to stress, and with the stress levels naturally experienced by each species (Feder & Hofmann, 1999). Hsp expression patterns can also vary within a species (Brown *et al.*, 1995; Otsuka *et al.*, 1997; Tomanek, 2010), with respect to both temporal and spatial differences in environmental stress. Consequently, the quantification of Hsp expression levels has been proposed as a standard metric for estimating stress responses in natural populations (Evans & Hofmann, 2012).

Both constitutive and inducible forms of Hsp70 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 contain higher levels of Hsp70 than those collected at cooler times (Dahlhoff & Rank, 2000). Sørensen *et al.* (2009) report that fruit flies that cannot induce heat shock proteins are incapable of finding food stations on hot days, whereas wild-type conspecifics can. In marine snails (*Nucella spp.*), a higher expression of Hsps is correlated with increased thermotolerance, and the level of total rather than stress-inducible Hsp70 is a better predictor of thermal tolerance (Sorte & Hoffmann, 2005). Conversely, individuals of the coral *Acropora hyacinthus* that regularly experience thermal extremes up-regulate stress response transcripts constitutively compared with conspecifics from less variable environments (Barshis *et al.*, 2013). Constitutive up-regulation of stress response genes is also observed in populations of springtails *Orchesella cincta* (Collembola, Entomobryidae) living in metal contaminated mining sites (Roelofs *et al.*, 2007, 2009). Studies such as these are important for establishing an ecological context for Hsp expression.

Because of the roles that Hsps have in mediating thermal tolerance, their expression is expected to feature in local adaptation to climate. Expected correlations between Hsps and thermal environment are indeed observed in clines based on elevation for *Drosophila buzzatii* (Sørensen *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 only occasionally report correlations with latitude (e.g. in the mussel *Mytilus galloprovincialis*, Dutton & Hofmann, 2009). These studies do not produce results as conclusive as those completed on smaller scales (e.g. microclimatic variation in temperature) and the results are often complex and affected by other unknown factors (Sørensen *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 chosen study organism, the non-migratory melitaeine butterfly *Melitaea cinxia* L, fulfils this description. The European latitudinal range of *M. cinxia* at low elevation extends 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 are not included in the present study, extend the range southwards to around 35°N. The physiological ecology of *M. cinxia* is becoming well known, especially with respect to the traits involved in dispersal and metabolic rate, which are likely to affect thermal tolerance (Haag *et al.*, 2005; Hanski, 2011). This is a species in which Hsp variation is already known to be important: within the Finnish population, natural variation in Hsp70 is associated with flight metabolic rate and thoracic temperature at take-off (Mattila, 2015) and *M. cinxia* from Finland have higher Hsp70 expression than those from China, both before and after thermal stress (Luo *et al.*, 2014).

In the present study, Hsp gene expression in *M. cinxia* is compared between populations sampled from the latitudinal range limits of the species at low elevation in Finland and Spain. Analysis of mitochondrial DNA haplotypes indicates that the most recent common ancestor of these study populations existed at least 500 000 generations ago (Wahlberg & Saccheri, 2007); hence, there is much potential for differences to have evolved between them. Indeed, they are already known to differ in a physiologically important trait: the frequency of alleles affecting tracheal development and oxygen delivery (Marden *et al.*, 2013). The expectation for the present study is that butterflies at the southern range limit, being more often exposed to extreme heat events, may have either a higher level of constitutive Hsp gene expression and/or stronger Hsp induction in response to thermal stress. This prediction is tested using a single life-history stage: last-instar (post-diapause) larvae.

Materials and methods

Gene selection and primer design

Heat shock protein and control genes were selected using the transcriptome for *M. cinxia* available at http://cinxiabase.vmhost.psu.edu/TextSearch2.html. A search for 'hsp' resulted in a number of potential sequences, and those that matched with genes from other Lepidoptera were selected as potential candidate genes. Sequences annotated as Hsps were verified using BLAST (Altschul *et al.*, 1997) against the nonredundant ('nr') Genbank database (NCBI). Primers were designed to target 150 bp of these sequences using PRIMER3 (http://primer3.sourceforge.net) (see Supporting information, Table S1). Three potential control genes were selected based on their stability in other expression studies across taxa (Czechowski *et al.*, 2005; de Kok *et al.*, 2005; Wang *et al.*, 2008; Pijpe *et al.*, 2011; Ponton *et al.*, 2011; Stephens *et al.*, 2011).

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 2 orders of magnitude of template amount [5 to 0.078 ng RNA equivalent per polymerase chain reaction (PCR) reaction]. These reactions were all conducted in duplicate. Crossing point (Cp) values for each dilution series were then plotted against the $\log_2$[cDNA] and the slope determined for each primer set (see

Supporting information, Table S2). The primer-specific amplification efficiency (*E*, amplification factor per PCR cycle) was then derived from the slope of the regression [$E = 2^{-(1/\text{slope})}$] (Pfaffl, 2001). The quantitative PCR (qPCR) assays accepted for the present study exhibited PCR efficiencies within the range 1.91–2.03 (r^2 values in the range 0.98–0.999). To test for primer specificity and genomic DNA contamination, a negative control was run, lacking reverse transcriptase. No amplification was observed in the absence of reverse transcriptase. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH), elongation initiation factor 5B and β-actin were selected as potential control genes, the stability of which was tested using GENORM (Vandesompele *et al.*, 2002). Hsp20.4, Hsp21.4 and Hsp90 were selected as the target genes of interest.

Background on experimental design

At the selected study sites (i.e. at both of the range extremes), *M. cinxia* has a single generation per year, and spends winter in diapause as partly-developed larvae. In early spring, these thermophilic larvae begin to bask in sunshine and feed on new leaves of their food plants: *Plantago lanceolata* in Spain, and both *P. lanceolata* and *Veronica spicata* in Finland (Van Nouhuys *et al.*, 2003). Because these insects are active at slightly different seasons in different latitudes, it is possible that they may be exposed to similar temperatures across the range at each life-cycle stage. To test this possibility, the long-term (1998–2015) monthly mean maximum temperatures were obtained for the study sites (www.wunderground.com), as well as air temperature logger data from a butterfly habitat in Viladrau, Spain (courtesy of J. H. Marden, Penn State University, Pennsylvania, U.S.A.). Data loggers were placed 25 cm above the ground, in the sun and shade, and air temperatures were recorded between 10.00 and 15.00 h over a 13-day period at the end of May 2011 within the Viladrau *M. cinxia* habitat. These measurements were then compared with data from the same dates at Sant Hilari, Spain (www.wunderground.com), which is the nearest weather station at approximately the same elevation as Viladrau.

A number of pilot experiments were conducted using different temperature regimes similar to those used in other studies (Sørensen *et al.*, 2005; Karl *et al.*, 2008; Shen *et al.*, 2011). The eventual experimental temperatures of 22, 38 and 42 °C were selected based on these pilot experiments, as well as on maximum temperatures to which the species is currently exposed. Although the experimental temperatures were higher than ambient air temperatures 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. This was tested by obtaining thermal camera images of larvae basking in the sun and in the growth chamber. Therefore, in this experiment, ambient temperatures were chosen in the growth chamber to mimic the body temperatures of the caterpillars basking in the sun, rather than natural ambient air temperatures.

Larvae for these experiments were obtained by field-gathering adults, eggs or very young (pre-diapause) larvae from the two populations. All larvae were raised under controlled laboratory conditions from pre-diapause (room temperature, 22 °C) through diapause (4 °C). However, it remains possible that their experience prior to being collected may have influenced their performance because the 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). Larvae from six family broods of *M. cinxia* and seven from Finland were then taken out of diapause in the spring, and allowed to feed on *Plantago lanceolata* at room temperature (22 °C). In their final instar, three caterpillars were sampled from each family, and each individual was assigned to one of three temperature regimes in a climate-controlled growth chamber with artificial lights and freshly cut leaves:

- 1 h at 22 °C, followed by 1 h of recovery at room temperature (22 °C) (control)
- 1 h at 38 °C, followed by 1 h of recovery at room temperature (22 °C)
- 1 h at 42 °C, followed by 1 h of recovery at room temperature (22 °C).

Out of the six family batches from Spain, two families had only two progeny. For these families, only the treatments at 22 and 42 °C were used. After the recovery period, caterpillars were cut in half and each half was placed in a separate vial of RNAlater (Ambion, Inc., Foster City, California). The head region of each larva was kept at room temperature and used for RNA isolation.

RNA isolation

RNA was extracted from the samples using RNAqueous 4PCR kits (Ambion, Inc.). The concentration of RNA was then quantified using the Nanodrop 2000 (Thermo-Fisher Scientific, Waltham, Massachusetts). 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 estimated again and another electrophoresis gel was 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, Japan). Fifty nanograms of RNA from each sample were brought to a total volume of 4 μL using Milli-Q water (Merck-Millipore, Billerica, Massachusetts). One microlitre of a 6 μM solution of an oligo-dT-containing primer (5′-CGCAG TCGGTACTTTTTTTTTTTV-3′) was added to each of the above sample dilutions, incubated at 65 °C for 3 min, and then 5 μL of a master mix (0.5 μL of H_2O, 1 μL of dNTPs, 1 μL of dithiothreitol, 2 μL of 5x buffer and 0.5 μL of SSII reverse

transcriptase) was added to each sample. A no-reverse transcriptase (RT) control was also synthesized for each sample, under the same conditions described above but lacking the RT, and instead containing 1 μL of H_2O. All samples were then incubated at 42 °C for 1 h, followed by 65 °C for 3 min. Finally, each of the samples was diluted to contain a cDNA equivalent of 1 ng μL^{-1} RNA, by adding Milli-Q water.

qPCR

The qPCR reactions were conducted using the LightCycler 480 (Roche Diagnostics, Indianapolis, Indiana). All qPCR reactions were conducted in duplicate. One nanogram of each cDNA template was mixed with 4.5 μL of H_2O and 7.5 μL of 2x SYBR-green Master Mix (Roche Diagnostics). This mixture was then added to the well plate (LightCycler 480 multiwell plate 384, white; Roche Diagnostics) and 2 μL of 1.5 μM 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 Diagnostics), spun down and run in the LightCycler 480 with the protocol: 1x pre-incubation (95 °C for 5 min), 45x amplification (95 °C for 30 s, 60 °C for 40 s, 72 °C for 40 s), 1x melting curve (95 °C for 5 s, 65 °C for 1 min, slow ramping up to 97 °C), 1x cooling (40 °C for 10 s). Visual inspection of product melt curves was used to confirm accumulation of specific target gene product.

Statistical analysis

The analysis of qPCR data was performed in R (R Foundation for Statistical Computing, Austria) using the MCMC.qpcr package (Matz *et al.*, 2013) (see Supporting information, Table S3). 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 as a result of template loading from biologically relevant gene expression changes without relying on control genes, its power is substantially enhanced when control genes are specified. The modelling was therefore performed using the 'classic' model that follows the established multigene normalization procedure, consisting of dividing all the gene expression values by the harmonic mean of the expression values of the two control genes to account for unequal RNA input across samples (Vandesompele *et al.*, 2002). The unit of biological replication used was a family brood, so the replication level was $n = 6–7$ per population per treatment. To boost statistical power, the qPCR model included scalar effect of the family as a random factor and, because every family contributed to control and heated treatments, this makes the analysis analogous to a paired design.

Results

Macroclimatic conditions

In Finland, last-instar *M. cinxia* larvae are typically active in May and butterflies in June (Kuussaari *et al.*, 1996). In Spain, the phenology is approximately 1 month earlier, with last-instar larvae typically active in April and butterflies in May (C. Stefanescu, personal communication). The present study examined whether this phenological difference in activity was sufficient to counter the underlying climatic differences between these distant sites. If so, such phenological shifting might expose populations to similar macroclimatic conditions across the range, at each life-cycle stage. Long-term (1998–2015) monthly mean maximum temperatures for May (12.9 °C) and June (17.5 °C) at Mariehamn in the Åland islands, Finland, and for April (19.7 °C) and May (23.2 °C) at Girona in Catalunya, Spain (www.wunderground.com), show that variation in phenology is insufficient to maintain a common macroclimate experience for either larvae or adult butterflies between the geographical areas comprising northern and southern range limits of *M. cinxia*. Despite their earlier phenology, the Spanish populations experience hotter macroclimates.

Local conditions

Macroclimatic conditions recorded at weather stations may not reflect air temperatures experienced by the butterfly populations, and the latitudinal difference in climate could be mitigated if the insects were to occupy cool habitats at their southern range margin and warm sites at the northern range margin (Suggitt *et al.*, 2012). Somewhat unexpectedly, the butterflies are observed to occupy hot local habitats at both northern and southern range extremes. At the Spanish study site in Sils, Catalunya, *M. cinxia* occupy open fields without shade, are not biased towards north-facing slopes, and lay eggs close to the ground, a behaviour that can increase egg space temperatures by as much as 20 °C above ambient air (Bennett *et al.*, 2015). A similar thermophilic behaviour for *M. cinxia* has been reported at a different Spanish study site in Viladrau, Catalunya (J. H. Marden, unpublished observations). In the U.K., adult *M. cinxia* are reported to choose particularly hot plants for oviposition (Curtis & Isaac, 2015). To check these anecdotal observations suggesting that Spanish insects select hot habitats, air temperature measurements (courtesy of J. H. Marden) were analyzed, as made over a 13-day period at the end of May 2011 within the Viladrau habitat of *M. cinxia*. From 137 measurements in the shade taken when butterflies were active, the mean temperature was 26.1 °C. For the same period, the mean maximum daily temperature at Sant Hilari (nearest weather station) was calculated as 20.7 °C, which is more than 5 °C cooler than the mean at Viladrau (*M. cinxia* microhabitat). These data support the anecdotal observations (J. H. Marden, unpublished observations) that the *M. cinxia* studied at Viladrau, Spain, are not mitigating their exposure to thermal stress by choosing cool habitats. The choice of habitat by the Spanish population of *M. cinxia* may fail to protect them from exposure to high local air temperatures. It strengthens

expectation of the evolution of physiological adaptation to locally-hot climatic conditions.

Gene selection and normalization

Hsp70 was chosen initially as the primary target gene for comparison with other studies. However, all primer pairs designed for the homologous sequence from *M. cinxia* (contig 56282; see Supporting information, Table S1) 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. Luo *et al.* (2015) have subsequently found multiple primers for Hsp70 in *M. cinxia*, using Hsp70 sequences from *Bombyx mori* to compare using the Basic Local Alignment Search Tool (www.blast.ncbi.nlm.nih.gov) against the now published genome of *M. cinxia*. Of the putative control genes, GENORM analysis (Vandesompele *et al.*, 2002) suggested that G3PDH is not sufficiently stable to serve as a control (GENORM $M = 1.57$). This result was confirmed by 'naïve' (control-free) Bayesian analysis using the MCMC.qpcr package, which indicated that G3PDH is differentially expressed among populations. Therefore, only β-actin and elongation initiation factor 5B were used as control genes for Bayesian modelling (GENORM $M = 0.99$) when analyzing G3PDH as a response gene along with the Hsps. No-RT controls were >40, indicating that genomic DNA contamination was negligible (see Supporting information, Table S4).

Gene expression

Gene expression changes are summarized in Fig. 1 and Table 1. Overall, the genes that were least inducible were those that showed the clearest constitutive differences between the study sites. Hsp21.4 exhibited significant constitutive differences ($P = 0.02$) between populations, being expressed 3.3-fold higher in the Spanish population (hotter climate). The other two Hsps (Hsp20.4 and Hsp90) exhibited the same trend towards higher expression in Spain, although between-population differences for these two genes were not statistically significant. G3PDH also exhibited significant constitutive differences ($P = 0.04$) between populations, with expression being 1.9-fold higher in the Finland population. Neither Hsp21.4, nor G3PDH responded significantly to heat stress treatment.

With respect to heat stress treatment, Hsp20.4 demonstrated significant up-regulation at 38 °C (63-fold, $P < 0.001$) and 42 °C (32-fold, $P < 0.001$) relative to the 22 °C control. Hsp90 also demonstrated up-regulation at 38 °C (7.6-fold, $P < 0.001$) and 42 °C (5-fold, $P < 0.001$) relative to the 22 °C control. There was no significant difference between 38 and 42 °C for any of the Hsps, although all three exhibited a trend towards reduced expression at 42 °C (relative to 38 °C). No interaction terms between population and temperature treatment were statistically significant for any of the genes tested.

Table 1. Summary of Markov chain Monte Carlo (MCMC) models for gene expression differences observed between different latitutinal populations of the Glanville fritillary butterfly, *Melitaea cinxia* and between different temperature treatments.

Gene	Population/treatment	Fold change[a]	*P* (MCMC)[b]
Hsp20.4	Spain : Finland	1.5	0.2
	T38 : T22	63.1	<0.001
	T42 : T22	32	<0.001
	T42 : T38	−2	0.5
Hsp21.4	Spain : Finland	3.3	0.023
	T38 : T22	2.2	0.31
	T42 : T22	−1.8	0.39
	T42 : T38	−3.8	0.39
Hsp90	Spain : Finland	1.9	0.15
	T38 : T22	7.6	<0.001
	T42 : T22	5	<0.001
	T42 : T38	−1.5	0.34
G3PDH	Spain : Finland	−1.9	0.04
	T38 : T22	−1.4	0.49
	T42 : T22	1	0.96
	T42 : T38	1.4	0.54

[a]Negative values imply fold-change of the listed amplitude in the opposite direction.

[b]Empirical two-tailed *P*-value derived from the results of Markov chain Monte Carlo (MCMC) sampling.

Significant differences are shaded in grey. Details of the genes analyzed and the temperature regimes are provided in the text (e.g. T38 : T22 = 1 h at 38 °C, followed by 1 h of recovery at 22 °C).

Discussion

Examining how species adapt to climate variation spatially is considered to be a good way of evaluating how they might respond to similar changes in time (La Sorte *et al.*, 2009). Spatial variation in thermal performance is widespread in insects (Sinclair *et al.*, 2012) and metabolically-important enzymes, such as glycolytic enzymes, can vary adaptively along latitudinal gradients (Place & Powers, 1978; Lin & Somero, 1995). The results of studies of Hsp expression across geographical clines in insects suggest how these organisms adapt physiologically to different climates. For example, the heat-tolerant desert species of fruit fly *Drosophila mojavensis* expresses Hsp70 at higher temperatures than two more cold-adapted species, *Drosophila melanogaster* and *Drosophila simulans* (Krebs, 1999). This supports the hypothesis that adaptation to high temperature can be manifested not as an adjustment of the constitutive Hsp expression level but, instead, as a diminished response to heat in heat-adapted populations (Sørensen *et al.*, 2001). The idea is that heat-adapted populations are more tolerant of sub-lethal heat exposure, and thus express lower amounts of stress proteins.

The present study investigates geographical variation in both inducibility and constitutive levels of three heat shock proteins and G3PDH, using populations from the northern and southern range limits of the butterfly *Melitaea cinxia*. Estimates of constitutive expression of all three Hsps are higher in Spain than in Finland, with the difference reaching significance ($P = 0.02$) for Hsp21.4 (Fig. 1 and Table 1). For G3PDH, there is a significant trend in the opposite direction, with higher expression in

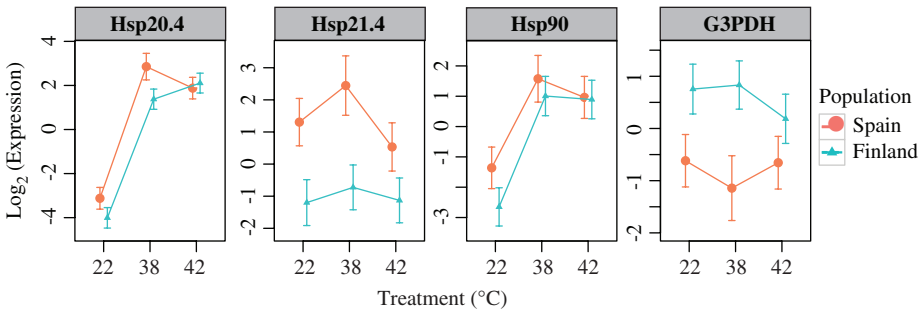

Fig. 1. Log_2-transformed relative gene expression values (mean ± SEM) of four genes (Hsp20.4, Hsp21.4, Hsp90 and G3PDH) from different latitudinal populations of the Glanville fritillary butterfly *Melitaea cinxia* shown with respect to temperature treatment and population origin (G3PDH, glyceraldehyde 3-phosphate dehydrogenase; Hsp, heat-shock protein).

Finland ($P = 0.04$). It is notable that Hsp21.4 and G3PDH do show significant between-population differences but do not respond to high temperature treatments. Conversely, the two Hsps that fail to show significant constitutive difference between Finland and Spain, Hsp20.4 and Hsp90, are highly inducible by heat stress at 38 and 42°C (Fig. 1 and Table 1). There is the suggestion that, in nature, differences between Finland and Spain would occur in the expression of all four traits studied, although these differences would be directly climate-induced in Hsp20.4 and Hsp90 and constitutive in Hsp21.4 and G3PDH. Inducibility may mitigate the need for constitutive variation. It is also interesting to note that all Hsps analyzed in the present study tend to decline (although not significantly) at 42 °C compared with 38 °C (Fig. 1 and Table 1), suggesting that Hsp synthesis itself may be limited by thermal stress (Tomanek, 2002).

Hsp21.4 and G3PDH

Hsp21.4 and G3PDH comprise the two proteins that are not induced in the present experiments. Working with the silkworm *Bombyx mori*, Li *et al.* (2009) similarly report that Hsp21.4 is not induced by thermal stress, and is expressed constitutively under nonstressful conditions in fat body and other tissues. It is speculated that Hsp21.4 may not be involved in the heat shock response and, instead, may 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.

However, in view of the present finding of increased constitutive expression of this protein in the Spanish *M. cinxia*, this question may not yet be resolved: there may, after all, be a role for Hsp 21.4 in protection from thermal stress. It is argued that constitutive over-expression is a common evolutionary response to abiotic stress across taxa (Roelofs *et al.*, 2010). The findings from the present study showing trends towards higher constitutive Hsp expression in *M. cinxia* from the hotter climate have parallels in other studies. For example, field-collected chrysomelid beetles from low altitudes express more Hsp70 than those from higher altitudes (Dahlhoff & Rank, 2000), corals from more thermally variable environments up-regulate Hsp expression constitutively (Barshis *et al.*, 2013) and springtails in mining-contaminated habitats over-express stress response genes (Roelofs *et al.*, 2007, 2009).

G3PDH has a well-established function not only in energy metabolism (glycolysis and gluconeogenesis), but also in arresting cell cycle under conditions of low metabolism (Seidler, 2013). Therefore, one possible explanation for the constitutive between-population difference found in the present study might be that it reflects differences in metabolism. 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, is strongly associated with fitness and performance in the same species of butterfly as that investigated in the present study (Haag *et al.*, 2005; Hanski & Saccheri, 2006) and correlates with temperature in several other organisms (Hoffmann, 1981; Watt, 1991).

Hsp20.4 and Hsp90

Working with the oriental leafworm moth *Spodoptera litura*, Shen *et al.* (2011) report up-regulation to the order of 67-fold for Hsp20.4 when exposed to 40 °C for 1 h. A similar result is reported in the present study for both Hsp20.4 and Hsp90, in which elevated temperature treatments induced significantly higher gene expression relative to the 22 °C treatment. It is predicted that organisms from low-stress environments might exhibit a reduced (rather than elevated) stress response compared with organisms from high stress environments (Feder & Hofmann, 1999) because of a lack of selection for stress-induced plasticity. Some experiments support this theory. For example, in the Copper butterfly (*Lycaena tityrus*), high altitude individuals exhibit much weaker Hsp70 induction in response to heat than low-altitude individuals (Karl *et al.*, 2009). However, in the present study, there is no difference between Finnish and Spanish insects in response of the inducible Hsps (Hsp20.4 and Hsp90) to heat stress, although such stress has presumably been more frequent in Spain. These findings do not support the hypothesis of differential inducibility between environments with different levels of thermal stress.

In general, studies of variation in traits involved in thermal adaptation can improve our understanding of how organisms cope with exposure to heat stress and contribute incrementally to predicting how poikilothermic species, such as *M. cinxia*,

will be affected by climate change. As a study insect, *M. cinxia* has already contributed novel insights into the physiological traits involved in evolution of dispersal, fecundity and oxygen metabolism (Haag *et al.*, 2005; Hanski & Saccheri, 2006; Hanski, 2011; Marden *et al.*, 2013; Mattila & Hanski, 2014). The results of the present study add a climate change dimension to prior work, demonstrating both the genetic and plastic physiological traits that underlie the adaptations of *M. cinxia* to climatic variation across its range.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: DOI: 10.1111/phen.12148

Table S1. Different primer pairs tested. An asterisk (*) indicates the primer pairs eventually used in the experiment.

Table S2. Primer efficiencies [$2^{-(1/\text{slope})}$] for Hsp20.4, Hsp21.4, Hsp90, G3PDH, elf5B and β-actin used for all quantitative polymerase chain reactions.

Table S3. Markov chain Monte Carlo R script (MCMC.qpcr) for analysis of quantitative polymerase chain reaction data.

Table S4. Raw CP values from quantitative polymerase chain reaction reactions.

Acknowledgements

We thank Eli Meyer for help with primer selection and design; Galina Aglyamova and Wayne Hall for help with the laboratory experiments; James Marden and Marjo Saastamoinen for help with obtaining habitat temperature data for *M. cinxia*; and Constanti Stefanescu and Saskya van Nouhuys for help with collecting insects from the field. Research was supported by National Science Foundation grant DEB-1054766 to M.V.M.

References

Altschul, S.F., Madden, T.L., Schaffer, A.A. *et al.* (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, **25**, 3389–3402.

Atkinson, D. & Sibly, R.M. (1997) Why are organisms usually bigger in colder environments? Making sense of a life history puzzle. *Trends in Ecology & Evolution*, **12**, 235–239.

Barshis, D., Ladner, J.T., Oliver, T.A. *et al.* (2013) Genomic basis for coral resilience to climate change. *Proceedings of the National Academy of Sciences of the United States of America*, **110**, 1387–1392.

Bennett, N.L., Severns, P.M., Parmesan, C. & Singer, M.C. (2015) High resilience to climate change predicted in a butterfly with strong local adaptation. *Oikos*, **124**, 42–53.

Brown, D.C., Bradley, B.P. & Tedengren, M. (1995) Genetic and environmental regulation of HSP70 expression. *Marine Environmental Research*, **39**, 181–184.

Curtis, R.J. & Isaac, N.J.B. (2015) The effect of temperature and habitat quality on abundance of the Glanville fritillary on the Isle of Wight: implications for conservation management in a warming climate. *Journal of Insect Conservation*, **19**, 217–225.

Czechowski, T., Stitt, M., Altmann, T. *et al.* (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*. *Plant Physiology*, **139**, 5–17.

Dahlhoff, E.P. & Rank, N.E. (2000) Functional and physiological consequences of genetic variation at phosphoglucose isomerase: heat shock protein expression is related to enzyme genotype in a montane beetle. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 10056–10061.

Dutton, J.M. & Hofmann, G.E. (2009) Biogeographic variation in *Mytilus galloprovincialis* heat shock gene expression across the eastern Pacific range. *Journal of Experimental Marine Biology and Ecology*, **376**, 37–42.

Evans, T.G. & Hofmann, G.E. (2012) Defining the limits of physiological plasticity: how gene expression can assess and predict the consequences of ocean change. *Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences*, **367**, 1733–1745.

Feder, M.E. & Hofmann, G.E. (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual Review of Physiology*, **61**, 243–282.

Haag, C.R., Saastamoinen, M., Marden, J.H. & Hanski, I. (2005) A candidate locus for variation in dispersal rate in a butterfly metapopulation. *Proceedings of the Royal Society of London Series B*, **272**, 2449–2456.

Hanski, I. (2011) Eco-evolutionary spatial dynamics in the Glanville fritillary butterfly. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 14397–14404.

Hanski, I. & Saccheri, I. (2006) Molecular-level variation affects population growth in a butterfly metapopulation. *PLoS Biology*, **4**, e129.

Hoffmann, R.J. (1981) Evolutionary genetics of *Metridium senile* .I. Kinetic differences in phosphoglucose isomerase allozymes. *Biochemical Genetics*, **19**, 129–144.

Hoffmann, A.A., Scott, M., Partridge, L. & Hallas, R. (2003) Overwintering in *Drosophila melanogaster*: outdoor field cage experiments on clinal and laboratory selected populations help to elucidate traits under selection. *Journal of Evolutionary Biology*, **16**, 614–623.

Karl, I., Janowitz, S.A. & Fischer, K. (2008) Altitudinal life-history variation and thermal adaptation in the copper butterfly *Lycaena tityrus*. *Oikos*, **117**, 778–788.

Karl, I., Sorensen, J.G., Loeschcke, V. & Fischer, K. (2009) HSP70 expression in the Copper butterfly *Lycaena tityrus* across altitudes and temperatures. *Journal of Evolutionary Biology*, **22**, 172–178.

Kenkel, C.D., Aglyamova, G., Alamaru, A. *et al.* (2011) Development of gene expression markers of acute heat-light stress in reef-building corals of the genus Porites. *PLoS ONE*, **6**, e26914.

de Kok, J.B., Roelofs, R.W., Giesendorf, B.A. *et al.* (2005) Normalization of gene expression measurements in tumor tissues: comparison of 13 endogenous control genes. *Laboratory Investigation*, **85**, 154–159.

Krebs, R.A. (1999) A comparison of Hsp70 expression and thermotolerance in adults and larvae of three *Drosophila* species. *Cell Stress & Chaperones*, **4**, 243–249.

Kültz, D. (2005) Molecular and evolutionary basis of the cellular stress response. *Annual Review of Physiology*, **67**, 225–257.

Kuussaari, M. (1998) *Biology of the Glanville fritillary butterfly* (Melitaea cinxia). PhD Thesis, University of Helsinki.

Kuussaari, M., Nieminen, M. & Hanski, I. (1996) An experimental study of migration in the Glanville fritillary butterfly, Melitaea cinxia. *Journal of Animal Ecology*, **65**, 791–801.

Lafranchis, T. (2004) *Butterflies of Europe*. Diatheo, France.

La Sorte, F.A., Lee, T.M., Wilman, H. & Jetz, W. (2009) Disparities between observed and predicted impacts of climate change on winter bird assemblages. *Proceedings of the Royal Society of London Series B*, **276**, 3167–3174.

Li, Z.W., Li, X., Yu, Q.Y. *et al.* (2009) The small heat shock protein (sHSP) genes in the silkworm, *Bombyx mori*, and comparative analysis with other insect sHSP genes. *BMC Evolutionary Biology*, **9**, 215.

Lin, J.J. & Somero, G.N. (1995) Thermal adaptation of cytoplasmic malate-dehydrogenases of eastern pacific barracuda (*Sphyraena* spp.) – the role of differential isoenzyme expression. *Journal of Experimental Biology*, **198**, 551–560.

Luo, S., Wong, S.C., Xu, C. *et al.* (2014) Phenotypic plasticity in thermal tolerance in the Glanville Fritillary butterfly. *Journal of Thermal Biology*, **42**, 33–39.

Luo, S., Ahola, V., Shu, C. *et al.* (2015) Heat shock protein 70 gene family in the Glanville fritillary butterfly and their response to thermal stress. *Gene*, **556**, 132–141.

Marden, J.H., Fescemyer, H.W., Schilder, R.J. *et al.* (2013) Genetic variation in signaling underlies quantitative variation in physiological and life-history traits within lowland butterfly populations. *Evolution*, **67**, 1105–1115.

Mattila, A.L.K. (2015) Thermal biology of flight in a butterfly: genotype, flight metabolism, and environmental conditions. *Ecology and Evolution*, **2**, 5539–5551.

Mattila, A.L.K. & Hanski, I. (2014) Heritability of flight and resting metabolic rates in the Glanville Fritillary butterfly. *Journal of Evolutionary Biology*, **27**, 1733–1743.

Matz, M.V., Wright, R.M. & Scott, J.G. (2013) No control genes required: bayesian analysis of qRT-PCR data. *PLoS ONE*, **8**, e71448.

Otsuka, Y., Takano, T.S. & Yamazaki, T. (1997) Genetic variation in the expression of the six hsp genes in the presence of heat shock in *Drosophila melanogaster*. *Genes & Genetic Systems*, **72**, 19–24.

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, **29**, e45.

Pijpe, J., Pul, N., van Duijn, S. *et al.* (2011) Changed gene expression for candidate ageing genes in long-lived *Bicyclus anynana* butterflies. *Experimental Gerontology*, **46**, 426–434.

Place, A.R. & Powers, D.A. (1978) Genetic bases for protein polymorphism in fundulus-heteroclitus (l). 1. Lactate-dehydrogenase (ldh-b), malate-dehydrogenase (mdh-a), glucosephosphate isomerase (gpi-b), and phosphoglucomutase (pgm-a). *Biochemical Genetics*, **16**, 577–591.

Ponton, F., Chapuis, M.-P., Pernice, M. *et al.* (2011) Evaluation of potential reference genes for reverse transcription-qPCR studies of physiological responses in *Drosophila melanogaster*. *Journal of Insect Physiology*, **57**, 840–850.

Roelofs, D., Marien, J. & Van Straalen, N.M. (2007) Differential gene expression profiles associated with heavy metal tolerance in the soil insect *Orchesella cincta*. *Insect Biochemistry and Molecular Biology*, **37**, 287–295.

Roelofs, D., Janssens, T.K.S., Timmermans, M.J.T.N. *et al.* (2009) Adaptive differences in gene expression associated with heavy metal tolerance in the soil arthropod *Orchesella cincta*. *Molecular Ecology*, **18**, 3227–3239.

Roelofs, D., Morgan, J. & Sturzenbaum, S. (2010) The significance of genome-wide transcriptional regulation in the evolution of stress tolerance. *Evolutionary Ecology*, **24**, 527–539.

Seidler, N.W. (2013) GAPDH: biological properties and diversity. *Advances in Experimental Medicine & Biology*, **985**, 1–291.

Shen, Y., Gu, J., Huang, L.H. *et al.* (2011) Cloning and expression analysis of six small heat shock protein genes in the common cutworm, *Spodoptera litura*. *Journal of Insect Physiology*, **57**, 908–914.

Sinclair, B.J., Williams, C.M. & Terblanche, J.S. (2012) Variation in thermal performance among insect populations. *Physiological & Biochemical Zoology*, **85**, 594–606.

Sørensen, J.G., Dahlgaard, J. & Loeschcke, V. (2001) Genetic variation in thermal tolerance among natural populations of *Drosophila buzzatii*: down regulation of Hsp70 expression and variation in heat stress resistance traits. *Functional Ecology*, **15**, 289–296.

Sørensen, J.G., Norry, F., Scannapeico, A.C. & Loeschcke, V. (2005) Altitudinal variation for stress resistance traits and thermal adaptation in adult *Drosophila buzzatii* from the New World. *Journal of Evolutionary Biology*, **18**, 829–837.

Sørensen, J.G., Loeschcke, V. & Kristensen, T. (2009) Lessons from the use of genetically modified *Drosophila melanogaster* in ecological studies: Hsf mutant lines show highly trait-specific performance in field and laboratory thermal assays. *Functional Ecology*, **23**, 240–247.

Sorte, C.J.B. & Hoffmann, G.E. (2005) Thermotolerance and heat-shock protein expression in Northeastern Pacific *Nucella* species with different biogeographical ranges. *Marine Biology*, **146**, 985–993.

Stephens, A.S., Stephens, S.R. & Morrison, N.A. (2011) Internal control genes for quantitative RT-PCR expression analysis in mouse osteoblasts, osteoclasts and macrophages. *BMC Research Notes*, **4**, 410.

Suggitt, A.J., Stefanescu, C., Paramo, F. *et al.* (2012) Habitat associations of species show consistent but weak responses to climate. *Biology Letters*, **8**, 590–593.

Tomanek, L. (2002) The heat-shock response: its variation, regulation and ecological importance in intertidal gastropods (genus *Tegula*). *Integrative and Comparative Biology*, **42**, 797–807.

Tomanek, L. (2010) Variation in the heat shock response and its implication for predicting the effect of global climate change on species' biogeographical distribution ranges and metabolic costs. *The Journal of Experimental Biology*, **213**, 971–979.

Vandesompele, J., De Preter, K., Pattyn, F. *et al.* (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, **3**, 0034.1.

Van Nouhuys, S., Singer, M.C. & Nieminen, M. (2003) Spatial and temporal patterns of caterpillar performance and the suitability of two host plant species. *Ecological Entomology*, **28**, 193–202.

Wahlberg, N. & Saccheri, I. (2007) The effects of Pleistocene glaciations on the phylogeography of *Melitaea cinxia* (Lepidoptera: Nymphalidae). *European Journal of Entomology*, **104**, 675–684.

Wang, G.-H., Xia, Q.-Y., Cheng, D.-J. *et al.* (2008) Reference genes identified in the silkworm *Bombyx mori* during metamorphism based on oligonucleotide microarray and confirmed by qRT-PCR. *Insect Science*, **15**, 405–413.

Watt, W.B. (1991) Biochemistry, physiological ecology, and population-genetics – the mechanistic tools of evolutionary biology. *Functional Ecology*, **5**, 145–154.

Accepted 15 April 2016
First published online 20 May 2016