were tested within the laboratory. These trends were consistent when both the 2013 and 2014 populations were first tested at the F2 stage (two generations after introduction to the laboratory) but not later. Furthermore, there appeared to be a uniform increase in heat resistance across all treatments when the populations were re-tested. An increase of ~70 minutes was found across all treatment groups was found when the 2013-NSW population was re-tested at the F6 stage compared to when it was tested at the F2 stage, whilst a ~60 minute increase was found for the 2014 populations. Such a uniform increase in resistance could point to signs of adaptation to the laboratory environment or systematic changes in conditions that in turn influenced resistance, even though all individuals were reared in a consistent manner. The time spent in the laboratory environmental appears to be a factor, but would need further analysis. Laboratory adaptation has been shown to be a factor in modifying life history traits in *Drosophila*; there is some evidence in the literature to support increases in stress resistance such as heat resistance in *Drosophila* (Krebs et al, 2001), with others suggesting the opposite (Griffiths et al, 2005; Kellermann et al, 2015). The issue is addressed further in the next chapter.
Chapter Three - Laboratory adaptation

3.1 Introduction

When assessing traits such as thermal resistance and those to do with life history under laboratory conditions, laboratory adaptation must come into consideration. Studies on both short and long term evolutionary processes in the laboratory have come to complement field and comparative studies, and the high level of control provided in the laboratory is important (Gibbs 1999). However, these benefits come at a cost. Stress resistance studies using freshly introduced Drosophila, compared to stocks which have been maintained in the laboratory for many generations, may lead to different results (Harshman & Hoffmann 1999). It is common practice within Drosophila laboratories to give freshly caught field flies two generations in the laboratory before undertaking any experimental manipulation. In doing so, this will eliminate any maternal or grandmaternal effects from the field which could influence the progeny in the laboratory (Watson and Hoffmann 1996; Mousseau and Fox 1998; Magiafoglou & Hoffmann 2003; Rako and Hoffmann 2006). Paternal effects from the field would also need to be monitored (Watson and Hoffmann 1995; Magiafoglou & Hoffmann 2003). Laboratory stocks of Drosophila introduced into a novel environment such as a laboratory will evolve along different evolutionary trajectories depending on their genetic background (Cohan & Hoffmann, 1989; Matos et al, 2002). This could influence levels of stress resistance and mechanisms underlying them, making extrapolations to the field difficult.

Much of the work that has been done on stress resistance in Drosophila has been undertaken on long established laboratory stocks. Many believe there are benefits to using long established stocks for these types of experiments (Harshman & Hoffmann 2000). It is thought that these long established stocks are very close to genetic equilibrium with their environment when compared to newly established stocks (Harshman & Hoffmann 2000; Service & Rose 1985). The consequence of using these lines could then be the removal of confounding factors associated with laboratory adaptation. The use of newly established stocks of Drosophila for selection experiments needs to be undertaken with caution as correlated responses to selection could be mistaken for, or masked by, traits that are being influenced by rapid adaptation to a novel environment. The loss of genetic variation is a major downside to working with long established Drosophila stocks (Harshman &
Hoffmann 2000). With knowledge of both the positives and negatives of using fresh versus well established stocks, it may be possible to combine these approaches.

Maintaining populations of Drosophila for multiple generations in a novel environment such as a laboratory can give rise to trait selection which would not have been normally experienced in the wild (Harshman & Hoffmann 2000; Matos et al, 2002; Huey & Rosenzweig 2008; Santos et al, 2010, Tobler et al, 2015). This phenomenon has been referred to as laboratory adaptation and has been studied thoroughly in some Drosophila species (Harshman & Hoffmann 2000; Tobler et al, 2015). A considerable amount of work has been conducted on both D. subobscura and D. melanogaster, in looking into the role laboratory adaptation has on various life history traits in driving convergent adaptive responses. Laboratory adaptation has been shown to affect life history traits such as increased early fecundity, larval competitiveness and development time in D. subobscura (Matos et al, 2000; Simões et al, 2008; Fragata et al, 2014). In D. melanogaster laboratory adaptation has also been proven to increase larval competitiveness and decrease development time, and produce larger bodied individuals when populations are maintained for several generations in the laboratory (Frankham & Loebel 1992; Latter & Mulley 1995; Sgrò & Partridge 2000; Tobler et al, 2015).

As well as influencing life history traits in Drosophila, laboratory adaptation can also affect stress resistance levels, potentially confounding experiments attempting to predict future environmental responses. It was shown in D. melanogaster that testing several lines from the same location over several years leads to a rapid loss of both starvation and desiccation resistance (Hoffmann et al, 2001). This loss of resistance in these traits corresponded to the time the lines had spent in the laboratory, with the results most pronounced in the oldest lines compared to the newer lines (Hoffmann et al, 2001). In D. birchii instead it was found that both desiccation and heat resistance were not affected by laboratory adaptation, but that starvation resistance and recovery time following a cold shock treatment did appear to be influenced by the amount of time spent in the laboratory (Griffiths et al, 2005). The effect of laboratory adaptation on heat resistance was investigated by Krebs et al, (2001). They were able to show that after introduction into the laboratory environment, Hsp70 and heat resistance levels increased initially in D. melanogaster, but that over time these traits levelled out and remained quite constant. Krebs et al, (2001) surmised that despite initial environmental variation influencing heat resistance after initial introduction, changes in this trait were minimal. However this is likely to be species specific, and also dependant on the trait which is being measured by the assay.

Whether thermal tolerance traits are being influenced by laboratory adaptation is unclear. The literature for laboratory adaptation for some Drosophila species such as D. melanogaster and D.
subobscura remains sparse, while for more restricted species of Drosophila living in tropical areas for instance, the literature is even more limited (Griffiths et al, 2005). Measuring the scope for which these restricted Drosophila species can adapt to future temperature and desiccation increases in the laboratory remains a vital task. Ensuring other factors such as laboratory adaptation are not confounding or masking experimental results needs to be investigated.

Adaptation to the laboratory or experimental evolution has been shown to occur in species of Drosophila when introduced into a laboratory environment. Adaptation to a novel environment has led to improvements in certain life history traits in Drosophila (Sgro & Partridge 2000). The majority of studies dealing with experimental evolution tend to favour either a comparative or an evolutionary trajectory approach. The comparative method of investigating experimental evolution involves testing populations at different stages of evolution and/or different timepoints from their collection from the field (Frankham & Loebel 1992; Latter & Mulley 1995; Hoffmann et al, 2001; Woodworth et al, 2002; Gilligan & Frankham 2003; Griffiths et al, 2005). In this sense, the evolutionary dynamics of a particular population is inferred by comparing both the length of time spent in the laboratory and the location in which the population originated. However, this approach has been criticised in not being capable of accurately predicting how individual populations will evolve over time, and instead creating only a simple snapshot at one point in time.

The second comparative approach involves studying the evolutionary trajectory of populations from their introduction into the laboratory through to their adaptation to their new surroundings (Matos et al, 2000, 2002, 2004; Krebs et al, 2001). Different conclusions of experimental evolution have been found when comparing these approaches (Simoes et al, 2009). However, Matos et al, (2004) utilised both methods to test for experimental evolution, and found that the comparative method was capable of predicting fecundity-related traits due to their robust evolutionary nature, but was not as reliable for other stress related traits such as starvation resistance. Starvation resistance was found to have both increased (Griffiths et al, 2005) and decreased (Hoffmann et al, 2001) in response to laboratory adaptation. This further illustrates the difficulty in using comparative approaches for some traits to accurately predict laboratory adaptation.

In this study I intend to explore the influence of adaptation to the laboratory on heat resistance. Results which were obtained during the cross generational studies in Chapter Two prompted the exploration of potential laboratory adaptation being responsible for the gradual increase in heat resistance over time noted in this Chapter. In exploring this theme of laboratory adaptation I wanted to also assess other sources of variation such as sex, developmental rearing temperature, pre-experimental hardening, population effects, and geographical differences. I expect rearing at two
generations at 19°C to lead to a decrease in heat knockdown time relative to rearing at a
temperature of 28°C. This trend should be consistent across different populations.

Apart from considering developmental rearing temperatures, hardening will also be investigated. The literature has stated that hardening treatments prior to heat often lead to higher heat resistance (Hoffmann et al, 2003), but that this effect is not universal (Mitchell & Hoffmann 2010). In this study I expect a one hour hardening treatment at a stressful temperature will in essence prime individuals for a heat shock. In assaying both hardened and non-hardened individuals at the same time, a level playing field is maintained and the non-hardened flies act as strict controls in determining the influence of hardening. Of further interest in this area is the performance of the two sexes to hardening treatments. There is evidence to suggest heat selected female Drosophila will benefit less from hardening pre-treatments compared to males when later subjected to a heat stress (Sejerkilde et al, 2003), but this result has not been systematically investigated. Perhaps such effects depend on size, as it is thought that larger body size and internal fat deposits better insulate the effects of thermal fluctuations in female Drosophila. Male Drosophila have smaller bodies, and thus any added advantage to be gained from being primed for a stressful situation is likely to be expressed. I hope to further explore this and I predict males will benefit from a hardening treatment compared to females, and I would expect this trend to be independent of developmental rearing temperature.

I expect to find differences between populations, both in the context of differing collection times as well as differing locations, for heat resistance. Differences in heat resistance could be expected to occur between populations sourced from the north of Australia (Queensland) compared to the south of Australia (Victoria), with studies showing heat resistance has strong clinal patterns (Hoffmann et al 2002; Sgro et al 2010), but the form in which heat resistance is measured may be important. To explore the influence of laboratory adaptation, I will be comparing heat resistance for populations which have been kept in a laboratory environment for differing periods of time. I hypothesise that the populations which have been maintained in the laboratory for the longest will display the highest heat resistance, whilst the opposite will be true for newly collected populations. Two comparative studies will give two time points.
3.2 Methods

3.2.1 Field Flies and rearing

This study comprised of two separate population comparisons with the first being conducted in February 2015, and the second in October 2015. Five populations were used in February, and six in October. The aim of this experiment was to compare the heat resistance of these different populations whilst considering both temporal and spatial differences. The role of time spent in the laboratory was also paramount. All flies collected in the field were sexed upon capture after which they were sent to the laboratory in Melbourne.

All flies received from the field were set up in the laboratory using 21 isofemale lines per population to create the mass bred generation. The parental generation was cultured at 25°C on a 12h:12h light:dark photoperiod to produce the F1 offspring. All flies were held and reared on a medium composed of dextrose (7.5% w/v), cornmeal (7.3% w/v), inactive yeast (3.5% w/v), soy flour (2% w/v), agar (0.6% w/v), nipagin (1.6% w/v), and acid mix (1.4% 10:1 propionic acid:orthophosphoric acid). Prior to testing, all flies were sexed using CO2 anaesthesia and allowed to recover on laboratory medium for a minimum of 24 hours.

3.2.2 Experimental populations

Table 3.1 - Experimental populations used in the February population study. The populations are labelled according to their collection date and location. The number of generations each population has spent in the laboratory are noted.

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</thead>
<tbody>
<tr>
<td>Generations in lab</td>
<td>F17</td>
<td>F7</td>
<td>F7</td>
<td>F2</td>
<td>F2</td>
</tr>
</tbody>
</table>

Populations were defined by both their collection location and collection date, and I utilised a coding system to allow consistent labelling of the populations. For the first population comparison, five distinct populations were used (Table 3.1). Three populations were sourced from Kulnura, NSW and collected in March 2013, March 2014, and October 2014, and labelled '2013-NSW', '2014-NSW', '2014a-NSW' respectively. For remaining two populations, one was sourced from Innisfail, QLD
(‘2014-QLD’), and the other from Melbourne, VIC (‘2014-VIC’). This study was conducted in February 2015.

For the second population experiment, six populations were used, as shown in Table 3.2. In addition to the populations used in the February study, a further population was collected both from Kulnura, NSW in March 2015 (‘2015-NSW’), and from Melbourne, VIC in April 2015 (‘2015-VIC’). The 2014-QLD population was not used in this study.

Table 3.2 - Experimental populations used in the October population study. The populations are labelled according to their collection date and location. The number of generations each population has spent in the laboratory are noted.

<table>
<thead>
<tr>
<th>Collection location</th>
<th>Collection date</th>
<th>Generations in lab</th>
</tr>
</thead>
<tbody>
<tr>
<td>2013-NSW</td>
<td>March, 2013</td>
<td>F23</td>
</tr>
<tr>
<td>2014-NSW</td>
<td>March, 2014</td>
<td>F13</td>
</tr>
<tr>
<td>2014a-NSW</td>
<td>October, 2014</td>
<td>F7</td>
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<tr>
<td>2014-VIC</td>
<td></td>
<td>F7</td>
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<tr>
<td>2015-NSW</td>
<td>October, 2015</td>
<td>F2</td>
</tr>
<tr>
<td>2015-VIC</td>
<td>April, 2015</td>
<td>F2</td>
</tr>
</tbody>
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3.2.3 Experimental design

Each population was split into three separate and independent sub-populations and was then put through a two generation temperature rearing regime. All populations (and by extension, all sub-populations) were subjected to two generations at 19°C and 28°C to form two treatments of either '1919' for two generations at 19°C or '2828' for two generations at 28°C.

Flies which emerged at the F2 stage from both the 19°C and 28°C environments were transferred to 25°C for maturation prior to testing, as temperature has been shown to influence maturation times (Gilchrist & Huey 2001; Johnstone unpublished data). To ensure that all treatments could be compared simultaneously, treatments were set up in a staggered manner to compensate for differences in developmental time.

Further to the rearing temperature regime, all sub-populations were also subjected to short term hardening prior to testing their heat resistance. This hardening procedure was undertaken to ascertain whether heat resistance is influenced by hardening procedures. It was also used to see whether temporal latitude differences were found in hardened compared to non-hardened flies.
For each treatment and sub-population, flies were placed individually in vials and held in a 37°C water bath where the temperature was kept constant. They remained in the 37°C water bath for one hour and were then allowed to rest in single holding food vials at 25°C for six hours prior to the heat resistance assays. All treatments and sub-populations also had non-hardened flies which were tested alongside those that were hardened.

All vials were randomised, and each treatment, sub-population, and hardening treatment were tested at the same time (condition). Males and females were however tested in separate assays. Due to both logistical constraints and water bath capacity, one replicate from each condition was tested per assay and per sex to give a total number of 60 per assay for the first study performed in February 2015 (five distinct populations), and 72 flies per assay for the second study performed in October 2015 (six distinct populations). This was repeated ten times for each sex to give a final replicate number of ten for each condition.

3.2.4 Heat knockdown procedure

For the heat assays, flies were randomly placed into numbered glass specimen vials (50 mm height x 12 mm diameter). The vials were placed on a custom-built Perspex frame before being immersed into a 28°C custom-built water bath. Temperature was controlled using a Ratek SP599 thermoregulator with a REX-P24 controller. The '0.2' ramping protocol (Schiffer et al, 2013) was used, which involved having the water at 28°C for the first 15 minutes of the experiment followed by an incremental increase of 0.2°C per 10 minutes until reaching 38°C, after which the temperature remained at this level for the remainder of the experiment. Heat knockdown was defined as the point at which a fly was rendered unconscious, and unable to hold itself upright or be seen to be moving its mouthparts, and it did not respond to a light stimulus (a beam of light from a 12 LED hand torch). Heat knockdown time was recorded to the nearest second and then converted to minutes for data analysis. Flies were tested between the ages of 9 and 12 days old to ensure flies were sexually mature upon testing (Markow 1985; Johnstone unpublished data).

3.2.5 Analysis

The statistical program R was used for these ANOVA analyses, with post hoc analysis performed by a Tukey HSD test to determine which groups differed significantly from one another. A block effect
was included in the ANOVAs. Initially, a five factor ANOVA was undertaken that included population, sex, treatment, hardening status, and subpopulation as factors. Because subpopulations did not differ in resistance, they were pooled for population comparisons. Subsequent analysis were undertaken on subsections of the data to clarify patterns. Sex effects and some others were large and therefore sexes were treated and analysed separately as well as together. To clarify the relative magnitude of main effect, the maximum mean difference between populations, hardening, rearing effects and so on were considered directly.
3.3 Results

Table 3.3 - ANOVA analysis for the two Population Experiments indicating the sources of variation which influence heat resistance in *D. hydei*. Statistically significant results are indicated by bold P values.

<table>
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<tr>
<th>Source</th>
<th>February 2015 Population Comparison</th>
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</table>
Table 3.4 - Average mean knockdown time in minutes (±SEM) for *D. hydei* during the February 2015 experiment. *n* ≥ 30 per population, *n* ≥ 150 for the population aggregates.

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</thead>
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<td><strong>Overall</strong></td>
<td>181.76 ± 0.86</td>
<td>187.98 ± 1.02</td>
<td>184.24 ± 0.99</td>
<td>188.80 ± 0.96</td>
<td>187.33 ± 0.94</td>
<td>186.02 ± 0.96</td>
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<td><strong>Male</strong></td>
<td>183.67 ± 1.30</td>
<td>189.70 ± 1.34</td>
<td>185.03 ± 1.38</td>
<td>190.53 ± 1.34</td>
<td>189.00 ± 1.30</td>
<td>187.59 ± 1.33</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td>179.86 ± 1.10</td>
<td>186.25 ± 1.53</td>
<td>183.44 ± 1.42</td>
<td>187.08 ± 1.37</td>
<td>185.69 ± 1.36</td>
<td>184.46 ± 1.36</td>
</tr>
<tr>
<td><strong>19°C reared</strong></td>
<td>177.51 ± 0.98</td>
<td>186.53 ± 1.23</td>
<td>180.51 ± 1.10</td>
<td>184.82 ± 1.27</td>
<td>184.95 ± 1.20</td>
<td>182.86 ± 1.16</td>
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<tr>
<td><strong>28°C reared</strong></td>
<td>186.01 ± 1.30</td>
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<td>192.68 ± 1.36</td>
<td>189.84 ± 1.44</td>
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<tr>
<td><strong>Non-Hardened</strong></td>
<td>181.53 ± 1.10</td>
<td>187.65 ± 1.33</td>
<td>183.08 ± 1.33</td>
<td>186.96 ± 1.31</td>
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<tr>
<td><strong>Hardened</strong></td>
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<td>188.83 ± 1.41</td>
<td>187.03 ± 1.43</td>
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</table>

Table 3.5 - Average mean knockdown time in minutes (±SEM) for *D. hydei* during the October 2015 experiment. *n* ≥ 30 per population, *n* ≥ 180 for the population aggregates.

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<tr>
<td><strong>Overall</strong></td>
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<td><strong>Male</strong></td>
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<td>201.68 ± 1.31</td>
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<td><strong>Female</strong></td>
<td>184.97 ± 1.04</td>
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<td>195.64 ± 1.36</td>
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<td><strong>19°C reared</strong></td>
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<td><strong>Hardened</strong></td>
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<td>194.71 ± 1.44</td>
<td>198.56 ± 1.37</td>
<td>193.51 ± 1.37</td>
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Table 3.6 - Effects of hardening on both males and females in *D. hydei* across all populations for the February population experiment. Heat knockdown is presented in minutes (±SEM), *n* ≥ 30 per population, *n* ≥ 150 for the population aggregates.

<table>
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<tr>
<td>Overall</td>
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<td>183.08 ± 1.90</td>
<td>186.20 ± 1.91</td>
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<td>19°C reared</td>
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<td>28°C reared</td>
<td>184.29 ± 2.20</td>
<td>192.67 ± 2.42</td>
<td>190.76 ± 3.44</td>
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<tr>
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<td>Overall</td>
<td>185.91 ± 2.06</td>
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<tr>
<td>Overall</td>
<td>181.63 ± 1.58</td>
<td>185.55 ± 2.03</td>
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<td>177.46 ± 1.88</td>
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<td>Overall</td>
<td>178.08 ± 1.51</td>
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<td>183.79 ± 2.13</td>
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Table 3.7 - Effects of hardening on both males and females in *D. hydei* across all populations for the October population experiment. Heat knockdown is presented in minutes (±SEM), n ≥ 30 per population, n ≥ 150 for the population aggregates.

<table>
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<td>198.82 ± 1.91</td>
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<tr>
<td>Overall</td>
<td>181.93 ± 1.36</td>
<td>196.34 ± 1.97</td>
<td>183.86 ± 1.79</td>
<td>188.01 ± 1.79</td>
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<td>19°C reared</td>
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<td>192.79 ± 3.10</td>
<td>198.46 ± 2.79</td>
<td>190.66 ± 1.18</td>
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Figure 3.1 - Mean (±SEM) heat resistance, as measured by heat knockdown time (minutes) for experimental populations with 19°C reared flies being represented on the left hand side of the graphs (light grey), and 28°C reared flies shown on the right (dark grey). Comparisons between a) male populations tested in February, b) female populations tested in February, c) male populations tested in October, and d) female populations tested in October are shown in this figure. n ≥ 60 for each population, and an asterisk indicates statistical significance between the 19°C and 28°C groups.
Knockdown time (minutes)

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<td>2014</td>
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**Figure 3.2** - Mean (±SEM) heat resistance, as measured by heat knockdown time (minutes) for the *February* population experiment showing the effects of hardening and developmental rearing conditions. Populations are grouped in pairs on the x axis with non hardened flies represented in light grey and hardened flies represented in dark grey. Comparisons between a) male 19°C reared populations, b) male 28°C reared populations, c) female 19°C reared populations, and d) female 28°C reared populations are shown in this figure. n ≥ 60 for each population, and an asterisk indicates statistical significance between the non hardened population compared to its hardened equivalent.
Figure 3.3 - Mean (±SEM) heat resistance, as measured by heat knockdown time (minutes) for the October population experiment displaying the effects of hardening and developmental rearing conditions. Populations are grouped in pairs on the x axis with non hardened flies represented in light grey and hardened flies represented in dark grey. Comparisons between a) male 19°C reared populations, b) male 28°C reared populations, c) female 19°C reared populations, and d) female 28°C reared populations are shown in this figure. \( n \geq 60 \) for each population, and an asterisk indicates statistical significance between the non hardened population compared to its hardened equivalent.
Figure 3.4 - The relative effect contribution for each source of variation investigated (average difference between treatments) in the two population experiments.
3.3.1 General summary

February experiment

All five populations had heat knockdown times ranging from 154 minutes through to 220 minutes. A five factor ANOVA revealed highly significant treatment, population, and sex effects (Table 3.3). Furthermore, a hardening effect was detected. As well as these overall effects, a significant interaction between sex and hardening was also found (Table 3.3).

The 2013-NSW population consistently exhibited the lowest heat resistance and was significantly different from all other populations (Tukey: P<0.001) (Tables 3.3 + 3.4). Remaining populations did not vary much, with heat resistance being within five minutes (Table 3.4). As was predicted, all populations reared at 19°C for two generations had on average lower heat resistance compared to populations reared at 28°C. On average for both sexes, the 2828 treatments performed 4% better in terms of heat resistance compared to the 1919 treatments. Males outperformed females by an average of three minutes (Table 3.4). Hardening flies had only a small impact on resistance, with hardened flies performing 2 minutes better in compared to flies which weren't hardened (Table 3.4).

October experiment

Heat knockdown times ranged from 167 minutes to 228 minutes. An ANOVA on the entire data set revealed highly significant effects of treatment, population, sex, and hardening (Table 3.3). Furthermore, there was an interaction between sex and hardening.

The 2013-NSW population maintained its status of being the least resistant population, differing significantly from all other populations (Tukey: P<0.0001) (Tables 3.3 + 3.5). The remaining populations were within five minutes of each other for heat resistance (Table 3.5). Pooling across treatment, sex and hardening, the 2013-NSW was on average ten minutes lower for heat resistance compared to the next population. Males outperformed females by an average of five minutes (Table 3.5). The 2014-NSW population was the most heat resistant for both sexes, but a difference of four minutes for males and seven minutes for females separated the five populations collected from 2014. For rearing temperature, heat resistance was lowest for each of the populations when they were reared at 19°C compared to 28°C (Table 3.5). Compared to the 4% decrease in February comparison, the average decrease in resistance for the 19°C rearing treatment was 2%. The 2013 March NSW population was again the least resistant. Hardening did not confer much advantage with
an average decrease of two minutes in heat knockdown time compared to non-hardened individuals (Table 3.5).

3.3.2 February population experiment

Effects of heat hardening on sex

While there was a significant overall effect of hardening, there was also an interaction between hardening and sex (Table 3.3). Males performed on average better for heat resistance compared to females (Figure 3.2), and males showed a positive effect of hardening when reared at either 19°C or 28°C when aggregated across populations (Figure 3.2 a + b). As seen in Table 3.6, hardened males displayed an improvement of five minutes for heat knockdown time compared to non hardened males. For females, positive effects were not evident regardless of rearing conditions (Figure 3.2 c + d) and hardened flies had resistance levels 1 minute lower than non hardened females (Table 3.6).

Developmental rearing conditions influenced by sex

While populations differed overall, separating the effects of both sex and rearing temperature revealed where population effects were evident. For males, the populations differed significantly when reared at 19°C ($F_{4,292} = 6.45$, $P<0.0001$), but not when reared at 28°C ($F_{4,250} = 1.10$, $P=0.36$). Females followed a similar trend with 19°C reared populations differing significantly from each other ($F_{4,293} = 4.73$, $P<0.001$), whilst those reared at 28°C did not differ significantly ($F_{4,250} = 2.21$, $P=0.07$). Post hoc analyses revealed similar results to what was found with the original overall comparison. For both males and females reared at 19°C, the 2013-NSW population had the lowest heat resistance and was significantly different to all other populations (Tukey: $P<0.05$) apart from the 2014-NSW population (Tukey: $P = 0.94$). No statistical differences were found for the 28°C reared
populations, but the 2013-NSW population did have the lowest heat resistance for both males and females (Figure 3.1 a + b).

As has been established previously, rearing at 28°C leads to an increase in heat resistance compared to rearing at 19°C. This trend is shown in Figure 3.1 (a +b) with populations reared at 19°C being significantly less heat resistant compared to when reared at 28°C.

### 3.3.3 October population experiment

**Effects of heat hardening on sex**

As shown in Table 3.1, a significant hardening effect was found for the October experiment. A difference of three minutes was found between non hardened and hardened flies (Table 3.7). Non hardened individuals performed better for heat knockdown time. A significant interaction was found between hardening and the sex of an individual (Table 3.1).

Performances of the two sexes differed when hardening was taken into consideration. Males performed on average only slightly better when hardened prior to heat assaying (compared to non hardened flies), with an increase of less than one minute (Table 3.7). Hardened females were on average six minutes less heat resistant compared to non hardened females (Table 3.7). For males, rearing temperature did not have an influence on hardening as seen in both Table 3.7 and Figure 3.3 (a + b). For females, rearing temperature did have an influence on hardening. Females reared at 19°C performed on average three minutes lower for heat knockdown time, whilst 28°C reared hardened females performed on average nine minutes lower (Table 3.7).

**Developmental rearing conditions influenced by sex**

For males, the six populations differed when reared at both 19°C (F5,354 = 13.23, P<0.0001) and 28°C (F5,354 = 10.28, P<0.0001), whilst the 2013-NSW population was significantly less heat resistant than
all other populations when reared at either temperature (Tukey: P<0.0001). A comparable situation was found for females with both 19°C and 28°C reared populations differing significantly from each other. Post hoc analysis revealed that for 19°C reared females, the heat resistance of the 2013-NSW population was significantly less than the 2014-NSW, 2015-NSW, and 2015-VIC populations (Tukey: P<0.01), whilst for 28°C reared females, the heat resistance of the 2013-NSW population was significantly less than all populations (Tukey: P<0.05) apart from the 2014-VIC population (Tukey: P = 0.38). These results are represented graphically in Figure 3.1 (c + d).

As is also seen in Figure 3.1 (c + d), the 2013-NSW population is consistently the least heat resistant population, with temperature and sex playing no role in this finding. An aggregate of all populations showed that populations reared at 19°C had reduced heat resistance compared to those reared at 28°C (Figure 3.1 c + d).

Sources of variation for heat resistance

Figure 3.4 displays the sources of variation which influence heat resistance over this study when maximum mean differences for the factors were compared. Differences between populations as well as those differences linked to potential adaptation to the laboratory remain the largest source of variation displayed in this study. The developmental rearing temperature also had a considerable effect on heat resistance, whilst sex and hardening status were the two variables which showed the least influence, however see above for specific discussion on how these two variables interact to influence heat resistance.

As has been mentioned already, the 2013-NSW population was consistently the weakest performing population for heat resistance, and this remained the case during both experiments (Tables 3.4 + 3.5). When populations were tested in the October experiment, and compared to how they performed for the February experiment, an increase of ~11 minutes was found for the 2014-NSW population, whilst an average of ~6.5 minutes was found for the 2014a-NSW and 2014-VIC (Tables 3.4 + 3.5).
3.4 Discussion

The two population comparison experiments were performed to explore the ways in which heat resistance might be influenced by lab adaptation, relative to hardening and rearing effects as well as origin differences. Differences were found between experimental populations, rearing temperature, pre-experimental hardening procedures, and sex (Table 3.1). A significant two-way interaction between sex and hardening revealed that males and females were responding differently to hardening procedures (Table 3.1). A summary of the sources of variation investigated during the two experiments in Figure 3.4 highlights that population differences and rearing temperature effects were the most important factors influencing heat resistance.

3.4.1 Rearing temperature differences

As expected, there was a strong developmental rearing effect for all populations and both sexes. This finding was similar to what was found in the cross generational study detailed in Chapter Two of this thesis. Flies which were reared at 19°C were invariably less heat resistant compared to those reared at the higher temperature of 28°C. This was by no means a new finding, as previous studies on ectotherms has demonstrated that developmental rearing temperature can influence adult thermal resistance (Jenkins & Hoffmann 1994; Rako & Hoffmann 2006; Schiffer et al, 2013). It did however account for the largest amount of variation found during the study (Figure 3.4), and was consistent during both experiments and was not (seemingly) influenced by population, sex, or hardening. It has been shown on many occasions that both developmental rearing (larval stage), as well adult hardening can cause a heat shock response (Feder et al, 1996; Krebs & Feder 1997a; Sørensen et al, 2003), leading to significant benefits when a later stressful event occurs (a heat shock for example).

Of further interest was the lack of difference between populations sourced from different parts of Australia. No discernible differences were found between Queensland flies which were reared at either 19°C or 28°C compared to those which were sourced from Victoria, despite over 2500 km separating the two locations. Clines for heat knockdown time measured via both static and ramping assays (0.06°C min$^{-1}$) have been shown for D. melanogaster (Hoffmann et al, 2002; Sgro et al, 2010), whilst a faster ramping rate of 0.1°C min$^{-1}$ did not result in a clinal pattern (Sgro et al, 2010). The ramping rate used in this study was even faster than this (0.2°C min$^{-1}$) and could be a reason why no obvious differences are found between populations from different locations. However, this needs to be considered in light of laboratory adaptation (see below).
Hardening was expected to directly result in flies directly benefiting from an increase in heat resistance, as it has been previously shown to increase heat resistance (Sejerkilde et al, 2003; Hoffmann et al, 2003; Norry et al, 2008). In my study, a net increase of zero minutes for heat knockdown time was found across both population experiments. However, I also found that male flies showed an effect of hardening unlike females. This finding has been previously described by Sejerkilde et al, (2003) for D. melanogaster. It is hard to pinpoint the exact reasons behind males gaining an increased benefit of hardening, but body size may be one possibility. Sexual dimorphism for body size is present for many species of Drosophila, including D. hydei, with females being larger compared to males, and this of particular significance in the early stages of post eclosion development. As a consequence of increased body size in females, their lipid content is thus greater than males, and also continues to increase up to days post eclosion, whereas in males lipid content is in decline post eclosion (Prasad & Joshi 2003). This benefit or larger body size and higher lipid content could be the reason female Drosophila are already at a greater advantage to stress resistance assays compared to males. As well as involving lipid changes, hardening treatments are able to influence other internal factors such as sugar or polyol concentration, as well as metabolic rate, which could also be a reason differences are found between the sexes (Hoffmann et al, 2003).

3.4.2 Laboratory adaptation

Some early studies which investigated the effects of laboratory adaptation demonstrated that Drosophila stocks which had been held in laboratory environments for long periods of time were very close to genetic equilibrium when compared to stocks which had been recently introduced into a laboratory environment (Service & Rose 1985; Harshman & Hoffmann 2000). In my study, I was able to draw upon populations which had been kept in a laboratory environment for differing periods of time, with one population in particular being long established. The 2013-NSW population provided the most insight for this study in how its heat resistance compares to other populations. The 2013-NSW population had been in the laboratory for two years for the first experiment in February, and then for over 2.5 years when tested in October, and at both time points it had significantly reduced heat resistance compared to all other populations. This observation stands in contrast to what was predicted prior to the experiment based on trends that were picked up during the cross generational effects study discussed in Chapter Two. The lack of change in the 2013-NSW population suggests that this population may have reached an equilibrium, which could be indirectly reflecting a plateau along the lines suggested by Service & Rose (1985).

In D. melanogaster, the process of heat resistance potentially plateauing after a certain number of generations in a laboratory environment has been discussed by Krebs et al, (2001). Krebs et al,
(2001) found a rapid increase in heat shock protein (HSP) production upon entry in the laboratory, but by generation two the HSP levels began to drop and plateaued by generation six. This is interesting compared to the results I obtained during both my cross generational and population experiments in that heat knockdown times were still rising when I re-tested my oldest population (2013-NSW) at a generation time of F6. As shown in the results section of Chapter Two, the average knockdown time for the 25°C reared 2013-NSW population was sitting around the 200-210 minute mark when tested at F6, compared to around 140-150 minutes when tested at the F2 stage. This same population was then involved in the two population experiments with heat resistance, taken as an average between the 19°C and 28°C reared non-hardened individuals, sitting at ~181 minutes at F17, and at ~186 minutes when tested at F23. Perhaps a loss of resistance has occurred over time, or resistance has reached a plateau and there is a lack of repeatability between successive tests.

More evidence for laboratory adaptation comes from comparing populations which were tested twice during this study. An increase of 11 minutes was noted when the 2014-NSW was tested in October compared to February, whilst an average increase of almost 7 minutes was evident for both the 2014-VIC and 2014a-NSW populations when re-tested.

However, a comparison of populations from the same location but tested at different times provided a different picture of laboratory adaptation. In the case of the NSW populations and to a lesser extent the Victorian population, there is a lower resistance of populations that have been in the laboratory for longer. A comparative approach to measuring how populations evolve in a laboratory environment in response to stress resistance traits utilises the testing of populations which are at different generational stages and using this as a proxy measure to show any effects of adaptation (Frankham & Loebel 1992; Latter & Mulley 1995; Hoffmann et al, 2001; Woodworth et al, 2002; Gilligan & Frankham 2003; Griffiths et al, 2005). This provides an alternative to tracking and measuring the evolutionary trajectories of populations as they enter a laboratory environment and adapt to their new surroundings (Matos et al, 2000, 2002, 2004; Krebs et al, 2001). The idea behind the second approach is that each newly introduced population, however close in origin to other distinct populations, will evolve along a different trajectory to another population due to their inherent underlying genetic differences (Cohan & Hoffmann 1989; Matos et al, 2002). Comparisons of these two approaches have been conducted to investigate laboratory adaptation with mixed results (Matos et al 2004; Simoes et al 2009).

The findings of this study need to placed in this context, and the overall outcome is unclear. The assays undertaken may be prone to variability in that heat resistance depends on minor differences in culture conditions and so on, which makes it difficult to follow evolutionary trajectories. On the
other hand it may also be the case that populations differ substantially for resistance to start and then follow different trajectories as they adapt to laboratory conditions.

Laboratory adaptation has been studied and identified in *Drosophila* and been found to influence life history traits (Frankham & Loebel 1992; Latter & Mulley 1995; Matos et al, 2000; Sgro & Partridge 2000; Tobler et al, 2015). Further to these findings, the implications for other traits such as thermal resistance and desiccation resistance remain less clear cut and consistent (Hoffmann et al, 2001; Krebs et al, 2001; Griffiths et al, 2005; Kellerman et al, 2015), and their presence (or lack thereof) deserves further investigation. In this study I provide some evidence that heat resistance might be influenced by time spent in laboratory environments. Whilst Griffiths et al, (2005) and Kellermann et al, (2015) found heat resistance to be unaffected by laboratory adaptation, Krebs et al, (2001) found an initial increase in heat resistance, which was not maintained. However, these studies have all used somewhat different measures of heat resistance, and at the moment a clear picture has not yet emerged.

### 3.4.3 Concluding remarks

This thesis has attempted to consider the importance of both plasticity and evolutionary approaches to heat resistance in *D. hydei* and the varying strength of these sources of variation. In Chapter One, I showed small, but non-directional cross generational effects for heat resistance. In testing three populations of *D. hydei* by using developmental rearing temperature as a variable, I was able to provide some evidence for how *D. hydei* populations may respond to changes in temperature in a relatively fast period of time. Cross generational effects were detected only in newly collected populations, and were not present when line were later re-tested, and a by-product of this investigation led to the possibility of laboratory adaptation being a factor. A comparative approach to assessing potential laboratory adaptation was devised using populations of *D. hydei* of differing times spent in a laboratory environment and comparing them using heat assays at two experimental time points. The addition of a hardening component to the experiment added to the variables of sex, and developmental rearing temperature with a view to assessing the varying significance of these variables. Evidence for potential laboratory adaptation was provided in that the oldest population used was significantly lower in heat resistance across both timepoints compared to all other populations. However, this was contradicted by early signs showing populations, which were newly initiated in the lab, increasing their resistance between timepoints. The effects of developmental rearing temperature were a significant plastic factor, which was noted in both Chapter Two and Chapter Three. Hardening was a small factor, and an interaction with sex was noted.
Further research on the topics discussed in this thesis could involve an expansion of the population comparison experiment I performed with the addition of further populations, and also a re-assessment of those populations that were still increasing in heat resistance and perhaps the introduction of new freshly collected populations for comparisons.

It appears cross generational effects for heat remain quite small, and further research on this topic could focus on trying to obtain consistent results using different heat assay measures or perhaps focus on a variable different to developmental rearing temperature. Hardening prior to testing for cross generational effects might be worth pursuing.
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