UNIVERSITY OF BIRMINGHAM

DOCUMENTING LONG-TERM IMPACT OF EUTROPHICATION AND CLIMATE CHANGE ON THE KEYSTONE SPECIES *DAPHNIA* USING RESURRECTION ECOLOGY AND PALEOGENOMICS.

By

Maria Cuenca Cambronero

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School of Biosciences
College of Life and Environmental Sciences
University of Birmingham
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Abstract

Dramatic loss of biodiversity in the last two decades has been associated with human activities. Yet, we understand little of the mechanisms that enable species persistence to anthropogenic environmental changes over evolutionary time. Here, we measured ecological and evolutionary responses of a population of *Daphnia magna* to multiple anthropogenic stressors over evolutionary time, and assessed the role of historical exposure in adaptive response to recurring environmental stress. *D. magna* is a keystone grazer in freshwater standing waters and a driver of ecosystem dynamics. As part of its life cycle, *Daphnia* produces dormant embryos that arrest their development entering dormancy and creating a long-term documentation of evolutionary responses to environmental change. Resurrected dormant stages are maintained as clonal lines in the laboratory, providing us with the unique opportunity of disentangling the role of phenotypic plasticity and genetic adaptation in population responses to environmental stress. We studied phenotypic, physiological, and molecular responses that enabled a population of *D. magna* to persist across major pollution events. We found that adaptive responses to multiple environmental stressors are not predictable from the responses to single stressors. We also discovered that historical exposure to stress prior to dormancy provides an evolutionary advantage when the stress recurs. However, this advantage is contingent upon the type and severity of environmental stressor. We discovered that response to environmental stress is underpinned by extensive epistasis and pleiotropy, suggesting that standing genetic variation is the clay of evolution in this species.

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Abbreviation

ANOVA	Analysis of variance
cDNA	(complementary) Deoxyribonucleic acid
CGE	Common Garden Experiment
CMLM	Compressed mixed linear model
COMBO	Freshwater culture medium for algae and zooplankton
CT	Control Temperature
CT	cycle threshold
Ctmax	Critical Thermal maximum
CWP	Clear water population
DE	Differential expression
DR	Read Depth
E	Environment
EP	Eutrophic population
G	Genotype
gDNA	(genomic) Deoxyribonucleic acid
GOT	Alloenzyme
GWAS	Genome-wide association
H	High concentration
Hb	Haemoglobin
HIF	Haemoglobin inducible factor
HSP	Heat shock proteins
HTS	Hyper-thermal stress
L	Low concentration
L:D	Light: Dark
LMMS	Linear mixed models
LOI	loss of ignition
M	Magnitude of plastic response
MAF	Minimum allele frequency
MANOVA	Multivariate analysis of variance
P	Phenotype
P	Population
PCA	Principal component analysis
PP	Pesticide population
PTA	Phenotypic trajectory analysis
qPCR	Real time polymerase chain reaction
RGCCA	Regularized generalized canonical correlation analysis
SNP	Single-nucleotide polymorphism
SOP	Standard operating procedure

Timm	Time to immobilization
tRNA	transfer RNA
TT	Treatment temperature
θ	direction of plastic response

GENERAL INTRODUCTION

1. Human-driven environmental change

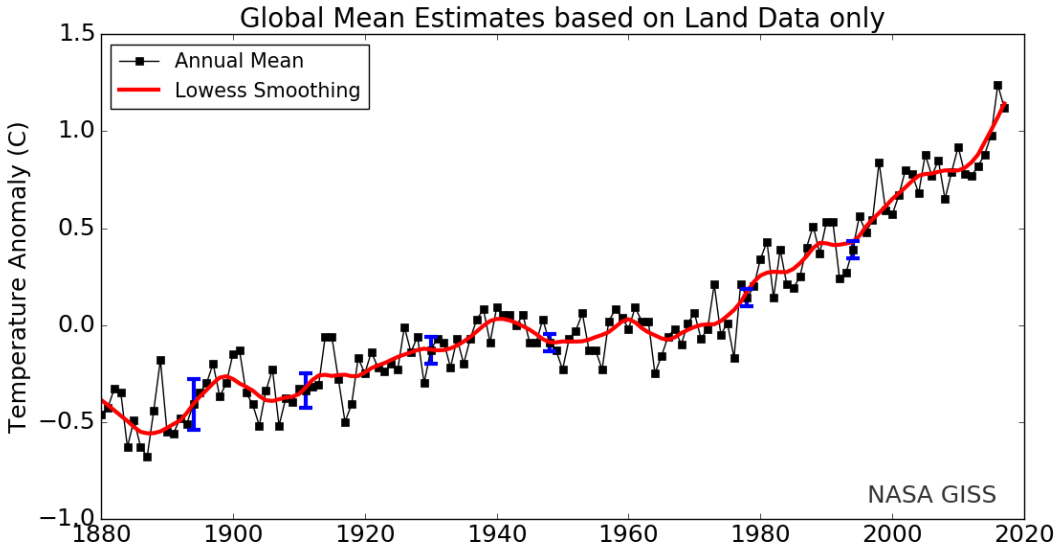
Twenty-years ago environmentalists predicted that more than 6,000 species would go extinct and many more would be impacted by altered synchrony with food and/or habitat requirements, largely because of human activities (Easterling *et al.* 2000; Parmesan *et al.* 2000; Bellard *et al.* 2012). Current trends in species extinction rates are many times greater than the direst predictions (Christensen *et al.* 2006; Pereira *et al.* 2010; Isbell *et al.* 2011), showing that human-driven environmental change has profoundly impacted natural ecosystems (Sala *et al.* 2000; Christensen *et al.* 2006) and the services they provide (Williamson *et al.* 2009). Recent reports document 75% loss of insect biodiversity, 36% of marine species, and 38% of terrestrial species, in the last 30 years alone (Sala *et al.* 2000; Hallmann *et al.* 2017; WWF 2014). Freshwater ecosystems, in particular lentic ecosystems, are among the most impacted by human-driven environmental change, suffering up to 81% of biodiversity loss between 1970 and 2012 (WWF 2016; Williamson *et al.* 2009a). A recent study focusing on North America freshwater ecosystems has revealed that more than 123 species went extinct in the 20th century, including 49% of mussels, 23% of gastropods, 33% of crayfishes, 26% of amphibians and 21% of fishes (Balanya *et al.* 2007; Becks *et al.* 2010). This loss of biodiversity is of high concern because lentic ecosystems are hotspot of biodiversity, hosting up to 10% of the world's biodiversity (Becks *et al.* 2010), and important landscape connectors (Stewart-Koster *et al.* 2015). In addition, lentic water ecosystems serve as critical resources for human societies providing water for drinking, recreation, irrigation, fishing and industrial services (Ansari *et al.* 2011).

One of the major concerns associated with human-driven environmental changes is the increase in average ambient temperature (Angilletta *et al.* 2003), recorded to be on average 0.6 ± 0.2 °C in the past 100 years (IPCC 2014). More importantly, a further increase of 4-6°C is forecasted for the next few decades if greenhouse gases emission continues as is (Fig. 1) (IPCC 2014). Changes in average ambient temperature has led to poleward shifts in

species ranges (Brommer 2004; Massimino *et al.* 2015), has increased local extinctions (Carpenter *et al.* 2008; Vaughn 2012), has accelerated changes in species phenology (Thackeray *et al.* 2008; Elliott & Defew 2012; Vadadi-Fülöp & Hufnagel 2014), has contributed to habitats loss for native species in favour of invasive species (Ormerod *et al.* 2010; Colautti *et al.* 2017), and has caused asynchrony of species with food (Charmantier & Gienapp 2014) and/or hosts (Singer & Parmesan 2010).

A concern for biodiversity is the higher recurrence and severity of extreme events, such as heat waves (Easterling *et al.* 2000; Parmesan *et al.* 2000). These events, co-occurring with average temperature increase, are predicted to be more severe in Europe and North America due to atmospheric circulation patterns intensified by greenhouse gases (Easterling *et al.* 2000), and to become more intense, longer lasting, and/or more frequent (Thomas R. Karl and Kevin E. Trenberth 2003). In line with these predictions, records of temperature anomalies confirm that seventeen of the 18 warmest years in the las 136-years were recorded from 2001 (https://climate.nasa.gov).

Figure 1. Annual average of land temperature between the 1980s and the present. The solid black line represents the mean annual temperature. The solid red line represents the five-year lowness smoothing. The blue bars account only for incomplete spatial sampling (95%confidence limit) (Source: NASA/ARGIS)



The combined effect of increasing average temperature and recurrence of extreme events has been shown to have a severe impact on aquatic inland waters and reservoirs (Bärlocher *et al.* 2008; Durance & Ormerod 2009). This is because of lower complexity of aquatic ecosystems biological ecosystems (Berklioglu 1999; Jacobs *et al.* 2008). Moreover, changes in water temperature are tightly associated with changes in oxygen solubility that can alter nutrient availability via changes in pH and salinity (Altshuler *et al.* 2011) and impact on the rate of metabolic processes (Hochachka 2002). For example, temperature increase in freshwater ecosystems has been shown to aggravate the consequences of eutrophication by altering the solubility of Nitrogen and Phosphorus, as well as by changing water conductivity (Feuchtmayr *et al.* 2009; Van Doorslaer *et al.* 2010).

Freshwater ecosystems are particularly vulnerable to climate change (Woodward 2010). Primary producers and invertebrate grazers in these ecosystems are unable to regulate their body temperature (Strayer & Dudgeon 2010). Hence, changes in ambient temperature have a profound impact on these species' phenology, population growth, behaviour, reproduction and metabolism (Durance & Ormerod 2009). For example, warmer springs lead to mismatches between algal blooms and zooplankton growth (Thackeray *et al.* 2008; Elliott & Defew 2012), and longer seasons favour extended reproduction periods for vertebrates leading to prolonged predation pressures for the invertebrate community (Rosenzweig *et al.* 2007; Greenhalgh 2009).

Most studies have focused on the short-term impact of temperature (Edwards *et al.* 2004; Durance & Ormerod 2009; Pearson *et al.* 2014). This is because it is generally challenging to assess the cross-generational effect of temperature stress and the impact of average temperature increase over evolutionary time. Yet, understanding long-term responses to temperature stress is key to make accurate predictions on the persistence of local populations under global warming (Pearson & Dawson 2003).

Rising temperatures associated with global warming present a challenge to the fate of many aquatic organisms. Rapid evolutionary responses may mediate local population persistence under global warming. Yet, proof that such evolutionary responses occur is rare (e.g. Van Doorslaer *et al.* 2009). Conversely, plastic responses figure prominently in adaptation to climate change (Knouft & Ficklin 2017). A typical response to temperature increase in freshwater organisms is reduction in body size, a fundamental biological characteristic linked to important ecological properties such as fecundity, population growth rate, and competitive abilities (Chopelet *et al.* 2008; Stock *et al.* 2014).

In addition to global warming, natural resources exploitation, agricultural land-use, and greenhouse gas emission have quickened the degradation of natural ecosystems (Bai *et al.* 2008; Giri & Qiu 2016). Eutrophication currently afflicts up to 50% of freshwater lakes and reservoirs in Europe, as a consequence of increased human activities in the last century, (Yan *et al.* 1996; Foley *et al.* 2005; Ansari *et al.* 2011), reducing provision of water resource, and affecting other services such as tourism and recreation (Dodds *et al.* 2009). Under natural conditions, two key nutrients for primary produces' growth, nitrogen (N) and phosphorus (P), are limiting, and eutrophication is a natural process by which water ecosystems are enriched with nutrients over time (Volterra *et al.* 2002). Depending on the geological and climate conditions, this process can take up to 1,000 years (EEA 1999). Conversely, human-driven eutrophication is characterised by an over-enrichment of nutrients occurring over much reduced time periods (decades). This rapid enrichment of nutrients causes excess growth of primary producers and can lead to anoxic conditions, responsible for generating methane and nitrous oxide, harmful greenhouse gasses (Abdel-Raouf *et al.* 2012). Anthropogenic eutrophication can also lead to a shift in primary producers favouring cyanobacteria over algae (Konopka & Brock 1978). Some cyanobacteria species are toxic, and may further accelerate degradation of water quality, with impact on human and environmental health, as well as on the economy (Pretty *et al.* 2003; Smith & Schindler 2009). Changes in bacteria and algae composition impact on the grazer community and

higher trophic levels by affecting species-species competition and consumer-resources dynamics (Kratina *et al.* 2012). For example, filamentous algae, which tend to grow in eutrophic conditions, have been shown to impact negatively on the grazer *Daphnia*'s life history traits, reducing growth rate, body size and fecundity (Bednarska *et al.* 2011; Ger *et al.* 2014). Moreover, the shift from algae to cyanobacteria linked can induce a shift in food quality impacting on grazers' growth rate (Tuchman *et al.* 2002). For example, human-driven eutrophication has been shown to induce changes in the population genetic composition of grazers as result of food quality changes (Frisch *et al.* 2014b).

Leaching of nutrients, pesticides and herbicides in freshwater ecosystems have contributed to alter water quality. Globally, between 1960 and 2010, the use of fertilizers and pesticides has increase 700% (Foley *et al.* 2005). Pesticides and herbicides have been shown to have an adverse effect on freshwater species (Malaj *et al.* 2014): in a continental scale monitoring program across Europe, it has been shown that organic chemicals (mainly pesticide) were present above environmental risk thresholds in more than 4000 freshwater ecosystems. Pesticides and herbicides have also been shown to impact negatively on lake's microinvertebrates biodiversity (Lang 2000). Whereas high concentrations of pesticides are lethal to biodiversity (Fleeger *et al.* 2003), sub-lethal concentrations can affect individual life history traits (e.g. fecundity in Daphnia (Jansen *et al.* 2011)), immune responses, behaviour, physiology, and morphology (Weis *et al.* 2001). Whereas the short-term impact of pesticides has been documented, the cross generational impact of pesticides is still poorly understood, but see Jansen *et al.* (2011), in which rapid evolution of insecticide resistance was shown in Daphnia.

Generally, biotic and abiotic stressors co-occur in the natural environment - pesticides, high temperature, food shortage, predators and parasites (Ha & Choi 2009; Altshuler *et al.* 2011; Garbutt *et al.* 2014; Schade *et al.* 2014). Importantly, the interaction of multiple stressors may result in antagonistic, additive or synergistic effect on wildlife (Coors & De

Meester 2008). Because of the complex nature of these interactions, it remains a great challenge to quantify the impact of multiple stressors on organismal fitness and to predicting the impact of multiple stressors from the response to single stress (Todgham & Stillman 2013). Recently, a meta-analysis on the impact of multiple stressors on freshwater organisms has identified recurrent trends (Jackson *et al.* 2016). According to this analysis most interactions between pair of stressors are antagonistic (41%) as opposed to synergistic, additive or reverse effect (Jackson *et al.* 2016).

2. Organisms' responses to environmental changes

Organisms respond to changes in the environment via different mechanisms, including geographical range shifts, plasticity and genetic adaptation (Hoffmann & Sgró 2011). Understanding these mechanisms is vital to assess resilience and/or persistence of species in the face of global change.

Geographic range shifts.

Migration has been a dominant response of species to climate shifts and habitat fragmentation (Huntley & Webb 1989; Schwartz 1993). Recent meta-analyses have shown a global latitudinal shift of up to 80% of species over a period of time as short as two decades (Parmesan *et al.* 2003). In support of these findings, the fossil record has provided abundant evidence of mostly poleward migration of many species following warming of the Palaeocene-Eocene Thermal Maximum (McInerney & Wing 2011). Geographical poleward shifts are ten times faster in marine than terrestrial ecosystems, reaching up to 19 km per year (Sorte *et al.* 2010). This rapid poleward geographic shift has led to many local extirpations for populations sitting at the boundaries of their distributional range and for sessile species (Aitken *et al.* 2008). Altitudinal shifts have affected up to 30% of the flora in some areas of the globe (Wolf *et al.* 2016) and have brought endangered species close to extinction (Yousefi *et al.* 2015). These shifts can be as severe as 17 kilometres in a decade (Chen *et al.* 2011).

Geographical shifts can be beneficial in the first instance, but generally lead to genetic instabilities over few generations as local adaptation can be disrupted by outbreeding depression (Frankham 2005), and alteration in species-species interactions (Aitken *et al.* 2008). In a stable environment, species found new equilibria. However, in rapidly changing environments this destabilization can lead to local extinctions (Visser 2016). For example, the Argentine ant (*Linepithema humile*) is controlled by a species-specific parasite that keeps local populations from monopolizing food resources. However, upon migration into new habitats not suitable for the parasite, the Argentine ant monopolizes food resources, leading to dramatic changes in ecosystem dynamics (Holway 1999). Poleward shift of this species, due to climate warming, caused asynchrony of the ant and the parasite population dynamics, leading to the complete extirpation of native ants' populations (Menke *et al.* 2018).

Geographic range shifts are a common mechanism that migratory species adopt to cope with environmental change (Sutherland *et al.* 2000; Reif & Flousek 2012; MacLean & Beissinger 2017). However, for long-lived, sessile species and species confined to discrete habitats (e.g. lakes), tracking warming climate depends on seedling/hatchlings colonization of new habitats (Monleon & Lintz 2015) and passive dispersal (Bilton *et al.* 2001). Passive dispersal may occur through transport by animal vectors or wind, often involving a specific desiccation-resistant stage in the life cycle (Figuerola & Green 2002; Figuerola *et al.* 2005; Vanschoenwinkel *et al.* 2011). Because of their limited migration scope, species living in discreet habitats are ideal to study in-situ adaptive responses to environmental change (Woodward *et al.* 2010; Dawson 2011; Stoks *et al.* 2014).

Adaptation

Genetic adaptation and plasticity are two of the main mechanisms mediating *in situ* adaptation.

Plasticity is a reversible response to short-lived and sudden changes in the environment that occur within the life span of an organism. Plasticity is defined as the capacity of a single genotype to produce different phenotypes in response to environmental cues (Pigliucci 2005). Plasticity can occur at the level of physiology, behaviour, phenology and phenotype (Parmesan 2006; Snell-Rood *et al.* 2010).

Phenotypic plasticity is one of the most common mechanism of response to environmental change (Hoffmann & Sgró 2011; Merilä & Hendry 2014). Because climate is warming at an unnatural pace, in recent years, the study of phenotypic plasticity in response to temperature changes has received much attention (Hoffmann & Sgró 2011; Hoffmann *et al.* 2015). Key phenotypes have been identified as target of temperature warming. For examples, decrease in body size has been associated with higher temperatures in many species, including arthropods, fish and mammals (Vidal 1980; Gardner *et al.* 2011; Baudron *et al.* 2014). Body size is a fundamental biological characteristic linked to important ecological properties such as fecundity, population growth rate, and competitive abilities (Millien *et al.* 2006; Gianuca *et al.* 2016). A recent meta-analysis showed that for each degree of temperature increase, body size is reduced from 4% in marine invertebrates to 14% in amphibians (Sheridan & Bickford 2011). A second phenotype generally affected by changes in temperature is the critical thermal maximum (CT_{max}), the reversible thermal endpoint where locomotor functions are compromised (Terblanche *et al.* 2007). Higher CT_{max} has been shown to evolve with temperature at different altitudes (García-Robledo *et al.* 2016; Oyen *et al.* 2016) and over evolutionary time in response to warmer climates in some species (Daufresne *et al.* 2009; Geerts *et al.* 2015; Brans *et al.* 2017) but to be phylogenetically constrained in others (Kellermann *et al.* 2012).

Behaviour can change more rapidly than most morphological traits and many physiological traits. A typical example of behavioural change in response to environmental stimuli is diel vertical migration in marine and freshwater zooplankton in response to predator cues

(Cousyn *et al.* 2001; Williamson *et al.* 2011). In the context of climate change, change in behaviour has been associated with change in temperature, especially in thermal-sensitive species (e.g. reptiles; (Caldwell *et al.* 2017)). Furthermore, changes in phenology– the timing of seasonal activities such as breeding or flowering – have been recorded in many species (Roy & Sparks 2000; Walther *et al.* 2002; Cleland *et al.* 2007). For example, increase in average ambient temperature has been associated with early breeding in birds (Williams *et al.* 2015), early arrival of migratory birds (Charmantier & Gienapp 2014), or early blooming in plants (Roy & Sparks 2000; Parmesan *et al.* 2003; Parmesan 2007). An important behavioural trait that has been affected by changes in temperature and, most importantly by the asynchrony between temperature and photoperiod, is dormancy; organisms have been recorded to enter diapause late and to emergence early from dormancy (Harada *et al.* 2011; Urbanski *et al.* 2012). Behavioural traits such as dormancy or flowering time are generally regulated by specific biochemical pathways, which are directly affected by physio-chemical cues. Changes in biochemical pathways and gene expression in candidate genes, such as heath shock proteins (HSP) have been linked to temperature changes (Sgrò *et al.* 2016). *HSPs* function as molecular chaperones protecting cells against accumulation of damaged proteins (Sørensen *et al.* 2003), and play a vital role in stress tolerance and survival under adverse conditions (Mayer & Bukau 2005). Both, plastic responses in the expression of HSP proteins (Mikulski *et al.* 2009, 2011; Brans *et al.* 2017) and evolution of HSPs over microevolutionary time scales (Bettencourt *et al.* 1999; Riehle *et al.* 2003; Ketola *et al.* 2004) have been associated with thermal stress.

A form of plasticity that is increasingly appreciated in a diverse array of taxa is the one across generations or transgenerational plasticity (TGP). Transgenerational plasticity (TGP) occurs whenever environmental cues experienced by either parent prior to fertilisation result in a modification of offspring reaction norms (Fox & Mousseau 1998). TGP has been documented in a number of organisms in response to environmental perturbations (Jablonka

et al. 1992; Jablonka & Raz 2009; Bonduriansky *et al.* 2012) and it is expected to favour offspring generations when environmental conditions are stable (Bonduriansky *et al.* 2012; Kuijper *et al.* 2014), favouring increased plasticity across-generations (Hoyle & Ezard 2012; Kuijper *et al.* 2014). Examples of TGP have been described in plants (Galloway & Etterson 2007), insects (Coleman *et al.* 2014) and freshwater invertebrates (Walsh *et al.* 2016) in response to many environmental perturbations.

Measures of phenotypic plasticity are typically expressed as linear correlations (e.,g. reaction norms), which describe the change in a genotype's phenotype in response to the environment (Nussey *et al.* 2007). The slope of the reaction norms quantifies the amount of plastic response, whereas the direction of change in the reaction norm quantifies whether the response to the environment is negative or positive. The quantification of plasticity via reaction norms is generally complicated by genetic variation, making it impossible to completely disentangle genetic adaptation from plasticity (Merilä & Hendry 2014). The only species for which it is possible to quantify the relative contribution of genetic and plastic response to environmental change are parthenogenetic species (e.g. Crustaceans), in which the same genotype can be exposed to multiple environmental cues and plastic response quantified. Furthermore, for parthenogenetic species that form dormant stages as part of their life cycle, **evolution of plasticity** can be quantified by comparing the slopes of the reaction norms from different phenotypes of the same population separated in time (Hairston & De Meester 2008; Baudron *et al.* 2014).

Genetic adaptation is the movement of a population towards a phenotype that best fits the environment (Orr 2005). Genetic adaptation can occur via two main mechanisms: selection on pre-existing genetic variation (standing genetic variation; copy number variation, duplication, inversion) and selection on new mutations (single nucleotide polymorphism (SNPs), indels, transpositions, chromosome mutations). Compared with new mutations, standing genetic variation is likely to lead to faster evolution, as beneficial alleles have

already passed through a 'selective filter', which increases the chance that large-effect alleles are advantageous (Schluter *et al.* 2004; Barrett & Schluter 2008; Barrett & Hoekstra 2011). On the other hand, standing genetic variation may hinder adaptation to new environmental selection pressures that have not been previously experienced, such as new abiotic stressors (Hedrick 2013).

Mutations are one of the fundamental forces of evolution because they fuel the variability in populations and thus enable evolutionary change (Barrett & Schluter 2008). The effects of mutations depend on many aspects, some of which are: i) the presence or absence of other mutations, (ii) selection pressure from the environment, and (iii) effective population size (Loewe & Hill 2010). The contribution of new mutation to adaptation is generally greater for population with large effective population's size and short generation times as the probability to increase beneficial allele frequency is higher (Hoffmann & Sgró 2011). Mutations are frequently classified as non-synonymous or synonymous according to whether or not they change the amino acid sequence and are commonly used in population genetics. D*e novo* mutations have and initial low frequencies, as they start out as single copies, and most are lost by chance or by purging via genetic drift before they establish in the population (Olson-Manning *et al.* 2012).

Although genetic adaptation is expected to be more important than phenotypic plasticity in long-term evolution, there are limited examples of genetic adaptation in natural populations (Cousyn *et al.* 2001; Jones *et al.* 2012b; Orsini *et al.* 2012, 2013a). The limited number of studies on genetic adaptation may be explained with the lack of genomic resources for non-model species (Merilä 2012). However, in recent years this issue has been alleviated with the application of next generation sequencing technologies to a number of ecological models species of arthropods (Toth 2011; Colbourne *et al.* 2011b); plants (Novaes *et al.* 2008); mammals (Li *et al.* 2010); birds (Romanov *et al.* 2009); and fish (Jones *et al.* 2012a). These species have well-documented histories of phenotype-environment

interactions and the development of genomic resources opens unprecedented avenues to unravel relationships among genotypes, phenotypes and environmental change.

Extinctions

If organisms cannot adapt to changes in the environment, or if they cannot migrate to more suitable habitats they will decline and eventually go extinct (Devictor *et al.* 2008). Human activities have been shown to expedite the extinction and the decline of many species (Pimm *et al.* 2014). In the last 60 years the International Union for Conservation of Nature (IUCN 2017) has assessed 91,523 species worldwide, of which 11,783 were found vulnerable, 8,455 endangered and 5,583 critically endangered (IUCN 2017). Habitat loss and degradation have been identified as the main causes of species extinction, being responsible for 85% of current species loss (IUCN 2017). Human consumption, production, and overexploitation of resources are among the strongest drivers of species extinction (IUCN, 2017). These factors directly impact species survival (Brook 2008). In addition, they are responsible for mismatches between food availability, and for interfering with breeding dynamics (Visser & Both 2005).

Rapid environmental change can also affect habitat resources favouring invasive species over indigenous ones (Sax & Gaines 2008; Colautti *et al.* 2017). Finally, high mortality or decline in species diversity is tightly associated with decrease in genetic diversity, which leads to population bottlenecks (Templeton *et al.* 1990). Reduced genetic diversity severely impairs adaptive responses to changing environments (see genetic adaptation above), eventually leading to species decline and extinction (Agashe *et al.* 2011).

Overall, freshwater ecosystems have lost 81% of the species diversity in the last four decades alone, ranging between 26% in mammals and fish to 39% in reptiles (Pimm *et al.* 2014); this trend corresponds to an annual average decline of 3.9% (WWF 2016). Species loss in terrestrial ecosystems ranges from 13% in birds to 41% of amphibian and

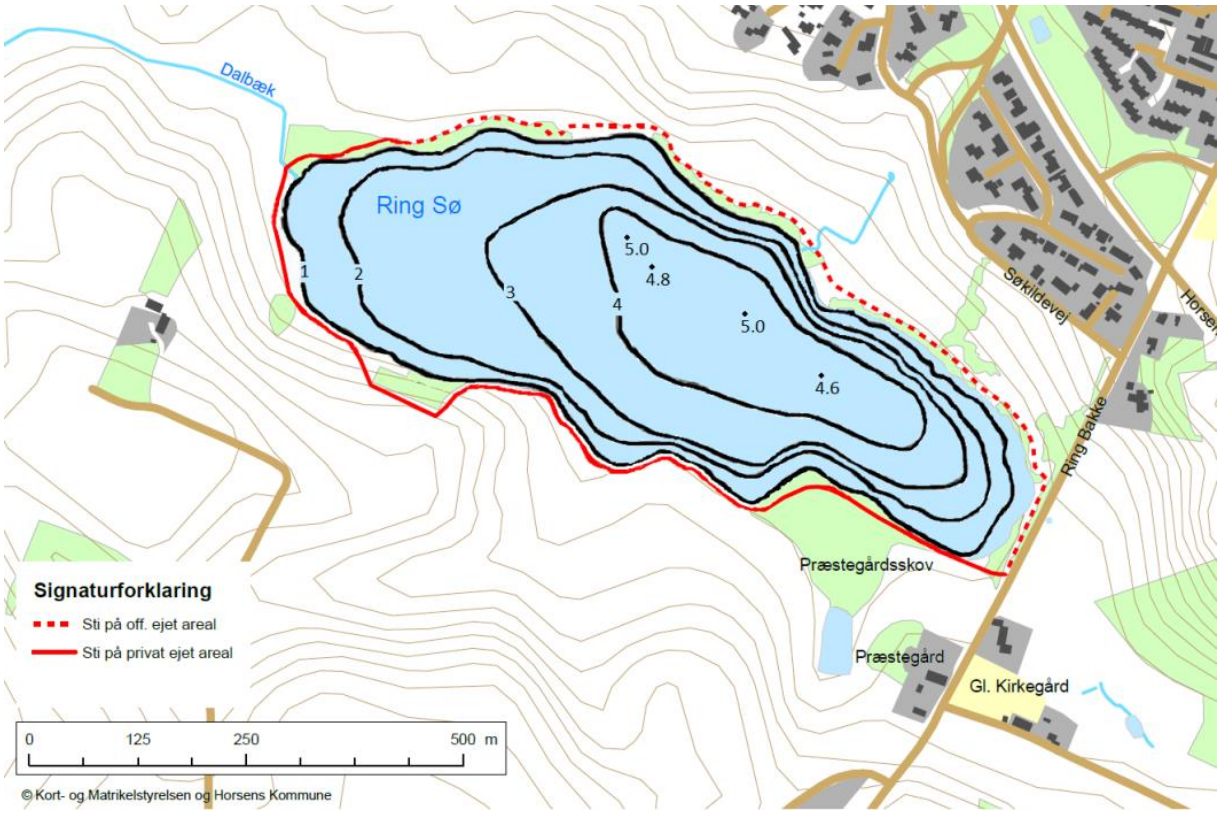
gymnosperms (Pimm *et al.* 2014). Although major efforts have been made to assess species loss in marine species, marine biodiversity loss has been more challenging to evaluate (Selig *et al.* 2014). Yet, severe decline in iconic species has been documented [e.g. coral reefs(Carpenter *et al.* 2008), marine mammals (Davidson *et al.* 2012).

3. Study system: Lake Ring

Lake Ring (Fig. 3) is a natural shallow pond located in Denmark (55° 57' 51.83'' N, 9° 35' 46.87'' E). It is a relatively small pond with a surface of 22.5 ha and an average depth of 2.9 m. According to historical records the lake suffered from hyper-eutrophication between 1950s and 1960s due a sewage inflow from a nearby town (4,000 inhabitants). At the end of the 1960s, sewage was diverted from the lake. From the late 1970s, increase in agricultural land use provoked intense pesticides and herbicides leaching in the lake. According to the local county authority, carbamate pesticides were among the most commonly sold in Denmark in the 1980s. Finally, in the late 1980s, the lake partially recovered due to a decrease in agricultural land-use. In 1989, the Lake was used as an open air experiment to assess the impact of fish predation on the local zooplankton community (Berg *et al.* 1994) Following the introduction of fish, Michels *et al*. 2004, investigated the effect of predation on diel vertical migration of the zooplankton grazer of *Daphnia magna*.

A detailed paleolimnological analysis of Lake Ring was conducted for this thesis and will be introduced in Chapter 2. Furthermore, historical data of temperature, water transparency, pesticide usage, records of phosphorus, nitrogen and water transparency were provided by the local county authority.

Fig 3. Bathymetric map of Lake Ring, Denmark. The dotted red line indicates public land and the solid red line indicates private land.



4. *Daphnia magna* as model organism

The water flea *Daphnia* (Crustacean, Cladocera) is a small planktonic crustacean. *Daphnia* are keystone species in a wide range of freshwater standing waters worldwide, including permanent and temporary ponds that can eventually dry or freeze, depending on the period of the year (Adamowicz *et al.* 2009). As filter feeder, *Daphnia* spp. graze on phytoplankton and bacteria; at the same time, they are the main food for both invertebrate and vertebrate predators (Ebert 2005). Hence, *Daphnia* spp. play a pivotal role in the pelagic food web of freshwater ecosystems (Lampert 2006; Stollewerk 2010) where they are drivers of ecosystem dynamics (Miner *et al.* 2012).

The waterflea *Daphnia magna* Straus occurs in lakes and ponds in Europe, Africa, Asia and America (Forró *et al.* 2008). It has a prominent ecological role in pelagic freshwater

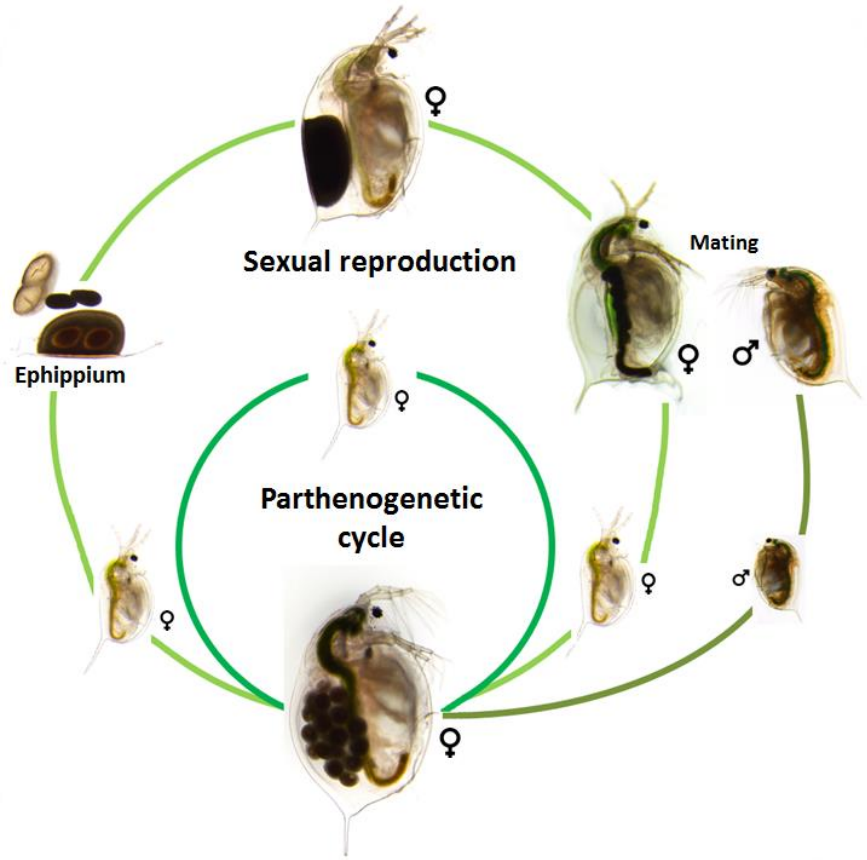
food webs, where it is the primary forage for many vertebrate and invertebrate predators, an efficient grazer of algae, and a strong competitor for other zooplankters (Lampert 2011).

D. magna has a parthenogenetic life cycle in which sexual and asexual reproduction alternate. Under favourable conditions, *D. magna* reproduces asexually, whereas harsh environmental conditions prompt male formation and a switch to sexual reproduction, which culminates in dormant embryos. Dormant embryos are protected from the environment by a chitin case called *ephippium*. Dormant embryos can survive to the most extreme condition, such as desiccation or freezing for extended time periods. A proportion of the dormant embryos hatches in the following growing season and contributes to fuel the local population genetic diversity. A good proportion of these dormant embryos remains buried in the sediment of lakes and ponds not having the chance to hatch, and accumulates to form stratified dormant egg banks. These are a valuable resource to reconstruct evolutionary changes over evolutionary time (e.g. across generations) (De Meester *et al.* 2007; Orsini *et al.* 2012, 2016b; Frisch *et al.* 2014).

Daphnia reproductive strategy (Fig. 2) offers the unique opportunity to 'resurrect' historical populations from lake sediment by the practise of resurrection ecology (Kerfoot & Weider 2004). Once dormant stages have been 'resurrected', *Daphnia* genotypes are clonally maintained in the laboratory, providing the opportunity to study responses of the same genotypes to multiple stressors (Stoks *et al.* 2016; Cuenca Cambronero *et al.* 2018) and to reveal the relative contribution of plasticity and genetic adaptation to complex environments through evolutionary time. *Daphnia* is one of the few ecological models that enables longitudinal studies in which genotypes of the same population sampled at different time points along environmental gradients can be used in the same experimental setting. By comparing the responses to environmental stress of historical populations to the one of their modern descendants, one can investigate how historical exposures shape stress response of modern populations and assess species evolvability.

Recently, genomics resources for *Daphnia spp*, including *D. magna* have become available (Colbourne *et al.* 2011a; Orsini *et al.* 2018). Omics studies on *Daphnia spp* have revealed ecoresponsive genome (Colbourne *et al.* 2011a) and the ability to adjust within few hours to environmental change via a plastic transcriptome (Orsini *et al.* 2016a, 2018). The combination of modern genomics tools with trait-based analysis make *Daphnia* an ideal model species to investigate adaptive responses to environmental change (Miner *et al.* 2012). High experimental tractability, short generation time, and small body size enable experimental approaches on large populations of *D. magna*. Moreover, the cyclic parthenogenetic life cycle enables the parallel analysis of functional and fitness changes in the same genotype in multiple environmental conditions.

Fig 2. *Daphnia magna* lifecycle showing both, sexual and asexual reproduction



5. Resurrection ecology and paleogenomics

The collection of long-term data set is pivotal to our understanding of evolutionary responses to environmental stress, as environmental change as well as organismal response to these changes occur across multiple generations. Generally, the collection of cross generational data requires effort and commitment beyond a researcher's life.

'Resurrection ecology', applied to species that produce long-lasting dormant stages, provides the means to reconstruct cross-generational responses to environmental change within the scope of a research project (Kerfoot *et al.* 1999). This approach provides the possibility to revive organism from biological archives spanning decades or even centuries and to compare historical populations with their modern evolved decedents (Franks *et al.* 2018). Species of the genus *Daphnia* have been among the most commonly studied organisms in the context of resurrection ecology (Burge *et al.* 2018). With light and temperature cues, *ephippia* collected from different time points along a sedimentary archive can be induced to hatch, making possible to perform common garden experiments with historical and modern populations in the same experimental setting (Miner *et al.* 2012). Importantly, these experimental settings allow the identification of the mechanisms that enable adaptive responses to environmental change. Furthermore, populations separated in time enables us to assess the adaptive potential of populations in function of their historical exposure to environmental stress (Franks *et al.* 2018).

The combination of resurrection ecology and paleolimnology enables us to establish a causal link between eco-evolutionary responses and environmental change. The application of omics technologies to revived or still dormant propagules enables us to investigate the molecular mechanisms that enable adaptive evolution (Orsini *et al.* 2013b). Understanding the genotype-phenotype interactions driven by environmental change is especially relevant in the light of rapid human-driven environmental change (Chowdhury *et al.* 2015).

6. Adaptation to changing environments: outstanding challenges

Despite great effort and progress in recent years associated with the genomicisation of ecological model species and the ecologisations of genetic model species (Stapley *et al.* 2010), outstanding challenges remain that affect our understanding of how natural populations adapt to changing environments.

First, environmental changes occur over time (decades or centuries). Ideally, long-term data are required to understand the impact of environmental change on organisms and to measure organisms' response to such change (Orsini *et al.* 2013b). Most studies analysing temporal dynamics are laboratory or controlled mesocosm experiments (Blount *et al.* 2012; Kawecki *et al.* 2012; Barrick & Lenski 2013) with exceptional studies that reconstruct evolution of natural populations using transplant experiments in the wild (Reznick *et al.* 2008). For species that cannot be easily manipulated experimentally, or for which temporal samples are inaccessible, the 'space-for-time' substitution (Fukami & Wardle 2005) is frequently adopted as a surrogate to study long-term evolutionary dynamics. Space-for-time substitution assumes that two different conditions at two points in space can be treated as though they are in the same region at two different time points. This approach has its limitations as rates of adaptation at different spatial scales can differ compared to temporal variation in the same population evolving in time (Merilä & Hendry 2014). A powerful alternative that allows studying evolutionary dynamics in natural populations over time is the analysis of species producing long-lived dormant stages. Dormant stages accumulate in soil, sediment and ice to build up biological archives providing a powerful resource to generate long-term data in natural systems (Bidle *et al.* 2007; Orsini *et al.* 2012; Frisch *et al.* 2014b).

A second major challenge that studies of adaptation to changing environments face is disentangling the role of phenotypic plasticity and genetic adaptation. This is because phenotypic change can be the result of either genetic change or phenotypic plasticity; moreover phenotypic changes may or not be adaptive (Merilä & Hendry 2014). Despite the

promises associated with using molecular approaches to detect evolution driven by environmental change (Reusch & Wood 2007), few studies have been able to link genetic changes to phenotypes and to confirm that the changes are adaptive (but see Umina *et al.* 2005; Balanya *et al.* 2007). Many studies have reported plasticity as main mechanism of adaptive response to environmental change (Gienapp *et al.* 2008; Hendry *et al.* 2008; Teplitsky *et al.* 2008; Charmantier & Gienapp 2014). However, in most cases this conclusion has been reached without direct evidence for genetic change or lack thereof.

Directly associated with the difficulty of identifying the mechanisms driving adaptation to changing environments is the difficulty of determining the specific environmental factor(s) causing a particular phenotypic/genetic change (MacColl 2011). This is a known and concerning problem, especially in the context of climate change, in which increasing temperatures often co-occur with other environmental stressors (Altenburger *et al.* 2015). Difficulties associated with linking mechanisms of adaptation to specific environmental factors derive from the fact that multiple environmental factors produce effects that are not predictable from the impact of single stressors ('ecological surprises' (Ormerod *et al.* 2010; Jackson *et al.* 2016)). Growing evidence suggests that interactions among global change drivers are often nonlinear (Sala *et al.* 2000; Spaak *et al.* 2017; Wu *et al.* 2017). Hence, the assessment of organismal response to multiple stressors is limited to a handful of studies (e.g. (Christensen *et al.* 2006; Brans *et al.* 2017)).

3. Thesis objectives and chapter's guide

A parallel temporal analysis conducted on multiple sedimentary archives distributed across a landscape is required for the reconstruction of the processes that drive evolutionary dynamics at regional and continental scales (Orsini *et al.* 2013b). My thesis focuses on a single well-characterized lake system to generate the first proof of concept in the field of

paleogenomics by studying phenotypic, biochemical and genomic evolution through evolutionary time and across major environmental gradients.

We focus on a well-characterized lake with a documented history of anthropogenic impact (Berg *et al.* 1994a; Michels *et al.* 2007). The history of the lake was reconstructed via historical records (Berg *et al.* 1994a; Michels *et al.* 2007) and the limnological analysis of sediment (Chapter 1 and 2). In the late 1950s, sewage inflow from a nearby town initiated symptoms of severe eutrophication (Michels *et al.* 2007). The sewage inflow was diverted at the end of the 1970s, but this period coincided with agricultural land use intensification (>1975), leading to substantial pesticides and herbicide leaching in the lake (Michels *et al.* 2007). The lake partially recovered from hyper-eutrophication in modern times (>1999s) but, as every lake in Europe, it experienced an increase in average ambient temperature (IPCC 2014). Against this backdrop of well-documented environmental change, we show that the invertebrate community was responsive to pollution events, and that eutrophication, driven by sewage inflow, and pesticide leaching were major pollution events in the lake (Chapter 2). These events co-occurred with a modest average increase in ambient temperature, documented by a weather station located in proximity of the lake. The persistence of *D. magna* in the lake through these major pollution events (Chapter 1 and 2) suggests that this species adopted coping mechanisms to respond to environmental stress.

We investigated mechanisms of response to environmental stress, using common garden experiments, in which we tested the impact of temperature as single stressor and in combination with food levels and insecticide loads on the *D. magna* population, measuring changes in fitness-linked life history traits in controlled experimental settings (Chapter 2). In follow-up experiments, we studied physiological and molecular mechanisms of response to thermal and hyper-thermal stress (Chapter 3 and 4).

A central regulatory element compensating for changes in oxygen supply and ambient temperature is the respiratory protein haemoglobin (Hb). In chapter 3, we quantified haemoglobin (Hb) plastic and evolutionary responses of the *D. magna* population resurrected

from the sedimentary archive, and interpreted these responses in function of average temperature increase and occurrence of heat waves. In this chapter, we also tested competitive abilities of genotypes as a function of their Hb content. In chapter 4, we investigated trade-offs between constitutive and induced thermal tolerance and studied multiple physiological and molecular mechanisms of thermal tolerance over evolutionary times in response to temperature alone and in combination with a second stressors, capitalizing on the experiments in Chapter 2 and 3. Specifically, we quantified genetic and plastic differences in critical thermal maximum (CT_{max}), body size, haemoglobin (Hb) content and heat shock proteins (HSP) expression between the historical and the modern genotypes of the *D. magna* population resurrected from Lake Ring, which experienced increase in average temperature and occurrence of heat waves, in addition to dramatic changes in water chemistry over five decades. In Chapter 5, we investigated the genetic basis of adaptation to multifarious environments using genome-wide association analysis, genome scans and regularized generalized canonical correlation analysis. In this chapter I identified genome polymorphisms underpinning adaptive phenotypic changes driven by environmental selection.

The thesis is organized in seven chapters including a general introduction and a general discussion. Each chapter is presented as a manuscript, published, in review or unpublished, which content I outline below.

The Introduction (present chapter) to the thesis is unpublished.

In this chapter I provide a state-of-the-art on the anthropogenic impact on natural ecosystems and on mechanisms of organismal response to such changes. I describe outstanding challenges in our understanding of adaptive evolution to multifarious environments and the aim of my thesis. Finally, I outline the thesis chapters, and how they contribute to address the overall scope of the thesis.

Author's contribution: MCC conceived and wrote the chapter.

Chapter 1 is published in the *Journal of Visualized Experiments*: Cuenca Cambronero, M., and Orsini, L. (2017) **Resurrection of dormant *Daphnia*: protocol and applications.** Doi: 10.3791/56637.

A standard operating procedure (SOP) is provided to revive dormant *Daphnia magna* from dated sedimentary archives to dramatically advance the state-of-the-art of longitudinal data collection in natural systems. The field of Resurrection Ecology was defined in 1999. However, the methodology of resurrecting zooplankton species has been propagated among laboratories only via direct knowledge transfer. Here, an SOP is described that provides a step-by-step protocol on the practice of resurrecting dormant *Daphnia magna* eggs. This chapter provides a first description of the paleolimnological analysis of Lake Ring.

Author's contribution: MCC carried out the experiments and performed data analysis. LO conceived the study and coordinated data analysis. LO and MCC wrote the paper.

Chapter 2 is under review in the *Proceeding of Royal Society B*: Cuenca Cambronero, M., Marshall, H., De Meester, L., Thomas, A. D., Beckerman, P. A., and Orsini, L. **Multiple stressors in a changing world and ecological unpredictability.**

In this chapter, empirical evidence is provided to quantify the long-term impacts of multifarious environments on the keystone grazer *Daphnia magna.* Mechanisms of response to multiple anthropogenic stressors are studied through evolutionary time using common garden experiments; specifically, the impact of temperature as single stressor and in combination with food levels and insecticide loads is shown on life history traits of *D. magna* populations resurrected from Lake Ring.

Author's contribution: MCC and HM carried out the experiment. MCC and APB performed data analysis. LDM provided the sedimentary archive of Lake Ring and contributed to the experimental design. TAD contributed with information on the ecological background of Lake Ring and performed the community analysis. LO

conceived the study and coordinated data analysis. LO and MCC wrote the first version of the paper; all authors contributed to the editing of later versions.

Chapter 3 is published in *Evolutionary Applications* and it is part of the special issue *Evolutionary Aspects of Resurrection Ecology: Progress, Scope, and Applications*: Cuenca Cambronero, M., Zeis, B., and Orsini, L. (2017) **Haemoglobin-mediated response to hyper-thermal stress in the keystone species *Daphnia magna.*** Doi: 10.1111/eva.12561b

In this chapter, coping mechanisms to thermal and hyper-thermal stress are investigated by quantifying Haemoglobin (Hb) plastic and evolutionary responses and competitive abilities of genotypes as a function of their Hb content. *Daphnia magna* subpopulations resurrected from the same sedimentary archive used in previous chapters are used.

Author's contribution: BZ carried out the experiments. MCC performed data analysis. LO conceived the study and coordinated data analysis. LO and MCC wrote the first version of the paper. All authors contributed to the editing of the final version of the manuscript.

Chapter 4 is under review in *Molecular Ecology*: Cuenca Cambronero, M., Beasley, J., Kissane, K., and Orsini, L. (2018) **Evolution of thermal tolerance in multifarious environments.**

In this chapter, I expand on the findings of Chapter 3 and investigate physiological and molecular mechanisms underlying thermal tolerance over evolutionary times in multifarious environments. Trade-offs between constitutive and induced thermal tolerance are assessed in presence of warming alone and in combination with biotic and abiotic stress. This is done by quantifying genetic and plastic differences in critical thermal maximum (CT_{max}) and heat shock proteins (HSP) expression between historical and modern genotypes of the same population that experienced increase in

average temperature and occurrence of heat waves, in addition to dramatic changes in water chemistry over five decades.

Author's contribution: MCC carried out the experiments and performed data analysis with input from JB. SK and JB generated the HSP data. LO conceived the study and coordinated data analysis. LO and MCC wrote the first version of the paper. All authors contributed to the editing of the final version of the manuscript.

Chapter 5 is unpublished: Cuenca Cambronero, M., Dhandapani, V., Zhou, J. Colbourne, J. and Orsini, L. **Paleogenomics reveals genome variants underpinning plastic and constitutive phenotypic divergence in multifarious environments**.

In this chapter, high throughput sequencing technology is applied to the populations of *D. magna* resurrected from the biological archive of Lake Ring to identify the genomic basis of constitutive and plastic differences in life history traits measures in Chapter 3. Genome variants underpinning plastic and constitutive phenotypic responses to single and multiple stressors are identified via traditional GWAS analysis as well as using advanced computational tools developed in the research group.

Author's contribution: MCC carried out the experiments. MCC and VD performed bioinformatic analyses. JZ provided a newly developed pipeline for genome wide association studies. JKC contributed to the experimental design and advised on data analysis. LO conceived the study and coordinated data analysis. MCC wrote the chapter.

Discussion and conclusions are unpublished. In this chapter I provide an overall discussion of results, and a critical assessment of strengths and weaknesses of the thesis. Further, I discuss the advances that the thesis has provided in the field of paleogenomics identifying perspectives for future developments.

Author's contribution: MCC conceived and wrote the chapter.

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CHAPTER 1

Resurrection of Dormant *Daphnia magna:* Protocol and Applications

Information:

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Overview: A standard operating procedure (SOP) is provided to revive dormant *Daphnia magna* from dated sedimentary archives to dramatically advance the state-of-the-art of longitudinal data collection in natural systems. The field of Resurrection Ecology was defined in 1999. However, the methodology of resurrecting zooplankton species has been propagated among laboratories only via direct knowledge transfer. Here, an SOP is described that provides a step-by-step protocol on the practice of resurrecting dormant *Daphnia magna* eggs. This chapter provides a first description of the paleolimnological analysis of Lake Ring.

Author's contribution: MCC carried out the experiments and performed data analysis. LO conceived the study and coordinated data analysis. LO and MCC wrote the paper.

CHAPTER 2

Predictability of the impact of stressors mixtures on the keystone species *Daphnia magna*

Information:

Authors: Cuenca Cambronero, M., Marshall, H., De Meester, L., Thomas, A. D., Beckerman, P. A., and Orsini, L.

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Overview: In this chapter, empirical evidence is provided to quantify the long-term impact of mixture stressors on the keystone grazer *Daphnia magna.* Mechanisms of response to multiple anthropogenic stressors are studied through evolutionary time using common garden experiments; specifically, the impact of temperature as single stressor and in combination with food levels and insecticide loads is shown on life history traits of *D. magna* populations resurrected from Lake Ring.

Author's contribution: MCC and HM carried out the experiment. MCC and APB performed data analysis. LDM provided the sedimentary archive of Lake Ring and contributed to the experimental design. TAD contributed with information on the ecological background of Lake Ring and performed the community analysis. LO conceived the study and coordinated data analysis. LO and MCC wrote the first version of the paper; all authors contributed to the editing of later versions.

Predictability of the impact of stressors mixtures on the keystone species *Daphnia magna*

Maria Cuenca Cambronero[1], Hollie Marshall[1], Luc De Meester[2], Thomas Alexander Davidson[3], Andrew P. Beckerman[4#] and Luisa Orsini[1*#]

[1] Environmental Genomics Group, School of Biosciences, the University of Birmingham, Birmingham B15 2TT, UK

[2]Laboratory of Aquatic Ecology, Evolution and Conservation, University of Leuven, Ch. Deberiotstraat 32, 3000 Leuven, Belgium.

[3] Freshwater Ecology, Department of Bioscience, Aarhus University, Vejlsøvej 25, P.O. Box 314, DK-8600 Silkeborg, Denmark

[4]Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, U.K

[#]these authors contributed equally

Running head: Multiple stressors in a changing world

Corresponding author:

Miss Maria Cuenca Cambronero

Environmental Genomics Group, School of Biosciences, University of Birmingham, Birmingham B15 2TT, UK

Abstract

The impact of mixtures of anthropogenic stressors on biodiversity and organisms' response to such stressors are difficult to measure because interactions and responses may be nonlinear. Empirical evidence is needed to quantify the long-term impacts of multifarious environments on biodiversity.

Using 'resurrection ecology' of the keystone grazer *Daphnia magna*, we studied mechanisms of response to multiple anthropogenic stressors over evolutionary time. We resurrected three *D. magna* (sub)populations from a lake that experienced hyper-eutrophication due to sewage inflow, leaching of pesticides due to land use intensification and an increase in average ambient temperature over time. Using common garden experiments, we tested the impact of temperature as single stressor and in combination with food levels and insecticide loads on life history traits. We showed that populations' response to warming combined with biotic or abiotic stress is not predictable from the effect of warming alone. We also showed that historical exposure to stress prior to dormancy provides populations with an evolutionary advantage when the stress recurs. However, this advantage is contingent upon the type and severity of environmental stressor. Our study provides experimental evidence of evolutionary mechanisms of response to multiple stressors in a key indicator species in freshwater ecosystems.

Keywords: eutrophication, resurrection ecology, plasticity, adaptation, temperature

Background

Anthropogenic stressors have been shown to be responsible for 76% average population decline among freshwater species, and for 39% decline in terrestrial and marine species since 1970 [1, 2]. Their impact is more severe on freshwater biodiversity [3, 4] because freshwaters suffer from the effects of both local catchment and regional atmospheric processes [5]. In particular, human-driven hyper-eutrophication and global warming have been shown to affect up to 50% of freshwater biodiversity worldwide since the middle of the last century [6, 7], to reduce provision of water resource [8], and to affect other services such as tourism and recreation [9].

Multiple stressors produce effects on aquatic ecosystems that are not predictable from the impact of single stressors ('ecological surprises' [4, 10]). Growing evidence suggests that interactions among global change drivers are often nonlinear [11], making predictions of the impact of anthropogenic stress on biodiversity challenging [12, 13]. Furthermore, population level responses can vary dramatically because of differences in environmental sensitivity [14], tolerance among individuals [15], ecological trade-offs and patterns of local adaptation [16, 17]. Assessment of the complex organismal response to multiple stressors is limited to a few studies (e.g. [18, 19]). Yet, forecasts of the impacts of global change on biodiversity and related ecosystem processes will be unreliable without investigations focused on how multiple anthropogenic stressor effects manifest in different biomes [11, 20].

Here, we study ecological and evolutionary responses of the model freshwater habitat species *Daphnia magna* to multiple anthropogenic stressors. *D. magna* is a keystone grazer occurring in a wide range of freshwater standing waters worldwide [21]. *Daphnia* adopts a unique reproductive strategy, which alternates sexual and asexual reproduction, in response to environmental stimuli. Sexual reproduction culminates in the production of dormant stages, a proportion of which hatch in the following growing season and contributes to the local genetic diversity. The dormant stages that do not have the opportunity to hatch build up

biological archives. The properties of *Daphnia* offer the unique opportunity to 'resurrect' historical populations from lake sediment by the practise of resurrection ecology [22]. Once dormant stages have been 'resurrected' or hatched, *Daphnia* genotypes are clonally maintained in the laboratory, proving the opportunity to study responses of the same genotypes to multiple stressors [23, 24] (Chapter 2, 3 and 4). This is done by studying the response of the same genotypes to different environmental stressors. The properties of *Daphnia* provide us with the opportunity to reveal the relative contribution of plasticity and evolution to multi-stress response through evolutionary time. By comparing the responses to environmental stress of historical and modern populations in common garden experiments, we can investigate how historical exposures shape stress response of modern populations.

Our work focuses on Lake Ring in Denmark which has a well-defined history of anthropogenic impact [25, 26]. In the late 1950s, sewage inflow from a nearby town initiated symptoms of severe eutrophication [26]. The sewage inflow was diverted at the end of the 1970s, but this period coincided with agricultural land use intensification (>1975), leading to substantial pesticides and herbicide leaching in the lake [26]. The lake was stocked with white fish between 1989 and 1990 to study the impact of predation on the invertebrate community [25]. Finally, it partially recovered from hyper-eutrophication in modern times (>1999s) but, as every lake in Europe, experienced an increase in average ambient temperature [27]. From each of the lake phases – sewage, pesticide and recovery - we resurrected *D. magna* (sub)populations (hereafter referred to as populations) and performed common garden experiments in which we measured response of life history traits to temperature as single stressors and in combination with food levels and insecticide loads. The common garden experiments were designed to test four hypotheses.

1. The effect of temperature varies among populations and is less severe on the most recent population, which has experienced higher average ambient temperature than the historical population [28, 29].

2. Temperature and non-limiting resources have an antagonistic effect, which results in a negligible impact of temperature on life history traits [10, 30]. Conversely, temperature and food limitation have a synergistic (greater than individual stressors) effect on life history traits [10, 31].
3. The effect of insecticide varies with temperature, and effects are more severe at high concentrations. Concentrations of pesticides being equal, the impact on fitness is comparatively higher at low temperature, as some insecticides display higher volatilization and degradation at higher temperatures [32].
4. Population response to environmental stress depends on environmental exposure prior to dormancy [33]. Specifically, populations historically exposed to certain environmental stressors show an evolutionary advantage over naïve populations when the stress recurs.

Overall, our study provides insights into the response of a key grazer of the aquatic community to warming, in isolation and combined with other stressors, relevant in the context of global change. Moreover, it empirically tests the predictability of mixtures of stressors from single stressors in a freshwater keystone species.

Methods

Paleolimnological reconstruction of Lake Ring and historical records

Our study system is Lake Ring, a well characterized shallow mixed lake (without thermocline stratification) located in a typical peri-urban landscape in Jutland, Denmark (55°57'51.83'' N, 9°35'46.87'' E) [34]. A sedimentary archive was sampled from the lake in 2004 with a piston corer of 6 cm internal diameter as described in [35] (Chapter 1). After collection, the core was sliced in layers of 0.5 cm and stored in dark and cold (4 °C) conditions. A radiometric chronology of this sediment was completed in 2015 by ENSIS Ltd (UCL London) following standard protocols [36], and providing an accurate dating of the sediment layers to the year

1900. Dating of sediment was conducted by direct gamma assay, using ORTEC HPGe GWL series well-type coaxial low background intrinsic germanium detector. Sediment samples with known radionuclide profiles were used for calibration following [36].

Environmental changes through time occurring in Lake Ring were reconstructed from historical records and the paleolimnological analysis of sediment:

1. Historical records document severe eutrophication associated with sewage inflow between 1960 and 1970 [26]. Records of pesticides historically used in Denmark were available from the Danish national archives. Temperature records were collected by the Danish Meteorological Institute at a weather station located 80 km from the lake. Because air and water surface temperature have a positive correlation for shallow streams and lakes [37, 38], we used the data from the weather station as estimates of the lake water temperature. Data on total phosphorus, nitrogen, and water transparency were measured in the lake between 1971 and 1999 by the local county authority.
2. The paleolimnological analysis consisted of quantifying the organic matter content, the *Cladocera* assemblage and the *Daphnia* abundance throughout the sedimentary archive. Loss on ignition (LOI, the percentage sediment weight lost on ignition) was used to estimate the organic and carbonate content of the sediment [39]. The procedure for LOI consists of strongly heating ("igniting") a sediment sample at a specified temperature (550°C), allowing volatile substances to escape, until its mass ceases to change [40]. LOI is used in limnology as an indirect measure of eutrophication as Carbon is the main component of primary producers and a good estimate of their abundance [41]. The zooplankton community composition in each sediment layer was quantified form sub-fossil remains counted in each sediment layer using standard methods [42]. Changes in *D. magna* abundance were quantified from the dormant *D. magna* embryos count recovered from each sediment layer, and multiplied for the total lake surface (22.5 hectares).

Based on the paleolimnological and historical characterization of the lake, we identified three main lake phases: i) Eutrophication phase (*EP*, 1960-1970) characterized by an increase in primary production (high LOI) due to sewage inflow; ii) Pesticides phase (*PP*, 1980-1990) driven by increase in agricultural land use. This phase is associated with heavy usage of carbamate insecticides; and iii) Clear water or recovery phase (*CWP*, >1999) after diversion of sewage, associated with a decrease in primary production (low LOI), decrease in Phosphorus and Nitrogen and increase in water transparency.

The entire sedimentary archive was inspected for dormant *D. magna* and a total of 262 dormant *Daphnia* embryos were hatched following established protocols [35] (Chapter 1). Clonal lineages were established from individual hatchlings and maintained in standard laboratory condition (16:8 light: dark regime, 10°C and 0.4 mg C/L of *Chlorella vulgaris* be-weekly) as clonal lineages for several generations (up to 6 months) to reduce interference from extended dormancy. From the overall hatched embryos (262 distinct genotypes), 10 random genotypes were selected from each lake phase for a total of 30 genotypes to be used in experimental trials. The sample size per population was chosen based on previous results showing that 10 genotypes are representative of the local genetic diversity of *D. magna* populations [43].

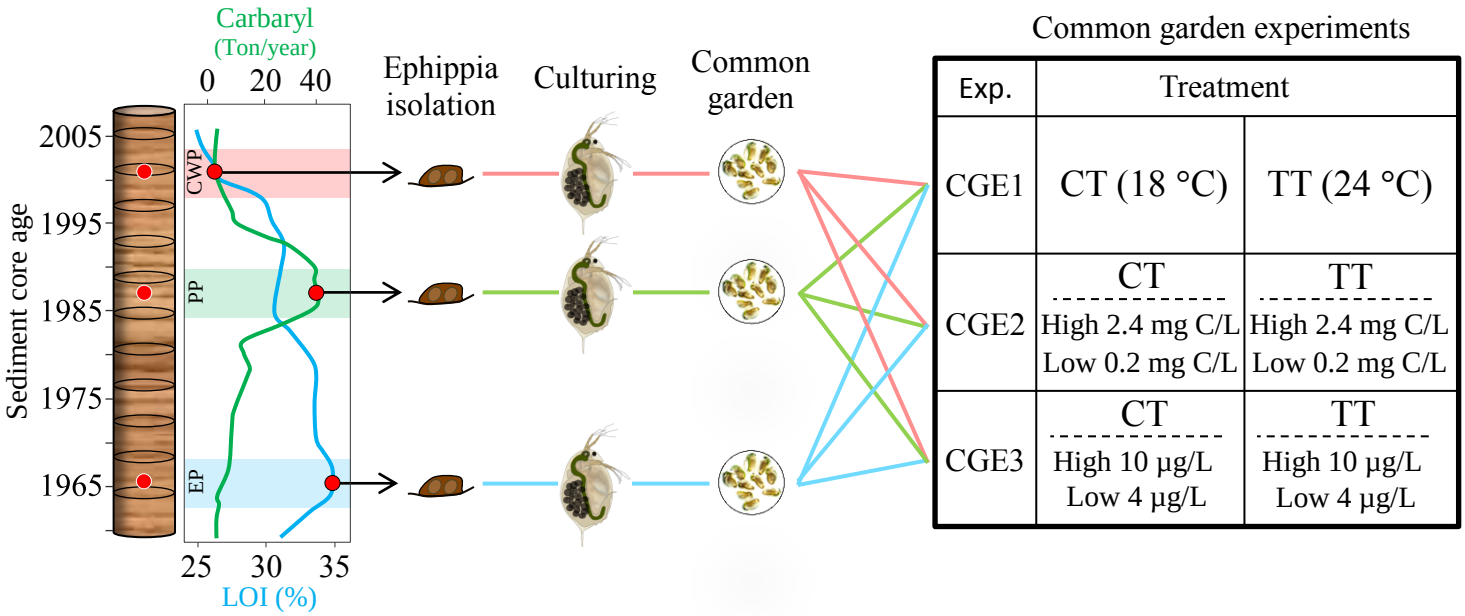
The hatched genotypes are an unbiased representation of the local population genetic diversity as hatching success fluctuated along the sedimentary archive but did not systematically decrease with the age of the sediment [35] (Chapter 1). Previous results on the genetic composition of *D. magna* in Lake Ring showed that genetic drift and selection did not have a detectable impact on the neutral genetic diversity over time, measured both on the hatched and unhached populations of *D. magna* throughout the sedimentary archive [43]. Negligible impact of drift and selection on neutral genetic diversity in presence of strong environmental selection is ideal to study evolution in life history traits over evolutionary time [43].

Common Garden Experiments

We performed three common garden experiments (CGEs) on the 30 genotypes (10 genotypes per population) “resurrected” from Lake Ring (Fig. 1). In these experiments, we measured genetic and plastic responses in key life history traits to temperature treatment by itself and in combination with either biotic stress (two food levels) or abiotic stress (two concentrations of the insecticide Carbaryl). We use Carbaryl, a carbamate insecticide, for our experiments, because carbamate pesticides were widely used in the 1980s across Europe and were widely applied in Denmark in the late 1980s (Fig. 2D).

Figure 1. Experimental design.

D. magna dormant embryos were sampled from a sedimentary archive of Lake Ring: EP - eutrophic population (blue), PP - pesticide population (green), and CWP - clear-water population (red). Ten hatchlings from each of these populations (N=30) were used in three common garden experiments (CGEs) and life history traits (mortality, size at maturity, age at maturity and fecundity) measured. CGE1: temperature treatment; CGE2: temperature and food levels treatment; CGE3: temperature and insecticide Carbaryl concentrations. Temperatures used are: control temperature (18±1°C) and temperature treatment (24±1°C); food levels are 0.2 mg C/L (low, L) and 2.4 mg C/L (high, H); Carbaryl concentration are 4 µg/L (low, L) and 10 µg/L (high, H).



Common garden experiments

Exp.	Treatment	
CGE1	CT (18 °C)	TT (24 °C)
CGE2	CT High 2.4 mg C/L Low 0.2 mg C/L	TT High 2.4 mg C/L Low 0.2 mg C/L
CGE3	CT High 10 µg/L Low 4 µg/L	TT High 10 µg/L Low 4 µg/L

D. magna dormant embryos were sampled from a sedimentary archive of Lake Ring in correspondence of major environmental shifts driven by changes in primary production (measured as Loss on Ignition (LOI)) and increase in land use. Carbaryl is a carbamate insecticide widely applied in Denmark in the late 1980s, as historical records show. The populations sampled from the archive are EP - eutrophic population (blue), PP - pesticide population (green), and CWP - clear-water population (red). Ten hatchlings from each of these populations (N=30) were used in three common garden experiments (CGEs) and life history traits (mortality, size at maturity, age at maturity and fecundity) measured. In CGE1 the populations were exposed to temperature treatment (TT=24±1 °C) and control (CT), defined as 18±1 °C; the experimental animals were fed *ad libitum* with *Chlorella vulgaris* (0.8 mg Carbon/L). In CGE2 the control and the temperature treatments were combined with two nutrient levels: 0.2 mg C/L and 2.4 mg C/L. In CGE3 the control and the temperature treatments were combined with two concentrations of the insecticide Carbaryl: 4 µg/L and 10 µg/L; in this experiment, the animals were fed *ad libitum* with *C. vulgaris*. Concentrations of food and insecticide imposing sub-lethal effects on *D. magna* life history traits were estimated in pilot experiments (Appendix 1).

Prior to starting the CGEs, the genotypes were acclimated and synchronized for two generations in common garden conditions (16:8 light: dark regime, 16±1°C and fed *ad libitum* 0.8 mg C/L of *C. vulgaris* daily) to reduce interference from maternal effect. After two generations in these conditions, individual juveniles of 24-48 hours from the second or following broods were randomly assigned to the experimental exposures in which life history traits were measured in the time spanning an individual life cycle (until release of the second brood). For each individual genotype we measured size at maturity (the distance between the head and the base of the tail spine), age at maturity (first time eggs were observed in the brood chamber), fecundity (total number of offspring released summing first and second brood), and mortality. For size at maturity, all animals were measured after releasing their first brood using image J software (https://imagej.nih.gov/ij/index.html). We use life history trait measurements to make inferences at population level throughout the study, using genotypes as replicates per population. Mortality rates per population were calculated with a survival model fit via the "psm" function in the rms R package V.3.3 [44]. A separate model was fitted to each treatment, in which the day of mortality and the mortality event itself were treated as dependent variables, whereas population was treated as fixed effect. All mortality curves were plotted using "survplot" function form rms package in R v.3.3.3 [44].

Population response to single and multiple stressors

We assessed evolutionary mechanisms - plastic, genetic or a combination thereof - mediating changes to temperature as single stress (CGE1), temperature combined with food levels (high and low, CGE2), and temperature combined with the insecticide Carbaryl (high and low, CGE3) via multivariate analysis of variance (MANOVA) followed by a univariate analysis per single trait (ANOVA). Prior to this analysis, the continuous dependent variables (fecundity, size and age of maturity) were log transformed to meet requirements of data normalization. Both analyses were performed using linear mixed models (LMMs) in R v.3.3.3

[44]. Genotypes were used as replicates per population and included as random effect in the models. Genotypes did not have a significant effect in the MANOVA and were, therefore, not included as random in the final model.

A significant population term in the variance analysis indicates genetic differences among populations and, hence, evolutionary responses in the life history traits. A significant response to treatment(s) indicates phenotypic plasticity in the life history trait. Finally, a significant interaction between the population and the treatment(s) indicates evolution of plasticity in the life history trait. In CGE2 and CGE3, we also assess the interaction terms between two treatments (temperature and food; temperature and insecticide) and between treatments and population. These terms provide us with the possibility to 1) detect whether multiple stressors are additive or interactive, and 2) whether evolution of plasticity to one stressor is modified by a second stressor.

We visualized main effects of population and treatments (temperature, food, and insecticide) as well as population x treatment interactions through univariate reaction norms, which describe the pattern of phenotypic expression of each genotype across treatments [45]. In addition, we visualized multivariate reaction norms via a phenotypic trajectory analysis (PTA) [46]. PTA quantifies for each population: 1) the amount of phenotypic change, calculated across all life history traits, between control and treatment(s) (e.g. plasticity); 2) and the direction of change of each population phenotype [46, 47]. Significant differences ($P < 0.05$) in magnitude and direction of phenotypic change between populations were derived from a residual randomization procedure following [48].

Results

Environmental profile of Lake Ring

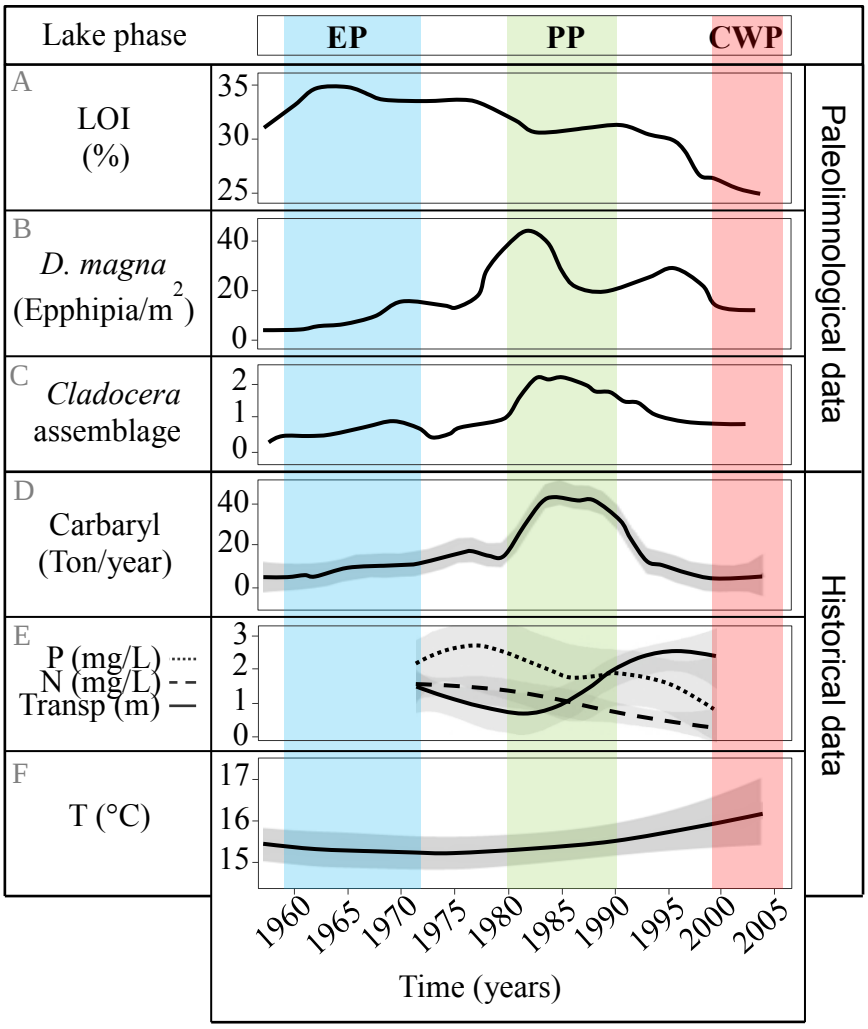
Historical records documenting eutrophication in coincidence of sewage inflow in Lake Ring were confirmed by a high level of primary production - high LOI (Fig. 2A, EP). The *D. magna*

population was less abundant at the beginning of the 1960s and increased towards the 1970s, in coincidence with sewage diversion from the lake (Fig. 2B). The *Cladocera* community followed the same trend (Fig. 2C). From the 1970s onwards, primary production fluctuated and remained overall high until mid-1990s (Fig. 2A, LOI). The LOI patterns for this time period were supported by low transparency, and high phosphorus levels (Fig. 2F). The *Daphnia* reached a peak of abundance in the early 1980s; the *Cladocera* community showed major changes in the same time period (Fig. 2B, C). In 1985, the use of carbamate pesticides, in particular of Carbaryl, was highest (Fig. 2D). Coincident with the peak of Carbaryl, the *Cladocera* assemblage changed (Fig. 2C) and the *Daphnia* population dramatically declined (Fig. 2B), even if primary production was high (Fig. 2A). Following the Carbaryl ban in 1990s (Fig. 2D), white fish was stocked in the lake for a year, imposing a short-lived negative effect on the *Cladocera* community (Fig. 2C), and the *Daphnia* population (Fig. 2B), which subsequently recovered. With the diversion of sewage and decrease in agricultural land use [49] in modern times a decline in primary production was observed later than 1990. Consistent with lower primary production, a shift in the grazer community (Fig. 2B, C), a decrease in phosphate and nitrates and an increase in water transparency is observed (Fig. 2F). Finally, a steady even if modest increase (~1°C) in environmental temperature was recorded over the five decades studied (Fig. 2E). According to these records the oldest population was sampled from the coldest and the most recent population from the warmest period of the 21st century [27].

Figure 2. Lake Ring historical profile.

The historical profile of Lake Ring is shown. A) loss on ignition (LOI, %); B) *Daphnia magna* ephippia abundance (m^2); C) *Cladocera* assemblage based on 33 taxa; D) Carbaryl usage (tons per year); E) average summer air temperatures (June-August); F total phosphorus (P, mg/L), total nitrogen (N, mg/L), and transparency calculated as Secchi disk depth (m) for the period 1970-1999. Populations' codes are as: EP - eutrophic population (blue), PP - pesticide

population (green), and CWP - clear-water population (red). Grey areas represent 95% confidence intervals.



Population response to single and multiple stressors

To investigate the impact of single and combined environmental stress on population fitness, we measured genetic and plastic responses in life history traits of the three *D. magna* populations exposed to temperature stress (CGE1), temperature and food levels (CGE2), temperature and insecticide Carbaryl loads (CGE3).

Temperature (CGE1) – The effect of temperature on life history traits did not vary by population (Table 1, MANOVA and ANOVA). Increasing temperature caused a significant

plastic response in all life history traits, except for mortality that did not change significantly between the control and temperature treatment (Table 1, Fig. 3). The interaction term between population and temperature was not significant, indicating no evolution of plasticity (Table 1).

The phenotypic trajectory analysis (PTA) showed no significant differences in magnitude and direction of change among populations in response to temperature stress (Fig. 4 – CGE1; Table 2 – CGE1). The univariate reaction norms revealed that plastic responses to temperature did not impose appreciable changes in CWP and PP, whereas imposed a decline in fecundity in the EP population (Fig. 3 – CGE1). Size at maturity declined in the temperature treatment as compared to the control temperature in the CWP and the EP population, whereas it did not appreciably change in the PP population (Fig. 3 - CGE1). Age at maturity declined in all populations in the temperature treatment as compared to the control temperature (Fig. 3 – CGE1).

Temperature and food (CGE2) – The effect of temperature combined with food levels varied significantly by population in the MANOVA (Table 1 – CGE2). However, the univariate statistics did not support evolutionary differences among populations at the individual traits (Table 1 – CGE2, ANOVA). We observed a significant plastic response to temperature and to food levels as single stressors but not to their interaction term (Table 1 – CGE2, MANOVA). The univariate statistics confirmed that size and age at maturity were responsive to temperature and that all traits, except for mortality, were responsive to food levels (Table 1 – CGE2, ANOVA). The population x treatment terms were non-significant, except for a significant interaction between population and temperature that explained differences in mortality (Table 1 – CGE2, ANOVA).

The PTA analysis showed no significant difference in magnitude and direction of plastic responses among populations in low food level combined with temperature (Fig. 4 – CGE2; Table 2 - CGE2). Conversely, significant differences were identified in the direction of

phenotypic trajectories between the most recent populations (CWP and PP) and the historical population (EP) in high food level combined with temperature treatment (Fig. 4 - CGE2; Table 2 - CGE2). The univariate reaction norms showed higher fecundity and larger size at maturity in high food levels at both temperature treatments (Fig. 3 – CGE2). We observed no appreciable difference in fecundity between control temperature and temperature treatment in high food level, whereas we observe a decline in fecundity in two of the three populations in the temperature treatment as compared to the control temperature in low food level (Fig. 3 – CGE2). Size at maturity did not change significantly between the control temperature and the temperature treatment in high food levels, whereas it declined in all populations in the temperature treatment in presence of limiting food level (Fig. 3 – CGE2). Finally, age at maturity declined in the temperature treatment at both food levels (Fig. 3 – CGE2).

Temperature and insecticide Carbaryl (CGE3) – The effect of temperature combined with insecticide varied significantly by population (Table 1 – CGE3, MANOVA). The ANOVA showed that this evolutionary difference was driven by differences in age at maturity (Table 1 – CGE3). We observed significant plastic response to temperature and to the insecticide as single stressors (Table 2 – CGE3). A significant interaction term temperature x insecticide was observed for fecundity, size at maturity and mortality (Table 1 – CGE3). The population per treatment terms were not significant (Table 2 – CGE3).

The PTA analysis showed no significant difference in the magnitude of plastic change in low insecticide level; conversely, it showed a significant divergence in the direction of change between the EP and PP population (Fig. 4 – CGE3; Table 2 – CGE3). In the high insecticide treatment, we observed a significant difference in the direction of phenotypic trajectories between the CWP and the EP population (Fig. 4 - CGE2, Table 2). In high insecticide treatment, the PP population went extinct at the control temperature treatment; hence, we were unable to assess changes in its phenotypic trajectory (Fig. S1).

The univariate reaction norms show comparable plastic response of the three populations to low insecticide level combined with warming, resulting in an increase in fecundity, a larger size at maturity, and an earlier age at maturation in the temperature treatment as compared to the control temperature (Fig. 3 – CGE3). Mortality was less severe at 24°C than at 18°C, in both insecticide concentrations, and significantly higher at 18 °C in presence of high insecticide level (Fig. S1). At 18°C and high insecticide, the PP population went extinct between day two and three (Fig. S1). The two surviving populations in the high insecticide treatment showed an earlier age at maturity in the temperature treatment than in the control temperature (Fig. 3 – CGE3, H). Conversely, they showed opposite plastic response for size at maturity and fecundity (Fig. 3 – CGE3, H). Specifically, the CWP population experienced a decrease in fecundity and a smaller size at maturity, whereas the EP population experienced an increase in fecundity and a larger size at maturity in the temperature treatment combined with high insecticide (Fig. 3 – CGE3).

Figure 3. Reaction norms

Univariate responses to temperature (CGE1), temperature combined with food levels (CGE2) and temperature combined with loads of the insecticide Carbaryl (CGE3) in the three populations of *D. magna* resurrected from Lake Ring. Population reaction norms based on population means (n=10) and SD, are shown for three life history traits. CT = control temperature; TT = Temperature treatment. High food and high Carbaryl are represented in 'H' panels; Low food and low Carbaryl concentrations are represented in 'L' panels. For the PP population, only mortality data are available at high concentrations of Carbaryl as the population experienced 100% mortality in this treatment three days after exposure (Figure S1). Populations' codes are as: EP - eutrophic population (blue), PP - pesticide population (green), and CWP - clear-water population (red).

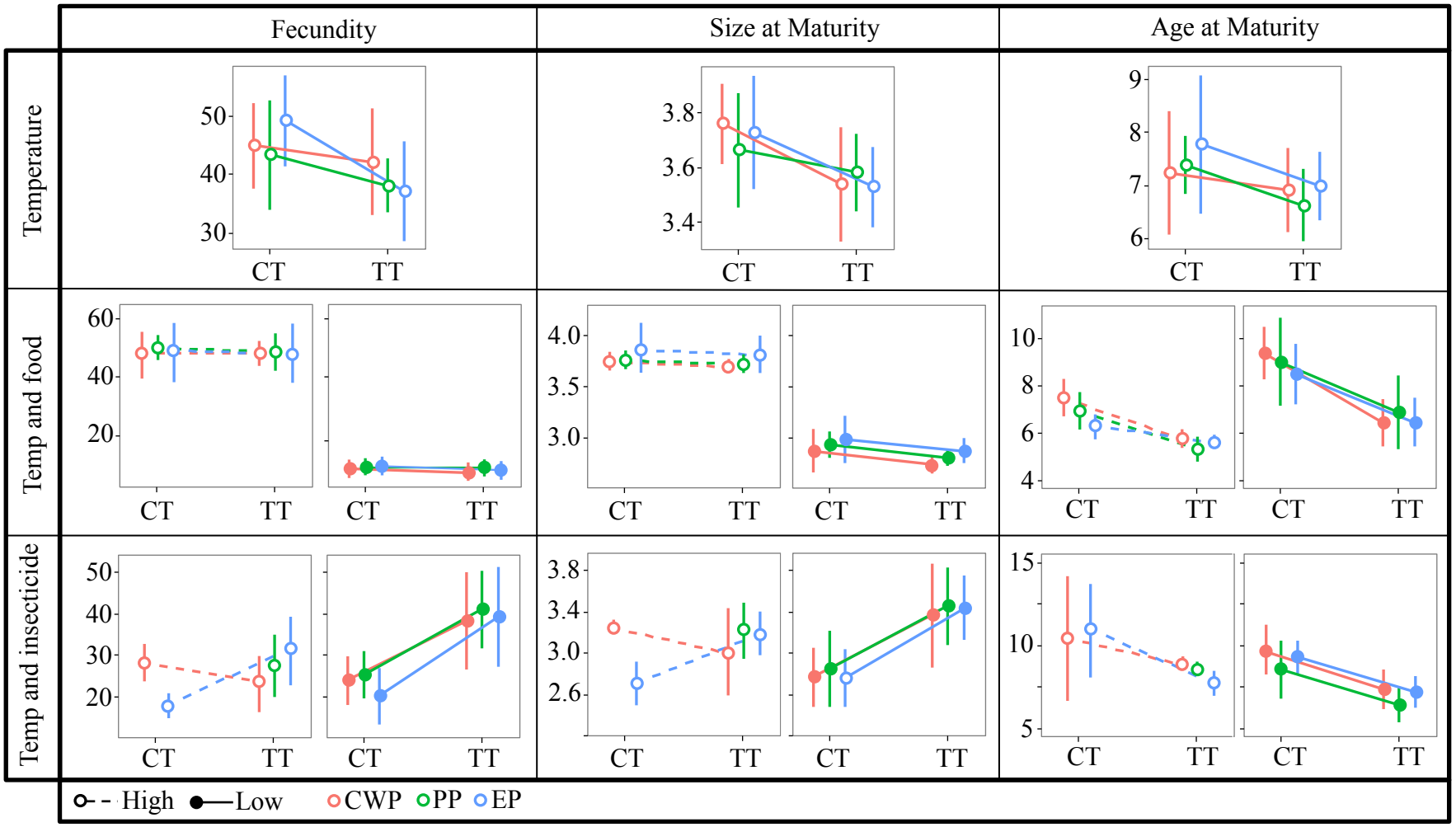

Fecundity
Size at Maturity
Age at Maturity
Temperature
Temp and food
Temp and insecticide
CT
TT
High
Low
CWP
PP
EP

Table 1. Analysis of variance

MANOVA (A) and univariate ANOVAs (B) per single life history trait (mortality, fecundity, size and age at maturity) testing for the effect of population, treatment and their interaction terms on three sets of 10 *Daphnia magna* genotypes representing different time periods in a natural lake. Difference among populations (P) are interpreted as genetic or evolutionary differences; trait response to treatment [temperature (T), food levels (F), insecticide loads (I), and their interaction terms] is interpreted as plasticity, and evolution of plasticity is expressed by the interaction terms between P and the treatments (T, F and I). Experimental temperatures are: 18°C and 24°C. Food levels are: 2.4 mg C/L and 0.2 mg C/L. Carbaryl loads are: 10 µg/L and 4 µg/L. Significant *P-values* are shown in bold.

	Population (P)		Temperature (T)		Food(F)-Insecticide (I)		P x T		T x F-I		P x F-I		P x T x F-I	
A. MANOVA	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*	F	*P*
CGE1 ($F_{-,45}$)	0.932	0.476	6.843	***0.001***			0.854	0.532						
CGE2-F ($F_{-,114}$)	3.390	***0.003***	29.650	***<0.001***	573.890	***<0.001***	0.600	0.729	1.530	0.212	0.860	0.525	0.470	0.832
CGE3-I ($F_{-,74}$)	2.162	***0.050***	20.189	***<0.001***	9.092	***<0.001***	1.204	0.308	1.750	0.164	0.971	0.447	0.879	0.456

B. ANOVA	Population (P)		Temperature (T)		Food(F)-Insecticide (I)		P x T		T x F-I		P x F-I		P x T x F-I	
CGE1	$F_{2,45}$	*P*	$F_{1,45}$	*P*			$F_{2,45}$	*P*						
Fecundity	1.120	0.571	8.648	***0.003***			3.329	0.189						
Size at maturity	0.066	0.968	12.730	***<0.001***			1.928	0.381						
Age at maturity	1.677	0.432	4.597	***0.032***			1.261	0.532						
Mortality	3.765	0.152	2.043	0.153			4.121	0.127						
CGE2 (Food-F)	$F_{2,114}$	*P*	$F_{1,114}$	*P*	$F_{1,114}$	*P*	$F_{2,114}$	*P*	$F_{1,114}$	*P*	$F_{2,114}$	*P*	$F_{1,114}$	*P*
Fecundity	3.223	0.200	1.235	0.266	1465.925	***<0.001***	0.759	0.684	0.400	0.527	2.526	0.283	0.814	0.666
Size at maturity	4.799	0.091	13.847	***<0.001***	1230.084	***<0.001***	0.195	0.907	2.992	0.084	2.086	0.352	0.153	0.927
Age at maturity	2.798	0.247	101.920	***<0.001***	61.217	***<0.001***	3.707	0.157	3.240	0.072	1.722	0.423	1.294	0.524
Mortality ($F_{-,126}$)	3.865	0.145	0.005	0.946	0.415	0.519	8.472	***0.014***	3.494	0.062	0.283	0.868	3.365	0.186
CGE3 (Insecticide-I)	$F_{2,74}$	*P*	$F_{1,74}$	*P*	$F_{1,74}$	*P*	$F_{2,74}$	*P*	$F_{1,74}$	*P*	$F_{2,74}$	*P*	$F_{1,74}$	*P*
Fecundity	1.007	0.605	48.766	***<0.001***	12.780	***<0.001***	6.460	***0.040***	4.006	***0.045***	0.430	0.806	2.111	0.146
Size at maturity	0.580	0.748	65.983	***<0.001***	2.315	0.128	2.145	0.342	4.931	***0.026***	1.335	0.513	1.705	0.192
Age at maturity	7.553	***0.023***	57.545	***<0.001***	17.281	***<0.001***	0.263	0.877	0.001	0.982	2.915	0.233	1.148	0.284
Mortality ($F_{-,130}$)	2.218	0.330	11.494	***0.001***	82.991	***<0.001***	0.462	0.794	4.857	***0.028***	1.662	0.436	0.463	0.793

Figure 4. Multivariate reaction norms

PCA plots showing phenotypic trajectories of the three populations of *Daphnia magna* "resurrected" from Lake Ring. The PC scores are derived from life history traits centred by the trait mean and scaled by the standard deviation in the following treatments: temperature (CGE1); temperature and food (CGE2); temperature and insecticide (CGE3). Control temperature (18±1°C) is represented with full circles; temperature treatment (24±1°C) is represented with open circles. Populations are colour coded as in Fig. 1. H – high food and Carbaryl; L – low food and Carbaryl. Concentrations for food and Carbaryl are shown in Figure 1 legend. Differences among populations, in terms of magnitude (M) and direction (θ) of plastic response, as calculated in the phenotypic trajectories analysis are shown for each exposure. ns=non-significant.

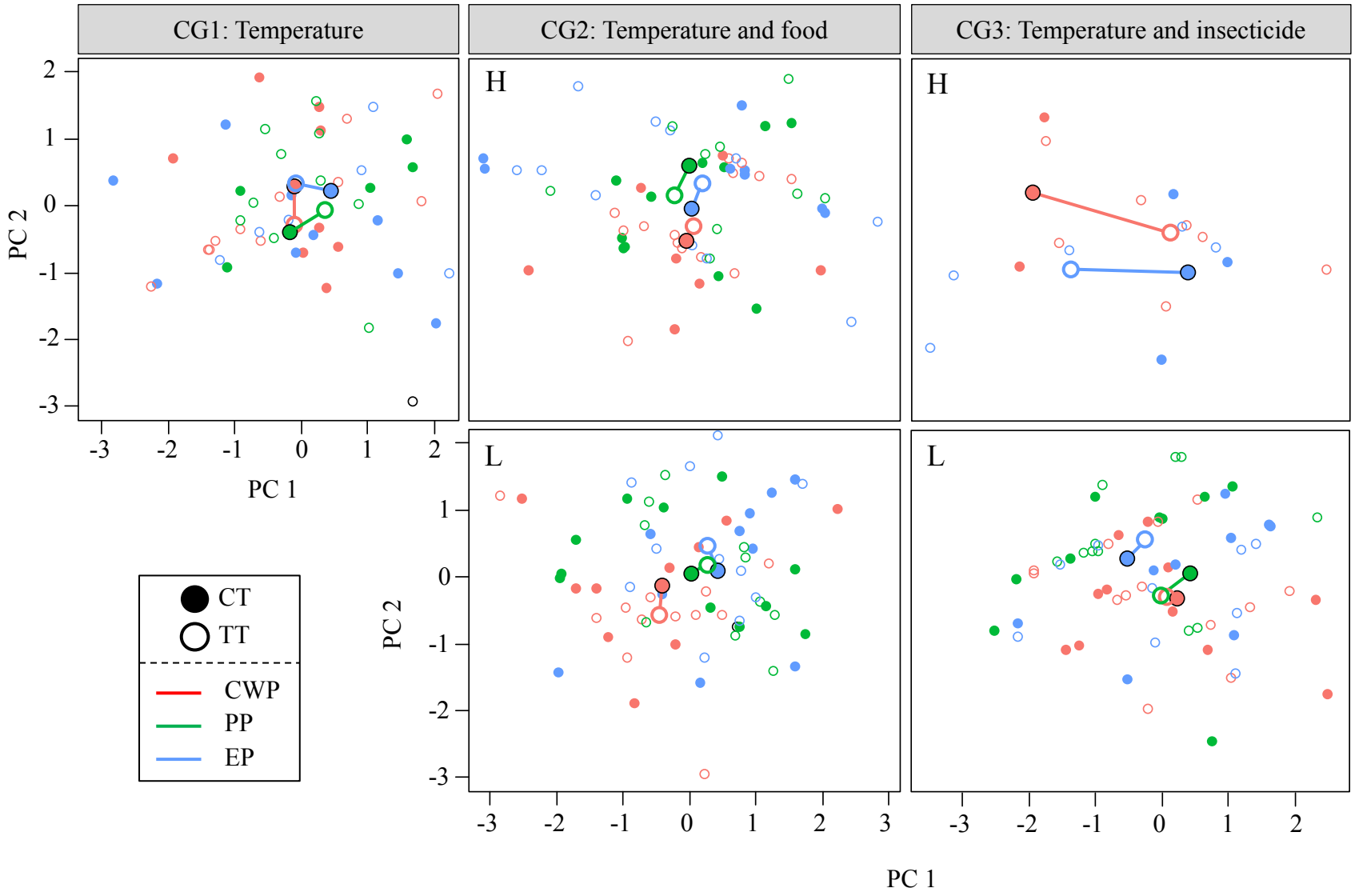

CG1: Temperature
CG2: Temperature and food
CG3: Temperature and insecticide
H
L
PC 1
PC 2
CT
TT
CWP
PP
EP

Table 2. PTA analysis

Phenotypic trajectories analysis testing for pairwise differences among the three *Daphnia magna* populations separated in time used in this study, in terms of magnitude (magnitude) and direction (θ) of plastic response. Tests are run for the five experimental exposures separately. CGE1 – Temperature treatment; CGE2H – High food level and Temperature; CGE2L - Low food level and Temperature; CGE3H – High insecticide concentration and Temperature; CGE3L - Low insecticide concentration and Temperature. In CGE3H tests are conducted on two populations as the PP population went extinct in this treatment. Significant P-values are shown in bold

PTA	Comparison	Magnitude	$P_{Magnitude}$	θ	P_{θ}
CG1	CWP-PP	0.11	0.81	107.39	0.36
	CWP-EP	0.02	0.97	130.06	0.19
	PP-EP	0.12	0.80	120.10	0.27
CG2H	CWP-PP	0.14	0.73	27.06	0.93
	CWP-EP	0.22	0.65	162.07	***0.04***
	PP-EP	0.08	0.88	169.12	***0.01***
CG2L	CWP-PP	0.06	0.88	137.06	0.15
	CWP-EP	0.13	0.76	53.22	0.83
	PP-EP	0.19	0.64	83.84	0.55
CG3H	CWP-EP	0.35	0.65	164.25	***0.03***
CG3L	CWP-PP	0.23	0.59	126.81	0.26
	CWP-EP	0.43	0.36	43.59	0.85
	PP-EP	0.19	0.67	170.19	***0.01***

Discussion

We investigated how a *Daphnia magna* population, exposed to a well-characterized history of anthropogenic impact responded to changes in temperature by itself and in combination with either biotic or abiotic stress. Our objectives were to assess the role of evolution and plasticity in the population response to these changes and to understand how historical exposure to stress impacts on recurring stress.

We first established that the invertebrate community was responsive to pollution events in Lake Ring. The paleolimonological analysis of sediment supported by historical records, identified sewage inflow and pesticide leaching as major pollution events. These events co-occurred with a modest average increase in temperature. We showed that decline in the *Daphnia* population and changes over time of the invertebrate community occurred in coincidence of sewage inflow and high pesticide use. The high primary production recorded until mid-1990s is likely explained by high fish stocking in 1989-1990 [25]. Although the lake may have suffered from more pollution events than our analysis revealed, the drastic decrease in *D. magna* abundance in correspondence of the peak of sewage inflow and the peak of insecticide Carbaryl usage suggest that these two stressors played a strong role in the population dynamics over time. The persistence of *D. magna* through times suggests coping mechanisms were adopted to respond to environmental stress.

We expected that the effect of temperature as single stressor would have less impact on the most recent population than on the other two populations, as a result of microevolutionary response to temperature increase. Our results suggest that the populations of *D. magna* accommodated changes in temperature via plasticity but not evolution. In contrast, two previous studies on *D. magna*, including the population from Lake Ring, showed evolution of the critical thermal maximum (CT_{max}) over few decades in response to temperature increase [28, 29, 50] (Chapter 4). In the population of Lake Ring, however, other life history traits, namely body size and haemoglobin content, did not show

evolutionary changes in response to temperature [50] (Chapter 4). Our and these previous results suggest ecological trade-offs among life history traits resulting in a differential response to the same environmental stressors [16].

We expected life history traits to be unaffected by non-limiting food levels combined with warming, and for temperature combined with low food to have an additive effect. Our results show non-additive effect of food and temperature treatment and an overall lower fitness - lower fecundity, smaller size at maturation and delayed age at maturity – in limiting food levels as compared to non-limiting food levels at both experimental temperatures. In addition, we observed the lowest overall fitness in low food level combined with temperature treatment. These results are in agreement with metabolic demands increasing faster than ingestion rates with higher temperatures [10] and confirm the additive effect hypothesis.

We expected the effect of the insecticide to vary with temperature because of higher volatilization and degradation of Carbaryl at higher temperature [32]. We observed a more severe effect of the insecticide in control than in temperature treatment on all life history traits, and in particular, on mortality. The antagonistic effect of temperature and insecticide provided higher fitness to all three populations in the temperature treatment as compared to the control temperature. In particular, the PP suffered the most severe impact at the control temperature (e.g. experiencing 100% mortality), whereas it responded like the other two populations in the temperature treatment combined with insecticide. Our findings confirm the hypothesis that temperature mitigates the effect of the insecticide Carbaryl.

We hypothesized that historical exposure to a recurring stress provides an evolutionary advantage. We discuss results from the different CGEs in this respect. We observed a significantly divergent response between the historical population (EP) and the two most recent populations (CWP and PP) in response to high food level (eutrophication), supported by evolutionary differences among populations (MANOVA and ANOVA). The EP population, historically exposed to sewage and experiencing the highest primary production,

showed the least change in plasticity in presence of high food level. This result suggest that prior exposure to eutrophication provides an evolutionary advantage to the historical population in presence of a recurring stress [33]. The evolutionary advantage in response to eutrophication is observed also in presence of warming, which the historical population did not experience. The evolutionary advantage of the historical population in presence of eutrophication combined with warming supports the evidence that plastic response to one stressor may favour plasticity in response to a second stressor [10].

Food limitation induced comparable plastic response among the populations resulting in non-significant evolutionary differences among populations. As all populations were naïve to limiting food levels, we expected that none of them would show an evolutionary advantage. Evolutionary differences were observed between the historical and the modern population in response to high insecticide loads. Surprisingly, the PP population, which historically experienced exposure to the insecticide Carbaryl, experienced 100% mortality in the high insecticide and control temperature treatment. Conversely, this population showed evolved differences as compared to the other two populations in response to low insecticide exposure. These findings suggest evolutionary differences between the historical population and the two more recent populations in response to the insecticide Carbaryl. The divergent response of the historical population and the two most recent populations in low but not high insecticide exposure suggest evolutionary advantage of historical exposure to abiotic stress, contingent upon the severity of the stress.

Overall, our study showed that the effect of multiple stressors is non-linear and unpredictable from the effect of single stressors (‘ecological surprises’ [4]). Therefore, the use of single stressors, such as temperature, as *proxy* for species response to global change can lead to wrong estimates of species evolvability and persistence.

Competing interests

The authors declare no competing interests

Author contribution

MCC and HM carried out the experiment.

MCC and APB performed data analysis

LDM provided the sedimentary archive of Lake Ring and contributed to the experimental design.

TAD contributed information on the ecological background of Lake Ring and performed the community analysis.

LO conceived the study and coordinated data analysis. LO and MCC wrote the first version of the paper; all authors contributed to the editing of later versions.

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Data accessibility

Data will be made available on the Dryad Digital Repository upon manuscript acceptance

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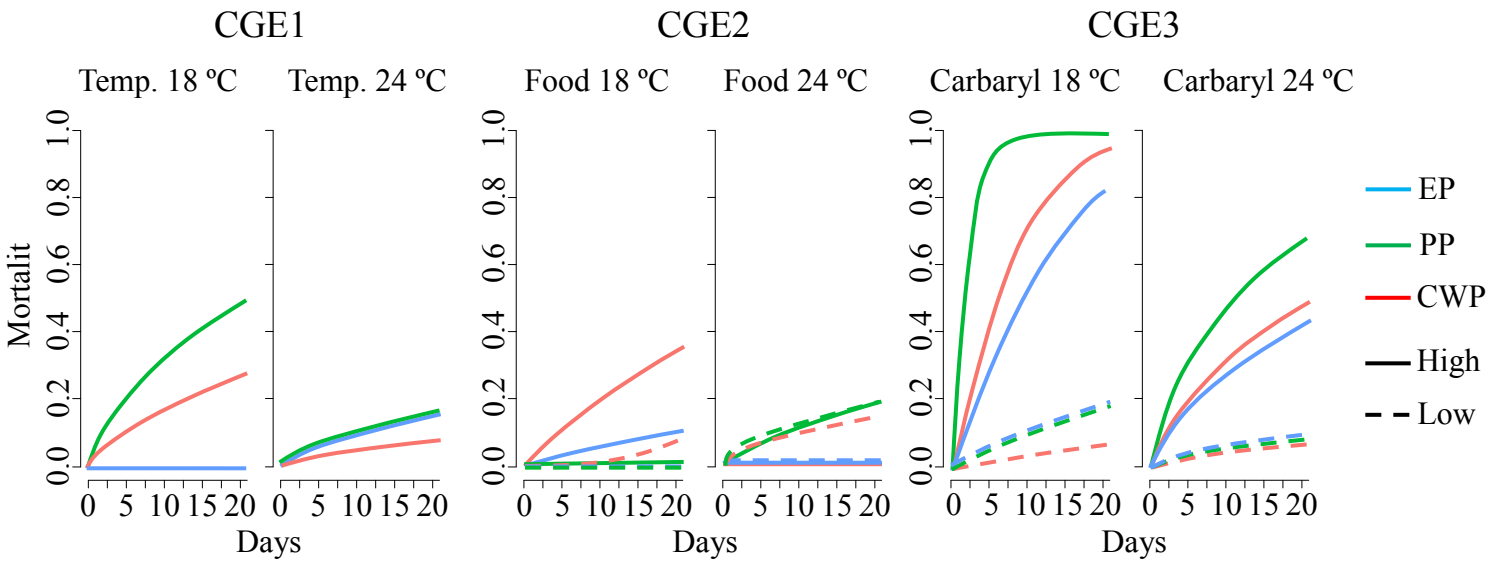
Figure S1. Mortality per population and experiment: CGE1 (Temperature), CGE2 (Temperature and Food) and CGE3 (Temperature and Carbaryl) measured over 21 days. For CGE2 and CGE3 high and low concentrations of either food or Carbaryl are shown. Mortality was calculated with survival analysis in R using the rms package (https://cran.r-project.org/web/packages/rms/rms.pdf). Populations are color coded as in Figure 1.



Appendix 1. Pilot experiment

Concentrations of food and insecticide imposing appreciable sub-lethal effects of *D. magna* life history traits were tested in pilot experiments at a constant temperature (20°C). Fecundity, age at maturity and mortality were measured at different concentrations of food (*Chlorella vulgaris*) and Carbaryl.

Food concentrations used were: 0.2 mg C/L, 0.4 mg C/L, 0.8 mg C/L, 1.6 mg C/L, and 2.4 mg C/L. Algae's carbon content was estimated by CH&N analysis carried out by combustion analysis (Medac Ltd).

Carbaryl concentrations used were: 4 µg/L, 8 µg/L, 10 µg/L, 12 µg/L, 14 µg/L, 16 µg/L, and 32 µg/L. These concentrations were tested in two separate experiments; in the first experiment we assessed the effect of 4 µg/L, 8 µg/L, 16 µg/L, and 32 µg/L of Carbaryl whereas we assessed the effect of 10 µg/L 12 µg/L, 14 µg/L in a second experiment. Carbaryl was dissolved in ETOH 0.005% and replaced every second day in the experimental vials. The experimental animals were fed *ad libitum* daily 0.8 mg C/L of *Chlorella vulgaris*.

Mortality did not significantly change in response to food treatments (Fig. A1). Conversely, we observed a delay in maturity, as well as a decrease in fecundity with decreasing food levels (Fig. A2). We used the lowest and highest concentrations tested for our main experiment: 0.2 mg C/L and 2.4 mg C/L.

Mortality increased mortality with increasing concentration of Carbaryl reaching 100% at 32 µg/L (Fig. A3). Increasing concentration of Carbaryl induced a decreased in fecundity and a delay in age at maturity (Fig. A4). We used 4 µg/L and 10 µg/L for our main experiment.

Figure A1. Mortality in response to food levels.

Mortality calculated per population over 21 days after exposure to the following concentrations of carbon: 0.2 mg C/L, 0.4 mg C/L, 0.8 mg C/L, 1.6 mg C/L, and 2.4 mg C/L. Population codes are as in Figure 1A.

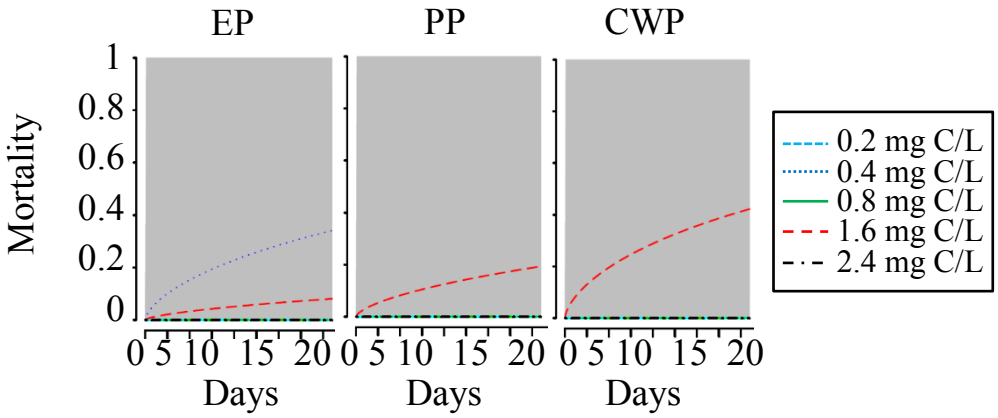


Figure A2. Univariate reaction norms of life history traits in response to food levels.

Reaction norms for age at maturity (days) and total number of neonates (cumulative for first and second brood) are plotted for different food concentrations: 0.2 mg C/L, 0.4 mg C/L, 0.8 mg C/L, 1.6 mg C/L and 2.4 mg C/L. Error bars in individual life history traits represent standard deviations from mean population values. Population codes are as in Figure S1

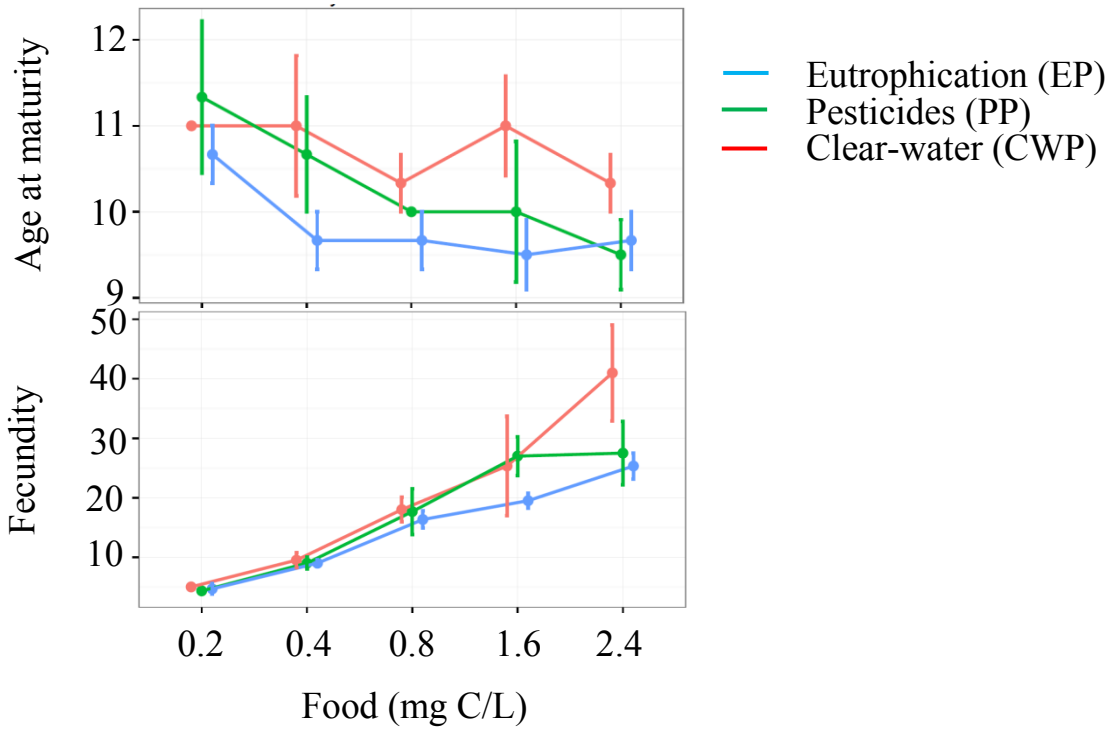


Figure A3. Mortality in response to Carbaryl concentrations.

Mortality calculated per population over 21 days after exposure to Carbaryl at the following concentrations: 4 µg/L, 8 µg/L, 10 µg/L, 12 µg/L, 14 µg/L, 16 µg/L, 32 µg/L. Because the different concentrations were tested in two separate experiments two controls are shown. Population names are as in Figure S1.

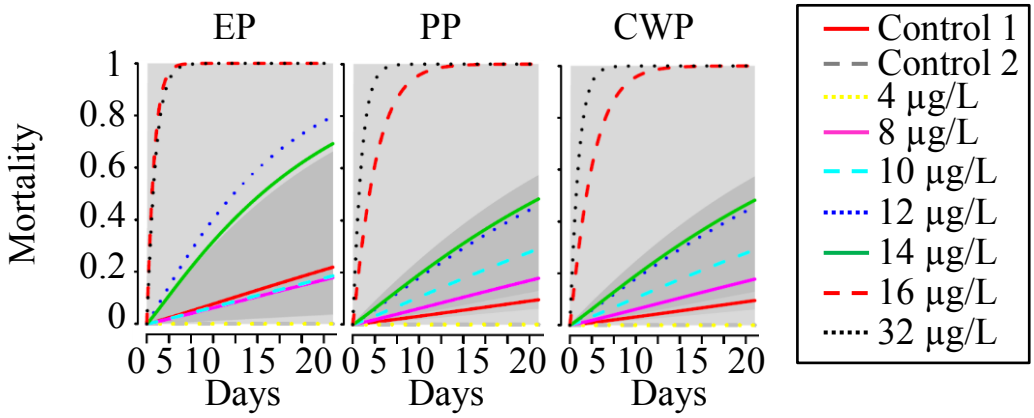
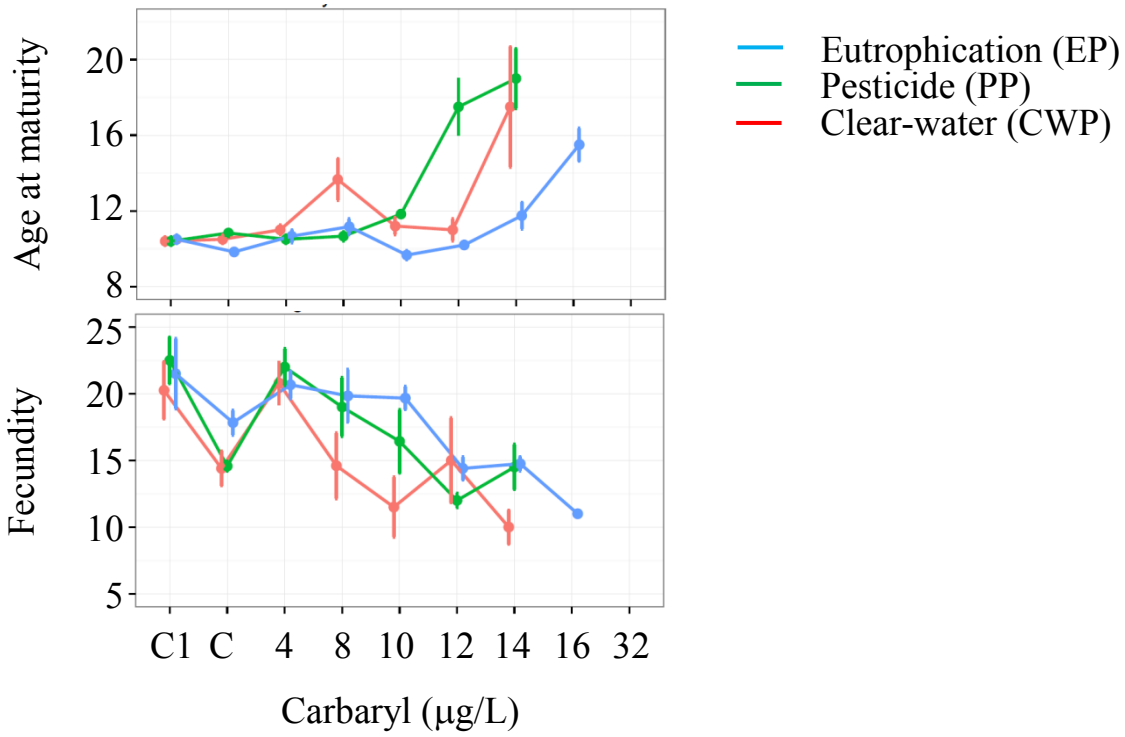


Figure A4. Reaction norms of life history traits in response to Carbaryl concentrations.

Reaction norms for age at maturity (days) and total number of neonates (cumulative for first and second brood) are plotted for different Carbaryl concentrations: 4 μg/L, 8 μg/L, 10 μg/L, 12 μg/L, 14 μg/L, 16 μg/L, 32 μg/L. Two controls (C1 and C2 – no Carbaryl) were used as the seven concentrations of Carbaryl were tested over two experiments. Error bars represent standard deviations from mean individual life history traits. Population names are as in Figure S1.



CHAPTER 3

Haemoglobin-mediated response to hyper-thermal stress in the keystone species *Daphnia magna*

Information:

Authors: Cuenca Cambronero, M., Zeis, B., and Orsini, L.

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Overview: In this chapter, coping mechanisms to thermal and hyper-thermal stress are investigated by quantifying Haemoglobin (Hb) plastic and evolutionary responses and competitive abilities of genotypes as a function of their Hb content. *Daphnia magna* subpopulations resurrected from the same sedimentary archive used in previous chapters are used.

Author's contribution: BZ carried out the experiments. MCC performed data analysis. LO conceived the study and coordinated data analysis. LO and MCC wrote the first version of the paper. All authors contributed to the editing of the final version of the manuscript.

Appendices

Appendix 1. Microsatellite multiplexes.

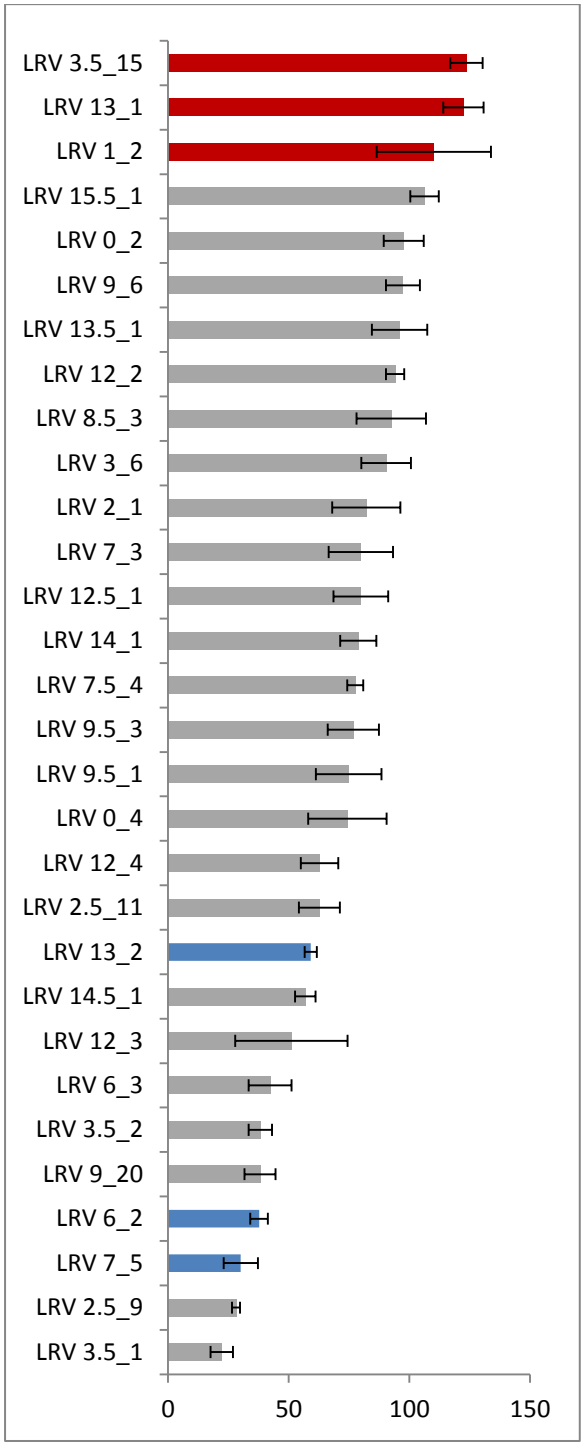
The NCBI Accession Number (AN), the multiplex information, the PCR primer sequences, the PCR size range, the repeat motif, the dye used to label the forward primer, the annealing temperature (Tm) and the multiplex (M) are shown.

Locus	AN	Size range (bp)	Primers (5'-3')	Dye label	Repeat motif	Tm	M
B008	HQ234154	150-170	F: TGGGATCACAACGTTACACAA	VIC	$(TC)^{9}$	56.0	1
			R: GCTGCTCGAGTCCTGAAATC				
B030	HQ234160	154-172	F: CCAGCACACAAAGACGAA	PET	$(GA)^{11}$	56.0	1
			R: ACCATTTCTCTCCCCCAACT				
B045	HQ234168	118-126	F: GCTCATCATCCCTCTGCTTC	NED	$(TG)^{8}$	56.0	1
			R: ATAGTTTCAGCAACGCGTCA				
B050	HQ234170	234-248	F: TTTCAAAAATCGCTCCCATC	6FAM	$(GAA)^{6}$	56.0	1
			R: TATGGCGTGGAATGTTTCAG				
B064	HQ234172	135-151	F: CTCCTTAGCAACCGAATCCA	6FAM	$(TC)^{8}$	56.0	1
			R: CAAACGCGTTCGATTAAAGA				
B074	HQ234174	196-204	F: TCTTTCAGCGCACAATGAAT	NED	$(GT)^{9}$	56.0	1
			R: TGTGTTCCTTGTCAACTGTCG				
B096	HQ234181	234-240	F: GGATCTGGCAGGAAGTGGTA	VIC	$(AC)^{15}$	56.0	1
			R: TTGAACCACGTCGAGGATTT				
B107	HQ234184	250-274	F: GGGGTGAAGCATCAAAGAAA	PET	$(CT)^{8}$	56.0	1

			R: TGTGACCAGGATAAGAGAAGAGG				
B087	HQ234178	174-200	F: CGAATTCGTTTGTCTGATAGGG	6FAM	$(CA)^{13}$	54.0	5
			R: CCTAACAGTCGCGTCATTCA				
A002	HQ234126	250-282	F: GTTCCACAGATAGACATTTGCT	6FAM	$(AG)^{8}$	54.0	5
			R: GTTGAAATGCAAATGAGTCG				
B052	HQ234171	277-305	F: AAGCTTGGGATCGTCTGCT	PET	$(CA)^{12}$	54.0	5
			R: CGAGATTTGGTGTGTGATGG				
B180	HQ234207	301-311	F: CAGCATCGCTCTGTAACTCG	VIC	$(GA)^{8}$	54.0	5
			R: GGATTTTCATGACCGGCTTA				
B033	HQ234163	96-114	F: AGGCATTCCTCAATTTCCAA	NED	$(TG)^{9}$	54.0	5
			R: GAAGACGGCGTGGTTAGTTT				

Appendix 2. **Haemoglobin content in crude extracts at 30°C.**

Distribution of haemoglobin content measured at 30°C in the 30 genotypes resurrected from the sedimentary archive of Lake Ring. Genotypes in red represent the Hb-rich and genotypes in blue represent Hb-poor, used in the microcosm competition experiment and in the T_{imm} assay. For a complete list of Hb content at both experimental temperatures see Appendix 3.



Appendix 3. Evolutionary and plastic response to hyper-thermal stress

Analysis of variance testing whether changes in haemoglobin content (response variable) of the resurrected (sub)populations can be explained by evolution (constitutive differences in Hb expression among (sub)populations - Pop), plasticity (differences among temperatures - Temp) or their interaction term. Genotype was included as random variable nested within population. Significant p-values (P<0.05) are shown in bold.

	Df	SS	*p-value*
(sub)population (Pop)	2	212.9	0.69
Temperature (Temp)	1	17131.6	<**0.0001**
Pop x Temp	2	2216.2	**0.05**

Appendix 4. **Haemoglobin content under experimental conditions**

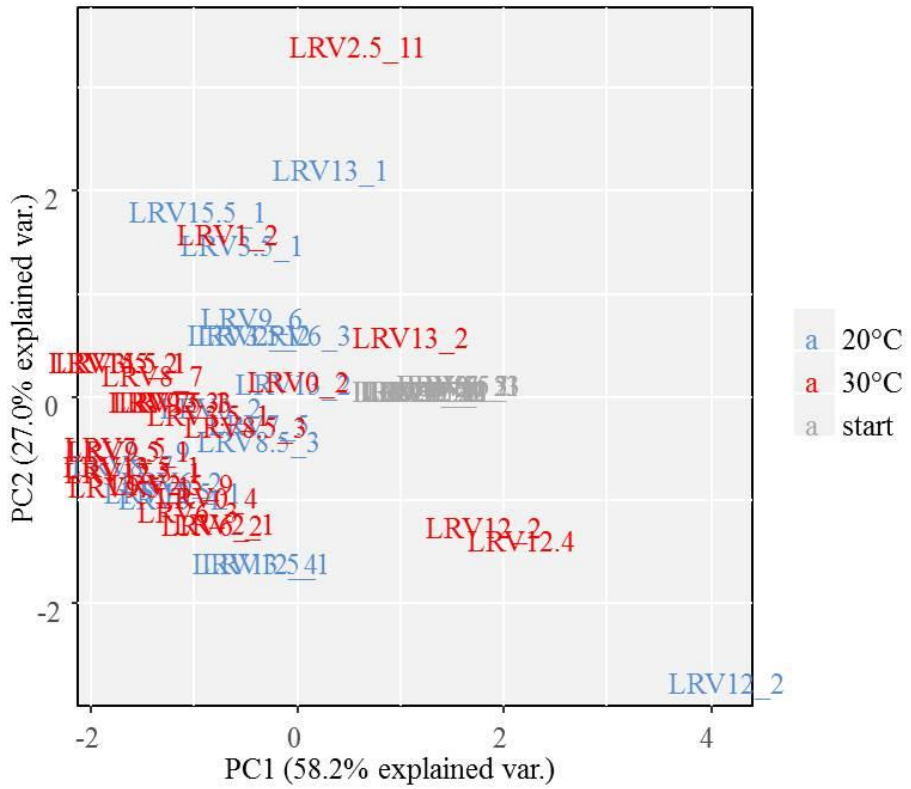
Haemoglobin content measured on the crude extract of 30 genotypes resurrected from Lake Ring under non-stressful temperature (20 °C) and hyper-thermal stress (30 °C). Values of Hb are shown for three replicates per condition in µmol/L. Average across replicates within experimental condition (mean) and log2 fold change between the mean values are also shown. An asterisk (*) indicates genotypes with the highest frequency in the mesocosm competition experiment (Fig. 3). Genotypes ID are as in (Orsini et al. 2016).

genotypeID	20 °C				30 °C				log2-fold change
	Hb (µmol/L)	Hb (µmol/L)	Hb (µmol/L)	mean	Hb (µmol/L)	Hb (µmol/L)	Hb (µmol/L)	mean	
LRV 0_2*	79.98	70.64	62.42	71.01	87.58	113.96	91.27	97.60	0.46
LRV 0_4	45.10	62.42	64.37	57.30	88.30	41.89	92.71	74.30	0.37
LRV 1_2*	26.70	35.52	44.87	35.70	146.40	65.61	118.28	110.09	1.62
LRV 2_1	70.40	59.14	55.65	61.73	109.20	61.40	75.77	82.12	0.41
LRV 2.5_9	26.69	20.12	19.40	22.07	25.15	30.70	28.54	28.13	0.35
LRV 2.5_11*	52.36	44.87	34.19	43.81	47.64	63.35	77.00	62.66	0.52
LRV 3_6	46.40	54.00	46.10	48.83	109.50	74.13	87.37	90.33	0.89
LRV 3.5_1	25.30	2.71	11.75	13.25	26.88	12.88	26.88	22.21	0.75
LRV 3.5_2	48.25	39.84	42.30	43.46	45.69	29.16	39.73	38.19	-0.19
LRV3.5_15	99.18	65.71	76.90	80.60	122.48	135.73	112.63	123.61	0.62
LRV 6_2	74.02	71.36	68.48	71.29	39.12	43.12	30.80	37.68	-0.92
LRV 6_3	64.40	47.33	48.25	53.33	59.30	29.16	38.40	42.29	-0.33

LRV 7_3	61.90	41.48	63.86	55.75	93.80	53.18	92.61	79.86	0.52
LRV 7_5	68.89	50.62	48.87	56.13	44.25	22.48	23.61	30.12	-0.90
LRV 7.5_4	61.50	61.19	79.26	67.32	71.87	77.21	83.37	77.48	0.20
LRV 8.5_3	55.95	46.30	75.05	59.10	64.17	102.36	110.88	92.47	0.65
LRV 9_6	56.90	39.53	29.36	41.93	83.80	100.92	107.29	97.34	1.22
LRV 9_20	57.91	48.67	42.30	49.62	50.92	30.08	33.26	38.09	-0.38
LRV 9.5_1	42.51	44.56	59.14	48.73	47.74	86.65	90.14	74.85	0.62
LRV 9.5_3	87.78	91.48	74.13	84.46	55.44	87.47	87.17	76.69	-0.14
LRV 12_2*	67.20	57.91	33.26	52.79	101.10	88.19	92.92	94.07	0.83
LRV 12_3	56.88	35.01	28.85	40.25	97.43	24.85	31.01	51.10	0.34
LRV 12_4*	33.47	50.62	58.01	47.36	52.46	77.93	57.91	62.77	0.41
LRV12.5_1	42.51	71.97	64.78	59.75	57.39	93.84	88.19	79.81	0.42
LRV 13_1	59.20	51.95	35.32	48.82	115.20	112.94	138.91	122.35	1.33
LRV 13_2*	16.94	26.88	25.07	22.96	63.47	54.89	58.95	59.10	1.36
LRV 13.5_1	53.70	55.44	76.39	61.84	73.31	110.37	104.11	95.93	0.63
LRV 14_1	73.30	51.95	68.58	64.61	64.80	80.90	90.66	78.79	0.29
LRV 14.5_1	82.55	54.21	46.10	60.95	57.60	49.18	63.86	56.88	-0.10
LRV 15.5_1	60.80	69.51	48.15	59.49	95.50	115.81	107.39	106.23	0.84

Appendix 5. PCA plot

PCA plot of genotype frequency changes after exposure to 20°C (blue) and 30°C (red) for four weeks as compared to the starting inoculum (start), in which an equal number of clones per genotype were inoculated.



Appendix 6. Time to immobilization.

Analysis of variance testing whether the time to immobilization (temperature tolerance as knockout time) is explained by constitutive differences in haemoglobin (Hb) expression (Hb-rich vs Hb-poor genotypes expression), the treatment (experimental temperature) and their interaction term. Significant p-values (P<0.05) are shown in bold.

	Df	SS	p-value
Hb (poor/rich)	1	136.1	0.59
Treatment (Temp)	1	30625	**2.11E-9**
Hb x Temp	1	1225	0.10

CHAPTER 4

Evolution of thermal tolerance in presence of multiple stressors

Information:

Authors: Cuenca Cambronero, M., Beasley, J., Kissane, K., and Orsini, L.

Submitted to *Molecular ecology*

Overview: In this chapter, I expand on the findings of Chapter 3 and investigate physiological and molecular mechanisms underlying thermal tolerance over evolutionary times in environments affected by multiple stressors. Trade-offs between constitutive and induced thermal tolerance are assessed in presence of warming alone and in combination with biotic and abiotic stress. This is done by quantifying genetic and plastic differences in critical thermal maximum (CT_{max}) and heat shock proteins (HSP) expression between historical and modern genotypes of the same population that experienced increase in average temperature and occurrence of heat waves, in addition to dramatic changes in water chemistry over five decades.

Author's contribution: MCC carried out the experiments and performed data analysis with input from JB. SK and JB generated the HSP data. LO conceived the study and coordinated data analysis. LO and MCC wrote the first version of the paper. All authors contributed to the editing of the final version of the manuscript.

Evolution of thermal tolerance in presence of multiple stressors

Running head: Thermal tolerance in multi-stress environments

Maria Cuenca Cambronero[1], Jordan Beasley [1,2], Stephen Kissane[1] and Luisa Orsini[1]

[1] Environmental Genomics Group, School of Biosciences, the University of Birmingham, Birmingham B15 2TT, UK

[2] Departments of Genetics, University of Leicester, Leicester LE1 7RH, UK

Corresponding author:

Dr Luisa Orsini

Environmental Genomics Group, School of Biosciences, University of Birmingham, Birmingham B15 2TT, UK

Abstract

Species extinction rates are many times greater than the direst predictions made two decades ago by environmentalists, largely because of human impact. Major concerns are associated with the predicted higher recurrence and severity of extreme events, such as heat waves. Although tolerance to these extreme events is instrumental to species survival, little is known about if and how it evolves in natural populations, and to what extent it is affected by other environmental stressors.

Here, we study physiological and molecular mechanisms of thermal tolerance over evolutionary times in multi-stress environments. Using the practise of 'resurrection ecology' on the keystone grazer *Daphnia magna*, we measured thermal tolerance after exposure to warming as single stressor and in combination with biotic and abiotic stress. This was done by quantifying genetic and plastic differences in critical thermal maximum (CT_{max}) and the expression of four heat shock proteins (HSP) bewteen historical and modern genotypes of the same population that experienced an increase in average temperature and occurrence of heat waves, in addition to dramatic changes in water chemistry, over five decades. We observed evolution of the critical thermal maximum in response to higher occurrence of heat waves and increase in average ambient temperature, in a warming only scenario. Molecular and physiological responses to extreme temperature in presence of multiple stressors were unpredictable from the response to warming as single stressor. This finding suggests that species persistence may be wrongly estimated if the role of multiple stressors is not assessed.

Keywords: heat shock proteins, CT_{max}, *Daphnia magna*, food limitation, insecticides, temperature

Introduction

Twenty-five years ago, the Convention on Biological Diversity predicted over 30% of multicellular species extinction by 2100, largely because of human activities. These predictions correspond to more than 6,000 species going extinct and many more being impacted by altered synchrony with food and/or habitat requirements (Bellard *et al.* 2012; Easterling *et al.* 2000; Parmesan *et al.* 2000). Current evidence shows that species extinction rates are many times greater than the direst predictions made two decades ago by environmentalists (Hallmann *et al.* 2017; Isbell *et al.* 2011). One of the major concerns associated with changing climate is the recurrence of extreme events, such as heat waves (Easterling *et al.* 2000; Parmesan *et al.* 2000). These events, co-occurring with average temperature increase, are predicted to be more severe in Europe and North America due to atmospheric circulation patterns intensified by greenhouse gases (Meehl & Tebaldi 2004), and to become more intense, longer lasting, and/or more frequent (Karl & Trenberth 2003).

Physiological and/or molecular plasticity have been shown to play an important role in organismal response to extreme temperatures. A well-documented response to thermal stress is the increase of the critical thermal maximum (CT_{max}), the upper temperature at which animals lose motor function (Angilletta 2009). Higher CT_{max} has been shown to evolve with temperature at different altitudes (Garcia-Robledo *et al.* 2016; Oyen *et al.* 2016) and over evolutionary time in response to warmer climates in some species (Daufresne *et al.* 2009; Geerts *et al.* 2015; Jansen *et al.* 2017; Kellermann *et al.* 2012) but to be phylogenetically constrained in others (Kellermann *et al.* 2012). Another typical response to sudden and extreme stress, including temperature stress, is the regulation of heat shock proteins (HSP) (Sorensen *et al.* 2003). *HSPs* function as molecular chaperones protecting cells against accumulation of damaged proteins (Sorensen *et al.* 2003), and playing a vital role in stress tolerance and survival under adverse conditions (Mayer & Bukau 2005). More specifically HSP20 plays and important role in modulating cellular defence under

environmental stress conditions (Seo *et al. 2006*); HSP60 controls modification of newly synthesized proteins, repairs damaged ones, and prevents peptides form accumulating (Pockley *et al. 2003*); HSP70 is responsible for cellular homeostasis under non-stress conditions and plays a vital role in stress tolerance and survival under adverse conditions (Mayer *et al.* 2005); HSP90 plays a major role in stress tolerance by removing proteins with incorrect structure and by mediating proper folding under stress conditions (Schnaider *et al. 2000*). Both, plastic responses in the expression of HSP proteins (Jansen *et al.* 2017; Mikulski *et al.* 2011; Mikulski *et al.* 2009) and evolution of HSPs over microevolutionary time scales (Bettencourt *et al.* 1999; Ketola *et al.* 2004; Riehle *et al.* 2003) have been associated with thermal stress. Yet, it is unclear how plasticity impacts on long-term evolutionary responses to thermal stress as plasticity can either help (Ghalambor *et al.* 2007; Mitchell *et al.* 2011) or hinder (Hendry 2016) evolutionary responses to environmental change.

Here, we study the evolution of molecular and physiological mechanisms of thermal tolerance in an invertebrate ectotherm common to European lotic freshwater ecosystems, *Daphnia magna* (Miner *et al.* 2012). *Daphnia* is a keystone species in lakes and ponds worldwide, and a driver of ecosystem dynamics (Altshuler *et al.* 2011; Miner *et al.* 2012). In the natural environment, it is exposed to severe spatial and temporal environmental changes, including temperature, food levels and other abiotic factors (e.g. pesticides) , to which it responds via an ecoresponsive genome (Colbourne *et al.* 2011; Orsini *et al.* 2017; Orsini *et al.* 2016a). In favourable environmental conditions, *D. magna* has a parthenogenetic life cycle that allows the rearing of populations of genetically identical individuals (clones) from a single genotype. In unfavourable environmental conditions, *Daphnia magna* switches from asexual to sexual reproduction, producing dormant embryos that arrest their development entering dormancy (Ebert 2005). These dormant embryos sink at the bottom of lakes and build up archives of living fossils that remain viable for decades or even centuries (Frisch *et al.* 2014; Orsini *et al.* 2016b; Orsini *et al.* 2012). With the practise of 'resurrection ecology' (Kerfoot & Weider 2004), the dormant embryos can be resuscitated and historical and

modern populations can be used in the same experimental settings. These properties provide us with the unique opportunity to study mechanisms of response to environmental changes through evolutionary time, and to disentangle the relative contribution of plastic and genetic response to environmental change. Previous studies have shown adaptive response of *D. magna* to temperature changes via physiological and molecular mechanisms (Cambronero *et al.* 2017; Geerts *et al.* 2015; Jansen *et al.* 2017) (Chapter 3 and 4). Geerts and co-workers provided evidence of evolution of temperature tolerance via CT_{max} across few decades in response to warmer climates (Geerts *et al.* 2015). In a follow up study, Jansen *et al.* showed that evolution in CT_{max} was mediated by both plastic and evolutionary changes in gene expression at a number of candidate genes (Jansen *et al.* 2017).

Three populations of *D. magna* separated in time were sampled from a well characterised lake in Denmark, Lake Ring, which experienced an increase in average temperature and occurrence of heat waves across five decades (1960-2005) (Orsini *et al.* 2016b). The lake also experienced changes in water chemistry transitioning from a severe event of eutrophication (1960-1970) (Sayer *et al.* 2010) to a partial recovery in modern times (Cambronero *et al.* 2017; Orsini *et al.* 2016b) (Chapter 1). In a previous study, Cambronero *et al.* (in review) (Chapter 2) used these three populations in common garden experiments to measure genetic and plastic response in life history traits (fecundity, size at maturity, age at maturity, and mortality) to temperature as single stress and in combination with insecticide loads and food levels, mimicking major stressors in the lake. Here, we measure the critical thermal maximum (CT_{max}) and quantify the differential expression of four heat shock proteins (*HSP20, HSP60, HSP70* and *HSP90*) immediately following the experimental exposures to assess the impact of multiple stressors on thermal tolerance. Our results provide important insights into coping mechanisms to thermal stress over evolutionary time.

Materials and methods

Sampling site and Lake Ring paleolimnological profile

The study site is Lake Ring, a well characterized peri-urban lake in Jutland, Denmark (55°57'51.83'' N, 9°35'46.87'' E). In 2004 a sedimentary archive was sampled from the Lake and stored in dark and cold (4 °C) conditions. In 2015 a radiometric chronology of this archive was completed by ENSIS Ltd (UCL London) following standard protocols (Appleby 2001). Environmental changes occurring in the Lake were reconstructed from the paleolimnological analysis of sediment and historical records as described in (Cambronero *et al.* in review) (Chapter 2). The paleolimnological analysis consisted of quantifying the organic matter in the sediment, measured as loss on ignition (LOI) (Heiri *et al.* 2001), and the invertebrate community (*Cladocera*) assemblage over time. Furthermore, total phosphorus, nitrogen, and water transparency, were collected by the local county authority between 1970 and 1990. Temperature records were collected by the Danish Meteorological Institute at a weather station located 80 km from the Lake. Because air and water surface temperature have a positive correlation for shallow streams and lakes (Preudhomme & Stefan 1992), especially for the summer months (e.g. (Livingstone & Lotter 1998), we used the data from the weather station as estimates of the monthly water temperature in the lake. Historical records of the most common pesticides marketed in Denmark were collected from the Danish national archives (Cambronero *et al.*, in review) (Chapter 2). According to the paleolimnological and historical data, three main phases were identified: Eutrophication phase (*EP*, 1960-1970) characterized by an increase of primary production (high LOI) due to sewage inflow; ii) Pesticides phase (*PP*, 1980-1990) driven by increase in land use; and iii) Clear water phase (*CWP*, >1999) after diversion of sewage, associated with a decrease in primary production and a partial recovery of the lake from eutrophication (Cambronero *et al.*, in review) (Chapter 2). From each lake phase Cambronero *et al.* (in review) (Chapter 2). resurrected *D. magna* populations separated in time following established protocols

(Cambronero & Orsini 2017) (Chapter 1). Common garden experiments were performed on the resurrected populations to assess evolutionary and plastic response in life history traits (size at maturity, age at maturity and fecundity) to temperature as single stress and in combination with loads of the insecticide Carbaryl and food levels. Among the total hatched embryos from the sediment core (N = 262), 10 random genotypes were selected from each lake phase for a total of 30 genotypes to be used in experimental trials.

The genotypes are an unbiased representation of the local population genetic diversity as hatching success fluctuated along the sedimentary archive but did not systematically decrease with the age of the sediment (Cambronero & Orsini 2017) (Chapter 1). Moreover, previous results on the genetic composition of *D. magna* in Lake Ring showed that genetic drift and selection did not have a detectable impact on the neutral genetic diversity over time, measured both on the hatched and unhatched local population of *D. magna* (Orsini *et al.* 2016b). Negligible impact of drift and selection on neutral genetic diversity in presence of strong environmental selection provide an ideal system to study evolutionary responses in physiological and molecular candidate traits. The sample size per population was chosen based on previous results showing that 10 genotypes are representative of the genetic diversity of *D. magna* populations (Orsini *et al.* 2016b).

Physiological response to thermal stress

To assess the impact of multiple stressors on tolerance to extreme temperatures (CT_{max}), we studied physiological responses to extreme temperature after exposure to temperature treatment by itself and in combination with either biotic stress (two food levels) or abiotic stress (two concentrations of the insecticide Carbaryl). Molecular responses (*HSPs* expression) to extreme temperature stress (CT_{max} treatment) were assessed after exposure to temperature treatment by itself and in combination with food limitation or one insecticide concentration, for feasibility. These combinations of stressors were previously identified as

imposing severe sub-lethal effects on life history traits (Cambronero *et al.*, in review) (Chapter 2).

The three populations resurrected from Lake Ring (N = 30; 10 genotypes per population) were previously used in three common garden experiments (CGEs) assessing the impact of temperature as single stressors and in combination with food levels and insecticide loads on life history traits. In CGE1 the populations were exposed to temperature treatment (24±1°C) and control temperature, defined as 18±1°C; the experimental animals were fed *ad libitum* with *Chlorella vulgaris* (0.8 mg C/L). In CGE2 the two experimental temperatures (18±1°C and 24±1°C) were combined with two nutrient levels: 0.2 mg C/L and 2.4 mg C/L. In CGE3 the two experimental temperatures (18±1°C and 24±1°C) were combined with two concentrations of the insecticide Carbaryl: 4 µg/L and 10 µg/L; the animals were fed *ad libitum* with *C. vulgaris*.

CT_{max}, defined as the reversible thermal endpoint where locomotor functions are compromised (Terblanche *et al.* 2007), was measured for the current study in all genotypes at the end of the three CGEs described above (Table S1). Following the protocol optimised by Geerts *et al.* (2015), we exposed individual adult *Daphnia* to temperature ramping of 1 °C increments every 20±5 seconds until the animal stopped swimming (Geerts *et al.* 2015). The assayed individuals were collected at the end of the CGEs, after the release of the second brood, and placed in eppendorf tubes (1.5 ml) within their own medium. The temperature ramping, starting at 16 °C, was performed in a thermal block (Eppendorf ThermoMixer C) where the swimming activity was continuously monitored by an operator, who recorded the temperature of ceased swimming activity using a voice recording device. The temperature was monitored both on the display of the thermal block and with a thermometer. A second operator assisted by flash freezing animals in liquid nitrogen immediately after they stopped swimming. Gene expression of four heat shock proteins was measured on the CT_{max} assayed genotypes (see below).

Molecular response to thermal stress (HSP)

We measured differential expression (DE) at four *HSPs* (*HSP20, HSP60, HSP70* and *HSP90*) on a subset of the CT_{max} assayed genotypes (3 genotypes per population) via qPCR, using three technical replicates per genotype. DE was measured after exposure to: 1) temperature treatment (24±1 °C); 2) temperature treatment (24±1 °C) combined with food limitation (low food, 0.2 mg C/L); and 3) temperature treatment (24±1 °C) combined with the insecticide Carbaryl (4µgL) without food limitation.

Total RNA was extracted using Agencourt® RNAdvance™ Tissue kit, following the manufacturer's instructions. RNA was quantified using Nanodrop® Technologies (USA) and cDNA synthesis was performed using the AffinityScript cDNA synthesis kit, following the manufacturer's instructions. qPCR was performed on three replicates per genotype using a Roche LC96 qPCR machine, following the SYBR® Premix Ex Taq (Tli, RNaseH Plus, Takara) protocol in 20 µL final volume. The cycling was as follows: initial denaturation for 1 minute at 95 °C, followed by 40 cycles consisting of 5" at 95 °C, 30" at 60 °C and 1' minute at 72 °C. A final extension of 5 minutes at 72 °C was used. We calculated the mean CT (cycle threshold) value per sample averaging among replicates before rescaling the value. CT values per samples and per protein were rescaled using an interplate calibrator consisting of a pool of RNA extracted from different genotypes at different developmental stages and including genotypes from the three populations studied here following (Jansen *et al.* 2017).

Mechanisms of heat tolerance

We performed an analysis of variance on CT_{max} and the four HSPs measured in the CGEs to assess evolutionary mechanisms of response - plastic, genetic or a combination thereof - to extreme temperature.

Results of the variance analysis are interpreted as follows: a) a significant population term indicates genetic differences among populations and, hence, evolutionary responses in

the molecular or physiological trait; b) a significant response to treatment(s) indicates plasticity in the life history or molecular trait; c) a significant interaction between the population and the treatment(s) indicates evolution of plasticity in the trait.

We analysed CT_{max} via ANOVAs using linear mixed models (LMMs) in R v.3.3.3 (Team 2017) and including genotypes as random effect. We visualized the main effects of population and the various treatments (temperature, food, Carbaryl) as well as population x treatment interactions on CT_{max} through reaction norms, which describe the pattern of phenotypic expression of each genotype across treatments (Roff 1997).

We assessed evolutionary mechanisms operating on HSP proteins via multivariate statistics (MANOVA) followed by a univariate analysis per single protein (ANOVA). Both analyses were performed using linear mixed models (LMMs) in R v.3.3.3 (Team 2017). For these analyses, we included a random error structure in each model to account for genotype-specific differences in gene expression within each population. We fitted one model per gene. The term 'CT_{max}' assesses whether the gene of interest displays a significant change in its expression level after CT_{max} treatment compared to the control condition. This term measures the short-term plastic response of gene expression when the animal is exposed to sudden and severe temperature increase. The term 'evolution' represents the constitutive difference in gene expression among populations. This term measures evolutionary or constitutive differences in gene expression among populations. The interaction term 'CT_{max}' x 'evolution' quantifies the difference in gene expression due to the CT_{max} treatment among populations and it reflects the evolution of plasticity in the expression of the HSPs.

We visualized multivariate analysis of HSPs via a phenotypic trajectory analysis (PTA) (Collyer & Adams 2007). For each population, we quantified the amount and the direction of change across the four HSPs between control and CT_{max} treatment (Collyer & Adams 2007; Dennis *et al.* 2011). Individual HSPs differential expression was visualized through univariate reaction norms.

Results

Physiological response to thermal stress

CGE1. The effect of temperature treatment on CT_{max} varied significantly by population (Table 1 – CGE1). . Exposure to temperature treatment induced a significantly higher CT_{max} response in the most recent population (CWP) as compared with the other two populations (Table 1 – CGE1). The temperature of maximum tolerance, $CT_{max,}$ was higher in the temperature treatment than in the control temperature in all populations. This effect was supported by a significant term of plasticity in the ANOVA analysis (Table 1)

CGE2. The effect of temperature combined with food levels varied significantly among populations.. We observed a significant plastic response to temperature and to food levels as single stressors (Table 1 – CGE2, T, F). A significant interaction between temperature and food (T x F) as well as a significant term of evolution of plasticity (P x T and P x F) were observed (Table 2 - CGE2).

The univariate reaction norms showed lower CT_{max} in low food levels than in high food levels and a different plastic response of the populations within food levels (Fig. 1 – CGE2). In high food levels (dotted lines), CT_{max} was higher in the temperature treatment (TT) than in the control temperature (CT) for EP and CWP, whereas remained unchanged in PP (Fig. 1 - CGE2). In low food levels (solid lines), CT_{max} was higher in the temperature treatment in the CWP population, whereas it was lower in the other two populations (Fig. 1 – CGE2).

CGE3. The effect of temperature combined with insecticide did not vary significantly by population (Table 1 – CGE3). We observed a significant plastic response to temperature but not to insecticide (Table 1 – CGE3). Significant evolution of plasticity was also observed (Table 1 - CGE3; P x I).

The univariate reaction norm showed comparable plastic responses of the three populations to CT_{max} at both insecticide levels (Fig. 1 – CGE3). The temperature of maximum tolerance was generally higher in the temperature treatment than in the control temperature, except for

the PP population in low Carbaryl (solid lines) (Fig. 3 – CGE3). At low insecticide concentration , the CWP population showed higher CT_{max} than the EP population In presence of high Carbaryl, the PP population went extinct at 18°C.

Figure 1. Reaction norms of physiological response

Population reaction norms, based on population means and SD (n=10), showing CT_{max} differences between control (18±1 °C) and temperature treatment (24±1 °C), after exposure to temperature increase (CGE1); temperature and food levels (CGE2); temperature and insecticide Carbaryl levels (CGE3). The populations are colour coded as follows: Eutrophic population (*EP*, 1960-1970) in blue; Pesticides population (*PP*, 1980-1990) in green; and Clear water phase population (*CWP*, >1999) in red. The PP population experienced 100% mortality in control temperature combined with high Carbaryl. High food and high Carbaryl are represented by circle symbols and dotted lines; Low food and Low Carbaryl are represented by triangle symbols and solid lines. CT = control temperature (18±1 °C); TT = Temperature treatment (24±1 °C).

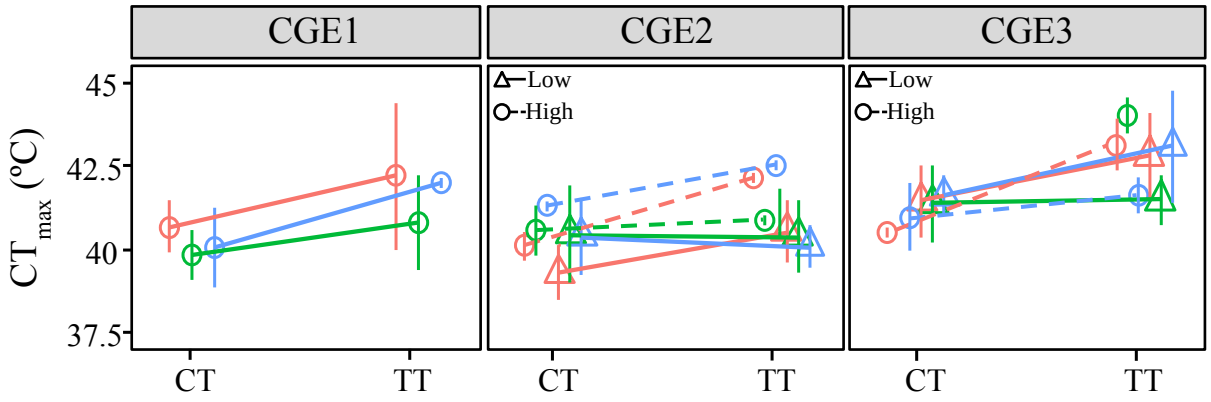


Table 1. ANOVA of physiological responses

Analysis of variance (ANOVA) after exposure to temperature (CGE1), temperature and food levels (CGE2) and temperature and insecticide loads (CGE3). The effect of the interaction between Temperature (T) and Population (P) (CG1), the interaction between Temperature (T) and Population (P) by Food level (F) (CG2) and the interaction between Temperature (T) and Population (P) by Insecticide loads (I) on CT_{max} is shown. Evolution is expresses as difference among populations (P), plasticity as trait response to treatment [temperature (T), food levels (F) and insecticide loads (I)], and evolution of plasticity is expressed by the interaction terms between P and the treatment(s) (T, F and I). Significant *P*-values (*P*<0.05) are shown in bold.

	Df	CT_{max} (°C)	
CGE1 (Temperature)		F	*P*
Evolution (P)	2	3.25	***0.048***
Plasticity (T)	1	12.86	***<0.001***
Evol. Plasticity (P x T)	2	0.43	0.651
CGE2 (Temperature and Food)	Df	F	*P*
Evolution (P)	2	2.96	***0.055***
Plasticity (T)	1	16.8	***<0.001***
Plasticity (F)	1	36.72	***<0.001***
Plasticity (T x F)	2	5.87	***0.017***
Evol. Plasticity (P x T)	2	6.88	***0.001***
Evol. Plasticity (P x F)	1	4.67	***0.011***
Evol. Plasticity (F x T x P)	2	0.86	0.43
CGE3 (Temperature and Insecticide)	Df	F	*P*
Evolution (P)	2	0.81	0.447
Plasticity (T)	1	18.4	***<0.001***
Plasticity (I)	1	0.26	0.614
Plasticity (T x I)	2	0.12	0.735
Evol. Plasticity (P x T)	2	2.41	0.096
Evol. Plasticity (P x I)	1	9.28	***<0.001***
Evol. Plasticity (I x T x P)	1	2.09	0.152

Molecular response to thermal stress

Warming. The effect of extreme temperature stress (CT_{max} treatment) on the HSPs expression did not vary significantly by population in the warming treatment (Table 2 – Warming). We observed a significant plastic response of the HSPs to CT_{max} treatment in both control temperature (CT) and temperature treatment (TT) (Table 2 – Warming). We did not observe significant evolution of plasticity in the HSPs expression in presence of warming as single stress (Table 2 – Warming).

The magnitude and direction of plastic change across the four HSPs in response to CT_{max} treatment was similar among populations in control temperature (Fig. 2 – Warming; CT; Table S3). In the temperature treatment (TT), the magnitude of change was comparable among populations, whereas the direction of change significantly differed between the most recent (CWP) and the historical (EP) population (Fig. 2 – Warming; Table S3).

The univariate statistics (ANOVA) confirmed that all HSPs, except for HSP20, did not vary significantly by population in CT, whereas HSP60, HSP70 and HSP90 differed significantly among populations in TT (Fig. 3 - Warming, Table S3). Significant plastic response of the HSPs after CT_{max} treatment observed in the MANOVA was confirmed by the ANOVAs on individual proteins. We observed significant upregulation of HSP20, HSP60, and HSP70 in the control temperature (CT), and of HSP60, HSP70 and HSP90 in the temperature treatment (TT) (Fig. 3 - Warming, Table S3). The univariate analysis also identified significant interaction between 'CT_{max}' and 'evolution' in HSP70 in TT (Fig. 3 - Warming, Table S3).

Warming and food limitation. The effect of CT_{max} treatment on the HSPs expression varied significantly by population in the warming and food limitation treatment in control temperature (CT), whereas it did not vary by population in the temperature treatment (TT) (Table 2 – Warming and food limitation). We observed a significant plastic response of the HSPs to CT_{max} treatment in both control temperature (CT) and temperature treatment (TT) (Table 2 –

Warming and food limitation). We observed significant evolution of plasticity in the HSPs in just in control conditions (CT) (Table 2 – Warming and food limitation).

In the phenotypic trajectories analysis (PTA), the magnitude of change in plastic response of the HSPs to CT_{max} did not significantly differ among populations at control temperature (CT). Conversely, the direction of change significantly differed in the pairwise comparisons involving the historical population and the other two populations (Fig. 2 – Warming and food limitation; Table S3). In the temperature treatment (TT), the magnitude of change in plastic response significantly differed between the historical population (EP) and the PP population (Fig. 2 Warming and food limitation, TT; Table S3). In this treatment, the direction of change did not significantly differ among populations (Fig. 2 Warming and food limitation, TT; Table S3).

The univariate statistics confirmed a significant constitutive difference (evolutionary differences among populations) in the expression of three HSPs in CT (HSP60, HSP70 and HSP90) and TT (HSP20, HSP60 and HSP70) (Fig. 3 – Warming and food limitation; Table S3). Significant plastic responses identified in the MANOVA were overall confirmed by the ANOVAs on individual proteins; however, whereas all proteins showed a significant downregulation in CT, only two of the four proteins (HSP60 and HSP90) were significantly differentially expressed in TT (Fig. 3 – Warming and food limitation; Table S3). A significant interaction term between 'CT_{max}' and 'evolution' was observed for all HSPs in CT and for HSP70 in TT (Fig. 3 – Warming and food limitation; Table S3).

W*arming and insecticide*. The effect of CT_{max} teatment on the HSPs expression did not vary significantly by population in the warming and insecticide treatment in both temperature treatments (Table 2 – Warming and insecticide). We observed a significant plastic response of the HSPs to CT_{max} treatment in both control temperature (CT) and temperature treatment (TT) (Table 2 – Warming and insecticide). We also observed significant evolution of plasticity of the HSPs in both temperature treatments (Table 2 – Warming and insecticide).

In the PTA, we observed that the magnitude of change of the HSPs significantly differed between the historical (EP) and the modern population (CWP), whereas the direction of change significantly differed between the PP and the EP population (Fig. 2 – Warming and insecticide; Table S3). In TT, there was no significant difference in the magnitude of plastic response among populations, whereas the direction of change was significantly different between the historical (EP) and the PP population (Fig. 2 – Warming and insecticide; Table S2).

The univariate statistics confirmed that the individual HSPs did not vary significantly by population in both CT and TT (Fig. 3 – Warming and insecticide; Table S3). Significant plastic responses identified by the MANOVA were confirmed by the univariate statistics. We observed significant upregulation of HSP20, HSP60, and HSP70 in CT, and significant differential expression of HSP70 and HSP90 in TT (Fig. 3 – Warming and insecticide; Table S3). A significant interaction term, 'CT_{max}' x 'evolution', was observed in HSP20 and HSP60 in CT, and in HSP60 and HSP70 in TT (Fig. 3; SC3).

Figure 2. Phenotypic trajectory analysis

PTA of the three populations of *Daphnia magna* resurrected from Lake Ring, resulting from multivariate response of four heat shock proteins to CT_{max} in control temperature (CT) and temperature treatment (TT). Patterns for PC1 and PC2 are shown for three combinations of stressors: 1) warming; 2) warming and food limitation; 3) warming and insecticide Carbaryl. Full circles represent control temperature (18±1 °C) and open circles CT_{max} treatment. Differences among populations, in terms of magnitude (M) and direction (θ) of plastic response are shown for significant pairwise population differences. ns = non-significant. Populations are colour coded as in Fig. 1. The statistics supporting this figure are in Table S2.

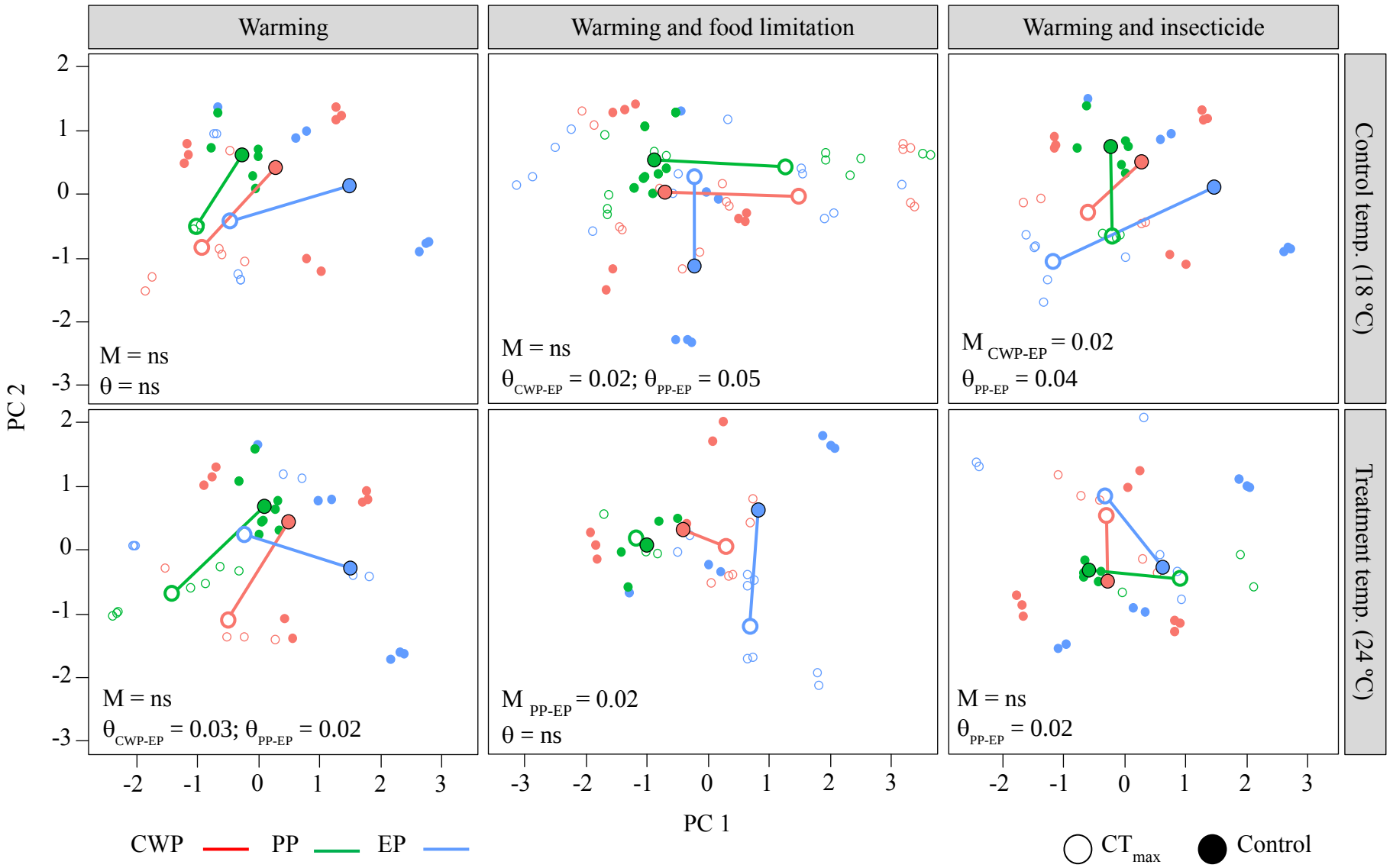

Warming
Warming and food limitation
Warming and insecticide
Control temp. (18 °C)
Treatment temp. (24 °C)
PC 2
PC 1
M = ns
θ = ns
M = ns
θCWP-EP = 0.02; θPP-EP = 0.05
MCWP-EP = 0.02
θPP-EP = 0.04
M = ns
θCWP-EP = 0.03; θPP-EP = 0.02
MPP-EP = 0.02
θ = ns
M = ns
θPP-EP = 0.02
CWP
PP
EP
CTmax
Control

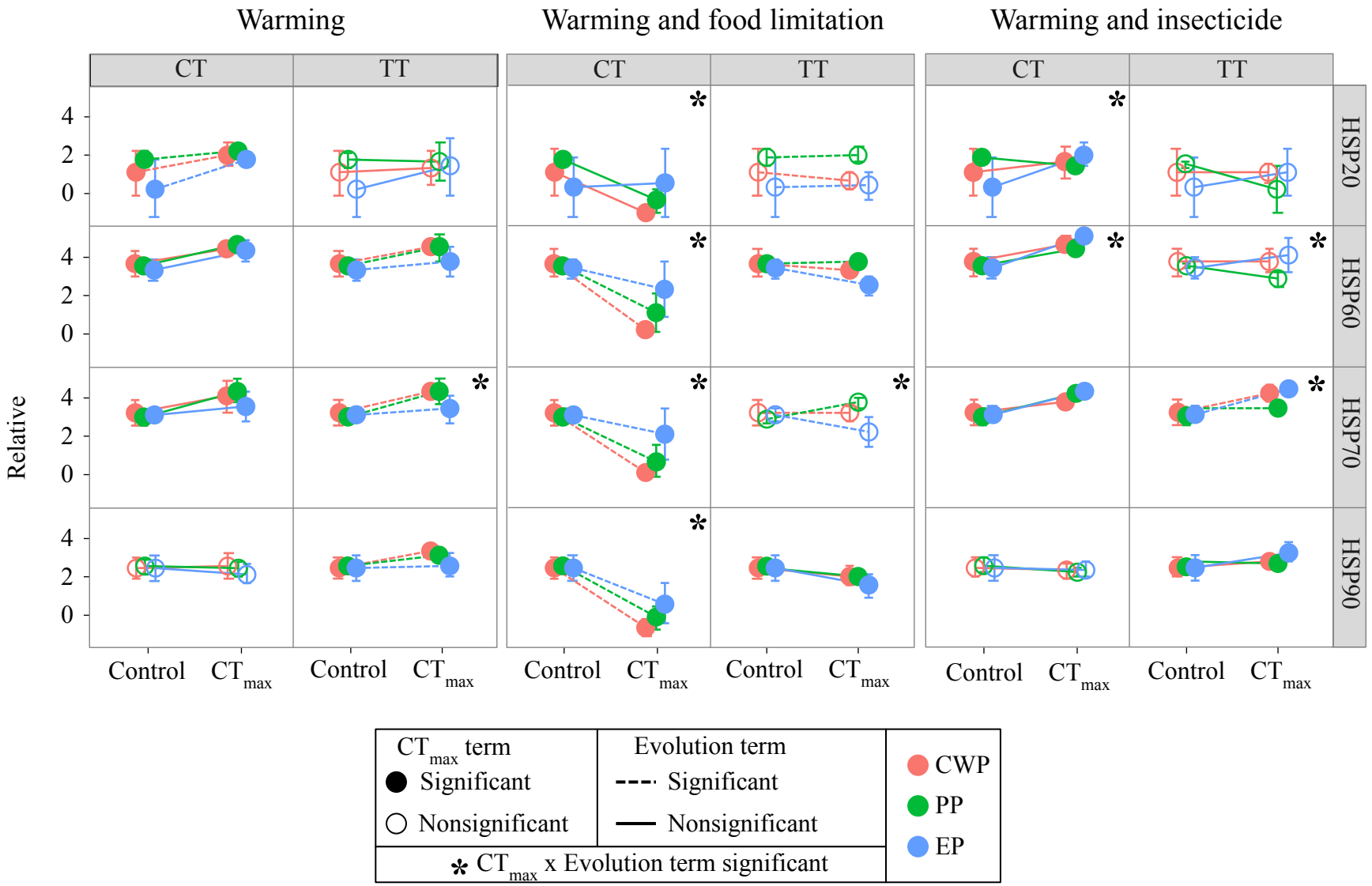
Table 2. Multivariate analysis of variance (MANOVA) of molecular responses

MANOVA showing multivariate response of four heat shock proteins to CT_{max}, in control conditions (CT) and after exposure to temperature treatment (TT) in three combinations of stress: Warming, temperature combined with food limitation (Warming and food), and temperature combined with insecticide Carbaryl (Warming and insecticide). The terms of the MANOVA are as follows: a) Evolution (P) indicates genetic differences among populations; b) plasticity indicates a plastic increase in HSPs in response to CT_{max} treatment; and c) evolution of plasticity is expressed as the interaction term between P and T. Significant *P*-values (*P*<0.05) are shown in bold.

Warming	Df	CT (18 °C)		TT (24 °C)	
		F	*P*	F	*P*
Evolution (P)	2	1.46	0.19	1.95	0.07
Plasticity (CT_{max})	1	17.94	**<0.001**	11.9	**<0.001**
P x CT_{max}	2	1.56	0.16	1.88	0.08
Warming and food limitation	Df	CT (18 °C)		TT (24 °C)	
		F	*P*	F	*P*
Evolution (P)	2	3.67	**<0.001**	1.99	0.07
Plasticity (CT_{max})	1	38.28	**<0.001**	6.14	**<0.001**
P x CT_{max}	2	3.33	**<0.001**	1.39	0.22
Warming and insecticide	Df	CT (18 °C)		TT (24 °C)	
		F	*P*	F	*P*
Evolution (P)	2	1.77	0.1	1.43	0.2
Plasticity (CT_{max})	1	26.21	**<0.001**	9.24	**<0.001**
P x CT_{max}	2	2.26	**0.04**	2.28	**0.04**

Figure 3. Differential expression of individual HSPs

Population average (n=3) and SD of the differential expression of four heat shock proteins after CT_{max} essay in control temperature (CT) and after temperature treatment (TT). Induction of HSP is measured in the three combinations of stressors listed in Figure 2. Dotted lines indicate significant constitutive differences in gene expression among populations (evolution term in Table S3). Full circles indicate a significant response in gene expression induced by CT_{max} (CT_{max} term in Table S3). Asterisks (*) indicates significant interaction terms 'evolution' x 'CT_{max}' (Evol plasticity in Table S3). CT = control temperature; TT = Temperature treatment. Populations are colour coded as in Figure 1. Statistics supporting the results summarized in this figure are reported in Supplementary Table S3.


Warming
Warming and food limitation
Warming and insecticide
CT
TT
HSP20
HSP60
HSP70
HSP90
Relative
0
2
4
Control
CTmax
CTmax term
Significant
Nonsignificant
Evolution term
Significant
Nonsignificant
CWP
PP
EP
* CTmax x Evolution term significant

Discussion

Despite the fact that tolerance to extreme temperature events is critical to survival and impacts on organisms' fitness, little progress has been made to elucidate if and how it evolves in natural populations. Many studies investigating heat tolerance tend to minimize confounding factors, searching for trends in relatively undisturbed systems (Anderson *et al.* 2012; Geerts *et al.* 2015; Hoffmann & Sgro 2011) but see Brans *et al.* 2017 for an exceptional study, where the authors explore the effect of "urban heat island" on 13 natural population of *Daphnia magna* from a well-defined urbanization gradient, and find that populations from urban areas have higher CT_{max} in than rural populations. Limiting confounding factors has obvious advantages, but can lead to wrong estimates of evolvability in the wild.

We studied the evolution of thermal tolerance of a population of the waterflea *Daphnia* exposed over five decades to an average increase in ambient temperature and occurrence of heat waves, as well as to changes in water chemistry. Using common garden experiments we tested the impact on thermal tolerance of warming as single stressors, as well as of warming in combination with biotic or abiotic stress. In experimental conditions mimicking warming as single stressor, the most recent population of Lake Ring showed a constitutive higher CT_{max} than the two historical populations in both control temperature and temperature treatment. This result shows that the most recent populations showed both a constitutively higher thermal maximum and a higher induced plasticity in this trait, suggesting a positive correlation between constitutive and induced thermal tolerance, at least in presence of warming as single stressor. These results suggest that the temperature of maximum tolerance (CT_{max}) evolved over time in the studied population. This evolutionary response occurred in response to increase in average temperature and occurrence of extreme temperature events. Indeed, climate records show an increase of ca. 1 °C in average ambient temperature and a higher occurrence of heat waves in the five decades

studied here (Change 2017). These trends were confirmed for the lake under study (weather data). In a previous study, the evolution of the thermal maximum over few decades was associated with the capacity of *D. magna* to tolerate higher temperatures, using a selection experiment and a resurrection ecology approach (Geerts *et al.* 2015). Our results corroborate these previous findings and provide a further line of evidence for the evolution of CT_{max} in *D. magna*.

Evolutionary differences in CT_{max} were not reflected in higher HSP induction in the most recent population, suggesting that other molecular mechanisms not considered here underlie evolutionary differences in this trait. A genome-wide transcriptome analysis and/or a genome-wide association study (GWAS) on the populations studied here will be required to identify the molecular mechanisms underlying evolutionary differences in $CT_{max.}$

The trajectory and magnitude of plastic change in the four HSPs in response to CT_{max} treatment did not differ among populations in control temperature, but significantly differed between the historical and the two most recent populations in the temperature treatment (PTA analysis). This finding suggests that after exposure to a temperature treatment, the two most recent populations show a divergent stress response as compared to the historical population. The recent past (PP) and the modern population (CWP) were resurrected from the 1980s and >1999, respectively. These time periods coincide with recorded higher average ambient temperature and higher occurrence of heat waves (Change 2017; Parmesan *et al.* 2000). It is possible that exposure prior to dormancy to these environmental conditions may be responsible for the divergent phenotypic trajectories of the two most recent populations and the historical population (Desai 2009). Furthermore, other environmental stressors occurring in the history of Lake Ring may have influenced the evolutionary response of the three populations.

In natural ecosystems and especially in enclosed habitats (e.g. lakes and ponds) other factors, such as eutrophication or by-products of land use, may play an important role in driving evolutionary responses by altering solubility of nutrient, conductivity and oxygen

levels (Feuchtmayr *et al.* 2009). There is increasing evidence that multiple factors may alter trait–environment and genotype–environment interactions influencing responses to climate change (Chown *et al.* 2010; Karl & Trenberth 2003); for example, CT_{max} has been shown to change with diet (Bujan & Kaspari 2017). We studied molecular and physiological responses to extreme temperatures in multi-stress environments, combining warming with a second stressor that occurred in the history of Lake Ring. In presence of multiple stressors, a complex interplay among plastic and evolutionary responses both at physiological and molecular level underpinned population responses to extreme temperatures. In addition, the evolutionary advantage of the most recent population, apparent in the constitutive higher CT_{max} in presence of warming as single stressor, was no longer detected when temperature co-occurred with food limitation or insecticide.

Overall, the direction and the magnitude of plastic changes at molecular and physiological levels in response to extreme temperatures in multi-stress environments was not predictable from populations' response to warming as single stressor. Our results contrasting physiological and molecular responses in single and multiple stress scenarios show that the co-occurrence of other environmental stressors with temperature has the potential to affect the evolution of thermal tolerance in natural populations of *D. magna* in ways not directly predictable from the response to warming alone.

Although our study investigates multiple mechanisms underlying thermal tolerance, it focuses on a single natural system, calling for a cautionary generalization of our findings. This is a common limitation of resurrection ecology studies. Whereas these studies provide important insights into the evolutionary processes underlying adaptation in the wild, they have the limitation of requiring large efforts, and, hence, to suffer from low replication. Despite these limitations, our study is pioneer in studying trade-offs between constitutive and induced thermal tolerance over evolutionary times and in investigating the effect of multiple stressors on thermal tolerance. Our results suggest that underestimating the effect of multiple stressors on thermal tolerance can lead to wrong estimates of species evolvability

and persistence to future global change. To develop more realistic predictions about the biological impacts of climate change on species persistence, interactions between the mean and variance of environmental temperature, as well as the impact of biotic and abiotic stressors on thermal tolerance should be considered (Bozinovic *et al.* 2016; Nadeau *et al.* 2017).

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The authors declare no conflicts of interest

Data Accessibility

Data associated with this study are deposited in the DRYAD databank at the following entries: XXX

Author contribution

MCC carried out the experiments and performed data analysis with input from JB.

SK and JB generated the HSP data

LO conceived the study and coordinated data analysis. LO and MCC wrote the first version of the paper; all authors contributed to the editing of later versions.

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Table S1. CT_{max} measurements

CT_{max} measured on the 30 genotypes resurrected from Lake Ring. Data were collected after exposure to temperature by itself (CGE1) and in combination with either food levels (CGE2) or insecticide Carbaryl loads (CGE3). CT – Control temperature is 18±1 °C. TT – Temperature treatment is 24±1 °C. The two nutrient levels are: 0.2 mg C/L and 2.4 mg C/L. The two concentrations of Carbaryl are: 4 µg/L and 10 µg/L.

	Warming		Warming and food				Warming and insecticide			
			High food		Low food		High insecticide		Low insecticide	
Indv ID	Control (18°C)	Heat treatment (24°C)	Control (18°C)	Heat treatment (24°C)	Control (18°C)	Heat treatment (24°C)	Control (18°C)	Heat treatment (24°C)	Control (18°C)	Heat treatment (24°C)
LRV 0_1	41	41		42	38	41.5				43
LRV 0_2		39	39	42	39			41.5	42.5	42
LRV 0_4			40.5	41	39.5	39.5			42	41
LRV 0.5_3	41	44		42	38	41.5		42	41	40
LRV 1_2		43	38.5	41.5		39.5		43	38.5	43
LRV 2_1	40		40	42.5	38.5	41.5			42	43
LRV 2_2		39		42.5					42	43
LRV 2.5_9	41	44		42.5	40			43	42	44.5
LRV 2.5_11		44			39.5	40				42
LRV 3_4	41.5		41.5	42.5	39.5	40	40	43.5	42	
LRV 3_6	39	40	40.5	42.5	40	39		44	40.5	43.5
LRV 3.5_1		44		42.5		41			42	44
LRV 3.5_2	41		40.5	42.5	40.5	41			41	44
LRV 3.5_15	41	44	40.5	42.5	40	41.5	41	45	42	44
LRV 6_2		40	42.5	41.5	41	40				40.5
LRV 6_3	40	40	40	41.5	39.5			43	41	

LRV 6.5_5			40	39	40.5			44	42	42
LRV 6.5_7			42.5	40.5	39	42.5		45	42.5	41
LRV 7_3	40	39	40	40	39	40.5			39	40
LRV 7_5	39	42	42		38.5			43	42	42
LRV 7.5_4	41	39		39.5		40				42
LRV 8_7		42	41.5	42	41	41			42	41.5
LRV 8.5_3		39	39.5		40			45		
LRV 9_6	40	42	39	42	41	40.5				42
LRV 9_20	39	42	39		44	39			42	
LRV 9.5_1		42	41.5	42	41.5	41			42	42
LRV 9.5_3		42	40		40.5	39			40	42
LRV 12_2	41		41	42	41	40.5			41	43
LRV 12_3			40.5		40.5	39			41	
LRV 12_4	40			43		40		42	42	40
LRV 12.5_1	40	42	40.5	42	40	40	40	42	42	41.5
LRV 13_1	40	42	41	41	42.5			40	42	45
LRV 13_2	38		42	42.5	40	39.5			42.5	44
LRV 13_3	39.5	42	42	43		41		42	42	44
LRV 13.5_1	39		42	43	40	40			41	45
LRV 14_1			41.5	43	39.5	40				43.5
LRV 14.5_1	41	42		43	38.5	41				
LRV 15.5_1	42	42	41.5	43	41.5	40	42	42	41	42

Table S2. Phenotypic trajectory analysis.

PTA analysis resulting from multivariate response of four heat shock proteins to CT_{max} in control temperature (CT) and temperature treatment (TT). Pairwise differences between populations, in terms of magnitude (magnitude) and direction (θ) of change are shown. Significant P-values are in bold. These statistics support plots in Figure 2.

		Warming				Warming and food limitation				Warming and insecticide			
	Comparison	Magnitude	$P_{magnitude}$	θ	P_{θ}	Magnitude	$P_{magnitude}$	θ	P_{θ}	Magnitude	$P_{magnitude}$	θ	P_{θ}
CT	CWP-PP	0.057	0.944	29.369	0.554	0.029	0.969	11.943	0.789	0.382	0.508	47.131	0.169
	CWP-EP	0.269	0.600	27.282	0.428	0.804	0.255	77.398	***0.019***	1.379	**0.007**	14.270	0.763
	PP-EP	0.212	0.779	40.290	0.381	0.775	0.294	75.617	***0.046***	0.997	0.062	61.335	***0.038***
TT	CWP-PP	0.276	0.637	18.237	0.682	0.084	0.883	74.568	0.312	0.411	0.531	83.871	0.197
	CWP-EP	0.329	0.605	66.391	***0.035***	0.927	0.061	65.398	0.568	0.358	0.530	45.905	0.512
	PP-EP	0.605	0.341	52.801	***0.016***	0.843	***0.018***	96.017	0.475	0.053	0.936	126.022	***0.025***

Table S3. ANOVAs of individual *HSPs*

Analysis of variance of four heat shock proteins assessed in three combination of stressors: 1) warming without food limitation; 2) warming and food limitation; 3) warming and insecticide Carbaryl. Evolutionary differences among populations (evolution, P), plastic response to the treatment (CT_{max}), and the interaction between these two terms (Evol. Platicity) is shown. Significant *P*-values (*P*<0.05) are shown in bold. These statistics support plots in Figure 3.

		Warming					Warming and food limitation					Warming and insecticide			
		CT (18 °C)		TT (24 °C)			CT (18 °C)		TT (24 °C)			CT (18 °C)		TT (24 °C)	
HSP20	Df	F	*P*	F	*P*	Df	F	*P*	F	*P*	Df	F	*P*	F	*P*
Evolution (P)	2	4.36	***0.02***	2.49	0.09	2	1.45	0.24	9.99	***<0.001***	2	1.44	0.25	0.5	0.61
CT_{max}	1	8.1	***0.01***	2.05	0.16	1	22.82	***<0.001***	0.13	0.72	1	4.6	***0.04***	0	0.95
Evol. Plasticity	2	0.76	0.48	1.35	0.27	2	6.29	***<0.001***	0.5	0.61	2	3.76	***0.03***	2.75	0.08
HSP60	Df	F	*P*	F	*P*	Df	F	*P*	F	*P*	Df	F	*P*	F	*P*
Evolution (P)	2	1.54	0.23	3.64	***0.03***	2	3.54	***0.04***	7.98	***<0.001***	2	1.07	0.35	3.85	***0.03***
CT_{max}	1	47.4	***<0.001***	22.08	***<0.001***	1	132.15	***<0.001***	11.11	***<0.001***	1	85.24	**<0.001**	0.2	0.66
Evol. Plasticity	2	0.54	0.59	1.09	0.35	2	9.82	***<0.001***	2.75	0.08	2	3.7	**0.03**	3.78	***0.03***
HSP70	Df	F	*P*	F	*P*	Df	F	*P*	F	*P*	Df	F	*P*	F	*P*
Evolution (P)	2	0.94	0.4	4.86	***0.01***	2	5.49	***0.01***	6.53	***<0.001***	2	0.82	0.45	1.55	0.22
CT_{max}	1	30.88	***<0.001***	49.18	***<0.001***	1	126.54	***<0.001***	0.65	0.43	1	75.9	**<0.001**	23.87	***<0.001***
Evol. Plasticity	2	2.51	0.09	5.33	***0.01***	2	8.71	***<0.001***	9.74	***<0.001***	2	2.54	0.09	3.98	***0.03***
HSP90	Df	F	*P*	F	*P*	Df	F	*P*	F	*P*	Df	F	*P*	F	*P*
Evolution (P)	2	0.85	0.43	3.65	***0.03***	2	4.56	***0.02***	1.17	0.32	2	0.03	0.97	0.89	0.42
CT_{max}	1	0.23	0.64	16.06	***<0.001***	1	210.32	***<0.001***	12.53	***<0.001***	1	2.36	0.13	8.64	***0.01***
Evol. Plasticity	2	0.46	0.63	2.39	0.1	2	4.31	***0.02***	1.01	0.37	2	0.4	0.67	3.23	0.05

CHAPTER 5

Paleogenomics reveals genome variants underpinning plastic and constitutive phenotypic divergence in multi-stress environments

Information:

Authors: Cuenca Cambronero, M., Dhandapani, V., Zhou, J. Colbourne, J. and Orsini, L.

In preparation

Overview: In this chapter, high throughput sequencing technology is applied to the populations of *D. magna* resurrected from the biological archive of Lake Ring to identify the genomic basis of constitutive and plastic differences in life history traits measures in Chapter 3. Genome variants underpinning plastic and constitutive phenotypic responses to single and multiple stressors are identified via traditional GWAS analysis as well as using advanced computational tools developed in the research group.

Author's contribution: MCC carried out the experiments. MCC and VD performed bioinformatic analyses. JZ provided a newly developed pipeline for genome wide association studies. JKC contributed to the experimental design and advised on data analysis. LO conceived the study and coordinated data analysis. MCC wrote the chapter.

Paleogenomics reveals genome variants underpinning plastic and constitutive phenotypic divergence in multi-stress environments

Maria Cuenca Cambronero[1], Vignesh Dhandapani[1], Jiarui Zhou[1,2] John K. Colbourne[1], and Luisa Orsini[1]

[1] Environmental Genomics Group, School of Biosciences, the University of Birmingham, Birmingham B15 2TT, UK
[2] School of Computer Science, University of Birmingham, B15 2TT, Birmingham, UK

Running head: Genome variation in multifarious environments

Abstract

Identifying genomic changes leading to phenotypic adaptation driven by environmental change in the wild is challenging. Despite growing evidence that plasticity is critical for short-term response to environmental change, the influence of plasticity on long-term evolution remains unclear, as it ideally requires sampling at multiple time points across environmental transitions.

Here, we apply high throughput sequencing and phenotyping to a population of the ecological model species and keystone aquatic crustacean *Daphnia magna* sampled across evolutionary time to investigate the genomics of adaptation to multifarious environments. *D. magna* provides the unique advantage of producing dormant stages as part of its life cycle. These stages can be 'resurrected' from biological archives and maintained as clonal lines in the laboratory. These features enable the study of plastic and evolutionary adaptive responses to environmental change. We studied genome polymorphisms underpinning constitutive and plastic phenotypic differences in life history traits in a population of *D. magna* resurrected from a lake with a well-described history of human-driven environmental change, effectively documenting genotype (G) – phenotype (P) -environment (E) correlations through evolutionary time. We use regularized generalized canonical correlation analysis and benchmark this approach against classic GWAS and genome scans approaches. We gain unprecedented insights into the genetics underpinning evolutionary responses and plasticity over evolutionary time in a natural system.

Keywords: environmental stress, evolution, resurrection ecology, plasticity, adaptation, polymorphism

Introduction

Uncovering the genetic basis of adaptation to multifarious environments is a central goal in ecological genomics [1]. Fundamental to this goal is elucidating how change at the genomic level underlies phenotypic change as populations adapt to their local environment [2]. However, demonstrating selection in the wild and linking difference in adaptive fitness traits to the underlying genetic variation is notoriously difficult [3]. Main challenges are associated with linking measurable fitness differences among wild populations with the underlying genetic variation, and identifying mechanisms of adaptive response over evolutionary time [4]. Main difficulties are associated with many fitness traits being complex and regulated by multiple genes. Understanding how multiple mutations interact to jointly impact multiple ecologically important traits is critical for creating a robust picture of organismal fitness and the process of adaptation [5]. However, this is complicated by both, environmental heterogeneity and the complexity of genotype-phenotype relationships generated by pleiotropy and epistasis [6,7]. Moreover, little is known about how pleiotropic and epistatic relationships themselves change over evolutionary time [7].

Quantitative genetics is the most common approach used to link fitness differences among populations to the underlying genetic variation [8]. However, this approach relies on measurable phenotypic differences previously implicated in environmental adaptation. When such phenotypic differences are not known *a priori* and when experimental crosses are not feasible, a reverse ecology approach is a viable alternative [9]. This consists of a genome-wide scan for regions of elevated sequence divergence between natural populations inhabiting divergent environments that are associated with environmental variation; this approach leads to gene–environment associations and hence to the identification of candidate loci potentially involved in the adaptive process [1,10,11]. The main limitation of gene–environment associations in ecologically relevant species is that the link between genome-wide variation and adaptive phenotypic traits driven by environmental selection pressure is often unknown [2,12]. Therefore, despite four or more decades of research effort trying to determine footprint of selection in

the wild, and the recent advances in sequencing technologies, only a few examples exist showing that genetic polymorphisms is maintained by environmental heterogeneity among natural populations (see [13,14] for recent reviews). Furthermore, genome-wide or genome scans can lead to the identification of false positives if the model specifications are not met by the empirical data, and gene-environment association may be confounded by shared demographic history among population, patterns of isolation by distance or historical colonization events followed by local adaptation [15,16]

Disentangling the mechanisms of adaptation to changing environments in natural populations remain a major challenge. Despite the growing evidence that plasticity is critical for short-term response to environmental change, the influence of plasticity on long-term evolution remains unclear [17,18]. To assess the relative contribution of plasticity and genetic adaptation in natural systems, sampling at multiple time points across environmental transitions is ideally required. Most studies analysing temporal dynamics involve experimental evolution in the laboratory or controlled mesocosm experiments [19,20] with exceptional studies that reconstruct evolution of natural populations using transplant experiments in the wild [21]. For species that cannot be easily manipulated experimentally, or for which temporal samples are inaccessible, the 'space-for-time' substitution [22] is frequently adopted as a surrogate to study long-term evolutionary dynamics. Space-for-time analyses assume that two different conditions at two points in space can be treated as though they are in the same region at two different time points. This approach has its limitations as rates of adaptation at different spatial scales can differ compared to temporal variation in the same population evolving in time [18]. A promising approach to overcome the limitations of sampling across environmental transitions in the wild is to apply genomic tools to 'resurrected' or still "dormant propagules in sediments, soils, and permafrost as they are convenient natural archives of population histories from which to trace adaptive trajectories along extended time periods" [23].

Here, we apply high throughput sequencing and phenotyping to a population of the ecological model species and keystone aquatic crustacean *Daphnia magna*, sampled across evolutionary time to investigate the genomics underpinning adaptation to multifarious environments. *D. magna* is a keystone specie in pelagic freshwater food webs, where it is the primary forage for many vertebrate and invertebrate predators [24-26], an efficient grazer of algae [27] and a strong competitor for other zooplankters [28]. Species of the genus *Daphnia* are key models in evolutionary biology and the study of adaptive responses to environmental change, to which they have been shown to respond both via genetic and plastic mechanisms [29-33]. *D. magna* offers the unique opportunity to 'resurrect' historical populations from lake sediment by the practise of resurrection ecology [34,35]. Once dormant propagules have been 'resurrected', *Daphnia* genotypes are clonally maintained in the laboratory, proving the opportunity to study responses of the same genotypes to multiple stressors and to disentangle the role of plasticity and evolution in adaptive responses to environmental change [36,37].

We previously resurrected a *D. magna* population from Lake Ring, a lake with a well-described history of human-driven environmental change [36,38,39]. The lake experienced hyper-eutrophication due to sewage inflow in the 1960s, leaching of pesticides due to land use intensification > 1975s and an increase in average ambient temperature over five decades [39]. The sewage inflow was diverted from the lake in 1970s and the lake partially recovered >1999. We resurrected *D. magna* populations from the lake main phases (described in Cambronero *et al.*, in review) (Chapter 2), and measured fitness-linked life history traits (fecundity, age at maturity, size at maturity and mortality) in responses to temperature as single stressor and in combination with food levels and insecticide loads (Cambronero, *et al.* in review). Here, we study genome-wide polymorphisms underpinning constitutive and plastic phenotypic differences in life history traits, gaining unprecedented insights into the mechanisms and processes leading to adaptation over evolutionary time in a natural system.

Materials and methods

Study system

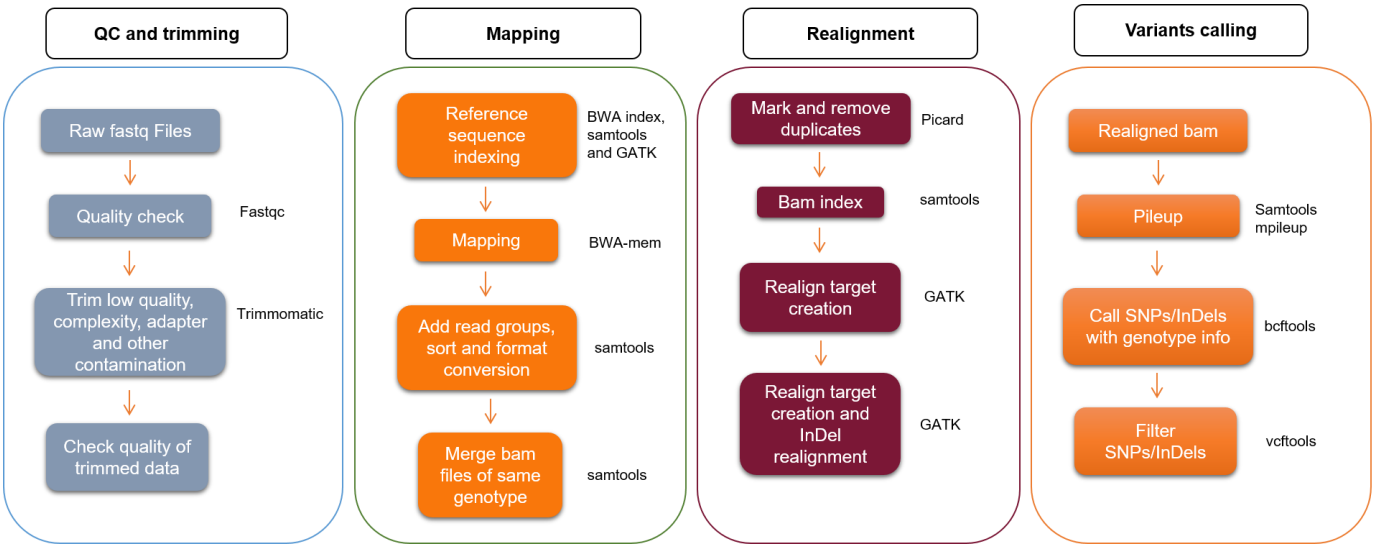
Our study system is Lake Ring, a well characterized shallow mixed lake (without thermocline stratification) located in a typical peri-urban landscape in Jutland, Denmark (55°57'51.83'' N, 9°35'46.87'' E) [40]. A sedimentary archive was sampled from the lake in 2004 and dated using radiometric chronology as described in [39]. Environmental changes through time occurring in the Lake were reconstructed from historical records and the paleolimnological analysis of sediment. The paleolimnological analysis and historical characterization of the lake identified three main phases associated with human-driven events: i) Eutrophication phase (*EP*, 1980-1990 associated with sewage inflow; ii) Pesticides phase (*PP*, 1980-1990) driven by increase in agricultural land use; and iii) Clear water or recovery phase (*CWP*, >1999) after diversion of sewage, and reduction in agricultural land use [41]. From the sedimentary archive, 262 dormant *Daphnia* embryos were hatched following established protocols [39]. Of these, 10 random genotypes were selected from each lake phase for a total of 30 genotypes to be used in experimental trials. The sample size per population was chosen based on previous results showing that 10 genotypes are representative of the local genetic diversity of *D. magna* populations [42].

The genotypes we collected are an unbiased representation of the local population genetic diversity as hatching success fluctuated along the sedimentary archive but did not systematically decrease with the age of the sediment [39]. Previous analysis of the neutral genetic composition of the *D. magna* population in Lake Ring showed that genetic drift and selection did not have a detectable impact on the system over time, [42]. Negligible impact of drift and selection on neutral genetic variation in presence of strong environmental selection provides an ideal system to study genome evolution over evolutionary time [42]. Three common garden experiments (CGEs) were previously performed on the 30 genotypes (10 genotypes per population) resurrected from Lake Ring (Cambronero *et al.* in review) (Chapter 2). In these experiments, genetic and plastic responses in key life history traits

(fecundity, age at maturity, size at maturity, and mortality) were measured in response to temperature treatment by itself and in combination with either biotic stress (two food levels) or abiotic stress (two concentrations of the insecticide Carbaryl) (Fig. 1). In CGE1 the populations were exposed to temperature treatment (24±1°C) and control temperature, defined as 18±1°C; the experimental animals were fed *ad libitum* with *Chlorella vulgaris* (0.8 mg C/L). In CGE2 the two experimental temperatures (18±1°C and 24±1°C) were combined with two nutrient levels: 0.2 mg C/L and 2.4 mg C/L. In CGE3 the two experimental temperatures (18±1°C and 24±1°C) were combined with two concentrations of the insecticide Carbaryl: 4 µg/L and 10 µg/L; the animals were fed *ad libitum* with *C. vulgaris*.

Figure 1. Genome sequence pipeline

The pipeline used for genome data preprocessing, mapping, realignment and variant calling is shown. For each process, the main steps and the tools used are listed.



Genome sequencing

The 30 genotypes of *D. magna* used in the common garden experiments were cultured in standard laboratory conditions (16:8 light: dark regime, 20°C and 0.8 mg C/L of *Chlorella vulgaris* daily) to generate sufficient material for extraction of high molecular weight gDNA. Two days prior to tissue collection, cultures were treated with a cocktail of antibiotics at a final concentration of 20 mg/L (Tetracycline-T, Streptomycin-S, Ampicillin-A) to reduce bacterial contamination in downstream analyses. Animals were also deprived of food the previous day to tissue collection to reduce contamination from alae. Genomic DNA was extracted using Agencourt DNA Advance (Beckman Coulter - A48706) with minor modifications. gDNA was quantified using a ND-8000 Nanodrop (Thermo Fisher Scientific - ND-8000-GL). Up to 1µg of gDNA per genotype was sheared using a Bioruptor® Pico ultrasonicator with integrated cooling module (Diagenode - B01060010), following cooling on ice for 10minutes. Sheared genomic DNA was assayed on a 2200 TapeStation (Agilent) with High Sensitivity DNA Screentapes to determine the distribution of sheared fragments. The sheared genomic DNA was then prepared into Illumina compatible DNA Sequencing 250bp paired-end libraries using KAPA HyperPrep Kit (Roche - KK8504), without amplification step. Following library construction, libraries were assayed and quantified on a 2200 TapeStation (Agilent) with High Sensitivity DNA Screentapes. Libraries were normalized to an average concentration of 2nM prior to pooling. Library construction, quantification, normalization, and pooling were performed on a Biomek FXP dual hybrid automated liquid handler (Beckman Coulter - A31844). Libraries were sequenced on two lanes of a HiSeq2500 (Illumina) using HiSeq Rapid SBS Kit v2 200 cycles (Illumina - FC-402-4021), HiSeq PE Rapid Cluster Kit v2 (Illumina - PE-402-4002), and HiSeq Rapid Duo cBot Sample Loading Kit (Illumina - CT-403-2001) following manufacturer instructions aiming to a final depth of coverage of 20X per genotype. Sample preparation and sequencing were completed at the Environmental Omics sequencing Facility at the University of Birmingham.

Genome data processing

The genome sequence data were subjected to quality check by mapping raw reads onto the draft genome of *D. magna* v2.4 (Accession: LRGB00000000). The reads were then aligned and assembled in individual genomes; SNP variants were called on the assembled genomes (Fig. 1). Read sequences basepair quality was assessed with Fastqc tools (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Trimmomatic ver.0.33 was applied for adapter trimming and to remove low quality sequences [43]. Paired-end reads with Q>30 and read length of 50bp were retained and mapped against the reference genome of *D. magna* using BWA-mem algorithm [44] (Fig. 1). Samtools were used for format conversion, sorting, indexing and merging of mapping files from multiple runs of the same genotype [45]. Picard tools were used to mark and remove duplicated reads and realign the trimmed dataset with GATK [46]. Allelic variants and indels were called via bcftools (https://samtools.github.io/bcftools/) after applying the samtools mpileup command (samtools ver. 0.1.19, 45). Finally, SNP and indel variants were filtered with vcftools v0.1.14. Filtering criteria were as follows: minimum read depth (DP) > 10; SNP calls Quality (Phred score) for each sample Q> 30; minor allele frequency (MAF) > 5%; minimum allele count > 5; maximum missing values 50%; Avg. genotype quality > 50.

Genome scan analysis: linking genes to environmental variables (G x E)

Genome scan analysis distinguishes 'outlier loci', loci hitchhiking with genes under the effect of selection or directly involved in adaptive population divergence, from strictly neutral loci representing genome-wide demographic effects [1]. We performed a genome scan analysis on the populations separated in time using a pipeline that combines *pcadapt* [47] and *outFLANK* [48]. Based on PCA-based method that doesn't need prior population information, *pcadapt* performs genome scans assuming that candidate markers are outliers with respect to how they are related to population structure [47]. *outFLANK* uses F_{ST} metric, working on the assumption that loci responsible for local adaptation are likely to have more genetic

differentiation among populations than neutral loci [48]. F_{ST} outliers were inferred from the distribution of neutral F_{ST}, using a cut-off values of 0.05 and a minor allele frequency of 5%. Outliers common to *pcadapt* and *outFLANK* were identified and correlated to environmental variables, obtained from the historical records and the paleolimnological analysis of sediment (Table S1). Missing values in the environmental variables were imputed to improve fitting using the k-nearest neighbour (k-NN) imputation tool [49] in the R package DMwR, [50]. The outliers showing a significant correlation with environmental variables were mapped onto the transcriptome of *D. magna* [51] to identify putative genes associate with environmental variation. In addition, we used the Genome Association and Prediction Integrated Tool (GAPIT) [52] to identify significant linear correlations between all SNPs found within genes and environmental variables (Table S1). This tool implements advanced statistical methods including the compressed mixed linear model (CMLM) and CMLM-based genomic prediction and selection [52]. After applying an FRD = 0.05, adjusted P-values for significant associations are provided.

Genome-wide association study: linking genes to life history traits (G x P)

We performed a genome-side association analysis (GWAS) to identify genes underlying constitutive differences in life history traits among populations and to identify plasticity genes. To identify constitutive differences in life history traits we performed association analysis between SNPs sitting within introns and fitness-linked life history traits measured in control conditions (18±1°C, feed *ad libitum*) using GAPIT[52] and applying an RDF 0.05 to reduce false positives. To identify plasticity genes we performed correlations between SNPs sitting within introns and the variation in fitness-linked life history traits measured in the following experimental conditions as compared to the control treatment (18±1 °C) (Table 1): 1) temperature (24±1 °C); 2) high nutrients (18±1 °C and 2.4 mg C/L); 3) low nutrients (18±1 °C and 0.2 mg C/L); 4) high insecticide Carbaryl (18±1 °C and 10 µg/L); 5) low insecticide

Carbaryl (18±1 °C and 4 µg/L);6) temperature and high nutrients (24±1 °C and 2.4 mg C/L); 7) temperature and low nutrients (24±1 °C and 0.2 mg C/L); 8) temperature and high insecticide Carbaryl (24±1 °C and 10 µg/L); 9) temperature and low insecticide Carbaryl (18±1 °C and 4 µg/L). We corrected for multiple testing applying a FDR = 0.05. To visualize shared genes between the different traits measured during the experiments, we performer Venn diagrams using VennDiagram package in R v.3.3.3 [50].

Regularized generalized canonical correlation analysis (RGCCA): linking genes underlying life history trait variation to environmental variables (G x E x P)

To visualize the relationships and amount of correlation among gene variants, life history traits and environmental variables in a unique framework, we used regularized generalized canonical correlation analysis (RGCCA), a generalization of regularized canonical correlation analysis to three or more sets of variables with bootstrap resampling [53]. RGCCA is a powerful tool for integrating complex datasets in the biological sciences [54]. While most forms of machine learning struggle in small sample size regimes, this tool is ideal for discovering complex, group-wise patterns between high-dimensional datasets with only dozens or hundreds of observations [55]. This technique enables symmetric matrix-on-matrix regression, and can be thought of as “supervised PCA”. To reduce false positives, we run the matrix correlations 15 times and plotted variables with the highest first principal component loadings robustly identified across runs.

Table 1. Experimental design.

Representative table showing the different treatments used in the experiments. Temperatures used are: control temperature (18±1°C) and temperature treatment (24±1°C); food levels are 0.2 mg C/L (low) and 2.4 mg C/L (high); Carbaryl concentration are 4 µg/L (low) and 10 µg/L (high). Control condition is represented in orange.

Temperature	Treatment
18 °C	**CONTROL:** Medium nutrients (0.8 mg C/L) - No Insecticide
24 °C	**TEMPERATURE:** Medium nutrients (0.8 mg C/L) - No Insecticide
18 °C	**HIGH NUTRIENTS:** 2.4 mg C/L - No Insecticide
	LOW NUTRIENTS: 0.2 mg C/L - No Insecticide
24 °C	**HIGH NUTRIENTS AND TEMPERATURE:** 2.4 mg C/L - No Insecticide
	LOW NUTRIENTS AND TEMPERATURE: 0.2 mg C/L - No Insecticide
18 °C	**HIGH INSECTICIDE:** Medium food - 10 µ/L
	LOW INSECTICIDE: Medium food - 4 µ/L
24 °C	**HIGH INSECTICIDE AND TEMPERATURE:** Medium food - 10 µ/L
	LOW INSECTICIDE AND TEMPERATURE: Medium food - 4 µ/L

Results

Genome scan analysis: linking genes to environmental variables (G x E)

The total number of raw reads obtained from the genome sequencing of the 30 genotypes was 944,821,902 (Table S2). After trimming, the total number of reads was 693,896,458 (Table S2). The average insert length was 244bp and the average depth of coverage was 24x (Table S2). The coverage per genotype ranged between 8.8x (individual 13.5_1) and 44x (individual 3.5_1) (Table S2).

SNP variant calls within intron totalled 1,502,577. The divergence among populations amounts to a total F_{st} of 0.01972. Of the total SNP variants, 15,591 were identified as outliers by the *pcadapt* software. The analysis conducted with *outFLANK* identified 425 outliers. The outliers common to *pcadapt* and *outFLANK* were 19 (Table S1). Five of these outliers were significantly correlated with environmental variables (Table 2) and two of them mapped to the same gene family, the tRNA-methyltransferase, whereas the remaining three mapped onto proteins of unknown function (Table 2). The tRNA-methyltransferase significantly correlated with multiple pesticides and herbicides. Cloud coverage in the summer months and maximum temperature significantly correlated with the tRNA-methyltransferase and one of the uncharacterized proteins (Table 2). The linear correlation analysis using GAPIT identified significant correlations between more than 96,000 SNPs and environmental variables (Table S3). Discussing each correlation in turn is beyond the scope of this manuscript, and hence, all correlations (Table S3) as well as the mapping of SNPs onto known genes, are provided in Supplementary material as a community resource. A quick scan of the correlation results revealed that temperature significantly correlated with 4,679 SNPs; some of these SNPs mapped onto heat shock proteins and CWF19 proteins, which regulate cell cycle (Table S3). A total of 1,034 SNPs significantly correlated with Carbamate insecticides and many more to multiple pesticides (Table S3).

Table 2. Correlation between outlier SNPs and environmental variables

Outlier SNPs identified in the genome scan analysis that significantly correlated with environmental variables. ScaffoldID position on the D. magna transcriptome, *D. magna* geneID and gene name as annotated on the D. magna transcriptome are shown. Environmental variables significantly associates with the genes are listed (Padj-val 0.05). The list of environmental variables including pesticides, herbicides, paleolimnological data and weather data are listed in Table S1. A represents the outlier SNPs with uncharacterized proteins and B represent the outlier SNPs with a known function.

A	**Uncharacterized protein**					
ScaffoldID_Position **GeneID** **GeneName**	SSCAFFOLD00327_11730 Dapma1EVg013745 Uncharacterized protein	Padj-val	SSCAFFOLD00781_196695 Dapma1EVg018324 Uncharacterized protein	Padj-val	SSCAFFOLD00341_178324 Dapma1EVg013878 Uncharacterized protein	Padj-val
Environmental variables	chlorflurenol	0.04	linuron	0.01	chlorfenson	0.05
	petroleum-distillate	0.05	highest temperature recorded °C	0.04		
	propargit	0.05				
	tri-allat	0.04				

B	**Characterized protein**			
ScaffoldID_Position **GeneID** **GeneName**	SSCAFFOLD00687_19505 Dapma1EVg017139 tRNA-methyltransferase	Padj-val	SSCAFFOLD00687_19666 Dapma1EVg017139 tRNA-methyltransferase	Padj-val
Environmental variables	betacyfluthrin	0.04	azinphos-methyl	0.04
	bromacil	0.03	barban	0.04
	bromophos	0.03	Boric-acid	0.04
	carbaryl	0.04	bromacil	0.03
	chlorflurenol	0.02	bromophos	0.01
	chlorphenamidine	0.05	buprofezin	0.03
	chlorthiamid	0.04	carbaryl	0.02
	dalapon	0.03	chlorflurenol	0.02

	dazomet	0.02	dalapon	0.03
	desmedipham	0.03	denatoniumbenzoat	0.04
	difethialon	0.04	desmedipham	0.02
	dinatrium-octaborat (natriumborat)	0.04	EPTC	0.02
	dithianon	0.03	fenpropathrin	0.02
	EPTC	0.03	ferrosulfat	0.04
	ethofumesat	0.03	flamprop-M-isopropyl	0.04
	fenpropathrin	0.01	fluazifop-P-butyl	0.04
	flamprop-M-isopropyl	0.02	fosetyl-Al	0.05
	fosetyl-Al	0.02	guazatin	0.04
	l-naphthyleddikesyre	0.02	maleinhydrazid	0.04
	mercaptodimethur	0.05	methylenisothiocyanat	0.02
	methylenisothiocyanat	0.03	metribuzin	0.04
	metribuzin	0.02	petroleum-distillate	0.02
	N-cyclohexyldiazeniumdioxi-kalium	0.05	propargit	0.02
	petroleum-distillate	0.02	sodium-nitrite	0.02
	prochloraz-Mn-Complex	0.05	sulfotep	0.03
	propargit	0.02	TCA	0.03
	sodium-nitrite	0.04	terbacil	0.05
	tecnazen	0.03	summer mean cloud cover%	0.01
	zineb	0.04		
	summer mean cloud cover%	0.03		

Genome-wide association study: linking genes to life history traits (G x P)

The GWAS analysis identified a number of significant correlations between life history traits and SNPs, associated to which we identified putative genes by mapping onto the reference transcriptome of *D. magna* [56]. All the gene-trait associations are presented in supplementary information (Table S4). We summarized these results using Venn diagrams: for each experimental conditions, we show the number of genes underlying each trait, as well as the number of shared genes among traits within treatment (Fig. 2). Overall in all conditions studied, traits are regulated by multiple genes (epistasis) and multiple traits are regulated by the same genes (pleiotropy).

Figure 2. GWAS between SNPs and life history traits

Venn diagrams showing genes shared among four fitness-linked life history traits measured in experimental set ups. The life history traits are: fecundity, mortality, age at maturity and size at maturity. The treatments are: 1) control (18±1°C); 2) temperature (24±1°C); 3) low nutrients (18±1°C and 0.2 mg C/L); 4) high nutrients (24±1°C and 2.4 mg C/L); 5) low insecticide Carbaryl (18±1°C and 4 µg/L); 6) high insecticide Carbaryl (18±1°C and and 10 µg/L); 7) temperature and low nutrients (24±1°C and 0.2 mg C/L); 8) temperature and high nutrients (24±1°C and 2.4 mg C/L); 9) temperature and low insecticide Carbaryl (18±1°C and 4 µg/L); 10) temperature and high insecticide Carbaryl (24±1°C and 10 µg/L). The list of phenotypic trait, environmental variables and SNPs, with associated geneID is in Table S5.

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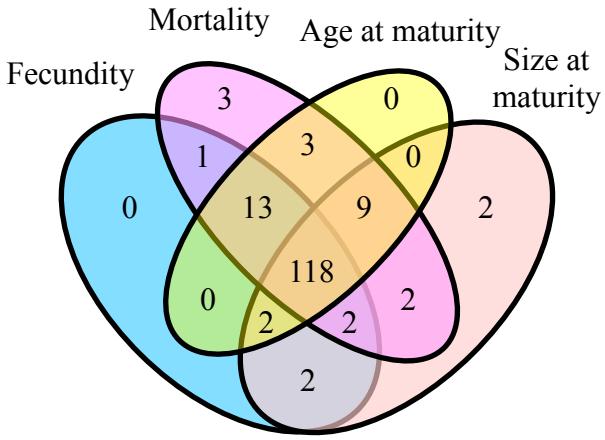	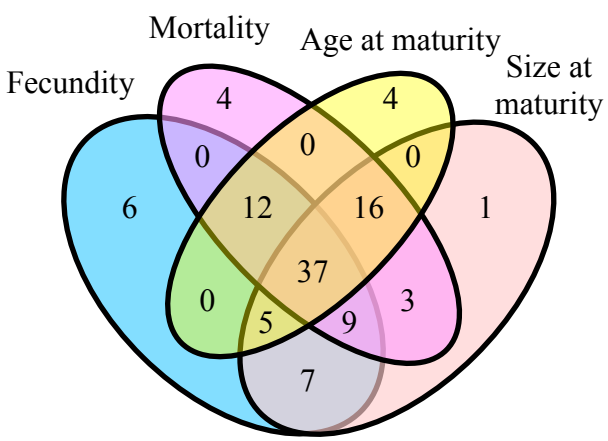
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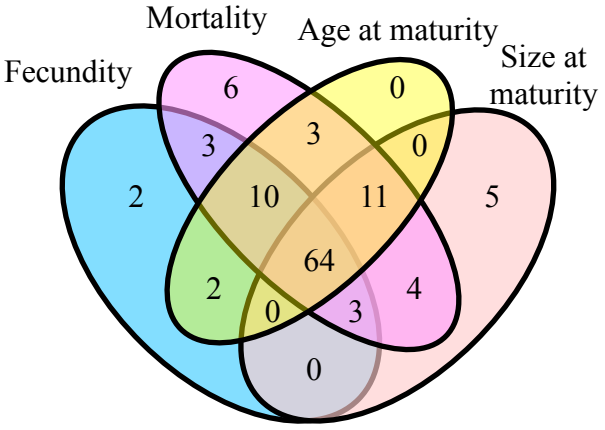	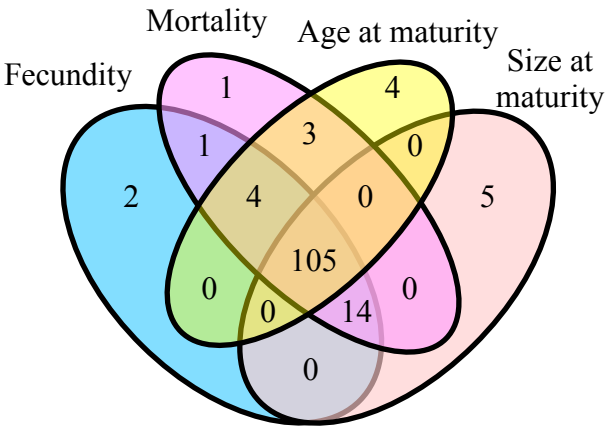
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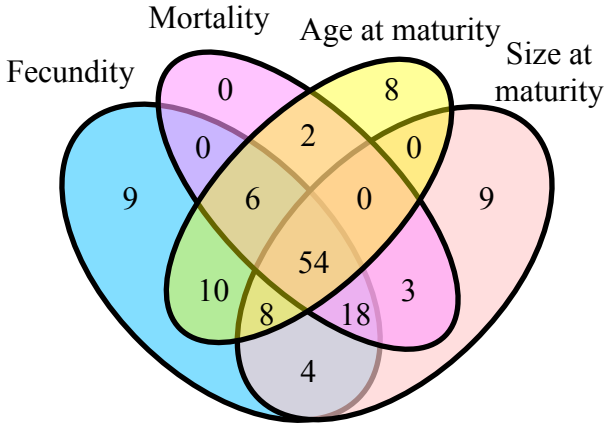	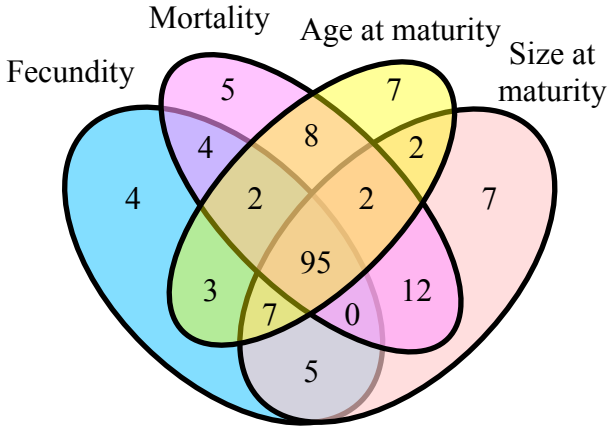

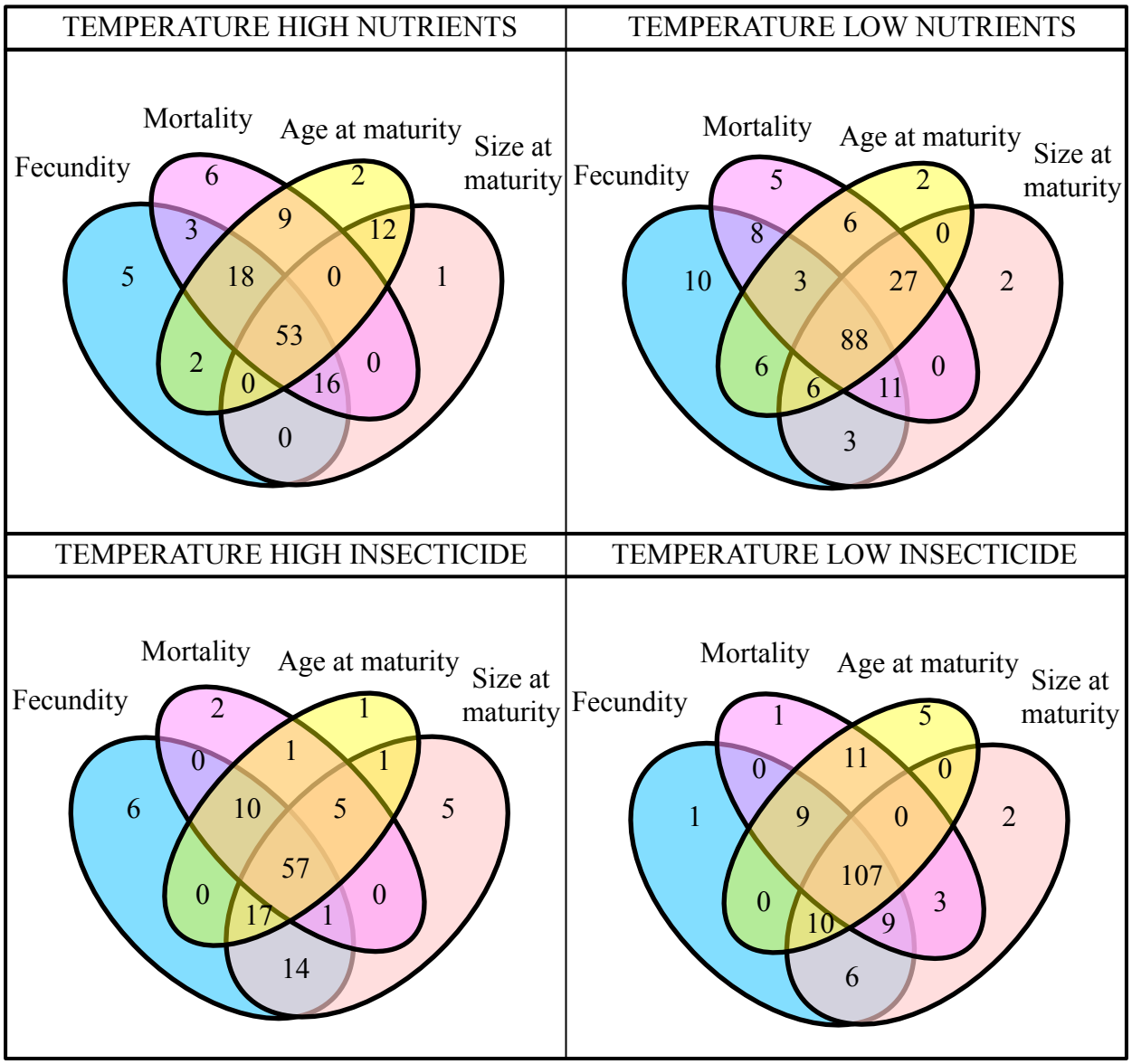

Regularized generalized canonical correlation analysis (RGCCA): linking genes underlying life history trait variation to environmental variables (G x E x P)

In *control* experimental conditions (18°C, no resource limitation) we measured constitutive phenotypic differences among populations. Evolutionary differences in size at maturity and fecundity (Table S5 - Phenotypic traits) separated the pesticide population (PP) from the other two populations (Fig. 3 - Control). These differences were explained by significant correlation with 28 pesticides (Table S5 – Environmental variables). Genes associated with constitutive differences in phenotypic traits are involved in central metabolic functions, exoskeleton remodelling and transcription (Table S5 – SNPs).

We measured changes in fitness-linked life history traits in response to temperature, food levels and insecticide Carbaryl as single stressors in comparison with control conditions. In addition, we measured changes in life history traits in experimental conditions that combined temperature (24°C) with either food levels or Carbaryl loads compared with control conditions. Differences identified in these experiments are indicative of evolved plastic responses in life history traits in function of their response to environmental insults. In all treatments, a number of pesticides explained plastic responses in life history traits (Table S5 – Environmental variables).

In the *temperature* treatment differences in fecundity and age at maturity (Table S5 – Phenotypic traits) explained divergence between the historical population (EP) and the two most recent populations (Fig. 3 – Temperature). Genes associated with different response to temperature include nutrient transporter and signalling proteins (Table S5 – SNPs).

In the *food treatments* (high and low nutrients), the historical population showed a divergent response from the other two populations (Fig. 3). These divergent responses were mainly explained by difference in fecundity (Table S5 – Phenotypic traits). Genes associated with responses to nutrients differed between high and low food levels; in high nutrients, energy sensor protein kinases were common, whereas in low food levels a number of nutrient transporters proteins were identified (Table S5 – SNPs).

In the *insecticide treatments*, the historical population showed a divergent response in life history traits, driven by differences in fecundity and age at maturaty (Fig. 3, Table S5 – Phenotypic traits). Genes associated with high insecticide treatment included remodelling proteins, growth factors and drug resistant proteins (Table S5 – SNPs). Genes associated with low insecticide treatment included remodelling proteins as well as regulatory proteins for physiological processes (Table S5 – SNPs).

The treatment combining *temperature and low level of nutrients,* showed populations responses similar to the low nutrients treatments as single stressors (Fig.3), where fecundity

was identified as a driver of population divergence. Nutrient transporters and gene regulating growth were associated with temperature and low nutrient treatment (Table S5 – SNPs).

In the treatment *temperature and high level of nutrients,* age of maturity was identified to be responsible for the divergent response among the historical population and the two more recent populations (Table S5 – Phenotypic traits). Nutrient transporters and gene regulating growth were associated with temperature and low nutrient treatment (Table S5 – SNPs). In the temperature and high nutrient treatment, we identified mostly proteins regulating basic metabolic processes (Table S5 – SNPs).

The treatment combining *temperature and low insecticide* showed population divergence similar to the low insecticide treatment by itself, in which the historical population showed divergent phenotypes from the ones of the other two populations (Fig. 3). This divergence was mainly associated with differences in fecundity and size at maturity among populations (Table S5 – Phenotypic traits). Many proteins associated with low insecticide as single stressor also appeared in the combined treatment (Table S5 – SNPs).

The *temperature and high insecticide* treatment showed a different population response as compared to the high insecticide treatment alone (Fig. 3). In the combined treatment the PP population showed a divergent phenotype as compared to the other two populations, associated mainly with fecundity and size at maturity (Table S5 – Phenotypic traits). The combined treatment activated mostly transmembrane proteins and metal transporters (Table S5 – SNPs).

Figure 3. RGCCA analysis

3D plots obtained from the first principal component of the regularized generalized canonical correlation analysis. The three axes are: Pheno-phenotype; Env-environmental variables; SNPs. Population are colour coded as follows: CWP-red; PP-grey; EP- orange. The treatments are as in Figure 3. The list of phenotypic trait, environmental variables and SNPs, with associated geneID, is in Table S5.

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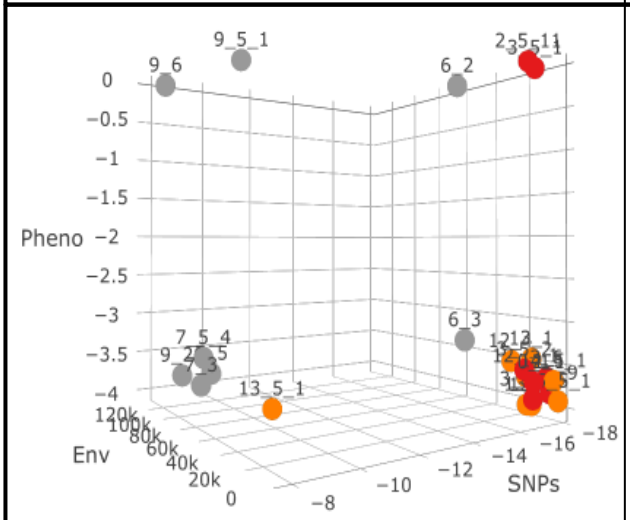	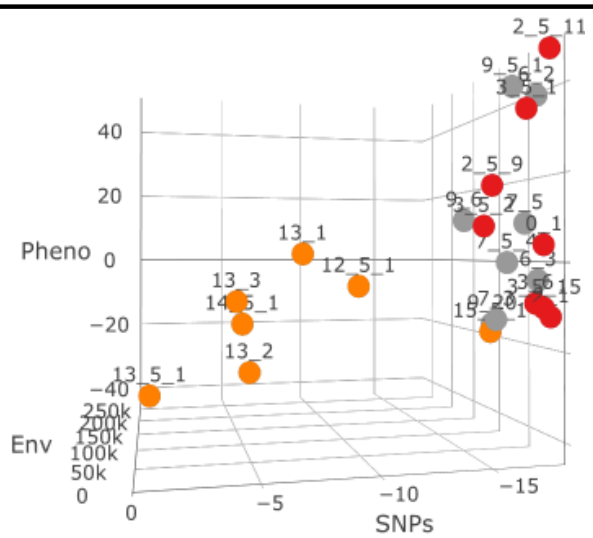
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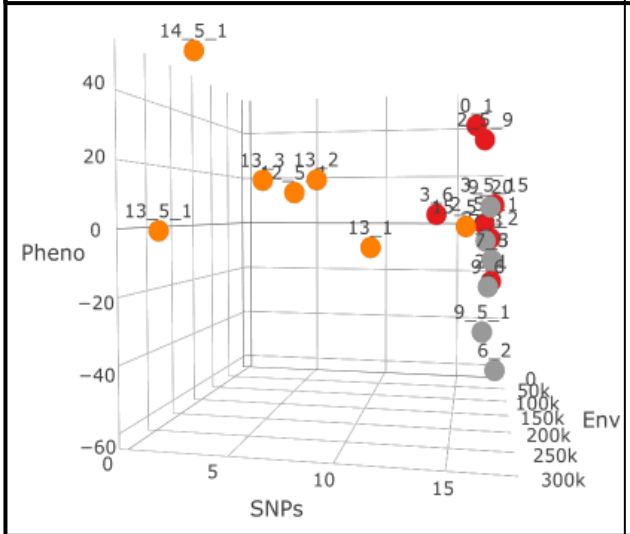	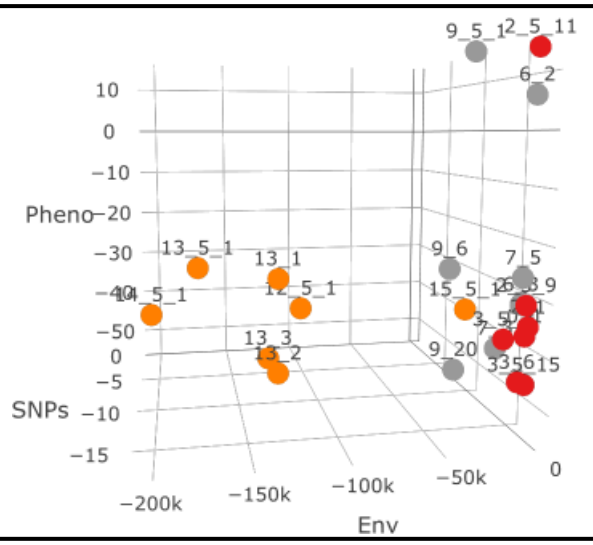
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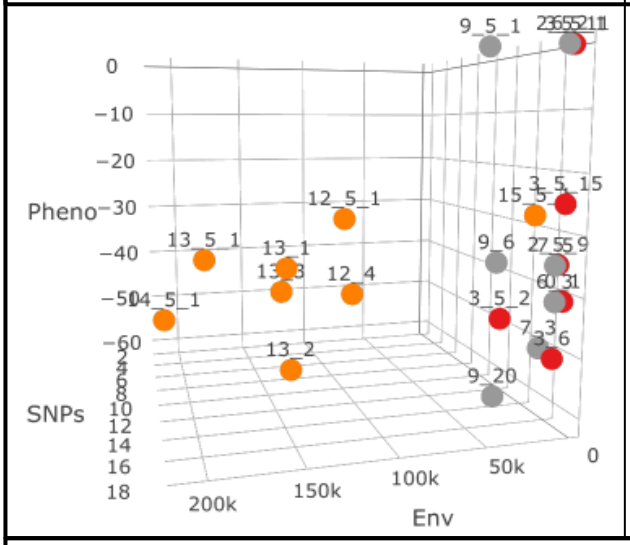
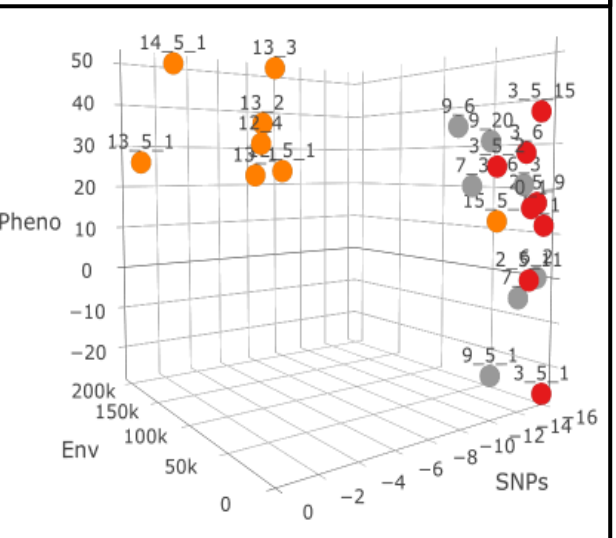
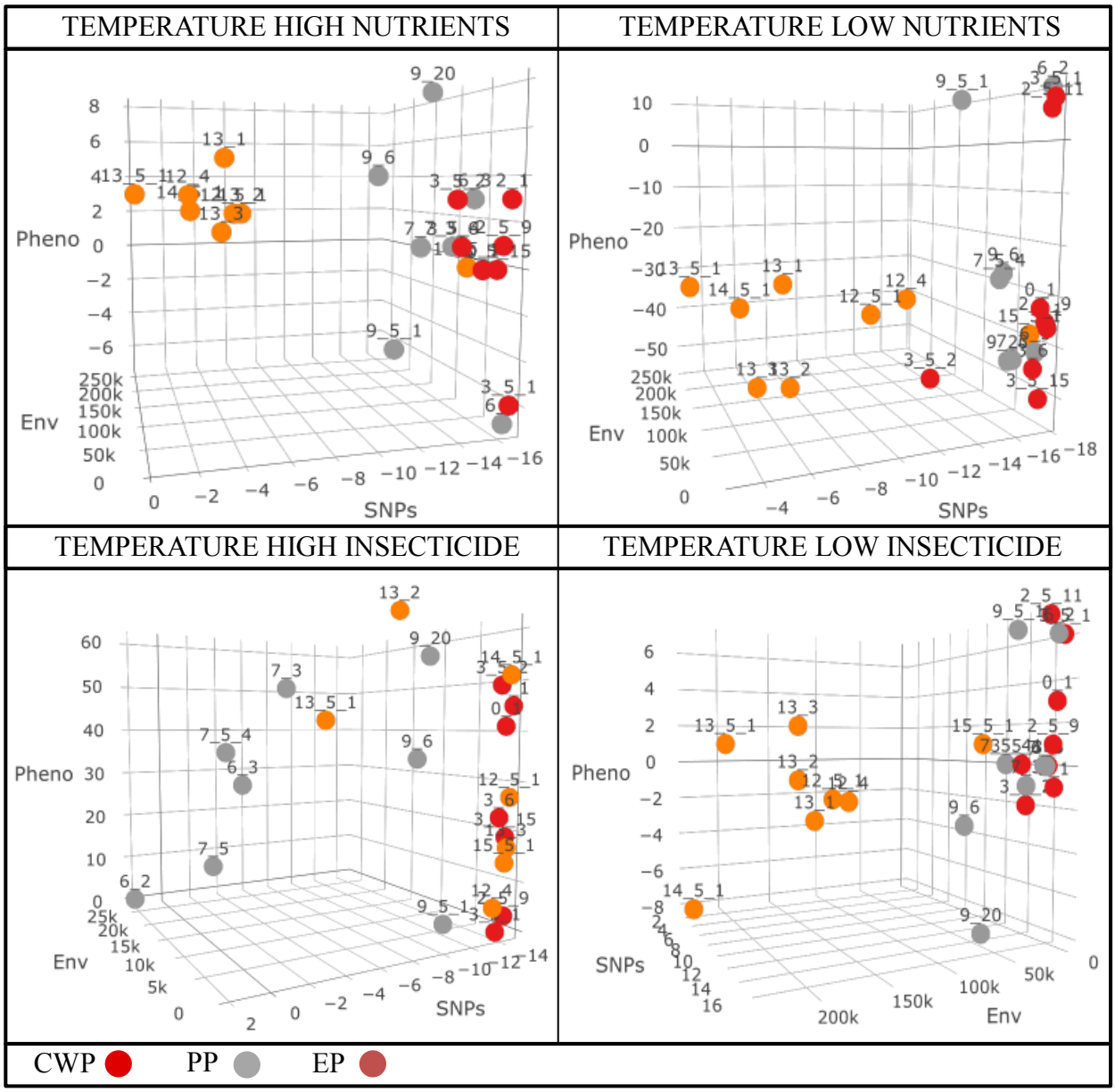
	
CWP PP EP	



Discussion

We investigated the genetic basis of constitutive and plastic divergence in life history traits and linked this variation to environmental variables measured via paleolimnological analysis or collected through historical archives. Environmental selection pressure changing fitness-linked life history traits via allelic changes was assessed using genome scans and genome-wide association analysis. The former allows to link genetic variance putatively under

directional selection with variation in environmental variables. The latter enables us to link genetic variants to phenotypic variation. Both approaches suffer from limitations.

Genome scans are biased towards genes of major effect and, therefore, have low power in identifying genes of minor effects and epistatic interactions among genes [1]. These limitations are obvious in our study. Out of 1.5M SNPs, we identified five outliers significantly linked to environmental variables. These outliers were supported by both a PCA method, which takes into consideration population structure and an F_{ST} based method. Importantly, to reduce false positives we identified as 'real' outliers only loci identified by both approaches and applying false discovery rate above 0.05. The low number of outliers identified may be the result of the stringent criteria adopted [15] or may indicate a low number of loci affected by divergent selection in our study system. Selection on complex polygenic traits is expected to lead to soft sweeps, which result in moderate changes in allele frequencies, often stemming from standing genetic variation, leading to low detection of outliers and modest significance values [57]. As oppose to the outlier analysis, the regularized generalized canonical correlation analysis is ideal for discovering complex, group-wise patterns between high-dimensional datasets with only dozens or hundreds of observations. in this approach the limitations of genome scans are alleviated by reducing the rate of false discovery by iteratively running the matrix on matrix correlations multiple times and by picking the first principal component loadings that are robustly identified across multiple runs.

Three the five outliers mapped to genes of unknown function. This is a typical limitation of genomics studies in non-model species, for which genome annotation is generally poor. The remaining two SNPs mapped onto tRNA-methyltranferase, and were significantly associated with a number of pesticides. Previously, Carbamate pesticides were identified as have a negative impact on *D. magna* life history traits (Cambronero *et al.*, in review) (Chapter 2). tRNA molecules undergo extensive post-transcriptional processing to generate the mature functional tRNA species that are essential for stress defence mechanisms in all organisms [58,59]. In particular, tRNA methyltransferases has been shown to

help cells survive insults by damaging agents [60]. Mutations in tRNA methyltransferases have been associated with human disease [58], temperature changes [61] and immune responses [62]. When we expanded the search for association between gene variants and environmental variables to all SNPs within exons, we found a much larger number of gene polymorphism to be correlated with pesticides and temperature. High land use starting from the 1975 and change over time of average ambient temperature was documented by our historical and paleolimnological analysis of Lake Ring [36,39,63,64] (Chapter 1).

The Genome Wide Association Study (GWAS) revealed extensive pleiotropy, which occurs when an allele has phenotypic effects on multiple traits [7], and epistasis, in which traits are affected by the interaction among multiple genes [6]. This may be expected for complex life history traits. Extensive epistasis and pleiotropy found here, supported by stable neutral genetic variation over evolutionary time in Lake Ring [42], strongly suggests that *D. magna* evolutionary adaptation was largely mediated by standing genetic variation, which enabled the local population to adjust to the dramatic environmental changes within few generations.

GWAS approaches have the intrinsic limitation of assuming that the phenotype is linearly determined by a single genetic factor. However, ecological and genetic complexities result in ubiquitous and complex departures from this simplest case [5]. In the biological reality of varying environments, the genotype–phenotype map must account for the multitude of genetic factors in the genome and the phenotypes of many organismal traits.

To overcome the limitation of current GWAS approaches and to visualize the relationships and amount of correlation among gene variants, life history traits and environmental variables we used regularized generalized canonical correlation analysis (RGCCA). This analysis allowed us to identify evolutionary and plastic population phenotypic divergence in function of environmental variation. We found that the PP population, which experienced the strongest impact of pesticides, diverged from the other two populations showing evolutionary difference in size at maturity and fecundity. Plastic response of the historical population (EP) to nutrient levels or loads of the insecticide Carbaryl were

significantly different from the one of the two other populations. This divergent response in the temperature treatment may be explained by the fact that the historical population was resurrected from the coldest period of the last century, whereas the other two populations experienced higher average ambient temperature and occurrence of heat waves.

The population response to food treatments combined with temperature was phenotypically comparable to the response to food treatments in isolation. The evolutionary advantage of the historical population in presence of nutrification combined with warming supports the evidence that plastic response to one stressor may favour plasticity in response to a second stressor [65] and confirms previous results of phenotypic divergence based on the sole analysis of life history traits in this population [64]. However, the RGCCA analysis presented here showed that the genomics underpinning the response to different food levels combined with temperature is mediated by different genes.

Previously, Cambronero *et al.* (in review) (Chapter 2) showed that evolutionary differences in life history traits between the historical population and the two more recent populations in response to the insecticide Carbaryl was not predictable from the response to either stressors by itself [64]. Here, we show that the phenotypic response of the three population and the associated genes are comparable in the low Carbaryl treatment and in the low Carbaryl combined with temperature treatment. Conversely, the phenotypic and molecular response to high Carbaryl differs from the one to high Carbaryl combined with temperature. These findings are relevant in the context of ecological unpredictability in multi-stress environments [66].

Understanding how multiple mutations interact to jointly impact multiple ecologically important traits is critical for creating a robust picture of organismal fitness and the process of adaptation. This is generally complicated by both environmental heterogeneity and the complexity of genotype-phenotype relationships. Using RGCCA we were able to gain important insights into the role of pleiotropy and epistasis over evolutionary time. These discoveries are generally limited to model organisms studied in artificial fitness landscapes [67].

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Author's contribution

MCC performed the experiments, prepared sampled for genome sequencing and performed the outlier analysis.

VD performed the GWAS analysis.

JZ performed the RGCCA Analysis

JKC contributed to the experimental design and advised on data analysis

LO conceived the study and coordinated data analysis. LO and MCC wrote the first version of the paper; all authors contributed to the editing of later versions.

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Supporting information

Table S1. Lake Ring environmental data.

The following data are shown: paleolimnological data obtained from the analysis of sediment spanning five decades; lake chemistry collected by the Danish county authority between 1971 and 1999; climate data collected from a weather station in proximity of Lake Ring, historical records on pesticides retrieved from the national Danish archive.

See excel file Cambronero *et al*. TableS1

Table S2. Read mapping statistics

Raw number of reads (RNoR), number of reads after trimming (TNoR), average read length (AvRL) and individual genome coverage (GenCov) after quality trimming are shown per individual genotype (genID). Each genotype is linked to a temporal population code: EP - 1960-1970; PP -1980-1990 ; CWP >1999.

popID	genID	RNoR	TNoR	Av RL	GenCov
CWP	0_1	42898046	26366374	244	27
CWP	0_2	34899888	21324534	244	21.9
CWP	0_4	36727180	27069518	245	28
CWP	1_2	35328130	26944648	245	27.8
CWP	2_1	34625074	25993730	243	26.5
CWP	2_5_11	40579974	31006408	244	31.8
CWP	2_5_9	22307196	16726166	244	17.1
CWP	3_5_1	60055488	42532156	245	43.8
CWP	3_5_15	27234930	20927250	244	21.4
CWP	3_5_2	40445206	30651302	245	31.6
CWP	3_6	49376564	36375140	244	37.3
PP	6_2	27527048	21715952	245	22.3
PP	6_3	30692420	21829186	242	22.2
PP	7_3	31388594	23931904	244	24.6
PP	7_5	31742684	24019282	244	24.7
PP	7_5_4	24275836	18879190	245	19.4
PP	8_5_3	21854486	16666546	245	17.1
EP	9_20	27899044	20786936	245	21.4
EP	9_5_1	40056842	30612348	244	31.4
EP	9_5_3	38418886	28890670	245	29.8
EP	9_6	24025744	18055324	244	18.6
EP	12_3	25175908	18357528	246	18.9
EP	12_4	24718840	18802592	245	19.3
EP	12_5_1	27141634	18495254	244	19
EP	13_1	30388750	23132518	244	23.7
EP	13_2	27843638	21213576	244	21.8
EP	13_3	21093966	16162106	244	16.7
EP	13_5_1	10789580	8479548	244	8.8
EP	14_5_1	27103542	20463522	245	21.1
EP	15_5_1	28206784	17485250	244	17.9

Table S3. Correlation between gene variants and environmental variables

SNPs significantly correlated to environmental variables were identified using GAPIT. The ScaffoldID on which the SNP was found, the list of environmental variables associated with each SNP and associated P-value, GeneID I which the SNP sits as identified by mapping onto the *D. magna* transcriptome and gene names are shown. Only the variables with P-val <0.01 are listed.

See excel file Cambronero *et al*. Table S3

Table S4. GWAS between SNPs sitting within exons and life history traits

Life history traits significantly associated with within introns SNPs. Trait, scaffoldID, geneID and gene function are shown.

Multiple spreadsheets are shown: 1) control: traits diverging among populations measured in control conditions. These are constitutive difference among life history traits; 2-9) plastic response in life history traits in response to the following treatments: 1) temperature (24±1°C); 2) high nutrients (24±1°C and 2.4 mg C/L); 3) low nutrients (18±1°C and 0.2 mg C/L); 4) high insecticide Carbaryl (18±1°C and and 10 µg/L); 5) low insecticide Carbaryl (18±1°C and 4 µg/L);6) temperature and high nutrients (24±1°C and 2.4 mg C/L); 7) temperature and low nutrients (24±1°C and 0.2 mg C/L); 8) temperature and high insecticide Carbaryl (24±1°C and 10 µg/L); 9) temperature and low insecticide Carbaryl (18±1°C and 4 µg/L). Traits with P-val <0.01 are shown.

See excel file Cambronero *et al*. Table S4

Table S5. Regularized Sparse Generalized Canonical Correlation Analysis

The first principal component of the RSCCA for phenotypic traits, environmental variables and SNPs, with associated gene function is shown in three separate tabs of the same spreadsheet. Experimental exposures are: 1) temperature (24±1°C); 2) high nutrients (24±1°C and 2.4 mg C/L); 3) low nutrients (18±1°C and 0.2 mg C/L); 4) high insecticide Carbaryl (18±1°C and and 10 µg/L); 5) low insecticide Carbaryl (18±1°C and 4 µg/L);6) temperature and high nutrients (24±1°C and 2.4 mg C/L); 7) temperature and low nutrients (24±1°C and 0.2 mg C/L); 8) temperature and high insecticide Carbaryl (24±1°C and 10 µg/L); 9) temperature and low insecticide Carbaryl (18±1°C and 4 µg/L)These data support plots in Figure 3.

DISCUSSION AND CONCLUSIONS

We investigated ecological and evolutionary responses of a population of *Daphnia magna* resurrected from a well-characterized lake system to multiple anthropogenic stressors over evolutionary time. We studied phenotypic, physiological, and molecular responses that enabled this population to persist across major pollution events. Moreover, we discovered genome variants underpinning plastic and constitutive phenotypic divergence.

Our objectives were to assess the role of evolution and plasticity in the population response to key environmental changes and to understand how historical exposure to stress impacts on recurring stress. We first established that the invertebrate community was responsive to pollution events in Lake Ring. The paleolimonological analysis of sediment supported by historical records, identified sewage inflow and pesticide leaching as major pollution events. These events co-occurred with a modest average increase in temperature (~ 1 °C) (Chapter 2). We showed that the *Daphnia* population and the invertebrate community declined in coincidence with sewage inflow and high pesticide use (Carbamate insecticide) (Chapter 2). These dynamics suggest that the two stressors played a strong role in the population dynamics over time, even if we cannot exclude that other environmental factors contributed to these dynamics. The persistence of *D. magna* through time suggests that coping mechanisms were adopted to respond to environmental stress. We set out to investigate these coping mechanisms using a blend of classic approaches in ecology and evolutionary biology as well as state-of-the-art high throughput sequencing technologies.

Because the effect of multiple stressors is non-linear and unpredictable from the effect of single stressors, we studied adaptive responses to single and combined stressors and investigated what mechanisms may have enabled evolution and persistence of the population thorough time (Chapter 2). We found that the *D. magna* population could accommodate changes in average ambient temperature via plasticity (Chapter 1, 2 and 3). However, exposure to temperature stress affected the overall population fitness, imposing a reduction in fecundity, later maturation and smaller size at maturity (Chapter 2). Our findings

confirm that *Daphnia* fecundity declines above optimal temperature, but it is compensated by faster maturation and smaller body size, as a result of increased metabolism rate [54]. Importantly, changes in *Daphnia* body size have the potential to alter predator-prey dynamics involving visual predators (e.g. fish), affecting the dynamics of food webs [55]. Our results on the impact of temperature on *Daphnia* indicate a potential negative impact of global warming forecasted for the next few decades on freshwater communities [26].

In response to hyper-thermal stress, we found that different mechanisms of response affected different traits (Chapter 3 and 4). Whereas, the population separated in time did not show significant constitutive differences in Hb content, they showed higher induction of Hb under temperature stress (30 °C). This finding suggests that plasticity in haemoglobin expression enabled the population to cope with hyper-thermal stress (Chapter 3). They also suggest that the modest increase in average temperature occurring over the past five decades did not trigger an evolutionary response in the haemoglobin response. Interestingly, three generations of acclimation to hyper-thermal stress provided Hb-rich genotypes with superior competitive abilities under hyper-thermal stress and a significant increase in the time to immobilization (T_{imm}) (Chapter 3). These competitive abilities were dampened in absence of acclimation, confirming previous observations [2, 3]. These findings supported by a significant interaction between constitutive Hb protein content and population under hyper-thermal stress, suggest that evolution of plastic response to hyper-thermal stress may be key to regulate haemoglobin metabolism in *D. magna* over evolutionary times. They further suggest that that long-term adjustment to higher occurrence of heat waves may require a combination of plasticity and genetic adaptation.

Whereas plasticity played a key role in the *D. magna* population in response to temperature as single stress, microevolutionary responses were observed in response to extreme temperature events mimicking heat waves (Chapter 4). We found that the temperature of maximum tolerance (CT_{max}) evolved over time in response to the combined

effect of increase in ambient temperature and occurrence of heat waves. Indeed, the most recent population showed a higher constitutive temperature of maximum tolerance (Chapter 4). These results are in line with previous findings showing the evolution of the thermal maximum over few decades, using a selection experiment and a resurrection ecology approach [4]. Microevolutionary responses in CT_{max} were not reflected in higher constitutive expression of individual HSPs in the most recent population, even though the three populations often showed constitutive difference in HSPs expression. However, the multivariate analysis performed of the four heat shock proteins to CT_{max} (phenotypic trajectory analysis) provided further insights. After temperature treatment, the two most recent (sub)populations showed significant differences in the trajectory and magnitude of plastic change in HSPs as compared to the historical population (EP). This difference was not observed in control temperature. In the warming and food limitation treatment, the historical population showed a significant divergent trajectory in response to CT_{max} treatment as compared to the other two populations in control temperature. This same population had a significantly higher magnitude of change than the other populations in temperature treatment (Chapter 4). Finally, I the warming and insecticide treatment the pesticide population (PP) showed a different magnitude and direction of phenotypic trajectory as compare to the other two populations in control temperature. This same population had a significantly different direction of change in temperature treatment. Exposure prior to dormancy to warmer climate and higher frequency of extreme events may be responsible for the divergent phenotypic trajectories in the temperature treatment [5]. Similarly prior exposure to different food regimes and pesticides may have influenced the population response to these stressors in the multi-stress exposure (Chapter 4).

By studying responses to single stressors using different markers we learned that life history traits and molecular biomarkers may respond to the same environmental stressors differently, likely because of ecological trade-offs. By studying physiological and molecular

responses in control and heated experimental conditions, we discovered evolutionary mechanisms of response to thermal and hyper-thermal stress.

In natural ecosystems and especially in enclosed habitats (e.g. lakes and ponds) other environmental factors, such as eutrophication or by-products of land use (pesticides), may play an important role in driving evolutionary responses by altering solubility of nutrient, conductivity and oxygen levels [6]. There is increasing evidence that multiple factors may alter trait–environment and genotype–environment interactions influencing responses to climate change [7, 8]; for example, CT_{max} has been shown to change with diet [9]. To understand the impact of multi-stress environments on the population of *D. magna*, we studied the impact of multiple stressors on molecular and physiological responses to extreme temperatures, as well as on fitness-linked life history traits by combining temperature with either biotic or abiotic stress.

In presence of multiple stressors, a complex interplay among plastic and evolutionary responses, both at physiological and molecular level, underpinned population responses to extreme temperatures. Importantly, the evolutionary advantage of the modern (sub)population, apparent in the constitutive higher CT_{max} in presence of warming as single stressor, was no longer evident when temperature co-occurred with food levels or insecticide loads (Chapter 4). Populations exposed to high concentration of food and temperature treatment showed significantly different responses. Specifically, the historical population (EP) showed the largest body size and higher maximum temperature tolerance (CT_{max}), but comparable fecundity and age at maturity than the other two populations in presence of high food level (Chapter 2 and 4). These superior advantages in some of the phenotypic traits measured may be explained by historical exposure of the EP population to high nutrient levels linked to sewage inflow in Lake Ring. Interestingly, at low food concentration we did not observe differences among populations for any or the phenotypic trait measured. This may be explained by the fact that all three populations never experienced limiting food levels in the natural environment (Chapter 2 and 4). In presence of warming and low Carbaryl

concentration we observed significant differences among populations in age at maturity but not in the rest of the phenotypic traits measured (fecundity, size at maturity and CT_{max}). However, this different did not translate in the pesticide population having an overall superior fitness (Chapter 2 and 4). These results were corroborated by the trajectory of phenotypic change across life history traits where the pesticide population (PP) did not differ significantly from the one of the other two populations (Chapter 2). However, in high pesticides concentration and control temperature, the pesticide population showed 100 % mortality.

Overall, our results suggest that historical exposure to stress provides an evolutionary advantage when the stress recurs, but the evolutionary advantage is contingent upon the type and intensity of stress. Furthermore, the impact of multiple stressors on life history traits (size at maturity, age at maturity, fecundity and mortality), matched our prediction based on the interaction between two stressors. The effect of food limitation combined with temperature was more severe than the effect of either stressor in isolation, in agreement with metabolic demands increasing faster than ingestion rates with higher temperatures [10] and with the additive effect hypothesis. The insecticide Carbaryl and temperature showed non-additive effects as expected from higher volatilization of Carbaryl at higher temperatures [11] (Chapter 2).

Our results contrasting physiological and molecular responses in single and multiple stress scenarios show that the co-occurrence of multiple environmental stressors has the potential to affect the evolution of natural populations, in ways not directly predictable from the response to singles stressors, and particularly from the response to temperature alone. Therefore, the use of single stressors, such as temperature, as *proxy* for species response to global change can lead to wrong estimates of species evolvability and persistence.

The analysis of the genomics underpinning of constitutive and plastic responses in life history traits revealed extensive pleiotropy and epistasis [12, 13]. Fitness-linked life history traits were associated with multiple SNPs within genes and with multiple genes, as it may be expected for complex traits. Furthermore, the same suite of genes was found to

underpin multiple life history traits, as it may be expected from ecological trade-offs affecting fitness-linked life history traits [14, 15]. Because the *D. magna* genome annotation is poor, we were unable to identify the function of a large proportion of the genes underpinning phenotypic changes. However, we were able to assess that constitutive and plastic changes in life history traits were regulated by different gene sets, suggesting different evolutionary mechanisms underlying plasticity and genetic adaptation. Further work is required to gain a better understanding of these complex dynamics.

Concluding remarks and future perspectives

Understanding the role of environmental change on population-level genetic and phenotypic variation is required to understand the ecological dynamics of natural populations, and ultimately to develop more informed management plans for protection and restoration of threatened species and biodiversity [16, 17]. Identifying environmental factors that drive phenotypic evolution and hence adaptation poses a few challenges. Although, next generation technologies can be applied to non-model species and the genome of many species has been or is in the process of being sequenced, the genetic basis of evolutionary adaptation to environmental change is still poorly understood [18]. The reasons are several folds.

Evolutionary changes occur over time and across generations. To study those changes long-term data are required to observe rather than infer past evolutionary processes. These long-term data can be obtained from elevation or latitudinal gradients, or from resurrection studies, in which the genetic/genomic signature of environmental change on ancestors and descendants in natural populations is measured. The advantage of resurrection ecology over other approaches is that patterns of adaptive evolution documented at genome level in the wild can be validated in experimental evolution studies, which allow the genetic basis of evolutionary change under controlled conditions to be

determined. The disadvantage of resurrection ecology studies is that they require a major effort to obtain large sample size ideal for population genetics.

The identification of the genetic elements underpinning evolutionary adaptation is complicated by the fact that evolution is often mediated by the interaction among multiple mutations (epistasis) as well as mutations at loci that affect more than one trait (pleiotropy) [19]. Studies on epistasis and pleiotropy are challenging, even in genetic model species, because they require the characterization of the recombination landscape of an organism in a quantitative genetics approach [20]. High resolution genetic maps and a physical map of the genome are needed to assess epistasis and pleiotropy; both require major efforts and laboratory crosses, which are not feasible for species that cannot he reared in the laboratory.

Many studies on the mechanisms of adaptive evolution in non-model species focus on one omics approach, generally genomics or transcriptomics, and phenotyping [21]. Ideally, multiple line of evidence should be used to discover the molecular targets of natural selection and their contributions to the process of adaptation. These discoveries can be accomplished by using a multi-omics and quantitative genetic approach across multiple generations to detect the elements of the genome, their products and interactions in shaping fitness traits (Fig. 1). Applying multiple lines of evidence in a resurrection ecology context will enable the reconstruction of historical patterns of evolutionary changes and the unravelling of the mechanisms of genetic and plastic response of natural populations to environmental change (Fig. 1).

Key to our understanding of the processes of adaptation is studying plasticity and genetic polymorphism within a common framework, and in the context of ‘whole organism’, rather than ‘single trait’, because organisms are integrated complex phenotypes [22]. Assessments of plasticity are typically focussed on traits with established significance tested in the context of ecologically important environmental gradients. With this reductionist approach, there is a risk that we might arrive at a biased view of, and perhaps even overestimate, the importance of plasticity [23]. However, the study of plasticity and genetic

adaptation in a common framework is confined to species for which mutation accumulation lines can be generated [24], species for which long-term experimental evolution is possible [25, 26], and species that produce dormant stages [27].

Phenotyping is generally a bottleneck in studies that try to establish a causal link between environmental selection and adaptation. More accurate and high throughput phenotyping strategies are needed to demonstrate causal relationships and mechanisms linking either variation in the capacity for plasticity itself or plasticity-induced phenotypic variation. Automated phenotyping is available for model species, such as the FlyCatwalk used to measure fly morphometric traits [29] and for economically relevant species, such as crop [30]. Progress in sensors, including remote sensing, and high-performance computing are paving the way to the next generation of phenotyping but being able to perform high throughput phenotyping still require major efforts. The use of extended phenotypes, such as transcriptomes, epigenomes, and metabolomes may alleviate the current limitations of phenotyping as these omics are now routinely applied to non-model species.

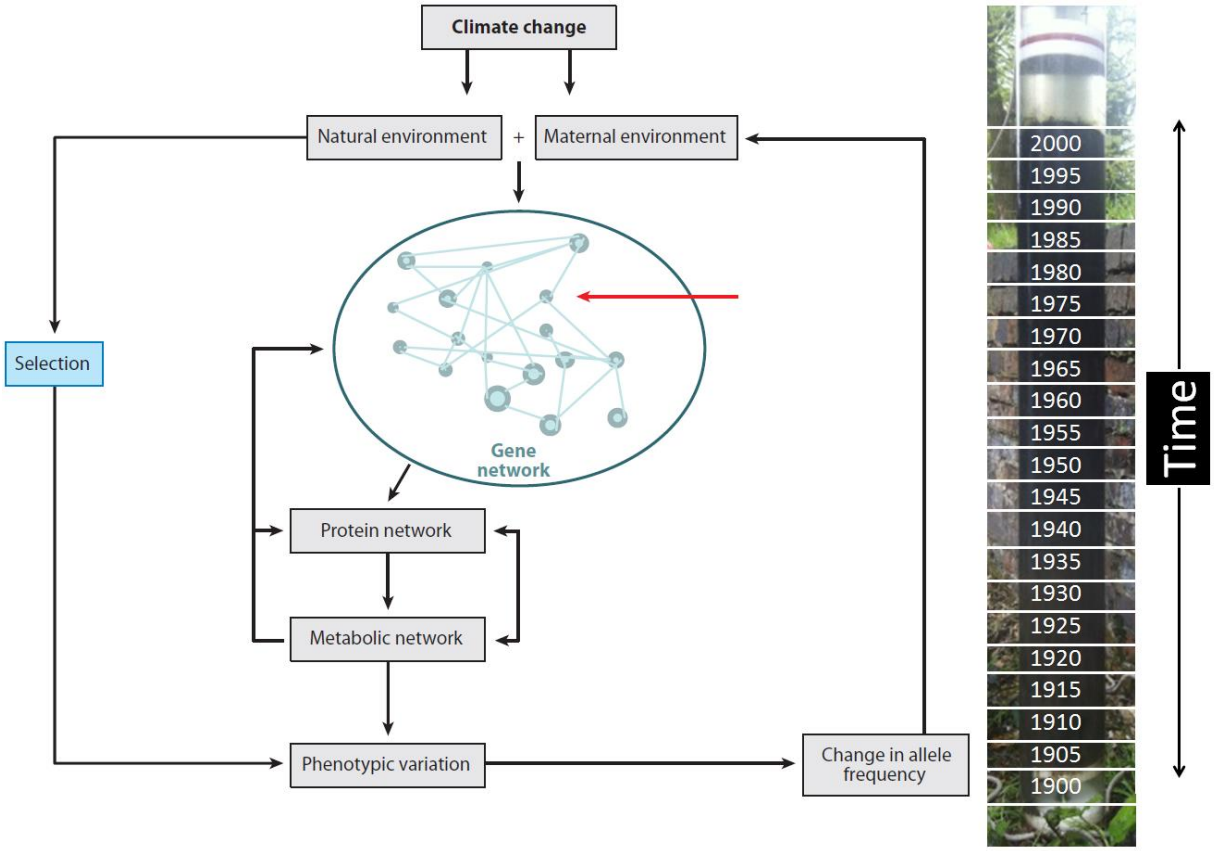
In the presented work, we provide empirical evidence of evolutionary response to multi-stress environments over evolutionary times of a central zooplankter in freshwater ecosystems. The limitation of our study is that it focuses on a single natural system, and on carefully selected environmental variables. Whereas resurrection ecology studies provide important insights into the evolutionary processes underlying adaptation in the wild, they require large efforts, and, hence, tend to suffer from low replication, the main bottleneck being the hatching of dormant stages from sediment [31]. Regardless of these limitations, our work is pioneer in collecting multiple line of evidence to study evolutionary adaptation in a natural population. In addition to the data presented, we are collecting other omics data to be analysed in a unique framework, which will reveal the molecular targets of natural selection and their contributions to the process of adaptation.

The approach used here, replicated across multiple longitudinal data sets, will be a major breakthrough in our understanding of the mechanisms and processes of evolution.

High dimensional data including high throughput phenotyping and genotyping over evolutionary time replicated across multiple environments are a powerful resource for forecast models to obtain more accurate predictions of species persistence.

Figure 1. Evolutionary adaptation to changing environments.

Environmental change imposes selection pressure on natural populations which respond via change in phenotypes. Adaptive phenotypic changes are underpinned by changes in genetic material (alleles), which changes population dynamics and feeds back to the environment. The environment drives genetic changes altering transcriptional and metabolic networks (*red arrow*). Applying multiple line of evidence in a resurrection ecology context will enable the reconstruction of historical patterns of evolutionary changes (Modified from [32]).



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