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ORIGINAL ARTICLE

How consistent are the transcriptome changes associated with cold acclimation in two species of the *Drosophila virilis* group?

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For many organisms the ability to cold acclimate with the onset of seasonal cold has major implications for their fitness. In insects, where this ability is widespread, the physiological changes associated with increased cold tolerance have been well studied. Despite this, little work has been done to trace changes in gene expression during cold acclimation that lead to an increase in cold tolerance. We used an RNA-Seq approach to investigate this in two species of the *Drosophila virilis* group. We found that the majority of genes that are differentially expressed during cold acclimation differ between the two species. Despite this, the biological processes associated with the differentially expressed genes were broadly similar in the two species. These included: metabolism, cell membrane composition, and circadian rhythms, which are largely consistent with previous work on cold acclimation/cold tolerance. In addition, we also found evidence of the involvement of the rhodopsin pathway in cold acclimation, a pathway that has been recently linked to thermotaxis. Interestingly, we found no evidence of differential expression of stress genes implying that long-term cold acclimation and short-term stress response may have a different physiological basis.
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INTRODUCTION

Insects have a range of tactics to deal with the onset of seasonal cold (Salt, 1961). One widespread tactic, cold acclimation, is an increase in an organism's cold tolerance that occurs over days or weeks when exposed to sub-lethal low temperatures, allowing insects to remain active for longer as the seasons change (Lee, 2010). The physiological changes leading to increased cold tolerance during cold acclimation have been well studied in insects and are known to primarily involve a shift in the metabolic profile of the insect, particularly sugars, polyols and amino acids (Lee, 1991; Lee, 2010). Accompanying this is a compositional change of the cell membrane primarily involving changes in phospholipids (Hazel, 1995; Koštál *et al.*, 2003; Overgaard *et al.*, 2008). These changes are cryoprotective, allowing cells to maintain their osmotic balance and thus to continue to function at low temperatures.

Cold acclimation is likely to be a homologous trait for many species of closely related insects as it has clear fitness benefits (Salt, 1961; Lee, 2010) and is thus unlikely to be lost and gained independently. Homology can be defined at several levels of biological organisation including genes, gene networks, and phenotypes, which may not necessarily coincide as the different levels may evolve at different rates. As a result it could be that although a trait is homologous and phenotypically similar across species, the genes that contribute to the production of the trait could be different as a result of evolutionary turnover, whereby genes involved in similar biological processes are co-opted in or out of influencing a trait as a result of orthologous selection or drift. This process of evolutionary turnover can thereby allow the genetic basis of a trait to change, whilst the trait itself remains conserved (see Canestro *et al.* (2007) and McCune and Schimenti (2012) for reviews).

Candidate gene approaches, whereby genes of interest identified in a model species are functionally examined in others, have often been successful and have indicated that gene function tends be conserved across species (Fitzpatrick *et al.*, 2005; Reaume and Sokolowski, 2011; Martin and Orgogozo, 2013). In contrast, quantitative genetics studies have often suggested that traits important to adaptation are polygenic, and quantitative trait loci (QTL) may have low repeatability between species (Arbuthnott, 2009; Huang *et al.*, 2012; Rockman, 2012). One reason for this disparity may be that candidate genes are typically those that have a large influence on a particular trait, and thus the evolutionary forces affecting them are likely to be stronger and more consistent than those for smaller effect loci. In particular, we expect that genes with a large influence on a particular trait are more likely to be conserved in a particular role (by purifying selection) than those with a smaller influence, which can be more easily co-opted in or out of influencing a trait.

As cold acclimation is an induced response, the gene expression changes involved with the trait can be studied using an RNA-seq approach. RNA-seq allows examination of the changes in gene expression independent of the strength of influence they have on cold acclimation, identifying both genes that have a major controlling influence on cold acclimation and genes involved in smaller secondary

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changes. Furthermore, it allows assessment of the proportion of genes that respond to cold acclimation that are the same between the two species, and what biological processes they are involved in.

Here we investigated gene expression changes in response to cold acclimation in *Drosophila* (*D.*) *montana* and *D. virilis* using an RNA-seq approach. These species belong to the *D. virilis* species group, and diverged from each other approximately 9–11 mya (Reis *et al.*, 2008; Morales-Hojas *et al.*, 2011). Both species show a high level of cold tolerance compared to other drosophilids, and *D. montana* is significantly more cold tolerant than *D. virilis* (Kellermann *et al.*, 2012; Vesala *et al.*, 2012b). These species have adapted to live in quite different environmental conditions (*D. montana* is found at high latitudes (30–70°N) and altitudes while *D. virilis* is a human commensal found at lower latitudes (south from 35°N) (Throckmorton, 1982)) but both exhibit a similar cold acclimation phenotype, whereby flies kept at sub-optimal temperatures show increased cold tolerance as measured by chill-coma recovery time (Vesala *et al.*, 2012b). When insects are placed in cold ($<0\,^{\circ}C$) temperatures a chill-coma is induced whereby insects lose the ability to move temporarily, due to a loss of nerve and muscle excitability (MacMillan and Sinclair, 2011). The time it takes for an insect to regain its ability to move is referred to as chill coma recovery time, which can be used as an estimate of an insect's cold tolerance (see David *et al.* (1998)). The cold acclimation responses of *D. montana* and *D. virilis* are likely to represent homologous traits as this trait is present in both species and across *Drosophila* species in general (see Hoffmann *et al.* (2003)). Thus our aims were: (i) to identify the genes that are differentially expressed in response to cold acclimation in *D. montana* and *D. virilis* and (ii) to identify the molecular pathways and processes underlying the cold acclimation response in each species, allowing us to determine to what extent to which these are consistent between the species.

MATERIALS AND METHODS

Samples

We used a single isofemale line established from the progenies of wild-caught fertilized females for each species: *D. montana* line 175OJ8 (originated from Oulanka, Finland, 2008) and *D. virilis* line TOY3F9 (originated from Toyama, Japan, 2003). The use of isofemale lines will reduce confounding effects of genetic variation within lines and cold acclimation responses (see discussion). Stock cultures have been maintained since their establishment on malt bottles in continuous light at $19\pm1\,^{\circ}C$, 65% humidity. Flies for the experiment were collected within one day of eclosion using light CO_2 anesthesia. Female flies were put into malt medium vials and transferred into a climate chamber (Sanyo MLR-351H) for 15 days at 16 °C for *D. montana* and 19 °C for *D. virilis*; see rationale for the temperature selection in discussion. Flies of each species were then split into two groups: a control group where flies were left in the same conditions for an additional 6 days, and a cold acclimation group where flies were maintained for 6 days at +5 °C. The light:dark (LD) cycle was 22:2 for all experimental groups and the samples for RNA extraction were flash frozen in liquid nitrogen 5 h after lights were turned on in the chambers (Zeitgeber = 5).

RNA extraction and sequencing

Frozen flies were pooled into 6 samples for *D. montana* (3 acclimation group samples and 3 control group samples, with 10 whole flies in each sample) and 4 samples for *D. virilis* (2 acclimation group samples and 2 control group samples, with 20 whole flies in each sample). Different numbers of pooled individuals per sample is unlikely to influence our analysis as all flies are from isofemale lines, but this design could have more power to detect changes in *D. montana* than *D. virilis* (but, see later). RNA was extracted from each sample using Tri Reagent (Sigma-Aldrich) followed by RNeasy Mini kit purification with DNase treatment (Qiagen). Purity of the RNA was checked using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) and integrity with 2100 Bioanalyzer (Agilent Technologies). Total RNA for each sample was approximately equal prior to sequencing.

Extracted RNA was sequenced using the SOLiD platform. For *D. montana* we used the SOLiD 5500 XL to produce 46 million 75+35 bp paired end reads and for *D. virilis* we used SOLiD V4 to produce 49 million 50 bp single end reads. Raw sequence reads were then trimmed using SOLiD TRIM (with run options: -p 3 -q 22 -y y -e 2 -d 10) to remove polyclonal errors from the data (Sasson and Michael, 2010). The reads that passed this filter were then error corrected using SOLiD Accuracy Enhancer Tools (SAET) to reduce the amount of color calling errors, or erroneous bases, in the sequence. Remaining low quality bases at the end of the reads were then trimmed using CLC Genomics Workbench 5.0.1 (CLC Bio http://www.clcbio.com/) (quality score: 0.02). Differences in the number of replicates and read type used between samples could influence our power to detect differential expression (DE), however we expect these issues to be small as we used a single model to detect DE and interactions. In addition we also repeated the analysis for *D. montana* using two replicates and with reads trimmed to be 50 bp and single end only (to make the samples comparable to those obtained for *D. virilis*). This produced very similar results as those with three replicates and paired reads (numbers of genes found to be DE 162 vs 177; correlation of the gene expression for DE genes from both analyses (r_s) = 0.94, $P<2.2\times10^{-16}$, see Supplementary Materials 1 for more details).

Mapping

Reads for each sample were mapped individually to the *D. virilis* genome (r1.2, available from http://flybase.org/) using CLC Bio. We also used this method to map reads to a *de novo* assembly of the *D. montana* transcriptome (see Supplementary Materials 2 for more details). HTSeq (Anders *et al.*, 2014) was used to quantify the read counts mapping uniquely to the reference using the reference annotation available from Flybase (r1.2).

Expression analysis

Gene expression analysis was performed using the Bioconductor package EdgeR (Robinson *et al.*, 2010) in R (R Core Team, 2013). Normalisation factors for each sample were computed using the TMM method. TMM normalisation allows variation in read depth (due to RNA quality, variation in sequencing reaction, etc.) to be accounted for, to prevent differences in read depth from influencing the detection of DE (Robinson and Oshlack, 2010). We then fitted a generalized linear model (GLM) with negative binomial distribution with the terms species, treatment and species * treatment (full model), and estimated dispersion using the Cox-Reid profile-adjusted likelihood (CR) method. We used a GLM likelihood ratio test to determine significance of a treatment effect for each gene by comparing appropriate model contrasts: the effect of treatment on *D. montana*, the effect of treatment on *D. virilis* and an interaction between species and treatment. The interaction term tests the extent to which gene expression changes differ between the species. The *P*-values from the GLM likelihood ratio tests were corrected for multiple testing using Benjamini and Hochberg's algorithm to control for false discovery rate (FDR) (Benjamini and Hochberg, 1995) with significance taken here to be $<5\%$ (FDR <0.05).

Functional classification

In order to functionally classify genes we used Gene Ontology (GO) annotation for orthologous genes in *D. melanogaster* (available from Flybase, version: FB2013_06). We used this approach instead of using GO terms from the *D. virilis* annotation due to the superior GO annotation available in *D. melanogaster* both in terms of the number of annotations and their specificity (Tweedie *et al.*, 2009). Significant enrichment of single GO terms were determined using the 'Gene Ontology Enrichment' function in FlyMine (www.flymine.org, v.37). The *D. melanogaster* orthologs of DE genes were also analysed using DAVID (Database for Annotation, Visualization and Integrated Discovery) v. 6.7 (Huang *et al.*, 2009a; Huang *et al.*, 2009b). DAVID clusters genes into functional groups using a 'fuzzy' clustering algorithm, and then uses a Fisher's exact test to identify significantly enriched functional groups. A functional group was considered to be significantly enriched if its enrichment score (the geometric mean (in -log scale) of the *P*-values of the GO terms in the group) was >1 ($P<0.1$).

RESULTS

We obtained approximately 46 million reads for *D. montana* and 49 million reads for *D. virilis*, of which approximately 10% were found to map uniquely to a gene in the *D. virilis* reference genome. The number of reads mapping to each gene was highly correlated between the species ($r_s = 0.90$), suggesting the mapping efficiency per gene is approximately equal for the two species. The number of genes that were differentially expressed (DE) during cold acclimation was fewer in *D. montana* (177) than in *D. virilis* (458) at 0.05 FDR (representing 593 total unique genes DE across both species, see Supplementary Materials 3). Among these genes only 42 genes (7%) were DE in both species. The genes DE in both species had very similar expression changes in response to cold acclimation in terms of direction ($r_s = 0.85$, $P < 2.2 \times 10^{-16}$) (Figure 1a) and fold change (Figure 1b) though changes were on average somewhat higher for *D. virilis*. One gene (*Dvir\GJ10437*) showed a significant interaction effect, being up-regulated in *D. montana* but down-regulated in *D. virilis*. The remaining genes, i.e. the genes that were DE in only one of the species, showed a much smaller correlation in expression levels ($r_s = 0.20$, $P = 1.18 \times 10^{-6}$) (Figure 2) and 132 (24%) of them showed a significant species by treatment interaction, whereby genes which showed a strong response to acclimation in one species do not show a response in the other (Figure 3) (i.e. these genes showed species-specific responses).

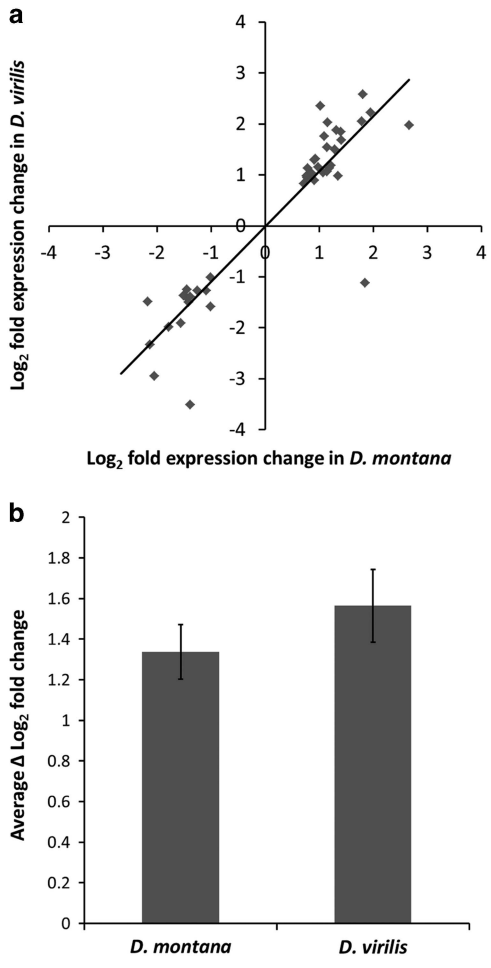

Figure 1 (**a**) Plot of $\log_2$ fold changes between control and cold acclimated flies for each of the genes that were DE in both *D. montana* and *D. virilis* (**b**) Average change in gene expression (Δ $\log_2$ fold change) between control and cold acclimated flies for genes that were DE in both species. Error bars represent approximate 95% confidence intervals.

Gene function

To examine the biological processes and pathways of the genes DE due to cold acclimation, we examined the genes in four subsets: those DE in *D. montana*, those DE in *D. virilis*, those DE in both species, and those that showed a significant treatment by species interaction. Broad level (2) GO term functional classification revealed that the majority of genes that showed significant differential expression due to cold acclimation in each of the subsets were involved in metabolic and cellular processes. We also found that the proportion of genes involved in a particular biological process was similar for each of the subsets (Figure 4). When the metabolic GO term was split into its constitute

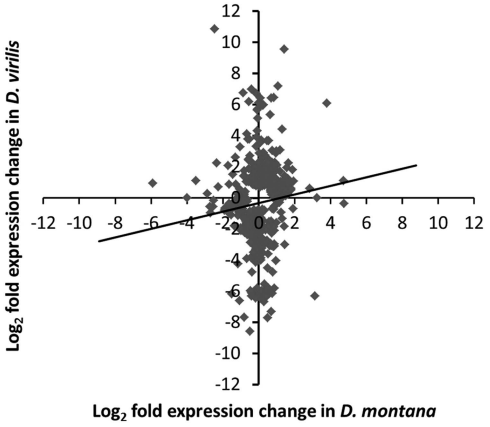

Figure 2 Plot of $\log_2$ fold changes between control and cold acclimated flies for each of the genes that are DE in either *D. montana* or *D. virilis*.

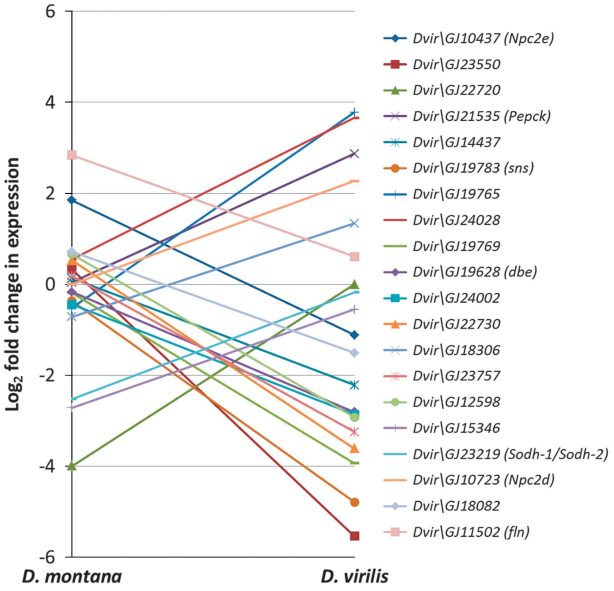

Figure 3 $\log_2$ fold change between control and cold acclimated flies for the top 20 genes which showed a significant species by treatment interaction in *D. montana* and *D. virilis*. *D. virilis* gene names are given in the legend with *D. melanogaster* orthologs in parentheses.

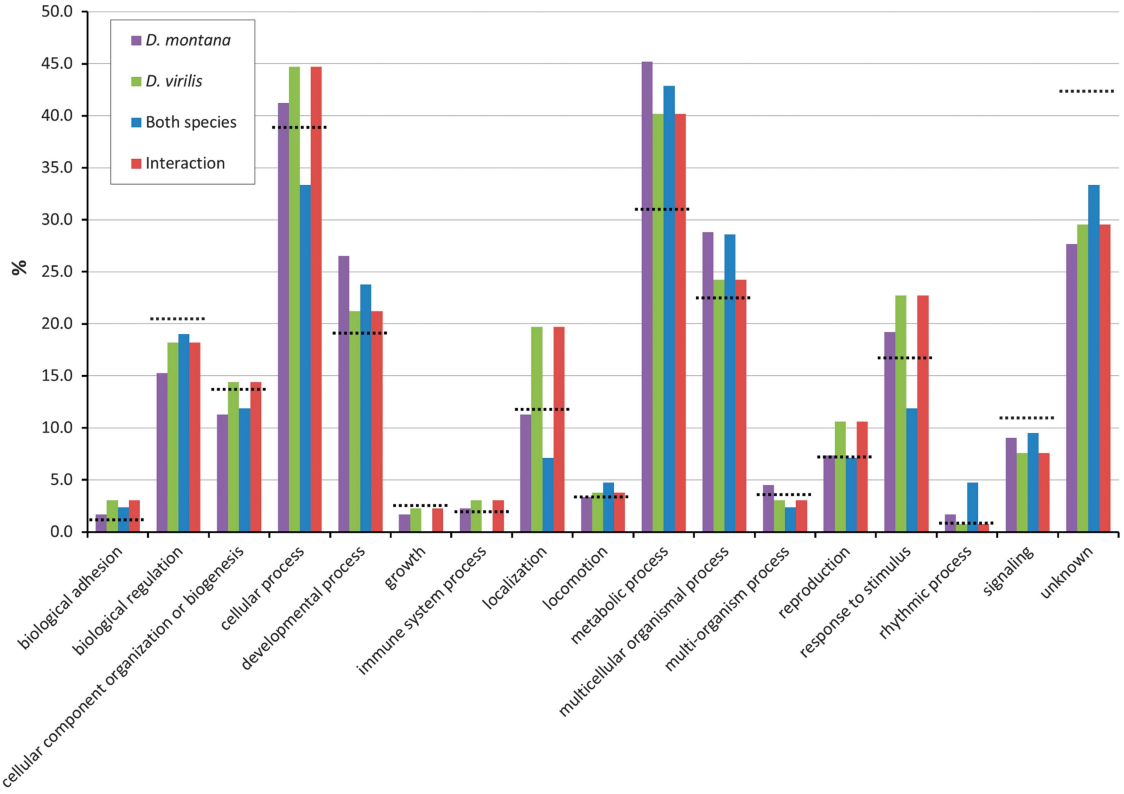

Figure 4 Proportion of genes annotated for each of the level 2 GO terms identified for each of the gene subsets (*D. montana, D. virilis*, both species and interaction). Black dashed lines indicate proportion of genes annotated for each of the level 2 GO terms in the *D. virilis* genome.

parts (Figure 5, Supplementary Materials 4–8) we found the proportion of genes annotated with each metabolic GO term was similar for each of the subsets.

GO term enrichment analysis

The single GO terms oxidation-reduction process, and single-organism metabolic process were significantly enriched in *D. montana* ($P=0.002$ and $P=0.016$ respectively). No single GO terms were significantly enriched in *D. virilis*. By using the functional clustering program DAVID we were able to identify significantly enriched functional clusters in three of the four subsets (*D. montana, D. virilis*, and interaction (Table 1)) but none were identified for the genes DE in both species. Significant clusters involved in metabolism or producing metabolites were found in the *D. montana, D. virilis*, and interaction subsets (Table 1, yellow) as well as in transmembrane transport/ion transport in *D. montana* and *D. virilis* (Table 1, green). 14 (8%) of the genes DE in *D. montana* and 39 (9%) of the genes DE in *D. virilis* were annotated with the GO terms for transmembrane transport/ion transport, with 2 genes DE in both species and 9 showing a significant interaction (Supplementary Materials 9).

In addition we identified a number of enriched gene clusters which were different between the species. In *D. montana*, we found two gene clusters which showed significant enrichment for muscle protein (Table 1, orange), while in *D. virilis* there were several clusters significantly enriched for protein signalling (Table 1, pink). Although the above mentioned functional clusters were only significantly enriched in one species, these processes are likely to be a component of cold acclimation in both of the species as we also found genes

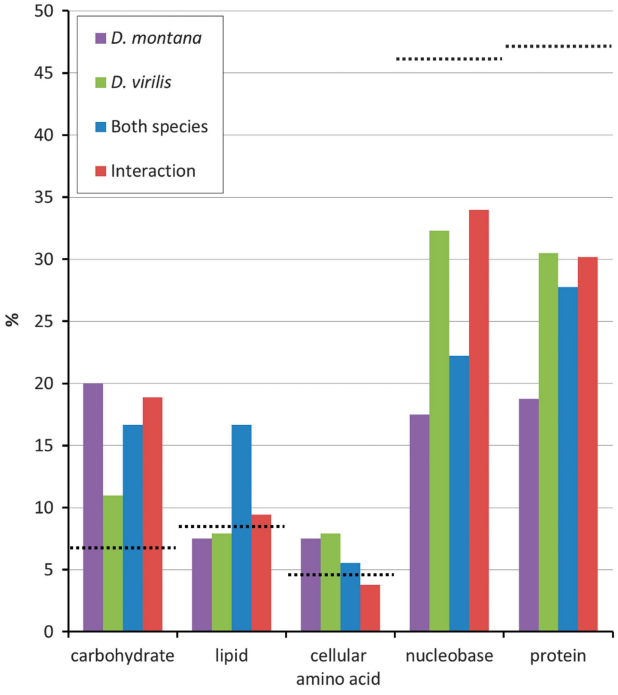

Figure 5 Proportion of genes annotated for each of the primary metabolic processes for each of the gene subsets (*D. montana, D. virilis*, both species and interaction). Black dashed lines indicate proportion of genes annotated for each of the primary metabolic processes in the *D. virilis* genome.

Table 1 Functional clusters identified by DAVID for each of the gene groups for enrichment scores with $P<0.1$

Cluster	Enrichment Score	P	Term summary
D.montana			
1	2.45	0.004	actin cytoskeleton, acetylation, myosin complex, muscle protein
2	1.97	0.010	sarcomere, actin cytoskeleton, myofibril, actin filament-based process, muscle protein
3	1.74	0.018	ion transmembrane transporter activity, mitochondrial, generation of precursor metabolites and energy, cytochrome-c oxidase activity, oxidoreductase activity, oxidative phosphorylation, phosphate metabolic process, phosphorylation
4	1.55	0.028	alkaline phosphatase, folate biosynthesis
5	1.45	0.035	mitochondrion, transit peptide
6	1.20	0.063	organic acid biosynthetic process, cellular amino acid biosynthetic process, amine biosynthetic process
7	1.11	0.078	calcium-binding EF-hand, calcium, calcium ion binding
8	1.11	0.078	oxidoreductase activity, nitrogen compound biosynthetic process, heterocycle biosynthetic process
9	1.01	0.098	ion transmembrane transporter activity, mitochondrial, generation of precursor metabolites and energy, oxidative phosphorylation, ATPase activity, hydrolase activity, phosphorylation
D. virilis			
1	1.97	0.011	integral to membrane, intrinsic to membrane, transmembrane, membrane
2	1.61	0.025	CHK kinase-like, CHK
3	1.45	0.035	identical protein binding, protein dimerization activity, protein homodimerization activity
4	1.29	0.051	metabolism of xenobiotics by cytochrome P449, glutathione transferase activity, transferase activity, glutathione S-transferase/chloride channel, glutathione metabolism, posttranslational modification, protein turnover, chaperones
5	1.2	0.063	PAS fold, PAS
6	1.19	0.065	odorant binding protein, hormone binding, JHBP
7	1.18	0.066	glutamate receptor-related, NMDA receptor, ion transport, passive transmembrane transporter activity, metal ion transmembrane transporter activity, cell membrane
8	1.12	0.076	pteridine and derivative biosynthetic process, aromatic compound biosynthetic process, pteridine and derivative metabolic process, heterocycle biosynthetic process
Interaction			
1	1.27	0.054	carbohydrate catabolic process, alcohol catabolic process
2	1.27	0.054	hexose metabolic process, monosaccharide metabolic process, glucose metabolic process

Cluster colour code: orange = muscle-related, yellow = metabolic, green = transmembrane transport/ion transport, pink = protein signalling. A full color version of this table is available at the *Heredity* journal online.

annotated with these functions DE in both species (Supplementary Materials 10).

DISCUSSION

While our understanding of the genetic basis of many traits has advanced greatly in many model systems, whether these results translate across species remains an open question. Numerous candidate gene studies have demonstrated that genes appear to be conserved in their function between taxa (Fitzpatrick *et al.*, 2005; Reaume and Sokolowski, 2011; Martin and Orgogozo, 2013). Candidate gene studies however generally focus upon a subset of genes which have a large influence on a particular trait. By using an RNA-Seq approach we were able to examine genes which altered expression in response to cold acclimation independent of the strength of influence they have on cold acclimation in two closely related *Drosophila virilis* group species where the trait is likely to be homologous. This approach captures both the genes that have a major influence on cold acclimation but also genes of smaller influence and those involved in secondary changes, which may be more susceptible to evolutionary turnover. Using this approach enabled us to identify genes that show differential expression in response to cold acclimation in both or only one of the species and to identify the molecular pathways and processes involved. Our study found evidence for both conservation and divergence in gene expression in response to cold acclimation between *D. montana* and *D. virilis*.

We identified 42 genes that were differentially expressed in both species. These genes (with one exception) showed very similar changes in terms of fold change and direction of expression changes, and thus may represent a 'core set' of genes, which appear to be evolutionary conserved in response to cold acclimation. We also identified a large number of genes that were DE in one species but not the other with around a quarter of these showing a significant species by treatment interaction. Gene expression changes in these genes were only weakly correlated with each other, with only one of the 132 genes which showed a significant interaction DE in both species. This suggests that a large proportion of the genes that were DE in response to cold acclimation were different between *D. montana* and *D. virilis*. We also observed that many more genes were DE in *D. virilis* than *D. montana*. One potential reason for this may be that *D. virilis* is less cold tolerant than *D. montana* (Kellermann *et al.*, 2012; Vesala *et al.*, 2012b) and thus the cold acclimation treatment may be more stressful for *D. virilis* than *D. montana*, promoting a stronger cold acclimation response. In support of this we also note that the average fold change in genes DE in both species was also slightly larger in *D. virilis* than *D. montana* (Figure 1b).

Our focus in this study was to look at temperature-induced changes in gene expression in *D. montana* and *D. virilis*. Therefore we chose the conditions for the control treatments to represent non-cold-acclimating 'summer' conditions for each species. A complication is that identical long day conditions for both species may have led to *D. montana* flies entering reproductive diapause which would have complicated the results markedly as *D. virilis* does not have such photoperiodic diapause (Throckmorton, 1982). According to temperature data obtained for the collection site of *D. montana* flies used in this experiment (years 2000–2009, Oulanka research station, University of Oulu, Finland), the average temperature of summer months was: June: 12.2 °C, July: 15.5 °C and August 12.6 °C. Based on this data we chose 16 °C to represent non-acclimating (control) temperature for *D. montana* strain and 19 °C for more southern and less cold tolerant *D. virilis*. As such, the temperature difference between the control (non-acclimating) and the acclimation temperatures was slightly larger for *D. virilis* than *D. montana* (14 and 11 °C respectively) which could partly influence the larger number of genes DE in *D. virilis* compared to *D. montana*. However, given the relatively small difference between the two control non-acclimating temperatures compared to the large difference between the control and the acclimation temperatures, we expect the potential influence of this to be small.

As we were primarily interested in species-specific responses to cold acclimation we used isofemale lines for both species to minimise confounding intra-population genetic variation within species. However, as we have only one line per species we cannot distinguish intra- and inter-specific sources of genetic variation. Yet, as the species separated 9–11 mya (Reis *et al.*, 2008; Morales-Hojas *et al.*, 2011) it seems most likely that interspecific differences will dominate the main differences described here. Future population-level work is required to explore the extent of differences within species.

Functional processes

As we found that the genes DE due to cold acclimation were often different between the species, we examined the functional processes involved in four subsets: genes DE in *D. montana*, in *D. virilis*, in both species, and genes which showed a significant treatment by species interaction. We discuss the main functional processes found in the DE gene subsets below (for full list of processes for all the genes found to be DE see Supplementary Materials 11).

Metabolic profile. Cold acclimation is known to involve a shift in the metabolic profile as well as the production of cyroprotectants which act to maintain osmotic balance and stabilise the membrane structures of a cell (Lee, 1991; Lee, 2010). Consistent with this previous work, we found that the majority of genes DE in both species were metabolic in function. Splitting the metabolic GO term into its constitute parts showed that there were a similar proportion of genes involved in each of the metabolic processes for the studied subsets (Figure 4). The single GO-terms 'oxidation-reduction processes' and 'single-organism metabolic processes' were significantly enriched in *D. montana*. We did not find any significantly enriched single GO terms for *D. virilis*, though *D. virilis* showed a similar (though slightly lower) proportion of metabolic genes involved in the cold acclimation response. Examination of the enriched functional clusters identified clusters of genes involved in metabolism/production of metabolites for three of the subsets (*D. montana*, *D. virilis*, and the species by treatment interaction, Table 1). We did not find any significantly enriched functional clusters for genes DE in both species, likely due to the small number of genes in this group. Although we identified a number of metabolic gene clusters for three of the subsets, the metabolic pathways implicated for each set were different. This suggests that although both species show significant enrichment for metabolic changes, the exact metabolic pathways involved differ.

Cell membrane composition. Changes to the composition of phospholipids in the cell membrane are thought to be particularly important for cold acclimation, as it allows cells to maintain their physiological function in sub-optimal temperatures (Hazel, 1995; Koštál *et al.*, 2003). Our previous work has shown the major metabolite produced in overwintering *D. montana* flies to be *myo*-inositol (Vesala *et al.*, 2012a), which functions in many processes including regulation of cell development and growth (Loewus and Loewus, 1983). It also is a precursor of inositol phospholipids leading to production of inositol phosphates that function as second messengers (Downes and Macphee, 1990). *Myo*-inositol has not been found to play a role in cold acclimation in other *Drosophila* species, but it has been shown to accumulate seasonally in few other insect species (Block and Sømme, 1983; Koštál *et al.*, 1996). It has also been correlated with an increase in cold tolerance in some Coleoptera and Lepidoptera species (Watanabe and Tanaka, 1999; Watanabe, 2002; Liu *et al.*, 2009) and we hypothesise that its accumulation in overwintering *D. montana* would act as a cryoprotectant. In the present study the ortholog of *inos* (*Dvir\GJ20549*) was upregulated in both *D. montana* and *D. virilis*. This is interesting as *inos* encodes the enzyme *myo*-inositol-1-phosphate synthase, which is part of the inositol biosynthetic pathway (GO:0006021), catalysing the conversion of D-glucose-6-phosphate into 1L-*myo*-inositol-1-phosphate, the first committed step into production of all inositol compounds (Majumder *et al.*, 1997). It is not known what the major metabolite produced in overwintering *D. virilis* flies is but given the DE of *inos* ortholog in response to cold acclimation we suggest that the production of 1L-*myo*-inositol-1-phosphate is likely to be important for both species. Another gene annotated in the inositol metabolic pathway, *Dvir\GJ15346* (*D. melanogaster* ortholog: *CG6910*), showed a significant species by treatment interaction during acclimation. This gene, which was significantly down-regulated only in *D. montana*, is annotated with the GO term inositol catabolic process and has been indicated to have the same function as myo-inositol oxygenase, an enzyme that is the first committed step in inositol catabolism in eukaryotes (Jones *et al.*, 2012). Thus the downregulation of this gene may lead to higher concentrations of inositol compounds, enabling *D. montana to* accumulate proportionally more inositol compounds, than *D. virilis*. *D. virilis*, on the other hand, may increase its cold tolerance with the aid of sphingolipid and sterol compounds, which have previously been shown to act together to alter cell membrane fluidity (Guan *et al.*, 2009), as genes annotated for the metabolism of these compounds were found to be DE in *D. virilis* but not in *D. montana* (Supplementary Materials 5).

An additional group of genes that may influence cell membrane composition are *Niemann-Pick type C* (*npc*) genes. In *D. melanogaster npc* genes have been shown to be involved in the homeostatic regulation of cholesterol, which influences the permeability and fluidity of cell membranes (Huang *et al.*, 2007; Niwa and Niwa, 2011). We found that *npc2e* was DE in both species, though it was upregulated in *D. montana* and downregulated in *D. virilis* (interaction P-value < 0.001). In addition, *npc1b* and *npc2d* were DE in *D. virilis* but not in *D. montana* (interaction P-values for both genes < 0.001). This suggests that the changes in the regulation of cholesterol are important for cold acclimation in both species, though the genetic basis used to produce the change is different in each of the species.

Ion transport/transmembrane transport. Changes in temperature are known to affect the transport mechanisms involved in the maintenance of cellular ion balance (Heitler *et al.*, 1977; Kivivuori *et al.*, 1990). Failure to maintain the ionic balance of cells can lead to metabolic perturbations which can cause a wide range of negative consequences, in particular, the loss of nerve excitation (Hochachka, 1986; Kostal *et al.*, 2004). We found enrichment of several functional gene clusters involved in ion transport/transmembrane transport in both species (Table 1) which suggests that changes in gene expression of these genes are acting to maintain cellular ion concentration at low temperatures. Although both species showed enrichment for ion transport/transmembrane transport the genes responsible were largely different, with only two genes shared between the species and nine showing a significant interaction (Supplementary materials 9).

Rhodopsin. The ortholog of a key gene in the rhodopsin bio-synthesis pathway *santa-maria, Dvir\GJ17608,* was up-regulated in both species in response to cold acclimation. Rhodopsin has long been known to be the primary pigment for phototransduction (see Katz and Minke (2009) for a review), however recently it has been shown that the rhodopsin signalling pathway also has a several additional light-independent roles including hearing (Senthilan *et al.*, 2012) and thermosensory signalling (Shen *et al.*, 2011). Shen *et al.* (2011) showed that by knocking out *santa-maria* in *D. melanogaster,* flies were unable to discriminate between differences in temperature. Our finding that *Dvir\GJ17608* is upregulated in both species is intriguing as it suggests the rhodopsin pathway may act to detect changes in temperature and thus may cue the cold acclimation response. None of the other genes involved in the rhodopsin bio-synthesis pathway were found to be DE in either of the species suggesting that *santa-maria* may be the key gene involved in the detection of temperature change. We did, however, find that an ortholog of *CdsA (Dvir\GJ13151)*, a gene involved in the regulation of lipid storage, was DE in *D. virilis* but not *D. montana*. *CdsA* also belongs to a rhodopsin mediated signalling pathway and thus could play a role in linking rhodopsin signalling and downstream metabolic changes.

Circadian clock. Consistent with previous candidate gene approaches in *D. montana* and *D. virilis* (Vesala *et al.*, 2012b) we found that two of the core circadian clock genes, *period* and *vrille*, were DE in both species. These genes have a well-studied role in the regulation and maintenance of circadian rhythms in insects (Bell-Pedersen *et al.*, 2005). In addition, we also found four other peripheral clock genes to be DE in either *D. montana* or *D. virilis* including one which showed a significant species by treatment interaction (Table 2). The circadian clock has been previously implicated in cold acclimation in several taxa (Fowler and Thomashow, 2002; Magnone *et al.*, 2005; Vesala *et al.*, 2012b). It is not clear however if the changes in these genes have a direct influence on the increase in cold tolerance or if changes of the expression levels of these genes affect changes to the circadian rhythms of the fly in anticipation of the change in seasonal photoperiod. Further functional genetic studies will allow disentanglement of these hypothesis but given extensive work showing how the clock genes have a large influence on the regulation of metabolic processes in *Drosophila* (Xu *et al.*, 2008; Xu *et al.*, 2011; Sahar and Sassone-Corsi, 2012) we suggest that clock genes are good candidates for orchestrating the shifts in metabolic profile seen during cold acclimation.

Differences between the species. Despite the overall pattern of broadly similar processes occurring in both species we also identified differences between the species. In *D. montana* we found significant enrichment for actin filaments/muscle protein, while in *D. virilis* we found significant enrichment for several clusters of genes involved in protein signalling/modification (Table 1). Changes in actin filaments /muscle protein have previously been implicated in insects to prevent cold injury by preventing the depolymerisation of the cell membrane during diapause (Kim *et al.*, 2006; Robich *et al.*, 2007; Koštál, 2010). Thus, these changes may help to maintain cellular physiology at sub-optimal temperatures. The four clusters of genes involved in protein signalling/modification indicate that these processes may also be important in cold acclimation. These differences seen between the species are unlikely to represent the absence of one process in one species and presence in another, but rather a greater or lesser relative importance in the context of the other processes occurring during cold acclimation for that species. For each of these clusters we also found several genes in the other species involved in the same biological process (Supplementary Materials 10) suggesting that these processes are likely to be a component of cold acclimation in both of the studied species.

Cold acclimation and cold shock

Previous work on cold tolerance in insects (particularly in *D. melanogaster*) has mostly focused upon the response to short-term cold shock rather than longer periods of cold acclimation (Colinet and Hoffmann, 2012). These processes, although related, are distinct and likely to produce differing transcriptional responses. This is important to consider when comparing our findings to previous work. Broadly, work on short-term cold shock gene expression changes has shown that the predominant group of genes showing expression changes are those involved in the stress and immune response (e.g. heat shock proteins (Hsps), *Turandot genes*, etc) (Zhang *et al.*, 2011; Sinclair *et al.*, 2013; Storey and Storey, 2013). In addition, the neuropeptide CAPA has been shown to play an important role in both cold and desiccation resistance in several species of *Drosophila*, including *D. montana,* when assessed using cold shock (Terhzaz *et al.,* submitted). We do not find these genes or others involved in heat-shock response to be DE during cold acclimation. This is consistent with the findings by Colinet *et al.* (2013) who did not detect changes in heat shock protein levels after a 5-day cold acclimation in *D. melanogaster*. One explanation for this may be that the Hsps are important in the early part of thermal stress but are not involved in longer-term cold acclimation *per se* (Colinet and Hoffmann, 2012; Teets and Denlinger, 2013; but see Vesala *et al.* 2012b).

Functional modules

We were able to identify several functional processes that contribute to the cold acclimation response, the most important of which are likely to include: cold detection, circadian genes, and metabolic and cellular membrane profile shifts. For each of these functional processes we found genes that are conserved between the species and others that have diverged. We suggest that the genes that were found to be DE in both species are likely to represent key genes with a large influence on the cold acclimation phenotype. Further functional genetic studies are needed to confirm this, although it should be noted that several of the genes DE in both species have previously been demonstrated to have a large influence on processes implicated in cold acclimation. For example, we found that orthologs of two of the major genes involved in the circadian clock (*period* and *vrille*) were DE in both species, whereas genes more peripheral to the clock were DE in one species but not in the other (Table 2). We also observed the ortholog of the gene *inos*, which is responsible for producing the main overwintering metabolite in *D. montana*, and thus presumably has a large influence

Table 2 Circadian genes differentially expressed in response to cold acclimation

Gene	*D. melanogaster ortholog*	*Species*	*Interaction?*
Dvir\per	*per*	Both species	
Dvir\GJ17539	*vri*	Both species	
Dvir\GJ11819	*dysc*	*D. virilis*	
Dvir\GJ16719	*CG2650*	*D. virilis*	
Dvir\GJ19065	*na*	*D. virilis*	Yes
Dvir\GJ11425	*Pdp1*	*D. montana*	

on the cold acclimation, is DE in both species. Interestingly, we found *santa-maria* (*Dvir\GJ17608*) was DE in both species. As stated above, *santa-maria* has a large role in the rhodopsin bio-synthesis pathway (Shen *et al.*, 2011). Although this pathway has been previously linked to the ability to discriminate between differences in temperature (Shen *et al.*, 2011), it has not been implicated in the cold acclimation response. One possibility is that rhodopsin bio-synthesis pathway may act to detect the onset of cold and to subsequently cue the DE of genes which increase cold tolerance.

CONCLUSIONS

We examined the effect of cold acclimation on gene expression in two *Drosophila virilis* group species to determine what genes and pathways were involved and if these were the same for both species. We found that the transcriptional changes associated with cold acclimation were broadly different with only 42 genes DE in both species and 132 showing a significant species by treatment interaction. This suggests that when comparing homologous phenotypes the underlying genetic basis of the trait may differ. The genes that were DE in both species are likely to have been conserved in their role in cold acclimation since at least the time of the species split. These evolutionarily conserved genes are likely to have a large influence on cold acclimation, as the selective forces maintaining cold acclimation will be stronger on these genes, than on genes that have a smaller influence.

Although we found that many of the genes DE due to cold acclimation in each of the species were different, we also found that the biological processes they were involved in were broadly similar. This is consistent with the idea of evolutionary turnover co-opting functionally related genes of smaller influence in and out of the cold acclimation response. The result of such evolutionary turnover is that although a trait may be homologous and appear phenotypically similar between species, many of the genes underlying the trait can be different.

DATA ARCHIVING

All data has been deposited in NCBI's Gene Expression Omnibus (GEO). Accession ID: GSE58925 Databank URL: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE58925

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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