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Document Version
Peer reviewed version

Citation for published version (Harvard):
Jansen, M, Geerts, A, Rago, A, Spanier, KI, Denis, C, De Meester, L & Orsini, L 2017, 'Thermal tolerance in the keystone species Daphnia magna –a candidate gene and an outlier analysis approach', *Molecular Ecology*.

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Accepted Article

Received Date : 16-Sep-2016

Revised Date : 16-Jan-2017

Accepted Date : 19-Jan-2017

Article type : Original Article

Thermal tolerance in the keystone species *Daphnia magna* –a candidate gene and an outlier analysis approach

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Running title: Thermal tolerance in *Daphnia magna*

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/mec.14040
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Abstract

Changes in temperature have occurred throughout Earth's history. However, current warming trends exacerbated by human activities impose severe and rapid loss of biodiversity. Although understanding the mechanisms orchestrating organismal response to climate change is important, remarkably few studies document their role in nature. This is because only few systems enable the combined analysis of genetic and plastic responses to environmental change over long time-spans. Here, we characterize genetic and plastic responses to temperature increase in the aquatic keystone grazer *Daphnia magna* combining a candidate gene and an outlier analysis approach. We capitalize on the short generation time of our species, facilitating experimental evolution, and the production of dormant eggs enabling the analysis of long term response to environmental change through a resurrection ecology approach. We quantify plasticity in the expression of 35 candidate genes in *D. magna* populations resurrected from a lake that experienced changes in average temperature over the past century and from experimental populations differing in thermal tolerance isolated from a selection experiment. By measuring expression in multiple genotypes from each of these populations in control and heat treatments we assess plastic responses to extreme temperature events. By measuring evolutionary changes in gene expression between warm and cold adapted populations we assess evolutionary response to temperature changes. Evolutionary response to temperature increase is also assessed via an outlier analysis using EST-linked microsatellite loci. This study provides the first insights into the role of plasticity and genetic adaptation in orchestrating adaptive responses to environmental change in *D. magna.*

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Introduction

Natural populations currently face an increase in average temperature as well as an increase in frequency of extreme temperature events (Hoffmann *et al.* 2015). Projections predict an average temperature increase over the next 100 years in the order of 2 to 6°C (IPCC 2013). In addition, human-driven climate change increases the frequency of extreme events such as droughts, floods and heat waves, imposing further strain on natural populations (Moss 2012; Pachauri & Reisinger 2007). The speed of these changes and severity of extreme events represent a major threat to biodiversity (Urban 2015).

Organisms can respond to changes in temperature by migrating to more suitable habitats, via plasticity or genetic adaptation (e.g. Geerts *et al.* 2015; Merila & Hendry 2014; Stoks *et al.* 2016). However, because of the speed and severity of modern climate change, the ability of current populations to cope with the change is under debate (Cardinale 2012; Cardinale *et al.* 2012; Smith *et al.* 2007). So far, a number of studies explored the limits of thermal tolerance in animals and plants to understand if they will persist beyond their current habitat distribution (Deutsch *et al.* 2008; Overgaard *et al.* 2011; Tewksbury *et al.* 2008). Most studies conclude that the main mechanism of population response to climate change is phenotypic plasticity (Merila & Hendry 2014), allowing for rapid adjustments to novel environmental conditions (Chevin & Lande 2010; Chevin *et al.* 2010; Kovach-Orr & Fussmann 2013). As opposed to studies on phenotypic plasticity, studies reporting genetic adaptation to climate change are scarce (but see Hoffmann & Sgro, 2011; Merilä & Hendry, 2014). This is because it is challenging to link phenotypic to genetic variation either in nature or under controlled laboratory conditions (Merila 2012). Moreover, it is challenging to document transgenerational evolution, except for species with short generation time such as unicellular organisms (Bennett & Lenski 2007; Illingworth *et al.* 2012), in systems for which long time series data are available (e.g Darwin finches, Grant & Grant 2002) and algae,

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(Lohbeck *et al.* 2012) or for species that produce dormant stages that can be resurrected and used in common garden experiments (e.g. Franks *et al.* 2014; Geerts *et al.* 2015).

A direct approach that allows to measure evolutionary dynamics in natural populations and communities across multiple generations is in most cases impossible, as adaptive response to environmental change cannot be measured in individuals from different times experiencing different selection pressure (Merila & Hendry 2014). Exceptional are species that produce resting stages, such as aquatic invertebrates or plants (Franks *et al.* 2014; Geerts *et al.* 2015). These species offer the unique opportunity to compare genetic and plastic responses of historical populations to their modern (evolved) descendants in common garden experiments, enabling the reconstruction of evolutionary dynamics over time in response to environmental change (resurrection ecology: Kerfoot & Weider 2004).

Here, we focus on the aquatic invertebrate *Daphnia magna*, a species that produces dormant eggs which remain viable in layered lake sediments for decades or even centuries (Frisch *et al.* 2014). *Daphnia* are keystone species central to the food-web of inland waters (Miner *et al.* 2012), drivers of community dynamics (Pantel *et al.* 2015), and responsive to environmental changes either via genetic adaptation or phenotypic plasticity (e.g. (Decaestecker *et al.* 2007; Latta *et al.* 2012; Orsini *et al.* 2012; Yampolsky *et al.* 2014). *Daphnia* dormant eggs can be resurrected to resume development and maintained via clonal reproduction in the laboratory. The short generation time coupled with clonal reproduction provides the unique advantage of rearing populations of identical genotypes from a single individual and to enable common garden experiments in which historical and modern populations can be analysed.

In a recent study, *D. magna* has been shown to adapt to temperature changes via the evolution of the critical thermal maximum, documented experimentally in populations exposed to controlled thermal conditions (mesocosms) and in (sub)populations from a lake sediment

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core resurrected from the coldest (1960s) and the warmest (2005s) period of the last century (Geerts *et al.* 2015). The critical thermal maximum was measured by exposing animals from the mesocosms and the sediment core to increasing temperature until they lost motor function (critical temperature maximum CTmax: Geerts *et al.* 2015; Kristensen *et al.* 2007). In the present study, we follow up this earlier work and use the same populations to assess plastic and genetic responses orchestrating adaptation to extreme temperature events and warming. We measure gene expression changes in candidate genes associated with thermotolerance in other species to test whether the expression of these genes is altered in our populations. Changes in gene expression between warm and cold-adapted populations are discussed in the context of evolutionary differences between populations adapted to different temperature regimes, whereas changes in gene expression following the CTmax treatment are discussed in the context of adaptive response to extreme temperature events. Additionally, we use an outlier analysis (Luikart *et al.* 2003) to identify loci potentially under selection and underlying genetic responses to temperature increase. This combined approach allowed us to gain insights into the mechanisms of adaptation to warming trends combined with extreme temperature events. We discuss our findings in light of other confounding factors, such as additional environmental stressors, that may have influenced the response observed in the natural populations.

Materials and methods

Study populations

The populations of this study (Fig. 1) have been used in an earlier study on thermal tolerance testing for the evolution of the critical thermal maximum (CTmax) (Geerts *et al.* 2015). The first set of populations consisted of animals resurrected from *D. magna* dormant eggs obtained from an artificial selection experiment (Feuchtmayr *et al.* 2007; Feuchtmayr *et al.*

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2009). In this selection experiment, forty-eight tanks were used to create 3,000 L mesocosms of shallow lake communities, in which the effect of multiple stressors was studied, including warming (+4°C), nutrient loading, and predation. This experiment was designed in a full factorial manner so that all combinations of the three stressors were analysed (Feuchtmayr *et al.* 2007). Here, we sample four of these mesocosms, of which two were exposed to a warming treatment (ambient +4°C) and two were maintained under control conditions (ambient temperature). To accomplish the heating treatment in the selection experiment, hot water (60 °C) was pumped through submerged pipes in the mesocosms. Temperature was measured electronically every 15 minutes by sensors submerged in each mesocosm, and a computer-controlled system ensured a 4 °C higher water temperature in the heated as compared to the non-heated mesocosms. In both the ambient and the heated mesocosms natural fluctuations of the ambient temperature were allowed to reflect natural conditions (Feuchtmayr *et al.* 2007; Feuchtmayr *et al.* 2009). The choice of imposing a 4°C increase in temperature was inspired by climate change predictions under the A2 scenario (Intergovernmental Panel on Climate Change, (IPCC 2007).

In the original experiment, the mesocosms were filled in October 2005 with ground water pumped from a borehole and a 20cm deep sediment layer containing (by volume) 50% garden loam and 50% organic material (47.5% chopped organic oat straw and 2.5% rotted organic cow manure to simulate a sediment of a eutrophic lake or pond) (Feuchtmayr *et al.* 2007; Feuchtmayr *et al.* 2009). This sediment did not contain dormant embryos of *D. magna*. Active zooplankton communities from several ponds in Ness Botanic Garden and a nearby shallow lake (Rostherne Mere: 53.354°N 2.387°W) were collected and mixed in equal proportion to create an artificial community sample. All mesocosms were inoculated with this zooplankton community sample. *D. magna* dormant eggs were collected from a natural pond (Brown Moss pond: 52.570°N 2.390°W) and added to the mesocosm in October 2005. Two

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and three weeks after the initial inoculation (October 2005), the invertebrates community inclusive of *Daphnia* from all mesocosms was thoroughly cross-mixed using sweep net samples; all communities were pooled and redistributed in the mesocosms to ensure comparable starting community conditions. The whole set-up and procedure resulted in mesocosms providing semi-natural conditions, mimicking the ecology of highly eutrophic small ponds. In March 2006, an additional set of 150 *D. magna* genotypes hatched from the same natural pond used for the initial inoculation (Brown Moss) was added to each mesocosm to ensure high abundance of a standardized and genetically diverse sample of *D. magna*. The experiment tested for the capacity of a single *D. magna* population to respond to experimentally controlled warming.

All mesocosms were exposed to controlled experimental conditions for two years (October 2005 – September 2007), which corresponds to approximately 30 *D. magna* parthenogenetic and two sexual generations (typically *D. magna* has one round of sexual reproduction at the end of the growing season). For the current study as well as for the earlier study on thermal tolerance (Geerts *et al.* 2015), *D. magna* dormant eggs were isolated from the top 2 cm sediment layer of two ambient and two ambient +4°C mesocosms and hatched by exposure to fresh medium, long-day photoperiod (14h light: 10h dark) and 20°C (Fig. 1).

The second set of populations used in this study and in Geerts *et al*. (2015), termed core populations, was hatched from dormant eggs obtained from the sediment of Felbrigg Hall Lake (North Norfolk, UK, 52°54.10'N, 1°15.19'E), a shallow lake (0.9 m average depth) with a documented continuous presence of *D. magna* since ±1940 (Sayer *et al.* 2010). The dormant eggs were isolated at this location from two cores (FELB5 and FELB7) sampled in 2005 using a wide-bore (diameter: 140 mm) Livingston-type core sampler (Patmore *et al.* 2014). The sediment was kept in a dark cold room (+4 °C) until hatching. Core FELB5 was dated by radiometric analysis and core FELB7 by cross-matching its loss-of-ignition

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stratigraphic profile with FELB1, a 'master core' dated by radiometric and spheroidal carbonaceous particle analysis (Sayer *et al.* 2010). *D. magna* dormant eggs were hatched from the sediment layers of two time periods: 1955-1965 (cold) and 1995-2005 (warm), corresponding to the lowest and highest temperatures of the last century (Fig. 1) (IPCC, 2013).

All genotypes obtained from the mesocosms and the sediment core were kept in monoclonal cultures for several generations (up to a year) under standard laboratory conditions (20 °C, long day photoperiod 14:10 L: D and fed *Acutodesmus obliquus*). These genotypes were then used for the outlier and the gene expression analysis described below. For the outlier analysis the genotypes were harvested for DNA extraction to obtain sufficient tissue. For the qPCR assays the strains were synchronized for two generations under standardized laboratory conditions prior to RNA extraction to reduce interference from maternal effect. The laboratory conditions for the control samples were as follows: 20 °C, 14h light:10h dark, and fed daily *Acutodesmus obliquus* at a concentration of $1x10^5$ cells ml^{-1}; the medium was refreshed every two days. For the CTmax treatment we followed Geerts *et al*. (2015). Briefly, after two generations in standardized laboratory conditions, the animals were individually subjected to a heating treatment. This consists of heating the medium in which the animals swim at incremental intervals of 1°C each 20 seconds until the animals lose locomotion. This is not a permanent status as the animals recover when placed at room temperature. CTmax thus quantifies the highest temperature at which animals still retain motor function (Geerts *et al.* 2015; Kristensen *et al.* 2007) and measures tolerance of a given individual to extreme temperatures. We collected animals immediately after they lost locomotion and flash froze them in liquid nitrogen. The Ctmax values reported by (Geerts *et al.* 2015) for the two population sets are in Table S1.

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Identification of candidate genes from literature

Our goal was to test whether the expression of genes previously associated with thermotolerance in other species was altered in our populations. We screened the literature for studies on thermotolerance in *Drosophila,* the closest arthropod to *Daphnia* with genetic resources. We identified a list of candidate genes associated with temperature stress in arthropods (Telonis-Scott *et al.* 2009; Telonis-Scott *et al.* 2011). For completeness we also identified genes previously associated with stress response in *Daphnia* (Heckmann *et al.* 2006; Labbe & Little 2009; Schwarzenberger *et al.* 2010). The 138 candidate gene sequences obtained from this search were blasted for homology to the genome sequence of *D. magna* including the sequence of some house-keeping genes. The community resources used for homology searches was the genome assembly of *D. magna* v2.4 (NCBI accession: LRGB00000000 and http://arthropods.eugenes.org/EvidentialGene/daphnia/daphnia_magna/Genome/dmagna-v2.4-20100422-assembly.fna.gz; genome-modelled gene catalogue:http://arthropods.eugenes.org/EvidentialGene/daphnia/daphnia_magna/Genes/modelled_on_genome/daphmagna_201104m8.gff.gz).

Of the total genes screened for homology (N=138), 35 were retained (Table S2). For 17 of these 35 genes the primer regions were not conserved between our species and *Drosophila*. Hence, we designed specific primers using primer 3 (v4.0.0) (Untergasser *et al.* 2012) (genes highlighted in grey in Table S2).

qPCR

Differential expression between control and CTmax treatment was measured on the total set of 35 candidate genes in the mesocosm populations (Table S2). This first analysis showed that only a subset of 15 candidate genes was differentially expressed. Based on this evidence

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we decided to perform qPCR only on this subset of genes in the core populations, in addition to two reference house-keeping genes (Atb, Xbp1) and a few candidate genes that were unresponsive in the mesocosm populations (CT802, itpr, NOS2, spag, T152). This design resulted in a total of 22 genes analyzed in the core populations. Gene expression was assessed via qPCR assays on nine to ten genotypes per population and using two technical replicates per genotype (Table S3). Total RNA was extracted from a pool of six individuals per genotype to ensure sufficient yield using the Trizol® extraction method (Invitrogen, Belgium) and following the manufacturer's instructions. Total RNA was quantified using Nanodrop® Technologies (USA) and denaturing formaldehyde-agarose gel-electrophoresis. cDNA synthesis was performed using the Perfect real-time kit (1x prime Script RT reagent - clontech) for samples from the mesocosm populations (all genes except the Heat Shock Proteins, HSP) or the 'RevertAid H Minus First Strand cDNA Synthesis kit' (Fermentas) for samples from the core populations and the HSP genes of the mesocosm populations, following the manufacturer's guidelines. We changed the cDNA synthesis kit in the course of the experiment because we experienced low repeatability of results with a batch of the Perfect real-time kit that could not be resolved. The batch of Perfect real-time kit used for cDNA synthesis in the mesocosm populations produced repeatable results. qPCR was performed on cDNA on an Abi Prism 7000 system (Applied BioSystems, Belgium) following the SYBR® Premix Ex Taq (Tli, RNaseH Plus, Takara) protocol in 20µl final volume. The amplification program was as follows: initial denaturation for 30s at 95°C, followed by 40 PCR cycles consisting of 5s at 95°C and 31s at 60°C followed by a dissociation step of 15s at 95°C, 20sat 60°C, and 15" at 95°C. A sequence detection software v1.2.3 (7000 System Software, Applied Biosystems) was used to collect amplification and melting curves. We calculated the mean CT value (cycle threshold) per sample averaging between replicates before rescaling the value. CT values per samples and per primer-pair were rescaled using an interplate

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calibrator consisting of a pool of RNA extracted from different genotypes at different developmental stages and including multiple genotypes from the population sets studied here. We refer to this rescaled value in the remaining of the manuscript when reporting CT values.

Statistical analysis of differential gene expression

We tested whether change in gene expression was explained by heat treatment (CTmax versus control), evolutionary adaptation to a temperature regime (evolution, increased versus ambient temperature in the selection experiment populations; recent versus historic population in the core), and their interaction term by using linear mixed models (LMMs). As our experimental setup included the analysis of up to ten genotypes per population (Table S2), 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 independently in the mesocosm and core populations.

The term 'CTmax' assesses whether the gene of interest displays a significant change in its expression level after CTmax 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 between warm and cold-adapted populations (warm and cold mesocosm populations; young and old core populations). This term measures evolutionary differences in gene expression due to adaptation in the field or under experimental conditions. The interaction term 'CTmax' x 'evolution' quantifies the difference in gene expression due to the CTmax treatment between cold and warm-adapted populations. As such, it reflects the evolution of plasticity in gene expression of the candidate genes.

We measured changes in gene expression between heat treatment (CTmax) and control (20°C) in all populations and genotypes. Based on our initial literature search, the

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housekeeping genes Atb and Xbp1 were expected to be unresponsive. However, our analysis of the mesocosm populations (see qPCR above) confirmed only Xbp as invariant after heat treatment, whereas Atb was differentially expressed between control and heat treatment. Given this variation and the limited information on *Daphnia* gene expression under thermal stress, we adopted a within-gene normalization procedure that uses individual mean expression levels across the whole data as a reference point (intercept) and measures deviations from this mean in each gene. This approach enabled an unbiased identification of variation in gene expression.

We use model-averaged effect sizes to quantify the impact of each term (CTmax, evolution and their interaction) on gene expression. The use of effect size provides several advantages over model selection, whether based on p-values or Akaike Information Criterion (AIC) (Burnham & Anderson 2002). Moreover, the use of average-model effect size allowed us to minimize errors on parameter estimates even when it was uncertain which model was the most parsimonious (Forstmeier & Schielzeth 2011; Lukacs *et al.* 2010). Effect size estimates in best models have been shown to perform poorly on parameters with small effects, either failing to find them significant (type II error) or overestimating their effect (Forstmeier & Schielzeth 2011; Lukacs *et al.* 2010). Conversely, model-averaged effect sizes avoid the issues of best model selection by averaging information across all models considered in a set, weighted by their relative ability to explain the data (measured via AIC). To obtain model-averaged effect sizes for each term on our candidate genes, we fitted the main LMM including all factors to each gene separately in the mesocosm and the core populations (Table S4). For each of those main LMM we generated a complete model-set by fitting all models represented by subsets of the terms and ranked them via AICc (Akaike Information Criterion, adjusted for sample size). Finally, for each model set we obtained model averaged estimates

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of each term by calculating the mean of each term effect size across models. The effect size of each term is weighted by its relative AICc score as compared to the total model set. This procedure generated 95% confidence intervals for the effect sizes of each term on each gene; terms whose 95% effect size estimates did not cross the intercept were considered significant. A False Coverage Ratio correction (FCR, (Benjamini & Yekutieli 2005) was applied to correct for multiple testing before assessing the significance of each term ($P \leq 0.05$). Throughout the paper we refer to the FCR penalized confidence intervals of effect sizes when discussing the results. The LMM analysis was performed using R version 3.0.3 (Team 2013); more specifically the package plyr was used for data handling (Wickham 2011), lme4 for model fitting (Bates *et al.* 2013) and MuMIn for model-set generation and averaging (Barton 2011).

We investigated whether each term (CTmax, evolution and their combination) affected the expression of multiple candidate genes by performing a single value decomposition principal component analysis (SVD PCA) on all genes across mesocosm and core populations. Previous to PCA, all gene expression data were averaged across replicates (genotypes within population) by using centered and scaled medians. The PCA analyses were performed using the prcomp function in the R package stats and plotted using the ggbiplot package.

Outlier analysis and neutral genetic stability

An outlier analysis was performed to identify loci putatively under selection for temperature adaptation. Capitalizing on a microsatellite panel previously developed for *D. magna* (Jansen *et al.* 2011; Orsini *et al.* 2012), we genotyped the populations from the sediment core and the mesocosm experiment using 84 microsatellites, fifty of which are EST-linked loci (Orsini *et al.* 2012). To identify outlier loci departing from neutral expectations we contrasted warm-

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adapted and cold-adapted populations in the two population sets (mesocosm and sediment core) independently, using Lositan (Antao *et al.* 2008) and retained as outliers only loci conserved between these two population sets. This conservative approach which reduces false positives has been reliably used in previous *D. magna* population genomics studies (Orsini *et al*., 2011, 2012).

In parallel to the outlier analysis and to exclude drift as cause of allele frequency changes in the two population sets, we studied the neutral genetic stability of the mesocosm and core populations following (Orsini *et al.* 2016). We studied changes in genetic diversity between old and young core populations and warm and cold mesocosm populations, by quantifying observed and expected heterozygosity (H_o and H_e), allelic richness (AR) and F_{st} using MSA analyser (Dieringer & Schlötterer 2003). Furthermore, we reconstructed the genetic structure of the two population sets to detect major shifts in genotypic composition over time using individual-based STRUCTURE analysis (Falush *et al.* 2003; Pritchard *et al.* 2000). The following parameters were used based on the stability of the MCMC parameters: 1,000,000 burn-in period, 100,000 MCMC iterations, uncorrelated loci and admixture model. Different values of K were tested. Clusters of individuals with the highest likelihood were identified using the Evanno method (Evanno *et al.* 2005) implemented in HARVESTER (Earl & vonHoldt 2012).

Results

Candidate genes

The CTmax treatment had a significant effect on the expression of a large proportion of the candidate genes analysed even though in some cases this effect differed in sign between the population sets (e.g atb Fig 2 and Table S5,). More precisely, 20 of the 35 (57%) tested genes, including some heat shock proteins, showed a significant downregulation in expression in the mesocosm populations (Fig. 2. Table S5), whereas only 5 of the 22 (23%)

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tested genes showed a significant change in expression in the core populations in response to the heat treatment (Table S5). Three of these genes (atb, T152 primer1, and T152 primer2) were significantly upregulated, whereas two were downregulated (DamVTG1 and AnxB11, Fig. 2). Differential expression at AnxB11 and vitellogenin (DamVTG1) was observed in both population sets (Fig. 2 and Table S5).

We find three genes (Cyp, Lip, Sema-1a) whose constitutive expression was significantly different between warm and cold adapted populations in the mesocosm experiment, whereas two genes (DamVTG1 and GPX) showed different constitutive expression between young and old populations in the sediment core. The former three genes show non-significant plastic response to heat treatment, whereas the latter two show a significant plasticity in addition to significant evolutionary difference in constitutive expression. Finally, our analyses did not identify a significant interaction between the terms 'CTmax' and 'evolution' in any of the genes analysed. The lack of significant interaction between the terms 'CTmax' and 'evolution' suggests lack of evolution of plasticity. However, we cannot exclude that this result may be due to lack of power, as the identification of significant interaction between terms requires more replications than first-order terms.

Co-expression of candidate genes

We performed a single value decomposition principal component analysis (SVD PCA) to investigate co-variation of the expressed genes in respect to both the short term plastic response (CTmax) and the evolutionary differences between warm and cold-adapted populations (Fig. 3). We find that a large proportion of the observed variance (84%) is explained by PC1 which, however, captures very little of the differences between population sets or experimental treatments (Fig. 3A). PC 2 explains only 9.7% of the total variance but clearly separates the core and mesocosm populations (Fig. 3A). PC3 (3.3% var explained, Fig

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3B) and PC4 (1.3% var explained, Fig 3C) both separate CTmax treatment from control, but whereas PC3 separates CTmax treatment from control in both population sets, PC4 only separates CTmax treatment from control in the mecososm populations. This suggests that PC3 represents the small-scale correlated response to CTmax treatment shared between populations. As expected, this correlated response was not observed in single-gene expression analysis within population sets. A closer examination of PC4 reveals a nested ordering of mesocosm populations, which are sorted first by CTmax treatment and then by evolutionary response (Fig. 3C). This suggests that genes expressed under CTmax treatment also increase their expression in the warm-adapted mesocosm population. That is changes in expression identified along PC4 are a mixture of plasticity and evolution in the mesocosm populations (e.g. GPX and DamVTG1 in Fig. 3C). Conversely, PC4 depicts only evolutionary responses in gene expression in the core populations, with a minor plastic component only in the old population.

PC4 also separates old and young core populations (Fig 3C). A separation between CTmax treatment and control is only observed in the old core population, whereas the young population shows identical levels of expression along this PC (Fig. 3C). PC5 shows a nested ordering of the mesocosm populations sorting them first by selection regime (cold/warm and young/old populations) and then by CTmax (Fig 3D). Conversely, this PC has no explanatory power on the core populations (Fig. 3D). It is noteworthy that this PC, as PC4 (Fig. 3C), shows collinearity in gene expression between plastic and evolutionary response in the mesocosm populations (Fig. 3D).

Outlier analysis and neutral genetic stability

The microsatellite locus B088 was identified as an outlier in both population sets (Table 1). The major allele at this locus (allele with the highest frequency) is the same in both sets and

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between populations within core and mesocosm (Fig. S1). This finding suggests that the locus itself or a gene tightly linked to it is under selection in both populations. Regretfully, a blast search of the sequence of this locus using the gene models built for *D. magna* (Orsini *et al*. 2016) did not identify a gene with known function in other species.

Neutral genetic stability was observed in both population sets (Fig. 4). More specifically, the two population sets were in Hardy-Weinberg equilibrium at neutral loci (data not shown), heterozygosity and allelic richness were comparable between warm and cold adapted mesocosm populations as well as between old and young core populations (Fig. 4 A-B). Genetic differentiation between old and young core populations was in the range previously observed for *D. magna* (Orsini *et al.* 2013; Orsini *et al.* 2012) (F_{st} = 0.01; P = 0.09). Genetic differentiation was higher between the mesocosm populations (F_{st} = 0.04; P = 0.001) as expected for a more diverse genetic pool.Population genetic structure was comparable before and after selection in the mesocosm populations (Fig. 4 C), and over time in the sediment core (Fig. 4D). In both population sets the most likely number of cluster identified by the STRUCTURE analysis corresponded to the expected number of groups of individuals (two, Table S6). Furthermore, we observe a stable genotypic composition over time in the core and between mecosoms.

Discussion

Understanding the interplay between short-term plastic and long-term evolutionary response to climate change is critical to assess the ability of natural populations to respond to extreme events such as heat waves combined with global warming. A consensus on the relative importance of phenotypic plasticity and genetic responses to climate change has not yet been reached, mostly because systems in which both processes can be observed in concert are rare. The large majority of studies on climate change identified phenotypic plasticity as a common

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mechanism of response (Chevin & Lande, 2010; Chevin *et al*., 2010; Kovach-Orr & Fussmann, 2013). However, an increasing number of case studies reports evolutionary changes in response to climate change (reviewed in Hoffmann & Sgro 2011; Merila & Hendry 2014).

Our study provides evidence of plasticity in the expression of several candidate genes for thermotolerance in other species in response to sudden and drastic temperature increase (CTmax) in *D. magna*. Although CTmax assays may be expected to induce a shut-down of the regulatory machinery because of the rapid and extreme increase in temperature maintained until the animals lose locomotion (Kristensen *et al.* 2007), we observe both a downregulation of some genes, and an upregulation of some other genes after the heat treatment. The number of co-responsive genes to CTmax treatment, including some heat shock proteins, was higher in the mesocosm than in the core populations. However, responsive genes in the core populations showed a more pronounced change in expression in repose to the CTmax treatment. This pattern may indicate a stronger and more selective regulation of response genes in the natural as compared to the mesocosm population. This may be expected considering that the mesocosm population consists of a more diverse genetic pool as compared to the natural population. Interestingly, a different suite of genes responds to the CTmax treatment in the population sets analysed. This difference may be explained by the different genetic background of the populations as we discuss below.

We identify different genes whose constitutive expression changes between warm and cold adapted mesocosm populations and young and old core populations (evolutionary differential expression). Additionally, some of the genes showing plastic response to heat treatment (DamVTG1 and GPX) in the core populations also show evolutionary differential expression between young and old populations, whereas plastic genes in the mesocosm do not show an

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evolutionary difference in expression between the warm and cold-adapted populations. These findings are consistent with the results of inspecting PC4 in the PCA plot, which represents both evolutionary responses in natural populations and plastic differential gene expression in the mesocosms. A positive correlation between plastic and evolutionary responses in gene expression has been shown to facilitate adaptation during the early phases of colonization to thermal environments (Makinen *et al.* 2016). Conversely, selection for increased or decreased gene expression leads to loss of plastic responses , a process known as genetic assimilation (Scoville & Pfrender 2010). The analysis of individual genes in our study suggests that plasticity enhanced adaptation in the core population, whereas it was lost in the selection experiment populations. However, investigating patterns of co-expression among the candidate genes (PCA plots) provided further insights. PC4 clearly separates CTmax and control animals in the old but not in the young core population. This result combined with the individual gene analysis suggests that whereas some key genes showing evolutionary differences tend to maintain plasticity, the large majority of genes tend to lose plasticity under evolutionary constraints. The patterns of co-expression in the experimental populations reveal a different scenario; that is the majority of genes showing plasticity also show some degree of evolutionary difference between warm and cold-adapted populations.

The differences in the number and type of genes co-expressed in the two population sets, as well as the different mechanisms of response may be expected because of the different genetic background and the selection regimes the populations experienced. The natural population (core population) evolved over 40 years from the same initial genetic pool which became locally adapted to the specific environmental conditions of the lake, whereas the mesocosm populations evolved from an artificial more diverse genetic pool obtained from the mixing of sediment collected from several lakes. Concerning the selection regime of the two populations, the mesocosm populations experienced a strong selection regime with an

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increase in temperature of 4°C in a relatively shorter time frame (2 years) as compared to the natural population which experienced an average increase in temperature of 1.2°C over a time period of 40 years. It has been shown that the strength of selection and the speed of environmental change may strongly impact evolutionary trajectories (Grant & Grant 2002). An alternative explanation to the differences observed is that the two population sets do not respond to the same selection pressure. Whereas the genes identified in the mesocosm experiment can be directly linked to temperature adaptation, this signal may be confounded by other environmental stressors in the natural population. Although evolution to temperature increase in the natural populations has been previously associated with increased CTmax (Geerts *et al.* 2015), we do not have sufficient evidence to directly link the genes analysed here to temperature adaptation. It is striking, however, that a common gene polymorphism (B088 in the genome scan analysis) was shared between the two population sets, in absence of drift and gene flow from immigrant genotypes. This findings suggest that evolutionary responses in the populations studied are mediated by standing genetic variation, supporting previous results (Orsini *et al.* 2016; Orsini *et al.* 2013; Orsini *et al.* 2012) and that we have potentially captured adaptive response to warming trends in the two populations. However, to firmly conclude that the patterns observed are due to temperature adaptation in the natural population will require a more comprehensive analysis of genome-wide gene expression.

The two populations studied here respond to environmental change regulating different sets of genes. Although it is not surprising that populations of the same species with different genetic backgrounds respond to the same stressors using a different suite of genes, it is possible that this difference is due to the response to different selection pressures, as discussed previously. It noteworthy, however, that the genes differentially expressed in the two population sets belong to the same functional categories, including central metabolic

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functions, immunoregulation and oxidative stress response. More specifically, genes showing evolutionary differential expression between warm and cold-adapted mesocosm populations include genes associated with immunosuppression (Cyp), metabolism (Lip) and signal transduction (Sema-1a) (Heckmann *et al.* 2006; Heckmann *et al.* 2008). Genes showing evolutionary differential expression in the core populations are associated with egg formation (DamVTG1) and oxidative stress response (GPX). Finally, DamVTG1 and AnxB11are shared among population sets. AnxB11 is involved in lipid transport, whereas DamVTG1 encodes the egg yolk precursor protein vitellogenin, which has been shown to be upregulated in asexual females of *Daphnia pulex* as compared to sexual, resting-egg bearing females (Raborn *et al.* 2016). The downregulation of vitellogenin documented in this study might thus be indicative of a switch from asexual to sexual reproduction and in the induction of dormant eggs under heat stress. As these eggs are resistant to environmental hardship, it is reasonable to assume that temperature stress triggers dormant egg formation to enable survival to temperature stress. The responsiveness of many central metabolic genes in our study is not unexpected. The correlation between temperature and metabolism is well-known in studies on climate change (Tewksbury *et al*., 2008), suggesting an important indirect role of metabolic genes in temperature adaptation. Moreover, this finding is in agreement with the previously recorded differentiation in growth between warm and cold-adapted populations from the core and the mesocosm: smaller animals showed a higher CTmax than larger animals, suggesting a link between growth and thermal tolerance (Geerts *et al.* 2015). As we gained more insights on the similarities between population sets by studying co-expression patterns among the candidate genes, a higher-level analysis, such as pathway or network-based analysis on a larger suite of genes is likely to reveal stronger homologies between the populations studied.

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Conclusions

Our study identified five candidate genes (DamVTG1, GPX, Cyp, Lip and Sema-1a) potentially linked to temperature adaptation in *Daphnia*. This list is not exhaustive and the genes are likely only indirectly linked to temperature adaptation or underlie response to more stressors than just temperature. However, they represent good candidates for future functional studies. Our results reveal plasticity in the expression of several genes in response to extreme temperature events. In addition, we document evolutionary changes in gene expression and divergence in gene polymorphism occurring both over short (two years, mesocosm populations) and long (40 years, core populations) time spans. While we were able to link these evolutionary changes to temperature adaptation in the experimental (mesocosm) population, the co-occurrence of other environmental stressors with temperature prevents us to establish a direct link between temperature and candidate genes in the natural (core) population. Studying the relative contribution of plastic and genetic response to temperature changes at candidate genes provided us with the first insights into the architecture of adaptation to environmental stressors in *D. magna.* An exploratory analysis of co-expression patterns revealed that higher-level homologies in response to the same environmental stressor may be uncovered by studying patterns of co-variation in a larger number of genes.

Acknowledgments

This study was supported by KU Leuven Research Fund excellence center funding PF/2010/07, by Belspo IAP project SPEEDY, by BiodivERsA project TIPPINGPOND and by the University of Birmingham NERC highlights grant deCODE (NE/N016777/1). MJ is a postdoctoral researcher with the National Fund for Scientific Research, Flanders. AG and KIS enjoyed an IWT PhD fellowship while carrying out this work.

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Data Accessibility

Microsatellites genotypes for mesocosm and core populations are deposited at dryad entry

doi:10.5061/dryad.q7c00.

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Table 1. **Outlier analysis**.

The selection detection workbench Lositan (Antao *et al.* 2008) was used to identify outlier loci in the Fellbrigg Hall Lake (core) and the mesocosm populations by contrasting warm and cold-adapted populations within each data set. Each comparison was performed in triplicates and the outlier loci identified using a FDR=0.1. The locus B088 was identified as outlier in both pairwise comparisons. Other outliers identified within one population set are also reported. The loci names are as in (Jansen *et al.* 2011; Orsini *et al.* 2012).

Felbrigg Hall Lake			Mesocosm		
Run 1	Run 2	Run3	Run 1	Run 2	Run3
B088	**B088**	**B088**	**B008**	**B008**	**B008**
		B065		A009	A009
		B031		WFes0006310	
B150	B150	B150	WFes0001245	WFes0001245	WFes0001245
B133	B133	B133	WFes0003187	WFes0003187	

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B180	B180	B180	WFes00011411		WFes00011411
B179		B179	WFes00011982	WFes00011982	WFes00011982
WFes0006310	WFes0006310	WFes0006310	WFes00011318	WFes00012318	
	WFes0009489	WFes0004129			WFes0001770
WFes0001770	WFes0001770	WFes0001770			B030
WFes00011039	WFes00011039	WFes00011039	B150		
B050	B050				S6-199
	B021	B021			
	A001				
B96					
	WFes0004129				
	WFes00011345				

Figure legends

Figure 1. Sampling and experimental work-flow

(A) Felbrigg Hall Lake, Norfolk, UK, was sampled with a piston corer. The sampled sediment was dated and dormant eggs of *Daphnia magna* were hatched and cultured as isoclonal lines from two time periods: a colder time period (1955-1965) and a more recent, warmer period (1995-2005). The average temperature difference between these two time periods is about 1.2°C. (B) The upper 2cm of the sediment from four mesocosms exposed to either ambient or ambient +4 °C temperature treatment were sampled for *D. magna* resting eggs. Hatched *D. magna* from the two population sets were used in an outlier analysis and a candidate gene analysis.

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Figure 2. Candidate genes analysis

Reaction norms of the differential expression measured at the candidate genes between heat treatment (CTmax) and control (20°C). In the heat treatment animals were exposed to temperature increase until they lost locomotor function. Thirty-five candidate genes were studied in the populations from the artificial selection experiment (mesocosm) whereas a subset of 22 genes was analyzed in the populations resurrected from Felbrigg Hall Lake (core). Triangles represent the core population and circles represent the mesocosm population. Blue symbols represent cold (mesocosm) or old (core) populations, whereas red symbols represent warm (mesocosm) or young (core) populations. Solid lines indicate a significant change in gene expression between the heat treatment and control (CTmax term), whereas large symbols indicate significant changes in gene expression between warm- and cold-adapted mesocosm populations or old and young core populations (evolution term in Table S5).

Figure 3. Factor analysis

Single value decomposition principal component analysis (SVD PCA) on all genes in the mesocosm and the core populations. The decomposition of variance is represented in the following panels: (A) PC1 - PC2; (B) PC2 - PC3; (C) PC2 - PC4; and (D) PC2- PC5. The gene names are as in Figure 2. The terms ‘old’ and ‘young’ refer to the core population whereas the terms ‘warm’ and ‘cold’ refer to the mesocosm populations. Control and CTmax refer to the heat treatment. Care was taken to avoid overlapping gene names; this may have slightly altered the position of some genes in the plots.

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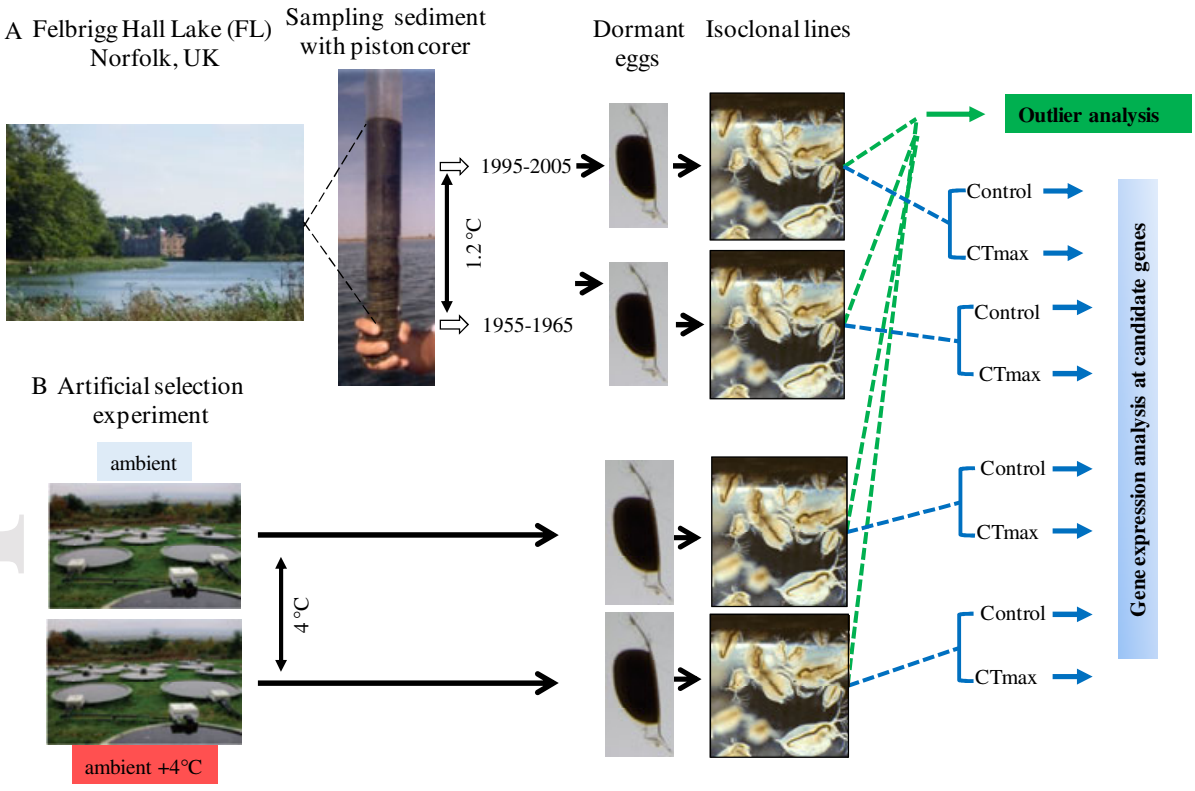
Figure 4. Neutral genetic stability.

(A,B) Genetic diversity indices and (C, D) population genetic structure in the core and mesocosm populations. Observed heterozygosity (Ho), expected heterozygosity (He), and allelic richness (AR) are shown. The color code for the alleles in the STRUCTURE analysis is randomly generated, hence identical colors may represent different alleles in the two population sets. The population names are as in Figure 2.



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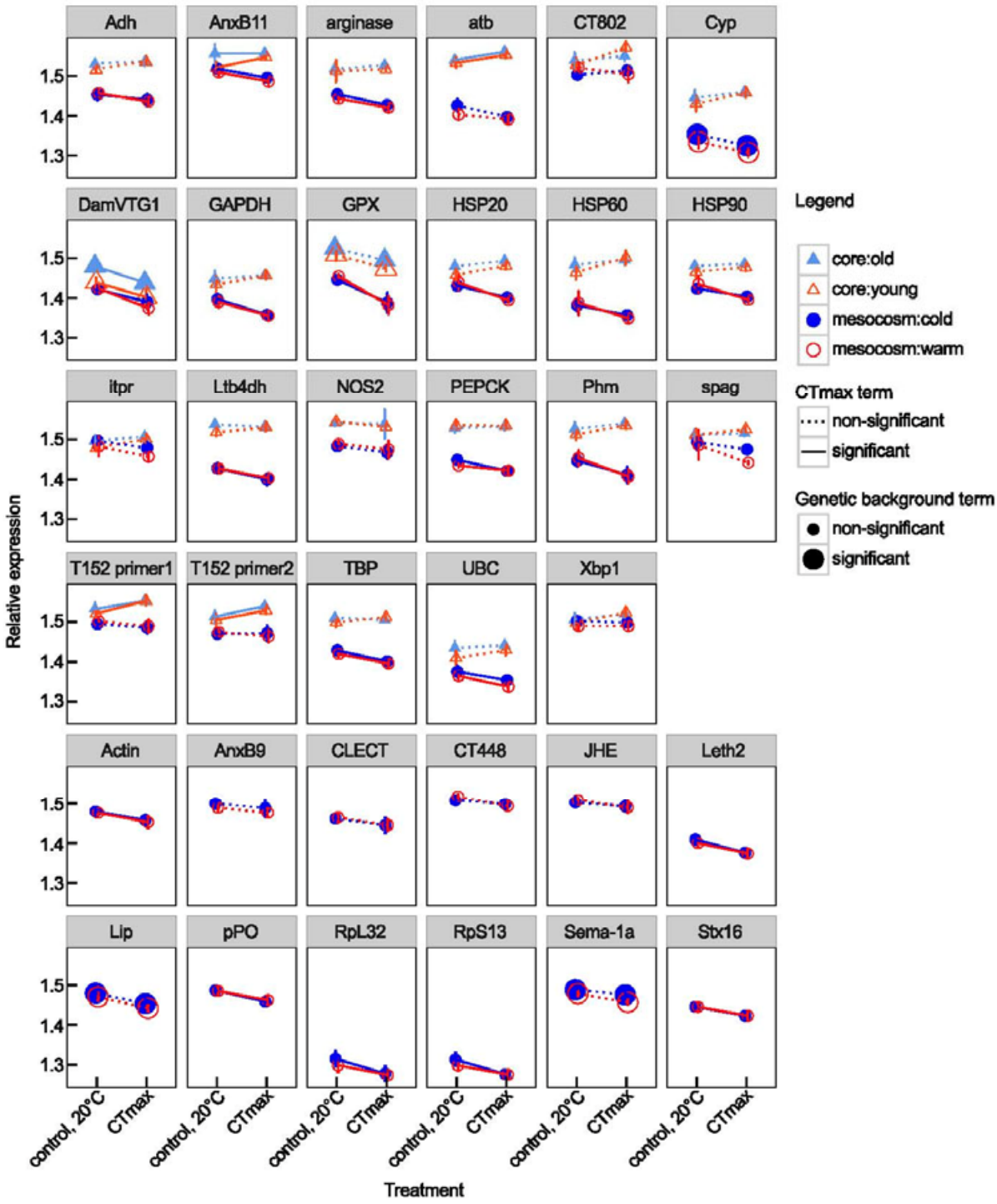

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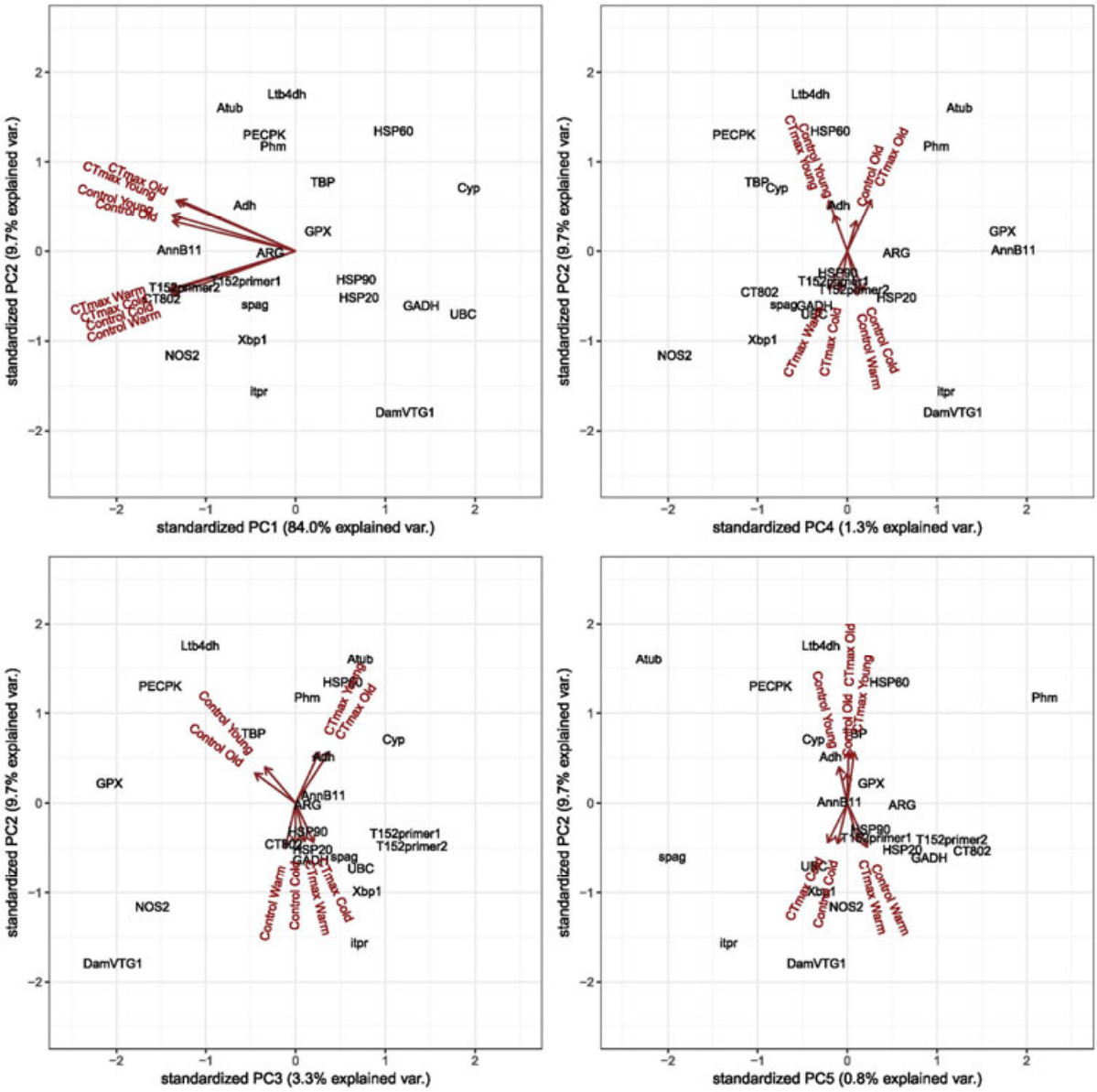

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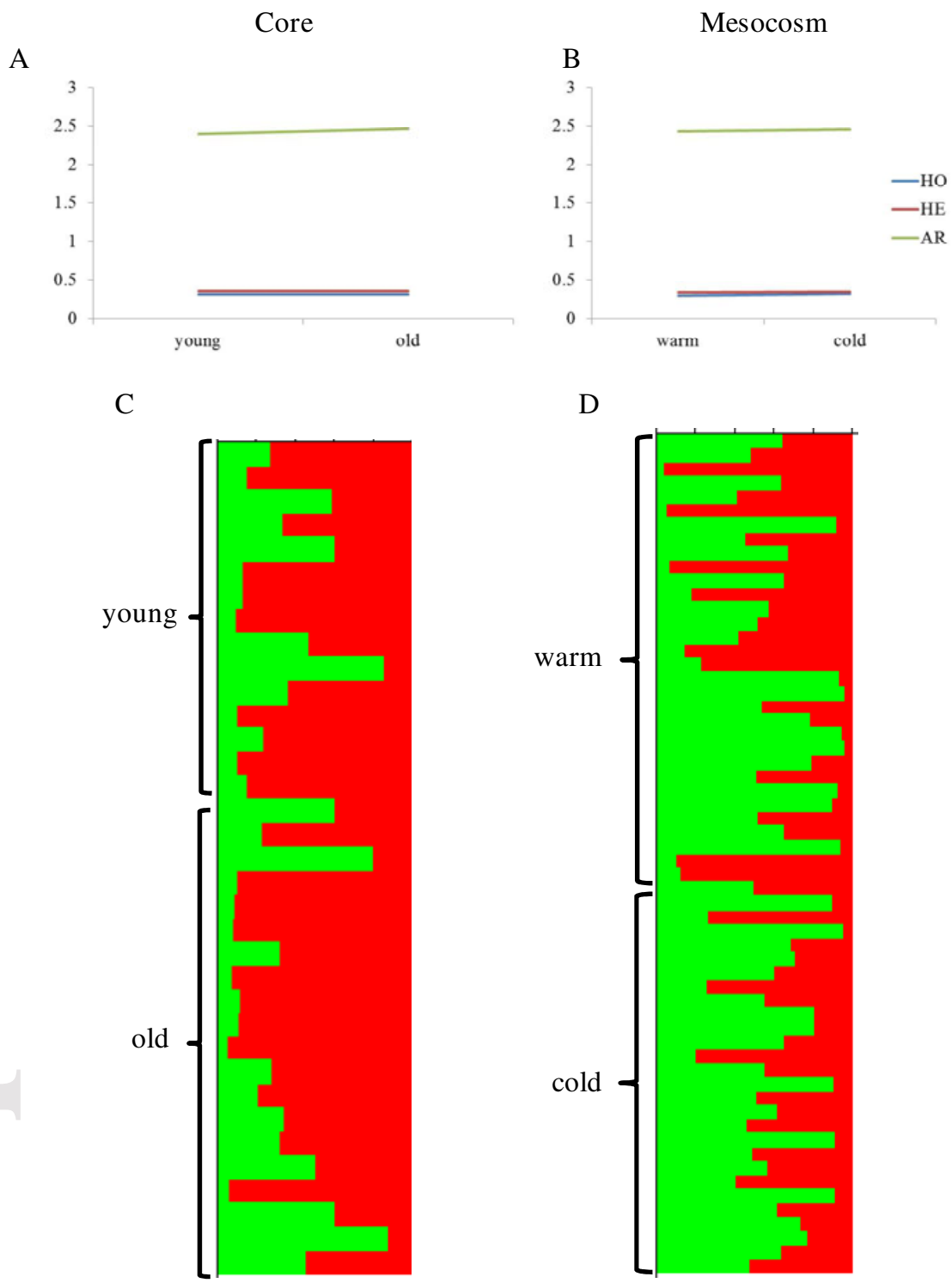

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