2016-05-20

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10.1111/phen.12148
Physiological Entomology
Wiley

This is an accepted manuscript of an article published by the Royal Entomological Society (Wiley journals) in *Physiological Entomology*, published in "Early View" on-line May 20, 2016, available at DOI: 10.1111/phen.12148

Variation in heat shock protein expression at the latitudinal range limits of a wide-ranging species, the Glanville fritillary butterfly (*Melitaea cinxia*)

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Keywords: Hsp 20.4, Hsp 21.4, Hsp 90, latitude, qPCR, constitutive adjustment hypothesis

Abstract

Studies of Hsp expression have shown correlation across thermal clines, though more often across altitudinal gradients, and less so across large latitudinal gradients. Here we investigate the response of three heat shock proteins to thermal stress, in populations from the northern range limit (Åland Islands, Finland) and the low elevation southern range limit (Catalunya, Spain) of the Glanville fritillary butterfly, *Melitaea cinxia*. Hsp 20.4 and Hsp 90 demonstrated dramatic up-regulation at higher temperatures, however there were no significant expression differences between the two populations. Hsp 21.4 showed no significant up-regulation in response to increased temperatures, however it did exhibit a significant constitutive difference between populations, with insects from Catalunya having 4-6-fold higher levels than those from the Åland Islands. Interestingly, the key metabolic enzyme and cell cycle modulator glyceraldehyde-3-phosphate dehydrogenase (G3PDH), which was originally selected as a control gene, was consistently expressed 1.5-2-fold higher across all temperature treatments in Finnish compared to Spanish butterflies. Differences in putatively homeostatic Hsp 21.4 and G3PDH suggest that the analyzed populations might exhibit differences in energetic homeostasis. This type of data has potential to provide greater understanding of the mechanisms underlying adaptation of poikilotherms to regional climate and to help predict how they may be affected by a changing climate.

Introduction

Heat shock proteins (Hsps) are molecular chaperones well-known as stabilizers of protein integrity under stressful conditions but also important as folding catalysts for protein maturation under non-stressful conditions (Feder & Hofmann, 1999). Levels of Hsps are often useful indicators of heat stress, as their induction represents the earliest step in an organism's response to environmental stress (Kültz 2005). In addition, Hsps are evolutionarily ubiquitous: the majority of species studied have Hsp genes that exhibit varying patterns of expression, which often correlate with resistance to stress, and with the stress levels naturally experienced by each species (Feder & Hofmann 1999). Hsp expression patterns can also vary within a species (Tomanek, 2010; Otsuka et. al., 1997,

Brown et. al., 1995), with respect to both temporal and spatial differences in environmental stress. Consequently, transcriptomic analyses, such as quantification of Hsp expression levels, have been proposed as a standard metric for quantifying stress responses within and among natural populations (Evans & Hofmann 2012).

The genes encoding Hsps are highly conserved, and are often named after the molecular weight of the protein (e.g. Hsp70). Much work on Hsps has focused on insects (King & MacRae, 2015), in particular *Drosophila* (e.g. Sorensen et. al., 2005; Krebs & Holbrook, 2001) and Hsp70 (Krebs & Feder, 1997; Benedict et. al., 1993), since this is the primary inducible Hsp found in *Drosophila* (Krebs & Bettencourt, 1999). Hsp70 plays a role in a number of stress responses, including tolerance of hyperthermia (Wischmeyer et. al., 1997), tolerance of hypoxia (Heads et. al., 1995) and regulation of heat shock response (Solomon et. al., 1991). Hsp90 performs similar functions. The larger Hsp families (Hsp100, Hsp90, Hsp70 and Hsp60) tend to have more highly conserved sequences, and are some of the most highly conserved protein families known (Waters et al., 2008). Small heat shock proteins, with molecular weights ranging from 12 to 42 kDa, tend to be more diverse than large Hsps, but their functions appear to be similar (Basha et al., 2012; Li et. al., 2009).

Both constitutive and inducible forms of Hsp 70 play a role in thermotolerance, and temporal fluctuations in Hsp expression represent plastic responses of individuals to their immediate environments. For example, beetles collected at the warmest time of day contained higher levels of Hsp70 than those collected at cooler times (Dahlhoff & Rank 2000). Sorensen et al. (2009) found that fruit flies that could not induce heat shock proteins were incapable of finding food stations on hot days, while wildtype conspecifics could. Studies such as these are important for establishing an ecological context for Hsp expression. Nikinmaa et al. (2008) found geographical differences in constitutive expression of Hsp 70 in the frog *Rana temporaria*. Sorensen et al. (2009[2]) found variation in constitutive levels of Hsp expression as well as a temperature-induced increase in expression levels in the same species. Studies of *Nucella* snails found that higher expression of Hsps was correlated with increased thermotolerance, and that the level of

total rather than stress-inducible Hsp 70 was a better predictor of thermal tolerance (Sorte & Hoffmann 2005).

Much research has demonstrated clinal variation in stress resistance and life history traits (Angilletta et. al., 2003; Hoffmann et. al., 2003; Sorensen et. al., 2009[2]). Because of the roles that Hsps play in mediating thermal tolerance, their expression is expected to feature in local adaptation to climate. Expected correlations between Hsps and thermal environment have indeed been observed in clines based on elevation for *Drosophila buzzatii* (Sorensen et. al., 2005), the copper butterfly *Lycaena tityrus* (Karl et. al., 2008) and the montane beetle *Chrysomela aeneicollis* (Dahlhoff & Rank, 2000). However, studies of thermal clines occurring across large latitudinal gradients have only occasionally found correlations with latitude (e.g. in the mussel *Mytilus galloprovincialis*, Dutton & Hofmann, 2009). These studies have also not produced as conclusive results as those completed on smaller scales (e.g. microclimatic variation in temperature) and the results have often been complex and affected by other unknown factors (Sorensen et. al., 2009). Therefore, further investigation of latitudinal variation of Hsp expression presents an interesting avenue of study.

Variation of physiological traits across thermal clines is expected to be most pronounced in species with large geographical ranges and low rates of dispersal. The species studied here, the melitaeine butterfly *Melitaea cinxia*, fits this description. This study investigates Hsp expression in populations of *M. cinxia* sampled from its latitudinal range limits at low elevation. The expectation is that butterflies at the southern range limit, being more often exposed to extreme heat events, may have either higher levels of constitutive Hsp expression, and/or stronger Hsp induction in response to thermal stress.

Methods

Study system

This study covers the latitudinal range of *M. cinxia* at low elevation, which stretches from 41.5°N in Catalunya, Spain to 60.4°N in the Åland islands, Finland (Lafranchis, 2004). Isolated montane populations in Spain and Morocco, which were not included in the work, extend the range southwards to around 35°N. At all study sites the insects spend winter as partly-developed larvae. In early spring, these thermophilic larvae begin to bask in sunshine and feed on new leaves of their hosts, *Plantago lanceolata* in Catalunya, and both *P. lanceolata* and *Veronica spicata* in Finland (Van Nouhuys et al. 2003).

In Catalunya, post-diapause larvae and adult butterflies are typically active in April and May. In the Åland Islands, post-diapause larvae and adult butterflies are typically active in May and June. Since the insects are active at different seasons in different latitudes, it is possible that each life stage may experience similar climatic conditions across the range. To examine this possibility, we obtained the monthly mean maximum temperatures and highest maximum temperatures for both study areas for the period 1980-2013 (figure 1). The Figure shows that variation in phenology is insufficient to maintain a common climate experience between northern and southern populations for either larvae or adults.

Gene selection and primer design

Hsp and control genes were selected using the transcriptome for *M. cinxia* available at http://cinxiabase.vmhost.psu.edu/TextSearch2.html. A search for "hsp" brought up a number of potential sequences, and those that matched other Lepidoptera were selected as potential candidate genes. Sequences annotated as Hsps were verified using BLAST (Altschul, 1997) against the non-redundant ("nr") Genbank database (NCBI). Primers were designed to target 150bp of these sequences using Primer3 (http://primer3.sourceforge.net/). Three potential control genes were selected from those frequently used in other studies (de Kok et. al., 2005).

Primer validation

Primer validation followed the protocol described by Kenkel et al. (2011). The specificity of each primer pair for its target gene was tested using gel electrophoresis and melt curve analysis of the amplification product obtained with *M. cinxia* cDNA as a template. Primer efficiencies were determined by amplifying a series of two-fold dilutions of *M. cinxia* cDNA covering two orders of magnitude of template amount (5ng to 0.078ng RNA equivalent per PCR reaction). These reactions were all conducted in duplicate. CP values for each dilution series were then plotted against the $\log_2$[cDNA], and the slope determined for each primer set (supplementary Table 1). The primer-specific amplification efficiency (*E*, amplification factor per PCR cycle) was then derived from the slope of the regression ($E = 2^{-(1/slope)}$) (Pfaffl, 2001). The qPCR assays accepted for this study exhibited PCR efficiencies within the range 1.91-2.03 (R^2 values ranging from 0.98-0.999). In order to test for primer specificity and genomic DNA contamination, a negative control was run, lacking reverse transcriptase. No amplification was observed here. G3PDH (Glyceraldehyde-3-Phosphate Dehydrogenase), elF5B (Elongation Initiation Factor 5B) and Beta Actin were selected as potential control genes, the stability of which was validated using GeNorm (Vandesompele et al 2002). Hsp 20.4, Hsp 21.4 and Hsp 90 were selected as the target genes of interest.

Background on experimental design

A number of pilot experiments were conducted using different temperature regimes similar to those used in other studies (Sorensen et. al., 2005; Karl et. al., 2008; Shen et. al., 2011). The eventual experimental temperatures of 22°C, 38°C and 42°C were selected based on these pilot experiments, as well as on maximum temperatures to which the species is currently exposed (figure 1). While the experimental temperatures are higher than those to which the species is currently exposed in the field, it is important to note that the black *M. cinxia* larvae achieve significantly higher body temperatures when basking in the sun, relative to ambient temperature (Kuussaari, 1998). The lights in the growth chamber fail to mimic this effect. Therefore, in this experiment we chose ambient temperatures in the growth chamber to mimic the body temperatures of the caterpillars basking in the sun, rather than natural ambient air temperatures.

Larvae for the experiments were obtained by field-gathering adults, eggs, or very young (pre-diapause) larvae. All larvae were raised under controlled lab conditions for the majority of their life cycle; from pre-diapause through both diapause and post-diapause development. However, it remains possible that their experience prior to being collected may have influenced their performance, since thermal environments of ectotherms experienced early in the life cycle may have effects later on, and thus may be expected to affect traits such as Hsp expression (Atkinson & Sibly, 1997; Hoffmann et. al., 2003).

Experimental design

Eggs from the two populations were collected from adult butterflies caught in the field, and caterpillars were then raised to diapause in the lab and kept over winter at 4°C. Caterpillars from six Catalunya families and seven Åland Island families were then taken out of diapause in the spring, and allowed to feed on *Plantago lanceolata* at room temperature (figure 1). In their final instar, three groups of N=3 caterpillars were sampled from each family. Each group was subjected to one of the following temperature regimes in a climate controlled growth chamber with artificial lights and freshly cut leaves:

1 hour at 22°C, followed by 1 hour recovery at room temperature (22°C) (control)
1 hour at 38°C, followed by 1 hour recovery at room temperature (22°C)
1 hour at 42°C, followed by 1 hour recovery at room temperature (22°C)

Out of the 6 families from Catalunya, 2 families had only 2 caterpillars. For these families, only the 22°C and 42°C treatments were used.

Following the recovery period, caterpillars were cut in half, and each half placed in a separate vial of RNAlater (Ambion). The head region was kept at room temperature and used for RNA isolation. The other half was stored at -80°C.

RNA isolation

RNA was extracted from the samples using RNAqueous 4PCR kits (Ambion). The concentration of RNA was then quantified using the Nanodrop 2000 (Thermo-Fisher). RNA quality was assessed through gel electrophoresis, and evaluated based on the presence of ribosomal RNA bands. After DNAse treatment the concentration of RNA was again estimated, and another electrophoresis gel run to check the integrity of the RNA and confirm the disappearance of the genomic DNA band.

cDNA synthesis

Synthesis of first strand cDNA was conducted using the SmartScribe Reverse Transcriptase kit (Takara-Clontech). 50ng of RNA from each sample was brought to 4µl total volume using milliQ H_2O. 1µl of 6µM of an oligo-dT-containing primer (5'-CGCAGTCGGTACTTTTTTTTTTTTV-3') was added to each of the above sample dilutions, incubated at 65°C for 3 minutes, and then 5µl of a master mix (0.5µl H_2O, 1µl dNTPs, 1µl DTT, 2µl 5xBuffer and 0.5µl SSII Reverse Transcriptase) was added to each sample. A no-RT control was also synthesized for each sample, under the same conditions described above but lacking the reverse transcriptase, and instead containing 1µl of H_2O. All samples were then incubated at 42°C for 1 hour, followed by 65°C for 3 minutes. Finally, each of the samples was diluted to contain a cDNA equivalent of 1ng/µl of RNA, by adding milliQ H_2O.

Quantitative PCR

qPCR reactions were conducted using the LightCycler 480 (Roche). All qPCR reactions were conducted in duplicate. 1ng of each cDNA template was mixed with 4.5µl of H_2O and 7.5µl of 2x SYBRgreen Master Mix (Roche). This mixture was then added to the well plate (LightCycler 480 multiwell plate 384, white, Roche), and 2µl of 1.5µM F+R primer was added to each well. No-RT controls were checked for genomic DNA contamination by amplification with G3PDH. The well plate was then covered by sealing film (Roche), spun down, and run in the LightCycler 480 under the following program: 1

x pre-incubation (95°C for 5 min), 45 x amplification (95°C for 30 sec, 60°C for 40 sec, 72°C for 40 sec), 1 x melting curve (95°C for 5 sec, 65°C for 1 min, slow ramping up to 97°C), 1 x cooling (40°C for 10 sec).

Statistical Analysis

The analysis of qPCR data was performed in R (R Development Core Team, 2008) using package MCMC.qpcr (Matz et al., 2013). Briefly, the analysis involves fitting a single Bayesian linear mixed model to the complete set of qPCR measurements (corrected for amplification efficiency) using a Markov chain Monte Carlo (MCMC) procedure and inferring the expression changes for all genes from the joint posterior distribution of parameters. The statistical significance of these changes is evaluated by estimating the empirical two-tailed p-value (P_{MCMC}), which is twice the fraction of sampled parameter values that cross zero with respect to the mean. Although this analysis is able to disentangle variation due to template loading from biologically relevant gene expression changes without relying on control genes, its power is substantially enhanced when control genes are specified. The modeling was therefore performed using the "classic" model that follows the established multi-gene normalization procedure (Vandesompele et al., 2002).

Results

Gene selection and normalization

Hsp70 was initially chosen as the primary target gene for comparison with other studies. However, all primer pairs designed for the homologous sequence from *M. cinxia* (Contig 56282) failed to yield specific amplification products (i.e. multiple peaks were observed in the melt curve analysis). In addition, primer efficiencies were outside the acceptable range. As a result, Hsp20.4, Hsp21.4 and Hsp90 were selected as target genes. Of the putative control genes, GeNorm analysis suggested that G3PDH (Glyceraldehyde-3-Phosphate Dehydrogenase) is not stable enough to serve as a control (GeNorm M=1.57).

This result was confirmed by "naive" (control-free) Bayesian analysis using MCMC.qpcr package, which indicated that G3PDH is differentially expressed among populations. We therefore used only Beta Actin and eIF5B (Elongation Initiation Factor 5B) as control genes for Bayesian modeling (GeNorm M=0.99), while analyzing G3PDH as a response gene along with the Hsps. All CP values for the no-RT controls were >35, indicating that genomic DNA contamination was negligible.

Gene expression

Gene expression changes are summarized in figure 2 and table 1. Hsp21.4 exhibited significant constitutive difference between populations, being expressed 3.3-fold higher in the Catalunya population. The other two Hsps exhibited the same trend, although the between-population differences for these two genes were not statistically significant. Hsp20.4 demonstrated dramatic up-regulation at 38^0C (63 fold) and 42^0C (32 fold) relative to the 22^0C control. Hsp90 also demonstrated up-regulation at 38^0C (7.6 fold) and 42^0C (5 fold) relative to the 22^0C control. Hsp21.4 showed no significant response to treatment. There was no significant difference between 38^0C and 42^0C for any of the Hsps, although all three of them exhibited a trend towards diminished expression at 42^0C (relative to 38^0C). G3PDH was expressed constitutively higher in Finland by 1.9-fold, and did not respond significantly to heat stress. No interaction terms between population and temperature treatment were statistically significant for any of the genes.

Discussion

With changes in climate projected to increase in the coming years (IPCC, 2013), there is a growing need to gain a better mechanistic understanding of how different species and populations may respond. Trait-based vulnerability assessments of species, including thermal tolerance, are becoming increasingly valuable (Advani, 2014). To this end, interest in the molecular and physiological functions of Hsps has increased (Karl et. al., 2009). Studies such as that presented here are now beginning to venture out to non-model organisms in ecological contexts relevant to climate change biology. Looking at how

species adapt to climate variation in space is a good way to evaluate how they might respond to similar changes in time (La Sorte et al., 2009). This study investigated the response of three heat shock proteins to thermal stress, in populations from the northern range limit (Åland Islands, Finland) and the southern range limit (Catalunya, Spain) of *Melitaea cinxia*.

Summary Hsp expression

Working with the oriental leafworm moth (*Spodoptera litura*), Shen et. al. (2011) found upregulation on the order of 67-fold in Hsp20.4 when exposed to 40°C for 1 hour. We have a similar result for both Hsp20.4 and Hsp90, in which elevated temperature treatments induced significantly higher gene expression relative to the 22°C treatment, confirming the role of these proteins in heat stress response in *M. cinxia* caterpillars. It is also interesting to note that all Hsps analyzed here tended to decline (though not significantly) at 42°C compared to 38°C (Table 1, Figure 2), suggesting that Hsp synthesis itself may be limited by thermal stress (Tomanek, 2002).

Working with the silkworm, *Bombyx mori*, Li et. al. (2009) found that Hsp21.4 was not induced by thermal stress, and was expressed constitutively under non-stressful conditions in fat body and other tissues. The authors speculate that Hsp21.4 may not be involved in the heat shock response, and may instead be involved in basic metabolic processes in insects. Shen et. al. (2011) also suggest that Hsp21.4 may have no direct relationship with thermal response. While the function of the small Hsps is not yet fully understood, they have been shown to be involved in the organization of cytoskeletons and the protection of insects during diapause (Rinehart & Denlinger, 2000; Yocum et al., 1998). In the current study we also found that Hsp21.4 showed no significant upregulation in response to increased temperatures. However, the population effect was significant for Hsp 21.4. Catalunya had a 3.3-fold higher constitutive expression than the Åland Islands, suggesting that there may, after all, be a role for this protein in protection from thermal stress.

Possible divergence in energetic homeostasis

It is notable that the genes that did show significant between-population differences in our experiment (Hsp 21.4 and G3PDH) were not the ones that responded to high temperature treatments. G3PDH has a well-established function in energy metabolism (glycolysis and gluconeogenesis) but also in arresting cell cycle under conditions of low metabolism (Seidler 2013). Hsp21.4 has also been hypothesized to serve general homeostatic function in insects (Li et al., 2009). Their constitutive between-population difference might therefore reflect differences in metabolism and its effect on growth. Although such metabolic adjustments are not necessarily related to adaptation to local conditions, previous results suggest such a possibility. For example, polymorphism in another glycolytic enzyme, phosphoglucose isomerase (*pgi*), is strongly associated with fitness and performance in the same species of butterfly that we studied (Hanski & Saccheri, 2006; Haag et al., 2005), and correlates with temperature in several other organisms (Hoffmann, 1981; Watt, 1991). Other glycolytic enzymes have been reported to vary adaptively along latitudinal gradients (Lin & Somero, 1995b; Place & Powers, 1978).

Are heat-shock proteins involved in adaptation to local temperature?

Studies of Hsp expression across geographic clines in insects have found variable results. Sorensen et. al. (2005) working with *Drosophila buzzatii* found no differences in Hsp70 expression between populations from different altitudes tested at 36.5°C. However, at a higher temperature of 38°C there was a positive relationship between gene expression and altitude. Likewise, working with three different *Drosophila* species, Krebs (1999) found that the more heat tolerant desert species (*D. mojavensis*) expressed Hsp70 at higher temperatures than the other, more cold adapted species (*D. melanogaster* and *D. simulans*). These results support the hypothesis that adaptation to high temperature is manifested not as adjustment of the constitutive Hsp expression level, but as diminished response to heat in heat-adapted populations (Sorensen et. al., 2001). The idea here is that

heat-adapted populations are more tolerant of sub-lethal heat exposure, and thus express lower amounts of stress proteins.

It has also been predicted that organisms from low-stress environments might exhibit reduced (rather than elevated) stress response compared to organisms from high stress environments (Feder & Hofmann, 1999) because of lack of selection for stress-induced plasticity. Some experiments support this theory as well. For example, in the Copper butterfly (*Lycaena tityrus)* high altitude individuals exhibited much weaker Hsp70 induction in response to heat than low-altitude individuals (Karl et al. 2009).

Finally, at least in some cases the adaptation to a different temperature regime might involve adjustments of constitutive level of Hsp expression: for example, chrysomelid beetles from low altitudes have been shown to constitutively express more Hsp70 than those from high altitudes (Dahlhoff & Rank, 1998).

Our results do not support the hypotheses implying differential Hsp response, as we detected no difference in response magnitude between populations (figure 2). A tendency of both heat-responsive Hsps (Hsp20.4 and Hsp90) to be constitutively expressed at a higher level in animals from the warmer location (Catalunya, figure 2) could be interpreted in favor of the constitutive adjustment hypothesis; however, these trends were not statistically significant.

Conclusion

While there is still no consensus on how different thermal tolerances across the species range are determined at the gene expression level, correlations between physiological traits and environmental stresses offer clues to future responses of organisms to global change. Studies such as this provide insight into how organisms cope with exposure to acute heat stress, and give us an idea of the range over which a physiological stress response is observed. From these data we may then understand the critical threshold for

such organisms, and gain some insight into how poikilothermic species, such as *M. cinxia,* may be affected by climate change.

Acknowledgements

Galina Aglyamova

Eli Meyer,

Wayne Hall

National Science Foundation grant DEB-1054766 to M.V.M.

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Figure 1: Map showing the study populations, monthly mean maximum temperatures (1981-2013) during the post-diapause larval stage/adult butterfly flight season and highest observed temperatures (Source: National Climatic Data Center, National Oceanic and Atmospheric Administration (NOAA).

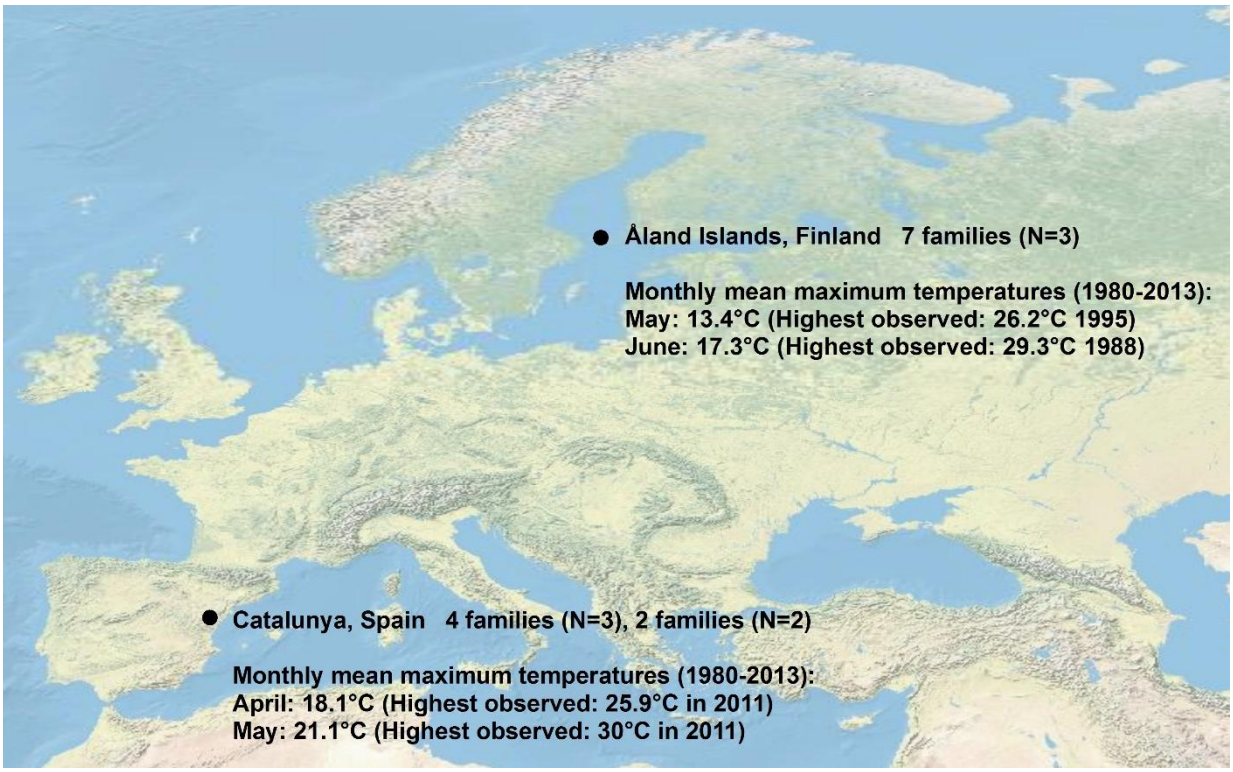

Figure 2: Gene expression changes

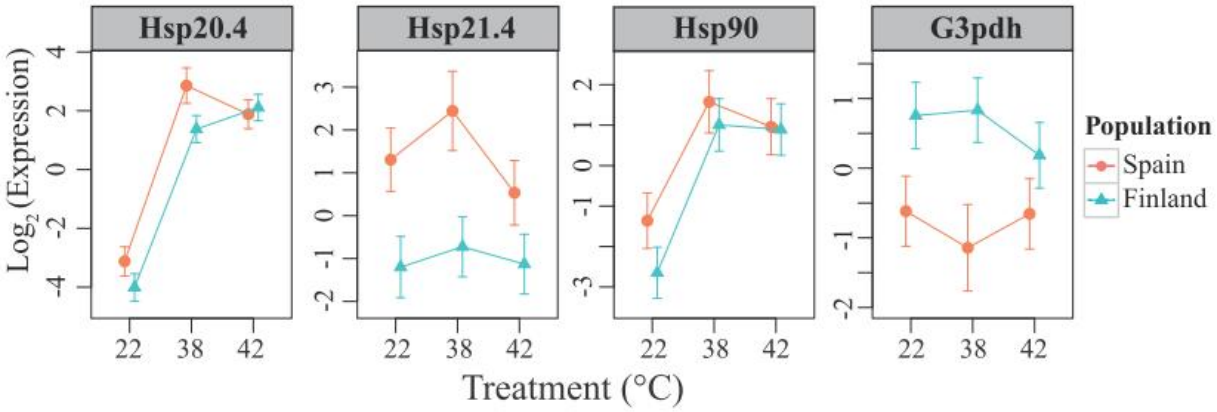

Table 1: Summary of MCMCglmm models for gene expression differences observed between different populations and between different temperature treatments. Significant differences are shaded in grey.

Gene	Population/Treatment	Fold Change*	P(MCMC)**
Hsp20.4	Catalunya:Finland	1.5	0.2
	T38:T22	63.1	<0.001
	T42:T22	32	<0.001
	T42:T38	-2	0.5
Hsp21.4	Catalunya:Finland	3.3	0.023
	T38:T22	2.2	0.31
	T42:T22	-1.8	0.39
	T42:T38	-3.8	0.39
Hsp90	Catalunya:Finland	1.9	0.15
	T38:T22	7.6	<0.001
	T42:T22	5	<0.001
	T42:T38	-1.5	0.34
G3pdh	Catalunya:Finland	-1.9	0.04
	T38:T22	-1.4	0.49
	T42:T22	1	0.96
	T42:T38	1.4	0.54

* negative values imply fold-change of the listed amplitude in the opposite direction

** empirical two-tailed p-value derived from the results of MCMC sampling

Supplementary Table 1: Primer efficiencies ($2^{-(1/slope)}$) for Hsp 20.4, Hsp 21.4, Hsp 90, G3PDH, elf5B and Beta Actin used for all qPCR reactions

Gene	Trial	Slope	R^2	Primer Efficiency
Hsp20.4	1	-1.066	0.998	
	2	-1.017	0.997	
	Average slope	-1.0415		1.95
Hsp21.4	1	-1.01	0.99	
	2	-1.03	0.99	
	Average slope	-1.02		1.97
Hsp90	1	-0.954	0.989	
	2	-0.998	0.993	
	Average slope	-0.976		2.03
G3PDH	1	-0.96	0.98	
	2	-1.03	0.99	
	Average slope	-0.995		2.01
elF5B	1	-1.021	0.997	
	2	-1.026	0.999	
	Average slope	-1.0235		1.97
Beta Actin	1	-1.1	0.98	
	2	-1.05	0.99	
	Average slope	-1.075		1.91