Effects Of Acclimation, Population, And Sex On Behavioral Thermoregulation, CT_{Max}, Symptoms Of Heat Stress, And Gene Expression of *Melanoplus Differentialis*, A Generalist Grasshopper - Does Temporal Thermal Heterogeneity Prepare Populations For A Warming World?

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Abstract

Insects thermoregulate using both canalized and plastic mechanisms. Populations of insects utilize these mechanisms to different extents, and while it is posited that the degree of thermal fluctuation a population experiences can determine the optimal combination of mechanisms to utilize, this is still being elucidated. We used three populations of the generalist grasshopper, *Melanoplus differentialis* (Thomas, 1856), from sites experiencing different degrees of thermal heterogeneity to test for correlations between thermal heterogeneity and 1) behavioral thermoregulation, 2) upper temperature tolerance, 3) the ability to thermally acclimate, and 4) gene expression. We found that 1) behavioral thermoregulation did not differ among sites, 2) CT_{Max} of males, but not females, was higher at more thermally heterogeneous sites, 3) there was acclimation in some of the tested traits, but thermally heterogeneous sites did not always have the most plastic individuals, and 4) there were differences in gene expression among sites, but these differences were not between the most and least thermally heterogeneous sites. We concluded that thermal heterogeneity may play a selective role in some, but not all, of the measured thermoregulatory traits and their plasticity.

Introduction

All insects experience fluctuating temperatures. While fluctuation within a range of temperatures around a thermal optimum is tolerated and can be beneficial (Adamo and Lovett 2011), exceeding this range in either direction can also result in detrimental effects (Colinet et al. 2015). One of the mechanisms insects have evolved to enhance thermal tolerance is canalized physiological thermoregulation. This has been demonstrated in the lab, where artificial selection can produce individuals with increased cold tolerance (Condon et al. 2015), and in the field, where extreme thermal specialists can utilize canalized thermoregulation to withstand temperatures as high as 62.2 (Bernstein 1979) and as low as -24° C (Slabber et al. 2007). The ability to thermoregulate in this manner can vary among species and populations (Slatyer et al. 2016). While a canalized ability to withstand hot or cold temperatures can alleviate some of the negative effects of temperature stress on populations, evidence indicates that a canalized response alone is not enough to alleviate effects of hazardous temperatures. For example, Mitchell & Hoffmann (2010), who tested *Drosophila melanogaster* for heritability of thermal resistance, concluded that there was little evolutionary potential to widen their thermal tolerance via canalized thermoregulation. Thus, plastic responses (e.g. Andrew et al. 2013) may be favored as complementary mechanisms to offset deleterious temperatures, as thermotolerant species can also be plastic (Verberk et al. 2018).

Plastic responses have an advantage over canalized responses in that they can be induced when needed and are adaptive in situations of relatively sudden and extreme shifts in temperature (Angilletta 2009), as in the current situation of climate change. Acclimation is a plastic response that has been defined as an adaptive compensatory change in an individual caused by a shift in an environmental variable or variables (Bullock 1955, Precht 1955, Prosser 1955). Thermal acclimation can enhance performance near the low (Mutamiswa et al. 2018) and high (Sømme 1982, Dietz & Somero 1992) ends of an individual's

thermal tolerances or expand thermal tolerance, and has been demonstrated in insects (e.g. Allen et al. 2012; Piyaphongkul et al. 2018). Thermal acclimation can be induced by long-term exposure to a new temperature which imparts irreversible changes (developmental acclimation; Angilletta 2009). Developmental acclimation is defined as the irreversible plasticity of a physiological trait in response to an isolated environmental variable, such as temperature, which is experienced during ontogeny (Angilletta et al. 2006). It permanently affects insects physiologically (Hercus et al. 2003), and has been documented in a variety of insect species (Meats 1976, Fischer et al. 2011, Andrew et al. 2013, Van Dooremalen et al. 2013, Gerken et al. 2015), producing individuals that have increased tolerance to hot and cold temperatures (Piyaphongkul et al. 2014, Javal et al. 2016). Both populations and individuals can vary in their ability to acclimate (Tufto 2000, Kristensen 2004, Ellers et al. 2008).

Insects can also use behavioral thermoregulation (i.e. thermoregulation that is under voluntary control by the organism) to combat extreme temperatures. This has been shown to be effective in alleviating non-optimal environmental temperatures in orthopterans (Clissold 2013, Harris et al. 2015) and evolves in response to climate change-induced thermal heterogeneity in other taxa (Nussey et al. 2005). Behavioral thermoregulation is widespread in acridids (e.g. Forsman 2000, Coggan et al. 2011, Harris et al. 2015), and Forsman et al. (2002) concluded that differences in behavioral thermoregulation between individual orthopterans were genetically, rather than environmentally, determined.

It is tempting to assume that an organism that behaviorally thermoregulates does not need to utilize other types of thermoregulation, but this is not necessarily true. In a thermophilic ant, an extreme form of foraging restriction optimizes its available time between periods of high predator exposure and heat stress (Wehner 1992). However, this ant species is also able to maintain production of heat shock proteins at a temperature 6° C higher than another ant species adapted to a temperate climate (Gehring &Wehner 1995). Thus, insects in a thermally stressful environment may utilize both behavioral and physiological mechanisms to maintain homeostasis. As insects achieve maintenance of optimal temperatures by multiple responses, it is important to use more than one metric when determining a population's ability to persist in a situation of fluctuating temperatures.

In addition to physiological and behavioral mechanisms, individuals may also respond to temperature via differential gene expression. For example, variable temperatures can induce differential translation levels of heat shock proteins (HSPs), which can be constitutive or induced by thermal stress (Wang et al. 2019). The patterns of genetic expression and thresholds can be correlated to the amount of thermal stress that individuals are subjected to in the field (Benoit et al. 2019), and species can alter their gene expression seasonally (Semmouri et al. 2020) or daily (Mishra et al. 2019) to match fluctuating environmental stressors. Heat shock protein production has been detected in all organisms where tested for, including insects (see Sørensen et al. 2003 for a review), and its evolution has been artificially induced in the lab (Scheiner 1993, Bettencourt et al. 1999).

Plasticity in a battery of traits has been demonstrated to respond to artificial selection (reviewed in Scheiner 1993; Mallard et al. 2020), and the ability of insects to evolve increased plasticity is not under

debate. However, what drives the evolution of increased plasticity in the field is less clear. Extreme temperatures alone are not expected to drive the evolution of plasticity, as, in some species, selection for enhanced thermal tolerance can reduce plasticity in thermal tolerance (Kelly et al. 2017). Theory posits that populations will be more plastic when they experience environmental heterogeneity (Lynch & Gabriel 1987, Moran 1992, Scheiner & Holt 2012, Scheiner 2013), particularly when it is predictable (Reed et al. 2010). This has been supported by results from modeling experiments (e.g. Scheiner & Holt 2012) and multiple field experiments (e. g. Semlitsch et al. 1990, Richter-Boix et al. 2006, Lind & Johansson 2007, Lind et al. 2010). Empirical data from field experiments indicate spatial (Richter-Boix et al. 2006, Lind & Johansson 2007) and temporal (Semlitsch et al. 1990, Lind et al. 2010) environmental heterogeneity as drivers of the evolution of developmental (i.e. irreversible) plasticity. While spatial variation may be important, a modeling experiment by Moran (1992) indicated that physiological plasticity was more readily maintained under a situation of temporal variation as compared to a situation of spatial variation, and that species that inhabit narrower geographic ranges and experience less thermal fluctuation generally do not possess the physiological machinery necessary to maintain homeostasis across more broad temperature ranges (e.g. Strickland et al. 2016). However, Scheiner (2013) conducted an individual-based simulation of the evolution of plasticity that incorporated both spatial and temporal variation at different times and concluded from the results that spatial heterogeneity was more important than temporal. Thus, the most important type of heterogeneity driving phenotypic plasticity is not universally agreed upon and may not be generalizable.

In a previous experiment, we quantified thermal heterogeneity at five sites across the Midwest of the United States over a 101-year period during our experimental species' active season (Preston and Johnson 2020). The animals we used in this experiment were from three of those five sites; Minneapolis, Kansas (KS), Marshall, Missouri (MO), and Mount Vernon, Illinois (IL; Suppl. Figure 1). In that study, we determined that KS had a higher daily temperature range (Suppl. Figure 2 and more variance in daily maximal temperatures than MO and IL. Missouri and IL were more comparable, but MO did have more variance in daily maxima than IL (Suppl. Figure 3).

We raised an F2 generation of the generalist grasshopper *Melanoplus differentialis* (Thomas, 1856) bred from F1 individuals (described in Preston and Johnson 2020) in a warm and cold rearing environment to test the hypotheses that populations experiencing more thermal heterogeneity will: 1) be able to more effectively behaviorally thermoregulate, 2) have a greater ability to withstand extremely high temperatures, 3) display more pronounced acclimation effects in one or more physiological and behavioral thermoregulatory traits in response to variable rearing temperatures, and 4) show differences in gene expression in response to stressfully high temperatures when compared to populations with less thermal heterogeneity.

Materials And Methods

All animals used in this experiment were treated in accordance with the U.S. National Research Council's Guide for the Care and Use of Laboratory Animals, the U.S. Public Health Service's Policy on Humane

Care and Use of Laboratory Animals, and the National Research Council's Guide for the Care and Use of Laboratory Animals.

The experimental population in this study was an F2 generation produced from F1 individuals from three states in the United States: Kansas (KS), Missouri (MO), and Illinois (IL; described in Preston and Johnson 2020). We allowed these individuals to mate randomly with others whose parents were from the same site. For convenience, we refer to these groups of F2 individuals (KS, MO, and IL) and their sites of origin interchangeably as populations hereafter. We subsequently followed one of two rearing protocols described in Preston and Johnson (2020), using the diapause treatment that subjected eggs to 0°C (as opposed to 22°C), as it yielded more hatches in that experiment. We only altered this protocol by raising the F2 generation in either a high or low temperature treatment (see *Experimental rearing temperatures* below) until they reached adulthood, after which we measured them for a variety of traits.

2.1 Experimental rearing temperatures

Immediately after F2 individuals from each site hatched, we divided them into cold- and warm-temperature rearing treatments and raised them to adulthood. For the cold rearing environment, we originally had planned to use 15.6°C. We chose this temperature because it 1) is close to what has been used for rearing *Melanoplus* species in the past (e.g. Harrison 1988), 2) approximates the 101-year mean May (when *M. differentialis* nymphs are developing) daily minimum temperature (DTMin) that our experimental populations have experienced (11.2°C), and 3) was the coolest temperature we were able to maintain with a reasonable amount of error. However, in a pilot assay, we determined that mortality was too high during rearing at 15.6°C for these populations. Thus, we used 21°C instead.

For the warm rearing environment, we used 30°C. Similar to the temperature originally chosen for the cold-acclimated individuals, this temperature 1) is close to what has been used for rearing *Melanoplus* species in the past (Jonas & Joern 2013), 2) approximates these populations' previously experienced maximum 101-year mean June and July daily maximum temperature (31.4°C), and 3) was the warmest temperature we were able to maintain within a reasonable amount of error. For both acclimation treatments, we kept relative humidity at 40%.

2.2 Accuracy of Behavioral Thermoregulation

We conducted behavioral thermoregulation trials to determine individuals' accuracy of behavioral thermoregulation, i.e. the ability to keep their body temperatures (T_bs) close to their preferred temperatures (T_{pref}s). We calculated this using the metric d_b, after Hertz et al. (1993), by taking the absolute deviation of each individual's (T_b) from its T_{pref} using protocols described below. We obtained T_{pref}, d_b, and critical thermal maximum (CT_{Max}) from each individual, allowing a 24-hour period to pass between each type of trial to allow individuals to recover. We have listed the sample size for this and all other non-gene expression trials in Suppl. Table 1).

To determine T_{pref}, we placed each individual into a custom-made 10.16 x 91.44 x 10.16 cm wooden shuttle box (Suppl. Figures 4 and 5). We covered the shuttle box with a clear acrylic sheet secured onto the main body of the shuttle box with adhesive hook and loop fasteners. We divided the shuttle box into seven 10.16 x 13.06 x 10.16 cm partitions (7.62 x 9.8 x 4.92 cm of open space in each partition; Fig. 1). We placed a space heater on one side and a window air conditioning unit on the other and connected each to the shuttle box with aluminum duct and detachable fittings. We cut 2.54 cm square notches into the wooden dividers between each partition to allow individuals free passage. The partitions had a temperature range of 6.9 (closest to the air conditioning unit) to 72.9°C (closest to the space heater). The mean steady state temperatures of the partitions were, from the partition closest to the air conditioning unit to that closest to the space heater, 8.2, 9.2, 11.2, 15.4, 18.0, 44.3, and 67.2°C, respectively. For each T_{pref} trial, we placed an individual into the middle partition and started the trial immediately. After a 15-minute period, we recorded T_b of each individual by immediately inserting a hypodermic needle with an embedded thermocouple under the metasternum of the thorax.

To obtain d_b, we used a protocol identical to that used to determine T_{pref} described above, with the exception that, every three minutes, we rotated the shuttle box 180 ° such that the space heater was heating the end that the air conditioning unit was previously cooling and vice versa. This presented individuals with a microenvironment that varied drastically in temperature over time, similar to quick shifts in temperature an organism might experience in the field from a change in cloud cover, wind speed, an incoming front, etc. After four rotations over 15 minutes, we removed each individual from the shuttle box and recorded its thoracic T_b as in the T_{pref} trials. We used this T_b measurement and each individual's T_{pref} to calculate d_b. Being able to obtain this metric in the lab allowed us to obtain an estimate of T_{pref}s unbiased by field conditions. We carried out all behavioral trials between 10 AM and 2 PM and took care to keep lighting constant from one trial to the next.

Preferred temperatures were not normally distributed and were not easily transformed. Thus, we analyzed them using non-parametric analyses. As a preliminary analysis determined that there were sex differences in T_{pref}s (consistent with other studies, e. g. Forsman 2000), we analyzed the potential effects of weight, site, acclimation, and their interaction terms on sex-specific T_{pref}s using Kruskal-Wallis and Scheirer-Ray-Hare tests. Values of d_b were also not normally distributed, but conformed to a normal distribution when we applied a $\log_{10}$ transformation. We used a 4-way ANOVA to analyze them by using site, sex, rearing temperature, weight, and their interactions as predictor variables and d_b as a response variable. We used total residual sums of squares to obtain F values in this and all subsequent ANOVAs.

2.3 Upper Thermal Limits (CT_{Max})

We measured CT_{Max} with a thermal ramping experiment, following the protocol described in Preston and Johnson (2020). An important limitation to the CT_{Max} trials was that there was only one IL female raised in the cold environment that survived to be tested. As preliminary analyses indicated a significant effect of sex on CT_{Max}, we analyzed each sex separately with 3-way ANOVAs using CT_{Max} as a response variable and site, rearing temperature, weight, and their interactions as predictor variables. Where we

detected a significant interaction effect between site and rearing temperature, we visualized differences between mean CT_{Max} in the cold-reared individuals and mean CT_{Max} in the warm-reared individuals for each population with generalized reaction norms calculated among individuals originating from each population. We did not include IL females when visualizing generalized reaction norms due to having only one cold-acclimated IL female for that analysis.

2.4 Heat Stress Symptom Principal Component Analysis

While the ramping test to determine CT_{Max} was ongoing, we recorded air temperatures at which symptoms of heat stress manifested, one of each for each individual. These symptoms were 1) restlessness, defined as at least ten seconds of continuous movement after at least five minutes of motionlessness, 2) jumping, 3) onset of back leg spasms, 4) loss of coordinated movement, defined as the continued movement of extremities without ambulation after at least five minutes of continuous ambulation, and 5) heat coma. We analyzed air temperatures at which these symptoms manifested with a principal component analysis (PCA), using the air temperatures at which the symptoms above manifested to construct principal components (PCs). As a preliminary analysis indicated that there was a significant effect of sex, we analyzed the sexes separately and subsequently used PCs in 3-way ANOVAs with site, rearing temperature, and weight as predictor variables.

2.5 Differences in Gene Expression Among Males

For select individuals that we tested for upper thermal limits as described above, we used whole transcriptome profiling to assess differences in gene expression among treatments. For logistical and financial reasons, and to control for sex effects in gene expression, we only used males for this assay. Immediately after testing for CT_{Max}, we took whole-head samples and macerated them in Trizol. We stored each sample at -80°C before extracting total RNA using a standard Trizol protocol (Invitrogen; Thermofisher Scientific, Waltham, United States).

We assessed total purified RNA in each sample using a Qubit fluorometer (Thermofisher Scientific, Waltham, United States). Afterwards, we assessed RNA fragment size and integrity using a Bioanalyzer automated electrophoresis tool (Agilent, Santa Clara, United States). We outsourced cDNA library construction and transcriptome sequencing to the University of Texas at Austin, where we had the transcriptomes of two MO individuals sequenced using the NextSeq 500 system (Illumina, San Diego, United States) according to standard RNASeq protocols expressly for the purpose of building a reference transcriptome. The remainder of samples were processed by the HiSeq 2500 system (Illumina, San Diego, United States) using TagSeq (Meyer et al. 2011), a sequencing protocol that costs much less than RNASeq but can be just as or more accurate and informative (Lohman et al. 2016).

We performed the transcriptome assembly in a Linux environment using the RNASeq samples. We assessed the quality of raw reads with FastQC version 0.11.9 (Andrews 2010) and subsequently trimmed low-quality bases using Trimmomatic version 0.39 (Bolger et al. 2014). Afterwards, we used the Trinity package version 2.8.6 (Grabherr et al. 2011; Haas et al. 2013) to construct a *de novo* transcriptome. We then used BUSCO version 3.0.2 (Seppey et al. 2019) to validate the completeness of our assembly

against the Insecta database from the BUSCO website (https://busco.ezlab.org/), searching a total of 1658 BUSCO groups. Finally, we used Bowtie2 version 2.3.5 (Langmead & Salzberg 2012, Langmead et al. 2019) to index the assembled transcriptome.

We used the TagSeq samples to generate a count table in order to examine differential gene expression. We used FASTX-Toolkit's (version 0.0.14; Gordon & Hannon 2010) Fastx_clipper tool in conjunction with the Cutadapt tool (Martin 2011) to remove common TagSeq adapters and primers. We then used Bowtie2 version 2.3.5(Langmead & Salzberg 2012, Langmead et al. 2019) to map the TagSeq reads to the reference transcriptome. We generated read-counts-per gene isoform using a custom perl script (Mikhail Matz, personal comm.), then counted hits per gene using SAMtools (Li et al. 2009).

We used the DESeq2 package (Love et al. 2014) in R (version 3.6; R Core Team 2019) to normalize and analyze reads from the count table for potential differences in gene expression. One of the reasons we chose this package is because of our low sample size; DESeq2 biases shrinkage by applying it more strongly where information for a gene is low (Love et al. 2014), thus reducing the probability of false positives. This package uses a generalized linear model, in which we specified site of origin and acclimation treatment as predictor variables, and $\log_2$ fold change (FC) for each sequence as response variables. Prior to analysis, we removed all rows (genes) that had < 10 reads. We used the package DEVis (Price 2019) to visualize differences in gene expression among sites and treatments by calculating dissimilarity measures for each pairwise comparison of samples to create a multidimensional scaling hull plot. After visualization, it was apparent that one sample (KS, warm-acclimated) was of poor quality and had to be removed from the analysis. Afterwards, we re-ran the analysis and re-visualized the results without the sample.

We used Blast2GO (https://www.blast2go.com/) to infer functionality of significantly differentially expressed (DE; $\alpha = 0.01$) genes (392 total). We used the discontiguous-MEGABLAST algorithm to compare significantly differently expressed sequences against the National Center for Biotechnology Information's (Wheeler et al. 2007) standard nucleotide collection (nr) database, applying the Orthoptera taxonomy filter (taxid: 6993). We used a BLAST expectation value of 10^{-1}, a word size of 11, and a high-scoring segment pair length cutoff of 33. We used Blast2GO and InterProScan (Quevillon et al. 2005) to obtain two separate lists of gene ontology (GO) terms. Afterwards, we merged the GO terms into a single list. Finally, we used Blast2GO to generate multi-level pie charts to categorize DE sequences by biological, cellular, and molecular GO terms.

After we performed the above steps, we failed to obtain a significant match for one of the genes with the most difference in expression between two treatments. Thus, we subjected it to another discontiguous MEGABLAST, but we extended the query to the Insecta database.

Results

3.1 Accuracy of Behavioral Thermoregulation

Weight did not affect male (Kruskal-Wallis test, df = 27, c^2 = 28.659, p = 0.38) or female (Kruskal-Wallis test, df = 36, c^2 = 37.892, p = 0.383) T_{pref}. Similarly, neither site, rearing temperature, nor the interaction between the two affected male or female T_{pref} (Scheirer-Ray-Hare test, all p values > 0.05). The only significant factor affecting $\log_{10} d_b$ was rearing temperature; cold-reared individuals had a lower mean value of $\log_{10} d_b$ (Table 1, Fig. 2).

Table 1
Results from a 4-way ANOVA run on select individuals of *Melanoplus differentialis* from three populations, reared at warm (30°C) and cold (21°C) temperatures. We used the $\log_{10}$ of d_b (the deviation of an individual's preferred from its actual body temperature) as a response variable and population (Site), sex, rearing temperature (Acclimation), and weight as predictor variables. Significant result highlighted in yellow.

$Log_{10}D_b$	DF	F	p	η_p^2
Site	2	0.655	0.524	0.003
Sex	1	1.347	0.251	0.002
Acclimation	1	10.843	0.002	0.065
Weight	1	0.177	0.676	0.001
Site x Sex	2	0.088	0.916	0.007
Site x Acclimation	2	1.203	0.309	0.063
Sex x Acclimation	1	0.042	0.838	0.013
Site x Weight	2	0.283	0.754	0.122
Sex x Weight	1	2.627	0.111	0.104
Acclimation x Weight	1	0.138	0.711	0.004
Site x Sex x Acclimation	2	1.355	0.267	0.086
Site x Sex Weight	2	1.875	0.164	0.025
Site x Acclimation x Weight	2	1.318	0.277	0.026
Sex x Acclimation x Weight	1	0.448	0.507	0.009
Site x Sex x Acclimation x Weight	1	0.32	0.574	0.006

3.2 Upper Thermal Limits (CT_{Max})

The only factor that significantly affected male CT_{Max} was population. Male CT_{Max} was significantly higher in KS than in IL males, but no other site comparisons were significant (Tukey HSD, p = 0.029 for the KS-IL comparison, p > 0.05 for all other comparisons; Table 2; Fig. 3). Female CT_{Max} was only affected by

population and the interaction between population and rearing temperature. The mean CT_{Max} of IL females (49.11 ± 0.15 SE ˚C) was higher than that of KS (48.47 ± 0.27 SE ˚C) and MO (48.02 ± 0.27 SE ˚C) females (Table 2, Fig. 4).

Table 2
Results from 3-way ANOVAs run on *Melanoplus differentialis* of both sexes from three populations, reared at warm (30°C) and cold (21°C) temperatures. We used critical thermal maximum (CT_{Max}) as a response variable and population (Site), rearing temperature (acclimation) and weight as predictor variables. Significant results highlighted in yellow.

Male CT_{Max}	**DF**	**F**	**p**	**η_p^2**
Site	2	4.951	0.04	0.419
Acclimation	1	4.743	0.061	0.01
Weight	1	2.754	0.136	0.13
Site x Acclimation	2	3.759	0.071	0.332
Site x Weight	2	0.588	0.578	0.22
Acclimation x Weight	1	0.818	0.392	0.064
Site x Acclimation x Weight	2	0.052	0.95	0.138
Female CT_{Max}	**DF**	**F**	**p**	**η_p^2**
Site	2	7.404	0.005	0.578
Acclimation	1	0.025	0.877	0.251
Weight	1	2.557	0.128	0.236
Site x Acclimation	2	3.726	0.046	0.139
Site x Weight	2	1.002	0.388	0.123
Acclimation x Weight	1	0.089	0.769	0.004
Site x Acclimation x Weight	1	2.984	0.102	0.332

3.3 Heat Stress Symptom Principal Component Analysis

In the male PCA, PC1 and PC2 had eigenvalues of 2.261 and 1.057, respectively. The remaining PCs had eigenvalues < 1 and were not considered further. The proportion of variance explained by PC1 and PC2 were 0.452 and 0.211, respectively. PC1 loaded positively for all factors. It loaded strongly for air temperatures at which restlessness and jumping began, and moderately for air temperatures at which loss of movement occurred. PC2 loaded very strongly and positively for air temperatures at which spasms began, and strongly and negatively for air temperatures at which coma set in (Table 3).

Table 3
Loadings for the two useful principal components resulting from a principal component analysis run on *M. differentialis* from three populations.

Loadings	PC1 (male)	PC2 (male)	PC1 (female)	PC2 (female)
Restlessness	0.572	-0.215	0.56	-0.116
Jumping	0.548	0.017	0.592	0.039
Spasm	0.209	0.807	0.572	0.061
Loss of Movement	0.489	0.181	0.069	0.733
Coma	0.292	-0.519	-0.065	0.666

The only factor significantly affecting male PC1 was weight, whereas only acclimation significantly affected PC2 (Table 4). Heavier males tended to have higher values of PC1 (r = 0.509), and warm-acclimated males had a higher mean value of PC2 (Fig. 5). Thus, heavier males withstood higher temperatures before manifesting most symptoms, and warm-acclimated males withstood higher temperatures before spasming.

Table 4
Results from 3-way ANOVAs run on select individuals of *Melanoplus differentialis* males from three populations reared at warm (30°C) and cold (21°C) temperatures using two principal components (PC1 and PC2) as response variables and population (Site), rearing temperature (Acclimation) and weight as predictor variables. Significant results highlighted in yellow.

PC1	DF	F	p	η_p^2	PC2	DF	F	p	η_p^2
Site	2	0.161	0.853	0.137	**Site**	2	0.975	0.401	0.112
Acclimation	1	2.16	0.164	0.005	**Acclimation**	1	24.139	< 0.001	0.553
Weight	1	7.221	0.017	0.38	**Weight**	1	0.002	0.963	0.001
Site x Acclimation	2	1.231	0.322	0.037	**Site x Acclimation**	2	0.238	0.791	0.017
Site x Weight	2	0.143	0.868	0.02	**Site x Weight**	2	0.461	0.64	0.035
Acclimation x Weight	1	0.006	0.941	< 0.001	**Acclimation x Weight**	1	0.491	0.495	0.034
Site x Acclimation x Weight	2	0.02	0.98	0.003	**Site x Acclimation x Weight**	2	0.357	0.706	0.049

In the female PCA, PC1 and PC2 had eigenvalues of 2.640 and 1.221, respectively. The remaining PCs had eigenvalues < 1 and were not considered further. The proportion of variance explained by PC1 and PC2 were 0.528 and 0.244, respectively. PC1 strongly and positively loaded for air temperatures at which restlessness, jumping, and spasm set in. PC2 loaded strongly and positively for air temperatures at which loss of movement and coma occurred (Table 3).

Warm-acclimated females had a significantly higher mean value of PC1 (Table 5; Fig. 6), and warm-acclimated MO females had a higher mean value of PC2 than cold-acclimated MO females, whereas warm-acclimated KS females had a lower mean value of PC2 than cold-acclimated KS females (Fig. 7). While this interaction effect was significant, its effect size (η_p^2) was only half a percent (Table 5). PC2 values of IL females are not visualized in Fig. 7, as there were no cold-acclimated females in this trial.

Table 5
Results from 3-way ANOVAs run on select *Melanoplus differentialis* females from three populations, reared at warm (30°C) and cold (21°C) temperatures using principal components 1 and 2 (PC1 and PC2) as response variables and population (Site), rearing temperature (Acclimation) and weight as predictor variables. Significant results highlighted in yellow.

PC1	DF	F	p	η_p^2	PC2	DF	F	p	η_p^2
Site	2	1.089	0.354	0.047	**Site**	2	2.233	0.131	0.137
Acclimation	1	25.41	< 0.001	0.394	**Acclimation**	1	0.787	0.385	0.043
Weight	1	0.81	0.378	0.036	**Weight**	1	1.038	0.319	0.06
Site x Acclimation	1	0.004	0.95	< 0.001	**Site x Acclimation**	1	5.791	0.025	0.005
Site x Weight	2	0.666	0.524	0.028	**Site x Weight**	2	1.37	0.275	0.125
Acclimation x Weight	1	0.074	0.788	0.003	**Acclimation x Weight**	1	0.403	0.532	0.018
Site x Acclimation x Weight	1	0.664	0.424	0.029	**Site x Acclimation x Weight**	1	0.436	0.516	0.02

3.4 Differences in Gene Expression Among Males

We had three populations, two treatments, and 2–4 biological replicates per treatment for a total of 19 cDNA libraries (Table 6). We obtained BUSCO metrics of 96.6% complete (52.2% single-copy, 44.4% duplicated), 1.6% fragmented, and 1.8% missing BUSCOs in the assembled transcriptome.

Table 6
Sample sizes for gene expression analysis trials run on *Melanoplus differentialis* males from three populations and reared at warm (30°C) and cold (21°C) temperatures.

# of samples	Cold	Warm
KS	3	3
MO	3	2
IL	4	4

MO had the greatest number of DE genes between acclimation treatments, with a greater number of genes downregulated than upregulated in the warm acclimation treatment when compared to the cold acclimation treatment. (Fig. 8). When we compared differences in expression among sites while keeping acclimation treatment constant, the largest difference in expression existed in the KSWarm-MOWarm comparison, with more genes upregulated in the MOWarm treatment (Fig. 9), with the total number of DE genes being approximately one and a half times that of the MOWarm-MOCold comparison (Fig. 8).

The pooled mean of adjusted p-values (Wald test) in the between-acclimation comparisons (μ of p values = 0.829 ± 0.099 SE) was comparable to that of the acclimation-specific among-site comparisons (μ = 0.856 ± 0.068 SE). The absolute $\log_2$ FCs in the between-acclimation comparisons had a higher pooled mean and were more variable (μ = 0.534 ± 0.113 SE) than the among-site comparisons (μ = 0.467 ± 0.028 SE; Figs. 10 and 11; the first treatment in each subfigure title indicates the direction of FCs, e.g. in "KS_warm_vs_KS_cold", a dot to the left of zero indicates a negative FC in KS_warm as compared to KS_cold.). When we examined acclimation-specific site comparisons, the warm-acclimated site comparisons had a lower mean of adjusted p-values (μ = 0.723 ± 0.071 SE) than the cold-acclimated site comparison (μ = 0.990 ± 0.003 SE). The warm-acclimated site comparison also had a higher mean absolute $\log_2$ FC (μ = 0.505 ± 0.044 SE) than the cold-acclimated site comparison (μ = 0.428 ± 0.021 SE).

Gene expression pairwise distances as determined by multidimensional scaling did not have any discernible pattern when grouped by acclimation temperature (Fig. 12). However, they appeared to group somewhat strongly by site of origin (Fig. 13).

Of the five sequences that had an absolute $\log_2$ fold change > 10 (μ = 10.88, SD = 0.93), all were downregulated in the KS treatments. Three occurred in the KSWarm-ILWarm comparison and one occurred in the KSCold-MOCold comparison. All of these had GO terms associated with mitochondrial electron transport, ATP synthesis, and the aerobic electron transport chain. The top matches for these four sequences came from *M. differentialis'* congener, *Melanoplus bivittatus* and had E values < 0.001. The final sequence was DE in the KSWarm-MOWarm comparison and had no BLAST results when we searched the Orthoptera database. In the Insecta database, the closest match (E value = 0.021) was a predicted mRNA in the aphid *Melanaphis sacchari* that played a role in tubulin polyglutamylation.

We obtained significant BLAST hits for 299 out of 392 sequences when running the discontiguous MEGABLAST. Of these, we were able to obtain GO annotation for 182 sequences. The three most common categories of GO terms assigned to DE sequences, when grouped by biological process, were 1) ATP synthesis, 2) mitochondrial electron transport, and 3) aerobic electron transport (Fig. 14). When grouped by cellular component, they were 1) integral component of membrane, 2) respiratory chain complex IV, and 3) mitochondrial proton-transporting ATP synthase complex, coupling factor (Fig. 16). Lastly, when grouped by molecular function, they were 1) heme binding, 2) hydrolase activity, and 3) cytochrome-c oxidase activity (Fig. 16).

Discussion

4.1 Accuracy of Behavioral Thermoregulation

No tested factors had an effect on T_{pref}s. It is possible that this was an artifact of our protocol; T_{prefs} of warm-acclimated individuals may actually been higher than that of cold-acclimated individuals, but steeper temperature gradients near the warm end of the shuttle box may have made it harder for individuals selecting warmer temperatures to maintain a constant temperature, whereas individuals selecting colder temperatures may have been able to more systematically choose and maintain T_{prefs} in the relatively gradual temperature gradient present near the colder end of the shuttle box. Contrary to our results, Forsman et al. (2002) found that acclimation affected orthopteran T_{pref}. Their study used an 800 x 350 x 3 mm copper plate with a heat retaining hot tray at one end and a polystyrene foam box with ice at the other. The plate had a temperature range of 14–50 ˚C, but they do not disclose the steepness of the temperature gradient. If their gradient was much smoother than the one we utilized, then it is likely that individuals used in their experiment were more easily able to maintain T_{pref}s regardless of rearing temperature. As T_{pref} was a critical component of our calculation of d_b, this may have been responsible for detection of a lower d_b in cold-acclimated individuals.

4.2 Upper Thermal Limits (CT_{Max})

Male CT_{Max} was higher in KS males than in IL males. Preston and Johnson (2020) found that the KS site had more variable temperatures and more days per month with extreme heat events than the IL site, supporting our hypothesis of a positive correlation between thermal tolerance and heterogeneity. However, that study did not detect significant effects of population on male CT_{Max}. It's possible that maternal effects had more of an effect on weight (which affected CT_{Max}) in that study, as those males were an F1 generation raised from eggs laid by field-collected individuals, and the males used in this study were F2 individuals raised from eggs laid by F1 individuals.

Illinois females had a higher mean CT_{Max} than MO females, which was surprising given that IL males had the lowest mean CT_{Max}. A study with another *Melanoplus* species found that males and females in a single population differed in their epicuticular lipid composition (Gibbs & Mousseau 1994), which affects water loss at high temperatures (Noble-Nesbitt 1991), which in turn could affect CT_{Max}. If *M. differentialis* also has sex differences in epicuticular lipid composition, and these differences are more pronounced in different populations, these differences may be responsible for the mismatch in relative CT_{Max} among males and females at each site.

There were no significant acclimation effects in males, agreeing with the conclusion by Gunderson and Stillman (2015) that ectotherms have limited plasticity in upper limits of thermal tolerance. However, the generalized reaction norm (from cold-to-warm acclimation treatment) for female CT_{Max} was negative in the and MO population, but positive in the KS population. Thus, results from females support our hypothesis of adaptive acclimation effects in thermally heterogeneous populations. That being said, a caveat to this conclusion is that we only used two rearing temperatures. Lyons et al. (2012), used three rearing temperatures (20 ˚C, 25 ˚C, and 30 ˚C) to examine potential effects of acclimation on thermal

limits of mosquitoes in lab and field settings. In that study, warm-acclimated mosquitoes generally had a higher CT_{Max} than cold-acclimated mosquitoes. Interestingly, where negative reaction norms (from colder-to-warmer temperatures) did occur, nearly all 'recovered' from the cold-to intermediate (20–25 ˚C) treatment and turned positive in the intermediate-to-warm (25–30 ˚C) treatment. Thus, had we used another level of acclimation, e.g. rearing individuals at 37 ˚C, in our experiment, we may have obtained similar results. Lastly, while the slope of the reaction norm in KS females was in the opposite direction of that of MO, the standard error bars for the KS and MO warm-acclimated females overlapped that of their cold-acclimated counterparts. Thus, the effects of acclimation on CT_{Max} in KS and MO females are likely small.

4.3 Heat Stress Symptom Principal Component Analysis

There were no significant interpopulation differences in male PC1 values. This was surprising, as population affected male CT_{Max}. Heavier males had higher values of PC1, indicating that heavier males became restless, began jumping, and experienced loss of movement at higher temperatures than lighter males. Furthermore, warm-acclimated males had higher values of PC2 than cold-acclimated males, i.e., lighter males and males reared in a cold environment generally experienced thermal distress at lower temperatures. These findings were expected as 1) body size in larger orthopterans is inversely related to rate of temperature change (Whitman 1987), 2) a previous study (Preston & Johnson 2020) indicated that heavier *M. differentialis* males have a higher CT_{Max}, and 3) acclimation to a warm temperature should confer higher temperature tolerance (e. g. Gibbs & Mousseau 1994).

Unlike the generalized reaction norm for CT_{Max}, the slope of the generalized reaction norm for PC2 (from cold-to-warm acclimation treatment) was positive for MO females but negative for KS females, indicating that severe symptoms of heat stress were ameliorated by acclimation in MO females, but not in KS females. While this indicates differential plasticity among populations in upper thermal limits due to acclimation, the fact that it was present in MO and not KS runs counter to the concept of the evolution of acclimation being induced by thermal heterogeneity; 101-year daily temperature range and variance in daily maximum temperatures are both higher at the KS site (Preston & Johnson 2020). The only significant effect on PC1 was acclimation treatment; cold-acclimated females had a lower value of PC1, indicating that mild symptoms of thermal distress set in at lower temperatures in cold-acclimated females. This was to be expected, as previous studies have found similar results (Gibbs & Mousseau 1994).

4.4 Differences in Gene Expression Among Males

Acclimation treatment had the greatest effect on the number of differentially expressed genes in MO males than on males from any other site; both upregulated and downregulated genes in this site comparison were more than three times that of any other acclimation treatment comparison. KS males had the least change in number of differentially expressed genes from one acclimation treatment to the other, while IL males were intermediate in this respect. However, there were no significant interaction effects detected between site and acclimation in male $Log_{10}D_b$, CT_{Max}, PC1, or PC2, as would be expected

if acclimation-induced changes in gene expression conferred thermoregulatory or thermotolerance benefits differentially among sites. Further, CT_{Max} of KS males did not differ from that of MO males and was higher than that of IL males. Thus, differences in gene expression of MO and IL males due to acclimation did not confer higher CT_{Max}. While we know of no other studies directly testing population-specific plasticity in orthopteran gene expression in response to heat stress, Roelofs et al. (2009) detected population-specific plasticity in arthropod (Collembola) gene expression in response to cadmium exposure. They observed a genome-wide response in non-tolerant individuals, whereas tolerant individuals maintained normal gene expression upon exposure. If a minimal genetic response to heat stress is adaptive, then the populations in this study may follow the same pattern as Collembola, albeit imperfectly. Molecular responses to heat extremes can come at a cost to other measures of performance (e. g. Silbermann & Tatar 2000), thus it behooves individuals to mount heat responses that are highly targeted and efficient. As KS males had the highest mean CT_{Max} and the smallest number of DE genes in response to heat stress, it seems likely that males in this population have the most adaptive heat response of the three populations tested.

The greatest intersite differences in gene expression were among the warm-acclimated treatments; the cold-acclimated comparison with the greatest number of DE genes had less DE genes than the warm-acclimated comparison with the lowest number of DE genes. Furthermore, more genes were significantly different and had higher absolute $\log_2$ FCs in the warm-acclimated comparison. Similar results were found by Spees et al. (2002), wherein warm-acclimated (13.6 ˚C) lobsters had higher levels of heat-shock protein transcription than cold-acclimated (0.4 ˚C) lobsters when exposed to heat stress. While identifying the function of every differentially expressed gene is beyond the scope of this study, it is reasonable to conclude that, as warm-acclimated males responding to stressfully high temperatures had more differences in gene expression, these genes may be relevant to thermoregulation and thermotolerance.

The largest (by far) number of DE genes were between KS and MO. Gene expression grouped more strongly by population than by acclimation treatment, with the notable exception of sample 15C, a cold-acclimated IL male. This agrees with the relative pooled mean values of the Wald tests as well as the relative amount of $\log_2$ FCs. This may be due to substantial genetic variation among these populations, minimal ability to acclimate in these populations, or a combination of the two. Dunning et al. (2014) observed similar results with stick insects and gene expression in response to low temperatures; while all populations showed a transcriptional response to cold, the majority of the unigenes identified were population specific. Thus, while populations (or groups of species) may mount a transcriptional response to a common stressor, the genes involved and the strength of the response can differ by virtue of genetic differentiation among populations. As populations in this study grouped more strongly by site than acclimation treatment, it may be that the transcriptomic responses of the populations we sampled were more influenced by population-specific evolutionary influences than they were by different rearing temperatures.

When categorized by biological process, the three most common roles of differentially expressed genes with GO terms were ATP synthesis coupled proton transport, mitochondrial electron transport, and facilitation of the aerobic electron transport chain. This indicates that males may have differed in their rate of cellular respiration. As temperature increases, insect respiration and metabolism are both expected to increase up to a critical thermal limit (Neven 2000). The oxygen limitation model posits that thermal limits of performance (in this case, CT_{Max}) are set by the point at which aerobic respiration fails to meet energetic needs. In the case of high temperatures, this would occur when ventilation and circulation fall below the level required to supply mitochondria with sufficient oxygen (Pörtner 2001). Thus, higher rates of cellular respiration at higher temperatures may confer higher thermal limits. However, while the oxygen limitation model is promising for explaining upper thermal limits in aquatic arthropods, results from experiments examining oxygen limitation as a definitive mechanism for upper thermal limits in terrestrial arthropods are mixed at best (Verberk et al. 2016).

4.5 Conclusions

Overall, these data indicate that, in this species, 1) the ability to track preferred temperatures more accurately does not vary among site of origin or sex, 2) CT_{Max} depends on sex, site of origin, and rearing temperature, 3) air temperatures at which symptoms of thermal stress manifest depend on sex, site of origin, and rearing temperature, 4) gene expression during thermal stress is affected by site of origin and, in some populations, rearing temperature, and 5) the majority of transcriptional products in males during heat stress come from genes involved in cellular respiration.

Declarations

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6. Declarations

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Ethics approval: Not applicable

Consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and material (data transparency): If this work is accepted, we will upload our transcript sequences to NCBI's GenBank. We have provided our raw behavioral and physiological data as

supplementary files.Code availability: We have provided our custom perl script (for TagSeq) as a supplementary file.

7. Author Contributions

Devin B. Preston: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing - original draft, Writing - review & editing.
Steven G. Johnson: Funding acquisition, Investigation, Project administration, Supervision, Writing - review & editing.

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Figures

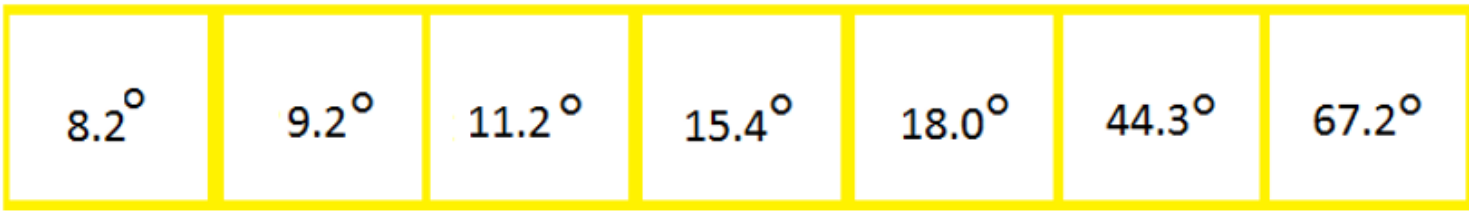

Figure 1

Diagram of shuttle box used for determining preferred temperatures and body temperature in a fluctuating environment showing the steady-state temperature in degrees Celsius in each of seven

partitions.

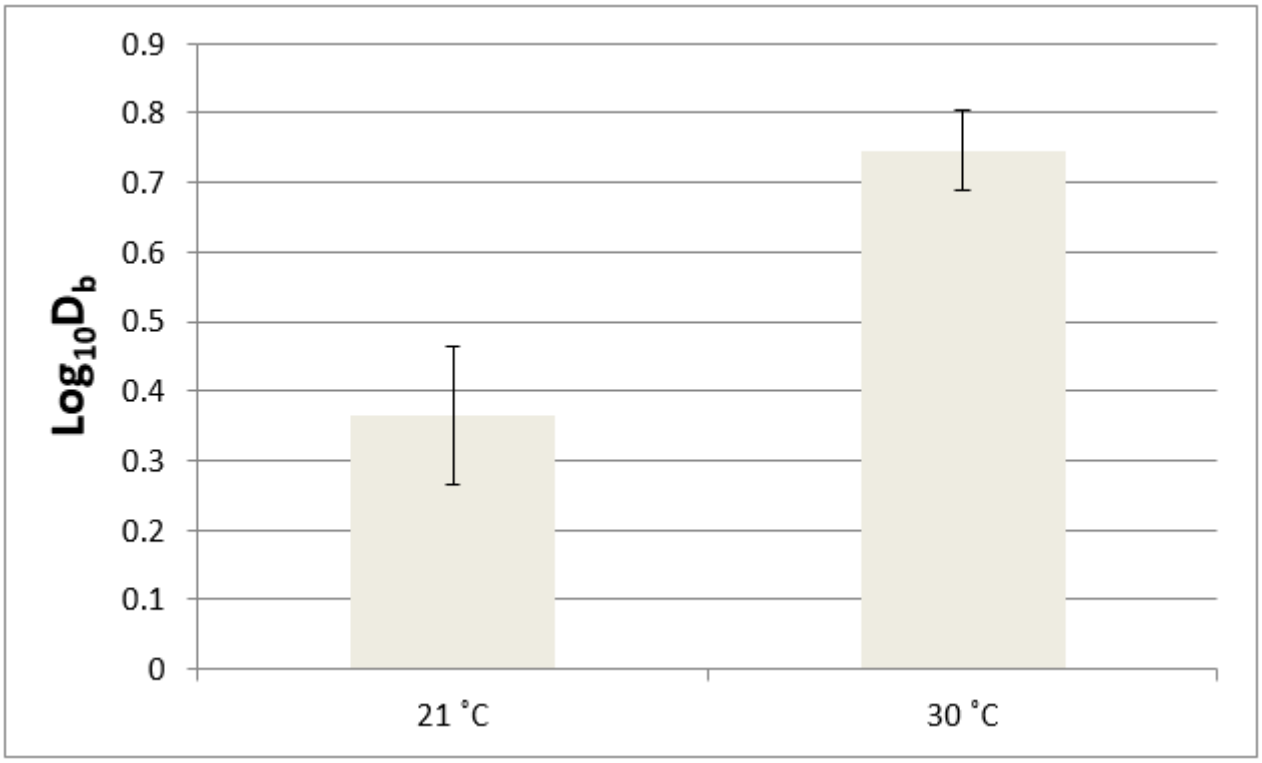

Figure 2

Mean log10 values of the deviation of individuals' actual body temperatures from their preferred body temperatures (db) of Melanoplus differentialis from three populations reared at warm (30 °C) and cold (21 °C) temperatures ± 1 SE. Values of Db were lower in individuals reared at 21 °C than at 30 °C (ANOVA: $F_{1,22} = 10.843$, $p = 0.002$).

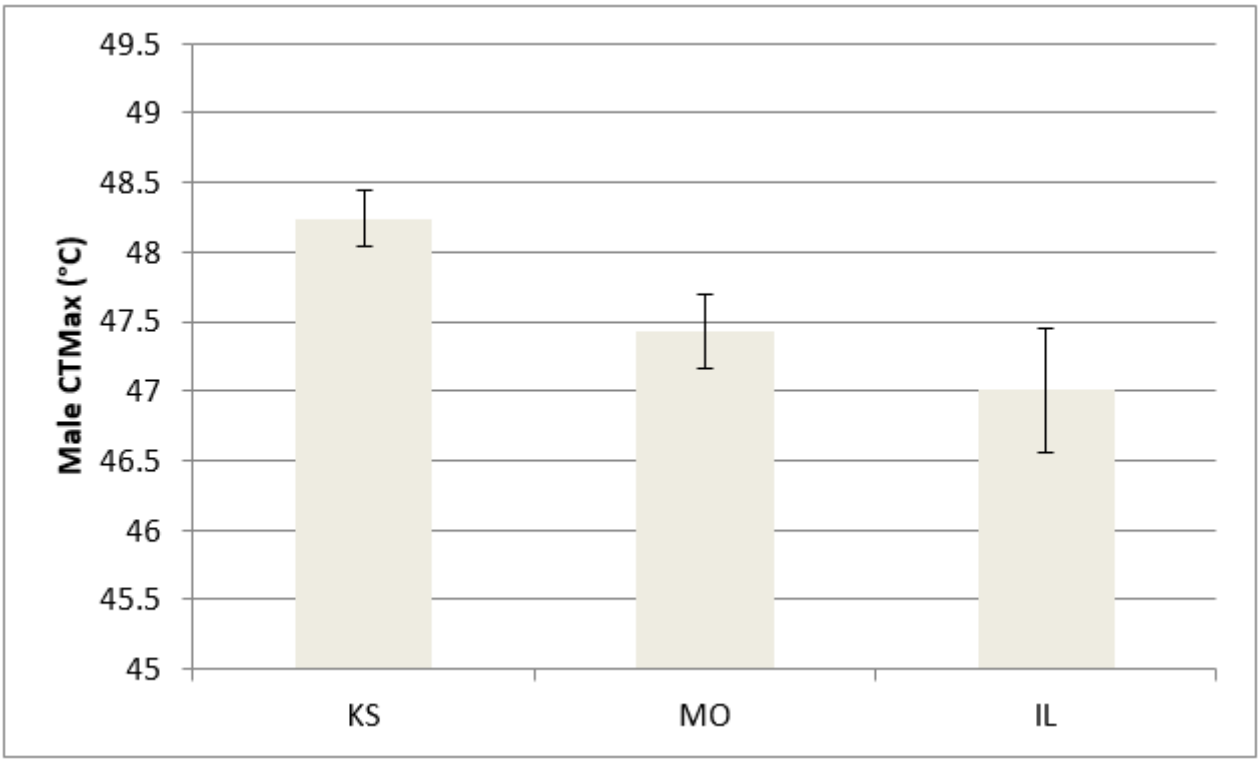

Figure 3

Mean critical thermal maximum (CTMax) of Melanoplus differentialis males from three populations ± 1 SE. Values of CTMax were higher in KS individuals than in IL individuals (ANOVA: $F_{2,11} = 4.951$, $p = 0.04$; Tukey HSD: $p = 0.029$).

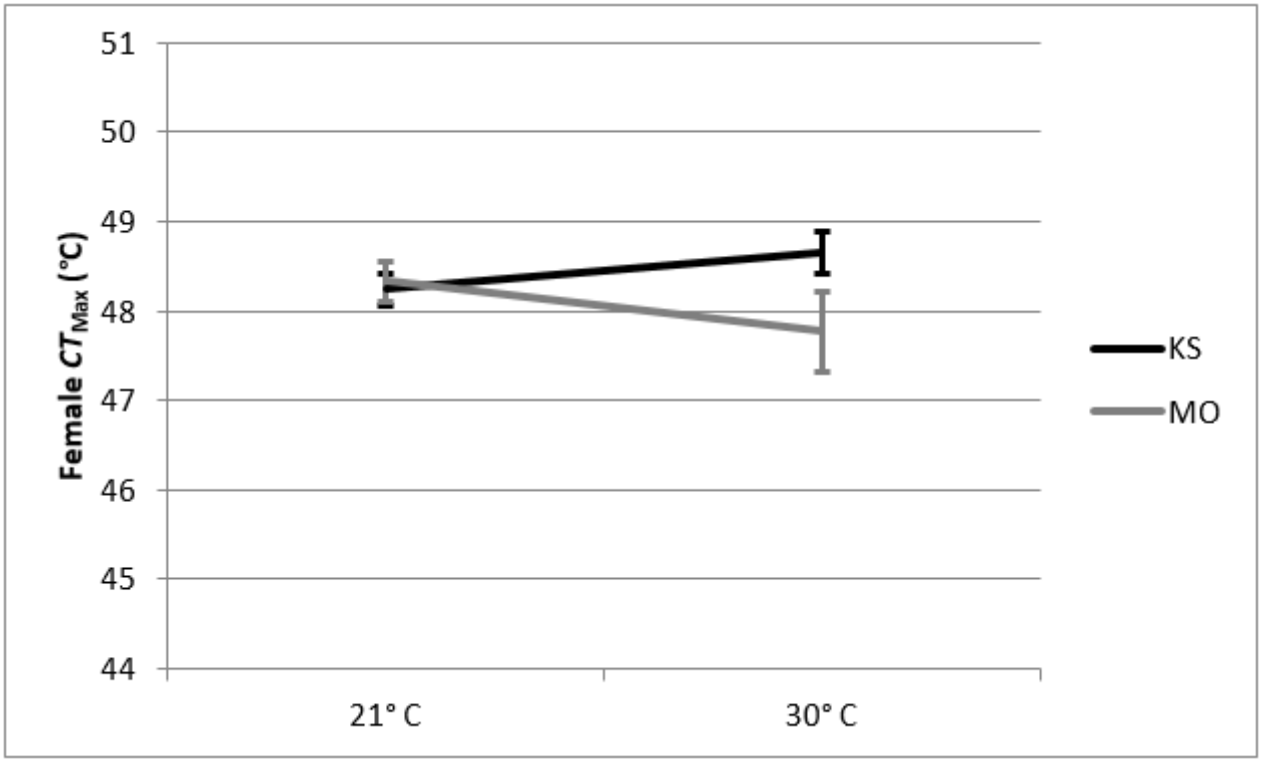

Figure 4

Generalized reaction norms for critical thermal maximum (CTMax) of Melanoplus differentialis females from two populations reared at warm (30 °C) and cold (21 °C) temperatures; ± 1 SE.

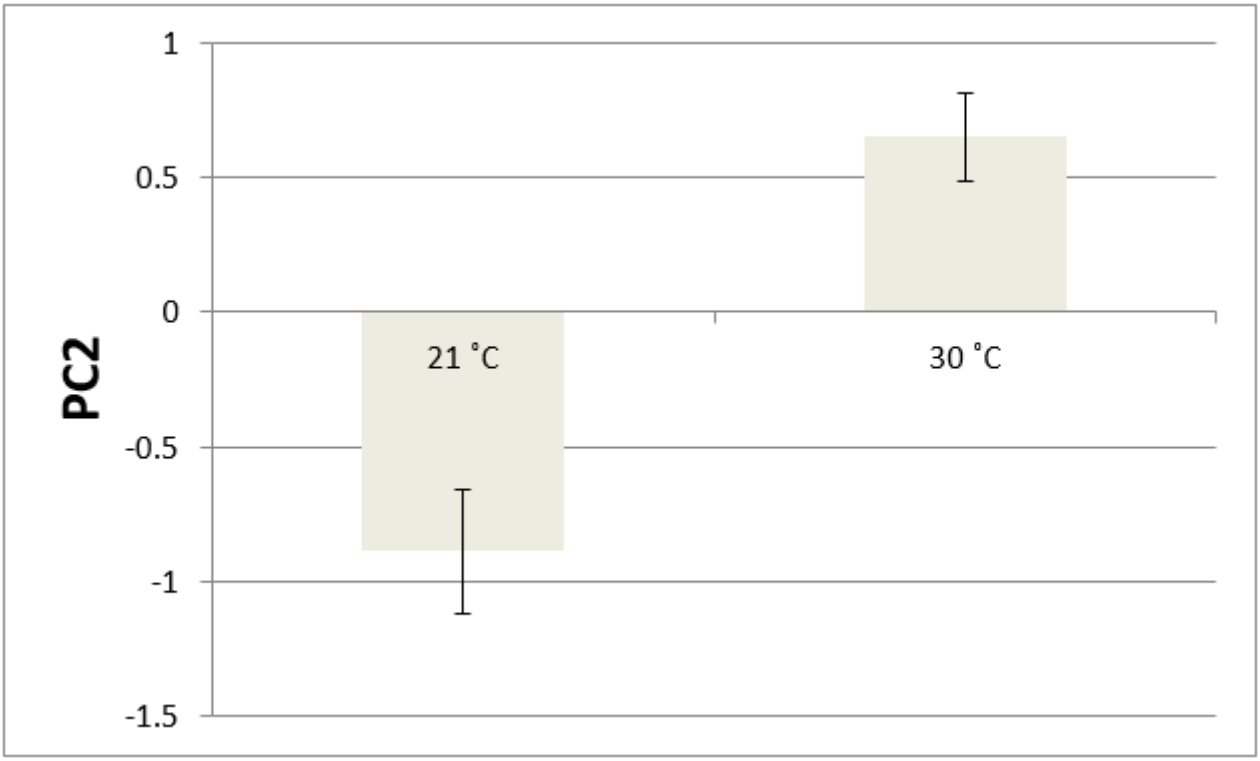

Figure 5

Mean values of PC2, calculated from lesser behavioral symptoms of heat stress of Melanoplus differentialis males from three populations reared at warm (30 °C) and cold (21 °C) temperatures ± 1 SE. Values of PC2 were lower in individuals reared at 21 °C than at 30 °C (ANOVA: $F_{1,11} = 23.139$, $p < 0.001$).

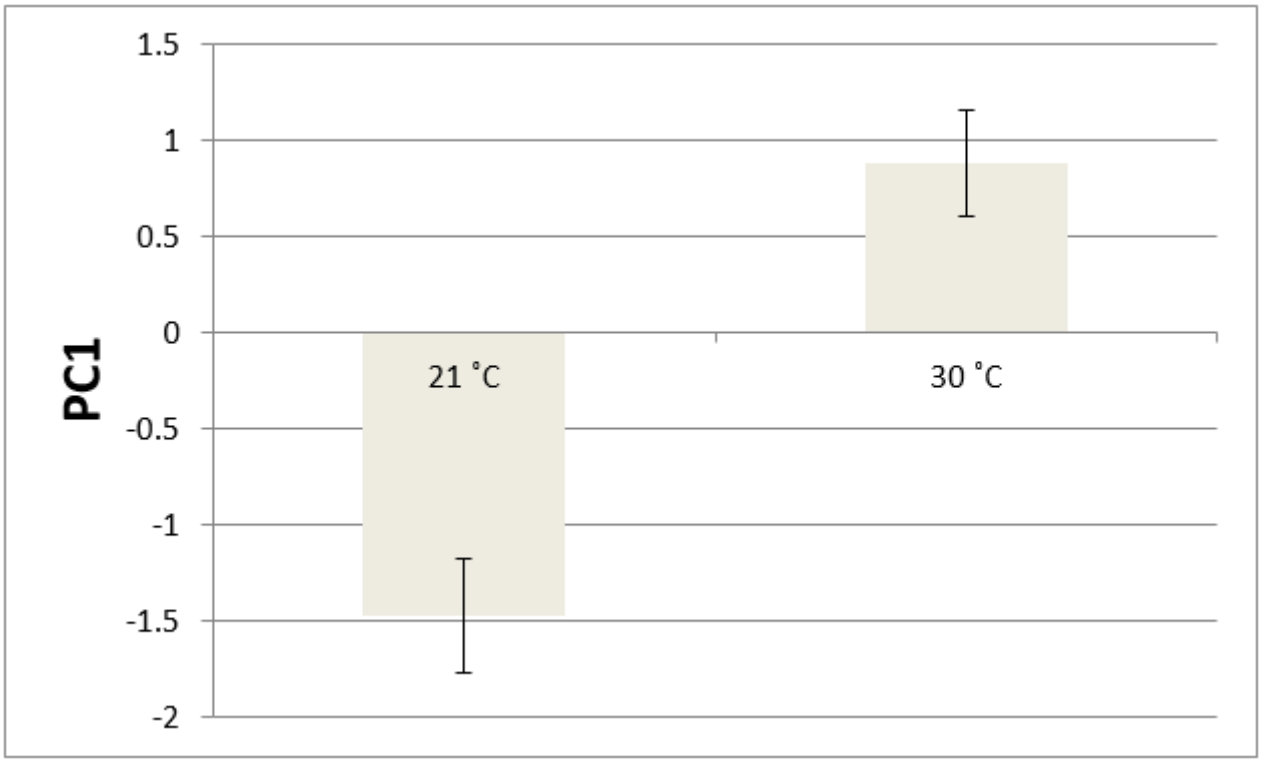

Figure 6

Mean values of PC1, calculated from greater behavioral symptoms of heat stress of Melanoplus differentialis females from three populations, reared at warm (30 °C) and cold (21 °C) temperatures ± 1 SE. Values of PC1 were lower in individuals reared at 21 °C than at 30 °C (ANOVA: $F_{1,11} = 25.41$, $p < 0.001$).

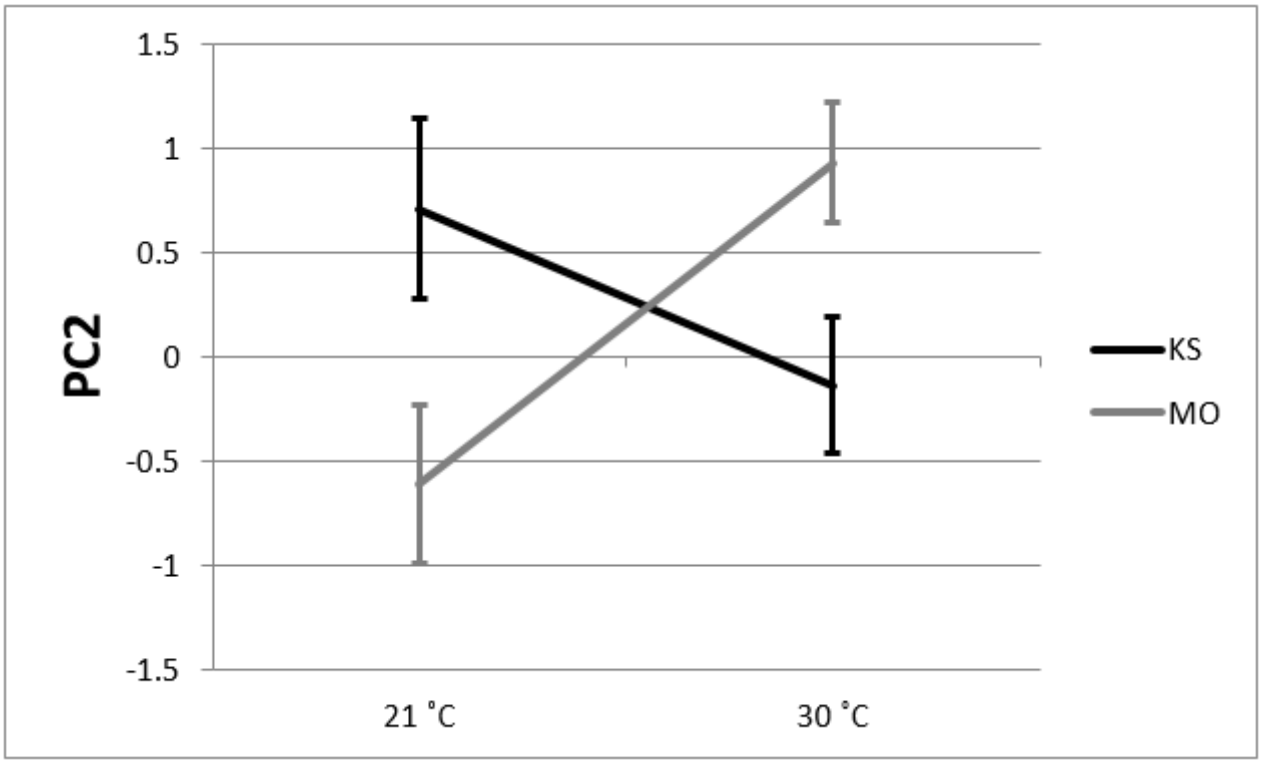

Figure 7

Generalized reaction norms for principal component 2 (PC2), calculated from lesser behavioral symptoms of heat stress values of unrelated Melanoplus differentialis females from two populations, reared at warm (30 °C) and cold (21 °C) temperatures ± 1 SE.

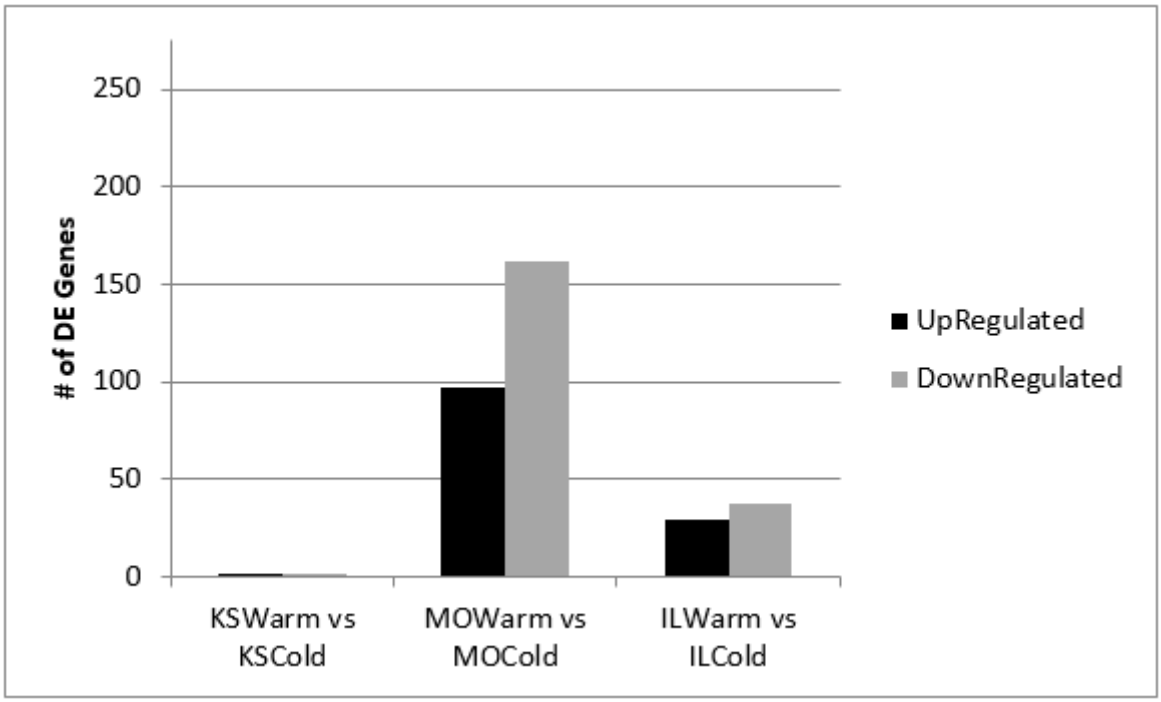

Figure 8

Number of significantly differentially expressed genes (Wald test; α = 0.05) of M. differentialis males in three population-specific comparisons between acclimation treatments. KS = Kansas, MO = Missouri, and IL = Illinois. Warm = reared at 30 ˚C, Cold = reared at 21 ˚C. The first treatment in the bar labels matches the direction of regulation. E. g. in the "MOWarm vs MOCold" comparison, 97 genes were upregulated in the "MOWarm" treatment over that of the "MOCold" treatment.

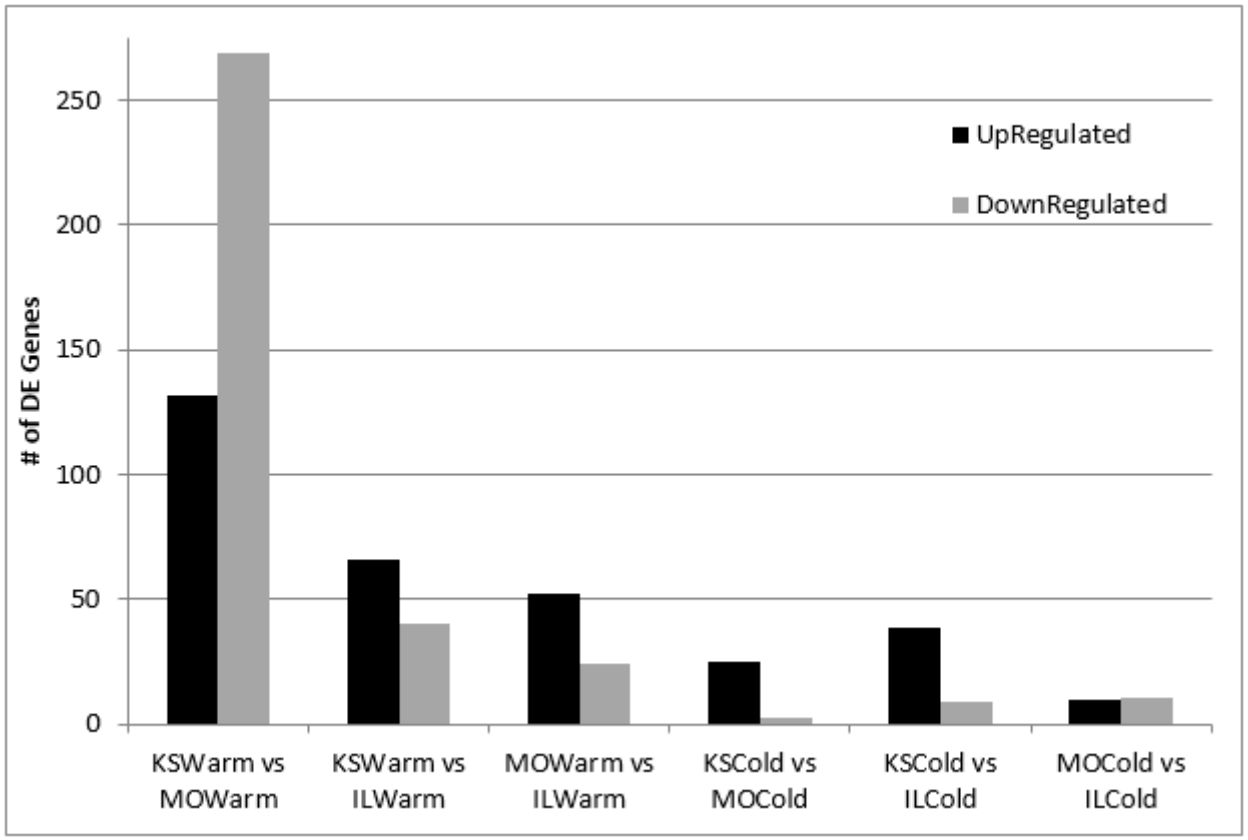

Figure 9

Number of significantly differentially expressed genes (Wald test; α = 0.05) of M. differentialis in acclimation-specific comparisons among sites. KS = Kansas, MO = Missouri, and IL = Illinois. Warm = reared at 30 ˚C, Cold = reared at 21 ˚C. The first treatment in the bar labels matches the direction of regulation. For example, in the "KSWarm vs MOWarm" comparison, 132 genes were upregulated in the "KSWarm" treatment over that of the "MOWarm" treatment.

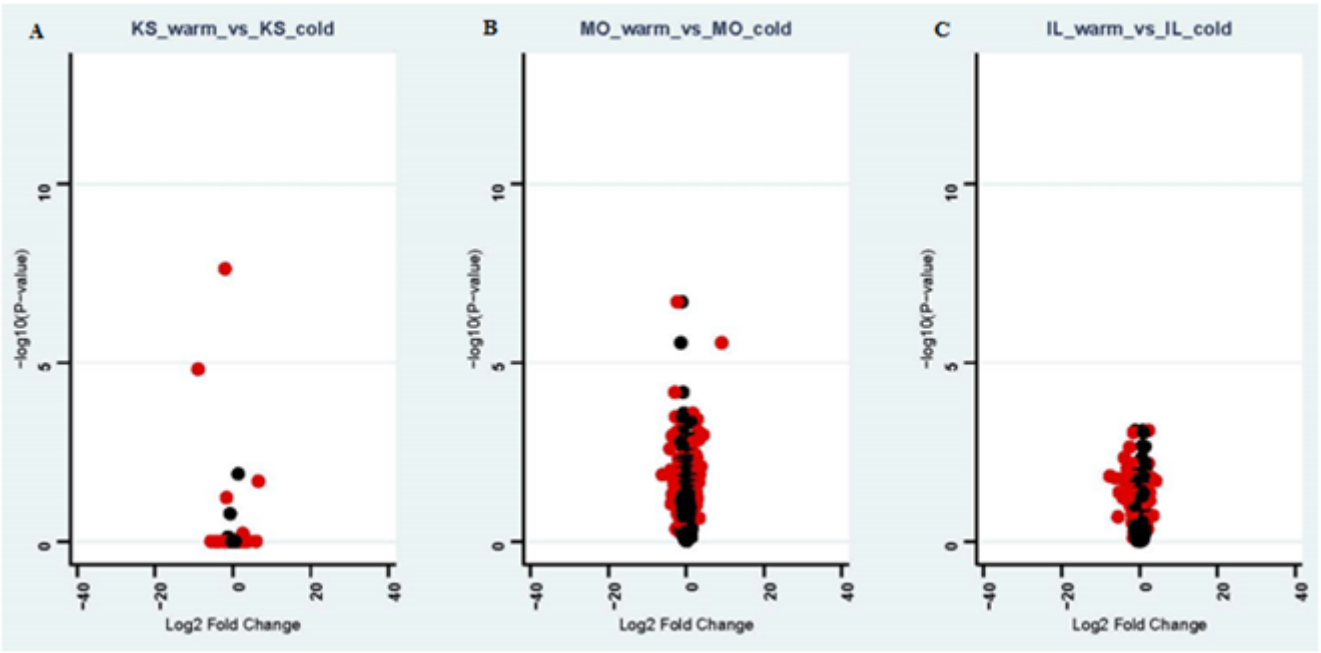

Figure 10

A-C: Volcano plots showing the negative log10 of adjusted p values (Wald test) and log2 fold changes (FCs) of putative gene products identified from head samples of male Melanoplus differentialis from three populations reared in warm (30 °C) and cold (21 °C) acclimation treatments. The figures below depict site-specific comparisons between acclimation treatments. Red dots are genes that have an absolute log2 FC > 1. KS = Kansas, MO = Missouri, and IL = Illinois. warm = reared at 30 ˚C, cold = reared at 21 ˚C. There were 5, 224, and 66 significantly differentially expressed (α= 0.05) genes in A, B, and C, respectively.

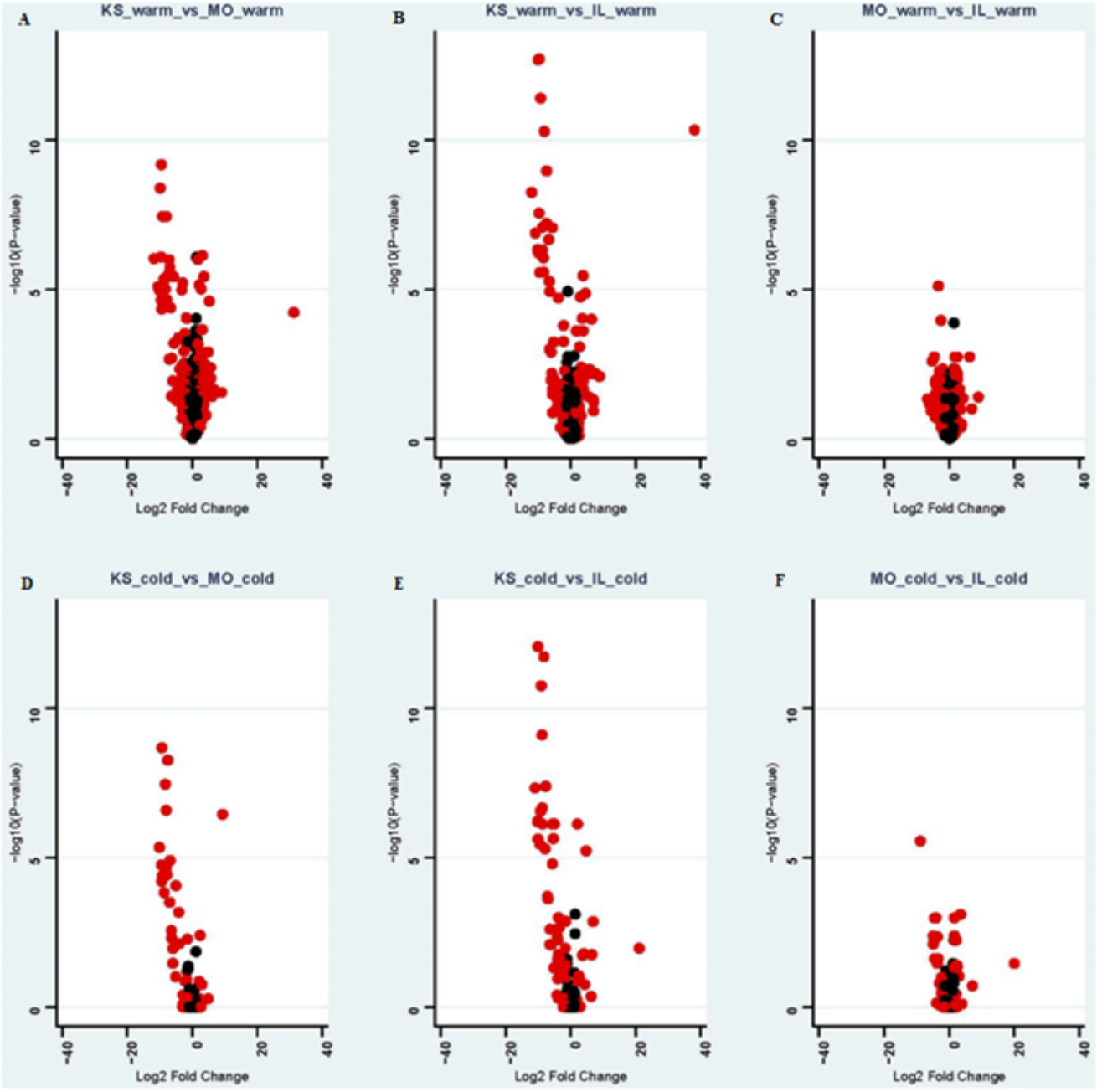

Figure 11

A-F: Volcano plots showing the negative log10 of adjusted p values (Wald test) and log2 fold changes (FCs) of putative gene products identified from head samples of male Melanoplus differentialis from three populations reared in warm (30 °C) and cold (21 °C) acclimation treatments. The figures below depict acclimation-specific comparisons among sites. Red dots are genes that have an absolute log2 FC > 1. KS = Kansas, MO = Missouri, and IL = Illinois. warm = reared at 30 ˚C, cold = reared at 21 ˚C. There were 385, 109, 74, 28, 54, and 21 significantly DE genes in A, B, C, D, E, and F, respectively.

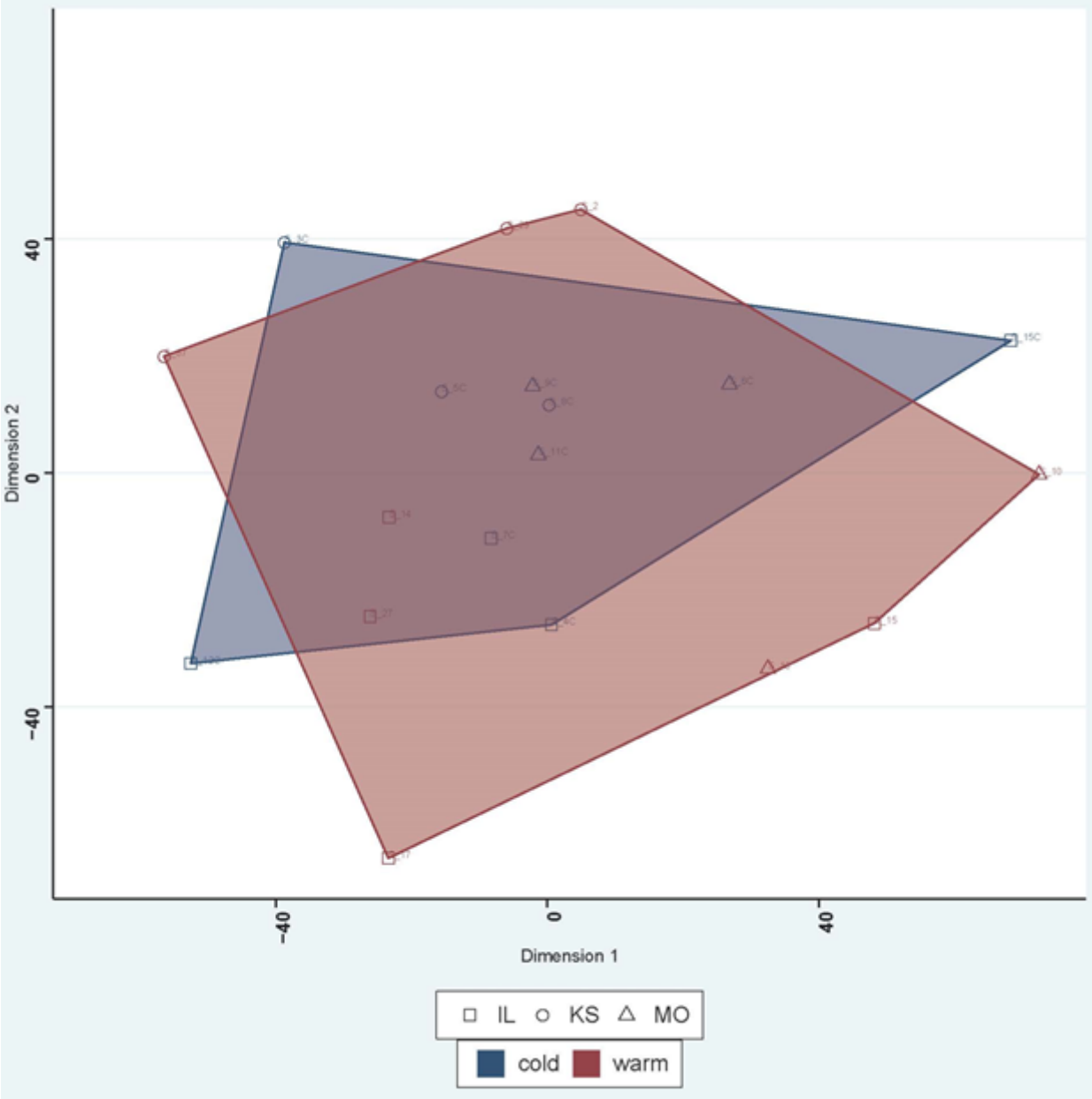

Figure 12

Multi-dimensional scaling hull plot showing expression of putative genes identified from head samples of male Melanoplus differentialis from three populations reared in warm (30 °C) and cold (21 °C) acclimation treatments, grouped by acclimation treatment. The x axis is the first ordination dimension; the y axis is the second ordination dimension.

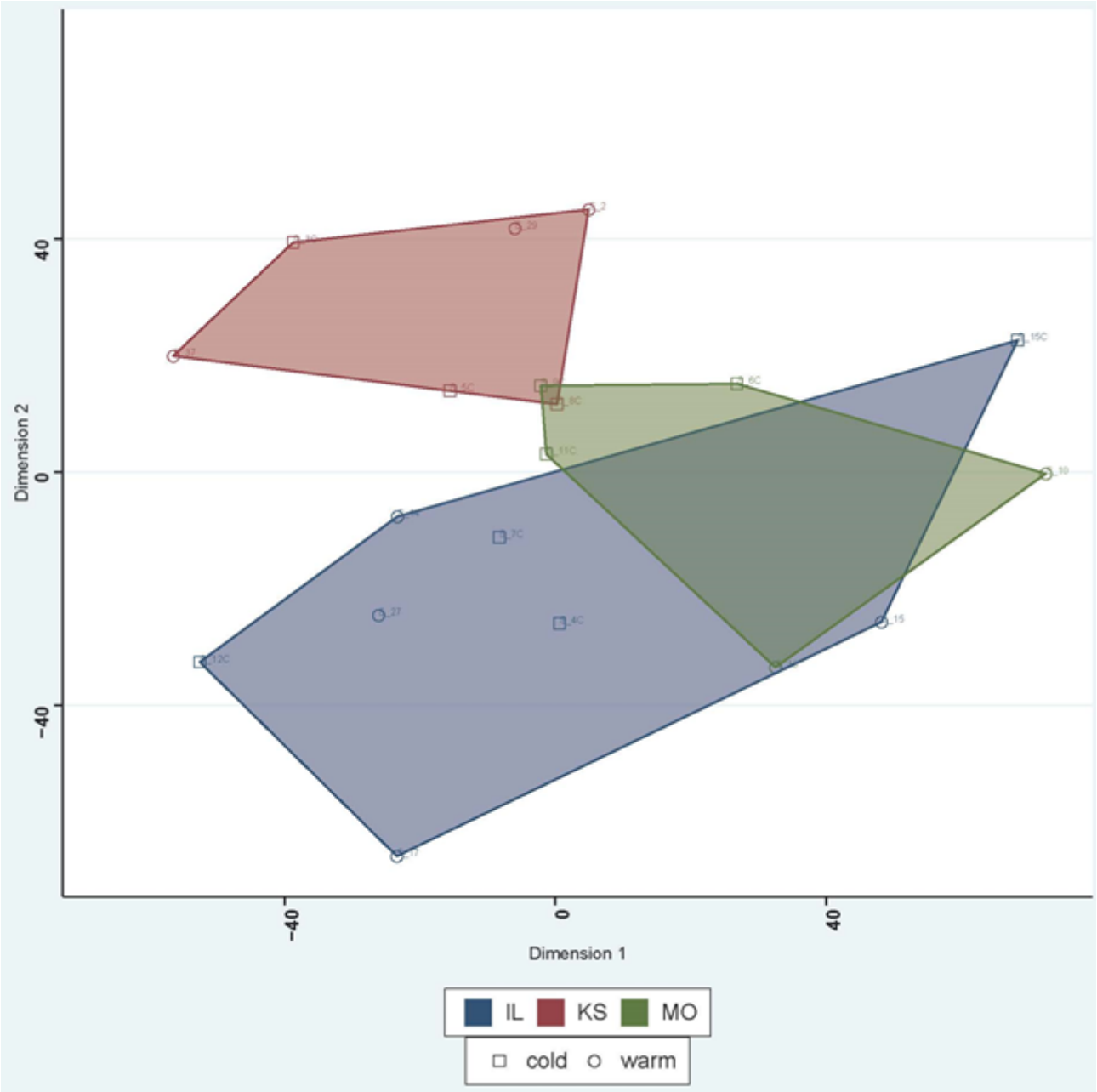

Figure 13

Multi-dimensional scaling hull plot showing expression of putative genes identified from head samples of male Melanoplus differentialis from three populations reared in warm (30 °C) and cold (21 °C) acclimation treatments, grouped by site of origin. The x axis is the first ordination dimension; the y axis is the second ordination dimension.

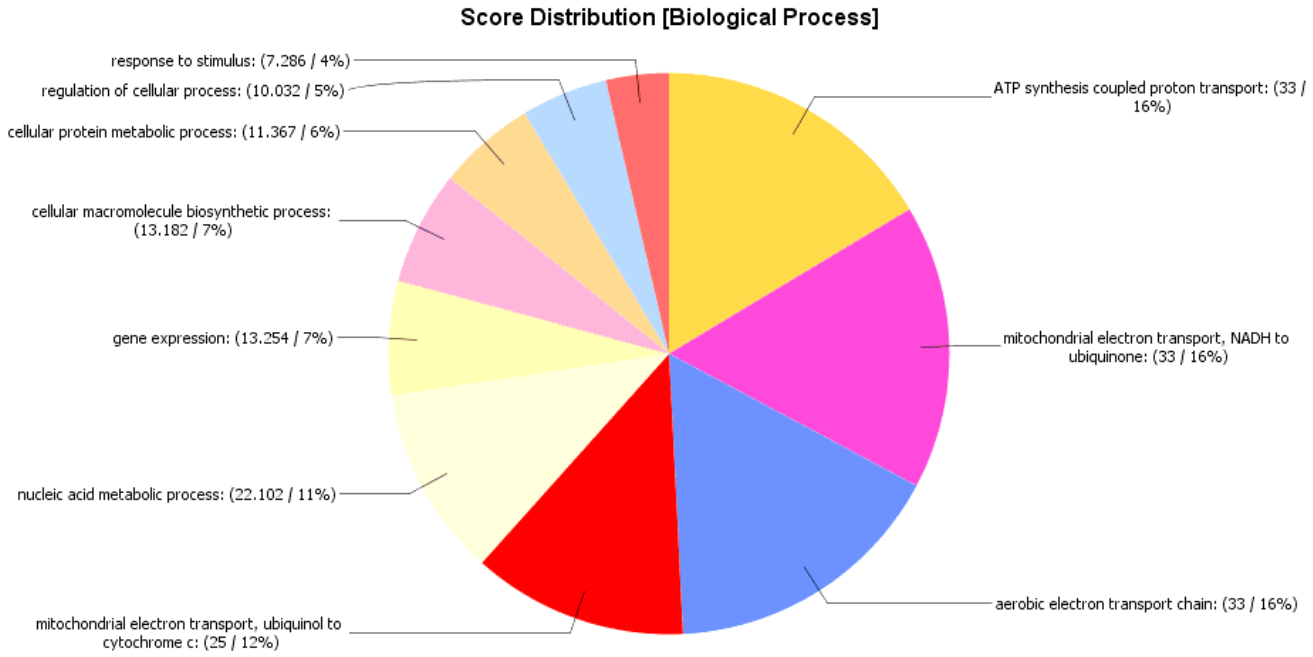

Figure 14

Distribution of node scores derived from differentially expressed sequences obtained from head samples of male Melanoplus differentialis from three populations reared in warm (30 °C) and cold (21 °C) acclimation treatments, grouped by biological function. These scores were determined from data generated by discontiguous-MEGABLAST and subsequent gene ontology annotation performed in Blast2GO.

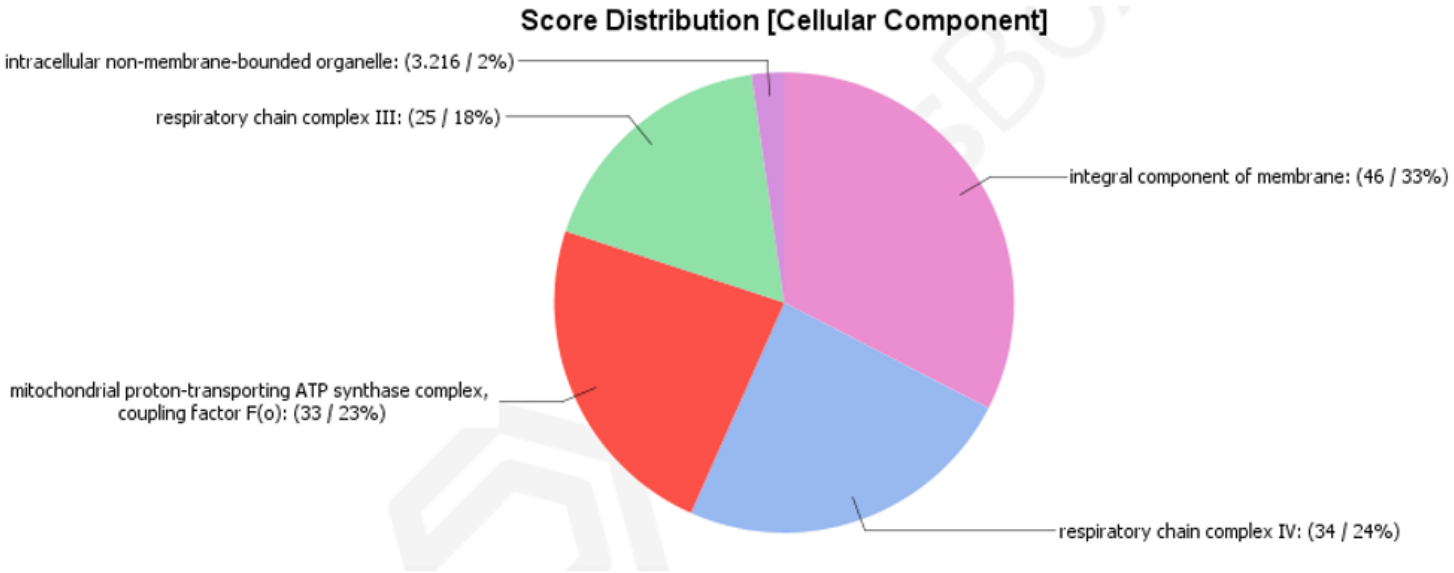

Figure 15

Distribution of node scores derived from differentially expressed sequences obtained from head samples of male Melanoplus differentialis from three populations reared in warm (30 °C) and cold (21 °C) acclimation treatments, grouped by cellular component. These scores were determined from data generated by discontiguous-MEGABLAST and subsequent gene ontology annotation performed in Blast2GO.

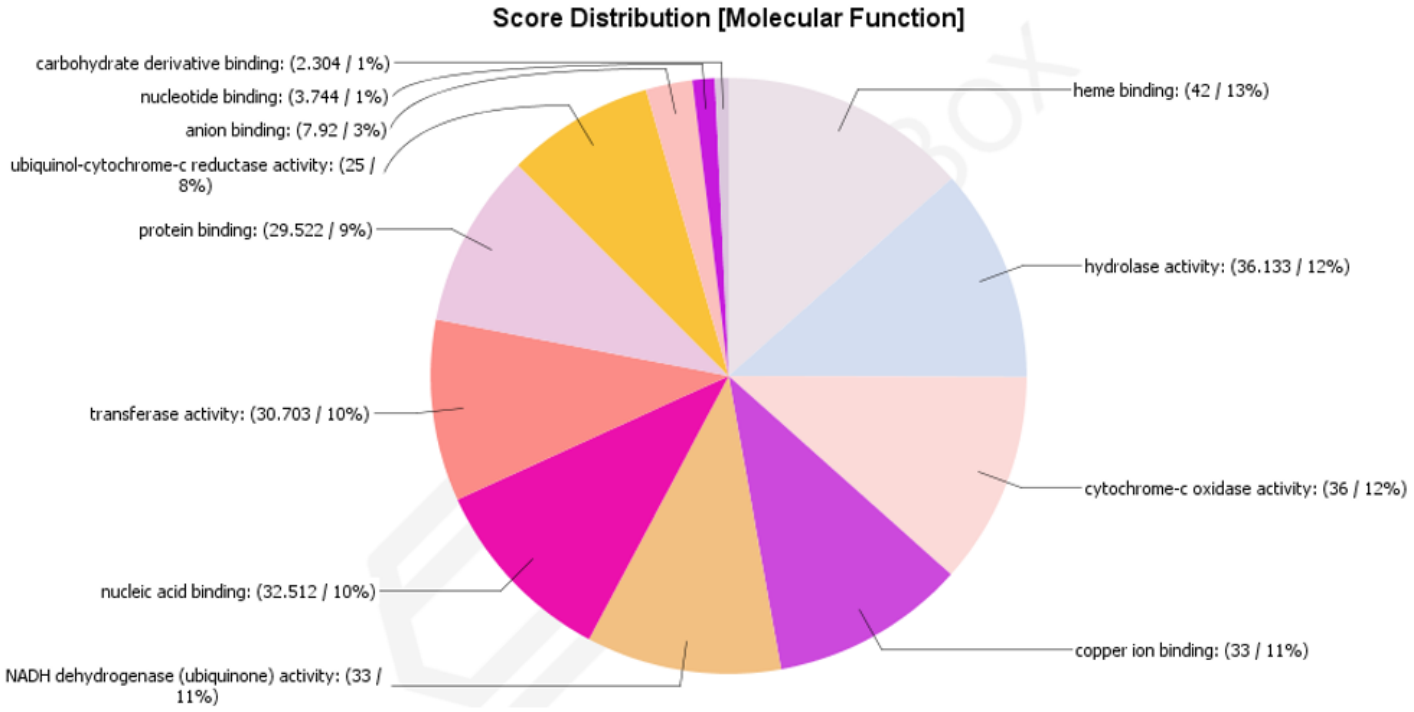

Figure 16

Distribution of node scores derived from differentially expressed sequences obtained from head samples of male Melanoplus differentialis from three populations reared in warm (30 °C) and cold (21 °C) acclimation treatments, grouped by molecular function. These scores were determined from data generated by discontiguous-MEGABLAST and subsequent gene ontology annotation performed in Blast2GO.

Supplementary Files

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