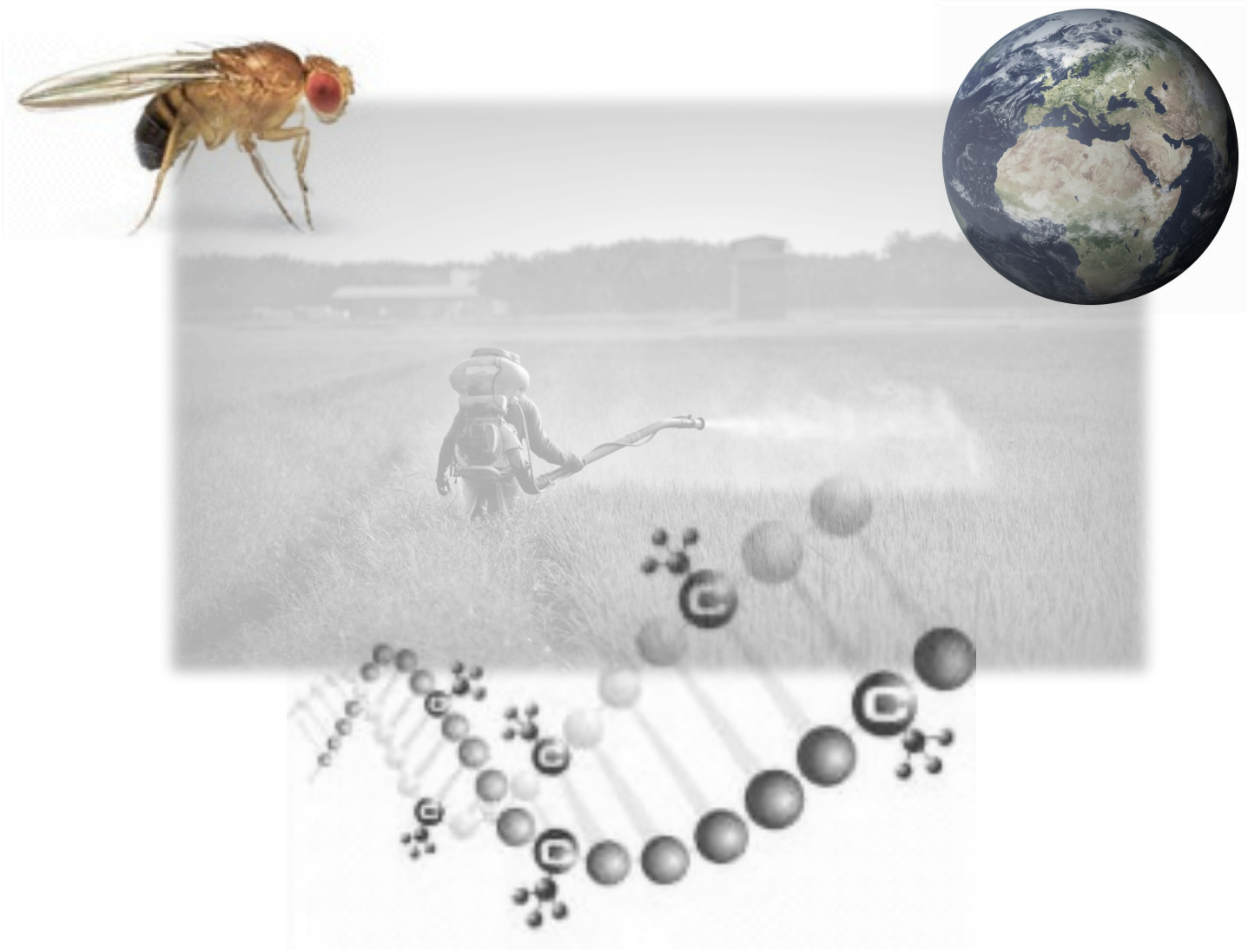
Crossgenerational effects of temperature and chemical stress in the model organism *Drosophila melanogaster*



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Resume

Fænotypisk plasticitet er et fænomen hvorved en enkelt genotype, under forskellige forhold, kan udvise forskellige fænotyper. Forskning viser, at organismer er særligt udsatte for permanente ændringer i fænotypen ved påvirkning af en miljøstress i den tidlige udvikling af organismen, og at en påvirkning på dette stadie også kan have videreækkende effekter transgenerationelt. I nærværende projekt undersøgte jeg om en miljøstress introduceret under udviklingen fra æg til voksen hos *Drosophila melanogaster* kunne spores i de efterfølgende generationer når disse ikke var udsat for samme stress. Udover en kontrol gruppe blev der generet tre grupper, der var udsat for hhv. 31°C varme, 13°C kulde og insekticidet dimethoat i den første generation. I de fire forsøgsgrupper blev der generet fire filial generationer, og i hver generation blev deres fitness testet på følgende parametre: æg til voksen overlevelse, varme- og kuldetolerance, samt frugtbarhed. Resultatet viste kun ringe transgenerationelle effekter ved 13°C kulde og dimethoat. Det var kun 31°C varme stress der havde en transgenerationel effekt på de efterfølgende generationers evne til at modstå temperaturstress. Resultatet viste at afkom af forældre opvokset ved varme temperature er bedre til at modstå kuldestress.

Introduction

Previously the phenotypic outcomes of an individual were thought to be a direct reflection of the individual's genotype, without the possibility of the individual displaying multiple phenotypes within the same genotype (West-Eberhard 2003; Uller 2008; Jablonka & Raz 2009). Studies of phenotypically different monozygotic twins questioned the idea of a single genotype being directly linked to display a single phenotype (Glastad et al. 2011; Sargant 2012; Bateson 2015). This gave rise to the idea of phenotypic plasticity, which led to a whole new understanding of genetics (Sargant 2012; Bateson 2015).

In the beginning of the 1940's the developmental biologist Conrad Waddington coined the term epigenetics, meaning "epi": "above" the genetics, which he defined as: *''the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being''* as an explanation for the varying phenotypic plasticity (Van Speybroeck 2002; Goldberg et al. 2007; Dupont et al. 2009; Skinner et al. 2010; Sadava et al. 2011). The meaning of epigenetics has undergone an immense transformation from its original meaning when Waddington first invented the term (Jablonka & Raz 2009; Sadava et al. 2011; Duncan et al. 2014; Deans & Maggert 2015). In *"What do you mean, "epigenetic"?"* by Deans and Maggert (2015), the focus is on how inconsistently the term epigenetic is used across different scientific fields, covering genetics to ecology, physiology and evolution but also phycology, making the concept of epigenetics difficult to synthesize and reconcile (Deans & Maggert 2015). This is also supported by Bird (2007) and Haig (2012). Haig (2012) states that we are in an epidemic of the use of the words *"epigenetic"* and *"epigenetics"* and by 2010 more than 13.000 articles on the subject were published, compared to the 2500 articles published on epigenetics in 2006.

Today the field of phenotypic plasticity is the field closest to the original meaning of epigenetics as defined by Conrad Waddington. Phenotypic plasticity, or developmental plasticity, is described as a phenomenon, where a single genotype of an organism can result in different phenotypic outcomes to enhance fitness, as a response to changes in external or internal stimuli (Zhou et al. 2014; Gilbert et al. 2015) e.g. environmental fluctuations (Snell-Rood et al. 2010; Colombo et al. 2014; Bateson 2015; Gilbert et al. 2015) or changes in hormone levels (Uller 2008; Colombo et al. 2014; Bateson 2015). Adaptive responses to environmental changes can be beneficial in temporarily fluctuating environments (Uller 2008; Dey et al. 2015; Sørensen et al. 2015). The phenotypic plasticity is

controlled by epigenetic modifications, and is thought to be reversible, but evidence suggests environmental impacts introduced under a critical point of development enhances the possibility or risk of the epigenetic modifications becoming heritable (Kota & Feil 2010; Iovino 2014; Jablonka & Lamb 2015). The crossgenerational effect of environment on offspring's plasticity could be beneficial for the offspring in terms of enhanced fitness, however a stressful environment could also cause the opposite. An example of that is seen in Linder and Promislow (2009), where offspring of immune-challenged female *Drosophila melanogaster* had a shorter lifespan than offspring from non-immune-challenged females (Linder & Promislow 2009).

Today, anthropogenic use of natural resources and the attempted effort to maximize the profit of natural resources is leading to an environment with multistressors, e.g. contaminants and global warming, which could influence the phenotypic plasticity experienced by the organism within the environment (Kimberly & Salice 2015). Therefore, research done within the field is of great importance to understand what consequences these changes might have to the phenotypic plasticity experienced by the individual, and what possible further reaching consequences it has for the ecology of species and the evolutionary adaptations of populations (West-Eberhard 2003; Jablonka & Raz 2009; Bateson 2015).

The hypothesis is that different environmental stimuli can induce an altered phenotype, and that stimuli introduced during early development could influence the fitness of multiple generations despite ceased stimuli. Therefore, the aim of this project is to investigate what consequences different stressors, introduced during early development, have for selected fitness traits in the directly affected generation and its successors. To test this, I will use the model organism *D. melanogaster*. I will create four different populations of flies where I will expose three of the groups to a single environmental stressor (environmental regimes) during the development from egg to adult fly in the first generation. The three stressors chosen, beside the control reared under benign conditions, include two temperature regimes and a chemical regime in the form of the insecticide dimethoate. Hereafter, I will generate four successive generations with flies exposed to the four different treatments in the first generation, all raised at benign conditions. In each of the five generations I will test four fitness components: egg-to-adult viability, upper thermal tolerance, lower thermal tolerance and total fecundity in the first ten days of living.

Phenotypic plasticity.

Within the last decade scientists have changed their view from the more gene centered and Mendelian regulation of phenotypic outcome, to focus on the influence of environmental control of the genes during development leading to a specific phenotype (Uller 2008; Bonduriansky 2012; Gilbert et al. 2015). Phenotypic plasticity can be viewed as an immediate response to environmental changes without changing the underlying genes (West-Eberhard 2003; Gluckman et al. 2009; Gilbert et al. 2015; Bateson 2015) or as a mediator, which promotes/delays an evolutionary change of the underlying gene sequence (Uller 2008; Gilbert et al. 2015).

Properties of phenotypic plasticity

Phenotypic plasticity is an adaptive response to accommodate environmental changes where the individual can enhance their fitness potential (Uller 2008; Snell-Rood et al. 2010; Zhou et al. 2014; Colombo et al. 2014; Bateson 2015; Gilbert et al. 2015). A phenotypic variation can be expressed in many different biological levels, from regulating respiratory pathways and hormones (Mukherjee et al. 2015) to morphological and behavioural changes (Rea et al. 2015; Yan et al. 2015). The variation is not necessarily persistent and visible throughout the life history of the individual, but can be expressed under specific environmental conditions (Czesak et al. 2006; Bateson 2015; Jablonka & Lamb 2015). With the understanding of phenotypic plasticity and the discovery of the epigenetic mechanisms, molecular science and genetics have moved away from the theory that genes have an on/off effect on expression, to having different levels of expression depending on epigenetic regulation (Jablonka & Lamb 2015).

Phenotypic plasticity is argued by Uller (2008) to be favoured under three conditions: 1) heterogeneity in the environment (also supported by Snell-Rood et al. (2010) and Dey et al. (2015)). 2) environmental cues are consistent in predicting future lifetime happenings and 3) the plasticity induced is of low energy cost to the organism exposed (also supported by Snell-Rood et al. (2010)). Despite the argument of Uller (2008) and Snell-Rood et al. (2010) that phenotypic plasticity is favored when it is of low cost to the organism, this is not always the case. Adapting to an environment can be costly for the individual. To accommodate for environmental changes, an organism often has to allocate resources to express phenotypic variation; this can result in a trade-off effect or maladaptive response where other trait expressions are compromised at expense (Sheldon & Verhulst 1996; Snell-Rood et al. 2010; De Loof 2011). A maladaptive response could

be changes in life strategy where the individual expresses phenotypic plasticity that accommodates environmental changes but comes with costs, e.g. lowered fecundity or longevity. In this example the phenotypic plasticity can be beneficial for the individual's present survival, but detrimental in terms of securing the overall fitness of the population. Another maladaptive strategy in fluctuating and extreme environments is bet hedging, wherein the parents' environment induces a progeny with a large variance in phenotypes, at the expense of a lowered general fitness (Dey et al. 2015; Sgró et al. 2016).

Phenotypic plasticity and heredity

Phenotypic plasticity is controlled and inherited by molecular epigenetic mechanisms (Uller 2008; Gilbert et al. 2015; Deans & Maggert 2015; Jablonka & Lamb 2015) or by passing along cytoplasmic material (Jenkins & Hoffmann 1994; West-Eberhard 2003). It has become evident that the phenotype expressed by the individual often depends on the environment experienced by their parents (Steigenga & Fischer 2007; Ferrer et al. 2013; Gilbert et al. 2015). A subclass of phenotypic plasticity is predictive adaptive responses (PAR's) where the phenotype of progeny is induced by cues given early in development by their parents or the environment itself (Duncan et al. 2014). This kind of heritability is often expressed as "soft heredity", where in one or both parents can induce developmental reprogramming, based on their own phenotype and experience of the environment, that can lead to the development of the same or a better suited phenotype in their progeny (Bonduriansky 2012; Ferrer et al. 2013; Kuijper & Johnstone 2015). There is clear evidence of gender specific inheritance, in which the female or the male can pass along different phenotypes according to their own physical status before and during the fertilization and oviposition of their offspring. Some research proves that maternal and paternal effects on the progeny have different results for the phenotypic fitness experienced in the progeny of both adaptive and maladaptive character (Bonduriansky 2012; Kuijper & Johnstone 2015).

Phenotypic plasticity, ecology and evolution

The hereditary effects on phenotypic plasticity raises the question of how phenotypic plasticity can influence the ecology and evolution of a species. The effect of phenotypic plasticity on ecology and evolution is equivocal, and a highly debated subject between scientists (West-Eberhard 2003; Gilbert et al. 2015; Kuijper & Johnstone 2015). Due to the environment's effect on phenotypic expression, phenotypic plasticity becomes a selective agent, which could influence the evolution of species dramatically (West-Eberhard 2003; Gilbert et al. 2015). Gilbert et al. (2015) states three

possible effects of phenotypic plasticity: 1) species can accommodate environmental changes in a broad variety of phenotypes leading to changed ecology of the species. An example of this is a changed geographical distribution as a response to climate changes (Townsend et al. 2008; Gilbert et al. 2015) 2) Phenotypic plasticity can facilitate a niche reconstruction of the species (also supported by Townsend et al. 2008) 3) An environmental change that facilitates a uniform expression of a phenotype can assimilate into a genomic change (also supported by West-Eberhard 2003; Duncan et al. 2014). The general assumption is that a uniform expression of a phenotype over multiple generations would prompt the variation to become embedded in the genome, whereas a phenotype with highly variable or reduced effect on the fitness would counteract the evolution (West-Eberhard 2003; Gluckmann et al. 2009; Duncan et al. 2014; Gilbert et al. 2015).

Molecular epigenetics

The understanding and use of the word epigenetics is broad. The most common field of epigenetics is genetics, where epigenetics refers specifically to the changes in gene expressions that do not stem from changes in the underlying nucleotide sequences of the DNA. It refers to changes in the epigenome. The epigenome is considered to be the software controlling how and when to read the hardware, which is the DNA. The physical changes in the epigenome are considered reversible, but research suggests that the changes are heritable and more stable than first anticipated (Jablonka & Raz 2009; Sadava et al. 2011; Bateson 2015).

The field of epigenetics has received massive attention in the second half of the 20th century (West-Eberhard 2003; Jablonka & Raz 2009). Today the word *epigenetics* is used inconsistently in literature describing several molecular mechanisms, making research within the field difficult. In this project I have chosen to distinguish between the two molecular fields *epigenetics* and *epigenetic inheritance* as in Jablonka & Raz (2009), Deans and Maggert (2015), and Mukherjee et al. (2015), although much literature still combines the two mechanisms under the term *epigenetics* (Skinner et al. 2010; Lyko & Maleszka 2011). Molecular epigenetics distinguishes between mechanical mechanisms, which concern the differentiation of a cell within a single organism, whereas epigenetic inheritance operates with the molecular mechanisms that concern passing along a phenotype within cell divisions or generations (Jablonka & Raz 2009; Duncan et al. 2014).

Epigenetics

Epigenetics include three overall processes: DNA methylations, chromosomal protein alterations (Baccarelli & Bollati 2009; Sadava et al. 2011; Duncan et al. 2014) and expression of Micro RNA (miRNA, non-coding RNA) (Baccarelli & Bollati 2009; Mukherjee et al. 2015). The majority of literature uses mammalian cells to describe epigenetic mechanisms, and although the mechanisms seems to be highly conserved in multicellular organisms, there are exceptions to the general rule (Glastad et al. 2011; Lyko & Maleszka 2011; Yan et al. 2015). There are different ways in which DNA methylations, chromosomal alterations and the expression of miRNA functions depending on the organism of interest. Scientist have discovered that even though the mechanism in general functions in the same way, there are some differences between taxa and even species (West-Eberhard 2003; Mukherjee et al. 2015). The expositions in this project will be based upon the mechanisms found in insect cells.

DNA Methylation

One mechanism linked to the control of the expression of the epigenome, and the first epigenetic mechanism to be discovered in the 1970s, is DNA methylation. DNA methylation involves modification to the DNA base by a covalent attachment of a methyl group ($-CH_3$) to 5-carbon of cytosine in the CpG denucleotide (Skinner et al. 2010; Sadava et al. 2011; Glastad et al. 2011; Lyko & Maleszka 2011; Yan et al. 2015). The general role of methylation is conserved and connected to gene silencing by changing the binding potential for transcriptional factors, making methylated genes inaccessible for expression (Feng et al. 2010; Sadava et al. 2011; Duncan et al. 2014; Yan et al. 2015). Although the function of methylation is conserved across taxa, the pattern of methylation seems to vary among organisms. Both vertebrates and plants are highly subject to methylation on the CpG dinucleotides in the regions between active genes. This is opposite to methylation of the CpG denucleotide in invertebrates, and especially insects, where the methylation seems to be sparse and limited to active gene sites only (Feng et al. 2010; Glastad et al. 2011; Lyko & Maleszka 2011; Yan et al. 2015).

The methylation of the CpG dinucleotide is catalyzed by a highly conserved family of enzymes called DNA-methyltrasferases (DNMTs) (Lyko & Maleszka 2011; Duncan et al. 2014; Mukherjee et al. 2015) and the process of methylation is reversible by an enzyme called demethylase (Sadava et al. 2011; Duncan et al. 2014). In vertebrates, DNMT1 and DNMT3 is of great importance in the methylation of the DNA (Sadava et al. 2011; Duncan et al. 2014; Mukherjee et al. 2015). DNMT1

ensures maintenance of methylations marks across cell division, whereas DNMT3 is responsible for *de novo* methyltransferase (Duncan et al. 2014; Mukherjee et al. 2015). Despite the high gene conservation of methylation, not all invertebrates have conserved the ability to maintain methylation as an epigenetic regulation. Model organisms like *D. melanogaster* and *Caenorhabditis elegans* have completely lost the gene encoding for DNMT1 and DNMT3 (Feng et al. 2010; Glastad et al. 2011; Lyko & Maleszka 2011; Yan et al. 2015), whereas the honey bee (*Ampis mellifera*) displays several copies of the genes expressing DNTM1 and DNMT3 (Feng et al. 2010; Lyko & Maleszka 2011; Yan et al. 2015). Examples of invertebrates wherein one of the two DNMTs genes is lost exist and the lineage-specific loss of DNA methylation has evolutionary explanations (Lyko & Maleszka 2011; Yan et al. 2015). One is that the cost of methylation might have outweighed its benefits compared to other epigenetic regulatory pathways (Lyko & Maleszka 2011).

Histone modification

Another epigenetic regulatory mechanism is histone modification. Histone modification can be inheritable across generations and the assumption is that the function of histone modification is conserved across higher eukaryotes (Spada et al. 2005). DNA is organized in chromatin structures, wherein DNA is packed in an octamer around four proteins called histones in a globular structure. The coiling around histones shapes the DNA into the well-known structure of a chromosome. The conformation of chromatin determines whether or not the DNA is accessible for transcription (Kouzarides 2007; Baccarelli & Bollati 2009; Sadava et al. 2011; Boros 2012; Duncan et al. 2014). The silent state of chromatin is called heterochromatin, whereas the active is named euchromatin (Kouzarides 2007; Boros 2012). Heterochromatin becomes euchromatin, and susceptible to transcription, when the affinity between the histones and the DNA is changed (Kouzarides 2007; Boros 2012, Duncan et al. 2014). Each of the four histone proteins (H2A, H2B, H3 and H4) have a compact structure wherefrom a "tail" called the N-terminal, consisting of 20-25 positively charged amino acids, sticks out (Kouzarides 2007; Sadava et al. 2011; Boros 2012). When adding an acetyl-, methyl- or phosphor group, the affinity of the N-terminal changes and the DNA string becomes more loosely attached to the histone complex, creating euchromatin. The process is reversible and transcription can thereby be regulated by histone modification (Kouzarides 2007; Boros 2012, Duncan et al. 2014).

miRNA regulation

miRNA is the last epigenetic regulation, which occurs post transcriptionally (Rea et al. 2015; Mukherjee et al. 2015). miRNA is small non-coding RNA which are around 21-23 bases long (Baccarelli & Bollati 2009; Sadava et al. 2011; Rea et al. 2015; Mukherjee et al. 2015). miRNA functions by downregulating gene expression by targeting complementary mRNA (Sadava et al. 2011; Rea et al. 2015; Mukherjee et al. 2015). Even though codes for miRNA only take up 1 % of the genome, they are thought to target up to 30 % of the genes (Rea et al. 2015) and miRNA are suspected to affect and regulate DNA methylation and histone modification to some degree (Mercer & Mattick 2013; Jenkins & Muskavitch 2015).

Epigenetic inheritance

Epigenetic inheritance is the science of understanding how epigenetic modifications can be heritable. The term epigenetic inheritance is loosely used in scientific articles, making it a subject that is difficult to comprehend. Jablonka & Raz (2009) distinguish between a broad and a narrow understanding of epigenetic inheritance. The broad concerns epigenetic inheritance as a part of understanding evolution, whereas the narrow sense concerns the molecular mechanism of genetic inheritance. In this section, the focus is on the narrow understanding of epigenetic inheritance.

Epigenetic modifications can be passed along generations, commonly referred to as trans- or cross-generational effects. There are two ways in which a changed fitness can be passed along to the next generation depending on the timeline. The phenotype could be inherited to the offspring by transferring somatic material from the parents, called cytoplasmic inheritance or soft inheritance, e.g. glandular secretions. Cytoplasmic inheritance, could only exist over one generation (Bonduriansky 2012). The second form of inheritance is if the epigenetic modification is imprinted in the germline of an organism that eventually is presented in the gametes of a sexually reproductive organism (most animals, plants and fungi). Because of the presence in the germline of an organism, it is possible to sustain the epigenetic modifications for more than one generation (Jablonkan & Raz 2009; Skinner et al. 2010; Duncan et al. 2014; Bateson 2015; Jablonka & Lamb 2015).

For an epigenetic inherited trait in cells to be continued to the next generation in diploid cells, it has to endure a range of complex transmission states, including meiosis, gametogenesis and the early embryonic state, in which the cells are rapidly deleting and reconstructing the chromatin structure

and DNA methylation (Jablonkan & Raz 2009; Kota & Feil 2010; Jablonka & Lamb 2015). The immense reconstruction happening under meiosis, gametogenesis and the early development of the embryo, makes the cells particularly vulnerable to environmental changes in these states, and the epigenetic markers created here are more likely to be irreversible and thereby persistent in the population (Kota & Feil 2010; Jablonka & Lamb 2015).

Materials and methods

Flies

In these experiments the model organism *D. melanogaster* was used. The species belongs to the genus of *Drosophila* (Jørgensen & Bundgaard 2006). *D. melanogaster* were chosen as the model organism for many reasons, but mainly because it is a cosmopolitan species which has a short generation time (Jørgensen & Bundgaard 2006; Fournier-Level et al. 2016). Furthermore, the choice of insects as model organisms in genetic and evolutionary studies is relatively cheap. The low cost in maintenance and the possibility of keeping more individuals in less space gives insects a huge advantage compared to other model organisms such as mice (Mukherjee et al. 2015).

The baseline population

The baseline population of *D. melanogaster* used in the experiments were kept in the laboratory for over 91 generations under normal laboratory conditions (benign conditions: 23°C, 50% RH, 12/12 hours light/dark cycle) on standard *Drosophila* food medium containing 30 g/L oatmeal, 40 g/L sugar, 60 g/L dry yeast, 16 g/L agar, 12 mL/L nipagen-solution (95 g methyl-4 hydroxybenzoat, 250 mL dest. H_2O, 700 mL 96% ethanol) and 1,2 mL/L of 80% acetic acid to 1 L of H_2O (hereafter referred to as Leeds medium). The laboratory baseline population is based on flies caught in the Karensminde Orchard at the Danish peninsula of Jutland (55°56´42 46”N, 10°12´45.31”E) in October 2010 (Schou et al. 2015).

Environmental regimes in the parental generation (1st) and in the 2nd to 5th generation.

Environmental impacts on populations can have a profound effect on the life history of a population in many traits e.g. lifespan, reproductive output and loss of coping abilities in a changing environment (Overgaard et al. 2011; Hunt et al. 2016). In the present study, I exposed flies to four different environmental regimes throughout the development of what I term the parental generation (1st generation). The parental generation was exposed to their respective regimes during the development from egg to hatched fly. At the adult stage and in the following generations, the flies from all four regimes were kept and reared under benign laboratory conditions (as described for the baseline population). For each of the four environmental regimes I created four filial generations: 2nd, 3rd, 4th and 5th generation, as a linage based upon the 1st generations population to the corresponding environmental regime.

The four environmental regimes in the 1st parental generations were: (1) A control regime equal to normal laboratory conditions. (2) A heat regime where the parental flies were exposed to 31°C. (3) A cold regime where the parental flies were exposed to development at 13°C. (4) A chemical regime, where the parental flies were exposed to (in the medium) 300 ppb of the organophosphate insecticide dimethoate ($C_5H_{12}NO_3PS_2$, purity 99,5% from SIGMA ALDRICH). Dimethoate is a nerve toxin, that acts as an acetylcholinesterase inhibitor in the insect's nervous system (Vontas et al. 2002; Van Scoy et al. 2016). The dimethoate is ingested through the food source and therefore applied to the medium dissolved in demineralized water. The concentration of 300 ppb dimethoate was chosen based on a pilot trail I conducted, where the concentration of 300 ppb dimethoate had a significantly negative effect in egg-to-adult survival. For all environmental regimes the photoperiod was kept constant at 12:12 hours throughout the 1st-, 2nd, 3rd-, 4th and 5th generation. To reduce the effect of inbreeding in the study, I designed the study to maintain a constant population size of approximately 500 to 1000 individuals, regime^{-1} generation^{-1}.

The experimental design

In the study I investigated the same four fitness traits in the 1st-, 2nd-, 3rd-, 4th and 5th generation. The traits investigated were: egg-to-adult viability, critical thermal maxima (CT_{max}), critical thermal minima (CT_{min}) and fecundity in the first ten days after hatchment. I incorporated the egg-to-adult viability test in the creation of the filial generations based upon the exposed parental generation, within each environmental regime. I designed the egg-to-adult viability test to maintain a high population size, wherefrom I could collect flies to test their fitness and continue the linage of inheritance to the following generations. In the parental generation the eggs for the egg-to-adult viability test were derived from flies in the laboratory baseline population. The eggs used in the egg-to-adult viability test in the 2nd-, 3rd-, 4th and 5th generations derived from flies that emerged in the previous generation.

The emerged flies from the egg-to-adult viability test in the 1st-, 2nd-, 3rd-, 4th and 5th generations in the four environmental regimes were counted and sorted for five different purposes: (1) Determining egg-to-adult viability for the generation, by counting the number of emerged offspring. (2) 20 females for determining CT_{max}. (3) 20 females for determining CT_{min}. (4) 20 pairs, consisting of one male and one female to test the fecundity in the first ten days after hatching. (5) >700 flies to produce the eggs used in following generation's egg-to-adult viability test. These procedures were

repeated until egg-to-adult viability-, thermal limits-, and the fecundity data were obtained from the 1st to the 5th generation in each environmental regime. **Figure 1** and **table 1** below illustrates the experimental design. The four environmental regimes were not conducted simultaneously due to an increased developmental time in the 13°C cold regime, and the time needed to determine the dimethoate concentration used. Therefore, the 23°C control regime and the 31°C heath regime were nearly two months ahead of the 13°C cold regime and the 23°C 300 ppb dimethoate regime.

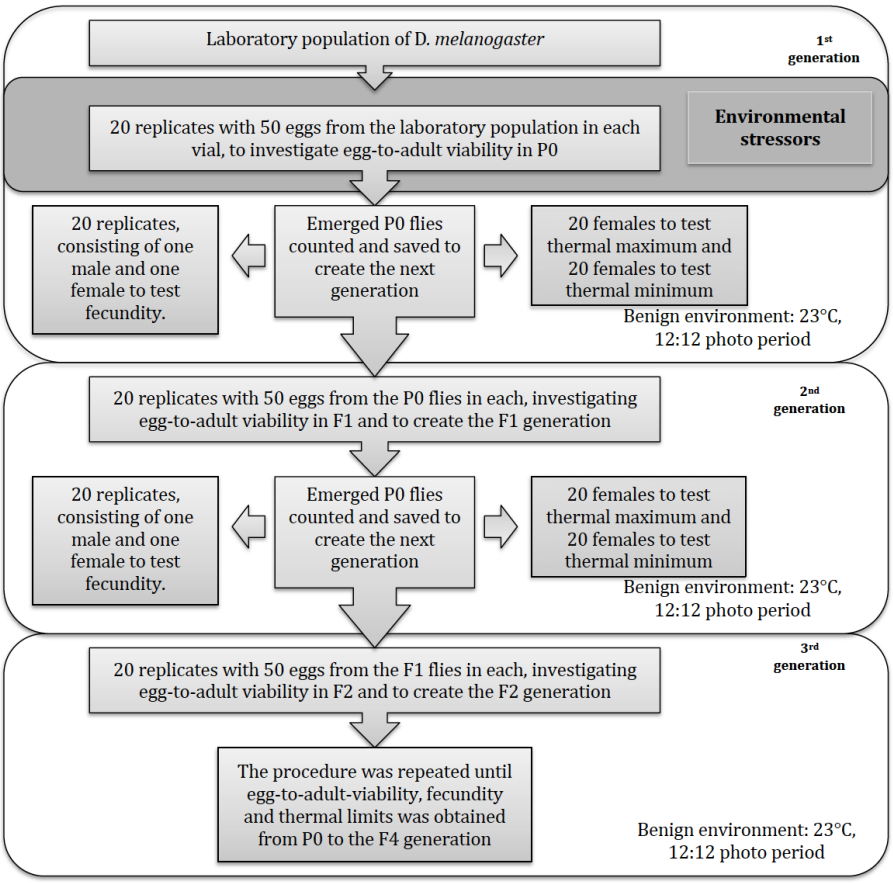


Figure 1. The flowchart illustrates the structure of the experimental design for all environmental regimes. It illustrates how the egg-to-adult viability test provides the flies used to generate the following filial generation, and the flies to test the critical thermal limits and fecundity. The figure also illustrates that the environmental impact only was present in the 1st generation during development, onwards from there the flies were kept at 23°C in a 12:12 light/dark photoperiod.

Table 1 Illustrates the terminology of generations and what conditions the traits in the generation were tested under. The flies were only directly exposed to the three environmental stresses in the development from egg-to-adult in the 1st generation (See *environmental regime*). Under benign conditions the flies were kept at 23°C, 50% RH, 12/12 hours light/dark cycle.

Generation	Trait tested	Environment stress (Exposed/Benign)
1st (P0/parental generation)	*Egg-to-adult viability*	Exposed
	CT_{max}	Exposed
	CT_{min}	Exposed
	Total fecundity	Exposed
2nd (F1/filial generation)	*Egg-to-adult viability*	Benign
	CT_{max}	Benign
	CT_{min}	Benign
	Total fecundity	Benign
3rd (F2/filial generation)	*Egg-to-adult viability*	Benign
	CT_{max}	Benign
	CT_{min}	Benign
	Total fecundity	Benign
4th (F3/filial generation)	*Egg-to-adult viability*	Benign
	CT_{max}	Benign
	CT_{min}	Benign
	Total fecundity	Benign
5th (F4/filial generation)	*Egg-to-adult viability*	Benign
	CT_{max}	Benign
	CT_{min}	Benign
	Total fecundity	Benign

Egg-to-adult viability

I conducted an egg-to-adult viability test to examine the percentage of eggs surviving to the adult stage in all generations across the four environmental regimes. I used different approaches in the design of the egg-to-adult viability test for the parental generation in the four environmental regimes, compared to the approach I used in 2nd-, 3rd-, 4th- and 5th generations in the four environmental regimes.

The differences between the approach used in the parental generation and the filial generations are based on: (1) The number of replicates. (2) The media used during incubation. (3) The conditions during incubation from egg-to-adult (4) The provenance of the eggs collected.

Every egg-to-adult viability test was conducted with a replica size of 20 vials generation^{-1} regime^{-1} with 50 eggs vial^{-1}, this gives a population size of up to a 1000 individuals generation^{-1} regime^{-1}. In the pilot trail I conducted prior to the study, to determine which concentration of dimethoate to use, I registered the concentration of 300 ppb of dimethoate to have a significantly negative effect on

egg-to-adult viability. I therefore made 75 replicates in the parental generation of the chemical regime to ensure population size of >700 individuals. In the following filial generations of the chemical regime, I only used 20 replicates with 50 eggs replica^{-1}generation^{-1} similar to the approach used in the three other environmental regimes.

In the parental generation of all environmental regimes I used Carolina media as the food source (Carolina Biological supply, Burlington, NC, USA) instead of the Leeds medium, which I used in the filial generations. I chose this approach because the Carolina media can be produced without the use of heating, which minimizes the risk of evaporation of dimethoate in the chemical regime, and thereby ensures the correct amount of chemical in the food source.

In the parental generation the eggs in the egg-to-adult viability were incubated under their respective environmental regime as described above. In the following filial generations flies from all four environmental regimes were incubated under benign laboratory conditions. In the parental generation the eggs used in the egg-to-adult viability test were collected from the baseline laboratory population. The eggs collected in the filial generations to create the egg-to-adult viability in the following filial generation, were collected among eggs laid by flies in the previous generation.

The procedure for collecting eggs was similar in all generations. The eggs collected in the egg-to-adult viability test were laid by the flies on teaspoons consisting of 2 mL of blue Leeds medium. I colored the Leeds media blue with fruit coloring for a better egg visualization. I transferred 20±5 flies, without the use of anesthesia, from the preceding generation to each *Drosophila* vial (VWR Drosophila vials) containing one teaspoon vial^{-1}. After eight hours of egg laying, I collected a maximum of 50 eggs teaspoon $^{-1}$ and transferred them to vials with an egg density of 50 eggs vial^{-1} to avoid crowding. To stimulate egg production in the female flies, I added a paste on top of the teaspoon containing water and yeast (Bass et al. 2007; Lee et al. 2008; Lee 2015). The eggs were incubated under their environmental regime in the parental generation and under benign conditions in the filial generations.

After incubation the number of emerged flies out of 50 eggs replica^{-1} were registered with the use of CO_2 anesthesia and thereafter transferred to vials with approximately 40±5 individuals vial^{-1} and

these vials were kept under benign laboratory conditions. From the emerged flies in the egg-to-adult viability test I collected flies to conduct the critical thermal limits and fecundity test. The next filial generation's egg-to-adult viability test was generated when the previous generation's egg-to-adult viability test had obtained a population size of >700 individuals or, earliest, at the third day of emergence in the previous generation.

Critical thermal limits (CT_{max} and CT_{min})

40 females regime^{-1} generation $^{-1}$ were tested for their ability to tolerate high and low temperatures. Within eight hours of emergence from the egg-to-adult viability test, the flies were sexed under CO_2 anesthesia. 40 females regime^{-1} generation^{-1} were selected and placed in a vial on Leeds medium and kept under benign laboratory conditions for three days post sedation, this was to ensure a full recovery from the anesthesia (Colinet & Renault 2012). On the third day of emergence the 40 flies were transferred individually to small screw-top glass vials (5 mL) without the use of anesthesia. The glass vials were randomly divided into two groups with 20 replicates in each, 20 replicas to tests critical thermal maxima (CT_{max}) and 20 replicas to test minima (CT_{min}). The two groups of flies were subsequently submerged into two water baths separately, pre-set at 23°C. A pump was added to each bath to circulate the water and thereby ensure homogeneity of the water temperature. The temperature was then increased with 0,1°C min^{-1} in the water bath containing the CT_{max} group and decreased with 0,1°C min^{-1} in the water bath containing the CT_{min} group. I then scored a knock down temperature for each fly, which was when the fly went into a thermal induced coma. The thermal induced coma was defined by when the fly was not standing and was totally immobilized despite physical stimuli and light.

Fecundity

I scored the fecundity in the parental and filial generations for all environmental regimes. I scored the fecundity as number of emerged offspring produced by a single pair of flies consisting of one male and one female in the first ten days of their lives. The ten days were divided into 48-hour intervals.

Within eight hours of emergence from the egg-to-adult-viability, 20 replicates regime^{-1} generation^{-1} were made consisting of one male and one female. Each replica was placed in a *Drosophila* vial with 7 mL of Leeds medium and over the next ten days I transferred the pairs to new vials, with the same amount of Leeds media, in 48-hour intervals. This created five time intervals of age replica^{-1}

(4-48 h, 48-96 h, 96-144 h, 144-192 h and 192- 240 h). I dismissed the replica if a fly died or escaped before the end of the 192-240 h time interval or there was no emerged offspring in the total time interval of a replica after incubation. The vials were incubated under benign laboratory conditions both in the parental and filial generations across all environmental regimes. The number of emerged adult offspring from each replica in each time interval was registered as a measure of the single pair's fecundity.

Statistical analysis

In this thesis the statistical program SPSS 23.0 were used for data processing. The data consisted of counts in the egg-to-adult viability test and total fecundity test and of temperature measurements in the assessment of critical thermal limits. The number of replicates regime^{-1} generation^{-1} is shown in appendix A.

Prior to the analysis, the number of scored flies in the egg-to-adult viability test was recalculated as a fraction of 50 eggs hatched. To enhance the normality and homogeneity of variance this data was arcsine transformed prior to the use of parametric tests. To increase the normality and homogeneity of variance of the data, in the critical thermal limits test and fecundity test were log transformed prior to the parametric analysis. The normality and homogeneity of variance of data from all three experiments were confirmed by a Sharpiro Wilk test and Kolmogorov-Smirnov test. To eliminate the variations observed in the 23°C control group, all comparisons with the remaining environmental regimes were made relative to the total mean of the five generations' mean in the 23°C control group.

To test the effect of generation and environmental regime in all experiments, a two-way nested ANOVA was used, where the measured variable (dependent variable) was compared with generation and environmental regime (fixed factors). The single comparison between a specific generation and environmental regime to the total mean of the 23°C control group, in all fitness traits tested, is based on a parametric ANOVA test followed by a Turkeys (HSD) post hoc test.

Results

In the following section the results obtained in the project will be presented. **Table** 1 gives an overall view of the three experiments, which illustrates the differences between each environmental regime's generations and the total mean of the 23°C control group with a significance level of 5%. It is seen that six out of twelve measurements in the 1st generation, in four different fitness traits, show a significantly different phenotype from the total mean of the control, and that two of the six measurements indicate crossgenerational effects to the successive generations.

1. The table illustrates the differences between the mean in the control and the single treatment within the same generation in the four fitness assays. It also illustrates the differences between each generation in the environmental regimes compared with the mean from 1st -5th in the control. The four assays are egg-to-adult viability, CT_{max} (°C), CT_{min} (°C) and Total fecundity in 244 h (10 days). The treatments are: 31°C heat, 13°C cold and 23°C 300 ppb dimethoate. Every treatment's mean value denoted with the difference to the mean value in the 23°C control from 1st -5th. A positive value from the control value corresponds with an improved fitness, if significant, within the measured trait. NS, *, ** and * denotes the significance level. NS: no significance; *: P<0,05; **: P<0,01 and ***: P<0001.**

Assay	Generation	23°C control mean	31°C - Heat Δ total mean	13°C - Cold Δ total mean	23°C - 300 ppb dimethoate Δ total mean
Egg-to-adult viability (%)	*1*	*75,4%*	*-2,9%^{NS}*	*-12,7%^{NS}*	*-52,82%****
	2	*94,4%*	*9,4%^{NS}*	*-1,4%^{NS}*	*4,7%^{NS}*
	3	*76,2%*	*2,0%^{NS}*	*-5,1%^{NS}*	*-2,4%^{NS}*
	4	*75,9%*	*6,6%^{NS}*	*-4,5%^{NS}*	*-11,3%^{NS}*
	5	*84,6%*	*10,8%^{NS}*	*10,1%^{NS}*	*9,1%^{NS}*
	Total mean.	*81,30%*			
CT_{max}°C	*1*	*39,88°C*	*0,39°C****	*-0,72°C****	*0,21°C^{NS}*
	2	*40,44°C*	*0,26°C ***	*0,18°C^{NS}*	*0,37°C****
	3	*40,41°C*	*0,16°C^{NS}*	*-0,06°C^{NS}*	*0,01°C^{NS}*
	4	*40,51°C*	*0,11°C^{NS}*	*-0,05°C^{NS}*	*-0,09°C^{NS}*
	5	*40,17°C*	*0,17°C^{NS}*	*-0,08°C^{NS}*	*0,00°C^{NS}*
	Total mean.	*40,28°C*			
CT_{min}°C	*1*	*6,06°C*	*-1,84°C****	*1,41°C****	*0,05°C^{NS}*
	2	*5,53°C*	*0,07°C^{NS}*	*0,15°C^{NS}*	*-0,06°C^{NS}*
	3	*5,46°C*	*0,50°C***	*0,19°C^{NS}*	*0,29°C^{NS}*
	4	*5,18°C*	*0,65°C****	*0,22°C^{NS}*	*-0,06°C^{NS}*
	5	*5,28°C*	*0,34°C^{NS}*	*0,14°C^{NS}*	*0,13°C^{NS}*
	Total mean.	*5,50°C*			
Total fecundity in 244 h	*1*	*129,16*	*-79,86^{NS}*	*-113,77****	*-5,64^{NS}*
	2	*176,14*	*5,42^{NS}*	*67,42^{NS}*	*31,27^{NS}*
	3	*181,32*	*9,55^{NS}*	*22,97^{NS}*	*47,76^{NS}*
	4	*247,70*	*63,20**	*21,96^{NS}*	*-13,14^{NS}*
	5	*256,21*	*57,82^{NS}*	*47,03^{NS}*	*71,32^{NS}*
	Total mean.	*198,11*			

Experiment one: Egg-to-adult viability

The results in **figure 1** and **table 2** show the egg-to-adult viability for the four environmental regimes: 23°C (control), 31°C (heat), 13°C (cold) and 23°C 300 ppb dimethoate over five successive generations. There was a significant effect (P<0,001) on egg-to-adult survivability of both environment and generation and the two combined (See **table 2**). In **table 1** the environmental regime 31°C (heat) and 13°C (cold) had no significant effect on survivability compared to the 23°C control group. The environmental regime of 23°C 300 ppb dimethoate lowered the survivability of the 1st generation (the directly affected generation) significantly by 52,82%, the effect was not crossgenerational to the successive generations (see **table 1**).

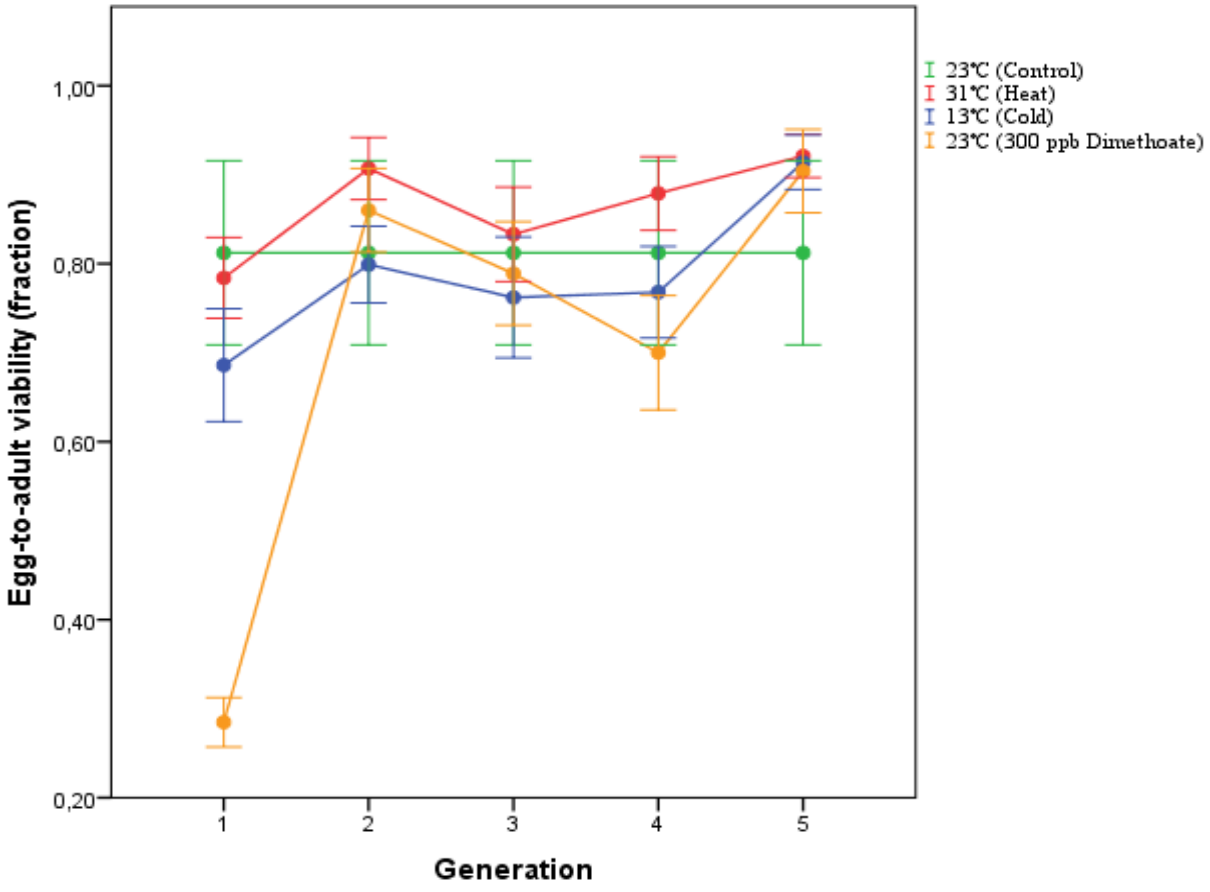


Figure 2. The panel illustrates the *D. melanogaster* egg-to-adult viability as a fraction of eggs developing successfully to the adult stage, over five generations in the respective four environmental regimes. 1st generation equals the parental generation where flies are exposed to the respective four environmental regimes and 2nd-5th generation denotes the generation that follows, where flies developed at control conditions. The control is based on the total mean over the five generations means. The green line; 23°C (control) - Red line; 31°C (heat) - Blue line; 13°C (cold) - and Orange line; 23°C (300 ppb dimethoate). Error bars represent 95% confidence intervals.

Table 2. Multifactor analysis of variance (ANOVA) on treatment and generation effect on egg-to-adult viability. Treatment and generation are the fixed factors and the egg-to-adult viability data were arc sin transformed prior to analysis to enhance normality. The treatment denotes the four environmental regimes; 23°C (control), 31°C (heat), 13°C (cold), 23°C (300 ppb dimethoate). The generation denotes the five generations within each environmental regime, the 1st, 2nd, 3rd, 4th and 5th generations. The flies were only affected by their respective environmental regime during development from egg to hatchling in the 1st generation, forwards all of the flies experienced normal laboratory conditions.

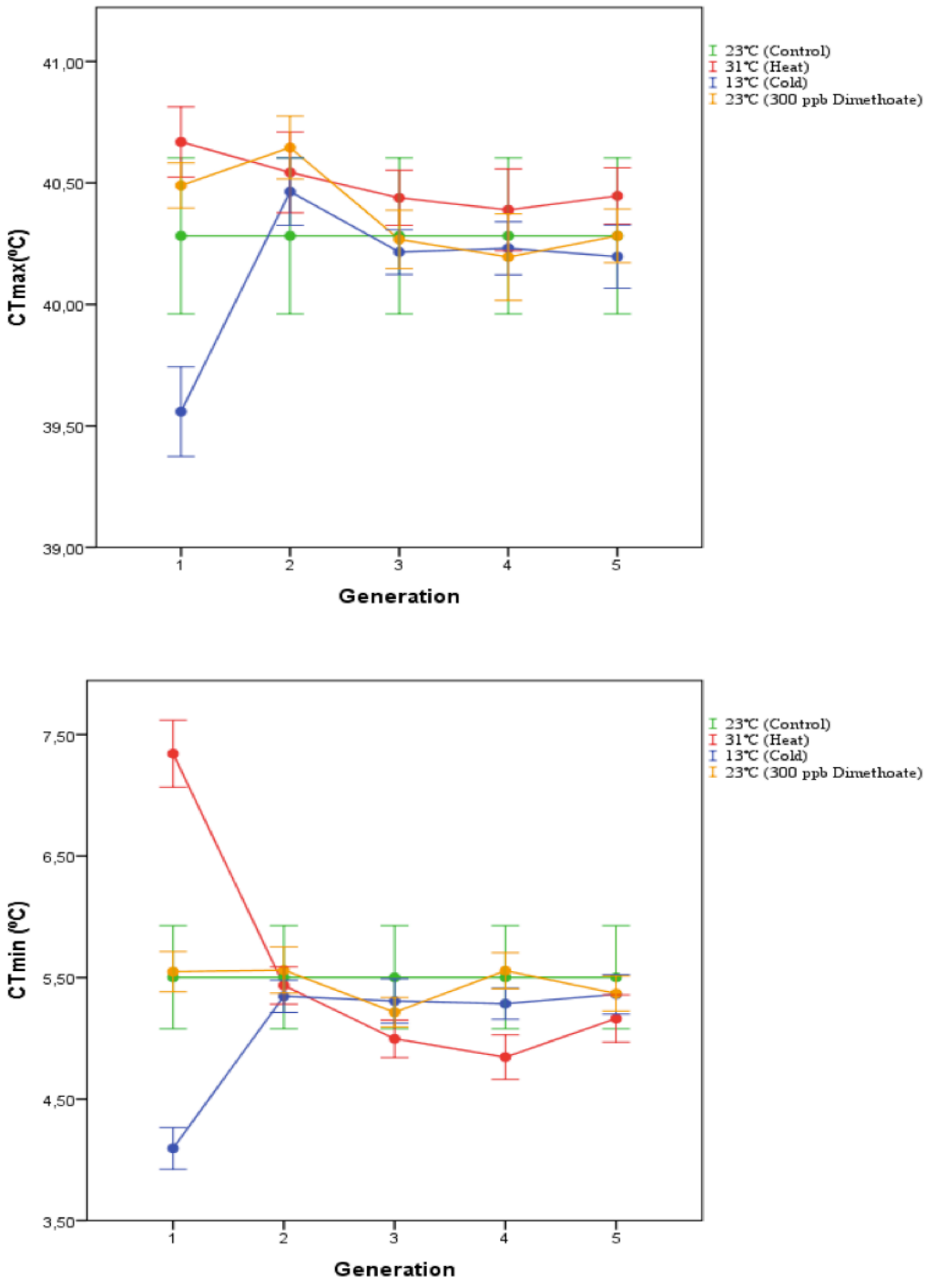
	df	MS	F	P
Generation	3	0,931	23,666	< 0,001
Treatment	4	2,948	75,780	< 0,001
Treatment x Generation	12	0,661	16,994	< 0,001
Error	435	0,039		

Experiment two: Critical thermal limits (CT_{max} and CT_{min})

The thermal critical limits for all environmental regimes across the five generations are illustrated in **figure 2**. Both generation and environmental regime and the two combined had a significant effect on critical thermal limits both in CT_{max} and CT_{min} (see **table 3** and **4**). The generational linage of the environmental regime 23°C 300 ppb dimethoate did not affect the critical minima (CT_{min}), whereas the 2nd generation in critical maxima (CT_{max}) showed a significantly increased heath tolerance of 0,37°C (see **table 1**).

The 31°C heat regime showed a significantly increased tolerance to heat exposure in both the 1st and 2nd generation at respectively 0,39°C and 0,26°C (see **table 1**). The same environmental regime showed a significantly decreased tolerance to cold exposure in the 1st generation, where after the cold tolerance increases significantly in the 3rd and 4th generation compared to the total mean of the 23°C control (see **table 1**). When looking at filial generations which had not been exposed directly, the 31°C heat regime showed a significantly increased fitness in tolerating both heat and cold (see **table 5** and **6**, and **figure 4**).

The 13°C cold regime showed a significantly decreased fitness in tolerance to heat exposure in the 1st generation by -0,72°C, compared to the total mean of the 23°C control group. The 13°C cold regime had a significantly increased fitness to cold tolerance in the 1st generation by 1,41°C (see **table 1**). There were no crossgenerational effects registered in critical thermal limits for the 13°C regime.



CT_{max} (°C)

CT_{min}(°C)

Figure 2. The panel illustrates critical thermal limits for *D. melanogaster* females, over five generations (1^{st}-5^{th}) to the respective four environmental regimes. 1 equals the parental generation where flies are exposed to the respective four environmental regimes and 2-5 denotes the generations that follows, where flies developed at benign conditions. The green line; 23°C (control) - Red line; 31°C (heat) - Blue line; 13°C (cold) - and Orange line; 23°C (300 ppb dimethoate). The top panel illustrates CT_{max} (°C) and the lower panel illustrates CT_{min} (°C). Error bars represent 95 % confidence intervals.

Table 3. Multifactor analysis of variance (ANOVA) of treatment and generations effect on CT_{max} (°C). Treatment and generation are the fixed factors and CT_{max} (°C) data were log transformed prior to analysis to enhance normality. Treatment denotes the four environmental regimes: 23°C (control), 31°C (heat), 13°C (cold), 23°C (300 ppb dimethoate) and generation denotes the five generations: 1st, 2nd, 3rd, 4th and 5th within each environmental regime. The flies were only affected by their respective environmental regimes during development from egg to hatchling in the 1st generation; forwards all of the flies experienced benign laboratory conditions.

	df	MS	F	P
Generation	3	< 0,001	29,083	< 0,001
Treatment	4	< 0,001	18,174	< 0,001
Treatment x Generation	12	< 0,001	12,868	< 0,001
Error	380	$9{,}499\times10^{-6}$		

Table 4. Multifactor analysis of variance (ANOVA) of treatment and generations effect on CT_{min} (°C). Treatment and generation are the fixed factors and CT_{min} (°C) data were log transformed prior to analysis to enhance normality. Treatment denotes the four environmental regimes: 23°C (control), 31°C (heat), 13°C (cold), 23°C (300 ppb dimethoate) and generation denotes the five generations: 1st, 2nd, 3rd, 4th and 5th within each environmental regime. The flies were only affected by their respective environmental regimes during development from egg to hatchling in the 1st generation; forwards all of the flies experienced benign laboratory conditions.

	df	MS	F	P
Generation	3	0,031	31,287	< 0,001
Treatment	4	0,016	16,495	< 0,001
Treatment x Generation	12	0,053	54,568	< 0,001
Error	380	0,001		

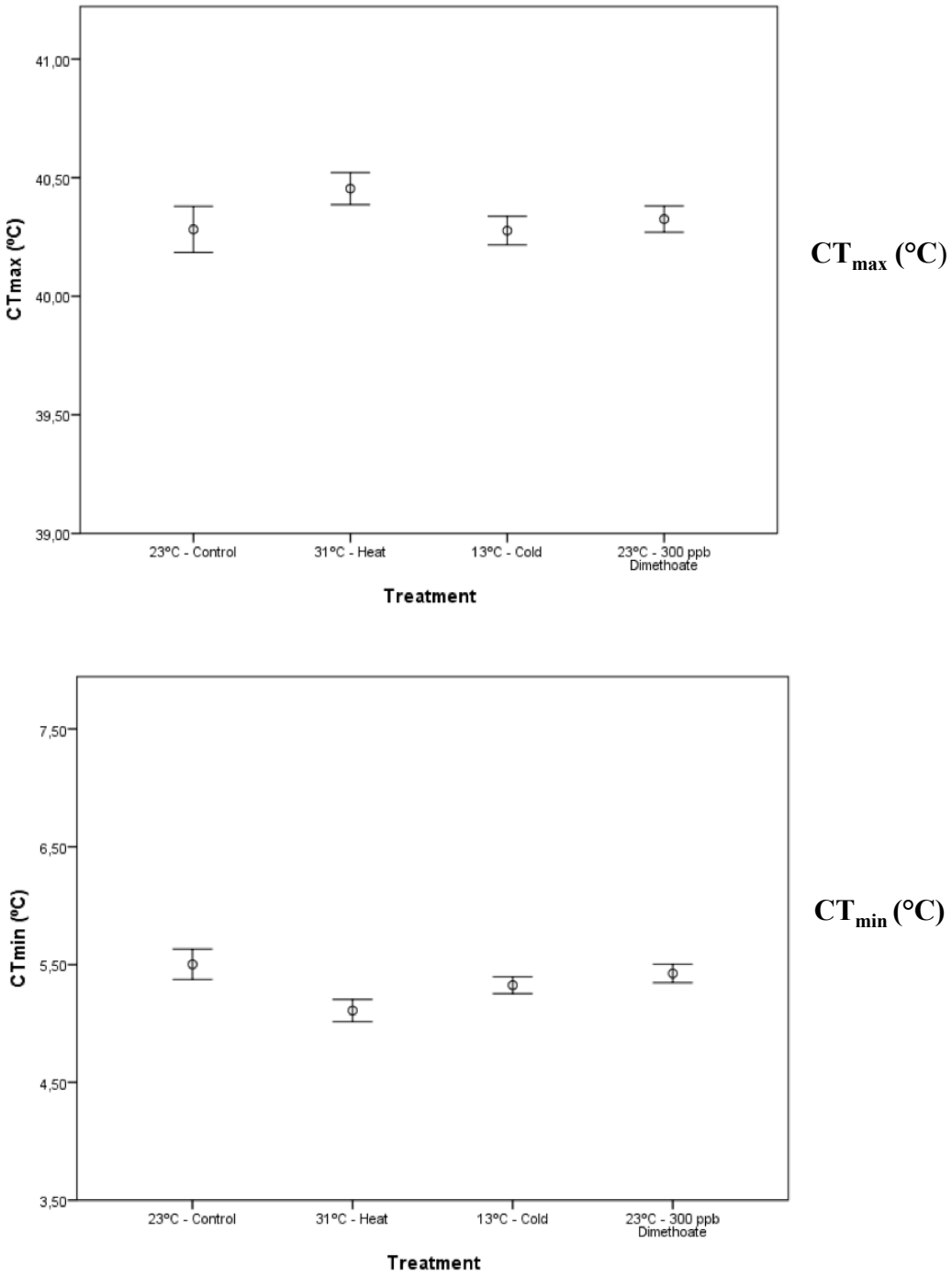


Figure 4. The panel illustrates the mean CT_{max} (°C) and CT_{min} (°C) value for the 2nd, 3rd, 4th and 5th generations for *D. melanogaster* females to the respective environmental regimes. Denote that all of the filial flies experienced normal laboratory conditions. The four environmental regimes that the 1st generation experienced are: 23°C (control), 31°C (heat), 13°C (cold) and 23°C (300 ppb dimethoate). The top panel illustrates mean CT_{max} (°C) value for the four environmental regimes in the filial generations and the lower panel illustrates mean CT_{min} (°C) value for the four environmental regimes in the filial generations. In both panels the control is represented as a mean of the five generations within the control group. Error bars represent 95% confidence intervals.

Table 5. A one-way ANOVA of the 1st generations impact on CTmax (°C) mean from the 2nd to the 5th generation, in their respective environmental regimes. The analysis is conducted on the total mean from the 2nd-5th generation, without the 1st generation, in their respective environmental regimes. Treatment denotes the four environmental regimes: 23°C (control), 31°C (heat), 13°C (cold), 23°C (300 ppb dimethoate). The data were log transformed prior to analysis to enhance normality.

	df	MS	F	P
Treatment	3	$5{,}026\times10^{-5}$	4,888	<0,01
Error	316	$1{,}028\times10^{-5}$		

Table 6. A one-way ANOVA of the 1st generations impact on CT_{min} (°C) mean from the 2nd to the 5th generation, in their respective environmental regimes. The analysis is conducted on the total mean from the 2nd- 5th generation, without the 1st generation, in their respective environmental regimes. Treatment denotes the four environmental regimes: 23°C (control), 31°C (heat), 13°C (cold), 23°C (300 ppb dimethoate). The data were log transformed prior to analysis to enhance normality.

	df	MS	F	P
Treatment	3	0,011	10,045	< 0,001
Error	316	0,001		

Experiment three: Total fecundity in the first 244 h of longevity.

Figure 5 shows the total fecundity in the number of adult offspring produced in the first 244 hours of the flies' longevity, in each generation of each of the four environmental regimes. The nested ANOVA in **table 7** indicates that environmental regime, generation, and the two factors combined significantly affect the total fecundity. The environmental regime of 23°C -300 ppb dimethoate did not affect the fecundity significantly in either the directly affected generation or in the successive generations (see **table 1**) which illustrates a non-existent crossgenerational effect for this specific fitness trait and environmental regime.

13°C cold regime significantly affected fecundity in the 1st generation by a decreased total offspring production. There is no significant effect on fecundity in the successive generations within the same environmental regime (see **table 1**). 31°C heat regimes shows no difference in fecundity compared to the total mean of the control group in the directly affected generation or in the 2nd, 3rd and 5th generation (see **table 1**). The fecundity significantly increases in the 4th generation, the increase does not significantly change the fecundity in the filial generation when gathered (see **figure 6** and **table 8**).

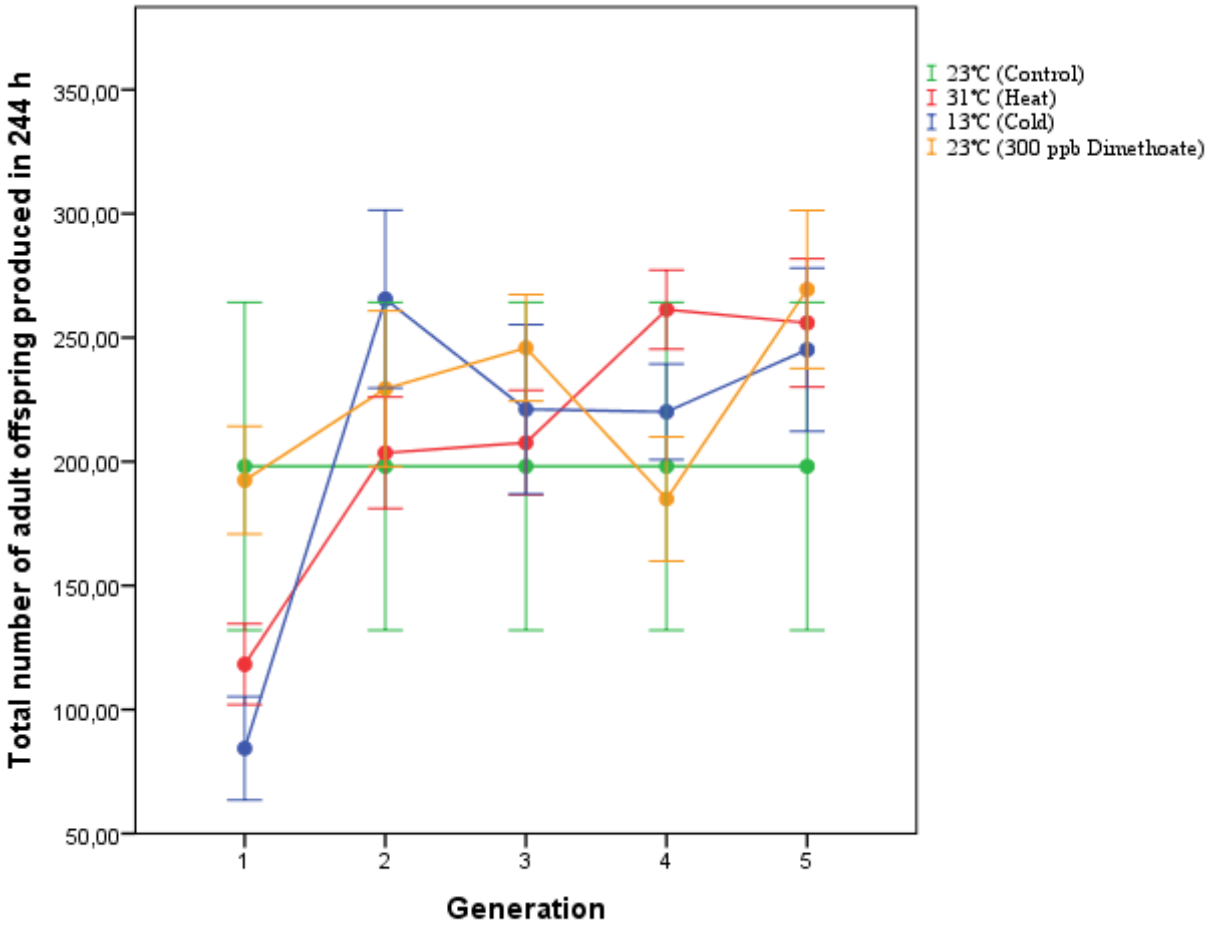


Figure 5. The panel illustrates the mean (based on 11-20 replicate vials with one male and one female) of the total number of emerged offspring after 244 hours of egg-laying for *D. melanogaster*, over five generations to the respective four environmental regimes. 1 equals the parental generation where flies are exposed to the respective four environmental regimes and 2-5 denotes the generations that follow where flies developed at control conditions. The green line; 23°C (control) - Red line; 31°C (heat) - Blue line; 13°C (cold) - and Orange line; 23°C (300 ppb dimethoate). The control is represented as a total mean over the fiver control generations. Error bars represent 95% confidence intervals.

Table 7. Multifactor analysis of variance (ANOVA) of treatment and generations effect on offspring produced in 244 h (figure 5). Treatment and generation are the fixed factors and the mean of total offspring produced data were log transformed prior to analysis to enhance normality. Treatment denotes the four environmental regimes: 23°C (control), 31°C (heat), 13°C (cold), 23°C (300 ppb dimethoate) and generation denotes the five generations: 1st, 2nd, 3rd, 4th and 5th generation within each environmental regime. The flies were only affected by their respective environmental regimes during development from egg to hatchling in the 1st generation, forwards all of the flies experienced normal laboratory conditions.

	df	MS	F	P
Generation	4	1,087	67,763	< 0,001
Treatment	3	0,090	5,592	= 0,001
Treatment x Generation	12	0,150	9,362	< 0,001
Error	300	0,016		

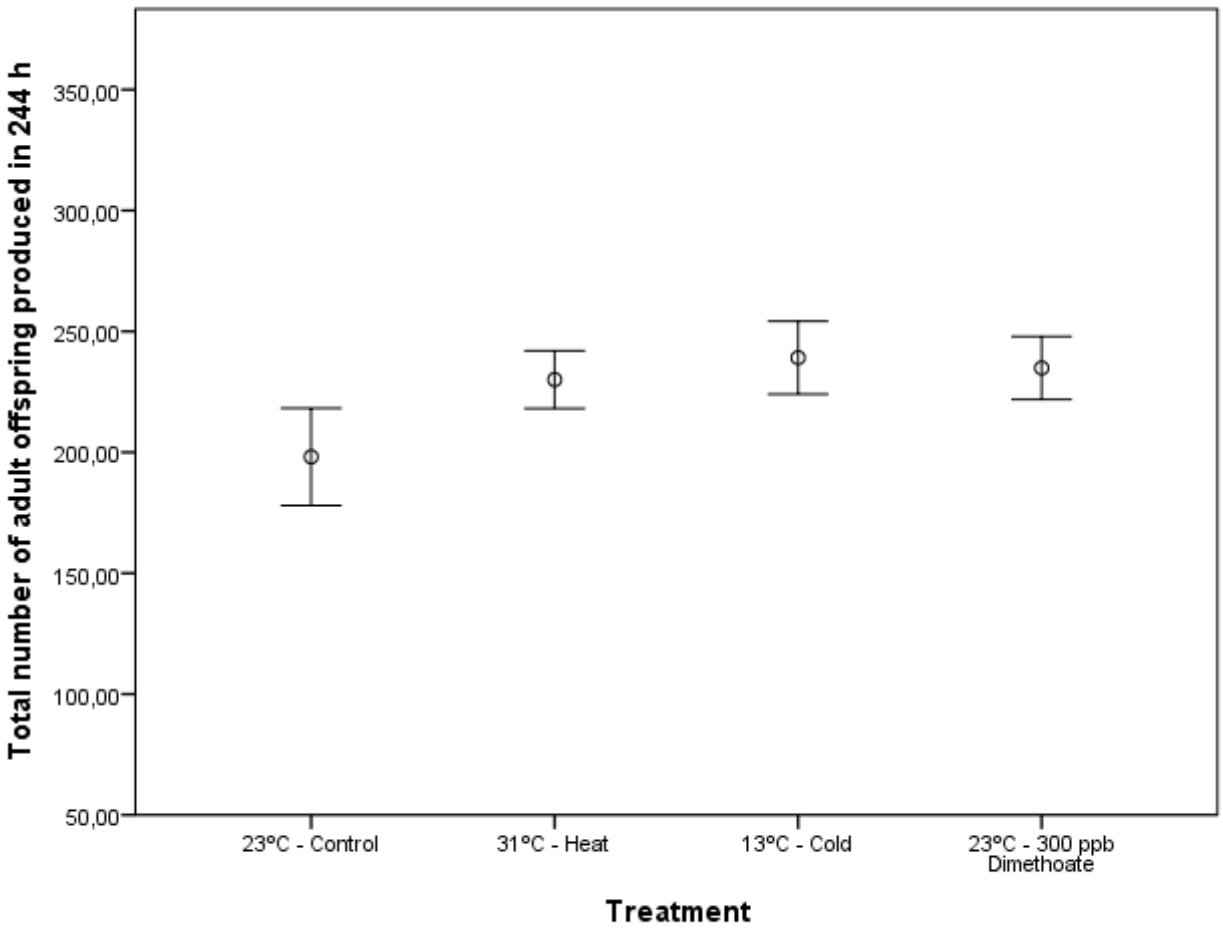


Figure 6. The Panel illustrates the total mean number of adult offspring for *D. melanogaster* after 244 h from the filial generations from 2nd to the 5th generation in their respective environmental regime. The environmental regimes are 23°C (control), 31°C (heat), 13°C (cold), 23°C (300 ppb dimethoate). Error bars with 95% confidence interval and the mean of the control is based on the total mean from the 1st to the 5th generation.

Table 8. A one-way ANOVA of the impact on total mean number of offspring in 244 h from the 2nd to the 5th generation in their respective environmental regimes. The analysis is conducted on the total mean from the 2nd-5th generation, without the 1st generation, in their respective environmental regimes. Treatment denotes the four environmental regimes: 23°C (control), 31°C (Heat), 13°C (cold), 23°C (300 ppb dimethoate). The fraction data were log transformed prior to analysis to enhance normality. NS denotes non-significant.

	df	MS	F	P
Treatment	3	0,021	1,562	NS
Error	240	0,014		

Figure 7 illustrates the mean number of offspring produced in each age interval with a 95% confidence interval in the first generation of the environmental regimes 23°C control, 31°C heat and 13°C cold, where the significant results were observed in total fecundity **table 1**. It is indicated, but not statically tested, that 13°C has lower fecundity in each age than 23°C control and 31°C heat in

the first 244 hours of longevity. It is seen that the difference between the 23°C control and 31°C increases with increasing age and that the environmental regime of 13°C cold has a lower fecundity in each age than 23°C control.

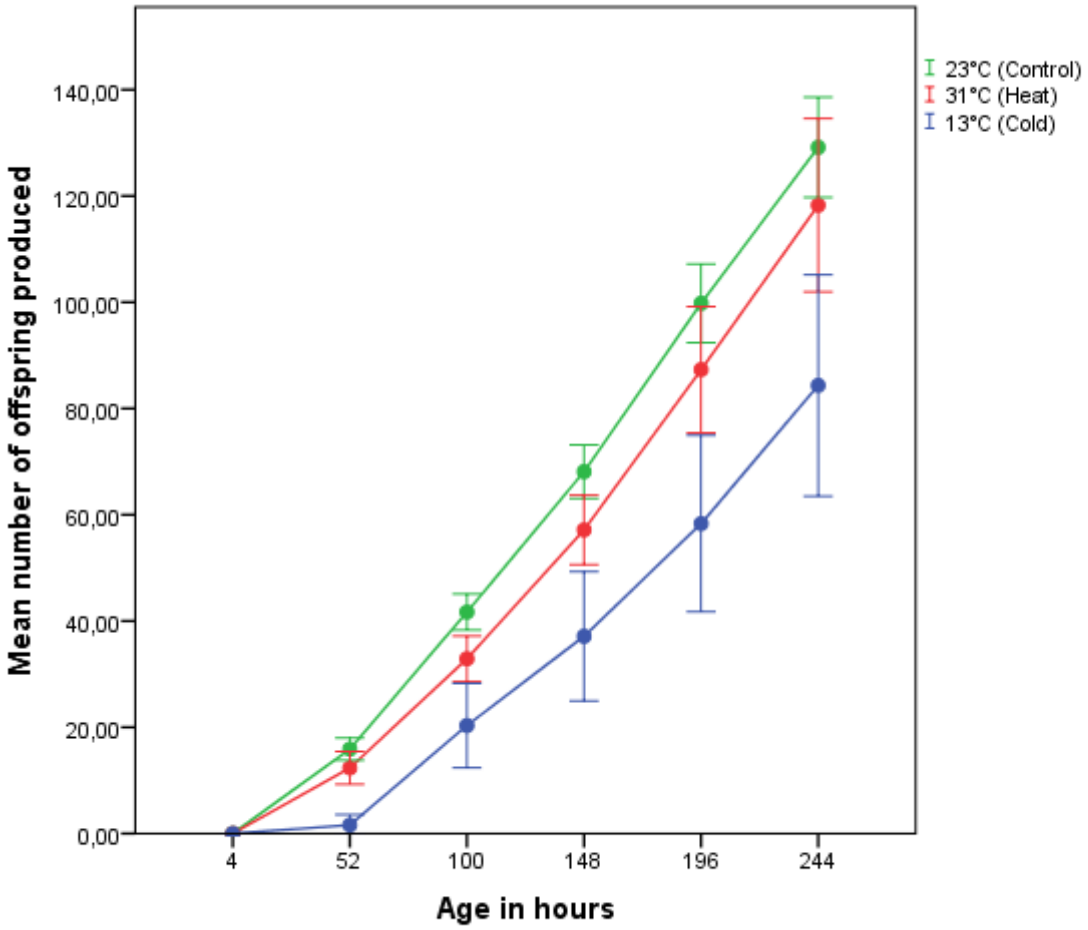


Figure 7. The panel illustrates the mean number of emerged adult offspring for *D. melanogaster* over five time periods in the first 244 hours of their lives in the first generation. The environmental regime of 23°C 300 ppb dimethoate was left out, since no significance was registered in table 1 in total fecundity. Green: 23°C (control), Red: 31°C (heat), Blue: 13°C (cold) with a 95% confidence interval.

Discussion

The aim of this master thesis was to investigate whether an environmental stressor during development could induce a phenotypic plasticity, that was epigenetically inherited by successive generations which experienced normal conditions without environmental stress. The understanding of phenotypic plasticity and epigenetically inheritance is an extensive and growing field with equivocal results and conclusions (Bird 2007; Haig 2012). Phenotypic plasticity and epigenetic inheritance is argued to be of great importance in the ecology of a species and in mediating or delaying evolutionary changes (Franks & Hoffmann 2012; Iovino 2014). The results obtained in this project give some insights into the inheritance of phenotypically plastic fitness traits, however a clear inheritance pattern was not identified. The environmental regime involving 31°C heat showed a surprising pattern within the fitness trait of testing critical thermal minima, were it in the successive generations showed a phenotype with a improved fitness.

Egg-to-adult viability

The purpose of the egg-to-adult viability test was to test the survival rate from egg to hatchling when affected by an environmental impact during development, and if a potential change in survival rate was traceable across generations. The survivability of the offspring from egg-to-adult is an important factor in the ecology of at species, to ensure a healthy population size and thereby minimize the risk of inbreeding (Townsend et al. 2008; Sadava et al. 2011).

Temperature fluctuations is a recurring factor in the ecology of a species (Berteaux et al. 2004; Townsend et al. 2008; Dey et al. 2015) and climate changes are argued by Bertaux et al. (2004) and Dey et al. (2015) to enhance the possibility of changes in phenotypic plasticity to accommodate for varying temperatures. Therefore, I expected the temperature regimes to display a phenotypic plasticity, since the organism should encounter a new environment which could be costly in terms of energy. In the egg-to-adult viability test, the multifactor analysis (ANOVA) (**table 2**) indicated that treatment, generation and the two factors combined had a significant effect on egg-to-adult survivability. In **table 1** it is shown that the two temperature regimes, 31°C heat and 13°C cold, did not have a significant effect on the survivability in any generations compared to the 23°C control group. This indicates that the plasticity in the fitness trait of egg-to-adult survivability is not significantly affected by temperature, and therefore there are no visible changes in phenotype to be inherited crossgenerationally.

In Schou et al. (2015) the survivability from egg-to-adult is fairly stable at around 90-100% from 14-31°C and in Petavy et al. (2001) the survivability from egg-to-adult were around 70 - 80% in the temperature range from 14-31°C (Petavy et al. 2001; Schou et al. 2015). I found a mean survivability from egg-to-adult in the control group, over five generations, at 81,30% (see **Table 1**), which is slightly below the 90-100% found in Schou et al. (2015), but still corresponds with the measurements found in Petavy et al. (2001). Although my control groups survivability from egg-to-adult is slightly below the value found by Schou et al. (2015), both Petavy et al. (2001) and Schou et al. (2015) found that the survivability was both stable and at its highest in the temperature range from 13-31°C, outside this temperature range survivability decreased.

In the 1st generation, the environmental regime of 300 ppb dimethoate significantly decreased the egg-to-adult survival by 52,82% compared to the mean control (see **table 1**). One explanation for the decreased survivability is that inhibiting acetylcholinesterase would lead to an overstimulation of the muscles and prevent the muscles from returning to a relaxed state, which would affect motor abilities in the individual (Sadava et al. 2011; Van Scoy et al. 2016). The negative effect on egg-to-adult survival was not transferred to the successive generations of the 300 ppb dimethoate environmental regime (see **table 1**). This does not imply a complete lack of epigenetic inheritance according to a changed survivability from egg-to-adult when exposed to dimethoate. Fournier (1993) and Vontas et al. (2002) found that, respectively, *D. melanogaster* and *B. oleae* (Olive fruit fly) had a hormesis effect, a phenomenon were small doses of a harmful condition improves the functionality of cells or the organism (Vaiserman 2011). The exposure to dimethoate could alter the expression of the enzyme acetylcholinesterase, making them less susceptible to be affected by organophosphate poisoning. The exposure of dimethoate in the 1st generation could have induced epigenetic markers or selected for genotypes with an increased expression of the acetylcholinesterase enzyme, which could enhance survival in successive generations when exposed to similar environmental conditions during development (Snell-Rood et al. 2010; Franks & Hoffmann 2012; Valtonen et al. 2012; Jablonka & Lamb 2015).

The reduced survival in 300 ppb dimethoate could not only be explained by its toxicity when ingested, but could also be a case of a reduced food intake or food deprivation in the presence of dimethoate. Changes in food consumption and composition could alter life history characteristics

e.g. survival, longevity, fecundity and body size (Rodrigues et al. 2015). Therefore, measurements taken in the present trial on bodyweight on the emerged flies could have out ruled the effect of malnutrition to some degree, due to a change in foraging pattern. Rodrigues et al (2015) also proved that different food compositions and foraging patterns could alter the phenotype expressed in different fitness traits, because individuals selectively choose between macronutrients to encounter changed environments, which possibly could result in a changed life history of the individual. Therefore, it cannot be rejected that the foraging patterns of the larvae could affect the results in all measured fitness traits in both a positive and negative direction.

Thermal tolerance

The aims of the trial were to detect a possible crossgenerational plasticity in temperature tolerance after a single environmental impact on the parental generation. There are different laboratory protocols to test thermal tolerance in ectotherms. One assay aims to investigate the acute knock down temperature, in which the LT_{50} value (the temperature is lethal to 50% of the test organisms) is determined (Overgaard et al. 2012). The other test is a ramping assay where the thermal tolerance is tested by a gradually changing the temperature over a time period, until the organism goes into a thermal induced coma or dies. The ramping assay is argued to be more ecologically relevant than the acute knock down temperature, since this the most realistic scenario in nature (Mitchell & Hoffmann 2010; Overgaard et al. 2011; Terblanche et al. 2011; Overgaard et al. 2012). Critics have highlighted that desiccation and/or starvation might influence the performance of the flies in ramping assays, but Overgaard et al. (2012) found no support for this. Therefore, I chose to proceed with the ramping assay for determination of thermal tolerance.

According to the results obtained in **table 1**, the environmental regime of 300 ppb dimethoate did not affect the critical thermal tolerance in the parental generation in either CT_{min} or CT_{max}. A single outlier is seen in the 2nd generation of 300 ppb dimethoate in CT_{max}. I found no literature supporting an altered thermal tolerance when exposed to dimethoate. Woods et al. (1999) found that high variability between replicates could affect the stress detected, from which randomization in replicates is of great importance. During the trial I kept each environmental regime gathered on four separately shelves in the incubator room, creating a difference in height between top and bottom shelf of approximately one meter horizontally. This creates possible differences between the shelves in humidity, light and temperature. Therefore, the single outlier is possibly more a result of missing

randomization, than an actual result of an increased thermal tolerance inherited crossgenerationally due to the 300 ppb dimethoate exposure in the first generation.

Despite the effect of missing randomization in the results, some patterns of phenotypic inheritance were indicated in the thermal tolerance test. This is seen in linages were one or more of the successive generations, besides the parental generation, showed a significantly different response in trait plasticity than the control group. That is the case for 31°C heat treatment in the CT_{min} assay, **table 1**, wherein two of the successive generations (the 3rd and 4th generation) showed an increased cold tolerance, compared to the control group. The heritability seen in 31°C temperature tolerance was a surprising result, since Mitchell & Hoffmann (2010) states that flies exposed to heat stress only experience a weak heritability.

The plasticity in 31°C heat CT_{min} assay is significantly different from the mean of the control group, even when the filial generations of the four environmental regimes are pooled together as one sample (see **figure 4** and **table 6**). Gilchrist & Huey (2001) showed that offspring fitness in *D. melanogaster* was significantly influenced by parental temperature during development. They found that offspring from parents raised at 29°C had a significantly better overall fitness than offspring derived from a lower parental temperature, which also could be the case for the 31°C heat treatment in the CT_{min} assay. However, some literature argues the opposite, that offspring derived from a low parental temperature would have an improved fitness in tolerating colder climates due to an enhancement in body size (Gilchrist & Huey 2001; Ferrer et al. 2013).

The epigenetic inheritance displayed in the ramping assays of the 31°C heat treatment could indicate different physiological patterns of inheritance. The way in which the inheritance is tested in the present study has its limitations in terms of determining the origin of the epigenetic plasticity or what molecular causes lie behind the results. Therefore, one can only speculate and argue for the most plausible path of epigenetic inheritance. In the CT_{max} trait the 31°C heat treatment experienced an improved fitness in the directly affected generation and a reduced, but still significantly better fitness, in the 2nd generation. Since the improvement in fitness does not occur over more than one successive generation, the alteration of the phenotype could be a case of soft heredity with a somatic cell inheritance passed along directly from their parents (Bonduriansky 2012; Ferrer et al. 2013; Kuijper & Johnstone 2015). This kind of heritance is expected to be favored in fluctuating

environments, because a uniform and persistent expression of a phenotype could reduce the general fitness of the population (Uller 2008; Ferrer et al. 2013). Since the flies have experienced several generations in the laboratory prior to the experiment, with a lesser changing environment, a uniform phenotypic expression for optimal fitness could have occurred (Hoffmann 2010). Therefore, I anticipated soft heredity in the experiment since the conditions are not recurring, making it illogical to use energy on making epigenetic changes persistent in the gametes. Therefore, I anticipated that the phenotypic differences seen would cease dramatically after the 1st generation, but this was not the case. In the CT_{min} trait of the 31°C heat treatment the phenotypic plasticity was displayed in several generations, which indicates a changed heredity where the epigenetic modifications are possibly persistent in the gametes of the organism (Jablonka & Raz 2009; Skinner et al. 2010; Duncan et al. 2014; Bateson 2015; Jablonka & Lamb 2015).

There was no visible crossgenerational effect on the environmental regime of 13°C cold in regard to an increased or decreased thermal tolerance in the successive generations, since the differentiation in thermal tolerance only were present in the 1st generation of both CT_{min} and CT_{max}. The result indicates that flies exposed to warmer temperature are more susceptible for the environmental impact in thermal tolerance to be persistent in the successive generations. Gillman et al. (2010) and Wright et al. (2011) found that evolutionary nuclear changes in the DNA, in respectively plants and marine fish, are increased in tropical climates. Another explanation for the persistence is that the phenotype has become fixed within the population, even though it has no selective advantages (Jablonka & Lamb 2015).

The two temperature regimes of 13°C cold and 31°C heat show a negative fitness response in the 1st generation in regard to CT_{max} 13°C cold and CT_{min} for 31°C heat and a positive fitness response in the opposite assay (See **table 1**). One explanation is that the individuals in this generation have received environmental cues from the baseline population kept at 23°C, and that 23°C was the first temperature they were exposed to until the eggs were collected and subjected to their respective environmental regime. This could affect the first generation's thermal tolerance (Duncan et al. 2014). However, it is more likely that the response shown in the first generation is an indication of acclimation. Sørensen et al. (2015) proved that *Drosophila subobscura* had an acclimation time of approximately ten days when transferred from nature to 20°C. Since the flies were transferred from their development temperature at respectively 13°C and 31°C to 23°C three days post hatchment

before testing, this is the most plausible explanation for the reduced fitness registered in 1st generation of CT_{max} 13°C and CT_{min} 31°C. Acclimation could also be the explanation for the improved fitness in the first generation of CT_{min} 13°C and CT_{max} 31°C. Jenkins and Hoffmann (1994) found that *D. melanogaster* that were pre-exposed to non-lethal temperatures during their larval state had an increased resistance to heat, which could be an argument for the enhanced fitness seen in the first generation for CT_{min} 13°C and CT_{max} 31°C.

Total fecundity in 244 hours

An important part of the persistence of a population is the ability to reproduce successfully. Therefore investigating a phenotype related to reproductive output is of great importance (Townsend et al. 2008). Reproduction is time consuming and energy costly process and may therefore be a mechanism that could be downregulated to allocate energy for survival in stressful environments thereby changing the phenotype (Lee et al. 2008; De Loof 2011). Such a trade-off effect between reproductive resources and survival is proved by Hunt et al. (2016) where *D. melanogaster* allocated reproductive resources for an increased resistance to a pathogen infection.

Reproductive success in flies is affected by multiple factors. For female flies, food quality and quantity is essential for the female's egg production (Jørgensen & Bundgaard 2006; Rodrigues et al. 2015). Also larvae density during the development could affect both the reproductive success of the female and her future offspring (Huey et al. 1995; Townsend et al. 2008). The population density the female experienced during her development was controlled in all generations, which minimized the differences in nutritional state. The density experienced by the offspring in the fecundity test could not be controlled and could affect survival from egg-to-adult. To counteract density as a problem, I only kept one pair of flies $vial^{-1}$ $replica^{-1}$ and I transferred the pair to new vials at 48-hour intervals. Despite the efforts of minimizing density as a factor, I still observed vials where the media were well eaten by larvae indicating higher densities in some replicas.

It is known that extreme temperatures can reduce the fecundity in *D. melanogaster* because it affects the spermatogenesis in the male flies and lowers sexual activities of both the male and female flies (Jenkins & Hoffmann 1994; Chakir et al. 2002; Tobler et al. 2015; Singh et al. 2015). Chakir et al. (2002) found that D. *melanogaster* males developed and kept outside the temperature range of 13°C–29°C are sterile, but that the sterilization is reversible to some degree when males are transferred to temperatures within the range. Therefore, I expected both temperature regimes to show a reduced fecundity in in the first generation and they did, except that the reduction seen in the first generation of 31°C was not significant. All generations were kept under normal conditions at 23°C in the fecundity trial, so one explanation for the non-significantly reduced fecundity in 31°C heat treatment could be that males from hotter environments are better at reversing the effect than males from a colder environment (Gilchrist & Huey 2001) Ferrer et al. (2013), however, argues the opposite. Chakir et al. (2002) showed that males from 14°C had a higher fertility percentage that was reversed faster when returned to a temperature of 21°C, than males from 31°C, which to some degree contradicts the findings in the present study.

The reproductive output of a female *D. melanogaster* is varying, because the ovariole number varies with population and temperature (Delpuech et al. 1995; Wayne et al. 2006) and Chakir et al. (2002) state that one ovariole in *D. melanogaster* can produce 2 egg day^{-1} under optimal conditions. Delpuech et al. (1995) found that developmental temperature dictates the number of ovaries in *D. melanogaster* and that the patterns of the maximum number of ovaries around the optimal temperature were the same despite the geographical location of the population. All locations showed a bell shaped curve from 11°C to 31°C. The three different populations tested in Delpuech et al. (1995) indicated that female *D. melanogaster* developed the same number of ovaries when raised in 13°C and 31°C, and therefore have the same opportunities for egg production independent of temperature. This does not explain the differences seen between the total fecundity 13°C and 31°C in the 1st generation.

The explanation could be that 31°C flies are fecund in an earlier age than flies 13°C. Chakir et al. (2002) state that the maximum production of offspring is at the age of 4-15 days, which might indicate the differences seen in total fecundity between the thermal regimes could have been equalized if the trial was continued longer than the first 10 days of longevity. In **figure 7** it is indicated, not statistically tested, that the reproduction accelerated to an earlier age in the 1st

generation when reared at 31°C than at 13°C, and that the difference between 13°C and 31°C was minimized with increasing age. Flies reared under hotter climates have a shortened developmental time and may therefore become fecund faster than flies reared in colder climates, therefore flies physically age faster when develop 31°C than 13°C (Gilchrist & Huey 2001; Steigenga & Fischer 2007). This is also indicated in **figure 7** since the difference between the 23°C control and the 31°C heat regime increases with an increasing age. To clarify early fecundity, and a faster aging in the 31°C heat treatment, intervals shorter than two days might be needed, since a method used to determine early fecundity runs in 24-hour intervals (Huey et al. 1995; Wayne et al. 2006). The early fecundity explanation is, however, contradicted by Huey et al. (1995), who found that parents developed at higher temperatures transferred to a lower egg laying temperature showed a later fecundity in number of eggs laid, than parents developed at lower temperature laying eggs at higher temperature, which contradicts my findings.

There was no visible inherited phenotype to the successive generations in total fecundity, except from 31°C heat's 4^{th} generation which could be a case of missing randomization as discussed in the section of thermal tolerance (Woods et al. 1999). Some epigenetic crossgenerational effect is, however, weakly indicated in the total fecundity trial. Cold is proven to reduce fecundity in the directly affected generation (Chakir et al. 2002; Tobler et al. 2015) and females derived from parents influenced by low developmental temperature were proved to have a significantly higher lifetime egg production than females derived from hotter parents (Ferrer et al. 2013). The results presented in this project could indicate some truth in the above mentioned thesis of Ferrer et al. (2013), since 13°C is the environmental regime with the biggest increase in number of offspring between the first and second generation (see **table** 1), without it being statistically tested.

Conclusion

The purpose of the project was to identify whether environmental stimuli in a given generation at a critical time of development had long lasting consequences that could be detected across generations in the model organism *D. melanogaster*. Only six out of twelve possible results in the 1st generation showed a different phenotype than the control, and out of the six only two of them showed that the change in phenotype was traceable in the successive non-exposed generations. From the results, I can conclude that the environmental regimes of 13°C cold and 300 ppb dimethoate did not significantly change the measured phenotype in the successive non-exposed generations, even though some traits showed a significantly altered phenotype in the first generation. From this it cannot be excluded that epigenetic changes have not happened, since the trial lacks the insights that molecular techniques could have contributed. This means that the possible changes due to the exposure could enhance future generations' fitness when encountering similar conditions.

The environmental regime of 31°C heat was the only treatment indicating crossgenerational effects in the successive generations. Both soft heritability and heritability through the gametes were indicated. However, the response seen in testing critical thermal minima was surprising, since successors from hotter parents had a significantly increased cold tolerance, despite the maladaptive response seen in the 1st generation. This result supports the theory that "*hotter parents are better*" but contradicts the assumptions that heredity in hotter climates is lower. This finding could be interesting to elaborate in further tests with molecular technics involved.

From the results I obtained I must concede and join that research done within this field is extensive and often contradictory. The combination of stressors and fluctuations in nature could be difficult to replicate in the laboratory, and even small changes could be of great significance in showing the "true" result of stressors' influence on the phenotype and its possibility to be present across generations. Therefore, it is difficult to argue that results could be conclusive without a comprehensive research covering all possible combinations and without the involvement from, and cooperation between, multiple biological research fields from ecology, genetics, evolutionary biology and molecular biology.

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Appendix

Appendix A – Number of replicates

23 °C control

Generation	**1st**	**2nd**	**3rd**	**4th**	**5th**	**Mean**	**Stanard diviation**
Egg to Adult vability	20	20	20	20	20	20	0
Fecundity	19	15	19	11	20	16,8/16,6	+-3,4/+-3,7
CT_{Max}	20	20	20	20	20	20	0
CT_{Min}	20	20	20	20	20	20	0

31 °C heat

Generation	**1st**	**2nd**	**3rd**	**4th**	**5th**	**Mean**	**Stanard diviation**
Egg to Adult vability	20	20	20	20	20	20	0
Fecundity	20	19	16	16	14	17,2	+-2,1
CT_{Max}	20	20	20	20	20	20	0
CT_{Min}	20	20	20	20	20	20	0

13 °C cold

Generation	**1st**	**2nd**	**3rd**	**4th**	**5th**	**Mean**	**Stanard diviation**
Egg to Adult vability	20	20	20	20	20	20	0
Fecundity	15	17	13	16	15	16,8/15,8	+-1,2/+-1,7
CT_{Max}	20	20	20	20	20	20	0
CT_{Min}	20	20	20	20	20	20	0

23 °C 300 ppb dimethoat

Generation	**1st**	**2nd**	**3rd**	**4th**	**5th**	**Mean**	**Stanard diviation**
Egg to Adult vability	75*[1]	20	20	20	20	31	+-22
Fecundity	15	16	15	16	14	15,6/15,2	+-0,5/+-0,7
CT_{Max}	20	20	20	20	20	20	0
CT_{Min}	20	20	20	20	20	20	0

*[1]=300 ppb dimethoat affects the egg to adult viability, therefor to ensure enough emerge offspring to continue to F1 with enough genetic variation, the replicat size was made larger.