Temporal Genetic Stability and Fitness Responses to Human-Driven Eutrophication in the Water Flea; Daphnia magna.

By

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Abstract

Climate change and anthropogenic impact affect natural environments driving species adaptation though migration, plasticity and genetic evolution. Investigation of multiple stressors is paramount to accurately represent the mosaic of stressors found in natural environments. The synergism between different stressors is not always predictable and further work is needed in this field. Resurrection ecology, the hatching of dormant life-stages of some species, provides a powerful method to study species adaptive mechanisms through time to environmental stressors. In this thesis I examine a combination of stressors on *Daphnia magna* resurrected from the Lake Ring system and assess neutral genetic diversity through time in the same sediment core. Preliminary analysis suggests there are no differences in fitness responses between the sub-populations to combinations of stressors. As neutral genetic variation was also stable over time it is possible standing genetic variation, and possibly the buffering effect of resting egg banks, allow this species to adapt and evolve to strong environmental selection pressures. Further work investigating the mechanisms underpinning this adaptive ability will be done using whole genome and transcriptome sequencing. Finally it was also found hatched and unhatched sub-populations of *Daphnia magna*, from the same sediment core, show the same neutral genetic diversity and structure suggesting resting stages can be confidently used to represent dormant populations through time.

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Collaborative Work Statement

Fitness Responses in *Daphnia magna* to Human-Driven Environmental Change. Luisa Orsini conceived the study and experimental design. Maria Cuenca Cambronero and Hollie Marshall conducted the experiments and designed and performed the pilot experiments. The experimental work was supported by Erasmus/ volunteer project students. Hollie Marshall carried out all statistical analyses, production of graphs and written work in this chapter.

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Temporal Genetic Stability in *Daphnia magna* Exposed to Eutrophication. Luisa Orsini conceived the study. Hollie Marshall and Monica Alfonso carried out all lab work. Luisa Orsini performed data analyses with input from Maria Cuenca Cambronero, Hollie Marshall and Monica Alfonso. Hollie Marshall wrote the chapter.

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Abbreviations

AMOVA	Analysis of Molecular Variance
ANOVA	Analysis of Variance
C	Carbon
cm	centimeters
CTmax	Critical Temperature Maximum
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E	East
g	Grams
GFP	Green Florescent Protein
GWAS	Genome Wide Association Study
ha	Hectare
IUCN	International Union for Conservation of Nature
Km	Kilometers
L	Litres
L:D	Light:Dark
m	Meters
mm	Millimeters
mM	Millimolar
Mb	Megabases
mg	Milligrams
ml	Millilitres
mins	Minutes
N	North
nM	Nanomolar
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
rpm	Revolutions per Minute
secs	Seconds
SNP	Single Nucleotide Polymorphism
°C	Degrees Celcius
μg	Microgram
μl	Microlitre
μM	Micromolar

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Chapter 1

Introduction

1.1 Anthropogenic Impact on Natural Ecosystems

Population growth and increase in human activities have caused significant changes to the majority of the earth's natural environment (Rosenzweig *et al.* 2008). Of particular concern for ecosystems and species are global warming and pollution (Hof *et al.* 2011).

Global temperatures are expected to rise between 0.4-7.5°C in the next 100 years (Figure 1.1) (Stocker *et al.* 2013, Purvis *et al.* 2004, IPCC 2014). This sort of increase will inevitably bring a cascade of further changes to the environment, such as season shifts, weather changes (Figure 1.1) (Parmesan and Yohe 2003) and ice melt, paired with a rise in sea levels (Figure 1.2). All of these factors interact; for example the rise in global temperature has been found to increase sea ice melt in the arctic, which in turn has been predicted to play a role in longer and colder winters in Europe (Vihma 2013). Seasonal shifts play a crucial role in ecosystems, modifying phenology (e.g. flowering time in plants) and life history traits (reproduction and growth) and pushing species to the edges of their adaptive landscapes (Willis *et al.* 2008).

Season changes and temperature increases also tie in to disease patterns. A pathogenic fungus, *Batrachochytrium dendrobatidis*, has been labelled the cause of mass extinctions of amphibian species in South American, Pounds *et al.* (2006) believe the prevalence of this fungus has been caused by an increase in air temperatures, associated with global warming,

meaning more regions are now closer to it's growth optimum. While these species have been dramatically affected by these changes others are less so. Usov *et al.* (2013) documented a 20-day increase in the hydrological summer period in the White Sea over the past 50 years, however they found that the zooplankton community remained constant with just one species altering its life history to start reproduction slightly earlier.

These downstream effects of climate change are ultimately the results of anthropogenic factors such as greenhouse gases (Mitchell *et al.* 1995). Increasing anthropogenic pressures also come in the form of habitat loss; through urbanization and deforestation, species relocation, such as the induction of invasive species and pollution in agricultural and industrial forms (Rosenweig *et al.* 2008). For example Jetz *et al.* (2006) predict around 400 bird species may lose greater than 50% of their habitats by 2050 due to human land-use change. In terms of invasive species introductions, Clavero and Garcia-Berthou (2005) highlight that of the 170 extinct species with known causes, on the IUCN red list, 54'% included the effects of invasive species.

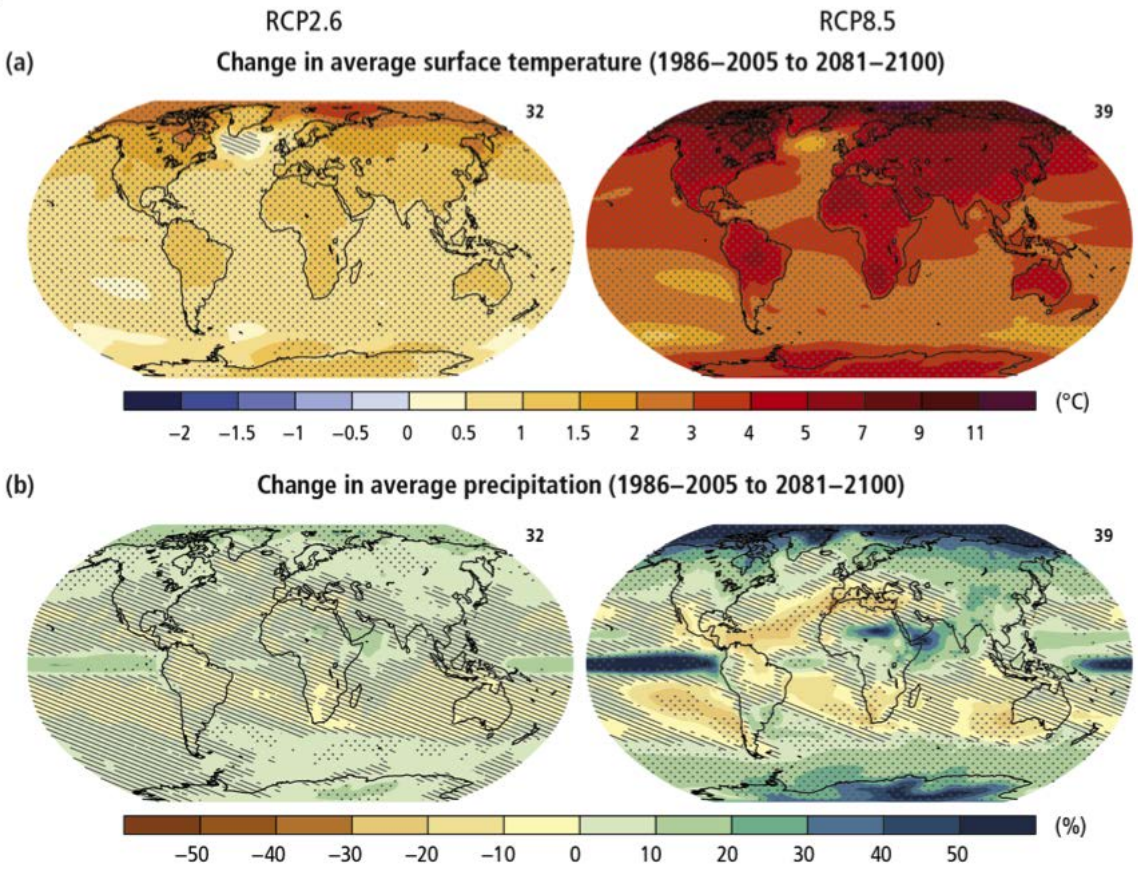

Figure 1.1: (a) Global changes in surface temperature between 1986-2005 and also the predicted change in temperature between 2081-2100. (b) Average precipitation between 1986-2005 and also the predicted precipitation between 2081-2100 [Sourced from IPCC 2014].

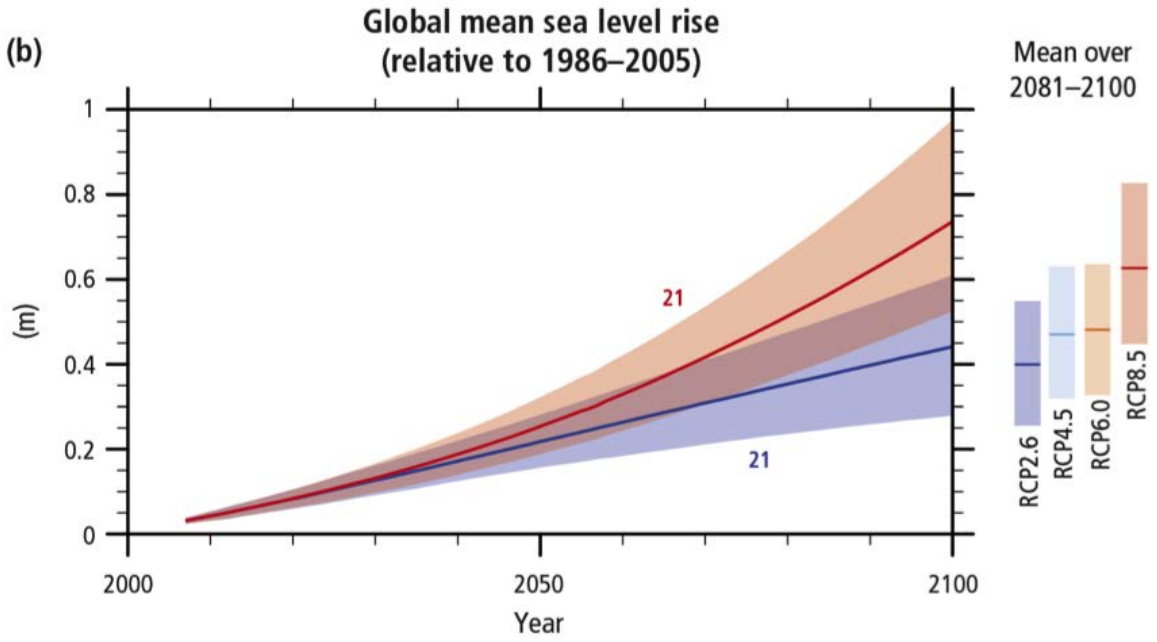

Figure 1.2: Actual and predicted increase in sea levels globally. [Sourced from IPCC 2014].

Climate change affects not just the species within an ecosystem but also the dynamics of the system as a whole (Figure 1.3). For example in a review by Grimm *et al.* (2013) they state a loss of productivity in dry forests is caused by occurrences such as wildfires and an increase in productivity is found in wetter forests due to the increase in air temperature. Marine and lake productivity are also affected by ice melt, warming and nutrient inflow (Grimm *et al.* 2013).

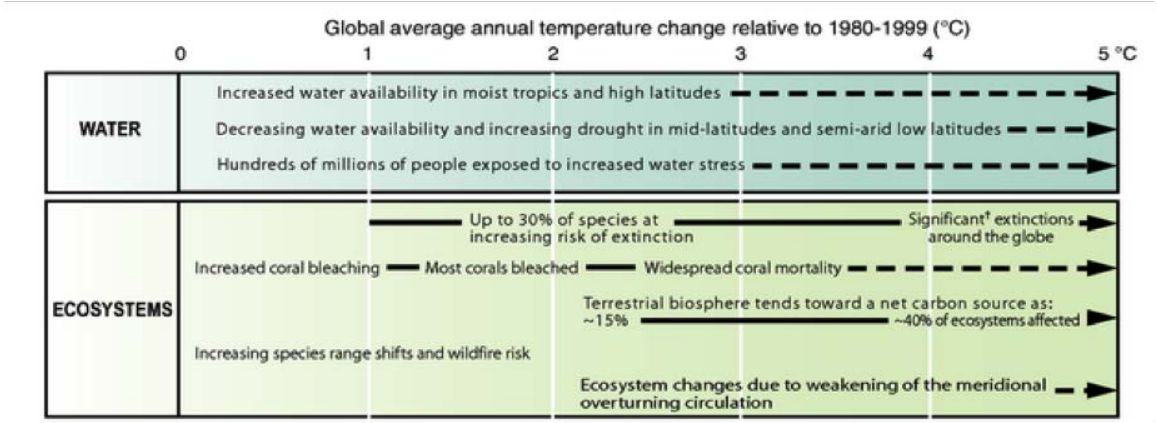

Figure 1.3: A summary of the likely effects of temperature increase on water (as a resource) and general ecosystems. † refers to over 40% [Sourced from IPCC 2014].

While temperature increases have been greatly studied as an independent stressor for both species and ecosystems (Anderson *et al.* 2012, Aubret and Shine 2009, Henning-Lucass *et al.* 2015, Sinervo *et al.* 2010), changes in land-use caused by human activity add further stressors to any given environment. The use of pesticides and fertilizers associated with agriculture are also known to be a major source of pollution and eutrophication respectively, specifically freshwater ecosystems are more affected by these agrochemicals (Gopal 2005).

The effects of agrochemical stressors have also been studied independently; a review by Relyea and Hoverman (2006) detailed the effect of pesticides on non-target species to include reduced survival, behavioural changes, morphological changes and changes in life history. More recently the European Union banned the use of neonicotinoids as pesticides due to the negative effects they have on wild bee populations (Gross *et al.* 2013). Eutrophication alone can also have a negative effect on freshwater species; Sandstrom and Karas (2002) report reduced grown in some fish species following a higher eutrophic gradient, it was thought this was caused by the lack of visibility in the water, decreasing hunting success.

In order understand how species cope with changing environments it is important to assess the combined effect of multiple stressors on their fitness. The importance of multiple stressors has been known for some time, for example cold tolerance and dessication have been greatly studied in *Drosophila* and found to be closely linked in terms of adaptation (Sinclair *et al.* 2007). However the synergistic effect of environmental stressors, related to climate change, has only recently been investigated. For example O'Neil *et al.* (2012) found an increase in temperature causes an increase in the occurrence of cyanobacteria and algal blooms leading to eutrophication. In another study, Lau *et al.* (2015) found that a higher temperature caused lower concentrations of pesticides to be more lethal to frog species than the same concentrations at lower temperatures. An increase in the susceptibility to pesticides with increasing temperature was also found in coho salmon (Laetz *et al.* 2014) and black tiger shrimp (Tu *et al.* 2012). It is important we understand how species deal with these combined stressors to enable accurate predictions of species survival and ecosystem management.

1.2 Freshwater Ecosystems

While anthropogenic activities affect all types of ecosystems, freshwater ecosystems are of particular concern because of their role in supporting high biodiversity and as a human resource (Figure 1.2). Human activities have been shown to produce some profound effects on water ecosystems, such as: a decrease in habitat for cool-water species, a reduction in water quality, and the migration of invasive species into new habitats (Mulholland *et al.* 1997). It is therefore important these effects are closely monitored and understood to not only maintain biodiversity but to also preserve water as a valuable resource. There is legislation in place in Europe called REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) that ensures regulatory measures are in place to help preserve water ecosystems by thoroughly testing and understanding new chemicals and their effects (www.hse.gov.uk/reach).

As well as REACH, the water framework directive (WFD) was imposed in Europe in 2000 to improve the quality of inland water; it highlights pollution and agricultural run-off as important problems affecting water quality and therefore ecosystems. Gopal (2005) also believes pollution and eutrophication caused by agricultural run-off are the next biggest threat to water ecosystems after destruction for changes in land-use. Species within these ecosystems can also be directly affected; Kiesecker (2002) documents pesticide exposure on amphibians, finding it increases their susceptibility to pathogens, which then cause limb deformities. O'Neil *et al.* (2012) also conclude harmful cyanobacteria algal blooms are increasing due to eutrophication and the increasing water temperatures associated with agricultural run-off and climate change respectively.

These effects at the species level can translate into whole ecosystem changes via the food chain. Marrugo-Negrete *et al.* (2008) found mercury introduced into a freshwater system was propagated through the food chain, affecting aquatic vertebrates and was finally transmitted into human populations. Although there is increasing evidence of anthropogenic factors affecting ecosystems, there are some cases where organisms cope well with the change; Bouetard *et al.* (2014) studied the effects of prolonged pesticide exposure on wild freshwater snails

and found little effect, however they do state other studies have shown key relationships in ecotoxicoloical models (Varian-Ramos *et al.* 2013, Belanger-Deschenes *et al.* 2013)and point out the importance of studying evolution in relation to anthropogenic impact and climate change.

Already knowing the sort of effects climate change and associated anthropogenic factors have on ecosystems, especially vital water systems, it is important we understand how species respond to cope with these pressures.

1.3 Mechanisms of Response to Environmental Stressors

When environments change previously adapted organisms face a new suite of stressors, this can cause extinction if they cannot migrate to a more favourable environment or adapt to cope with the stresses of the current one. Modern climate change has been predicted to cause mass species extinctions; some models even predict a 'sixth mass extinction' (Thomas *et al.* 2004, Bellard *et al.* 2012). This is highly contented however with very little evidence available for actual extinctions caused by temperature increases (Harte *et al.* 2004). The IUCN (2015) suggests only 24 out of 832 species extinctions have causes related directly to climate change (i.e. habitat shifts, temperature extremes, droughts). Human mediated land-use change however has been described as a major driving force behind many species extinctions (Jantz *et al.* 2015). Extinctions caused by land-use change have been predicted to massively increase when climate change predictions are also taken into account; a loss of around 220 species is predicted under the cooler temperature estimations and up to 21,000 under the highest temperature estimations (Jantz *et al.* 2015). To help prevent such species losses it is important the mechanisms species use to cope with environmental change are understood.

1.3.1 Migration

One of the ways organisms deal with the effects of climate change and anthropogenic stressors is to migrate into a more favourable environment. In a review by Walther *et al.* (2002) they detail a change in species distribution, towards the poles, in a huge variety of species including; plants, zooplankton, butterflies, birds and mammals. A similar meta-analysis found that of 434 species with range shifts, 80% were in line with predicted climate change patterns (Parmesan and Yohe 2003). Specifically butterflies seem to be able to keep up with the changing climate by altering their range (Parmesan *et al.* 1999).

These migrations can either be beneficial to the species and ecosystems or detrimental. Migratory populations may bring in new genes, increasing genetic diversity which gives a higher ability to cope with environmental changes. However they may also cause outbreeding depression, where the less well adapted migrants breed with the well adapted native population (Carlson *et al.* 2014). A review by Frankman (2005) found outbreeding depression alone tends not to cause species extinction; usually the original stressor driving outbreeding to occur is the cause. Model prediction by Aitken and Whitlock (2013) suggest that if only minor problems are caused by outbreeding, within the population, then after a few generations these may become eradicated.

At the ecosystem level these migratory species may outcompete native species or even hybridize with similar species causing a loss in diversity; Ayres *et al.* (2004) predict the extinction of California cordgrass in San Francisco due to the introduction of smooth cordgrass, which led to a hybrid able to outcompete the native species. Also the Argentine ant is inhibited from dominating food resources in its native range by a specific parasite (keeping the ecosystem in balance), however upon migration into new ranges, where this parasite does not exist, it monopolizes resources greatly outcompeting other species leading to a change in ecosystem dynamics (Orr and Seike 1998, Holway 1999).

While migration offers one solution to new environmental stressors, some organisms, such as reef-corals, have limited migratory abilities (Hoegh-Guldberg 1999). Also freshwater species such as fish or some freshwater invertebrates, such as Daphnia, are unable to migrate from

closed systems like ponds or lakes, meaning they will need to find other mechanisms to deal with new environmental stressors.

1.3.2 Tolerance

Tolerance to an environmental stressor is described as differences in sensitivity between species, life-stages (Hua *et al.* 2013) or populations. Tolerance allows organisms to cope with short-term changes in the environment such as heat-waves or one-off chemical exposures.

One of the most obvious forms of tolerance is thermo-tolerance, which natural organisms develop to cope with changes in temperature. CTmax (temperature of maximum tolerance) is a measure of an organism's ability to cope with high temperatures. It is defined as the temperature at which an organism loses it's motor function (Geerts *et al.* 2015). Hoffman *et al.* (2003) reviewed temperature tolerance in *Drosophila*, finding, via CTmax experiments, tolerance (in this case the ability of the flies to 'right' themselves) to extreme temperatures is usually associated with differential expression of genes coding for heat shock proteins, they also state while variation is seen in laboratory animals, it's important to explore these findings in natural populations.

CTmax has also recently been studied in natural populations of invertebrates, such as Bumblebees and *Daphnia* (Oyen *et al.* 2016, Geerts *et al.* 2015). Geerts *et al.* (2015) found *Daphnia* pre-acclimated to higher temperatures were able to tolerate higher thermal limits in a CTmax experiment than their lower temperature acclimated counter-parts.

It is important to be aware of thermal limits in natural populations to allow for better species management, avoiding species extinctions. It has been found in both lizard species and crab species that populations seemingly adapted to living in hotter areas are actually more prone to local extinction, due to already living so close to their thermal limits their ability to further acclimate is reduced (Sinervo *et al.* 2011, Somero 2010).

Species can also develop tolerances to various pesticides through exposure: Jones and Relyea (2015) managed to induce pesticide tolerance in Grey treefrog tadpoles, by exposing them to

sub-lethal concentrations, however the effects did not last into adulthood. An earlier study, found tolerance to a particular pesticide could also induce tolerance to other pesticides, even some with different modes of action (Hua *et al.* 2013). Tolerance to environmental changes can be costly in terms of fitness (Semlitsch *et al.* 2000) therefore other mechanisms of tolerance can be beneficial.

Indirect tolerance to environmental stressors is achieved by avoiding the stressor completely. Diapause (developmental arrest) is known widely throughout insect species as a mechanism to cope with cold winter environments (Saunders 2013). Crustaceans like *Daphnia* also produce 'resting eggs' in times of stress, these sexual eggs can lay dormant for 100's of years (Frisch *et al.* 2014) and will hatch only when conditions become favourable, i.e. avoiding environmental stress. Diapause can also be seen in species of killifish, where eggs are deposited into the sediment before the dry season and remain viable until water is once again present (Murphy *et al.* 1997). This mechanism allows species to 'tolerate' environmental stressors by ensuring some individuals are maintained in a protective state until conditions improve.

1.3.3 Adaptation: Phenotypic Plasticity and Genetic Adaptation

If the species cannot tolerate or migrate from a stressor then adaptation must occur in order for the species to survive. One mechanism of adaptation is plasticity, induced by changes in the environment sensed by the organism. Plasticity allows organisms to cope with stochastic or sudden changes in the environment and allows them to 'buy time' until genetic adaptation kicks in allowing long-term adaptation. Plastic responses can occur as changes in phenotype or as modulation of gene regulation. In both cases, changes are reversible and not accompanied by changes in the genotype (Merila and Hendry 2013). Evidence suggests that plasticity is widespread in natural populations (Merila and Hendry 2014, Hoffman and Srgo 2011). However it comes with costs, for example *Daphnia pulex* develop neck teeth as a predator induced defence, while this decreases their chances of predation it also increases the time it takes for them to reach maturity and become reproductively active (Hamill *et al.* 2008, Riessen 1999).

Plastic responses to temperature changes are renown; examples of phenotypic plasticity observed include birds, plants and fish which respond to season shifts and warming temperatures by adjusting their phenology; breeding, flowering and spawning to earlier in the season (Anderson *et al.* 2012, Charmantier *et al.* 2008, Crozier *et al.* 2008). Specifically it has been shown that birds that do not show this phenological response to climate change are declining (Moller *et al.* 2008). Plasticity can also take the form of behavioural change, for example Aubret and Shine (2010) found snakes adjusted their behaviour to reside in the most optimal temperature moving away from cooler and hotter areas.

Plastic responses to other anthropogenic stressors, such as pesticides, have also been documented; Relyea and Hoverman (2006) state changes in behaviour, physiology, life history and morphology can be seen in a number of non-target species. For example stream mayflies suffer higher predation rates by fish when they have been exposed to insecticides, this is because they exhibit a change in behaviour causing them to spend more time on rocks; increasing vulnerability (Schulz and Dabrowski 2001). Pesticides have also been found to affect the physiology of non-target species; wood frogs exposed to pesticides had lower levels of immune cells increasing their parasites loads (Kiesecker 2002).

Regulation of gene expression is a mechanism by which plastic changes, such as changes in morphology and physiology, occur. Individual genes can be up- or down- regulated in response to environmental clues, meaning organisms can alter entire genetic pathways in order to adapt to environmental demands (Yampolsky *et al.* 2014). Changes in gene expression have been observed in natural *Daphnia* populations to look at transcriptomic responses in both heat-sensitive and heat-tolerant individuals, with application to survival in increasing temperature conditions (Yampolsky *et al.* 2014). Rainbowfish have also been found to exhibit plastic responses to different temperatures by altering the gene expression of temperature-tolerance metabolic pathways (Smith *et al.* 2013).

While plasticity offers a 'quick fix' to environmental change it is not always able to stretch far enough to ensure long-term adaptation. Genetic adaptation can occur via structural rearrangements of the genetic material (e.g. copy number variation, insertions, deletions, inversions or duplications) or point mutations (single nucleotide polymorphisms: SNPs) (Baker

2012). It can spur from either standing genetic variation or from new mutations. Adaptation from standing genetic variation is a faster mechanism of response as the gene variants are already present in the genetic pool whereas new mutations causing protein-coding changes require longer evolutionary time (Barrett and Schluter 2008).

Little evidence exists for genetic adaptation to anthropogenic changes, however one example is reported by Karell *et al.* (2011), Tawny Owls (*Strix aluco*) were found to react to shorter winters by selecting for the darker coloured plumage in favour of the lighter coloured individuals. There is also some evidence for genetic adaptation to pesticide exposure; Orsini *et al.* (2012) found genes linked to adaptation to high intensity land use in a natural population of *Daphnia*, a genome scan (using neutral markers) was carried out to identify loci under selection, testing natural populations separated by space and time (dormant life stages were used). The limited number of studies looking at genetic adaptation to anthropogenic effects may be associated with the lack multi-generational data, which is necessary to observe genetic adaptation in action. Conversely many studies report plasticity as the most recurrent mechanism of adaptation to environmental change (Bellard *et al.* 2012, Carlson *et al.* 2014, Gienapp *et al.* 2008, Hoffmann and Sgro 2011).

In order to understand the way organisms cope with changes in the environment, it is paramount to disentangle the role that plasticity and genetic adaptation play in species adaptive responses.

1.4 Assessing Species Response to Stressors

A variety of methods are available to assess exactly how species are coping and adapting to environmental stressors. The availability and decreasing costs of genomic tools mean more studies are now able to gather larger data sets and share collaborative research efforts (Colbourne *et al.* 2011). This means combining multiple approaches to study the same question in depth is now feasible.

1.4.1 Experimental Evolution

One such approach is experimental evolution, this is where the environment is artificially manipulated to induce stress responses in various populations, allowing adaptive mechanisms to be quantified (Jansen *et al.* 2015). This approach requires a study species easy to manipulate in order to accommodate the number of individuals required for statistical analysis. It is argued that experimental evolution trials are not representative of the natural environment and therefore any results cannot be directly applied to natural populations. The use of mesocosms for some species avoids part of this bias (De Meester *et al.* 2011). Mesocosms are semi-natural experimental systems which aim to provide a stepping stone between fully controlled lab experiments and uncontrolled field studies. They have been used extensively to study adaptation in species such as fish, snails and *Daphnia* (Bassar *et al.* 2010, Bouetard *et al.* 2014, Geerts *et al.* 2015). Data from this approach could be purely phenotypic (such as life-history traits) or genomic/transcriptomic information could be collected from individuals during the experiment. Using this approach to study potential adaptive mechanisms however is limited by time. Long generation time of species and pressure for output from funders limits these studies to select organisms. Some long term studies (over many years) on species with a short generation time however do exist (Geerts *et al.* 2015) and provide a powerful tool for assessing adaptive potential.

Alongside mesocosms, another common experimental evolution approach is to 'transplant' species from different environments into a common environment and assess their fitness responses; this method is known as a 'common garden experiment' (Hoffman and Sgro 2011). These types of experiments allow the roles of plasticity and genetic adaptation to be identified; if plasticity plays an important role for adaptation to a given stressor then fitness would be less affected when exposed in all populations. However if genetic adaptation is important then one population may fair better than another. As with the above approach this method is limited to easily manipulable species such as plants or invertebrates (Hoffman and Sgro 2011), however generation time is unimportant as the space-for-time approach is usually taken (Dihn Van *et al.* 2013).

1.4.2 Space-for-Time

The 'space-for-time' method to study species adaptation overcomes the issues of long-term temporal studies. Populations of the same species geographically spread can serve to indicate how adaptation may occur over time to a specific stressor. For example adaptation to temperature has been studied in many species over a latitudinal gradient such as Damselfly (Dinh Van *et al.* 2014), *Daphnia* (Mitchell and Lampert 2000) and *Drosophila* (Hoffmann *et al.* 2003). Different populations can also be examined for adaptations to other stressors such as predation, parasitism and land-use (Orsini *et al.* 2012). However these studies can often lack power as many other naturally occurring variables cannot be taken into account; a problem overcome by experimental evolution approaches. It is also noted that the use of only a small number of populations is common (Hoffmann *et al.* 2003) meaning results are difficult to generalise to the species as a whole.

1.4.3 Resurrection Ecology

Whilst substituting space for time in adaptive research has some benefits, observing evolution in a temporal context is much more informative. Resurrection ecology is the study of hatched individuals from dormant life stages, such as eggs or seeds, from dated lake sediment cores (Kerfoot *et al.* 1999). This technique has been widely used to study phenotypic responses to environmental stressors (Decaestecker *et al.* 2007, Frisch *et al.* 2014, Oexle *et al.* 2016). It also allows for a comparison between populations from the same historic time period from different geographic locations, meaning it's possible to test the consistency of adaptive mechanisms to similar stressors (Angeler 2007).

Resurrection ecology isn't without flaws; the sediment containing the samples needs to be accurately dated, the history of the study site must be well known, and as not many species produce resting stages, the number of species for study is limited (Angeler 2007). Finally the older the sediment the more difficult it becomes to resurrect living individuals, for example; the oldest resurrected *Daphnia* was dated to come from around 700 years ago (Frisch *et al.* 2014) however resurrecting individuals this old is not common. It is known deleterious

mutations can accumulate in dormant seed banks (Chwedorzewska *et al.* 2006) however it's currently unknown if this process also occurs in *Daphnia* ephippia banks (Morton *et al.* 2015). This could explain why it is more difficult to resurrect older individuals.

Even if it isn't possible to resurrect some individuals/species to assess phenotypic differences, their dormant stages can still be used to assess genetic composition through time. Only a few studies however have examined genetic diversity through time and these used only a small set of genetic markers (Cousyn *et al.* 2001). However Orsini *et al.* (2013) believe evolutionary responses to environmental stressors can be well studied using these dormant stages, particularly with the development of new genetic tools. There is still some question as to whether the genetic diversity seen from dormant stages retrieved from these sediment cores actually represents the population from that time period, especially after strong selection pressures from an environmental change (Jankowski and Straile 2003).

1.4.4 Genetic Diversity as a Tool

In order to begin to understand how species are adapting to stressors researchers are identifying candidate genes under selection by using combinations of the above methods. For example Orsini *et al.* (2012) combine all three methods to draw strong conclusions on the adaptation of *Daphnia* to a suite of environmental stressors via a genome scan approach.

A genome scan works by identifying loci that deviate from neutral genetic parameters indicating they may be under selection or linked to genes being selected for; linked neutral loci are known as hitchhiking loci (Orsini *et al.* 2012). The analysis of neutral loci alone can also answer questions on the adaptive potential of a population. The amplification of neutral microsatellites (tandem repeats within the genome that differ in length) is common in population genetic analysis. This is because they're relatively common throughout genomes and as they don't contain coding regions, under selection, mutation rates can be high (D'Esposito *et al.* 2012).

These neutral loci can be used to assess parameters such as; allelic richness, effective population size, genetic structure, genetic variation and Hardy-Weinberg equilibrium. Allelic

richness is a measure of the number of alleles within a population, a higher allelic richness is linked with the ability of a species to adapt (Greenbaum *et al.* 2013). This is also the case with the effective population size, which has been shown to be linked to species extinction when low (Frankham 2005), it is the quantification of the number of breeding individuals within a population. Genetic structure is another method to assess population genetic variation, it is a measure of the number of 'sub-populations' within one population, defined by different frequencies of alleles present (Chakraborty 1993). A population with little structure indicates high gene flow and the ability to predict the genotype of the population from few individuals. Finally Hardy-Weinberg equilibrium assumes alleles within a population will remain constant when there is no; mutation, migration and selection, among other parameters. Deviation from Hardy-Weinberg equilibrium indicates some form of pressure on the alleles studied, such a strong selection (Vasemagi *et al.* 2005).

Measuring these parameters allows comparisons between and within populations meaning researchers can identify; populations for conservation (Coyer *et al.* 2010), populations experiencing selective pressures (Orsini *et al.* 2013) and populations nearing extinction (Frankham 2005).

1.5 *Daphnia* as a Model System

Daphnia is an example of a species well studied using the above techniques (Jansen *et al.* 2017). *Daphnia* are cladocerans (*Crustacea, Branchiopoda*) that can inhabit a variety of freshwater ecosystems from large lakes and shallow ponds to temporary seasonal pools across all continents including Europe, Asia and the Americas. They are classed as a keystone species due to their central position within the food chain of freshwater ecosystems being both a phytoplankton grazer (of unicellular algae and cyanobacteria) and a prey item for insects and fish (Van Doorslaer *et al.* 2009, Weetman and Atkinson 2004).

Daphnia reproduce by both asexual and sexual reproduction depending upon the environmental conditions (Figure 1.4). The asexual cycle (parthenogenesis) produces clones genetically identical to the mother when conditions are favourable (Miner *et al.* 2012). Sexual

reproduction occurs when environmental conditions start to become harsh (food depletion, drought, short photoperiod) mediated by the generation of males by parthenogenetic mothers and leading to the generation of embryos stored in resistant capsules (ephippia) (Miner *et al.* 2012). The ephippium protects the embryos from the environment until environmental conditions become favourable again allowing them to hatch (Pietrzak and Slusarczyk 2006). Populations have been known to survive dormant for decades, or even centuries when eggs have failed to hatch (Frisch *et al.* 2014), this shows the power of this strategy as a mechanism to survive environmental change. Species that produce dormant stages can provide a powerful resource for studying adaptive evolution through time in single populations from natural ecosystems (Frisch *et al.* 2014, Orsini *et al.* 2013). See resurrection ecology above.

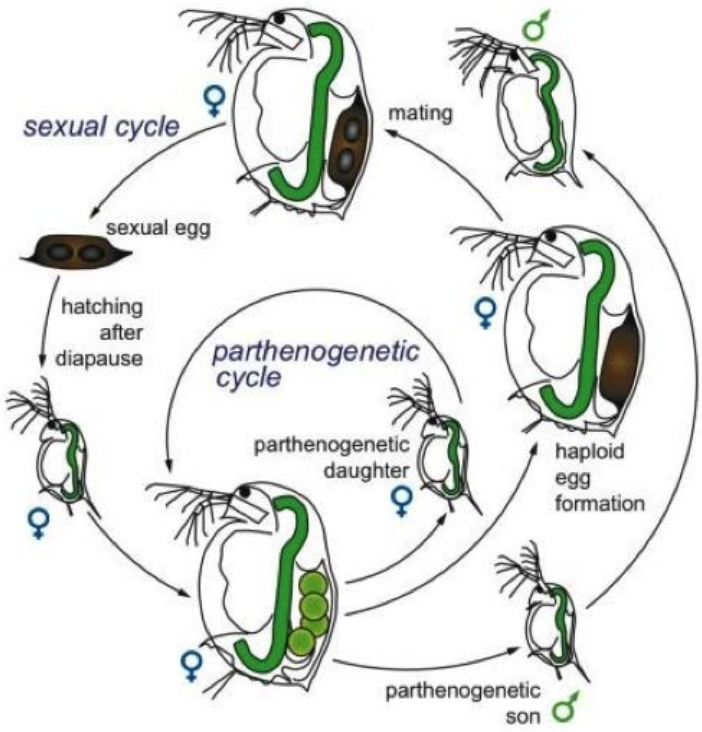

Figure 1.4: Daphnia lifecycle showing both sexual and asexual reproduction. Sourced from: $http://bioweb.uwlax.edu/bio203/2010/carroll_chri/reproduction.htm$.

Clonal propagation of individuals from the environment also means it's possible to use the same genotype in parallel experiments while investigating genetically diverse populations. A short generation time and high manipulability, means that large populations can be kept

in either lab or semi-natural conditions (mesocosms) with ease. In addition to this, there is a suite of genomic tools now available; the Daphnia pulex genome has been published (Colbourne *et al.* 2011) and there is a draft genome (NCBI 2016) and reference transcriptome (Orsini *et al.* 2016) in place for *Daphnia magna*.

The value of the above aspects and *Daphnia's* status as a key stone species mean it is starting to acquire model species status, specifically within the fields of ecotoxicology, ecology and evolution (Nakanishi *et al.* 2014). Decaestecker *et al.* (2007) resurrected individuals from a lake sediment core and cultured clonal lines within the lab to test for 'Red Queen' dynamics with *Daphnia's* parasite *Pasteuria ramosa*. They actually proved a co-evolutionary arms race with the parasite, something that would not have been possible without the ability to study many past generations at the same time (Decaestecker *et al.* 2007). Other fields are also benefitting from the unique opportunities this organism presents. Harris e*t al.* (2012) describe *Daphnia* as an important species for epigenetic studies; with epigenetics being the determining factor in gender differences and reproduction mode. The huge benefits of *Daphnia* as a study species are still being added to with Kato *et al.* (2010) successfully incorporating foreign DNA into the *Daphnia magna* genome using GFP (green fluorescent protein) and Nakanishi *et al.* (2014) successfully introducing heritable mutations using CRISPR technology.

1.6 Objectives and Study Site

A combination of experimental evolution and resurrection ecology will be used to assess adaptation to various anthropogenic stressors in a naturally occurring population of *Daphnia magna*. Neutral genetic diversity parameters will also be assessed in both resurrected and non-hatched individuals to determine if a hatching bias is present. The result of which would affect the interpretation of the main adaptation experiments.

1.6.1 Objectives and Hypotheses

Chapter 2 aims to assess patterns of adaptation to human-driven environmental change in populations of *Daphnia magna* using experimental evolution and resurrection ecology. Individuals from one population will be sampled through time (from a population that underwent a known agricultural chemical exposure) and their fitness responses will be assessed in a series of experimental trials to stressors mimicking those that would occur in nature. In particular global warming will be paired with pesticide exposure and then poor food quality to assess responses to a combination of multiple-stressors. Global warming (increased temperature) was chosen as a stressor due to the effects being seen in ecosystems and species across the world (see Anthrpogenic Impacts on Natural Ecosystems). Pesticide and poor food quality as stressors were chosen as one sub-population of the resurrected *Daphnia* have previous experience of these stressors allowing us to see if past exposure influences future responses (see below, Study Site and Samples).

Research Objectives for Chapter 2:

- Identify the adaptive capabilities of *Daphnia magna* to future climate change scenarios
- Understand if past exposure to a stressor provides benefit or handicap when re-exposed

Hypotheses for Chapter 2:

- Combinations of stressors will be more detrimental to *Daphnia* fitness than individual stressors
- *Daphnia* from older sub-populations will show reduced fitness when exposed to a higher temperature than those from more recent sub-populations
- *Daphnia* from sub-populations that have previously experienced an environmental stressor will cope better than those for which the stressor is novel

Chapter 3 aims to address the outstanding question of whether or not bias is introduced into resurrection ecology studies based on selective egg hatching. Neutral genetic diversity will be assessed in both hatched and unhatched individuals from the same sediment core, sampling over a 50 year period. This is particularly crucial to chapter 2 as it utilises a resurrection

ecology approach. If bias in indeed found then any results obtained from chapter 2 could be influenced by hatching/sampling bias. The samples used in chapter 3 come from the same core used in chapter 2 meaning the *Daphnia* also underwent a strong selective pressure (eutrophication) so the effect of this pressure on neutral genetic diversity can also be assessed.

Research Objectives for Chapter 3:

- Compare various neutral genetic diversity parameters between hatched and unhatched individuals from the same sediment core spanning 50 years
- Identify the effect eutrophication has on neutral genetic diversity in *Daphnia*

Hypotheses for Chapter 3:

- Neutral genetic diversity between hatched and unhatched *Daphnia* will not differ significantly
- Eutrophication will reduce the neutral genetic diversity seen in *Daphnia* during the exposure period and population genetic structure will be different before and after exposure

1.6.2 Study Site

All *Daphnia* genotypes used throughout this thesis have been isolated from a sediment core taken from Lake Ring in Denmark. This is a small (22.5ha) and shallow (maximum depth 5m) lake located in Mid Jutland, Denmark (55°57'51.83" N, 9°35'46.87" E) (Berg *et al.* 1994), (Figure 1.5).

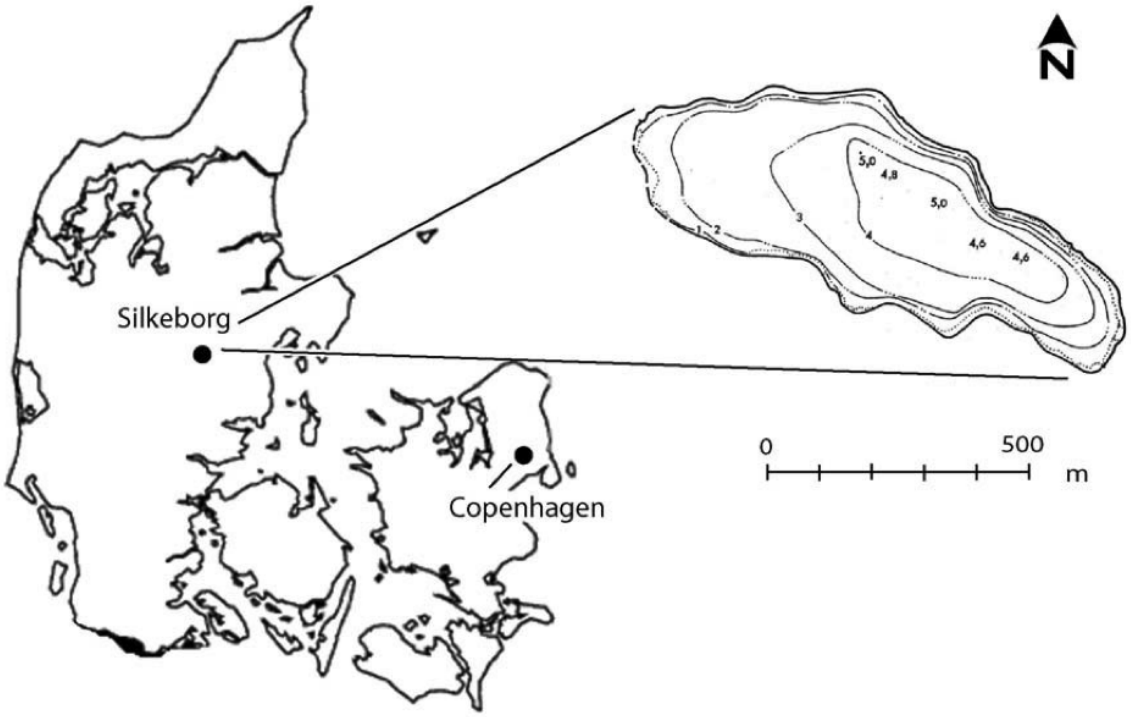

Figure 1.5: Map showing the location of Lake Ring within Denmark and a depth profile in meters, more specifically it forms part of the Gudena river. Image adapted from Berg *et al.* (1994).

Historical records exist detailing past anthropogenic effects on the lake (Berg *et al.* 1994). The recent history of the lake can be broken down into three distinct phases for study (Figure 1.6): the pond was pristine until the late 1950's (pristine phase), between 1960-1970 sewage in-flow from a near by town and agricultural run-off caused severe eutrophication in the lake (eutrophication phase), this was determined by increased levels of organic matter, estimated by loss of ignition analysis (Heiri 2001). During this time period agricultural land-use also intensified which corresponds with the increased use of carbamate pesticides such as carbaryl (Berg *et al.* 1994). The sewage in flow was then diverted from the lake in the late 1970s allowing the lake to recover from the severe eutrophication (recovery phase).

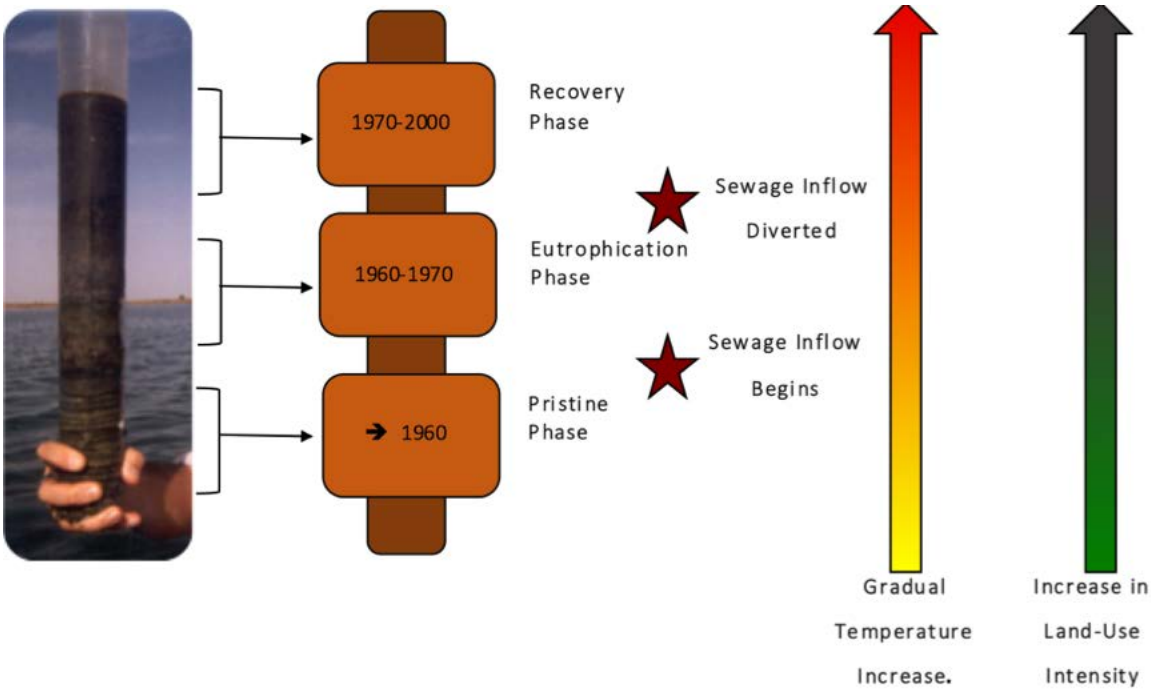

Figure 1.6: Overview showing how the three study phases relate to the sediment core taken from Lake Ring. The section of core closest to the surface is the most recent. Samples for the recovery sub-population were dated between 1970-2000. Samples from the eutrohpication phase were dated between 1960-1970 and samples from the pristine phase were between 1955-1960. The sewage inflow event occurred during the eutrophication phase. The more recent sub-populations were also subjected to increased land-use and a gradual temperature increase of around 1°C compared to the older sub-populations.

Water temperature records for Lake Ring are unavailable however air temperature profiles from Samso meteorological station (81km from Lake Ring) have been obtained. A near linear relationship between air temperature and temperature of freshwater lakes and streams has previously been reported (Schindler *et al.* 1990, Crisp and Howson 1982 respectively). However more recent findings suggest the relationship is not that clear and other factors like lake depth, cloud cover and solar radiation play a role (O'Reilly *et al.* 2015). O'Reilly *et al.* (2015) did however find a positive correlation between air temperature and lake temperature from lakes around the world. The means it is not possible to directly quantify the water temperature increase for Lake Ring over the sampling period. However it would be fair to assume, given the above findings, that extreme heatwaves at least are likely to cause an increase in lake temperature for a short period of time.

There is extreme fluctuation in annual highest air temperature from Samso meteorological station raging from around 27°C to 36°C. The overall average annual temperature also fluctuates between around 6.5°C to 9.5°C, overall there was around a 1°C increase in annual average air temperature between the these dates (Figure 1.7, Figure 1.8).

The recovery phase had an average annual temperature of 8.3°C which is 0.9°C higher than the eutrohpic phase (7.4°C) and 0.8°C higher than the pristine phase (7.5°C). The average highest temperature recorded during the recovery phase is also higher at 32.0°C compared to 30.7°C during the eutrophication and 30.9°C during the pristine, Appendix A. While there isn't a huge increase over the entire time period it is clear the recovery sub-population underwent a different temperature regime to the other two sampled sub-populations.

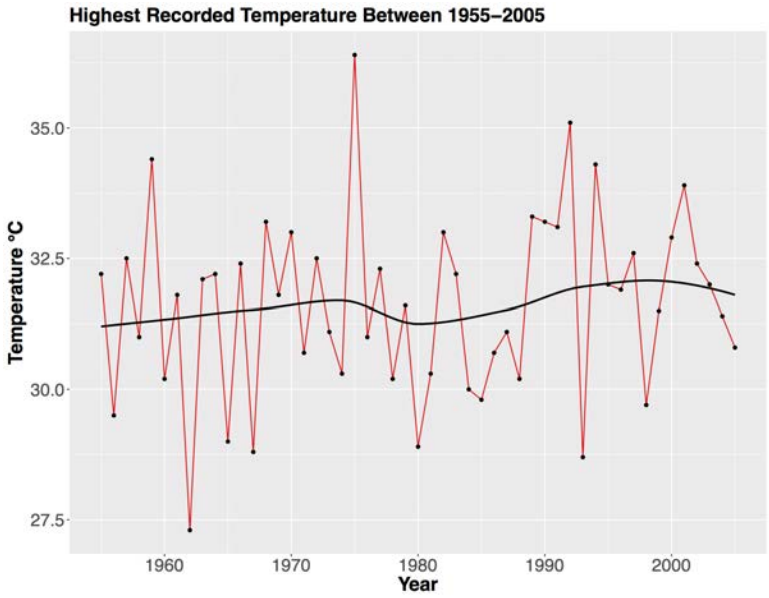

Figure 1.7: Highest recorded temperature per annum in degrees Celsius from Samso meteorological station between 1955-2005.

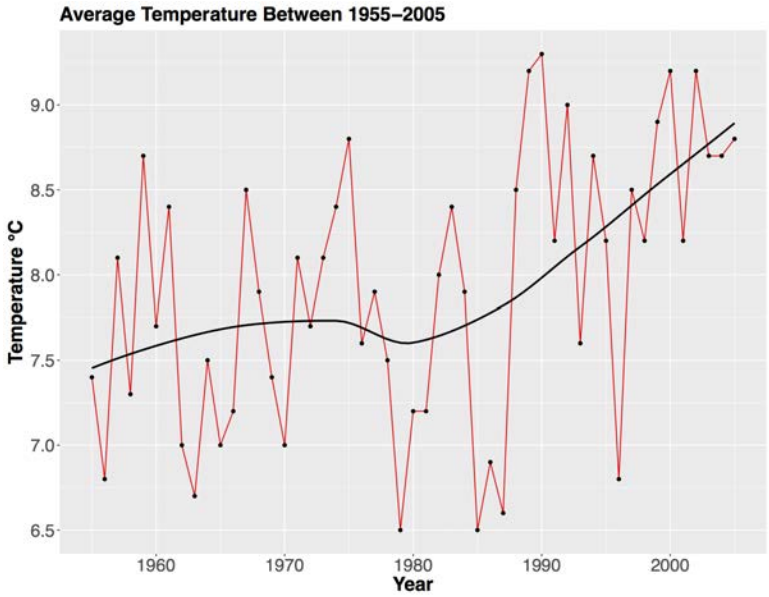

Figure 1.8: Average annual temperature in degrees Celsius from Samso meteorological station between 1955-2005. There is just over a 1°C increase in air temperature between 1955-2005.

1.6.3 Sample Collection

Sediment was collected from Lake Ring in 2004 using a piston corer; the core was then sliced into layers of 0.5cm and stored at 4°C in dark conditions. Sediment layers of the core were dated by radiometric chronology in 2015 by ENISS Ltd (UCL London) following standard protocols (Appelby 2001). *Daphnia* from each of the three lake phases were obtained by isolating ephippia from the sieved sediment core. They were hatched under standard conditions (Marcus 1990, Onbe 1978) by sieving the sediment and retrieving eggs by eye. The ephippia capsules were then opened under a dissecting microscope in a petri disk containing mineral water and left for 24hours at 20°C allowing *Daphnia* to hatch. Cultures of each genotype were established at 10°C by allowing the *Daphnia* to reproduce clonally. As *Daphnia* resting eggs are produced sexually each hatched animal represents a unique genotype. Throughout this study all animals were maintained in jars of mineral water (Table 1.1), fed *ab libitum* Chlorella algae (except for the food quantity experiment), kept under a long day photoperiod, 16:8 L:D, and water was changed three times per week at a rough ratio of 70% fresh water to 30% old water.

Table 1.1: Composition of mineral water used throughout the study.

Compound	**mg/litre**
Calcium	52.81
Magnesium	5.37
Nitrate	3.78
Potassium	2.87
Bicarbonate	162.0
Chloride	23.0
Sulphate	49.9
pH (at source)	7.93

Chapter 2

Fitness Responses in *Daphnia magna* to Human-Driven Environmental Change

Hollie Marshall, Maria Cuenca Cambronero and Luisa Orsini.

2.1 Rationale

The first objective of this thesis was to identify changes in fitness responses caused by human-driven environmental change. To reconstruct these changes experimental evolution trials were conducted mimicking changes that occurred over time in the study site (Lake Ring). From background knowledge of the lake (see Study Site and Samples) it is known that changes in temperature, pesticide loads and a shift from oligotrophic to eutrophic conditions occurred. To measure any shift in life history traits driven by these ecological changes three experiments were conducted: the first experiment examined changes driven by an increase in temperature over the past 50 years and to mimic predicted changes in temperature over the next 100 years, the second experiment tested the effect on fitness of this temperature combined with pesticide loads; the third experiment tested the effect on fitness of temperature combined

with food quality. The three experiments effectively measure adaptive responses to global warming, agricultural run-off and eutrophication, respectively.

2.2 Methods

2.2.1 Experimental Design

To assess fitness responses of natural *Daphnia magna* populations to human-driven eutrophication, key life history traits were quantified (fecundity, mortality, growth and overall population fitness) in common garden experiments. To document adaptive evolution to a past event of severe eutrophication followed by recovery and to document adaptation to hyper-eutrophication under future global warming scenarios, common garden experiments using temperature, oligotrophic *vs* eutrophic conditions and high vs low pesticide concentrations as variables were performed. The choice of these variables was guided by the ecological and paleolimnological analysis of Lake Ring (see Study Site). To be able to disentangle the effect of temperature from food and pesticides treatments we performed three experiments: in the first experiment the effect of temperature alone on fitness was documented, whereas in the other two experiments the effect of temperature combined with either food quality or carbaryl was documented.

2.2.2 Experimental Set-Up

Three experimental temperatures were used: 18°C, 21°C and 24°C. The first temperature reflects an average of the recent past and modern environmental conditions (See Objectives and Study Site), whereas the other two temperatures mimic a stepping stone increase in temperature forecasted for the next 100 years based on the predictions of the Intergovernmental Panel for Climate Change (IPCC 2014). In the second experiment two carbaryl concentrations, 4 μg/L (low) and 10 μg/L (high) were used following pilot exposures (Appendix B). Carbaryl was dissolved in 0.005% ethanol and replaced every second day, the experimental animals were fed *ab libitum* daily with 0.8mgC/L (carbon per litre) of *Chlorella*. In the third experiment two food regimes, 0.2mgC/L and 2.4mgC/L, were used as a low food source and high food source respectively, (see Appendix B for pilot experiments), low food quality reflecting that of eutrophic lake conditions (Richman and Dodson 1983). The carbon

content on the algae was estimated by CH&N analysis carried out by combustion analysis (Medac Ltd). These two concentrations were chosen after pilot experiments on different carbon concentrations (Appendix C).

Prior to the experimental phase, the 38 genotypes (N=14 for the recovery sub-population, N=13 for the eutrophication sub-population and N=11 for the pristine sub-population, from here on refereed to as sub-populations, of which more detail can be found in the Objectives and Study System section, Table 2.1) were grown for at least two generations at 16°C. By incubating all individuals at 16°C, acclimation to a common garden environment, prior to transferring them to the experimental temperatures, was achieved. When the second brood was observed in the pouch, all mothers were individually placed in 50ml jars until release of the neonates. Neonates from the second or following clutches, of the second generation, released over two days, were randomly assigned to each of the three experimental temperatures in which they were acclimated for at least a further two generations before commencing the experiment (Figure 2.1). In the acclimation phase to the specific temperatures the animals were maintained in standard food (0.8mgC/L) and light (16:8, L:D) regimes, and the medium was changed every second day. After the acclimation phase to the experimental temperatures, neonates released from the second or following clutches, of the second generation, over two days, were used to measure changes in life history traits in response to experimental treatments. During the experimental phase the animals were fed daily and the medium was refreshed every other day. In the experiment in which animals were exposed to temperature and carbaryl, freshly diluted carbaryl to a final concentration of 0.005% ethanol was added to the water every second day. Exact fitness traits recorded through out all experiments were; time of first and second brood, size of first and second brood, mortality, size at maturity (Figure 2.2) and number of molts.

Table 2.1: List of genotype names from each of the lake phases. The first number indicates the depth of the sediment core the resting egg from removed from, e.g. 7 would be equivalent to a depth of 7cm. The second number indicates the individual resurrected, e.g. 2 would be the 2nd individual from that layer to hatch. Note there are less genotypes in the pristine phase (oldest phase) as the older the sediment the more degraded it becomes and the more difficult it is to hatch animals from these layers.

Recovery Phase	**Eutrophication Phase**	**Pristine Phase**
0_1	6_2	12_2
0_2	6_3	12_3
0_4	6.5_5	12_4
0.5_3	6.5_7	12.5_1
1_2	7_3	13_1
2_1	7_5	13_2
2_2	7.5_4	13_3
2.5_9	8_7	13.5_1
2.5_11	8.5_3	14_1
3_4	9_6	14.5_1
3_6	9_20	15.5_1
3.5_1	9.5_1	
3.5_2	9.5_3	
3.5_15		

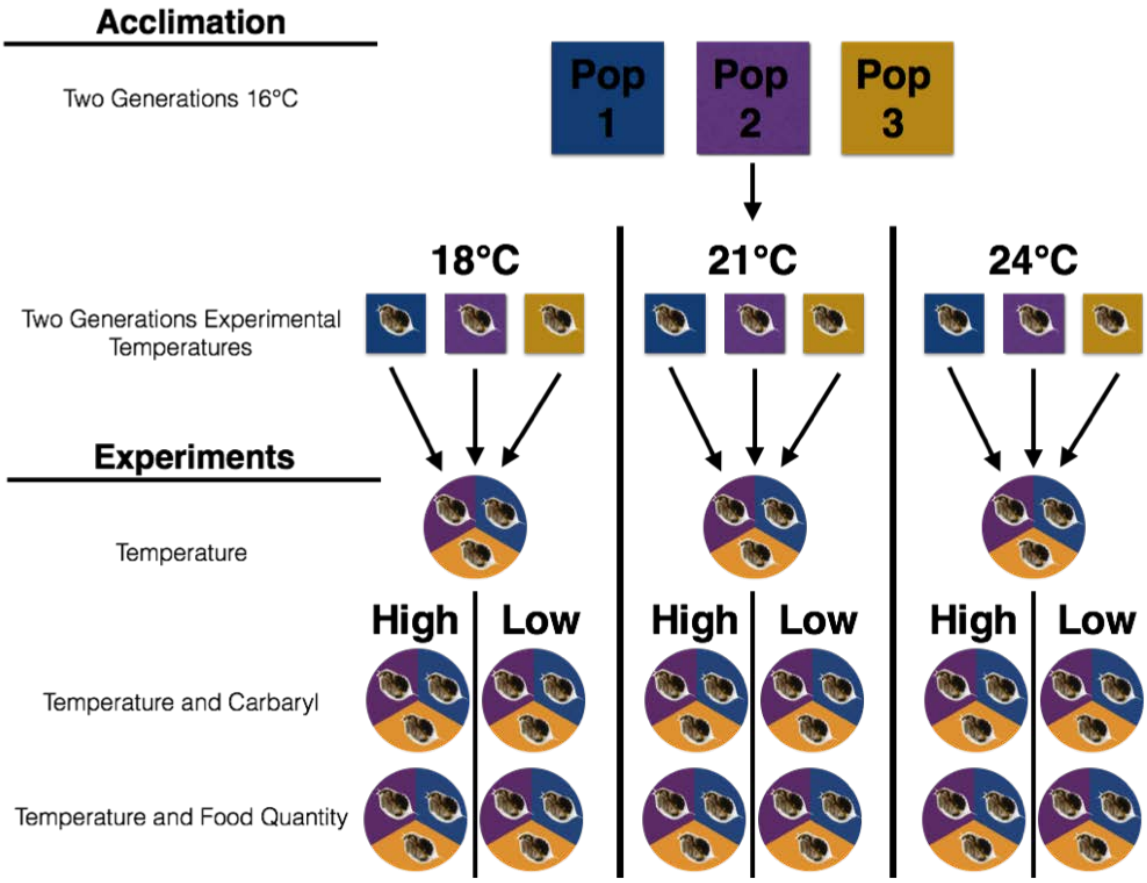

Figure 2.1: Experimental design. A minimum of 10 genotypes of each sub-population are represented by a single *Daphnia* image. Two stages of acclimation at 16°C and then each experimental temperature were carried out before each of the three subsequent experiments.

Figure 2.2: The blue arrowed line indicates where the measurement of length would be taken for each individual to generate the 'size' variable, i.e. directly through the eye from the top of the head to the base of the tail.

2.2.3 CTmax

After the completion of the common garden experiments, the experimental animals were tested for their tolerance to temperature increase via CTmax experiments. This was done after 21 days or after every individual had produced the second brood, which ever came first. This analysis allowed us to test whether animals pre-adapted to higher temperature regimes were more tolerant to rapid increases in environmental temperature, effectively mimicking heat waves. CTmax experiments were performed on a heated thermoblock on which the temperature was gradually increased from the experimental temperature until the animals lost locomotion (CTmax), the loss of locomotion is reversible once the animals are returned to room temperature. The temperature at which each individual 'fainted' (fell to the bottom of the water column and did not move) was recorded using a dictaphone. The observer would verbally call out the position within the thermoblock and the current temperature when each individual was observed to 'faint'. Each animal was then immediately frozen in liquid nitrogen for potential future transcriptome sequencing.

2.2.4 Statistical Analysis

All statistical analyses were carried out using R (version 3.2.3, http://www.R-project.org/) and all graphs were created using the *ggplot2* package also in R. Normality of each data set was determined using histograms and Q-Q normality plots in order to select either parametric or non-parametric tests.

Only three phenotypic traits along with survival were analysed in each experiment for inclusion in this thesis; number of neonates in the second brood (fecundity), size at maturity (growth) and CTmax (temperature tolerance). For the temperature experiment a One-Way ANOVA was used to identify differences in the number of neonates in the second brood between temperature conditions, this was the only normally distributed data obtained. The non-parametric equivalent, Kruskal-Wallis Rank Sum Test, was used to identify differences in CTmax and size at maturity between temperature conditions. Pair-way comparisons were then carried out for any significant differences found using 18°C-21°C, 18°C-24°C and 21°C-24°C (Tukey Comparison of Means for the number of neonates in the second brood and Wilcox Rank Sum for CTmax and size at maturity). The Kruskal-Wallis Rank Sum Test was then used to identify differences between sub-populations for each trait, for this analysis each temperature condition was treated as an individual experiment.

For the carbaryl-combined experiment and food quality-combined experiment all data appeared to be non-normally distributed. Therefore the Kruskal-Wallis Rank Sum Test was used to identify differences between high and low experimental conditions for each trait and for between sub-population differences (each temperature/ carbaryl or food quantity condition was treated as an independent experiment) for each of the three traits. Pair-way comparisons were then done for any significant differences found between sub-populations using Recovery-Eutrophic, Recovery-Pristine and Eutrophic-Pristine (Wilcox Rank Sum).

2.3 Results

The original aim of this chapter was to identify fitness responses to human-driven environmental change in sub-populations of *Daphnia magna* resurrected from a lake system with a known history. Preliminary results are presented in this thesis for only three fitness traits and survival, looking specifically at differences found between experimental conditions for all three sub-populations combined and then looking at differences between each sub-population for each experimental condition. This approach was taken to assess the following hypotheses:

- Combinations of stressors will be more detrimental to *Daphnia* fitness than individual stressors
- *Daphnia* from older sub-populations will show reduced fitness when exposed to a higher temperature than those from more recent sub-populations
- *Daphnia* from sub-populations that have previously experienced an environmental stressor will cope better than those for which the stressor is novel

2.3.1 Temperature Experiment- effect of temperature on the entire population

Whole population analysis was carried out in order to identify the fitness responses to temperature which can later be compared with fitness responses to temperature combined with an additional stressor. When comparing all populations across the three temperatures the 21°C and 24°C conditions appear to produce similar fitness responses compared to the 18°C condition (Figure 2.3). A significant difference between conditions was found for each trait; number of neonates in the second brood p= 9.85^{-e5} (One Way ANOVA, F=7.655, df=2), size at maturity p= 5.06^{-e3} (Kruskal-Wallis Rank Sum Test, chi-squared=10.57, df=2) and CTmax p= 1.66^{-e4} (Kruskal-Wallis Rank Sum Test, chi-squared=17.401, df=2).

Specifically the 18°C condition was found to be significantly different to both the 21°C and 24°C condition, with more neonates in the second brood, a larger size at maturity and a lower CTmax, (Table 2.2, Figure 2.3). The lowest survival was seen in the 18°C condition

with 78% still alive at 21 days, again 21°C and 24°C conditions show similar results to each other (Figure 2.4). The majority of mortality for all three conditions was observed within the first 7 days.

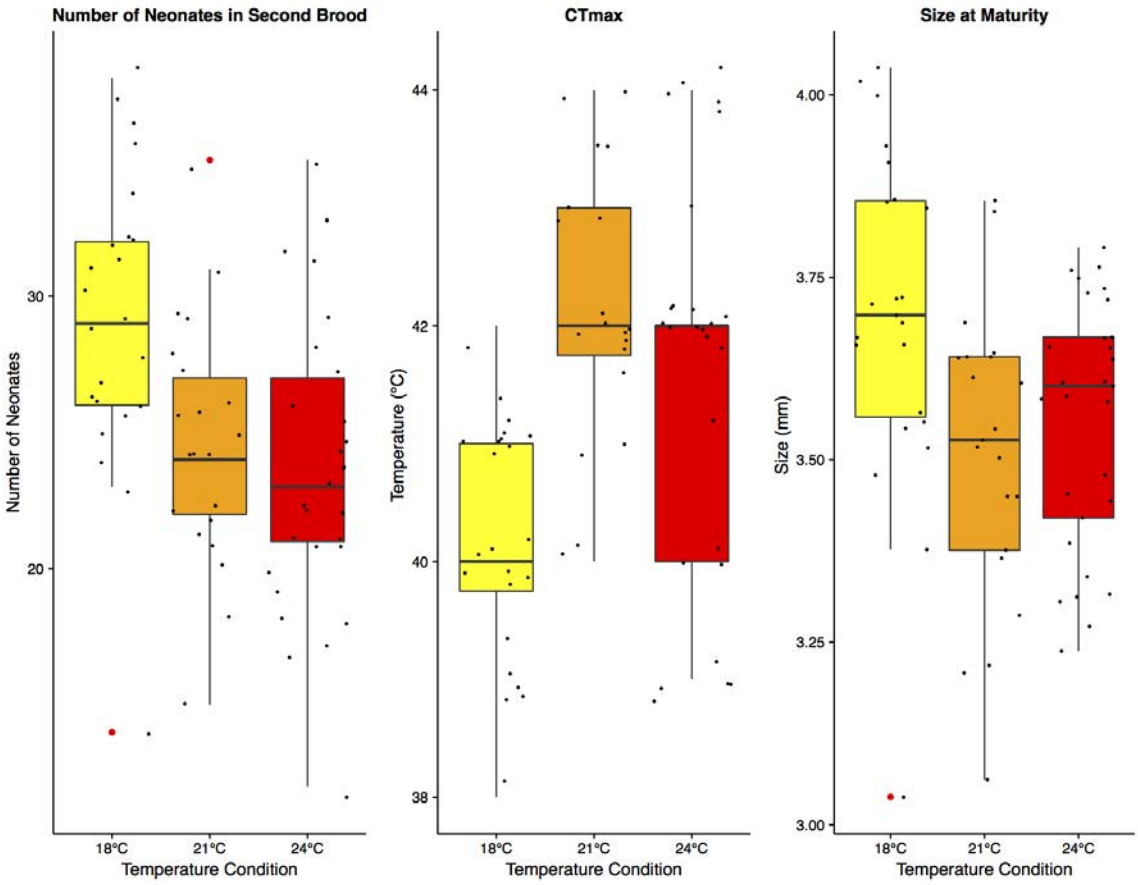

Figure 2.3: Box-plots for the number of neonates in the second brood, CTmax and size at maturity for all individuals across the three temperature conditions.

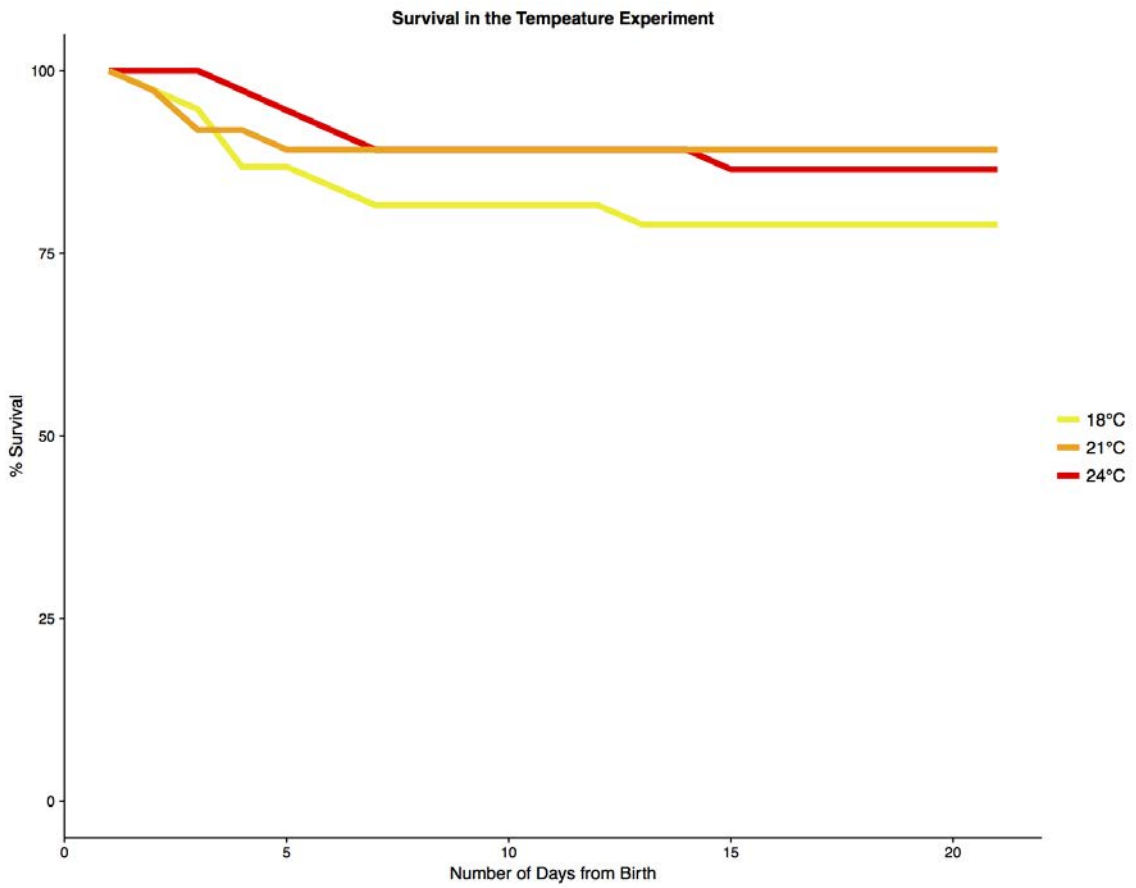

Figure 2.4: Cumulative mortality of all individuals (18°C N=32, 21°C N=28, 24°C N=33) across the three temperature conditions.

Table 2.2: Comparison of temperature conditions for three traits and significance values.

Trait Analysed	**Conditions Compared**	**Test**	**P-Value**	**W-Value**
Number of Neonates in Second Brood	18°C-21°C	Tukey Comparison of Means	0.011*	-
	18°C-24°C	Tukey Comparison of Means	0.001*	-
	21°C-24°C	Tukey Comparison of Means	0.877	-
Size at Maturity	18°C-21°C	Wilcox Rank Sum	0.000*	393
	18°C-24°C	Wilcox Rank Sum	0.017*	446
	21°C-24°C	Wilcox Rank Sum	0.161	329
CTmax	18°C-21°C	Wilcox Rank Sum	0.002*	110
	18°C-24°C	Wilcox Rank Sum	0.015*	201
	21°C-24°C	Wilcox Rank Sum	0.387	260

2.3.2 Temperature Experiment- effect of temperature between sub-populations

Each of the three temperature conditions were treated as individual experiments in order to analyse differences between the three sub-populations. No significant differences were found using the Kruskal-Wallis Rank Sum Test for CTmax, size at maturity or the number of neonates in the second brood (Appendix D). Survival however appears to be lowest in the eutrophic sub-population for both the 18°C and 21°C experiment, with survival being higher in the pristine sub-population. However in the 24°C experiment survival is lowest in the pristine sub-population (Figure 2.5).

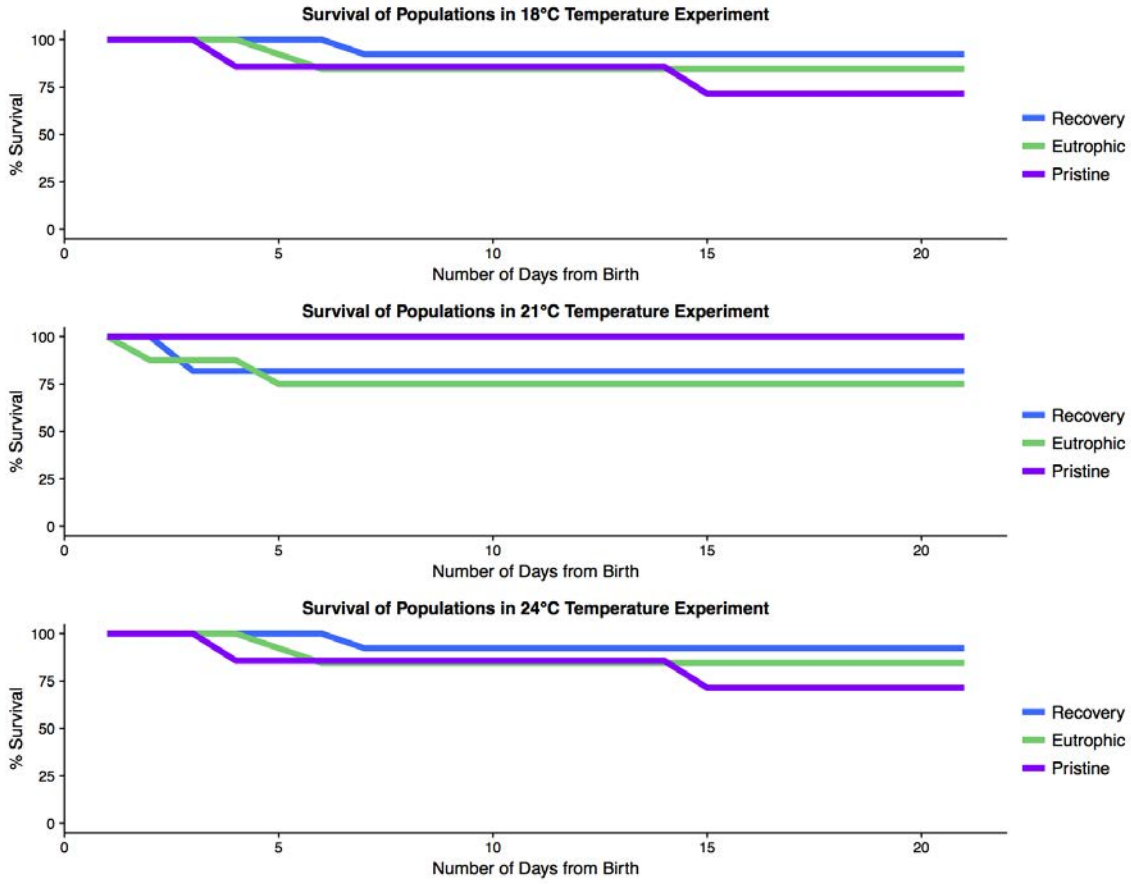

Figure 2.5: Cumulative mortality of each sub-population in the three temperature conditions. For 18°C: Recovery N=11, Eutrophic N=12 and Pristine N=9. For 21°C: Recovery N=11, Eutrophic N=7 and Pristine N=9. For 24°C: Recovery N=13, Eutrophic N=13 and Pristine N=7.

2.3.3 Carbaryl Experiment- effect of temperature combined with pesticide exposure on the whole population

Differences between high and low carbaryl conditions were statistically analysed for the 18°C and 24°C temperature condition independently for; the number of neonates in second brood, size at maturity and CTmax. Data from the 21°C condition was not analysed due to the experiment ceasing early at only 8 days; caused by incubator failure. The only difference found was for size at maturity in the 24°C condition, with size being significantly bigger in the low pesticide treatment (Kruskal-Wallis Rank Sum Test, chi-squared=8.9759, df=1, $p=2.73^{-e3}$, (Figure 2.6). The fourth trait; survival was much lower in the high pesticide treatment; decreasing as low as 23% in the 18°C condition. The lowest survival in the low pesticide treatment was also in the 18°C condition; dropping to 86%. However survival overall appears much higher in the low treatment compared to the high treatment (Figure 2.7).

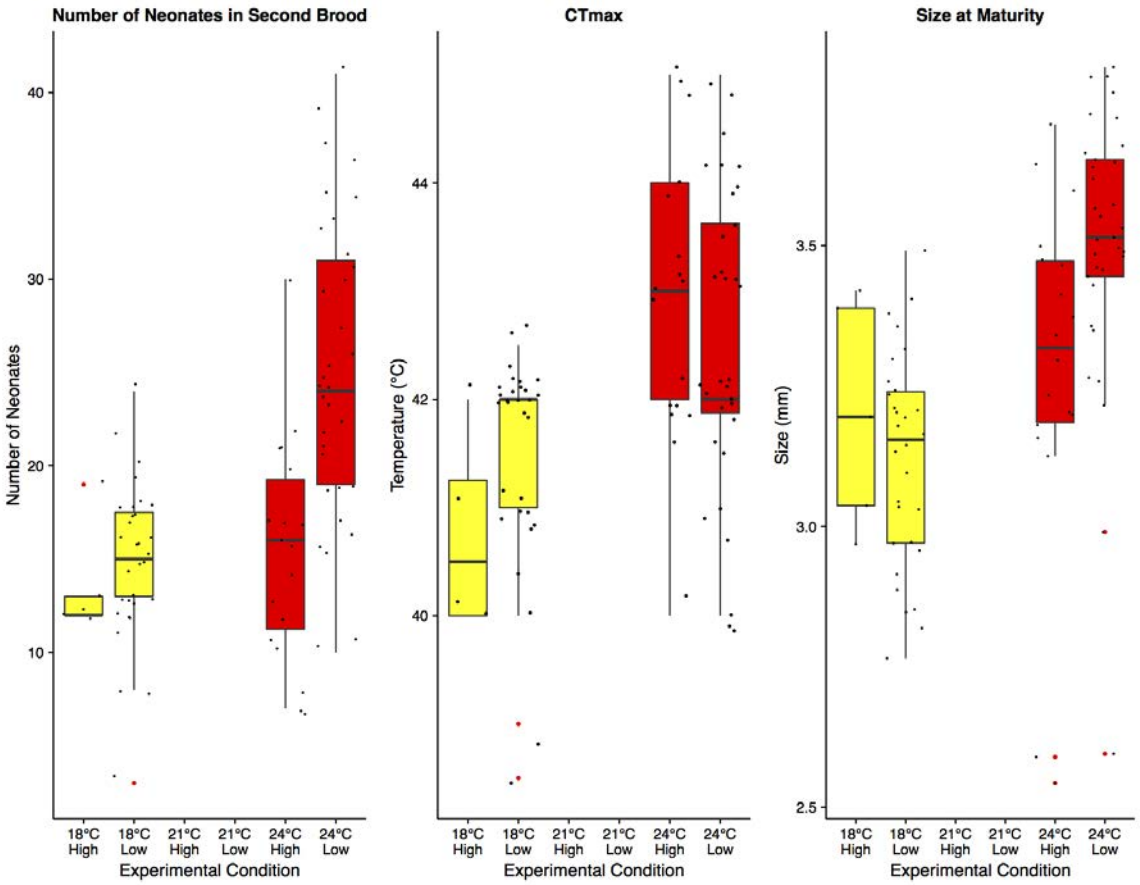

Figure 2.6: Box-plots for the number of neonates in the second brood, CTmax and size at maturity for all individuals across the three temperature conditions in both the high and low pesticide treatment. Data unavailable for 21°C.

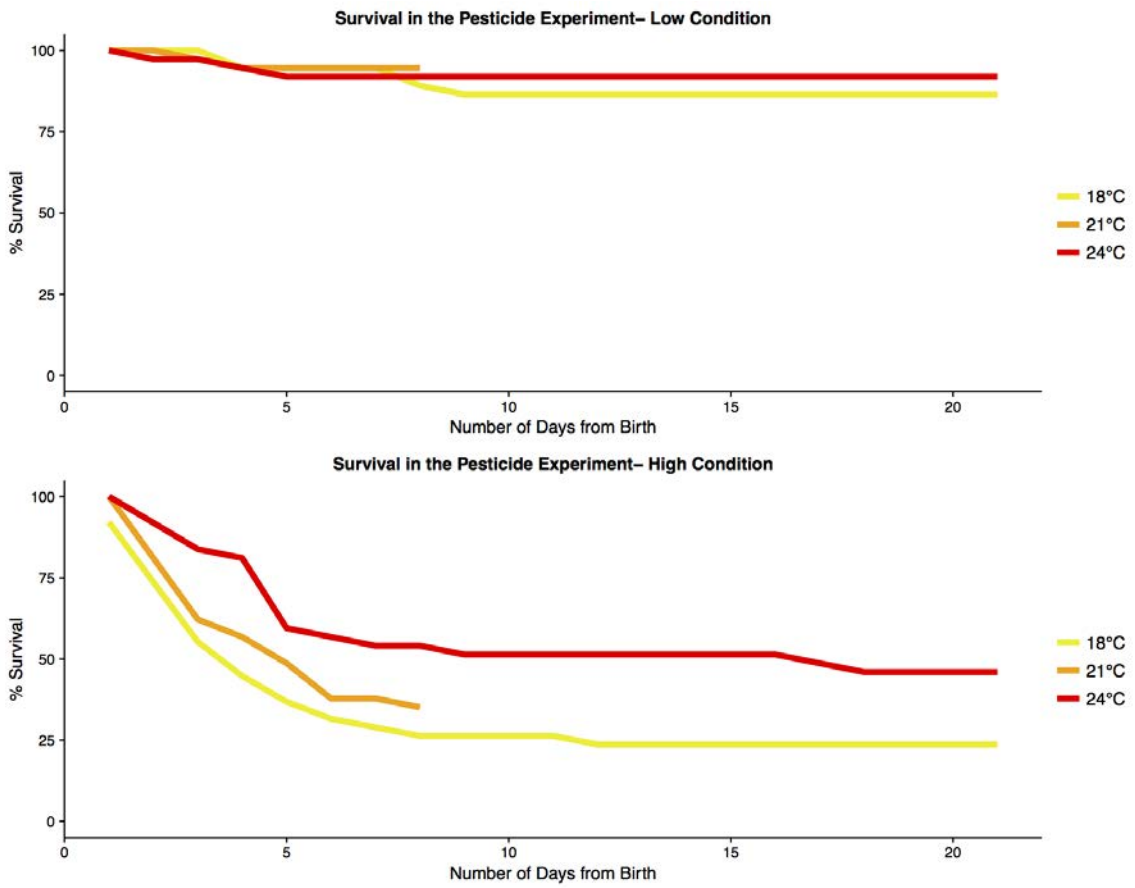

Figure 2.7: Cumulative mortality of all individuals across each temperature condition for both low and high pesticide treatments. (Low 18°C N=37, low 21°C N=35, low 24°C N=37, high 18°C N=34, high 21°C N=35, high 24°C N=37).

2.3.4 Carbaryl Experiment- effect of temperature combined with pesticide exposure between sub-populations

Each temperature condition and high/low treatment were considered as separate experiments for analysis between sub-populations. The only trait to show significant variation between sub-populations was CTmax and this was only found in the 24°C high and 24°C low conditions, p=0.019 (Kruskal-Wallis Rank Sum Test, chi-squared=7.9546, df=2) and p=0.018 (Kruskal-Wallis Rank Sum Test, chi-squared=7.9842, df=2) respectively. Specifically the eutrophic sub-population has been shown to have a significantly lower CTmax than the other two sub-populations in the 24°C low pesticide exposure experiment (Table 2.3, Figure 2.8).

However in the 24°C high pesticide experiment the only significant difference found was a higher CTmax in the eutrophic sub-population compared to the pristine sub-population (Figure 2.8). Survival appeared highest in the eutrophic sub-population in the high pesticide treatment consistently in all three temperature conditions (Figure 2.8).

Table 2.3: Difference in CTmax between sub-populations in both the high and low pesticide treatment for the 24°C condition. P and W values were derived using the Wilcox Rank Sum analysis.

Experimental Condition	**Conditions Compared**	**P-Value**	**W-Value**
Low Pesticide Treatment	Recovery-Eutrophic	0.008*	107
	Recovery-Pristine	0.588	50
	Eutrophic-Pristine	0.032*	19
High Pesticide Treatment	Recovery-Eutrophic	0.278	10.5
	Recovery-Pristine	0.063	29
	Eutrophic-Pristine	0.009*	25

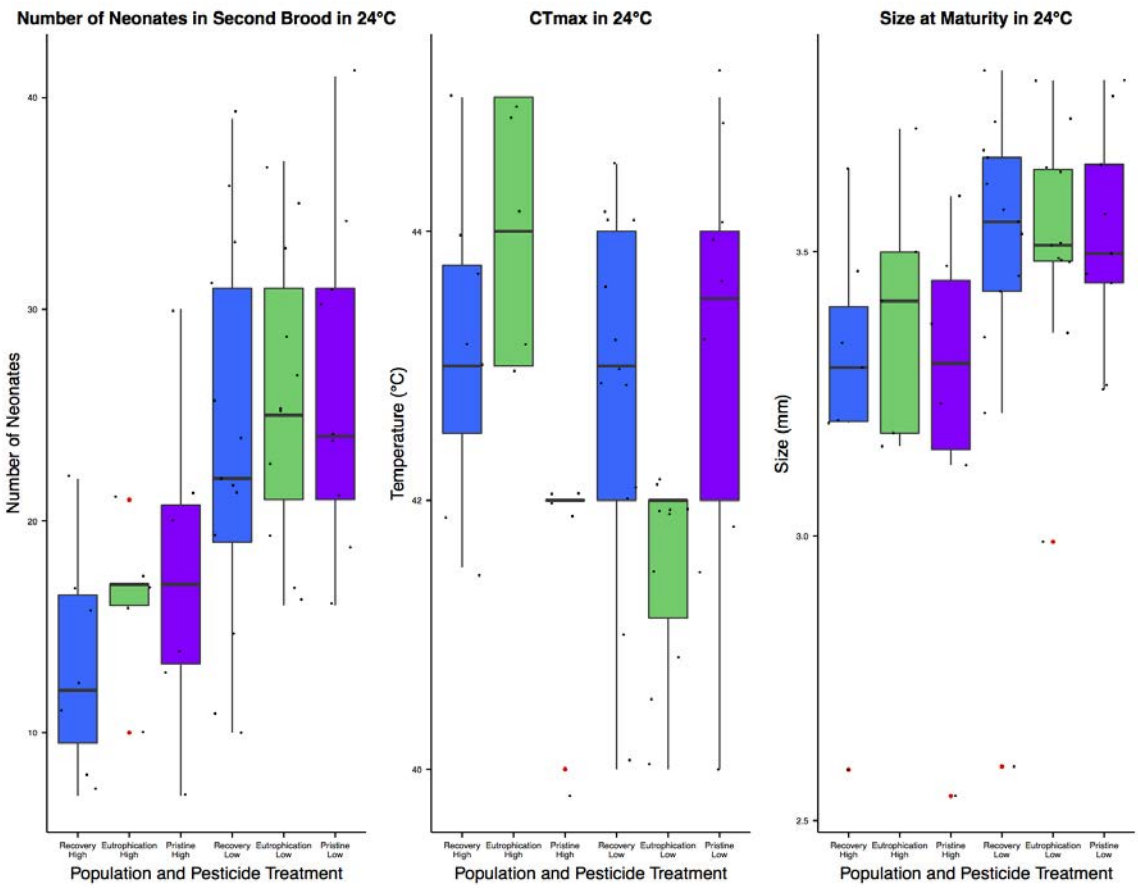

Figure 2.8: Box-plots for the number of neonates in the second brood, CTmax and size at maturity for each sub-population in both the low and high pesticide treatments in the 24°C temperature condition.

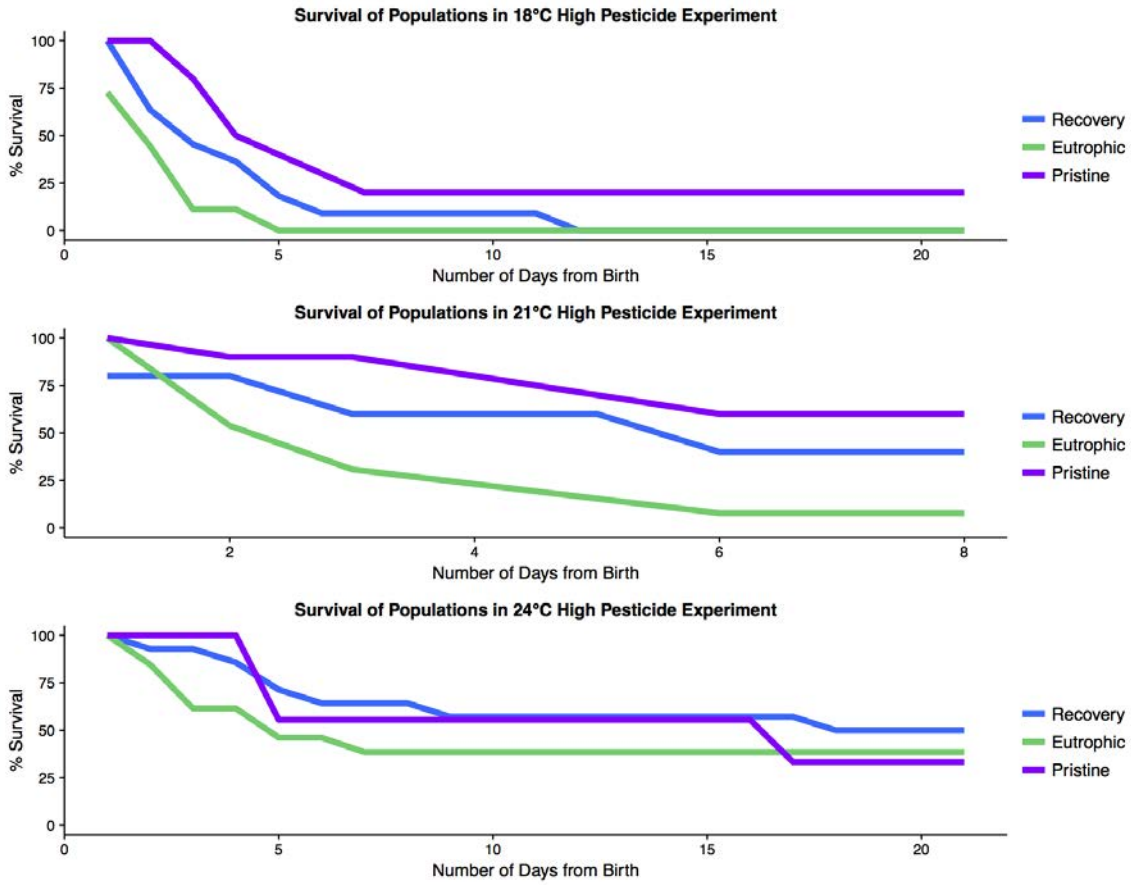

Figure 2.9: Cumulative mortality of each sub-population in the three temperature conditions and high pesticide treatment. For 18°C: Recovery N=13, Eutrophic N=10 and Pristine N=11. For 21°C: Recovery N=11, Eutrophic N=13 and Pristine N=9. For 24°C: Recovery N=14, Eutrophic N=13 and Pristine N=10.

2.3.5 Food Experiment- effect of temperature combined with food quality on the whole population

It seems food quality affects most of the traits observed (Figure 2.10), with low food quality negatively affecting the phenotype. Specifically in the three traits analysed further; number of neonates in second brood, size at maturity and CTmax, there was a significant difference found between treatments in all three temperature conditions (Table 2.4). Number of neonates in the second brood was significantly lower in the low food condition, size at maturity was significantly smaller in the low food condition and CTmax was significantly lower in the low food condition. Survival was lowest in 24°C low food quality experiment (83%

surviving), interestingly survival in the 18°C experiments was highest in the low food quality compared to the high food quality (86% and 97% respectively), (Figure 2.11).

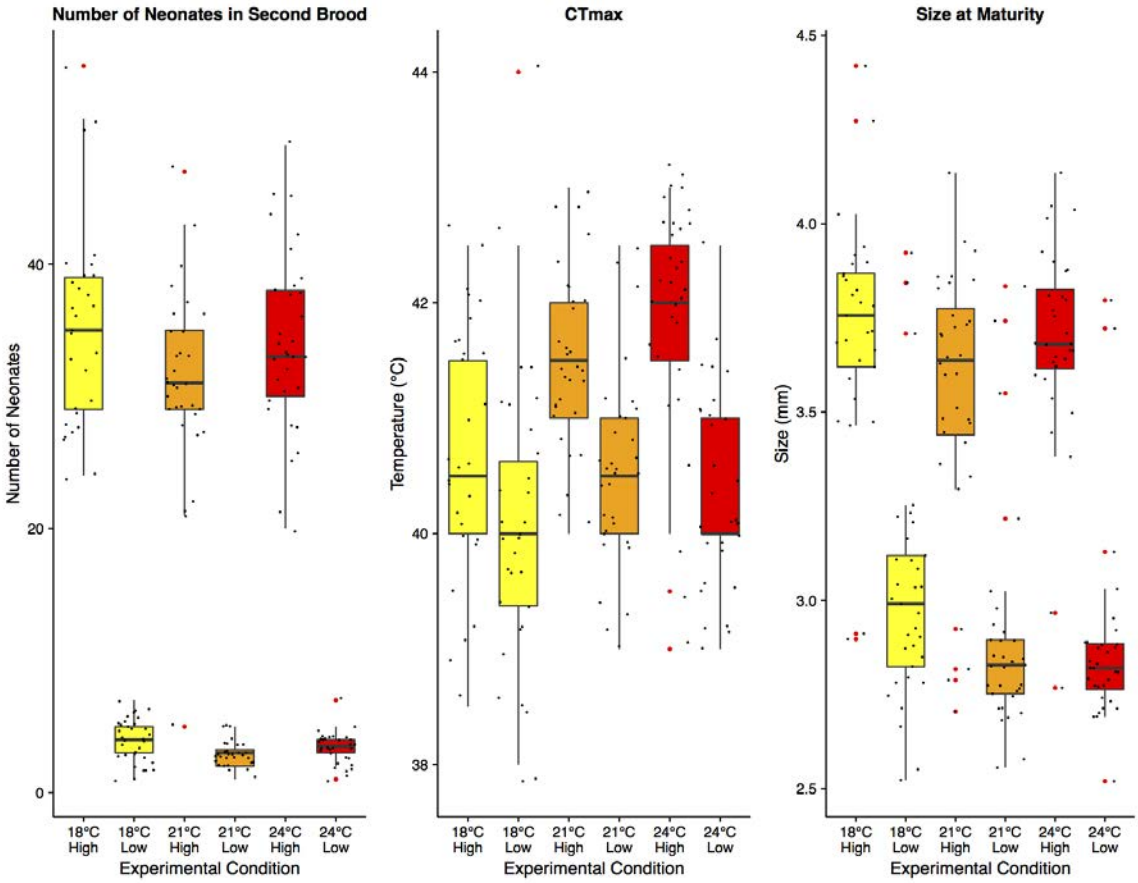

Figure 2.10: Box-plots for the number of neonates in the second brood, CTmax and size at maturity for all individuals across the three temperature conditions in both the high and low food treatment.

Table 2.4: Comparison of high and low food quality treatments in the three temperature conditions for each trait. Significance values obtained from the Kruskal-Wallis Rank Sum Test.

Temperature Condition	**Trait Analysed**	**Chi-Squared**	**P-Value**
18°C	Number of neonates in the second brood	45.81	$1.30^{e\text{-}11}$*
	Size at maturity	30.04	$4.22^{e\text{-}8}$*
	CTmax	5.56	0.018*
21°C	Number of neonates in the second brood	47.71	$4.93^{e\text{-}12}$*
	Size at maturity	28.38	$9.95^{e\text{-}8}$*
	CTmax	18.29	$1.89^{e\text{-}5}$*
24°C	Number of neonates in the second brood	47.85	$4.59^{e\text{-}12}$*
	Size at maturity	36.15	$1.79^{e\text{-}8}$*
	CTmax	26.02	$3.36^{e\text{-}0}$*

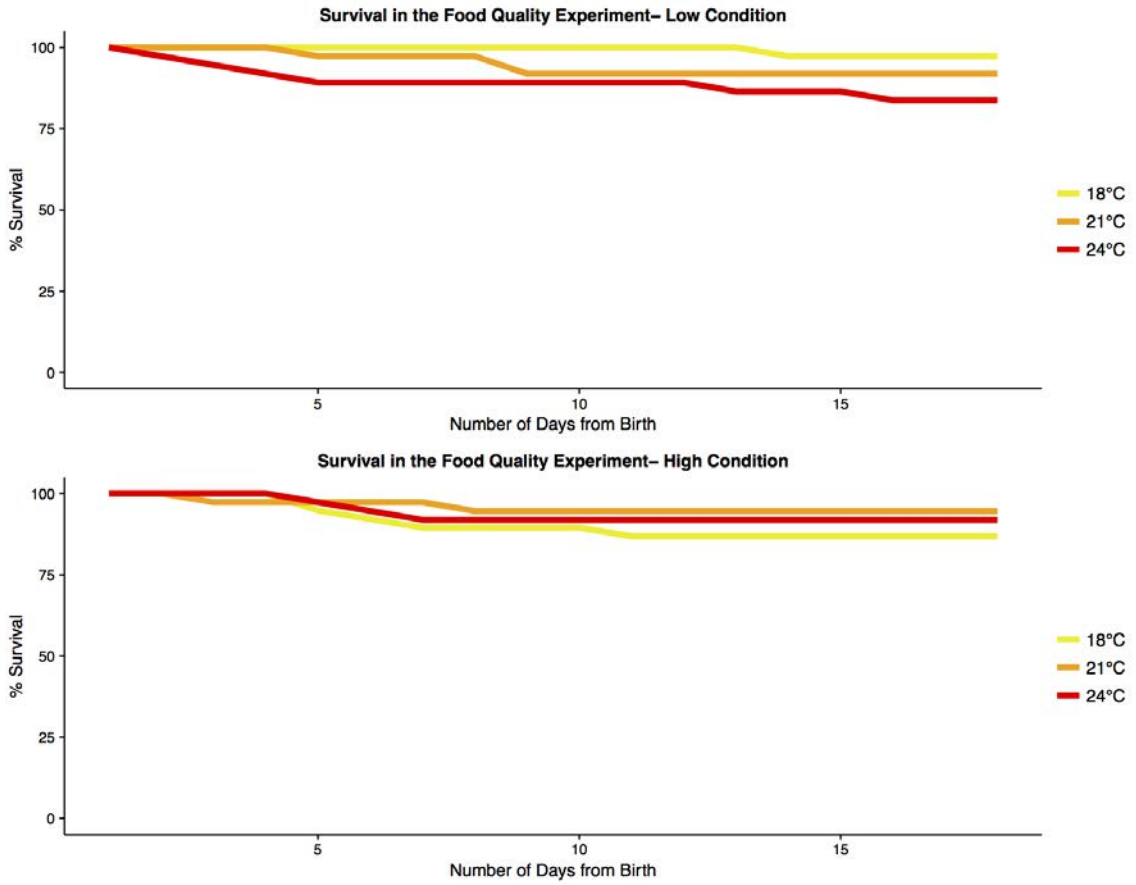

Figure 2.11: Cumulative mortality of all individuals across each temperature condition for both low and high food treatments. (Low 18°C N=35, low 21°C N=35, low 24°C N=36, high 18°C N=35, high 21°C N=35, high 24°C N=36).

2.3.6 Food Experiment- effect of temperature combined with food quality between sub-populations

Each temperature condition and high/low treatment were considered as separate experiments for analysis between sub-populations. The number of neonates in the second brood was significantly different between sub-populations in only the 21°C low food experiment (Kruskal-Wallis Rank Sum Test, chi-squared=6.4843 df=2, p=0.04). Specifically the pristine sub-population had significantly more neonates than the recovery sub-population (Table 2.5, Figure 2.12).

Size at maturity was significantly different between sub-populations in only the 21°C high

food quality experiment (Kruskal-Wallis Rank Sum Test, chi-squared=7.0161, df=2, p=0.03). The pristine sub-population was found to have a significantly larger size at maturity than the recovery sub-population (Table 2.5, Figure 2.12).

CTmax was significantly different between the three sub-populations in only the 24°C high food quality experiment (Kruskal-Wallis Rank Sum Test, chi-squared=13.519, df=2, p= 1.16^{-e3}). The eutrophic sub-population had a significantly lower CTmax than both the recovery and pristine sub-populations (Table 2.5, Figure 2.12). Finally survival was relatively similar in both the high and low food quality experiments (Appendix D).

Table 2.5: Experiments and traits that any significant values were found for from the high and low food quality experiment. P and W values were derived using the Wilcox Rank Sum analysis.

Experimental Condition and Trait	**Conditions Compared**	**P-Value**	**W-Value**
21°C Low Food Quality. Number of Neonates in the Second Brood.	Recovery-Eutrophic	0.844	62
	Recovery-Pristine	0.017*	27
	Eutrophic-Pristine	0.027*	18
21°C High Food Quality. Size at Maturity.	Recovery-Eutrophic	0.528	45.5
	Recovery-Pristine	0.026*	31
	Eutrophic-Pristine	0.053	25
24°C High Food Quality. CTmax.	Recovery-Eutrophic	0.002*	103.5
	Recovery-Pristine	0.148	42
	Eutrophic-Pristine	0.002*	8

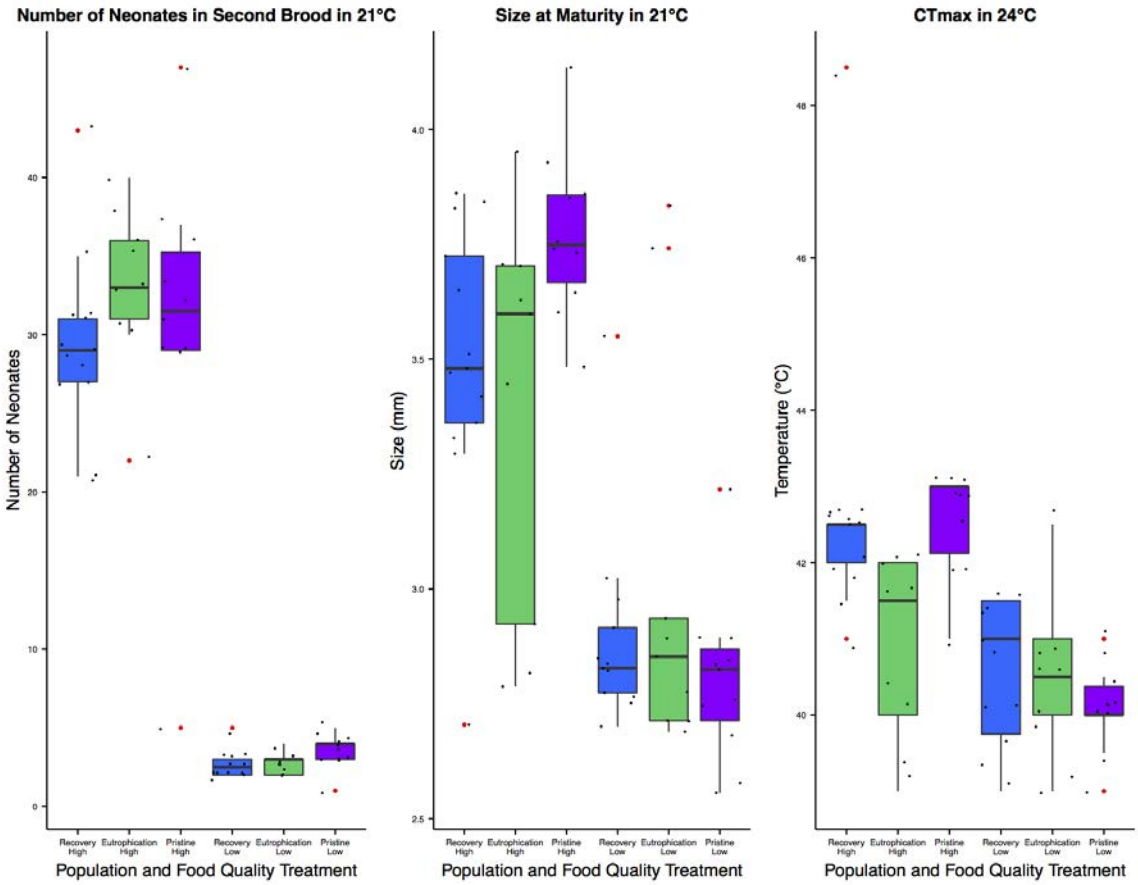

Figure 2.12: Left and centre: box-plots for the number of neonates in the second brood and size at maturity for each sub-population in both the low and high food treatments in the 21°C temperature condition. Right: box-plots for CTmax for each sub-population in both the low and high food treatment in the 24°C temperature condition.

2.4 Discussion

Three sub-populations of resurrected *Daphnia magna*, sampled before, during and after a strong environmental change (eutrophication) were subjected to a series of stressors in common garden experiments. These experiments focused on fitness responses of the population as a whole and responses of individual sub-populations to both an individual stressor (temperature) and combinations of stressors. Specifically preliminary analysis was designed to answer the following hypotheses:

- Combinations of stressors will be more detrimental to *Daphnia* fitness than individual stressors
- *Daphnia* from older sub-populations will show reduced fitness when exposed to a higher temperature than those from more recent sub-populations
- *Daphnia* from sub-populations that have previously experienced an environmental stressor will cope better than those for which the stressor is novel

2.4.1 Combined Effects of Stressors

Climate change paired with increasing human activities greatly effect the earth's natural environment; pushing species to migrate or adapt to new and increasing stressors (Hoffman and Sgro 2011). Specifically temperature as a single factor has been studied independently of other contributing factors (Anderson *et al.* 2012). This can lead to unrepresentative estimations of population and species adaptive capabilities, as the true mosaic of stressors, found in natural environments, is not represented. To more accurately predict the adaptive potential of species to climate change and human-driven eutrophication multiple stressors were combined in this study. Preliminary analysis of the data shows, in fact, fitness traits of *Daphnia magna* do differ when the same genotypes are exposed to either temperature as a single stressor or to temperature combined with either biotic or abiotic additional stressors.

Preliminary analysis however did not show an exact relationship between temperature and pesticide exposure, meaning the combined effect of the two stressors is not predictable from

the analyses carried out here. However it is known from previous work (Jansens 2014) that different temperatures can produce different levels of toxicity from the same chemical compound. This means individual populations experiencing seasonal changes and heat waves and, indeed, populations of the same species at different latitudes may suffer different levels of toxicity, potentially leading to local extinctions. Dinh Van *et al.* (2014) found higher temperatures did exaggerate the effects of pesticide exposure in freshwater damselfly larvae, concluding it was a trade-off favouring pesticide tolerance over growth rate that was observed. Jensen *et al.* (2011) also found developing resistance to the pesticide carbaryl in *Daphnia* resulted in other fitness trade-offs such as an increased risk to parasites.

What we found here would concur with these findings, *Daphnia* in the low pesticide treatment were significantly larger than their high treatment counterparts, this significant difference was only observed in the 24°C condition and not the two lower temperatures, indicating the higher temperature added to the effect of the pesticide exposure and potentially causing a trade-off with growth. However it was also found both low and high pesticide treatments in the 18°C condition had a negative effect on fecundity and size when compared to temperature alone. Interestingly the effect wasn't as large in the other two temperatures conditions, which is not in line with previous research above. However Muyssen *et al.* (2010) studied the effect of temperature combined with the pollutant; Cadmium, they found higher temperature did not further decrease the fitness of *Daphnia* exposed to Cadmium. Cuco *et al.* (2016) also found increasing temperature did not add to the negative fitness responses of *Daphnia* exposed to fungicides, they speculate it may be the period of acclimation that causes the lack of interaction. These studies highlight the non-linear relationship temperature and toxin exposure has on fitness traits of *Daphnia*. A more in-depth analysis of the data in this study, comparing the single stressor (temperature) with the results of the combined stressors would reveal if the differences observed in the 18°C condition are actually significant.

Temperature is also known to interact with food availability (Pinkney *et al.* 2015), higher temperatures are expected to have stronger effects on populations when food resources are low, impacting on survival. Interestingly a similar effect is seen with hyper-eutrophication where food quality, rather than quantity, is limited (Richman and Dodson 1983). Preliminary

results here only confirm the detrimental effect on fitness low food quality has, specifically low food quality is known to negatively effect growth rate and fecundity in *Daphnia*, (Frost *et al.* 2010, Persson *et al.* 2011) as was found here. Interestingly this effect cannot be generalised across *Daphnia* species, McFeeters and Frost (2011) found *Daphnia magna* show decreased fitness when exposed to low food quality whereas the same effects on fitness are not observed in *Daphnia pulex*. They also looked at the combined effect of low food quality and increasing temperature, finding it was in-fact the lower temperature (10°C) that produced the higher fitness costs caused by low food quality, the opposite to what has previously been observed (Persson *et al.* 2011). As with the pesticide combined experiments the data still need further analysis to uncover any interaction temperature may have with low food quality on the population examined in this study.

The initial hypothesis that combinations of stressors will be more detrimental to *Daphnia* fitness that individual stressors cannot be currently accepted. Previous literature supports this hypothesis, with some exceptions (McFeeters and Frost 2011, Muyssen *et al.* 2010). While it is clear from this study high pesticide exposure and low food quality negatively effect fitness responses of *Daphnia magna* at different temperatures, further statistical analysis is needed to untangle interactions between stressors. This full analysis will be carried out at a later date as part of the bigger project this thesis contributed to.

Being able to predict fitness responses and costs caused by combinations of environmental stressors will give further information on the ability of species to adapt to environmental changes. It is clear from the previous literature above that fitness responses caused by combinations of stressors are difficult to predict. Future work should include a complete and thorough analysis of the data from this study to attempt to identify the effect of increased temperature on pesticide exposure and low food quality. Further investigation should also be carried out to combine even more stressors to more accurately predict species responses by better representing the natural environment.

2.4.2 Fitness Responses Across Sub-Populations

As well as exploring the effects of combinations of stressors, it was also possible to examine evolution in time in a natural population to a severe environmental stressor. By using resurrection ecology we were able to sample the same population over many generations in the presence of known environmental changes (specifically eutrophication) allowing evolutionary changes to be tracked over time. This allowed fitness responses associated with high eutrophication to be explored under different global warming predictions, thereby assessing the ability of natural populations to adapt to possible future temperatures. There were no clear patterns observed in the preliminary analysis between different sub-populations to the different temperature regimes and additional stressors, i.e. one sub-population did not significantly out/under preform the others. This would suggest evolution to a stressor, such as severe eutrophication, could occur quickly enough to ensure populations maintain adequate levels of adaptive capabilities. Gonzales and Bell (2012) coin this 'evolutionary rescue', when a severe environmental change causes a large population decline, rapid evolution allows restoration of the affected population, i.e. 'rescuing it from extinction'.

The two initial hypotheses relating to sub-population differences would currently be rejected based on the current analysis. *Daphnia* from older sub-populations did not show reduced fitness when exposed to higher temperature than those from more recent sub-populations. Also *Daphnia* from sub-populations that had previously experienced an environmental stressor did not out perform those for which the stressor was novel. As mentioned above a more robust analysis of these data will be carried out which may elucidate subtle differences which were not detectable by the analysis in this thesis. As was predicted some previous work did find environmental effects on fitness can be passed on to future generations in *Daphnia* (Frost *et al.* 2010). However it's possible acclimation to a stable environment for many generations (which was carried out here prior to experiments) may effect the fitness responses of *Daphnia* (Cuco *et al.* 2016). This would explain why sub-populations were not found to have consistently different fitness responses to various stressors.

As well as the additional statistical analysis mentioned, future work will examine 'evolutionary rescue' theory further by using the same genotypes as used in this experiment to assess

genome wide variation of this population through time, using a GWAS approach (genome wide association studies) to link phenotypic differences with underlying genomic structure. This is possible as *Daphnia magna* has a relatively small genome (129Mb) and genomic tools such as a reference genome are available (NCBI 2016). RNA sequencing of individuals from each experiment will also be used, in this case to assess plastic responses determined by gene regulation (Jansen *et al.* 2013). There is already a bank of *Daphnia magna* transcriptomes, specific to 12 environmental stressors, available as reference (Orsini *et al.* 2016). Selection at the genome scale has already been found in *Daphnia* from other sediments cores, which had different environmental stressors (Orsini *et al.* 2013), this indicates it's likely such selective signatures will also be found in the population studied here. This future work will give further insight into the lack of differences observed here between sub-populations and elucidate the roles of plasticity and genetic adaptation in *Daphnia* after a considerable environmental event.

2.4.3 Conclusions

Climate change and anthropogenic impact affect natural environments causing species to adapt though migration, plasticity and genetic evolution. Investigation of multiple stressors is paramount to accurately represent the mosaic of stressors found in natural environments. The synergism between different stressors is not always predictable and further work is needed in this field. Resurrection ecology provides a powerful method to study species adaptive mechanisms through time to environmental stressors.

In this thesis I examine a combination of stressors on three sub-populations of *Daphnia magna* resurrected from the Lake Ring system. Preliminary analysis did not expose a clear relationship between temperature and pesticides or low food quality on the fitness responses of the population as a whole. Low food quality and pesticide exposure in general however did elicit effects on fitness in line with previous literature. There were also no clear differences observed between sub-populations to either; temperature as an individual stressor or to temperature combined with an additional stressor. This could be due to acclimation effects or a lack of power to detect subtle sub-population differences by the preliminary statistical

tests used.

Further work will involve a more in-depth statistical analysis of the data obtained. Work will also be done to investigate the mechanisms underpinning adaptive ability by using whole genome and transcriptome sequencing on the *Daphnia* in this study.

Chapter 3

Temporal Genetic Stability in *Daphnia magna* Exposed to Eutrophication

Hollie Marshall, Maria Cuenca Cambronero, Monica Alfronso and Luisa Orsini.

3.1 Importance to Chapter 2

This chapter works to complement the results of chapter 2. Showing hatching bias does not exist in resurrection ecology answers a major criticism of the technique. It means genotypes that were hatched and used in the experimental evolution trials in chapter 2 were a true representation of the population during each time period and not a selected sub-set of potentially fitter individuals. This chapter also shows shifts in fitness observed in chapter 2 were not the result of genetic drift or a population bottleneck.

3.2 Rationale

Studies monitoring changes in genetic diversity and composition through time allow a unique understanding of evolutionary dynamics and persistence of natural populations. However, such studies are often limited to species with short generation times that can be propagated in the laboratory or in a few exceptional cases in the wild (e.g. Darwin's Finches, Grant and Grant 2002). Species that produce dormant stages provide powerful models for the reconstruction of evolutionary dynamics in the natural environment. However it is unknown to what extent dormant egg banks are a biased representation of the population, and hence of the species' evolutionary potential, especially in presence of environmental change. To contribute towards answering this question, we investigate whether neutral genetic diversity declines in the sediment core of Lake Ring and whether it is maintained in the presence of a eutrophication. In addition, by comparing temporal genetic stability in hatched and unhatched populations, we attempt to show that dormant egg banks can be consulted to obtain a reliable measure of genetic diversity over time.

3.3 Methods

3.3.1 Daphnia Samples Isolated from Lake Ring

96 resurrected genotypes and 48 unhatched genotypes (dormant eggs) were sampled from the Lake Ring sediment core described in detail in the introduction. They were equally divided into four sub-populations spanning all three lake phases (pristine, eutrophic and recovery). This meant each sub-population spanned roughly the same amount of time; 6-8 years and contained roughly the same number of genotypes (Table 3.1). This was to attempt to make the sub-populations more comparable, dividing the population by lake phase would have meant vastly different sample size and time period per sub-population, potentially skewing any analysis. 28 hatched genotypes were used to create sub-population 1-3, sub-population 4 consisted of only 12 hatched genotypes (due to hatching difficulty in older dormant eggs). All four sub-populations of unhatched individuals consisted of 12 genotypes for each sub-population.

3.3.2 Genotyping

DNA was extracted from three clonal individuals per hatched genotype and from a single egg per unhatched genotype using the Agencourt DNAdvance genomic DNA isolation kit, the manufacturers protocol was modified for optimal extraction of both hatched and unhatched animals, (Appendix E). Only one egg from each unhatched ephippium was used (out of two present) in order to avoid sibling bias, as both eggs in each capsule originate from the same parents. Extracted DNA was then treated with 3μl of RNase A at a concentration of 5mg/μl (Appendix E). DNA was quantified using the Tecan Infinite M200 fluorescence machine and quality checked by gel electrophoresis, the Agilent TapeStation 2200 was also trailed for quantification and quality checking.

PCR reactions were carried out in 11 multiplexes consisting of a total of 74 primer pairs (Appendix F). PCR reactions were run as follows: 15mins initial denaturation at 95°C, 30 cycles of 94°C for 30secs, annealing temperature for 90secs and 72°C for 90secs and the final

extension for 30mins at 60°C in a PCR Thermocycler. PCR reaction success was confirmed used gel electrophoresis.

96-well sequencing plates were then made up by adding 1μl of PCR product to 10μl of a LIZ Size Standard/ formamide mix (1:19 ratio) per well. Sequencing was carried out by the University of Birmingham's genomics facility using an ABI 3730 machine.

Table 3.1: Structure of samples for the hatched and unhatched sub-populations used for microsatellite genotyping. Each individual represents a unique genotype as ephippia are formed from the sexual phase of *Daphnia*. The core was split into 4 sub-populations to try to represent an equal amount of time and samples. Sub-population 4 spans slightly more years and has less samples due to hatching success decreasing with core age.

Sub-population	**Sediment Layer (cm)**	**Year**	**Number of Years**	**Number of Hatched Individuals**	**Number of Unhatched Individuals**	**Total Hatched Individuals**	**Total Unhatched Individuals**
1	0	2000	6	6	2	28	12
	1	1997		4	3		
	2	1994		6	2		
	3	-		6	3		
	4	1991		6	2		
2	5	1989	6	9	4	28	12
	6	1986		10	4		
	7	1983		9	4		
3	8	1980	6	12	3	28	12
	9	1977		11	3		
	10	1975		3	3		
	11	1972		2	3		
4	12	1968	8	5	3	12	12
	13	1966		4	3		
	14	1963		2	3		
	15	1960		1	3		

3.3.3 Data Analysis

Alleles were called using Gene Mapper v4.0 (Applied Biosystsems, Foster City, CA). This software uses the previously collected sequence data to identify 'peaks' in each sample representing the number of base pairs (bp) that a particular microsatellite has. For example one large peak of '151' indicates a homozygote with 151bp in each allele, where as two smaller peaks of different microsatellite sizes such as '151' and '154' indicates a heterozygote carrying one allele with 151bp and one with 154bp.

Outlier loci linked to eutrophication were determined using Fdist (Beaumont and Nichols 1996) implemented in LOSITAN (Antao *et al.* 2008, Beaumont and Nichols 1996) following methods from Orsini *et al.* (2012). Of the 45 microsatellite loci successfully sequenced, 41 were identified as neutral and used for further analysis. Neutral loci are determined as those which do not deviate greatly from the average Fst generated by LOSITAN using the data provided (Antao *et al.* 2008). Outlier loci are excluded as they are supposedly neutral loci that show patterns of selection, indicated by a particularly high or low Fst value, this can either be because they are 'hitchhiking' with genes under selection or are themselves involved in adaptive processes (Orsini *et al.* 2012). It is important to exclude these loci from analyses in order evaluate just neutral markers.

To assess population genetic diversity and structure: heterozygosity, allelic richness and effective population size were quantified in both the hatched and unhatched samples. Heterozygosity and allelic richness were calculated with Microsatellite Analyser (MSA) (Dieringer and Schlotterer 2003). Colony (Wang 2009) was used to estimate changes in the effective population size, assuming random mating. Hardy-Weinberg equilibrium was calculated for each sub-population using GENEPOP (Rousset 2008). Changes in population genetic diversity were determined using a two-level AMOVA (Analysis of Molecular Varience) implemented in Arlequin (Excoffier *et al.* 2005), the two levels were within and among sub-population. MSA, Colony and Arlequin analyses were carried out following methods outlined in Orsini *et al.* (2013). GENEPOP analyses followed Orsini *et al.* (2012).

Finally changes in the genetic structure of the sub-populations were then assessed using

STRUCTURE (Falush *et al.* 2003, Pritchard *et al.* 2000). The allele frequencies of the 'start' population (the population from which our current samples were generated from) were predicted using 100,000 MCMC iterations using the current data following Pritchard *et al.* (2000) and various tests amounts, a burn-in period was set at 1,000,000. The value K was set to 10 after testing a range of K= 1-10, all K greater than 2 were stable, i.e. increasing K did not provide better population clusters. K in the STRUCTURE model refers to the number of potential ancestral populations that formed the current population under study (Falush *et al.* 2003).

3.4 Results

Population genetic parameters (Hardy-Weinberg equilibrium, effective population size, allelic richness, genetic variation and genetic structure) were assessed using neutral loci in both hatched and unhatched individuals from the Lake Ring sediment core. All sub-populations for both hatched and unhatched samples were found to be in Hard-Weinberg equilibrium over time (Figure 3.1). The effective population size was less consistent over time, however there is no clear increasing/ decreasing pattern (Figure 3.1). Allelic richness was also stable over time and comparable in both the hatched and unhatched samples (Figure 3.1).

AMOVA analysis indicates significant molecular variance both within and among sub-populations for both the hatched and unhatched samples, the variation within sub-populations being considerably higher (Table 3.2), again hatched and unhatched samples gave similar results. This result is in line with previous studies (Orsini *et al.* 2012, Orsini *et al.* 2013).

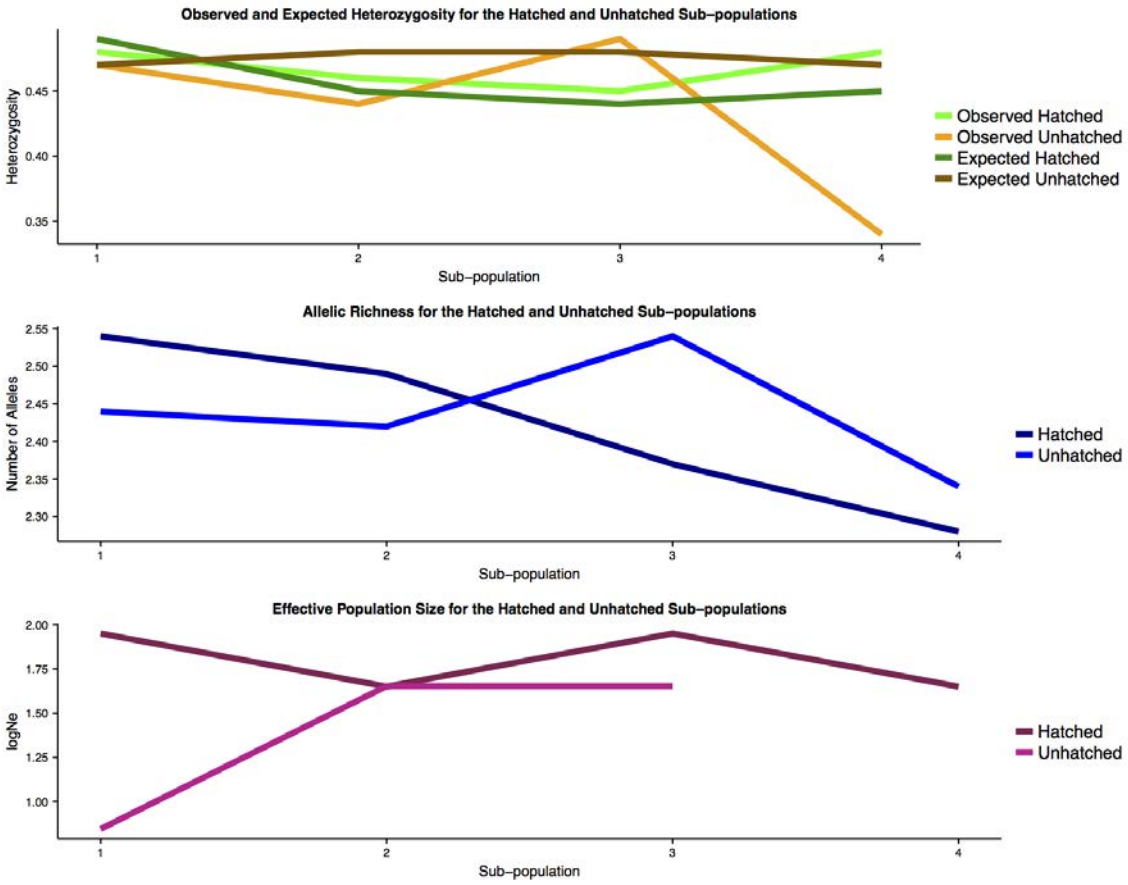

Figure 3.1: Variation at genetic diversity parameters across sub-populations in both the hatched and unhatched samples. Top: observed and expected heterozygosity for the hatched and unhatched samples determined by MSA. Centre: allelic richness for the hatched and unhatched samples determined by MSA. Bottom: effective population size for the hatched and unhatched samples on a log scale determined by Colony.

Table 3.2: Genetic variation within and among sub-populations for hatched and unhatched samples determined by AMOVA analysis. Statistically significant values (*, P<0.001) are based on 10,000 permutations.

	Among Sub-Populations	**Within Sub-Populations**
Hatched	1.08*	98.92*
Unhatched	1.75*	98.25*

Genetic structure analysis indicates very low genetic structure within both the hatched and unhatched sub-populations through time (Figure 3.2). There were no distinguishable clusters formed which indicates no large changes in microsatellite composition within the population over the time period sampled.

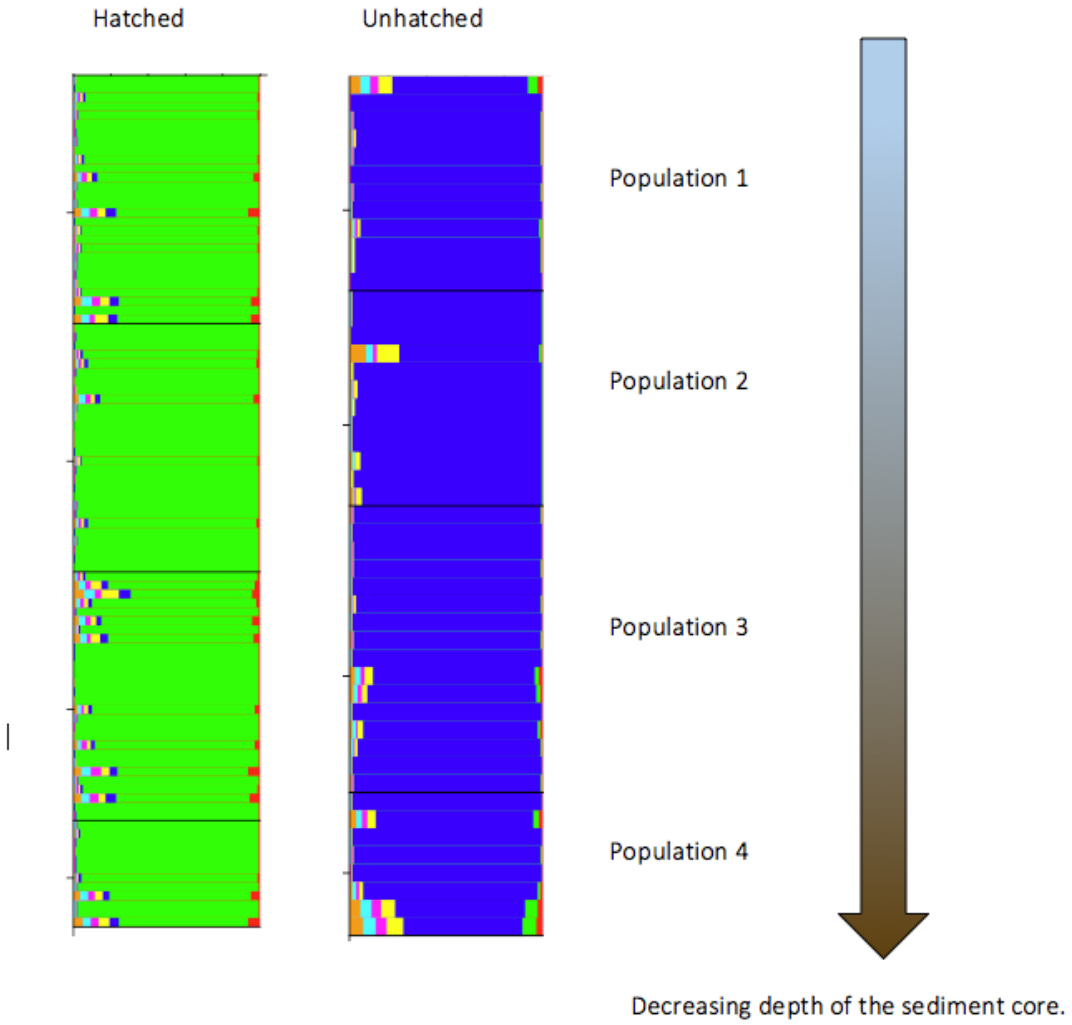

Figure 3.2: Population genetic structure analysis for the hatched and unhatched samples. Black horizontal lines separate the four sub-population and bars indicate individual genotypes. Each colour within a bar indicates a specific allele, therefore the more colours the more alleles are present. No clear pattern is visible for any particular colour indicating allele frequencies did not drastically change throughout the sub-populations or between hatched and unhatched samples. Note the colour coding is randomly assigned and each plot is generated independently, therefore identical colours in each plot may represent a different allele. Plots generated by STRUCTURE.

3.4.1 Summary of Results

Observed heterozygosity, expected heterozygosity, allelic richness, within and among sub-population genetic variance and population structure over time are all comparable between

hatched and unhatched samples. Effective population size however does fluctuate over time in both the hatched and unhatched populations but with no clear direction.

There is also little genetic variation in the overall population over time as shown by structural analysis and comparison of population genetic parameters. This demonstrates that there is no reduction in overall genetic diversity over time even in the presence of eutrophication.

Discussion

Neutral genetic parameters were assessed using microsatellites in hatched and unhatched *Daphnia magna* from a sediment core taken from Lake Ring (See Study System in the Introduction). Four sub-populations spanning before, during and after a strong environmental change (eutrophication) were defined for analysis to answer the following hypotheses:

- Neutral genetic diversity between hatched and unhatched *Daphnia* will not differ significantly
- Eutrophication will reduce the neutral genetic diversity seen in *Daphnia* during the exposure period and population genetic structure will be different before and after exposure

3.4.2 Hatching Bias in Resurrection Ecology

Resurrection ecology utilises dormant egg banks in sediment cores to examine traits of past populations and better understand evolution in time. However it is unknown whether hatching success causes bias in the samples obtained, meaning the original population is not fully represented, skewing any results and conclusions. This study addressed this issue for the first time by comparing neutral genetic diversity between hatched and unhatched populations of *Daphnia magna*, from the same sediment core. Examining a suite of population genetic parameters it was found genetic diversity and allelic composition through time is comparable between the hatched and unhatched sub-populations. This means the first hypothesis that neutral genetic diversity between hatched and unhatched Daphnia will not differ significantly can be accepted.

This suggests resurrection ecology is, indeed, a strong method for studying evolutionary responses through time, without introducing bias from hatching success. This has profound implications for the reliability of previous published work using *Daphnia magna* as well as paving the way for future studies. This also means there is no bias from hatching present in the results of Chapter 2 in this thesis.

However as a single sediment core and species were used in this study it may be the results obtained are unique to *Daphnia magna* in the Lake Ring system, therefore similar comparative analysis should be conducted on other species and further sediment cores to confirm the patterns shown here. If similar results were found this could also massively increase the time span of future studies, as hatching success decreases with sediment age, using only dormant eggs for evolutionary studies would then become an option. Currently studies are constrained to the recent past because of this limit, Frisch *et al.* (2014) is a rare example of a resurrection study spanning centuries.

3.4.3 Neutral Genetic Diversity Over Time

Hatching success was not the only possible bias of resurrection ecology. Waterflea species in general have a short generation time meaning in the time periods usually studied by resurrection ecology strong selection or genetic drift could occur. This was examined here using a sediment core from a lake with a known strong environmental change (eutrophication). There was no clear genetic erosion (decrease in genetic diversity) over time (from old to recent sub-populations) and no change in neutral diversity following a selection event in *Daphnia magna* from the Lake Ring system. This means the second hypothesis for this chapter can be rejected; eutrophication did not reduce the neutral genetic diversity seen in *Daphnia* during the exposure period and population genetic structure did not differ before and after exposure.

Rejection of the second hypothesis suggests genetic drift and selection do not influence neutral diversity in this particular population. Similar results have also been found in *Daphnia magna* populations from two other sediment cores from different lakes using SNPs as well as microsatellite markers (Orsini *et al.* 2017). This low level of genetic variation between resurrected sub-populations from the same lake is thought to be a result of the having one initial founding population with very little to no migration (Orsini *et al.* 2012).

Stable diversity over time in this species is particularly interesting because it has previously been shown there are considerable changes in population composition during the start of *Daphnia's* growing season and then as the season progresses clonal erosion decreases the

diversity seen (De Meester *et al.* 2006, Vanoverbeke and De Meester 2010). It is likely neutral diversity is maintained by the 'buffering effect' of resting egg banks themselves, it is thought these banks act as a mechanism to ensure populations do not become extinct under extreme environmental conditions (Hairston *et al.* 1999). This is possible as even though eggs hatch when favourable environmental cues occur (usually spring time), not all eggs will hatch, meaning some are left as a 'backup' in case of extreme environmental change (Pietrzak and Slusarczyk 2006).

A lack of genetic diversity has been linked with extinction risk (Frankham 2005), therefore the temporal stability of genetic diversity within populations could mean they maintain enough adaptive ability to deal with environmental change. Previous work in various species including fish and birds, has also found no reduction in genetic diversity over time, however many of these studies were limited in power by the number of generations available for study or the number of genetic makers used (Larsson *et al.* 2010, Welch *et al.* 2012). In this study the results span 40 years in an organism with a generation time of approximately two weeks meaning *Daphnia* provide a more powerful model organisms for studying genetic diversity over time.

While stability in neutral genetic diversity was observed here, even throughout an event thought to drive selection, earlier findings have shown a clear signature of selection and a decrease in diversity of loci 'hitchhiking' with genes under selection in two populations of *Daphnia magna*, sampled though time (Orsini *et al.* 2012). This would imply neutral diversity is conserved through selection events and it is target loci that undergo the selection process under environmental change. Further studies should be conducted to confirm these findings in other species allowing these results to be applicable across taxa.

3.4.4 Conclusions

Neutral genetic diversity through time was examined in four sub-populations taken from a sediment core from Lake Ring before, during and after a eutrophication event. Neutral genetic variation was stable over time meaning it is possible standing genetic variation, and

the buffering effect of resting egg banks, allow this species to adapt and evolve to strong environmental selections pressures. It was also found hatched and unhatched sub-populations of *Daphnia magna*, from the same sediment core, show the same neutral genetic diversity and structure suggesting resting stages can be confidently used to represent dormant populations through time. This has means the results in Chapter 2 aren't affected by hatching bias. This also has wider implications for the use of resurrection ecology as a technique for *Daphnia magna*.

Chapter 4

Thesis Implications and Future Suggestions

Anthropogenic impact, in the form of climate change and agricultural run-off, is affecting species in freshwater ecosystems. This is of particular concern due to the diversity of organisms these systems harbour and their value as a drinking-water source. Species within these systems deploy a variety of mechanisms to cope with these environmental stressors including; migration away from the stressor, tolerance by producing dormant life stages, adaptation through phenotypic plasticity or adaptation through genetic change. Researchers try to understand how species cope with these stressors in order to; enforce/create protective policies, predict species future responses and identify populations able to adapt. Combinations of methods are employed to assess species responses.

The first experimental chapter of this thesis (Chapter 2) set out to employ a combination of experimental evolution and resurrection ecology to identify fitness responses in the keystone aquatic invertebrate, *Daphnia magna* to combinations of stressors. Over 30 individuals were resurrected from a lake sediment core, from before, during and after a eutrophication event. These sub-populations were subjected to a range of temperatures chosen to mimic the next 100 year predicted increase combined with either pesticide exposure or low food quality. Preliminary analysis of data found combinations of stressors effected fitness traits in line

with previous literature. There was one occurrence where the lowest temperature condition combined with pesticide exposure seemed to have a greater effect on fitness than the higher temperature conditions, when compared to the effect of each temperature without pesticide exposure. This result is currently only an observation and full statistical analysis will be carried out independently of the thesis as part of a bigger project.

It was expected that combined stressors would affect fitness traits, it was also expected that previous exposure to a given stressor would elicit a less detrimental fitness response compared to individuals being exposed for the first time. The analysis here did not find this. The population which experienced agricultural run-off did not significantly out perform the other two populations studied. This could be due generations of acclimation in the lab prior to experiments, purging any adaptive abilities inherited. It may also be that the original stressor was not strong enough to cause direct adaptation. Future work will carry out further statistical analysis as above. Whole genome and transcription sequencing will also be conducted on the experimental genotypes used to try to identify genetic differences that may underlie any fitness responses identified.

The use of resurrection ecology in Chapter 2 is a power technique to study species adaptive capabilities through time. However there was doubt as to weather selective hatching induces bias in the individuals used for study. It was thought only fitter individuals would hatch meaning resurrection ecology does not provide an accurate representation of the population. The second experimental chapter of this thesis (Chapter 3) explored neutral genetic diversity through the same sediment core in Chapter 2 in both hatched and unhatched individuals. Analysis of neutral microsatellite data revealed comparable genetic parameters such as allelic richness, effective population size and genetic structure. This strongly shows there is no hatching bias introduced in the use of resurrection ecology for *Daphnia magna* from the Lake Ring sediment core.

It was also expected that the pressure of eutrophication may have caused a shift in the genetic diversity observed. This was not found to be the case with genetic structure remaining constant through out and no loss in genetic diversity observed. This indicates *Daphnia* may use standing genetic diversity and/or the resting egg bank as a buffer to cope with

environmental stressors.

Overall *Daphnia* is an ideal species to identify adaptation to anthropogenic stressors. The clonal stage of the life cycle means it's possible to subject the same genotype to many different experimental conditions for comparable analysis. Here combinations of stressors show varied fitness responses on the entire population level and also on the sub-population level through time. Future studies should also focus on combinations of stressors to more accurately represent the natural environment.

The sexual stage of the life-cycle and the production of resting eggs also means it's possible to study whole populations through time. This thesis has shown the use of resurrection ecology provides an unbiased representation of *Daphnia* populations through time. Further research should use other species and study sites to ensure this finding is applicable across space and taxa.

Appendix A

Temperature Summary for the Three Lake Phases

Tables showing the fluctuation and average in annual temperature and highest recorded temperature during the time period for each sub-population of *Daphnia* resurrected in chapter 2. Time periods are shorter than those represented in the introduction because they represent the actual periods of time for which *Daphnia* samples were taken from rather than the lake period as a whole.

Table A.1: The minimum, maximum and mean highest temperature recorded per year for the time period of each sub-population sampled. Data was obtained from Samso meteorological station.

Sub-Population	**Time Period**	**Minimum Temperature (°C)**	**Maximum Temperature (°C)**	**Mean Temperature (°C)**
Recovery	2000-1992	28.70	35.10	32.08
Eutrophication	1977-1986	28.90	33.00	30.90
Pristine	1960-1968	27.30	33.20	30.78

Table A.2: The minimum, maximum and mean annual temperature recorded for the time period of each sub-population sampled. Data was obtained from Samso meteorological station.

Sub-Population	**Time Period**	**Minimum Temperature (°C)**	**Maximum Temperature (°C)**	**Mean Temperature (°C)**
Recovery	2000-1992	6.80	9.20	8.34
Eutrophication	1977-1986	6.50	8.40	7.40
Pristine	1960-1968	6.70	8.50	7.50

Appendix B

Pesticide Pilot Experiments

Methods

Carbaryl Dilution

The high and low carbaryl concentrations were determined by two pilot studies. The first study examined the effects of the following concentrations; 4μg/L, 8μg/L, 16μg/L and 32μg/L on brood timing, brood size and mortality. The second study examined the same effects using; 10μg/L, 12μg/L and 14μg/L concentrations.

Carbaryl stocks were created by dissolving 32mg of carbaryl in 1ml of 100% ethanol. 100μl of this was diluted into 100ml of distilled water to create a stock solution. 100μl of this stock was added to the 100ml of mineral water in the jars of each experimental animal, giving a final exposure concentration of 32μg/L.

Serial dilutions were carried out to achieve the other stock concentrations of: 4μg/L, 8μg/L, 16μg/L in experiment one and 10μg/L, 12μg/L and 14μg/L for experiment two.

Study Animals

In both experiments 18 individuals were exposed to the above-mentioned conditions and to a control condition with no carbaryl. Each group of 18 consisted of three replicates of six different genotypes, two from each of the lake phases; 0.5_3 and 3_5 (recovery phase), 7_3 and 7_6 (eutrophic phase), 12_2 and 15.5_1 (pristine phase).

Experimental Set-up

All animals were maintained in individual 100ml jars of mineral water, fed ab libtum Chlorella algae and kept under a long day photoperiod, 16:8 L:D at 20°C. Water was changed every other day for all conditions and fresh carbaryl added according to exposure. Time of the first brood, time of the second brood, number of neonates in the first brood and number of neonates in the second brood were recorded until the day the second brood was released or 21 days, which ever was soonest.

Results

Experiment One

Mortality was extremely high in both the 16μg/L and 32μg/L condition. There was also some mortality in the 8μg/L condition however this was similar to the control condition (Figure B.1). The time to produce both the first and second brood increased with increasing carbaryl concentrations (Figure B.2). The number of neonates in both the first and second brood were seen to decrease with increasing carbaryl concentrations. Of only two surviving individuals (both genotype 15.5_1) in the 16μg/L condition, both produced a first brood and only one of those went on to produce a second brood.

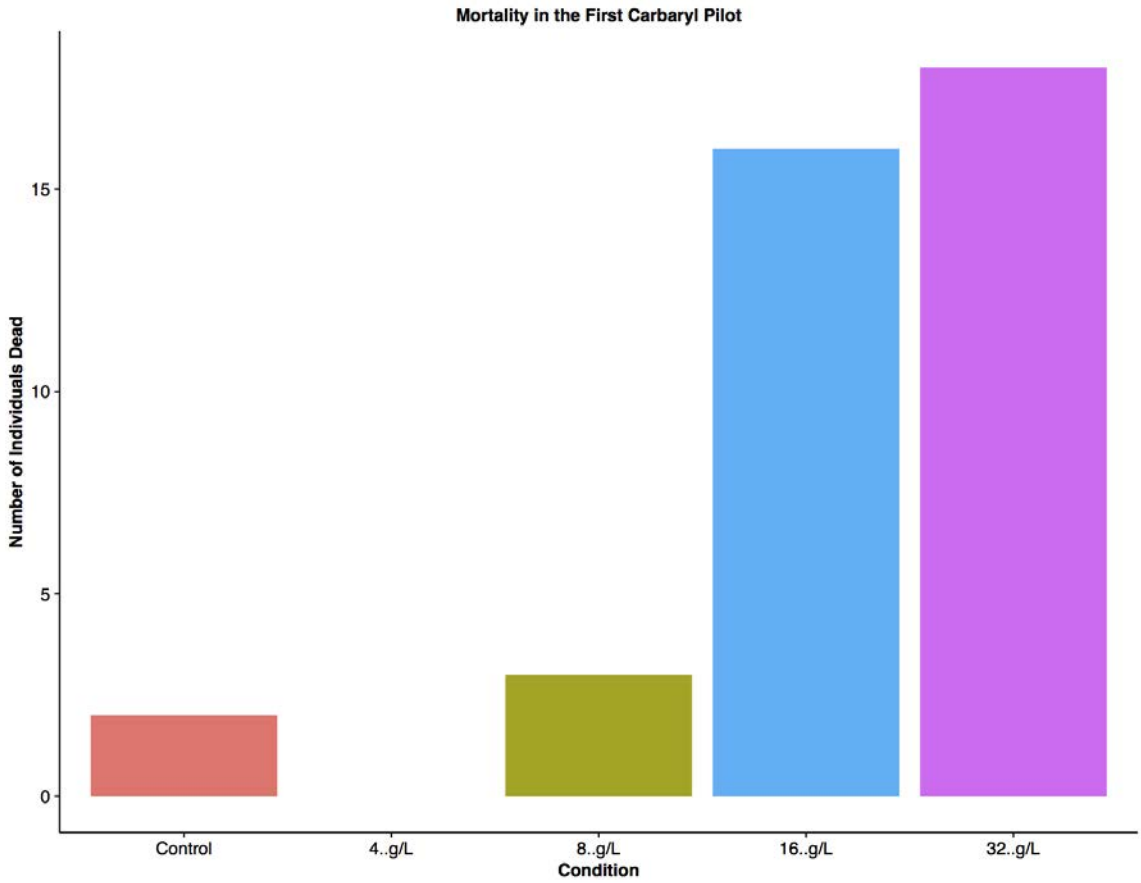

Figure B.1: Bar plot showing total mortality in the first carbaryl pilot experiment for each concentration, N=18 for each condition.

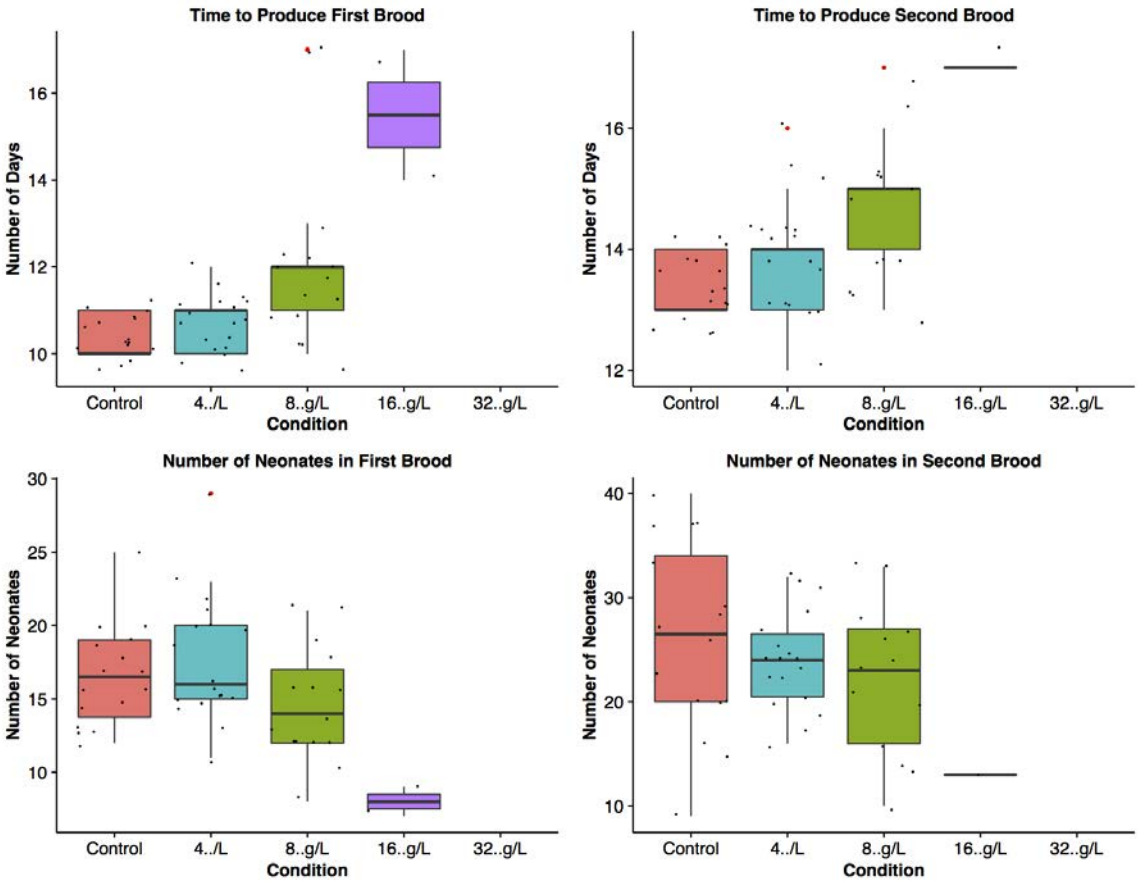

Figure B.2: Box-plots showing the time and number of neonates for the first and second brood in each carbaryl condition for the first experiment.

Experiment Two

Some mortality was seen in all three carbaryl conditions but none in the control. Both 12μg/L and 14μg/L show more mortality that 10μg/L (Figure B.3). The 10μg/L and 12μg/L show similar times to produce both first and second brood (slightly more delayed than the control), however 14μg/L shows a high variation in time to produce broods, the median being higher than the two lower carbaryl conditions (Figure B.4). The number of neonates in the first brood can be seen to decrease with increasing carbaryl concentrations, however the number of neonates in the second brood can be seen to be higher than the control in the 10μg/L condition and lowest overall in the 12μg/L condition (Figure B.4).

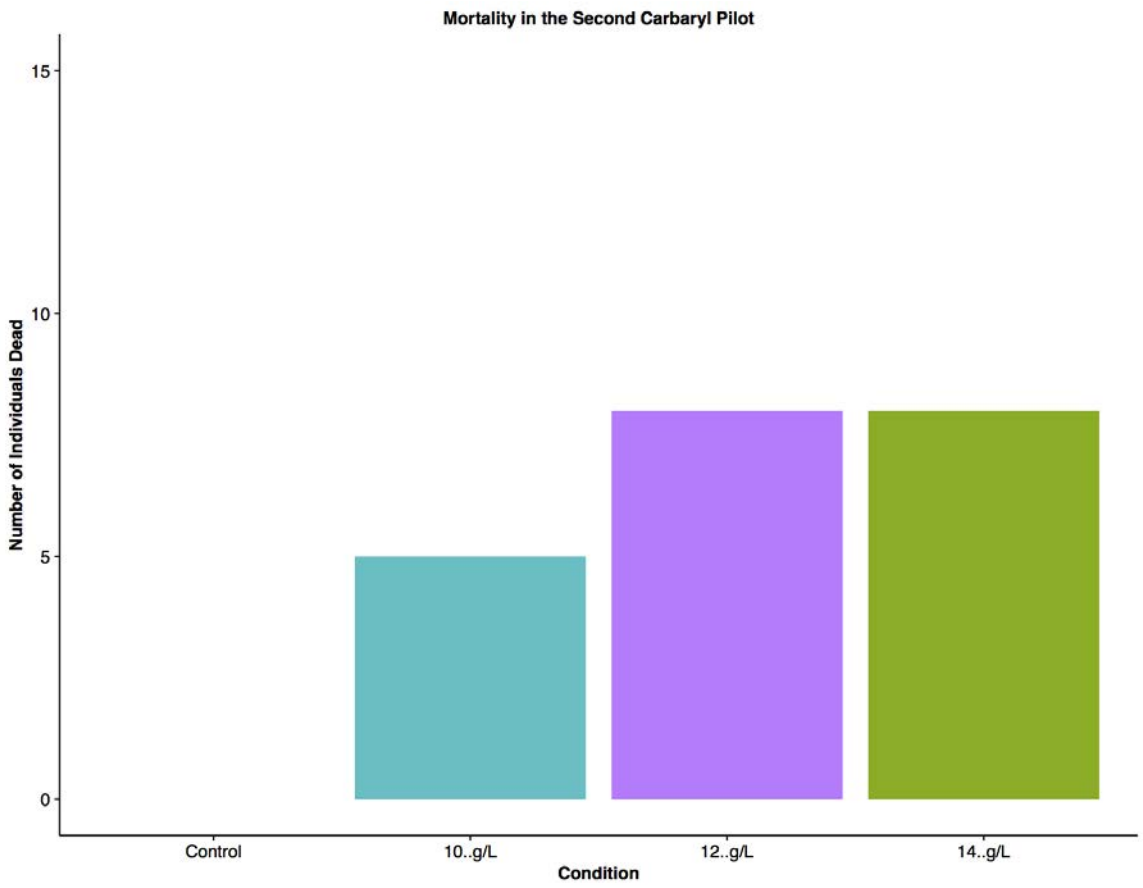

Figure B.3: Bar plot showing total mortality in the second carbaryl pilot experiment for each concentration, N=18 for each condition.

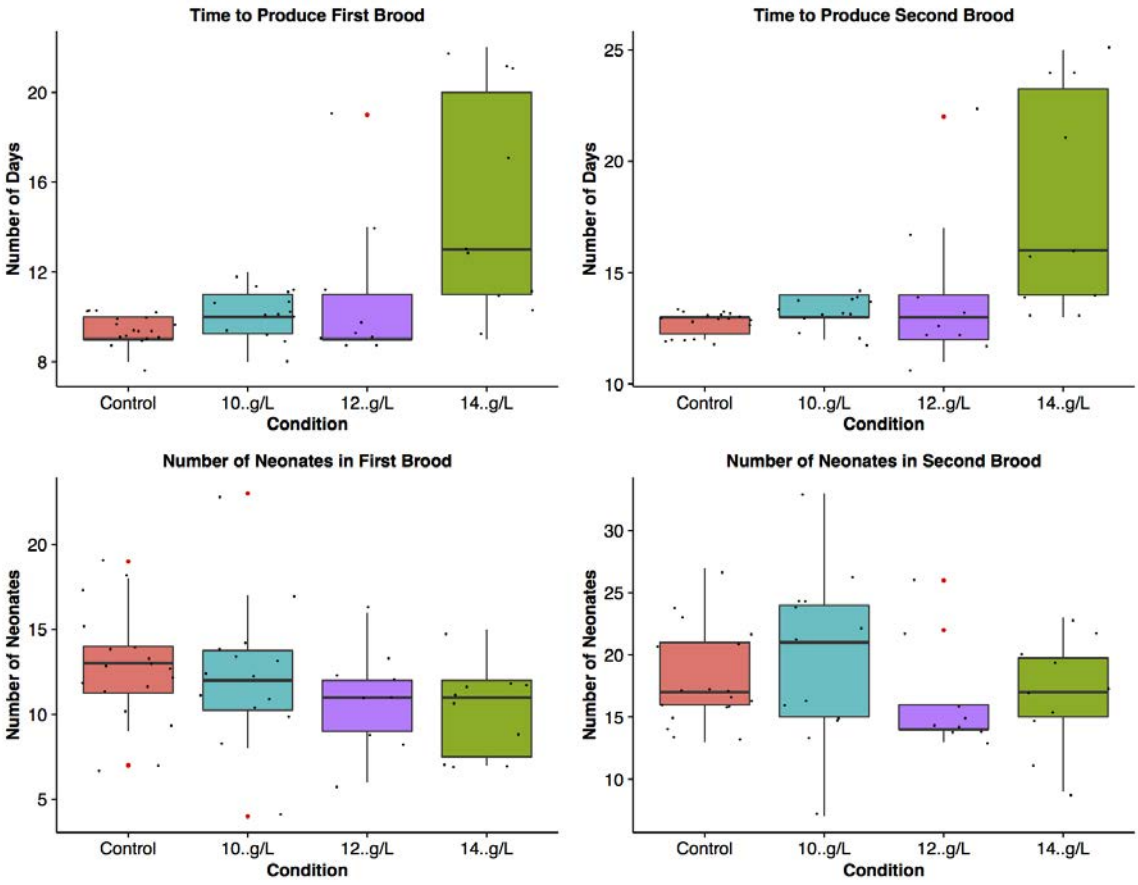

Figure B.4: Box-plots showing the time and number of neonates for the first and second brood in each carbaryl condition for the second experiment.

Conclusions

As seen in the first experiment mortality was particularly high in both of the high concentrations tested (16μg/L and 32μg/L) therefore a second experiment was carried out to determine a more suitable 'high' concentration. The aim of the main experiment is to assess sub-lethal concentrations on fitness related traits when combined with another stressor; temperature, therefore a concentration giving high mortality is not suitable. As mortality was still seen to be quite high in both the 12μg/L and 14μg/L conditions, the 10μg/L condition was selected along with the 4μg/L to give a big enough difference between the two conditions.

It is clear higher concentrations of pesticide cause longer time for brood production in Daph-

nia as well as smaller brood sizes. It is likely this is the result of a trade-off, decreasing reproduction to free more energy for toxin breakdown and survival.

Appendix C

Food Quantity Pilot Experiments

Methods

Algae Preparation

Two concentrations of phosphorous in algae were tested to determine the low food quantity condition: 2mM and 5mM of phosphorous along with a control of 50mM of phosphorous.

Scenedesmus acutus algae were cultured in COMBO media, as in Kilham et al. (1998). However in order to create the different phosphorous concentrations the following was adjusted: either 5ml of 50mM, 5mM or 2mM potassium phosphate was added, with an extra 5ml of potassium chloride added to the 5mM and 2mM.

150ml of previously cultured algae (at 50mM phosphorus) was inoculated into three flasks containing 1350ml of one of the above COMBO concentrations. After 3 days 40% of the medium was removed from each flask and a fresh 40% added containing the correct phosphorous levels. This method was maintained throughout the pilot experiment to ensure fresh algae were used. The concentration of algae fed to the animals was also maintained throughout by using a spectrophotometer to measure optical density at 400nm.

Study Animals

42 individuals were exposed in the 2mM, 5mM and 50mM condition. Each group consisted of seven replicates of six different genotypes, two from each lake phase: 0_3 and 3_5 (recovery phase), 7_3 and 7_6 (eutrophic phase), 12_2 and 15.5_1 (pristine phase).

Experimental Set-Up

Seven individuals of each genotype were maintained in 250ml jars of mineral water and kept under a long day photoperiod, 16:8 L:D at 20°C. Water was changed every other day for all conditions and 1.2ml of fresh algae were added with every water change. Mortality, time of the first brood, number of neonates in the first brood and number of neonates in the second brood were recorded until the day the second brood was released or 21 days, which ever was soonest.

Results

Mortality was highest in the 2mM condition at around 50%. Some mortality was also seen in the control and 5mM condition, the 5mM condition being slightly higher (Figure C.1). No reproduction was seen in the 2mM condition and only one individual produced a very small number of neonates in the 5mM condition and this was considerably later than first brood production seen in the 50mM condition (Figure C.2).

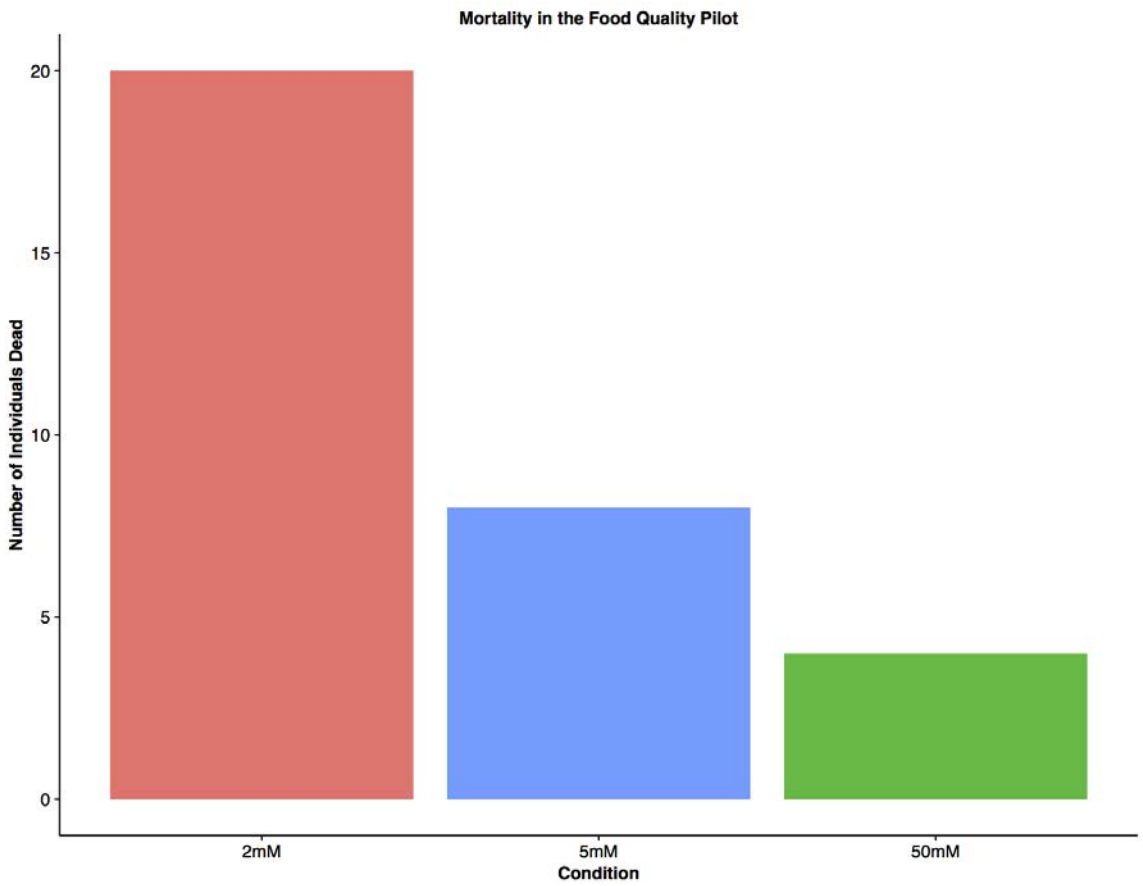

Figure C.1: Bar plot showing total mortality in the food quality pilot experiment for each concentration, N=18 for each condition.

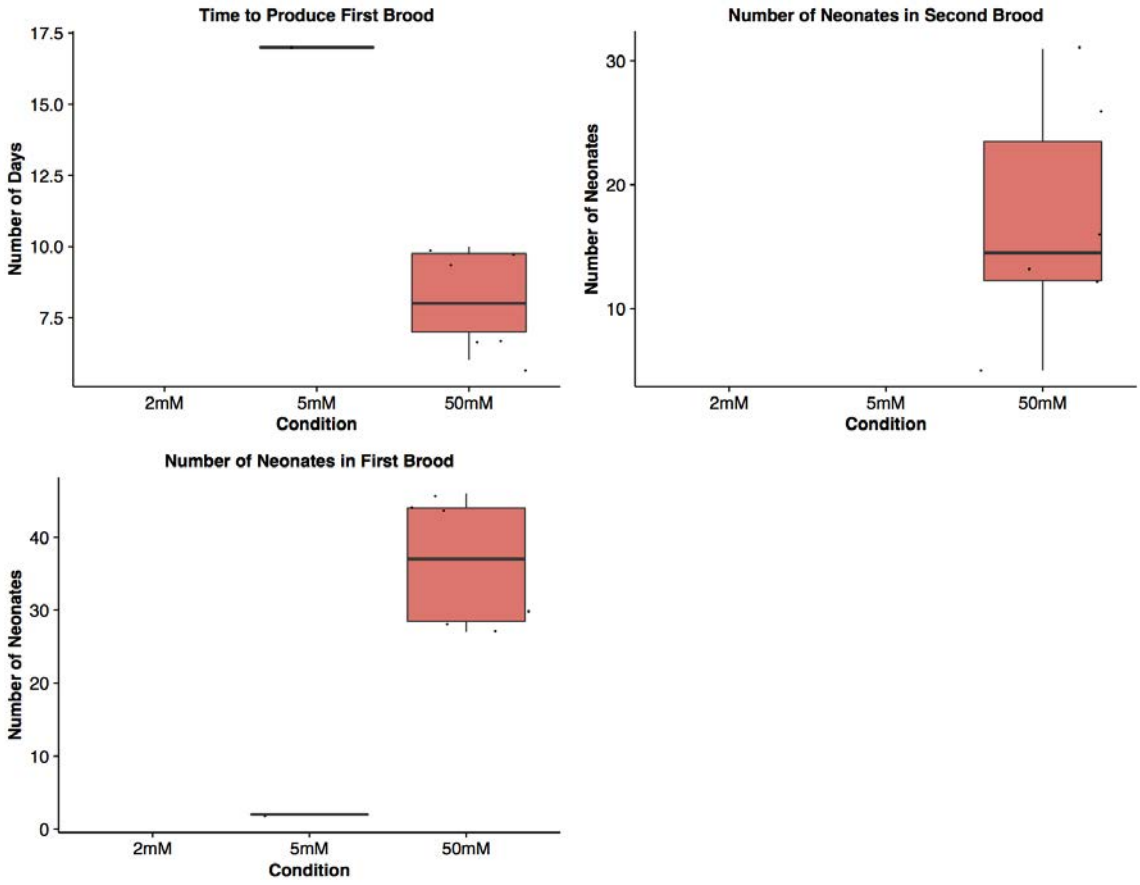

Figure C.2: Box-plots showing the time of the first brood and number of neonates for the first and second brood in each food condition.

Conclusions

It can be seen here that concentrations of phosphorous at 2mM and 5mM in algae massively affect reproduction and survival in *Daphnia magna*, with only one individual reproducing at the 5mM concentration. It is likely this is due to the lack of nutrients, meaning there are no spare resources for reproduction and any nutrients obtained need to be used primarily for survival.

Low phosphorous levels were chosen initially in order to mimic low food conditions as described in previous literature (Persson *et al.* 2011). However the actual concentrations used were determined in a later pilot experiment (carried out by Maria Cuenca Cambronero),

carbon levels in the algae were determined by combustion analysis by Medac Ltd, the level of carbon in algae more accurately indicates the food quantity when feeding specifically to *Daphnia* (Persson *et al.* 2011).

Appendix D

Graphs Not Referenced to in Text

Various graphs used to visualise data from all experiments, which were not referenced to in the main body of text. They also show phenotypic traits recorded throughout the experiments but not analysed further in this thesis.

Temperature Experiment

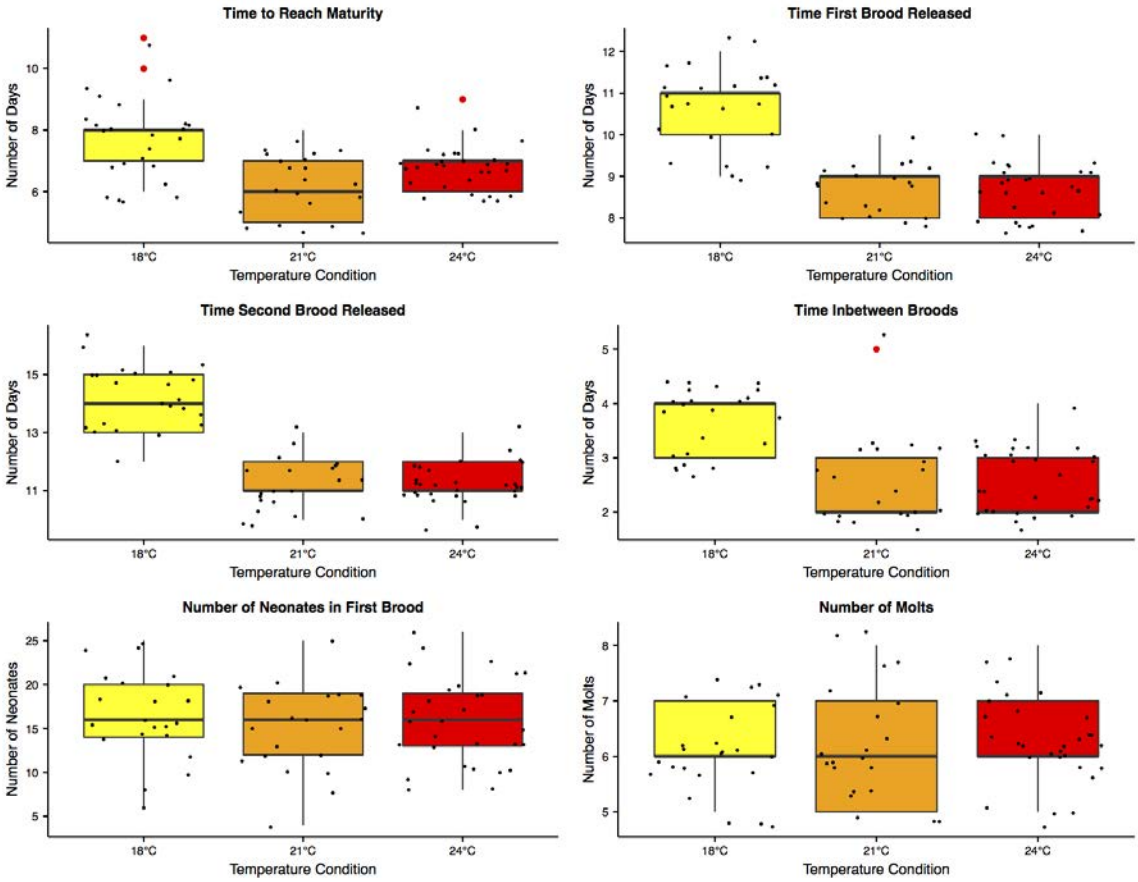

Figure D.1: Box-plots showing six of the nine recorded traits for all individuals in the temperature experiments.The other three graphs can be seen in the Chapter 2.

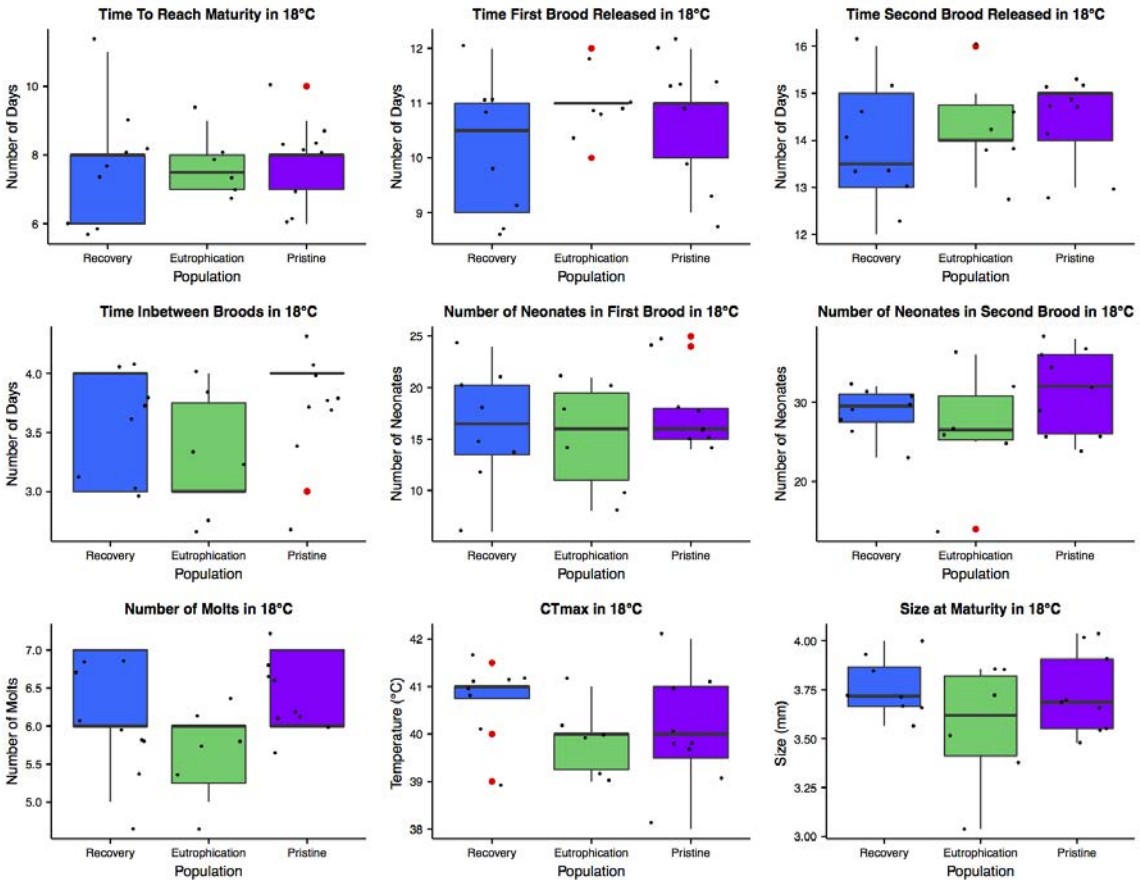

Figure D.2: Box-plots showing population level data for nine recorded traits in the 18°C temperature experiment.

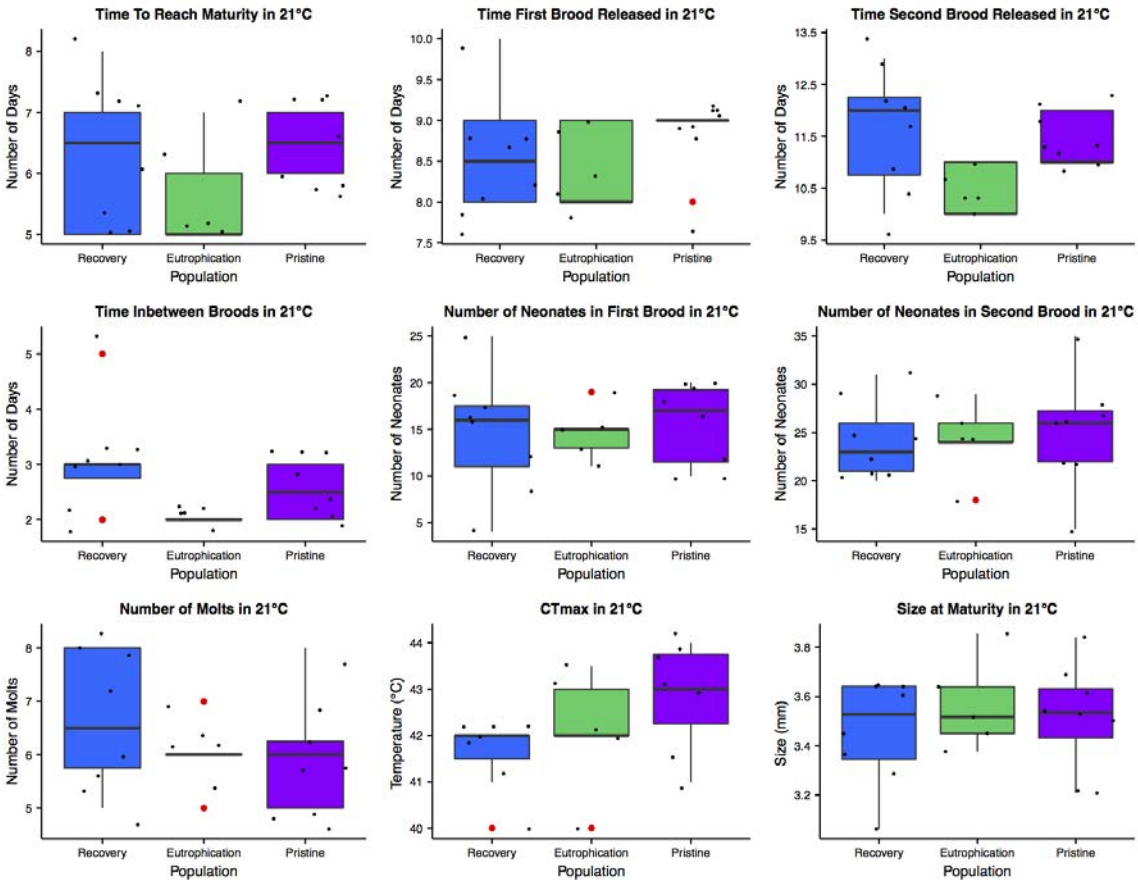

Figure D.3: Box-plots showing population level data for nine recorded traits in the 21°C temperature experiment.

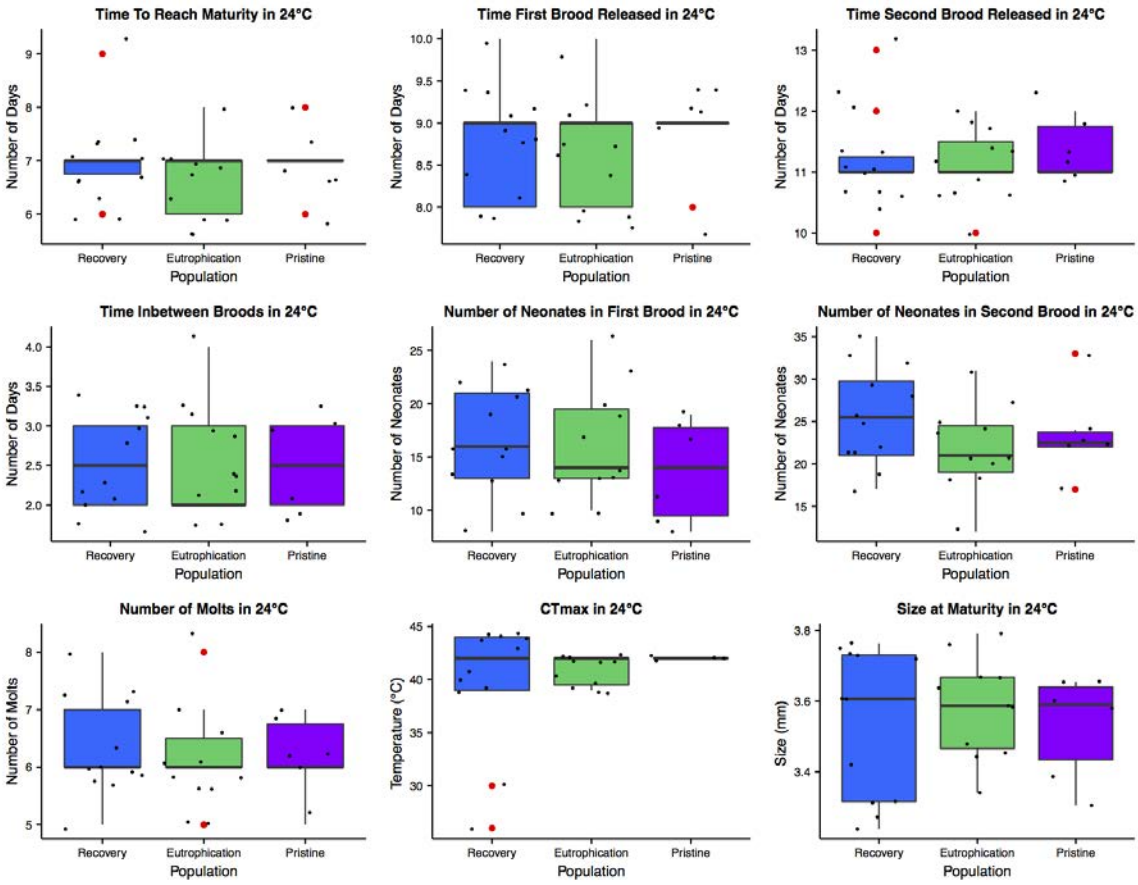

Figure D.4: Box-plots showing population level data for nine recorded traits in the 24°C temperature experiment.

Pesticide and Temperature Combined

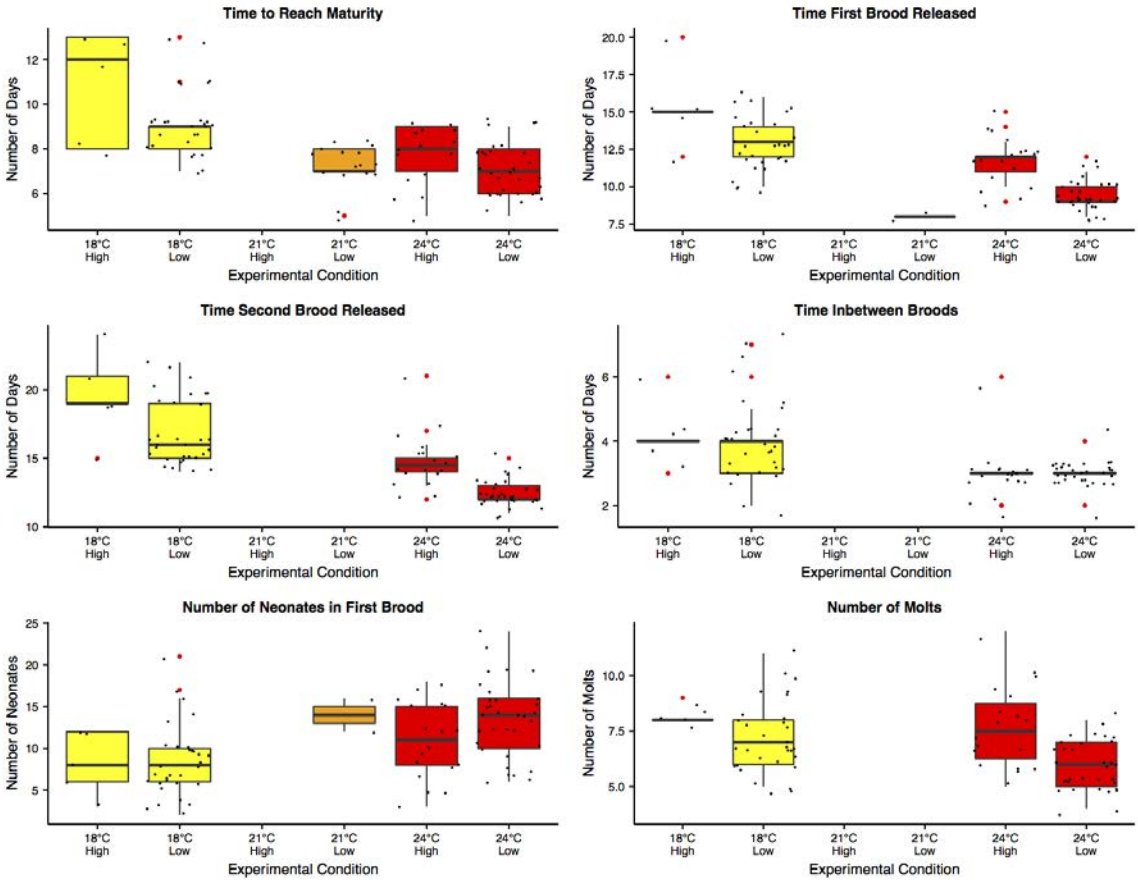

Figure D.5: Box-plots showing population level data for six of the nine recorded traits in the temperature and pesticide experiment.

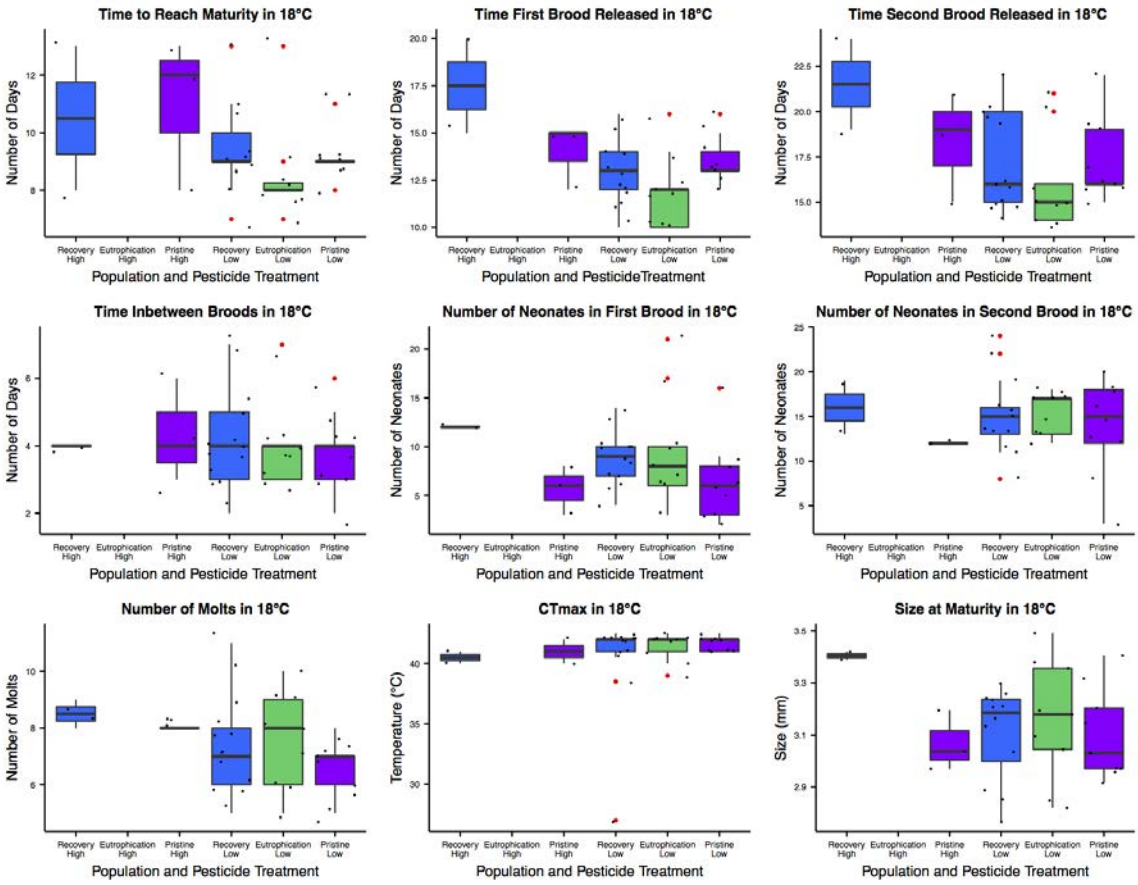

Figure D.6: Box-plots showing population level data for nine recorded traits in the 18°C temperature and pesticide experiment.

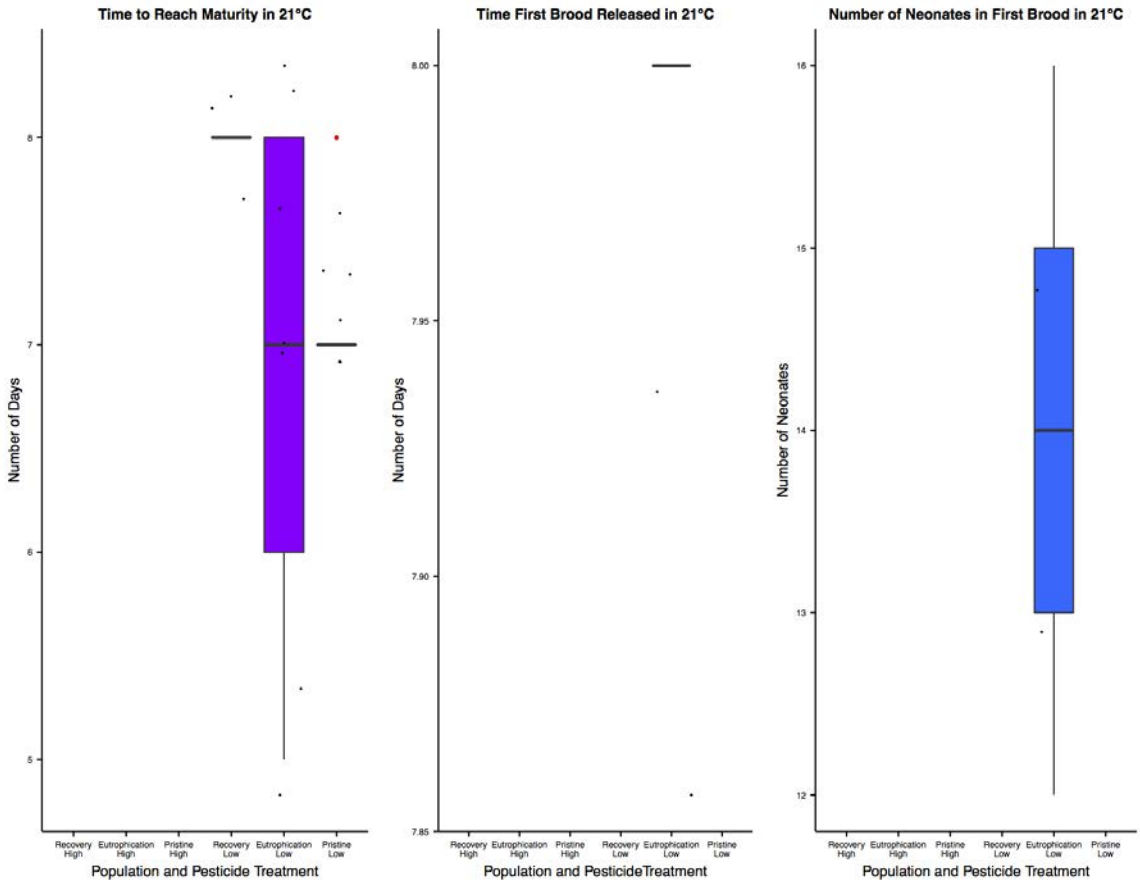

Figure D.7: Box-plots showing population level data for three recorded traits in the 21°C temperature and pesticide experiment. Note not all traits are shown as the experiment was stopped early due to incubator failure.

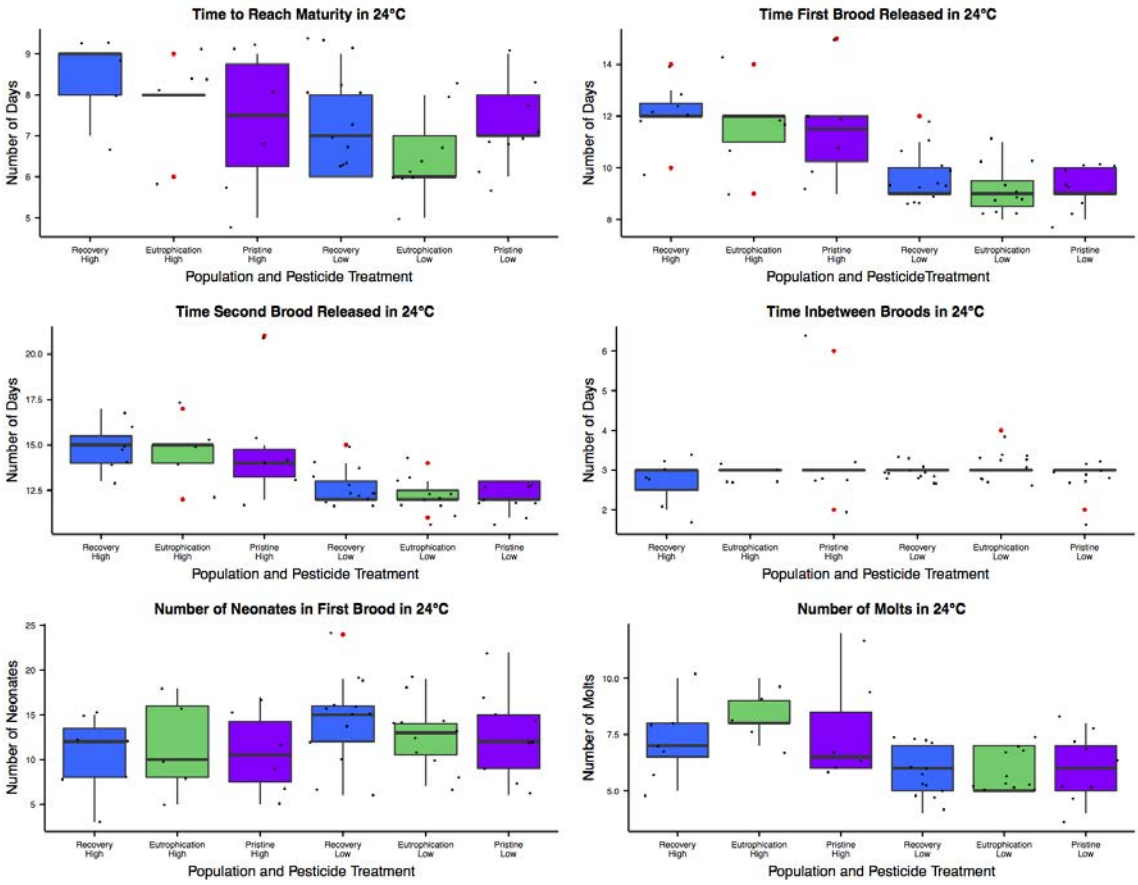

Figure D.8: Box-plots showing population level data for six of the nine recorded traits in the 24°C temperature and pesticide experiment. The other three graphs can be seen in the Chapter 2.

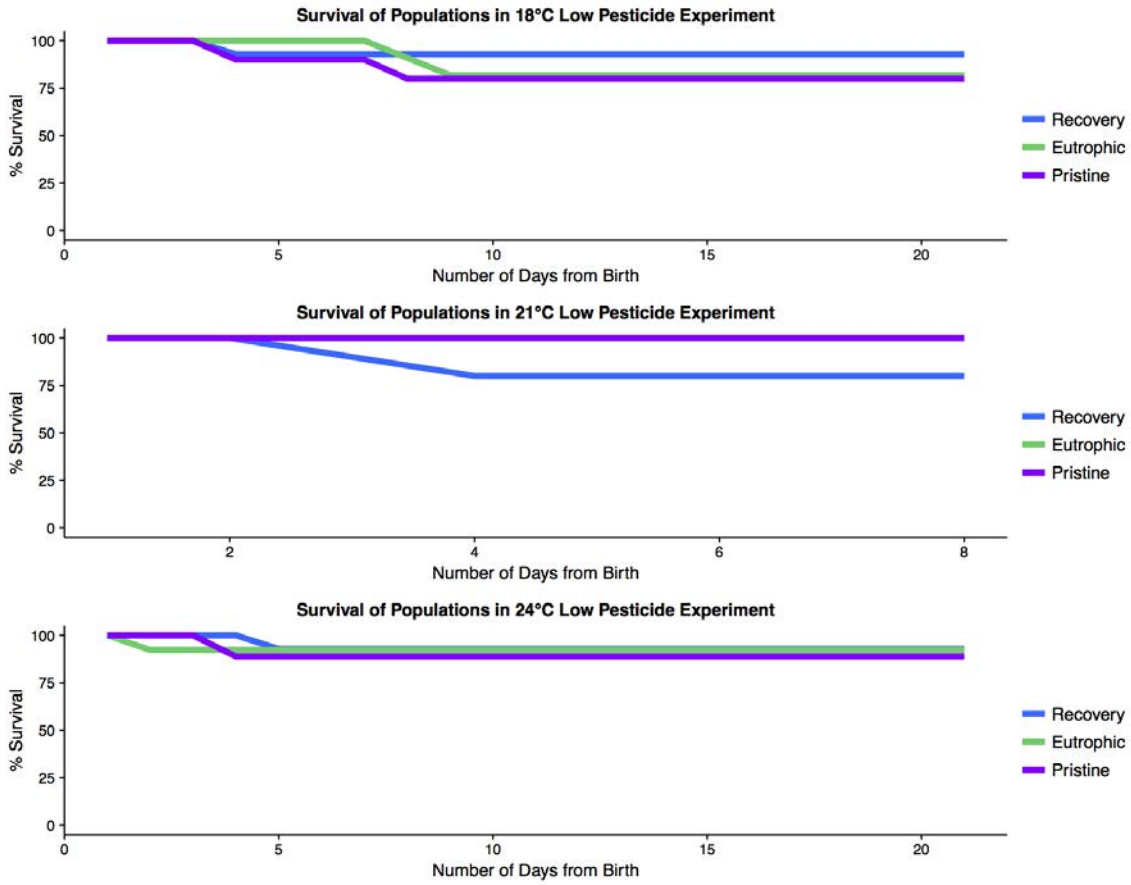

Figure D.9: Cumulative mortality for each population across each temperature regime for the low pesticide treatment. For 18°C: Recovery N=14, Eutrophic N=12 and Pristine N=11. For 21°C: Recovery N=11, Eutrophic N=13 and Pristine N=11. For 24°C: Recovery N=14, Eutrophic N=13 and Pristine N=10.

Food Quality and Temperature Combined

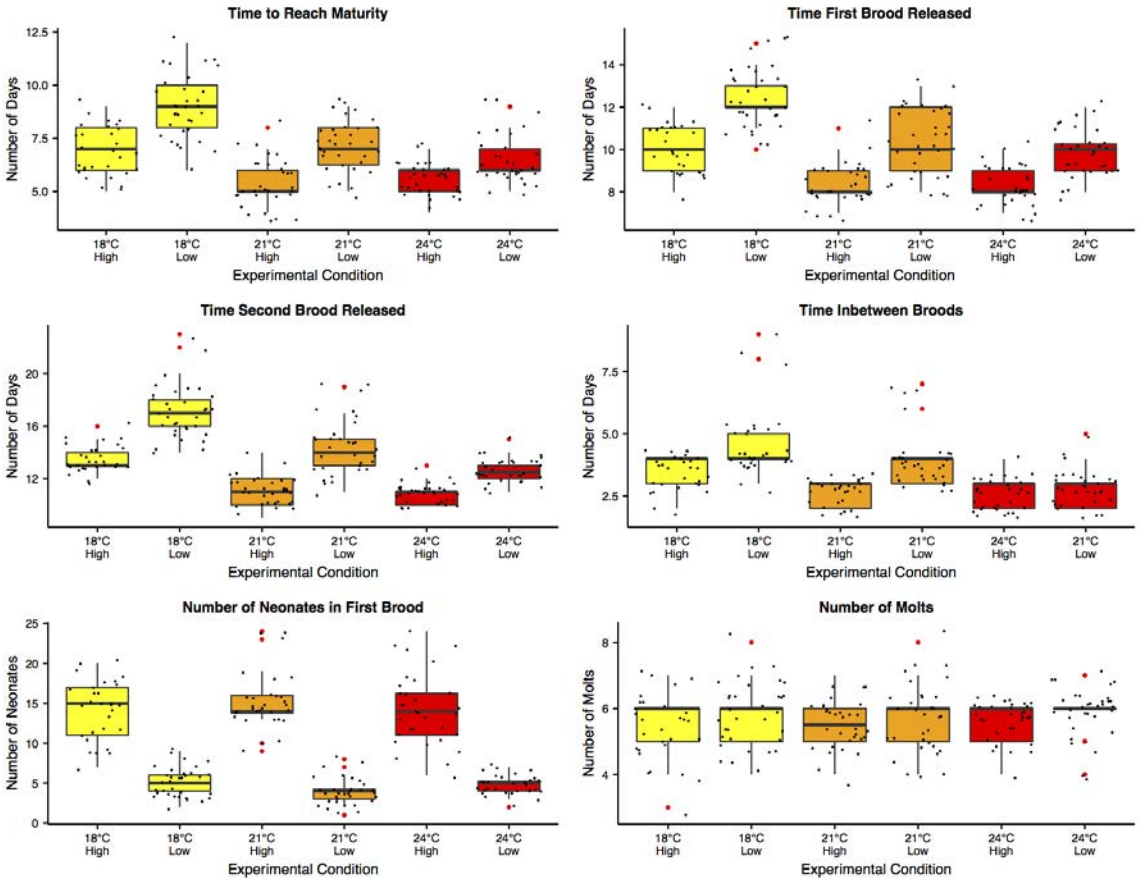

Figure D.10: Box-plots showing data for six of the nine recorded traits in the temperature and food quality experiments for all individuals.The other three graphs can be seen in the Chapter 2.

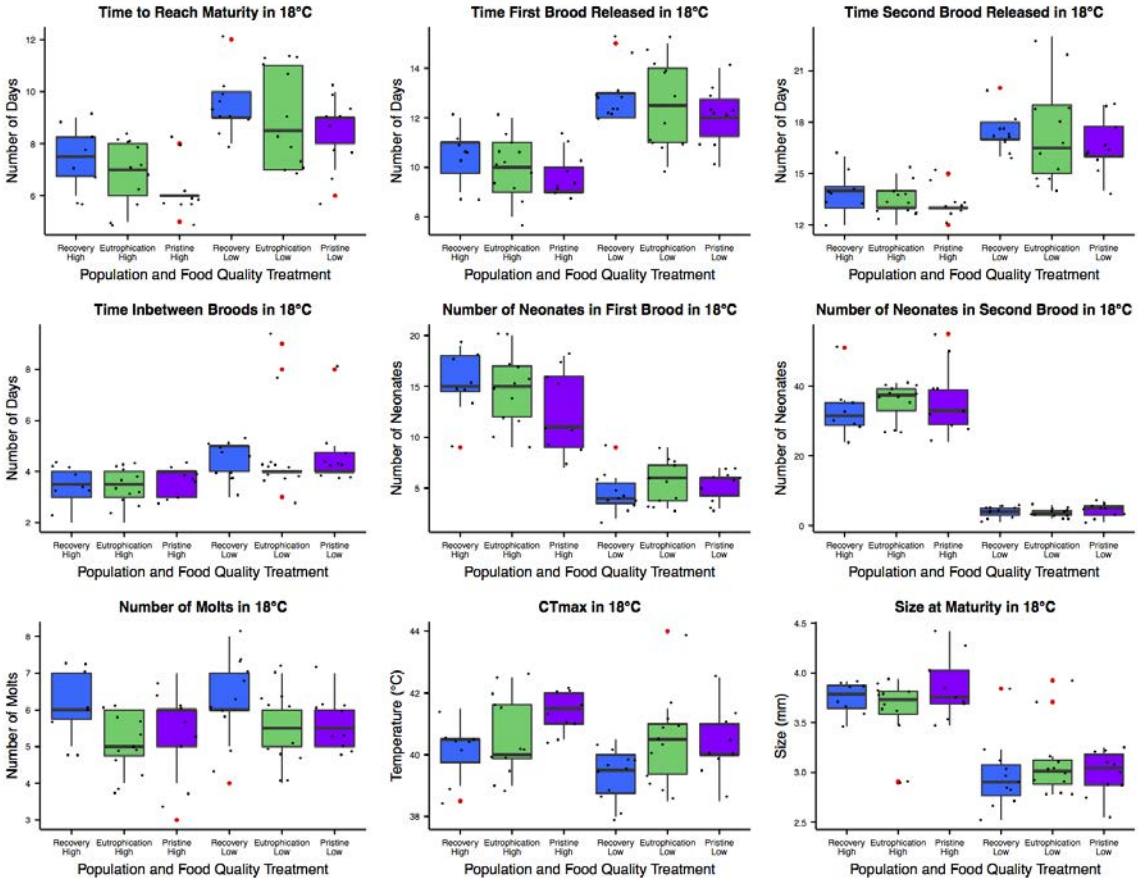

Figure D.11: Box-plots showing population level data for nine recorded traits in the 18°C temperature and pesticide experiment.

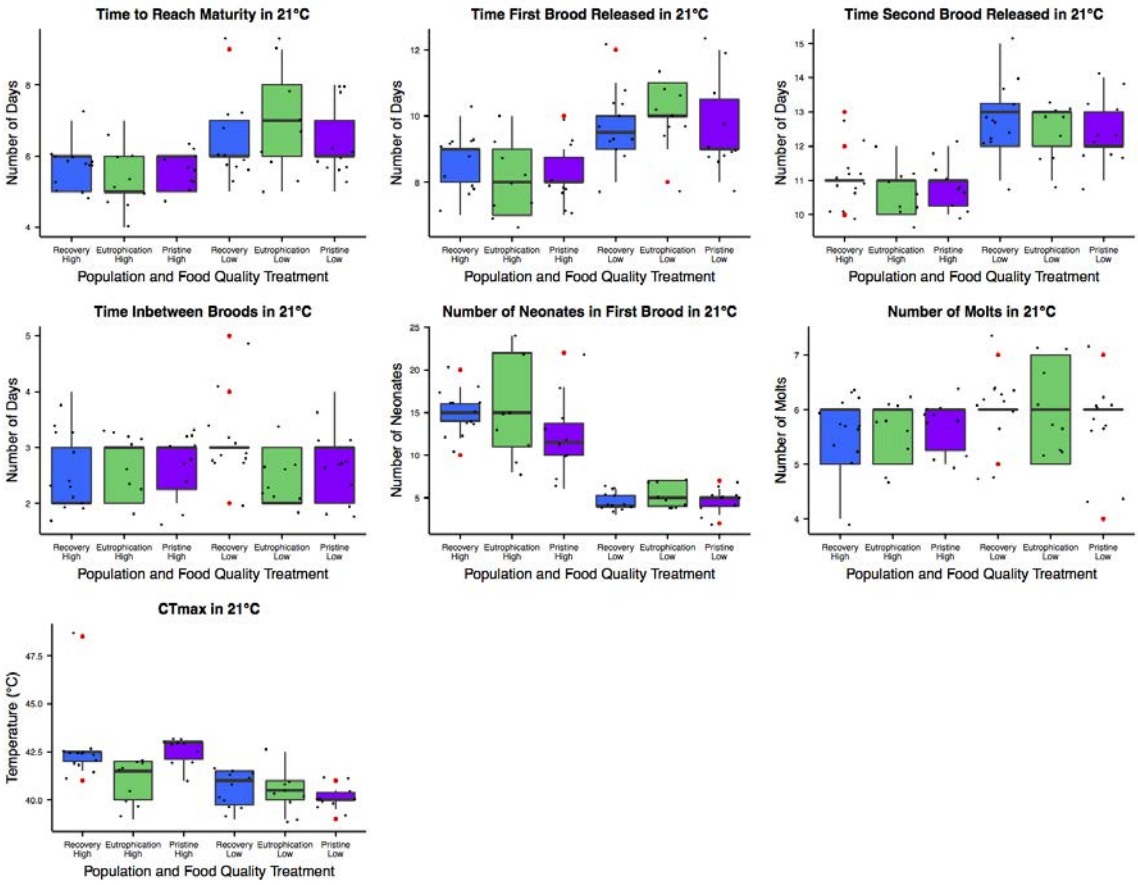

Figure D.12: Box-plots showing population level data for seven of nine recorded traits in the 21°C temperature and pesticide experiment. The other two graphs can be seen in the Chapter 2.

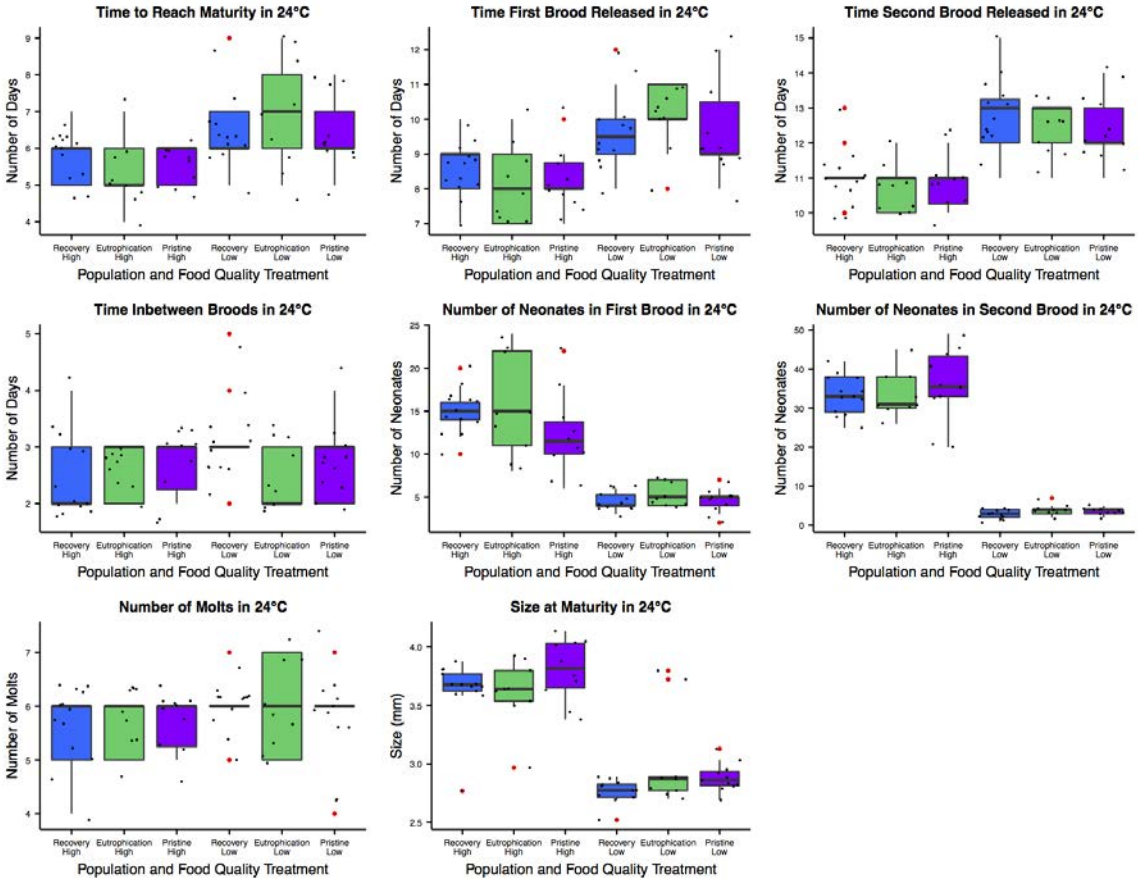

Figure D.13: Box-plots showing population level data for eight of nine recorded traits in the 24°C temperature and pesticide experiment. The other graph can be seen in the Chapter 2.

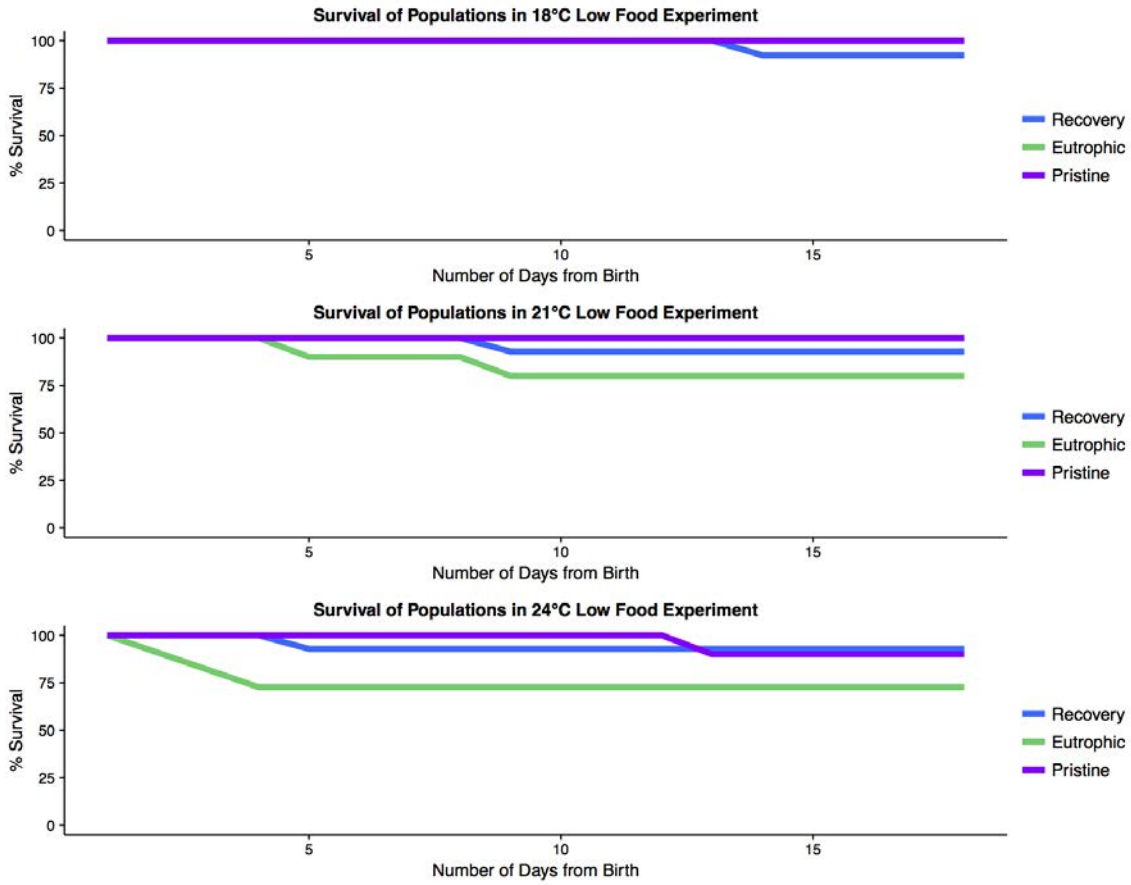

Figure D.14: Cumulative mortality for each population across each temperature regime for the low food quality treatment. For 18°C: Recovery N=13, Eutrophic N=12 and Pristine N=10. For 21°C: Recovery N=14, Eutrophic N=10 and Pristine N=11. For 24°C: Recovery N=14, Eutrophic N=11 and Pristine N=11.

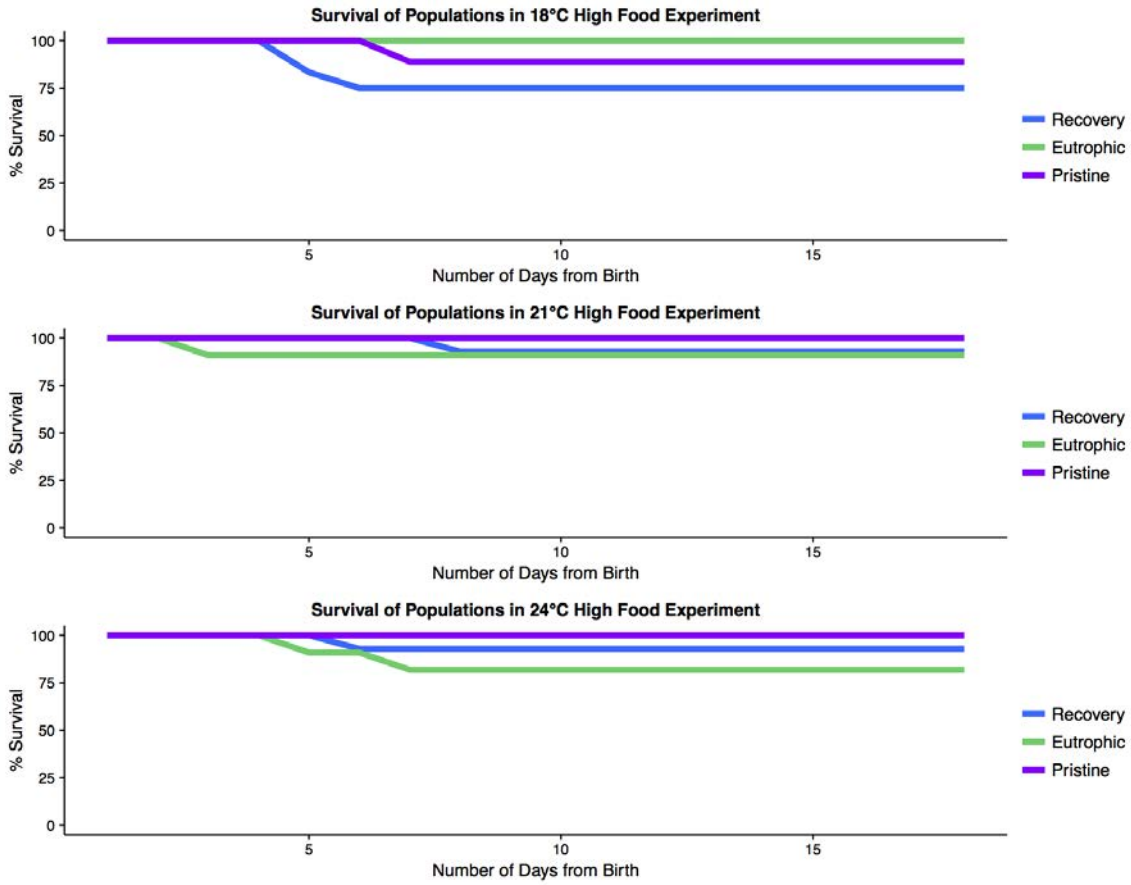

Figure D.15: Cumulative mortality for each population across each temperature regime for the high food quality treatment. For 18°C: Recovery N=13, Eutrophic N=12 and Pristine N=10. For 21°C: Recovery N=14, Eutrophic N=10 and Pristine N=11. For 24°C: Recovery N=14, Eutrophic N=11 and Pristine N=11.

Appendix E

DNA Extraction Procedure

Agencourt DNAdvance Optimised Procedure

Sample Preparation- Whole Daphnia

- Place 3x *Daphnia* into a 1.5ml eppendorf tube
- Freeze in liquid nitrogen
- Crush *Daphnia* immediately with a pestle inside the tube
- Proceed to overnight digestion

Sample Preparation- Resting Egg

- Open an ephippium under a dissecting microscope with tweezers
- Use a 1ml pipette to extract a single egg
- Place egg inside the Mastermix created in the 'Overnight Digestion' step below
- Burst the egg using a pipette tip

Overnight Digestion

- Create Lysis Mastermix (quantities per sample):
  - Lysis Buffer 188μl
  - 1M DTT 5μl
  - Proteinase K (40mg/ml) 7μl
- Add 200μl of Mastermix to each sample
- Incubate overnight (18-20hours) at 55°C shaking 100rpm

RNase Step

- Add 4μl of 5mg/μl to each sample
- Pipette to mix
- Incubate for 30mins at 37°C shaking 100rpm

DNA Binding

- Spin sample at 14,000rpm for 2mins
- Carefully remove 180μl of supernatant into a fresh tube leaving a pellet of debris behind
- Add 170μl of Bind1 buffer and pipette 10 times to mix
- Add 170μl of Bind2 buffer and pipette 15 times to mix
- **Shake Bind2 buffer well before each time you pipette and mix slowly to avoid air bubbles**
- Incubate at room temp for 1min
- Put on magnet for 4mins

- Discard supernatant while tubes are on the magnet

Ethanol Washing

- Take samples off magnet
- Add 340μl (freshly made) 70% ethanol
- Pipette 20 times to re-suspend beads
- Place back on magnet for 1min
- Remove ethanol without touching beads
- Repeat the above steps twice more for 3 washed total

DNA Elution

- Remove as much ethanol as possible- **Very Important**
- Leave tubes with lids open to air dry for 5mins covered over
- Warm elution buffer to 37°C before using
- Add 50μl of buffer and re-suspend the beads, pipette 10 times
- Place back on magnet for 5mins
- Transfer 43μl of supernatant into a fresh tube

Appendix F

PCR Multiplex Information

Autosomal Microsatellite Multiplexes

M01		
	1 reaction	100 reactions
Qiagenmix	5.00	500.00
B050 (20µM)	0.30	30.00
B064 (20µM)	0.10	10.00
B074 (20µM)	0.10	10.00
B045 (20µM)	0.10	10.00
B030 (20µM)	0.10	10.00
B107 (20µM)	0.15	15.00
B008 (20µM)	0.10	10.00
B096 (20µM)	0.05	5.00
RNase-free water	3.00	300.00
DNA	1.00	100.00
	10.0	1000.00

Primer	Label	Range
B050	6FAM	230 - 250
B064	6FAM	130 - 160
B074	NED	190 - 210
B045	NED	110 - 130
B030	PET	145 - 160
B107	PET	250 - 274
B008	VIC	160 - 175
B096	VIC	225 - 245

PCR	
Temp	Time
95 °C	15' 00''
94 °C	00' 30''
56 °C	01' 30''
72 °C	01' 30''
60 °C	30' 00''
4 °C	pause
Cycles	30

Figure F.1: M01 Multiplex and PCR Reaction.

M02		
	1 reaction	100 reactions
Qiagenmix	5.00	500.00
B031(20µM)	0.10	10.00
B065(20µM)	0.10	10.00
B088(20µM)	0.10	10.00
B174(20µM)	0.10	10.00
B075(20µM)	0.15	15.00
B155(20µM)	0.10	10.00
B135(20µM)	0.05	5.00
RNase-free water	3.30	330.00
DNA	1.00	100.00
	10.0	1000.00

Primer	Label	Range
B031	6FAM	98
B065	6FAM	184
B088	NED	180
B174	NED	276
B075	PET	176
B155	PET	290
B135	VIC	187

PCR	
Temp	Time
95 °C	15' 00''
94 °C	00' 30''
56 °C	01' 30''
72 °C	01' 30''
60 °C	30' 00''
4 °C	pause
Cycles	30

Figure F.2: M02 Multiplex and PCR Reaction.

M03		
	1 reaction	100 reactions
Qiagenmix	5.00	500.00
B010 (20µM)	0.30	30.00
B164 (20µM)	0.30	30.00
B133 (20µM)	0.30	30.00
B150 (20µM)	0.30	30.00
A001 (20µM)	0.30	30.00
RNase-free water	2.50	250.00
DNA	1.00	100.00
	10.0	

Primer	Label	Range
B010	NED	124
B164	NED	211
B133	PET	179
B150	VIC	190
A001	VIC	415

PCR	
Temp	Time
95 °C	15' 00''
94 °C	00' 30''
54 °C	01' 30''
72 °C	01' 30''
60 °C	30' 00''
4 °C	pause
Cycles	30

Figure F.3: M03 Multiplex and PCR Reaction.

M05					
	1 reaction	100 reactions	Primer	Label	Range
Qiagenmix	5.00	500.00	B087	6FAM	193
B087(20µM)	0.30	30.00	A002	6FAM	279
A002 (20µM)	0.30	30.00	B033	NED	92
B033 (20µM)	0.30	30.00	B011	PET	204
B011 (20µM)	0.30	30.00	B052	PET	297
B052 (20µM)	1.00	100.00	B180	VIC	309
B180 (20µM)	0.30	30.00			
RNase-free water	1.50	150.00			
DNA	1.00	100.00			
	10.0				

PCR	
Temp	Time
95 °C	15' 00''
94 °C	00' 30''
54 °C	01' 30''
72 °C	01' 30''
60 °C	30' 00''
4 °C	pause
Cycles	30

Figure F.4: M05 Multiplex and PCR Reaction.

M06					
	1 reaction	100 reactions	Primer	Label	Range
Qiagenmix	5.00	500.00	B097	6FAM	268
B097(20µM)	0.40	40.00	A009	NED	256
A009(20µM)	0.40	40.00	B021	VIC	168
B021(20µM)	0.40	40.00	B081	PET	199
B081(20µM)	0.40	40.00	B168	VIC	404
B168(20µM)	0.40	40.00	B179	VIC	350
B179(20µM)	0.40	40.00			
RNase-free water	1.60	160.00			
DNA	1.00	100.00			
	10.0	1000.00			

PCR	
Temp	Time
95 °C	15' 00''
94 °C	00' 30''
56 °C	01' 30''
72 °C	01' 30''
60 °C	30' 00''
4 °C	pause
Cycles	30

Figure F.5: M06 Multiplex and PCR Reaction.

EST-Linked Microsatellite Multiplexes

ESTM01					
	1 reaction	100 reactions	Primer	Label	Range
Qiagenmix	5.00	500.00	2936	6FAM	263-268
2936 (20µM)	0.30	30.00	8344	6FAM	116-130
8344 (20µM)	0.30	30.00	4276	NED	279-307
4276 (20µM)	0.30	30.00	5005	NED	119-128
5005 (20µM)	0.30	30.00	6166	VIC	164-173
6166 (20µM)	0.30	30.00	9235	VIC	311-314
9235 (20µM)	0.30	30.00	8608	PET	290-302
8608 (20µM)	0.30	30.00	1508	PET	137-145
1508 (20µM)	0.40	40.00			
RNase-free water	1.50	150.00			
DNA	1.00	100.00			
	10.00	1000.00			

PCR	
Temp	TIME
95 °C	15' 00''
94 °C	00' 30''
54 °C	01' 30''
72 °C	01' 30''
60 °C	30' 00''
4 °C	pause
Cycles	**30**

Figure F.6: ESTM01 Multiplex and PCR Reaction.

ESTM02					
	1 reaction	100 reactions	Primer	Label	Range
Qiagenmix	5.00	500.00	2528	NED	314-318
2528 (20µM)	0.30	30.00	5389	NED	176-180
5389 (20µM)	0.30	30.00	3015	6FAM	313-317
3015 (20µM)	0.30	30.00	8711	6FAM	173-179
8711 (20µM)	0.30	30.00	6310	VIC	313-325
6310 (20µM)	0.30	30.00	8371	PET	186-205
8371 (20µM)	0.30	30.00	9489	PET	315-318
9489 (20µM)	0.30	30.00			
RNase-free water	1.90	190.00			
DNA	1.00	100.00			
	10.00	1000.00			

PCR	
Temp	TIME
95 °C	15' 00''
94 °C	00' 30''
54 °C	01' 30''
72 °C	01' 30''
60 °C	30' 00''
4 °C	pause
Cycles	**30**

Figure F.7: ESTM02 Multiplex and PCR Reaction.

ESTM03					
	1 reaction	100 reactions	Primer	Label	Range
Qiagenmix	5.00	500.00	1245	VIC	347-353
1245 (20µM)	0.30	30.00	4129	VIC	230-239
4129 (20µM)	0.30	30.00	7834	6FAM	330-341
6418 (20µM)	0.40	40.00	6418	PET	329-361
4775 (20µM)	0.30	30.00	8210	PET	217-227
7867 (20µM)	0.30	30.00	4775	NED	221-223
7834 (20µM)	0.30	30.00	7867	NED	340-342
8210 (20µM)	0.30	30.00			
RNase-free water	1.80	180.00			
DNA	1.00	100.00			
	10.00	1000.00			

PCR	
Temp	TIME
95 °C	15' 00''
94 °C	00' 30''
54 °C	01' 30''
72 °C	01' 30''
60 °C	30' 00''
4 °C	pause
Cycles	**30**

Figure F.8: ESTM03 Multiplex and PCR Reaction.

ESTM05					
	1 reaction	100 reactions	Primer	Label	Range
Qiagenmix	5.00	500.00	2696	PET	365-371
2696 (20µM)	0.30	30.00	3196	PET	252-273
3196 (20µM)	0.30	30.00	9357	VIC	381-467
9357(20µM)	0.30	30.00	4827	NED	127-139
4827(20µM)	0.30	30.00	7148	NED	236-251
7148(20µM)	0.30	30.00	8693	6FAM	297-314
8693(20µM)	0.30	30.00			
RNase-free water	2.20	220.00			
DNA	1.00	100.00			
	10.00	1000.00			

PCR	
Temp	TIME
95 °C	15' 00''
94 °C	00' 30''
56 °C	01' 30''
72 °C	01' 30''
60 °C	30' 00''
4 °C	pause
Cycles	**30**

Figure F.9: ESTM05 Multiplex and PCR Reaction.

ESTM06					
	1 reaction	100 reactions	Primer	Label	Range
Qiagenmix	5.00	500.0	9449	VIC	195-208
9449(20µM)	0.30	30.00	6227	PET	403-439
6227(20µM)	0.40	40.00	2563	NED	238-268
2563(20µM)	0.30	30.00	7327	6FAM	361-375
7327(20µM)	0.30	30.00	8397	6FAM	328-338
8397(20µM)	0.30	30.00	8416	6FAM	250-272
8416(20µM)	0.30	30.00	7001	NED	393-397
7001(20µM)	0.40	40.00			
RNase-free water	1.70	170.00			
DNA	1.00	100.00			
	10.0	1000.0			

PCR	
Temp	TIME
95 °C	15' 00''
94 °C	00' 30''
56 °C	01' 30''
72 °C	01' 30''
60 °C	30' 00''
4 °C	pause
Cycles	**30**

Figure F.10: ESTM06 Multiplex and PCR Reaction.

ESTM07					
	1 reaction	100 reactions	Primer	Label	Range
Qiagenmix	5.00	500.0	2404	6FAM	167-274
2404(20µM)	0.20	20.00	1770	6FAM	382-412
1770(20µM)	0.15	15.00	11039	NED	278-359
11039(20µM)	0.50	50.00	10572	VIC	139-151
10572(20µM)	0.20	20.00	11375	VIC	381-415
11375(20µM)	0.10	10.00	3698	PET	338-414
3698(20µM)	0.40	40.00			
RNase-free water	2.45	245.00			
DNA	1.00	100.00			
	10.0	1000.0			

PCR	
Temp	TIME
95 °C	15' 00''
94 °C	00' 30''
55 °C	01' 30''
72 °C	01' 30''
60 °C	30' 00''
4 °C	pause
Cycles	**30**

Figure F.11: ESTM07 Multiplex and PCR Reaction.

ESTM08					
	1 reaction	100 reactions	Primer	Label	Range
Qiagenmix	5.00	500.0	3187	6FAM	379-391
3187(20µM)	0.25	25.00	11784	NED	273-285
11784(20µM)	0.20	20.00	4208	NED	363-368
4208(20µM)	0.10	10.00	10752	VIC	139-151
10752(20µM)	0.20	20.00	7705	VIC	295-342
7705(20µM)	0.10	10.00	11345	PET	225-233
11345(20µM)	0.5	50.00			
RNase-free water	2.65	265.00			
DNA	1.00	100.00			
	10.0	1000.0			

PCR	
Temp	TIME
95 °C	15' 00''
94 °C	00' 30''
55 °C	01' 30''
72 °C	01' 30''
60 °C	30' 00''
4 °C	pause
Cycles	**30**

Figure F.12: ESTM08 Multiplex and PCR Reaction.

ESTM09					
	1 reaction	100 reactions	Primer	Label	Range
Qiagenmix	5.00	500.0	2931	VIC	320-354
2931(20µM)	0.10	10.00	12318	VIC	233-246
12318(20µM)	0.10	10.00	11982	PET	465-467
11982(20µM)	0.35	35.00	11411	6FAM	206-268
11411(20µM)	0.10	10.00			
RNase-free water	3.35	335.00			
DNA	1.00	100.00			
	10.0	1000.0			

PCR	
Temp	TIME
95 °C	15' 00''
94 °C	00' 30''
55 °C	01' 30''
72 °C	01' 30''
60 °C	30' 00''
4 °C	pause
Cycles	**30**

Figure F.13: ESTM09 Multiplex and PCR Reaction.

References

Anderson, J. T., Inouye, D. W., McKinney, A. M., Colautti, R. I. and Mitchell-Olds, T. (2012). Phenotypic plasticity and adaptive evolution contribute to advancing flowering phenology in response to climate change. *Proceedings of the Royal Society of Biology.* [Online] $http://rspb.royalsocietypublishing.org/content/early/2012/07/05/rspb.2012.1051.short$

Antao, T., Lopes, A., Lopes, R. J., Beja-Pereora, A. and Luikart, G. (2008). LOSITAN: a workbench to detect molecular adaptation based on a Fst-outlier method. *BMC Bioinformatics.* [Online]. DOI: 10.1186/1471-2105-9-323

Aitken, S. N. and Whitlock, M. C. (2013). Assisted Gene Flow to Facilitate Local Adaptation to Climate Change. *Annual Review of Ecology, Evolution and Systematics.* 44: 367-388.

Altshuler, I., Demiri, B., Xu, S., Constantin, A., Yan, N. D. and Cristescu, M. E. (2011). An intergrated multi-disiplinary approach for studying multiple stressors in freshwater ecosystems: Daphnia as a model organism. *Integrative and Comparative Biology.* [Online] $http://icb.oxfordjournals.org/content/early/2011/08/27/icb.icr103.short$

Angeler, D. G. (2007). Resurrection ecology and global climate change research in freshwater ecosystems. *Journal of North American Benthological Society.* 26 (1); 12-22.

Appleby, P. G. (2001). Chronostratigraphic techniques in recent sediments. Kluwer Acedemic Publisher, The Netherlands. Aubret, F. and Shine, R. (2010). Thermal plasticity in young snakes: how will climate change affect the thermoregulatory tactics of ectotherms? *Journal of Experimental Biology.* 213; 242-248.

Auld, J. R., Agrawal, A. A. and Relyea, R. A. (2009). Re-Evaluating the Costs and Limits of Adaptive Phenotypic Plasticity. *Proceedings of the Royal Society B.* [Online] $http://rspb.royalsocietypublishing.org/content/early/2009/10/19/rspb.2009.1355.short.$

Ayres, D. R., Zaremba, K. and Strong, D. R. (2004). Extinction of a common native species by hybridization with an invasive Congener. *Weed Technology.* 18 (1); 1288-1291.

Baker, M. (2012). Structural Variation: the genomes hidden architecture. Nature Methods. 9 (2): p133-137. Beaumont, M. A. and Nichols, R. A. (1996). Evaluating loci for use in the genetic analysis of population structure. *Proceedings of the Royal Society B.* 263 (1377); 1619-1626.

Bassar, R. D., Marshall, M. C., Lopez-Sepulcre, A., Zandona, E., Auer, S. K., Travis, J., Pringle, C. M., Flecker, A. S., Thomas, S. A., Fraser, D. F. and Reznick, D. N. (2010). Local adaptation in Trinidadian guppies alters ecosystem processes. *PNAS.* 107 (8); 3616-3621.

Belanger-Deschenes, S., Couture, P., Campbell, P.G.C. and Bernatchez, L. (2013). Evolutionary change driven by metal exposure as revealed by coding SNP genome scan in wild yellow perch (*Perca flavescens*). *Ecotoxicology.* 22: 938-957.

Bellard, C., Bertellsmeier, C., Leadley, P., Thuillerz, W. and Courchamp, F. (2012). Impacts on climate change on the future of biodiversity. *Ecology Letters.* 15; 365-377.

Berg, S., Jeppesen, E., Sondergaard, M. and Mortensen, E. (1994). Environmental effects of introducing whitefish, Coregonus lavaretus (L.), in Lake Ring. *Hydrobiologia.* 275; 71-79.

Bouetard, A., Cote, J., Besnard, A. L., Collinet, M. and Coutellec, M. A. (2014). Envirnmental versus anthropogenic effects on population adaptive divergence in the freshwater snail Lymnaea stagnalis. *PLOS ONE.* 9 (9); 1-14.

Carlson, S. M., Cunningham, C. J., and Westley, P. A. H. (2014). Evolutionary rescue in a changing world. *Trends in Ecology and Evolution.* 29 (9); 521-530.

Clavero, M. and Garcia-Berthou. (2005). Invasive species are a leading cause in animal extinctions. *Trends in Ecology and Evolution.* 20 (3); 110.

Chakraborty, R. (1993). Analysis of genetic structure of populations: meaning, methods and implications. *Human population genetics.* Plenum Press, New York.

Charmantier, A., McCleery, R. H., Cole, L. R., Perrins, C., Kruuk, L. E. B. and Sheldon, B. C. (2008). Adaptive phenotypic plasticity in response to climate change in a wild bird

population. *Science*. 320; 800-803.

Chwedorzewska, K. J., Bednarek, P. T., Lewandowska, R., Krajewski, P. and Puchalski, J. (2006). Studies on genetic changes in rye samples (*Secale cereale* L.) maintained in a seed bank. *Cellular and Molecular Biology Letters*. 3: 338-347.

Colbourne, J. K., Pfredner, M. E., Gilbert, D., Thomas, W. K., Tucker, A., Oakley, T. H., Tokishita, S., Aerts, A., Arnold, G. J., Basu, M. K., Bauer, D. J., Caceres, C. E., Carmel, L., Casola, C., Choi, J., Detter, J. C., Dong, Q., Dusheyko, S., Eades, B. D., Frochlich, T., Geiler-Samerotte, K. A., Gerlach, D., Hatcher, P., Jogdeo, S., Krijgsveld, J., Kriventseva, E. V., Kultz, D., Lafrosch, C., Lindquist, D., Lopez, J., Manak, J. R., Muller, J., Pangilinan, J., Partwandhan, R. P., Pitluck, S., Pritham, E. J., Rechtsteiner, A., Rho, M., Rogozin, I. B., Sakarya, O., Salamov, A., Schaack, S., Shapiro, H., Shiga, Y., Skalitzky, C., Smith, Z., Souvorov, A., Sung, W., Tang, J., Tsuchiya, D., Tu, H., Vos, H., Wang, M., Wolf, Y., Yamagata, H., Yamada, T., Ye, Y., Shaw, J. R., Andrews, J., Crease, T. J., Tang, H., Lucas, S. M., Robertson, H. M., Bork, P., Koonin, E. V., Zdobnov, E. M., Grigoriev, I. V., Lynch, M. an Boore, J. L. (2011). The Ecoresponsive Genome of Daphnia pulex. *Science.* 331 (6017): p555-561.

Cole, P. c., Luecke, C., Wurtsbaugh, W. A. and Burkart, G. (2002). Growth and survival of Daphnia in epilimnetic and metalimnetic water from oligotrophic lakes: the effects of food and temperature. *Freshwater Biology.* 47 (11); 2113-2122.

Coors, A., Vanoverbeke, J., De Bie, T. and De Meester, L. (2009). Land use, genetic diversity and toxicant tolerance in natural populations of Daphnia magna. *Aquatic Toxicology.* 95 (1): 71-79.

Cousyn, C., De Meester, L. Colbourne, J. K., Brendonck, L., Verschuren, D. and Volckaert, F. (2001). Rapid, local adaptation of zooplankton behavior to changes in predation pressure in the absence of neutral genetic changes. *PNAS.* 98 (11); 6256-6260.

Crisp, D. T. and Howson, G. (1982) Effect of air temperature upon mean water temperature in streams in the north Pennines and English Lake District. *Freshwater Biology.* 12 (4); 359-367.

Crozier, L. G., Hendry, A. P., Lawson, P. W., Quinn, T. P., Mantua, N. J., Battin, J., Shaw, R. G. and Huey, R. B. (2008). Potential responses to climate change in organisms with complex life-histories: evolution and plasticity in Pacific Salmon. *Evolutionary Applications.* 1 (2); 252-270.

Cuco, A. P., Abrantes, N., Goncalves, F., Wolinska, J. and Castro, B. B. (2016). Toxicity of two fungicides in *Daphnia*: is it always temperature dependent? *Ecotoxicology.* 25 (7); 1376-1389.

D'Esposito, D., Dattolo, E., Badalamenti, F., Orsini, L. and Procaccini, G. (2012). Comparative analysis of genetic diversity of Posidonia oceanica along a depth gradient using neutral and selective/ non-neutral microsatellite markers. *Biol. Mar. Mediterr.* 19 (1); 45-58.

De Meester, L. and De Jager, H. (1993). Hatching of Daphnia sexual eggs. Intraspecific differences in the hatching responses of D.magna eggs. *Freshwater Biology.* 30 (2); 219-226.

De Meester, L., Van Doorslaer, W., Geerts, A., Orsini, L. and Stoks, R. (2011). Thermal genetic adaptation in the water flea Daphnia and its impact: an evolving metacommunity approach. *Integrative and Comparative Biology.* 51 (5); 703-718.

Decaestecker, E., Gaba, S., Raeymaekers, J. A. M., Stoks, R., Van Kerckhoven, L., Ebert, D. and De Meester, L. (2007). Host-parasite 'Red Queen' dynamics archived in pond sediment. Nature. 450: p870-874. DeWitt, T. J., Sih, A. and Wilson, D. S. (1998). Costs and Limits of Phenotypic Plasticity. *TREE.* 13 (2): p77-81.

Dieringer, D. and Schlotterer, C. (2003). Microsatellite analyser (MSA): a platform independent analysis tool for large microsatellite data sets. *Molecular Ecology Notes.* 3 (1); 167-169.

Dinh Van, K., Janssens, L., Debecker, S. and Stoks, R. (2014). Temperature- and latitude-specific individual growth rates shape the vulnerability of damselfly larvae to a widespread pesticide. *Journal of Applied Ecology.* 51 (4); 919-928.

Easterling, D. R., Meehl, G. A., Parmesan, C., Changnon, S. A., Karl, T. R. and Mearns, L. O. (2000). Climate Extremes: Observations, Modeling, and Impacts. *Science.* 289 (5487);

2068-2074.

Excoffier, L., Lavel, G. and Schneider, S. (2005). Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics.* [Online]. 1; 47-50.

Falush, D., Stephens, M and Pritchard, J. K. (2003). Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. *Genetics.* 164 (4); 1567-1687.

Fickel, J., Wagener, A. and Ludwig, A. (2007). Semen cryopreservation and the conservation of endangered species. *European Journal of Wildlife Research.* 53 (2); 81-89.

Frankham, R. (2005). Genetics and Extinction. *Biological Conservation.* 126: p131-140.

Frisch, D., Morton, P. K., Chowdhury, P. R., Culver, B. W., Colbourne, J. K., Weider, L. J. and Jeyasingh, P. D. (2014). A Millennial scale chronicle of evolutionary responses to cultural eutrophication in Daphnia. *Ecology Letters.* 17 (3): p360-368.

Geerts, A. N., Vanoverbeke, J., Vanschoenwinkel, B., Van Doorslaer, W., Feuchtmayr, H., Atkinson, D., Moss, B., Davidson, T. A., Sayer, C. D. and De Meester, L. (2015). Rapid evolution of thermal tolerance in the water flea Daphnia. *Nature Climate Chance.* 5: 665-668.

Gonzales, A. and Bell, G. (2014). Evolutionary rescue and adaptation to abrupt environmental change depends upon the history of stress. *Philosophical Transactions of the Royal Society of London.* 368 (1610); 20120079.

Gopal, B. (2005). Does inland aquatic biodiversity have a future in Asian developing countries? *Hydrobiologia.* 542; 69-75.

Grant, P. R. and Grant, B. R. (2002). Unpredictable evolution in a 30-year study of Darwin's Finches. *Science.* 296 (5568); 707-711.

Greenbaum, T., Templeton, A., Zarmi, Y. and Bar-David, S. (2014). Allelic richness following population founding effects - a stochastic modelling framework incorporating gene flow and genetic drift. *Plos One.* DOI:10.1371/journal.pone.0115203

Grimm, N. B., Chapin, S. F., Bierwagen, B., Gonzalez, P., Groffman, P. M., Luo, Y., Melton, F., Nadelhoffer, K., Pairis, A., Raymond, P. A., Schimel, J. and Williamson, C. E. (2013). The impacts of climate change on ecosystem structure and function. *Frontiers in Ecology and the Environment.* 11 (9); 474-482.

Gross, M. (2013). EU bans puts spotlight on complex effects of neonicotinoids. *Current Biology.* 23 (11): 462-464.

Hammill, E., Rogers, A. and Beckerman, A. P. (2008). Costs, Benefits and the Evolution of Inducible Defenses: A Case Study with Daphnia pulex. *Journal of Evolutionary Biology.* 21: p705-715.

Harris, K. D. M., Bartlett, N. J. and Lloyd, V. K. (2012). Daphnia as an emerging epigenetic model organism. *Genetics Research International.* [Online] *http* : *//dx.doi.org/*10.1155/2012/147892

Harte, J., Ostling, A., Green, J. L. and Kinzig, A. (2004). Climate change and extinction risk. *Nature.* [Online] *http* : *//www.nature.com/nature/journal/v*427/*n*6970/*full/nature*02121.*html*

Helmuth, B., Kingsolver, J. G. and Carrington, E. (2005). Biophysics, Physiological Ecology, and Climate Changes: Does Mechanism Matter? *Annual Review of Physiology.* 75; 177-201.

Henning-Lucass, N., Cordellier, M., Streit, B. and Schwenk, K. (2016). Phenotypic plasticity in life-history traits of Daphnia galeata in response to temperature – a comparison across clonal lineages separated in time. *Ecology and Evolution.* 6 (4); 881-891.

Hoegh-Guldberg, O. (1999). Climate change, coral bleaching and the future of the world's coral reefs. *Marine and Freshwater Research.* 50 (8); 839-866.

Hof, C., Araujo, M. B., Jetz, W. and Rahbek, C. (2011). Additive threats from pathogens, climate and land-use change for global amphibian diversity. *Nature.* 480; 516- 521.

Hoffman, A., Griffin, P., Dillon, S., Catullo, R., Rane, R., Byrne, M., Jordan, R., Oakeshott, J., Weeks, A., Joseph, L., Lockhart, P., Borevitz, J. and Sgro, C. (2015). A framework for incorporating evolutionary genomics into biodiversity conservation and management. *Climate Change Responses.* 2 (1); 1-23.

Hoffmann, A. A. and Sgro, C. M. (2011). Climate change and evolutionary adaptation.

Nature. 470; 479-485.

Hoffman, A. A., Sorensen, J. G. And Loeschcke, V. (2003). Adaptation of Drosophila to temperature extremes: bringing together quantitative and molecular approaches. *Journal of Thermal Biology.* 28 (3); 175-216.

Holway, D. A. (1999). Competative Mechanisms Underlying the Displacement of Native Ants by the Invasive Argentine Ant. *Ecology.* 80: p238-251.

Hu, C. W., Li, M., Cui, Y.B., Li, D. S., Chen, J. and Yang, L. Y. (2010). Toxicological effects of TiO2 and ZnO nanoparticles in soil on earthworm Eisenia fetida. *Soil Biology and Biochemistry.* 42; 586-591.

Hua, J., Morehouse, N. I. and Relyea, R. A. (2013). Pesticide tolerance in amphibians: induced tolerance in susceptible populations, constitutive tolerance in tolerant populations. *Evolutionary Applications.* [Online]. DOI:10.1111/eva.12083.

Hubbard, R. and Lindsay, R. M. (2008). Why P Values Are Not a Useful Measure of Evidence in Statistical Significance Testing. *Theory and Psychology.* 18 (1); 69-88.

IUCN (2015). *The IUCN Red List of Threatened Species.* [Online] *http* : *//www.iucnredlist.org/search.*

Jankowski, T. and Straile, D. (2003). A comparison of egg-bank and long-term plankton dynamics of two Daphnia species, D-hyalina and D-galaeta: Potentials and limits of reconstruction. *Limnology and Oceanography.* 48; 1948-1955.

Jansen, M., Geerts, A. N., Rago, A., Spanier, K. I., Denis, C., De Meester, L. and Orsini, L. (2017). Thermal tolerance in the keystone species *Daphnia magna* - a candidate gene and an outlier analysis approach. *Molecular Ecology.* DOI: 10.1111/mec.14040.

Jansen, M., Coors, A., Vanoverbeke, J., Schepens, M., De Voogt, P., De Schamphelaere, K. A. C. and De Meester, L. (2015). Experimental evolution reveals high insecticide tolerance in *Daphnia* inhabiting farmland ponds. *Evolutionary Applications.* 8 (5); 442-453.

Jansen, M., Vergauwen, L., Vandebrouck, T., Knapen, D., Dom, N., Spanier, K. I., Cielen, A. and De Meester, L. (2013). Gene expression profiling of three different stressors in the water flea *Daphnia magna*. *Ecotoxicology.* 22 (5); 900-914.

Jantz, S. M., Barker, B., Brooks, T. M., Chini, L. P., Huang, Q., Moore, R. M., Noel, J. and Hurtt, G. C. (2015). Future habitat loss and extinctions driven by land-use change in biodiversity hotspots under four scenarios of climate-change mitigation. *Conservation Biology.* 29 (4); 1122-1131.

Jensen, M., De Meester, L., Cielen, A., Buser, C. C. and Stoks, R., (2011). The interplay of past and current stress exposure on the water flea Daphnia. *Functional Ecology.* 25; 974-982.

Jetz, W., Wilcove, D. S. and Dobson, A. P. (2007). Projected impacts of climate change and land use change on the global biodiversity of birds. *PLOS Biology.* 9 (6); 1211-1219.

Jones, D. K. and Relyea, R. A. (2015). Here today, gone tomorrow: short-term retention of pesticide induced tolerance in amphibians. *Environmental Toxicology and Chemistry.* 999 (999); 1-7.

Karell, P., Ahola, K., Karstinen, T., Valkama, J. and Brommer, J. E. (2011). Climate change drives microevolution in wild bird. *Nature Communications.* [Online] DOI: 10.1038/ncomms1213.

Kato, Y., Kobayashi, K., Watanabe, H. and Iguchi, T. (2010). Introduction of foreign DNA into the water flea, Daphnia magna, by electroporation. *Ecotoxicology.* 19: p589-592.

Kerfoot, W. C., Robbins, J. A. and Weider, L. J. (1999). A new approach to historical reconstruction: Combining descriptive and experimental paleolimnology. *Limnology and Oceanography.* 44 (5): p1232-1247.

Kiesecker, J. M. (2002). Synergism between trematode infection and pesticide exposure: a link to amphibian limb deformities in nature? *Proceedings of the National Academy of Sciences.* 99; 9900–9904.

Kilham, S. S., Kreeger, D. A., Lynn, S. G., Goulden, C. E. and Herrera, L. (1998). COMBO: a defined freshwater culture medium for algae and zooplankton. *Hydrobiologica.* 377: 147-159.

Laetz, C. A., Baldwin, D. H., Hebert, V. R., Stark, J. D. and Scholz, N. L. (2014). Elevated temperatures increase the toxicity of pesticide mixtures to juvenile coho salmon. *Aquatic Toxicology.* 146: 38-44.

Lampert, W. (2001). Survival in a varying environment phenotypic and genotypic responses

in daphnia populations. *Limnetica.* 20 (1); 3-14.

Larsson, L. C., Laikre, L., Andre, C., Dahlgren, T. G. and Ryman, N. (2010) Temporally stable genetic structure of heavily exploited Atlantic herring (*Clupea harengus*) in Swedish waters. *Heredity.* 104; 40–51.

Lass,S. and Spaak, P. (2003). Chemically induced anti-predator defenses in plankton: a review. *Hydrobiologia.* 491; 221-239.

Lau, E. T. C., Karracker, N. E. and Leung, K. M. L. (2015). Temperature-dependent acute toxicity of methomyl pesticide on larvae of three Asian amphibian species. *Environmental Toxicology.* 34: 2322-2327. Limburg, P. A and Weider, L. J. (2002). 'Ancient' DNA in resting egg bank of a microcrustacean can serve as a palaeolimnological database. *Proceedings of the Royal Society of Biology.* 269: p281-287.

Lindsey, H. A., Gallie, J., Taylor, S. and Kerr, B. (2012). Evolutionary rescue from extinction is contingent on a lower rate of environmental change. *Nature.* 494; 463-467.

Loi, P., Ptak, G., Barboni, B., Fulka, J., Cappai, P. and Clinton, M. (2001). Genetic rescue of an endangered mammal by cross-species nuclear transfer using post-mortem somatic cells. *Nature Biotechnology.* 19; 962-964.

Marrugo-Negrete, J., Benitez, L. N. and Olivero-Verbal, J. (2008). Distribution of mercury in several environmental compartments in an aquatic ecosystem impacted by gold mining in northern Columbia. *Archives of Environmental Contamination and Toxicology.* 55; 305-316.

Markus, N. H. (1990). Calanoid copepod, cladoceran, and rotifer-eggs in sea-bottom sediments of northern Californian coastal waters: identification, occurrence and hatching. *Marine Biology.* 105; 413-418.

McLachlen, J. S., Hellmann, J. J. and Schwartz, M. W. (2007). A framework for debate of assisted migration in an era of climate change. *Conservation Biology.* 21 (2); 297-302.

McFeeters, B. J. and Frost, P. C. (2011). Temperature and the effects of elemental food quality on Daphnia. *Freshwater Biology.* 56; 1447-1455.

Merila, J. and Hendry, A. P. (2014). Climate change, adaptation, and phenotypic plasticity:

the problem and the evidence. *Evolutionary Applications.* 7; 1-14.

Miner, B. E., Meester, L. D., Pfrender, M. E., Lampert, M. and Hairston, N. G. (2012). Linking genes to communities and ecosystems: Daphnia as an ecogenomic model. *Proceedings of the Royal Society of Biology.* 279: p1873-1882.

Mitchell, S. E. and Lampert, W. (2000). Temperature adaptaion in a geographically widespread zooplanker, Daphnia magna. *Journal of Evolutionary Biology.* 13 (3); 371-382.

Mitchell, J. F. B., Johns, T. C., Gregory, J. M. and Tett, S. F. B. (1995). Climate response to increasing levels of greenhouse gases and sulfate aerosols. *Nature.* 376; 501-504.

Moller, A. P., Rubolini, D. and Lehikoinen, E. (2008). Populations of migratory bird species that did not show a phonological response to climate change are declining. *PNAS.* 105 (42); 16195-16200.

Morton, P. K., Frisch, D., Jeyasingh, P. D. and Weider, L. J. (2015). Out with the old, in with the new? Younger *Daphnia* clones are competitively superior over centuries-old ancestors. *Hydrobiologia.* 749: 43-52.

Mulholland, P. J., Best, G. R., Coutant, C. C., Hornberger, G. M., Meyer, J. L., Robinson, P. J., Stenberg, J. R., Turner, R. E., Vera-Herrera, F. and Wetzel, R. G. (1997). Effects of climate change on freshwater ecosystems of southern-eastern United States and the gulf coast of Mexico. *Hydrological Processes.* 11 (8): p949-970.

Murphy, W. J., Thomerson, J. E. and Collier, G. E. (1999). Phylogeny of the Neotropical killifish family Rivulidae (Cyprinodontiformes, Apolcheiloidei) inferred from mitochondrial DNA sequences. *Molecular Phylogenetic Evolution.* 13 (2): 298-301.

Muyssen, B. T. A., Messiaen, M. and Janssen, C. R. (2010). Combined cadmium and temperature acclimation in Daphnia magna: Physiological and sub-cellular effects. *Ecotoxicology and Environmental Safety.* 73; 735-742.

Nakanishi, T., Kato, Y., Matsuura, T. and Watanabe, H. (2014). CRISPR/Cas-Mediated Targeted Mutagenesis in Daphnia magna. *PLoS One.* [Online] doi:10. 1371/journal.pone.0098363.

Oexle, S., Jansen, M., Pauwels, K., Sommaruga, R., De Meester, L. and Stoks, R. (2016).

Rapid evolution of antioxidant defense in a natural population of Daphnia magna. *Journal of Evolutionary Biology.* [Online]. DOI: 10.1111/jeb.12873.

Onbe, T. (1978). Sugar floatation method for sorting the resting eggs of marine cladocerans and copepods from sea-bottom sediment. *Bulletin Japanese Society Scientific Fisheries.* 44; 1411.

O'Neil, J. M., Davis, T. W., Burford, M. A. and Gobler, C. J. (2012). The risk of harmful cyanobacteria blooms: the potential roles of eutrophication and climate change. Harmful Algae. 14: p313-334. Orr, M. R. and Seike, S. H. (1998). Parasitoids Deter Foraging by Argentine Ants (Linepithema humile) in Their Native Habitat in Brazil. *Oecologica.* 117: p420-425.

O'Reilly, C. M., S. Sharma, D. K. Gray, S. E. Hampton, J. S. Read, R. J. Rowley, P. Schneider, J. D. Lenters, P. B. McIntyre, B. M. Kraemer, et al. (2015), Rapid and highly variable warming of lake surface waters around the globe, *Geophys. Res. Lett.* 42 (10); 773-781

Orsini, L., Spanier, K. I. and De Meester, L. (2012). Genomic signature of natural and anthropogenic stress in wild populations of the waterflea Daphnia magna: validation in space, time and experimental evolution. *Molecular Ecology.* 21 (9); 2160-2175.

Orsini, L., Schwenk, K., Meester, L. D., Colbourne, J. K., Pfrender, M. E. and Weider, L. J. (2013). The evolutionary time machine: using dormant propagules to forecast how populations can adapt to changing environments. *Trends in Ecology and Evolution.* 28 (5): p274-282.

Oyen, K. J., Giri, S. and Dillon, M. E. (2016). Altitudinal variation in bumble bee (Bombus) critical thermal limits. *Journal of Thermal Biology.* [Online]. DOI: 10.1016/j.jtherbio.2016.04.015.

Parmesan, C. and Yohe, G. (2003). A globally coherent fingerprint of climate change impacts across natural systems. *Nature.* 421: p37-42.

Parmesan, C., Ryrholm, N., Stefanescu, C., Hill, J. K., Thomas, C. D., Descimon, H., Huntlet, B., Kailia, L., Kullberg, J., Tammaru, T., Tennent, W. J., Thomas, J. A., and

Warren, M. (1999). Poleward shifts in geographical ranges of butterfly species associated with regional warming. *Nature*. 399; 579-583.

Persson, J., Wojewodzic, M. W., Hessen, D. O. and Andersen, T. (2011). Increased rosk of phosphorous limitation at higher temperatures for Daphnia magna. *Oecologia.* 165 (1); 123-129.

Pietrzak, B. and Slusarczyk, M. (2006). The fate of ephippia- Daphnia dispersal in time and space. *Polish Journal of Ecology.* 54 (4); 709-714.

Pounds, J. A., Bustamante, M. R., Coloma, L. A., Consuegra, J. A., Fodgen, M. P. L., Foster, P. N., Marca, E., Master, K. L., Merino-Viteri, A., Puschendorf, R., Ron, S. R., Sa'nchez-Azofeifa, G. A., Still, C. J. and Young, E. (2006). Widespread amphibian extinctions from epidemic disease driven by global warming. *Nature.* 439; 161-167.

Pritchard, J. K., Stephens, M. and Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*. 155 (2); 945-959.

Purvis, A., Agapow, P. M., Gittleman, J. L. and Mace, G. M. (2004). Nonrandom extinction and the loss of evolutionary history. *Science.* 288; 328-330.

Reissen, H. P. (1992). Cost-Benefit Model for the Induction of an Antipredator Defense. *The American Naturalist.* 140 (2): p349-362.

Relyea, R. and Hoverman, J. (2006). Assessing the ecology in ecotoxicology: a review and synthesis in freshwater systems. *Ecology Letters.* 9: 1157-1171.

Richman, S. and Dodson, S. I. (1983). The effect of food quality on feeding and respiration by Daphnia and Diaptomus. *Limnol. Oceanogr.* 28 (5); 948-956.

Rosenweig, C., Karoly, D., Vicarelli, M., Neofotis, P., Wu, Q., Casassa, G., Menzel, A., Root, T. L., Estrella, N., Seguin, B., Tryjanowski, P., Liu, C., Rawlins, S. and Imeson, A. (2008). Attributing physical and biological impacts to anthropogenic climate change. *Nature.* 453 (7193); 353-357.

Rousset, F. (2008). GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources*. 8 (1); 103-106.

Sandstrom, A. and Karas. P. (2002). Effects of eutrophication on young-of-the-year freshwater fish communities in costal areas of the Baltic. *Environmental Biology of Fishes.* 63 (1): 89-101.

Saunders, D. S. (2014). Insect photoperiodism: effects of temperature on the induction of insect diapause and diverse roles for the circadian system in the photoperiodic response. *Entomological Science.* 17: 25-40.

Schulz, R. and Dabrowski, J.M. (2001). Combined effects of predatory fish and sublethal pesticide contamination on the behavior and mortality of mayfly nymphs. *Environmental Toxicology and Chemistry.* 20; 2537–2543.

Schindler, D. W., Beaty, K. G., Fee, E. J., Cruikshank, D. R., DeBruyn, E. R., Findlay, D. L., Linsey, G. A., Shearer J. A., Stainton, M. P. and Turner, M. A. (1990). Effects of climatic warming on lakes of the central boreal forest. *Science.* 250; 967.

Semlitsch, R. D., Bridges, C. M. And Welch, A. M. (2000). Genetic variation and a fitness tradeoff in the tolerance of grey treefrog (Hyla versicolor) tadpoles to the insecticide carbaryl. *Oecologia.* 125 (2); 179-185.

Sinclair, B. J., Nelson, S., Nilson, T. L., Roberts, S. P. and Gibbs, A. G. (2007). The effect of selection for desiccation resistance on the cold tolerance of Drosophila melanogaster. *Physiological Entomology.* 32 (4): 322-327.

Sinervo, B., Mendez-de-la-Cruz, F., Miles, D. B., Heulin, B., Bastiaans, E., Villagran-Santa Cruz, M., Lara-Resendiz, R., Martinez-Mendez, N., Calderon-Espinosa, M. L., Meza-Lazaro, R. N., Gadsden, H., Avila, L. J., Morando, M., De la Riva, I. J., Sepulveda, P. V., Rocha, C. F. D., Ibarguengoytia, N., Puntriano, C. A., Massot, M., Lepetz, V., Oksanen, T. A., Chapple, D. G., Bauer, A. M., Branch, W. R., Clobert, J. and Sites, J. W. (2010). Erosion of lizard diversity by climate change and altered thermal niches. *Science.* 328; 894-899.

Smith, S., Bernatchez, L. and Beheregaray, L. B. (2013). RNA-seq analysis reveals extensive transcriptional plasticity to temperature stress in a freshwater fish species. *BMC Genomics.* [Online]. DOI: 10.1186/1471-2164-14-375.

Somero, G. N. (2010). The physiology of climate change: how potentials for acclimation and genetic adaptation will determine 'winners' and 'losers'. *The Journal of Experimental Biology.* 213; 912-920.

Stocker, T. F., Qin, D., Plattner, G. K., Tignor, M. M. B., Allen, S. K., Boschung, J., Nauels, A., Xia, Y., Bex, V. and Midgley, P. M. (2013). *Climate change 2013: the physical science basis.* Cambridge University Press, New York.

Stuart, M., Lapworth, D., Crane, E. and Hart, A. (2012). Review of risk from potential emerging contaminates in UK groundwater. *Science of the Total Environment.* 416; 1-21.

Tejedo, M., Marangoni, F., Pertoldi, C., Richter-Boix, A., Laurila, A., Orizaola, G., Nicieza, A. G., Alvarez, D. and Gomez-Mestre, I. (2010). Contrasting effects of environmental factors during larval stages on morphological plasticity in post-morphometric frogs. *Climate Research.* 43; 31-39.

Thomas, C. D., Cameron, A., Green, R. E., Bakkeness, M., Beaumont, L. J., Collinghams, Y. C., Erasmus, B. F. N., Ferreira de Siqueira, M., Graingers, A., Hannah, L., Hughes, L., Huntleys, B., Van Jaarsveld, A. S., Midgley, G. F., Miles, L., Ortega-Huerta, M. A., Townsend-Peterson, A., Phillips, O. L. and Williams, S. E. (2004). Extinction risk from climate change. *Nature.* 427; 145-148.

Tu, H. T., Silvestre, F., De Meulder, B., Thome, J. P., Phuong, N. T and Kestemont, P. (2012). Combined effects of deltamethrin, temperature and salinity on oxidative stress biomarkers and acetylcholinesterase activity in the black tiger shrimp (Penaeus monodon). *Chemosphere.* 86 (1): 83-91.

Turvey, S. T., Pitman, R. L., Taylor, B. L., Barlow, J., Akamatso, T., Barrett, L. A., Zhao, X., Reeves, R. R., Stewart, B. S., Wang, K., Wei, Z., Zhang, X., Pusser, L. T., Richlen, M., Brandon, J. R. and Wang, D. (2007). First human-caused extinction of a cetacean species? *Biology Letters.* 3 (5); 537-540.

Urban, M. C., De Meester, L., Vellend, M., Stoks, R., Vanoverbeke, J. (2011). A crucial step toward realism: responses to climate change from an evolving metacommunity perspective. *Evolutionary Applications.* 5: p154-167.

Usov, N., Kutcheva, I., Primakov, I. and Martynova, D. (2012). Every species is good in its season: do the shifts in the annual temperature dynamics affect the phenology of the zooplankton species in the White Sea? *Hydrobiologia.* 706 (1); 11-33.

Van Doorslaer, W., Vanoverbeke, J., Duvivier, C., Rousseaux, S., Jansen, M., Jansen, B., Feuchtmayr, H., Atkinson, D., Moss, B., Stoks, R. and De Meester, L. (2009). Local adaptations to higher temperatures reduces immigration success of genotypes from a warmer region in the water flea Daphnia. *Global Change Biology.* 15; 3046-3055.

Varian-Ramos, C.w., Swaddle, J. P. and Cristol, D. A. (2013). Familial differencesi n the effects of mercury on reproduction in zebra finches. *Environmental Pollutants.* 182: 316-323.

Vasmagi, A., Nilsson, J. and Primmer, C. R. (2005). Expressed sequence tag-linked microsatellites as a source as gene-associated polymorphisms for detecting signature of divergent selection in Atlantic salmon (Salmo salar l.) *Molecular Biology and Evolution.* 22 (4); 1067-1076.

Vihma, T. (2013). Effects of arctic sea ice decline on weather and climate: a review. *Surveys in Geophysics.* 35; 1175-1214.

Walther, G., Post, E., Convey, P., Menzel, A., Parmesan, C., Beebee, T. J. C., Fromentin, J. M., Hoegh-Guldberg, O. and Bairlein, F. (2002). Ecological responses to recent climate change. Nature. 416; 389-395.

Wang, J. I. (2009). A new method for estimating effective population sizes from a single sample of multilocus genotypes. *Molecular Ecology.* 18 (10); 2148-2164.

Weetman, D. and Atkinson, D. (2004). Evalution of alternative hypotheses to explain temperature-induced life history shifts in Daphnia. *Journal of Plankton Research.* 26 (2); 107-116.

Welch, A. J., Wiley, A. E., James, H. F. et al. (2012). Ancient DNA reveals genetic stability despite demographic decline: 3,000 years of population history in the endemic Hawaiian petrel. *Molecular Biology and Evolution.* 29; 3729–3740.

Willis, C. G., Ruhfel, B., Primack, R. B., Miller-Rushing, A. J. and Davis, C. C. (2008).

Phylogenetic patterns of species loss in Thoreau's woods are driven by climate change. *PNAS.* 105 (44); 17029-17033.

Yampolsky, L. Y., Zeng, E., Lopez, J., Williams, P. J., Dick, K. B., Colbourne, J. K. and Pfrender, M. E. (2014). Functional genomics of acclimation and adaptation in response to thermal stress in Daphnia. *BMC Genomics.* 15; 859-871.