1 # How consistent are the transcriptome changes associated with cold

2 # acclimation in two species of the *Drosophila virilis* group?

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16

17 **Abstract**

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

34

35 **Introduction**

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

48 Cold acclimation is likely to be a homologous trait for many species of closely related
49 insects as it has clear fitness benefits (Salt, 1961; Lee, 2010) and is thus unlikely to
50 be lost and gained independently. Homology can be defined at several levels of
51 biological organisation including genes, gene networks, and phenotypes, which may
52 not necessarily coincide as the different levels may evolve at different rates. As a
53 result it could be that although a trait is homologous and phenotypically similar
54 across species, the genes that contribute to the production of the trait could be
55 different as a result of evolutionary turnover, whereby genes involved in similar
56 biological processes are co-opted in or out of influencing a trait as a result of
57 orthologous selection or drift. This process of evolutionary turnover can thereby allow

58 the genetic basis of a trait to change, whilst the trait itself remains conserved (*see*
59 Canestro et al. (2007) and McCune and Schimenti (2012) for reviews).

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

73 As cold acclimation is an induced response, the gene expression changes involved
74 with the trait can be studied using an RNA-seq approach. RNA-seq allows
75 examination of the changes in gene expression independent of the strength of
76 influence they have on cold acclimation, identifying both genes that have a major
77 controlling influence on cold acclimation and genes involved in smaller secondary
78 changes. Furthermore, it allows assessment of the proportion of genes that respond
79 to cold acclimation that are the same between the two species, and what biological
80 processes they are involved in.

81 Here we investigated gene expression changes in response to cold acclimation in
82 *Drosophila* (*D.*) *montana* and *D. virilis* using an RNA-seq approach. These species
83 belong to the *D. virilis* species group, and diverged from each other approximately 9-
84 11 mya (Reis et al., 2008; Morales-Hojas et al., 2011). Both species show a high
85 level of cold tolerance compared to other drosophilids, and *D. montana* is
86 significantly more cold tolerant than *D. virilis* (Kellermann et al., 2012; Vesala et al.,
87 2012b). These species have adapted to live in quite different environmental
88 conditions (*D. montana* is found at high latitudes (30-70°N) and altitudes while *D.*
89 *virilis* is a human commensal found at lower latitudes (south from 35°N)
90 (Throckmorton, 1982)) but both exhibit a similar cold acclimation phenotype,
91 whereby flies kept at sub-optimal temperatures show increased cold tolerance as
92 measured by chill-coma recovery time (Vesala et al., 2012b). When insects are
93 placed in cold (<0°C) temperatures a chill-coma is induced whereby insects lose the
94 ability to move temporarily, due to a loss of nerve and muscle excitability (MacMillan
95 and Sinclair, 2011). The time it takes for an insect to regain its ability to move is
96 referred as chill coma recovery time, which can be used as an estimate of an insect's
97 cold tolerance (*see* David et al. (1998)). The cold acclimation responses of *D.*
98 *montana* and *D. virilis* are likely to represent homologous traits as this trait is present
99 in both species and across *Drosophila* species in general (see Hoffmann et al.
100 (2003)). Thus our aims were: (i) to identify the genes that are differentially expressed
101 in response to cold acclimation in *D. montana* and *D. virilis* and (ii) to identify the
102 molecular pathways and processes underlying the cold acclimation response in each
103 species, allowing us to determine to what extent these are consistent between the
104 species.

105 **Methods**

106 **Samples**

107 We used a single isofemale line established from the progenies of wild-caught
108 fertilized females for each species: *D. montana* line 175OJ8 (originated from
109 Oulanka, Finland, 2008) and *D. virilis* line TOY3F9 (originated from Toyama, Japan,
110 2003). The use of isofemale lines will reduce confounding effects of genetic variation
111 within lines and cold acclimation responses (see discussion). Stock cultures have
112 been maintained since their establishment on malt bottles in continuous light at
113 19±1°C, 65% humidity. Flies for the experiment were collected within one day of
114 eclosion using light CO_2 anesthesia. Female flies were put into malt medium vials
115 and transferred into a climate chamber (Sanyo MLR-351H) for 15 days at 16 °C for
116 *D. montana* and 19°C for *D. virilis*; see rationale for the temperature selection in
117 discussion. Flies of each species were then split into two groups: a control group
118 where flies were left in the same conditions for an additional 6 days, and a cold
119 acclimation group where flies were maintained for 6 days at +5°C. The light:dark
120 (LD) cycle was 22:2 for all experimental groups and the samples for RNA extraction
121 were flash frozen in liquid nitrogen 5 hours after lights were turned on in the
122 chambers (Zeitgeber=5).

123 **RNA extraction and sequencing**

124 Frozen flies were pooled into 6 samples for *D. montana* (3 acclimation group
125 samples and 3 control group samples, with 10 whole flies in each sample) and 4
126 samples for *D. virilis* (2 acclimation group samples and 2 control group samples, with
127 20 whole flies in each sample). Different numbers of pooled individuals per sample is

128 unlikely to influence our analysis as all flies are from isofemale lines, but this design
129 could have more power to detect changes in *D. montana* than *D. virilis* (but, see
130 later). RNA was extracted from each sample using Tri Reagent (Sigma-Aldrich)
131 followed by RNeasy Mini kit (Qiagen) purification with DNase treatment. Purity of the
132 RNA was checked using NanoDrop ND-1000 spectrophotometer (NanoDrop
133 Technologies) and integrity with 2100 Bioanalyzer (Agilent Technologies). Total RNA
134 for each sample was approximately equal prior to sequencing.

135 Extracted RNA was sequenced using the SOLiD platform. For *D. montana* we used
136 the SOLiD 5500 XL to produce 46 million 75 + 35 BP paired end reads and for *D.*
137 *virilis* we used SOLiD V4 to produce 49 million 50BP single end reads. Raw
138 sequence reads were then trimmed using SOLiD TRIM (with run options: -p 3 -q 22 -
139 y y -e 2 -d 10) to remove polyclonal errors from the data (Sasson and Michael,
140 2010). The reads that passed this filter were then error corrected using SOLiD
141 Accuracy Enhancer Tools (SAET) to reduce the amount of color calling errors, or
142 erroneous bases, in the sequence. Remaining low quality bases at the end of the
143 reads were then trimmed using CLC Genomics Workbench 5.0.1 (CLC Bio
144 http://www.clcbio.com/) (quality score: 0.02). Differences in the number of replicates
145 and read type used between samples could influence our power to detect differential
146 expression (DE), however we expect these issues to be small as we used a single
147 model to detect DE and interactions. In addition we also repeated the analysis for *D.*
148 *montana* using two replicates and with reads trimmed to be 50 BP and single end
149 only (to make the samples comparable to those obtained for *D. virilis*). This produced
150 very similar results as those with three replicates and paired reads (numbers of
151 genes found to be DE 162 vs. 177; correlation of the gene expression for DE genes

152 from both analyses (r_s) = 0.94, $p < 2.2 \times 10^{-16}$, see Supplementary Materials 1 for
153 more details).

154 **Mapping**

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

161 **Expression analysis**

162 Gene expression analysis was performed using the Bioconductor package EdgeR
163 (Robinson et al., 2010) in R (R Core Team, 2013). Normalisation factors for each
164 sample were computed using the TMM method. TMM normalisation allows variation
165 in read depth (due to RNA quality, variation in sequencing reaction, etc.) to be
166 accounted for, to prevent differences in read depth from influencing the detection of
167 DE (Robinson and Oshlack, 2010). We then fitted a generalized linear model (GLM)
168 with negative binomial distribution with the terms species, treatment and species *
169 treatment (full model), and estimated dispersion using the Cox-Reid profile-adjusted
170 likelihood (CR) method. We used a GLM likelihood ratio test to determine
171 significance of a treatment effect for each gene by comparing appropriate model
172 contrasts: the effect of treatment on *D. montana*, the effect of treatment on *D. virilis*
173 and an interaction between species and treatment. The interaction term tests the
174 extent to which gene expression changes differ between the species. The p-values

175 from GLM likelihood ratio test were corrected for multiple testing using Benjamini and
176 Hochberg's algorithm to control for false discovery rate (FDR) (Benjamini and
177 Hochberg, 1995) with significance taken here to be <5% (FDR < 0.05).

178 **Functional classification**

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

193 **Results**

194 We obtained approximately 46 million reads for *D. montana* and 49 million reads for
195 *D. virilis*, of which approximately 10% were found to map uniquely to a gene in the *D.*
196 *virilis* reference genome. The number of reads mapping to each gene was highly
197 correlated between the species ($r_s = 0.90$), suggesting the mapping efficiency per
198 gene is approximately equal for the two species. The number of genes that were

199 differentially expressed (DE) during cold acclimation was fewer in *D. montana* (177)
200 than in *D. virilis* (458) at 0.05 FDR (representing 593 total unique genes DE across
201 both species, *see* Supplementary Materials 3). Among these genes only 42 genes
202 (7%) were DE in both species. The genes DE in both species had very similar
203 expression changes in response to cold acclimation in terms of direction ($r_s = 0.85$, p
204 $< 2.2 \times 10^{-16}$) (Fig. 1A) and fold change (Fig. 1B) though changes were on average
205 somewhat higher for *D. virilis*. One gene (*Dvir\GJ10437*) showed a significant
206 interaction effect, being up-regulated in *D. montana* but down-regulated in *D. virilis*.
207 The remaining genes, i.e. the genes that were DE in only one of the species, showed
208 a much smaller correlation in expression levels ($r_s = 0.20$, $p = 1.18 \times 10^{-6}$) (Fig. 2)
209 and 132 (24%) of them showed a significant species by treatment interaction,
210 whereby genes which showed a strong response to acclimation in one species do
211 not show a response in the other (Fig. 3) (i.e. these genes showed species-specific
212 responses).

213 **Gene Function**

214

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

223 GO term was split into its constitute parts (Fig. 5, Supplementary Materials 4 - 8) we
224 found the proportion of genes annotated with each metabolic GO term was similar
225 for each of the subsets.
226
227 **GO Term enrichment analysis**

228 The single GO terms oxidation-reduction process, and single-organism metabolic
229 process were significantly enriched in *D. montana* (p = 0.002 and p = 0.016
230 respectively). No single GO terms were significantly enriched in *D. virilis.* By using
231 the functional clustering program DAVID we were able to identify significantly
232 enriched functional clusters in three of the four subsets (*D. montana, D. virilis*, and
233 interaction (Table 1)) but none were identified for the genes DE in both species.
234 Significant clusters involved in metabolism or producing metabolites were found in
235 the *D. montana, D. virilis*, and interaction subsets (Table 1, yellow) as well as in
236 transmembrane transport/ion transport in *D. montana* and *D. virilis* (table 1, green).
237 14 (8%) of the genes DE in *D. montana* and 39 (9%) of the genes DE in *D. virilis*
238 were annotated with the GO terms for transmembrane transport/ion transport, with 2
239 genes DE in both species and 9 showing a significant interaction (Supplementary
240 Materials 9).

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

247 both of the species as we also found genes annotated with these functions DE in

248 both species (Supplementary Materials 10).

249

250 **Discussion**

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

269 We identified 42 genes that were differentially expressed in both species. These
270 genes (with one exception) showed very similar changes in terms of fold change and
271 direction of expression changes, and thus may represent a ‘core set’ of genes, which
272 appear to be evolutionary conserved in response to cold acclimation. We also
273 identified a large number of genes that were DE in one species but not the other with

274 around a quarter of these showing a significant species by treatment interaction.
275 Gene expression changes in these genes were only weakly correlated with each
276 other, with only one of the 132 genes which showed a significant interaction DE in
277 both species. This suggests that a large proportion of the genes that were DE in
278 response to cold acclimation were different between *D. montana* and *D. virilis*. We
279 also observed that many more genes are DE in *D. virilis* than *D. montana*. One
280 potential reason for this may be that *D. virilis* is less cold tolerant than *D. montana*
281 (Kellermann et al., 2012; Vesala et al., 2012b) and thus the cold acclimation
282 treatment may be more stressful for *D. virilis* than *D. montana,* promoting a stronger
283 cold acclimation response. In support of this we also note that the average fold
284 change in genes DE in both species is also slightly larger in *D. virilis* than *D.*
285 *montana* (Fig. 1B).

286 Our focus in this study was to look at temperature-induced changes in gene
287 expression in *D. montana* and *D. virilis*. Therefore we chose the conditions for the
288 control treatments to represent non-cold-acclimating 'summer' conditions for each
289 species. A complication is that identical long day conditions for both species may
290 have led to *D. montana* flies entering reproductive diapause which would have
291 complicated the results markedly as *D. virilis* does not have such photoperiodic
292 diapause (Throckmorton, 1982). According to temperature data obtained for the
293 collection site of *D. montana* flies used in this experiment (years 2000-2009, Oulanka
294 research station, University of Oulu, Finland), the average temperature of summer
295 months was: June: 12.2°C, July: 15.5°C and August 12.6°C. Based on this data we
296 chose 16°C to represent non-acclimating (control) temperature for *D. montana* strain
297 and 19°C for more southern and less cold tolerant *D. virilis*. As such, the temperature

298 difference between the control (non-acclimating) and the acclimation temperatures
299 was slightly larger for *D. virilis* than *D. montana* (14°C and 11°C respectively) which
300 could partly influence the larger number of genes DE in *D. virilis* compared to *D.*
301 *montana*. However, given the relatively small difference between the two control
302 non-acclimating temperatures compared to the large difference between the control
303 and the acclimation temperatures, we expected the potential influence of this to be
304 small.

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

313

314 **Functional Processes**

315

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

321 *see* Supplementary Materials 11).

322

323 *Metabolic profile*

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

343 *Cell membrane composition*

344

345 Changes to the composition of phospholipids in the cell membrane are thought to be
346 particularly important for cold acclimation, as it allows cells to maintain their
347 physiological function in sub-optimal temperatures (Hazel, 1995; Koštál et al., 2003).
348 Our previous work has shown the major metabolite produced in overwintering *D.*
349 *montana* flies to be *myo*-inositol (Vesala et al., 2012a), which functions in many
350 processes including regulation of cell development and growth (Loewus and Loewus,
351 1983). It also is a precursor of inositol phospholipids leading to production of inositol
352 phosphates that function as second messengers (Downes and Macphee, 1990).
353 *Myo*-inositol has not been found to play a role in cold acclimation in other *Drosophila*
354 species, but it has been shown to accumulate seasonally in few other insect species
355 (Block and Sømme, 1983; Koštál et al., 1996). It has also been correlated with an
356 increase in cold tolerance in some Coleoptera and Lepidoptera species (Watanabe
357 and Tanaka, 1999; Watanabe, 2002; Liu et al., 2009) and we hypothesise that its
358 accumulation in overwintering *D. montana* would act as a cryoprotectant. In the
359 present study the ortholog of *inos* (*Dvir\GJ20549*) was upregulated in both *D.*
360 *montana* and *D. virilis*. This is interesting as *inos* encodes the enzyme *myo*-inositol-
361 1-phosphate synthase, which is part of the inositol biosynthetic pathway
362 (GO:0006021), catalysing the conversion of D-glucose-6-phosphate into 1L-*myo*-
363 inositol-1-phosphate, the first committed step into production of all inositol
364 compounds (Majumder et al., 1997). It is not known what the major metabolite
365 produced in overwintering *D. virilis* flies is but given the DE of *inos* ortholog in
366 response to cold acclimation we suggest that the production of 1L-*myo*-inositol-1-
367 phosphate is likely to be important for both species. Another gene annotated in the
368 inositol metabolic pathway, *Dvir\GJ15346* (*D. melanogaster* ortholog: *CG6910*),

369 showed a significant species by treatment interaction during acclimation. This gene,
370 which was significantly down-regulated only in *D. montana*, is annotated with the GO
371 term inositol catabolic process and has been indicated to have the same function as
372 myo-inositol oxygenase, an enzyme that is the first committed step in inositol
373 catabolism in eukaryotes (Jones et al., 2012). Thus the downregulation of this gene
374 may lead to higher concentrations of inositol compounds, enabling *D. montana to*
375 accumulate proportionally more inositol compounds, than *D. virilis. D. virilis*, on the
376 other hand, may increase its cold tolerance with the aid of sphingolipid and sterol
377 compounds, which have previously been shown to act together to alter cell
378 membrane fluidity (Guan et al., 2009), as genes annotated for the metabolism of
379 these compounds were found to be DE in *D. virilis* but not in *D. montana*
380 (Supplementary Materials 5).

381 An additional group of genes that may influence cell membrane composition are
382 *Niemann-Pick type C* (*npc*) genes. In *D. melanogaster npc* genes have been shown
383 to be involved in the homeostatic regulation of cholesterol, which influences the
384 permeability and fluidity of cell membranes (Huang et al., 2007; Niwa and Niwa,
385 2011). We found that *npc2e* was DE in both species, though it was upregulated in *D.*
386 *montana* and downregulated in *D. virilis* (interaction p-value < 0.001). In addition,
387 *npc1b* and *npc2d* were DE in *D. virilis* but not in *D. montana* (interaction p-values for
388 both genes < 0.001). This suggests that the changes in the regulation of cholesterol
389 are important for cold acclimation in both species, though the genetic basis used to
390 produce the change is different in each of the species.

391 *Ion transport/ transmembrane transport*

392 Changes in temperature are known to affect the transport mechanisms involved in
393 the maintenance of cellular ion balance (Heitler et al., 1977; Kivivuori et al., 1990).
394 Failure to maintain the ionic balance of cells can lead to metabolic perturbations
395 which can cause a wide range of negative consequences, in particular, the loss of
396 nerve excitation (Hochachka, 1986; Kostal et al., 2004). We found enrichment of
397 several functional gene clusters involved in ion transport/ transmembrane transport
398 in both species (Table 1) which suggest that changes in gene expression of these
399 genes are acting to maintain cellular ion concentration at low temperatures. Although
400 both species showed enrichment for ion transport/ transmembrane transport the
401 genes responsible were largely different, with only two genes shared between the
402 species and nine showing a significant interaction (Supplementary materials 9).
403
404 *Rhodopsin*

405 The ortholog of a key gene in the rhodopsin bio-synthesis pathway *santa-maria,*
406 *Dvir\GJ17608,* was up-regulated in both species in response to cold acclimation.
407 Rhodopsin has long been known to be the primary pigment for phototransduction
408 (see Katz and Minke (2009) for a review), however recently it has been shown that
409 the rhodopsin signalling pathway also has a several additional light-independent
410 roles including hearing (Senthilan et al., 2012) and thermosensory signalling (Shen
411 et al., 2011). Shen et al. (2011) showed that by knocking out *santa-maria* in *D.*
412 *melanogaster,* flies were unable to discriminate between differences in temperature.
413 Our finding that *Dvir\GJ17608* is upregulated in both species is intriguing as it
414 suggests the rhodopsin pathway may act to detect changes in temperature and thus
415 may cue the cold acclimation response. None of the other genes involved in the

416 rhodopsin bio-synthesis pathway were found to be DE in either of the species
417 suggesting that *santa-maria* may be the key gene involved in the detection of
418 temperature change. We did, however, find that an ortholog of *cdsA (Dvir\GJ13151)*,
419 a gene involved in the regulation of lipid storage, was DE in *D. virilis* but not *D.*
420 *montana*. *cdsA* also belongs to a rhodopsin mediated signalling pathway and thus
421 could play a role in linking rhodopsin signalling and downstream metabolic changes.

422 *Circadian clock*

423 Consistent with previous candidate gene approaches in *D. montana* and *D. virilis*
424 (Vesala et al., 2012b) we found that two of the core circadian clock genes, *period*
425 and *vrille*, were DE in both species. These genes have a well-studied role in the
426 regulation and maintenance of circadian rhythms in insects (Bell-Pedersen et al.,
427 2005). In addition, we also found four other peripheral clock genes to be DE in either
428 *D. montana* or *D. virilis* including one which showed a significant species by
429 treatment interaction (Table 1). The circadian clock has been previously implicated in
430 cold acclimation in several taxa (Fowler and Thomashow, 2002; Magnone et al.,
431 2005; Vesala et al., 2012b). It is not clear however if the changes in these genes
432 have a direct influence on the increase in cold tolerance or if changes of the
433 expression levels of these genes affect changes to the circadian rhythms of the fly in
434 anticipation of the change in seasonal photoperiod. Further functional genetic studies
435 will allow disentanglement of these hypothesis but given extensive work showing
436 how the clock genes have a large influence on the regulation of metabolic processes
437 in *Drosophila* (Xu et al., 2008; Xu et al., 2011; Sahar and Sassone-Corsi, 2012) we
438 suggest clock genes are good candidates for orchestrating the shifts in metabolic
439 profile seen during cold acclimation.

440 *Differences between the species*

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

458 **Cold acclimation and cold shock**

459

460 Previous work on cold tolerance in insects (particularly in *D. melanogaster*) has
461 mostly focused upon the response to short-term cold shock rather than longer
462 periods of cold acclimation (Colinet and Hoffmann, 2012). These processes,
463 although related, are distinct and likely to produce differing transcriptional responses.

464 This is important to consider when comparing our findings to previous work. Broadly,
465 work on short-term cold shock gene expression changes has shown that the
466 predominant group of genes showing expression changes are those involved in the
467 stress and immune response (e.g. heat shock proteins (Hsps), *Turandot genes*, etc)
468 (Sinclair et al., 2013; Storey and Storey, 2013; Zhang et al., 2011). In addition, the
469 neuropeptide CAPA has been shown to play an important role in both cold and
470 desiccation resistance in several species of *Drosophila*, including *D. montana,* when
471 assessed using cold shock (Terhzaz et al., submitted). We do not find these genes
472 or others involved in heat-shock response to be DE during cold acclimation. This is
473 consistent with the findings by Colinet *et al*. (2013) who did not detect changes in
474 heat shock protein levels after a 5-day cold acclimation in *D. melanogaster.* One
475 explanation for this may be that the Hsps are important in the early part of thermal
476 stress but are not involved in longer-term cold acclimation *per se* (Colinet and
477 Hoffmann, 2012; Teets and Denlinger, 2013; but see Vesala et al. 2012b).

478 **Functional modules**

479 We were able to identify several functional processes that contribute to the cold
480 acclimation response, the most important of which are likely to include: cold
481 detection, circadian genes, and metabolic and cellular membrane profile shifts. For
482 each of these functional processes we found genes that are conserved between the
483 species and others that have diverged. We suggest that the genes that we found to
484 be DE in both species are likely to represent key genes with a large influence on the
485 cold acclimation phenotype. Further functional genetic studies are needed to confirm
486 this, although it should be noted that several of the genes DE in both species have
487 previously been demonstrated to have a large influence on processes implicated in

488 cold acclimation. For example, we find that orthologs of two of the major genes
489 involved in the circadian clock (*period* and *vrille*) are DE in both species, whereas
490 genes more peripheral to the clock are DE in one species but not in the other (Table
491 2). We also observe the ortholog of the gene *inos,* which is responsible for producing
492 the main overwintering metabolite in *D. montana*, and thus presumably has a large
493 influence on the cold acclimation, is DE in both species. Interestingly we find *santa-*
494 *maria* (*Dvir\GJ17608*) to be DE in both species. As stated above, *santa-maria* has a
495 large role in the rhodopsin bio-synthesis pathway (Shen et al., 2011). Although this
496 pathway has been previously linked to the ability to discriminate between differences
497 in temperature (Shen et al., 2011), it has not been implicated in the cold acclimation
498 response. One possibility is that rhodopsin bio-synthesis pathway may act to detect
499 the onset of cold and to subsequently cue the DE of genes which increase cold
500 tolerance.

501 **Conclusions**

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

511 cold acclimation, as the selective forces maintaining cold acclimation will be stronger
512 on these genes, than on genes that have a smaller influence.

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

520 **Conflict of interest**

521 The authors declare no conflict of interest.

522 **Data Accessibility**

523 All data has been deposited in NCBI's Gene Expression Omnibus (GEO), to be
524 made available upon acceptance.

525

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719 **Titles and legends to figures**

720 **Figure 1 |** A) Plot of $\log_2$ fold changes between control and cold acclimated flies for
721 each of the genes that were DE in both *D. montana* and *D. virilis* B) Average change
722 in gene expression ($\Delta \log_2$ fold change) between control and cold acclimated flies for
723 genes that were DE in both species. Error bars represent approximate 95%
724 confidence intervals.

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

727 **Figure 3|** Log_2 fold change between control and cold acclimated flies for the top 20
728 genes which showed a significant species by treatment interaction in *D. montana*
729 and *D. virilis*. *D. virilis* gene names are given in the legend with *D. melanogaster*
730 orthologs in parentheses.

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

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

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Table 1. DAVID clusters |Functional clusters identified by DAVID for each of the gene groups for enrichment scores with p < 0.1. Cluster colour code: orange = muscle-related, yellow = metabolic, green = transmembrane transport/ion transport, pink = protein signalling

D.montana

Cluster	Enrichment Score	*p*	Term summary
1	2.45	0.004	actin cytoskeleton, acetylation, myosin complex, muscle protein
2	1.97	0.01	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	1.2	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

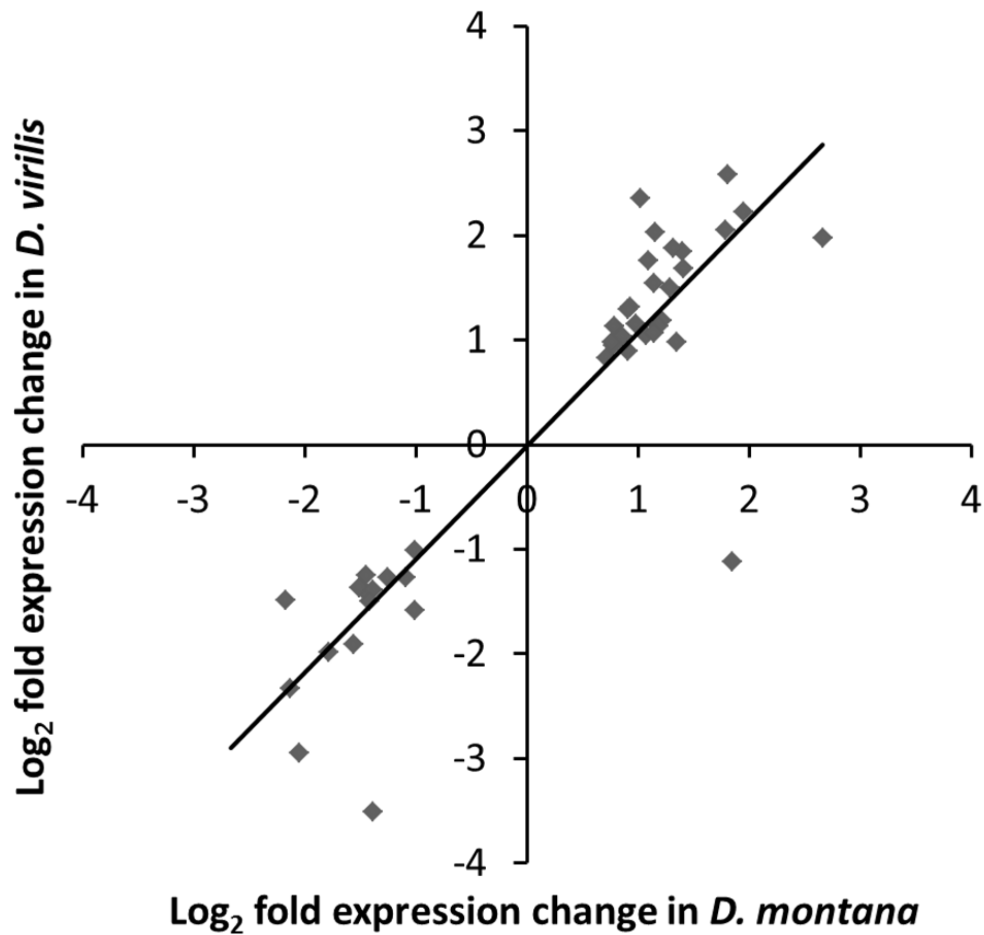
Cluster	Enrichment Score	*p*	Term summary
1	1.97	0.011	integral to membrane, intrinsic to membrane, transmembrane, membrane
2	1.61	0.025	CHK kinase-like, CHK
3	1.45	0.035	identical protein binding, protein dimerization activity, protein homodimerization activity
4	1.29	0.051	Metabolism of xenobiotics 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

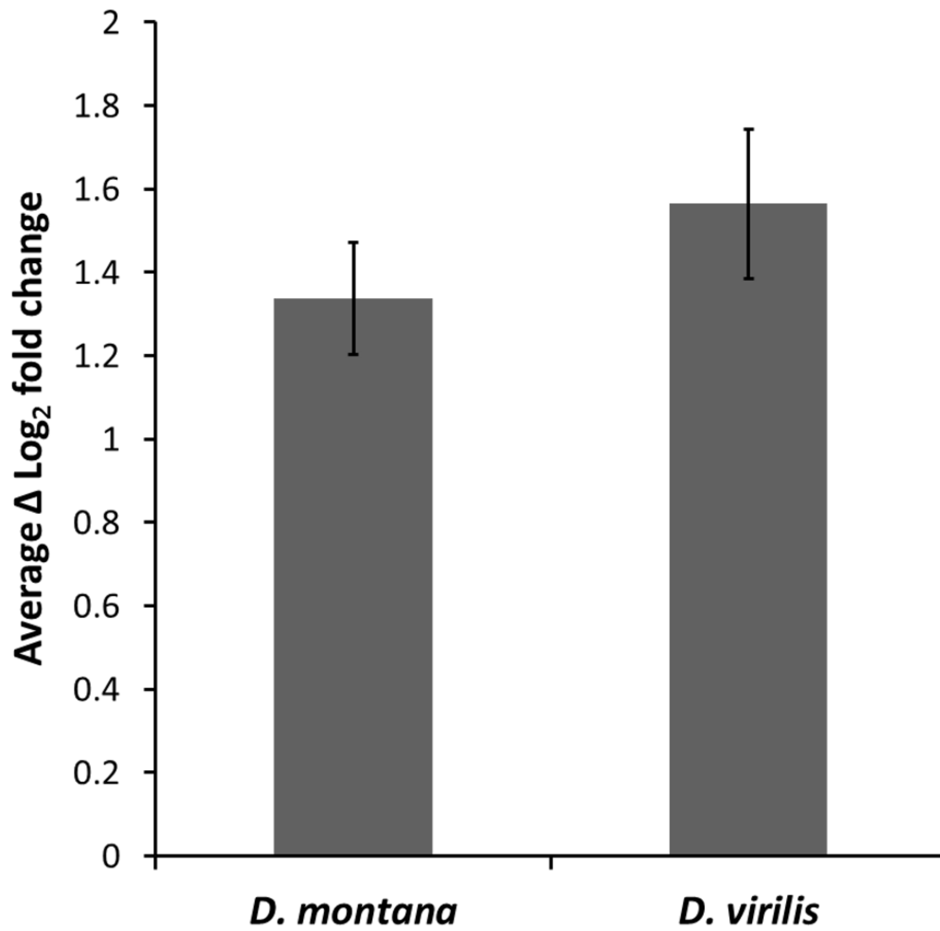
Interaction

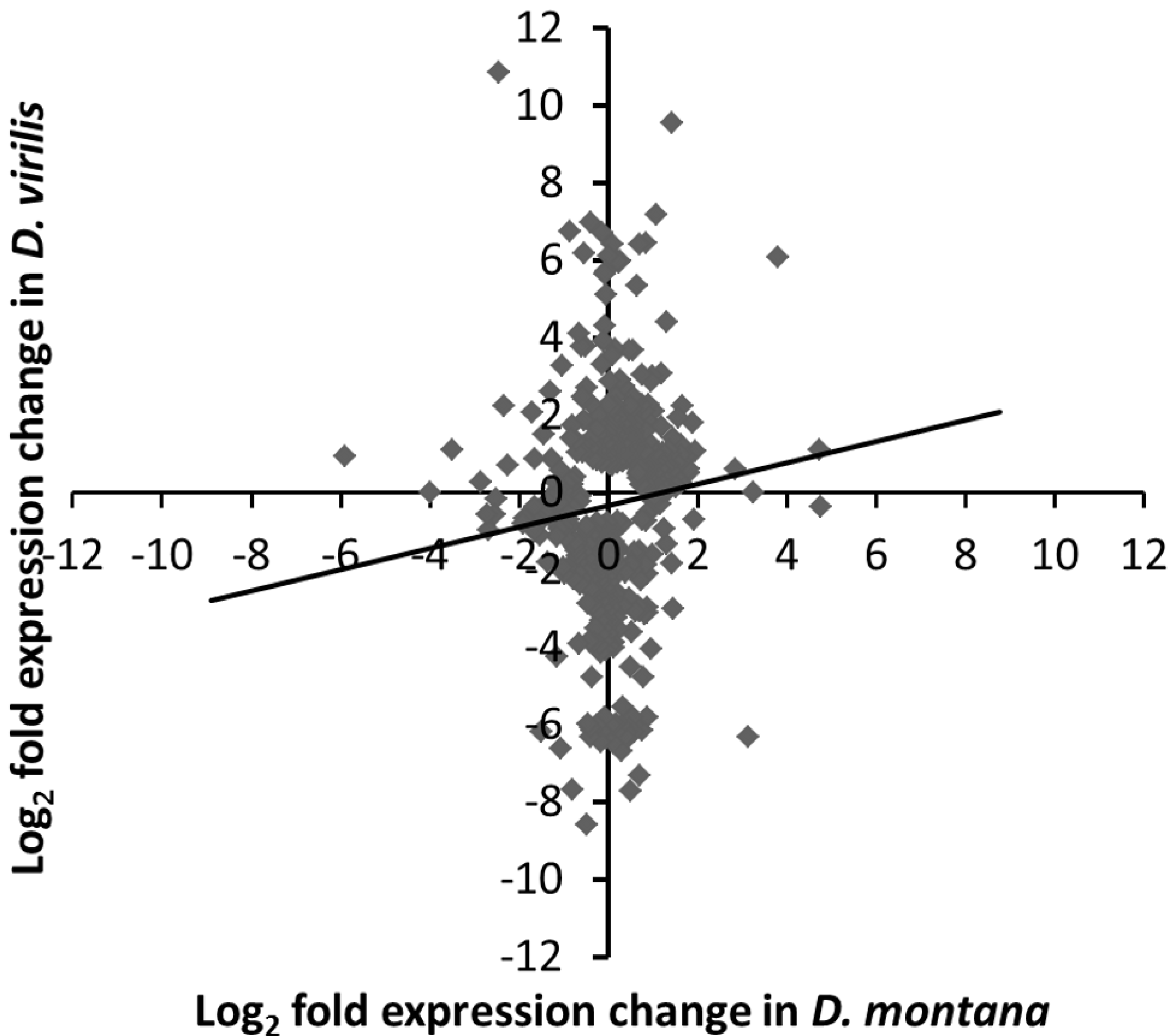
Cluster	Enrichment Score	*p*	Term summary
1	1.27	0.054	carbohydrate catabolic process, alcohol catabolic process
2	1.27	0.054	hexose metabolic process, monosaccharide metabolic process, glucose metabolic process

Table 2 | Circadian genes differentially expressed in response to cold acclimation

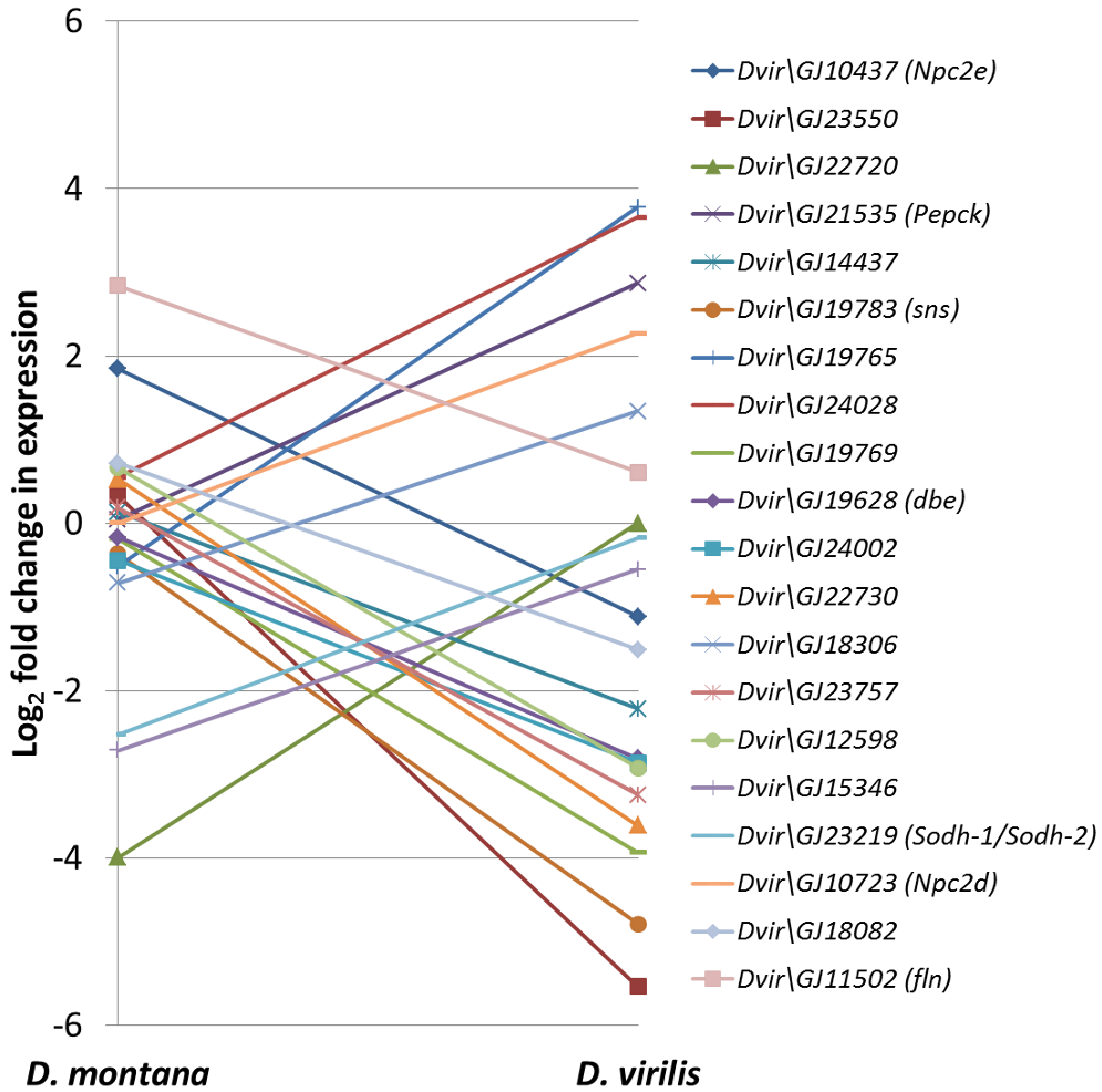
Gene	D. melanogaster ortholog	Species	Interaction?
Dvir\per	*per*	Both species	
Dvir\GJ17539	*vri*	Both species	
Dvir\GJ11819	*dysc*	*D. virilis*	
Dvir\GJ16719	*CG2650*	*D. virilis*	
Dvir\GJ19065	*na*	*D. virilis*	Yes
Dvir\GJ11425	*Pdp1*	*D. montana*	


A
Log2 fold expression change in D. virilis
Log2 fold expression change in D. montana
4
3
2
1
0
-1
-2
-3
-4
-4
-3
-2
-1
0
1
2
3
4

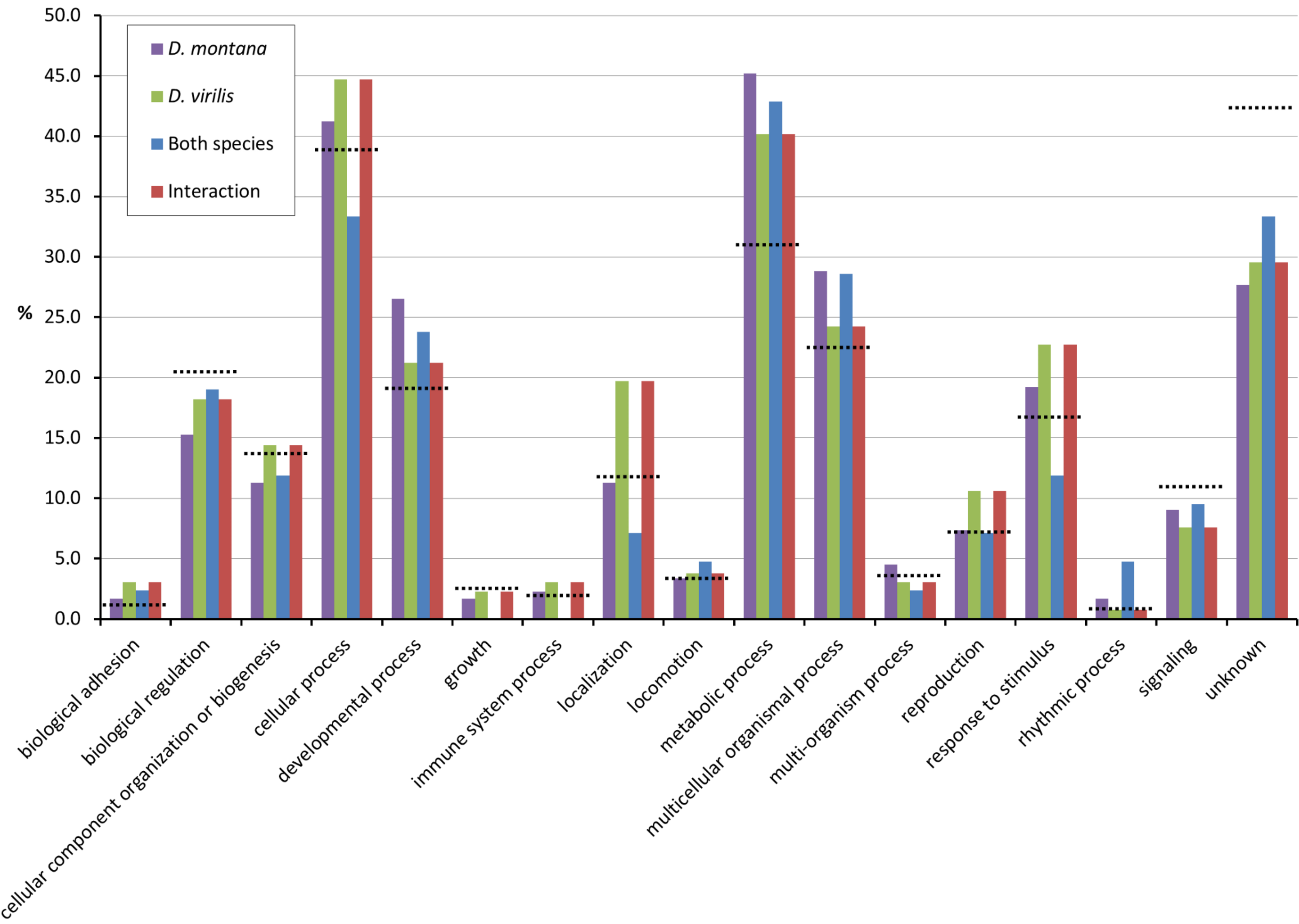

B
Average Δ Log2 fold change
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
D. montana
D. virilis



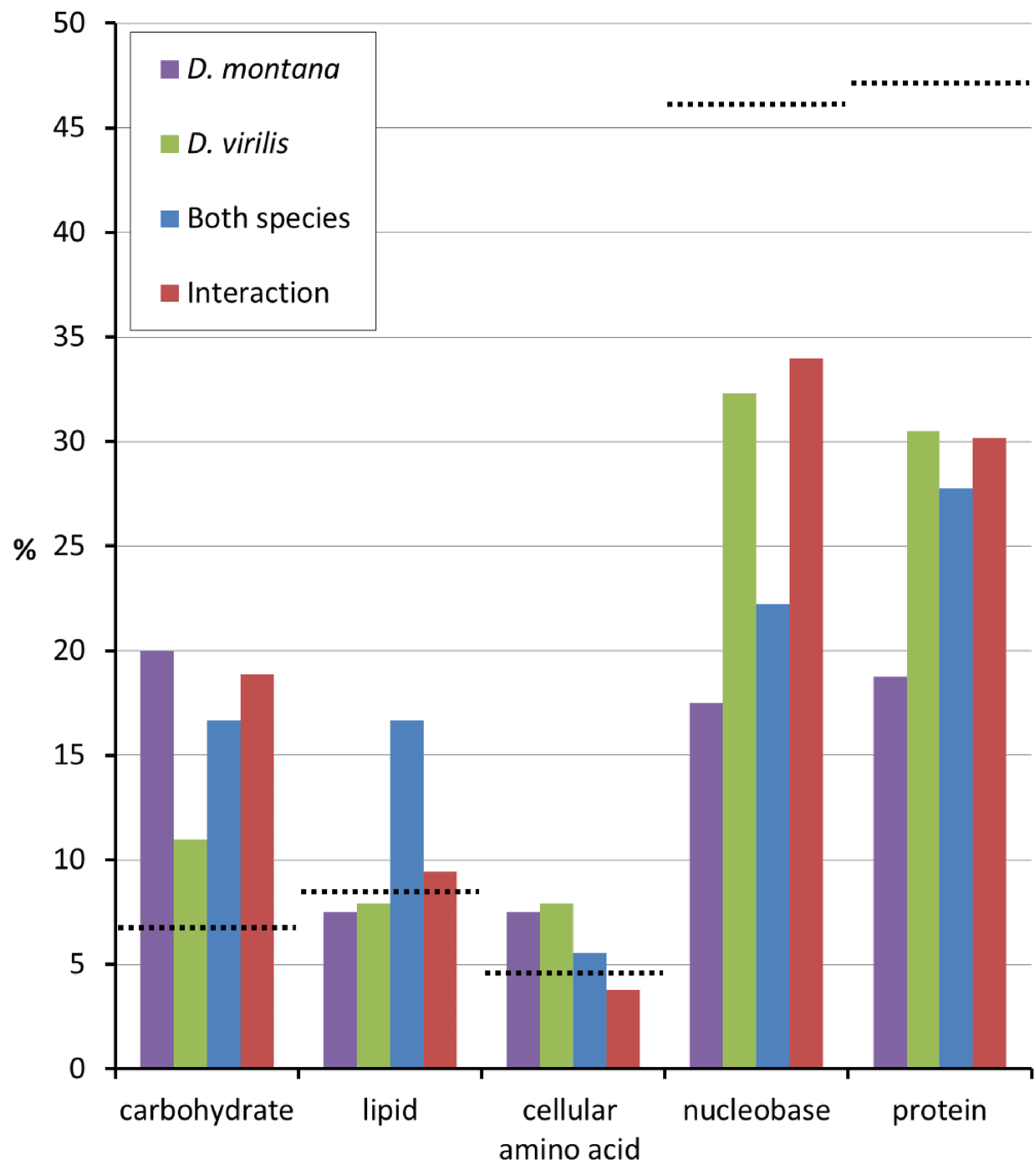
Log$_2$ fold expression change in *D. virilis*
Log$_2$ fold expression change in *D. montana*
12
10
8
6
4
2
0
-2
-4
-6
-8
-10
-12
-12 -10 -8 -6 -4 -2 0 2 4 6 8 10 12



6
4
2
0
-2
-4
-6
Log2 fold change in expression
D. montana
D. virilis
Dvir\GJ10437 (Npc2e)
Dvir\GJ23550
Dvir\GJ22720
Dvir\GJ21535 (Pepck)
Dvir\GJ14437
Dvir\GJ19783 (sns)
Dvir\GJ19765
Dvir\GJ24028
Dvir\GJ19769
Dvir\GJ19628 (dbe)
Dvir\GJ24002
Dvir\GJ22730
Dvir\GJ18306
Dvir\GJ23757
Dvir\GJ12598
Dvir\GJ15346
Dvir\GJ23219 (Sodh-1/Sodh-2)
Dvir\GJ10723 (Npc2d)
Dvir\GJ18082
Dvir\GJ11502 (fln)



50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
%
D. montana
D. virilis
Both species
Interaction
biological adhesion
biological regulation
cellular component organization or biogenesis
cellular process
developmental process
growth
immune system process
localization
locomotion
metabolic process
multicellular organismal process
multi-organism process
reproduction
response to stimulus
rhythmic process
signaling
unknown



D. montana
D. virilis
Both species
Interaction
%
0
5
10
15
20
25
30
35
40
45
50
carbohydrate
lipid
cellular amino acid
nucleobase
protein