UC Berkeley
UC Berkeley Previously Published Works

Title

Cold adaptation increases rates of nutrient flow and metabolic plasticity during cold exposure in Drosophila melanogaster

Permalink

https://escholarship.org/uc/item/1sr8f9fv

Journal

Proceedings of the Royal Society B, 283(1838)

ISSN

0962-8452

Authors

Williams, Caroline M
McCue, Marshall D
Sunny, Nishanth E
et al.

Publication Date

2016-09-14

DOI

10.1098/rspb.2016.1317

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

PROCEEDINGS B

Cold-adaptation increases rates of nutrient flow and metabolic plasticity during cold exposure in *Drosophila melanogaster*

Journal:	*Proceedings B*
Manuscript ID	RSPB-2016-1317.R1
Article Type:	Research
Date Submitted by the Author:	n/a
Complete List of Authors:	Williams, Caroline M.; University of California Berkeley, Integrative Biology McCue, Marshall; St. Mary's University, Department of Biological Sciences Sunny, Nishanth; University of Florida, Department of Medicine Szejner Sigal, Andre; University of Florida Morgan, Theodore; Kansas State University, Division of Biology Allison, David; University of Alabama at Birmingham, Section on Statistical Genetics, Department of Biostatistics Hahn, Daniel; University of Florida, Entomology and Nematology
Subject:	Physiology < BIOLOGY, Biochemistry < BIOLOGY, Evolution < BIOLOGY
Keywords:	chill coma, metabolism, ectotherm, insect, stable isotopes, stress hardiness
Proceedings B category:	Physiology

SCHOLARONE™
Manuscripts

1 # Cold-adaptation increases rates of nutrient flow and metabolic plasticity
2 # during cold exposure in *Drosophila melanogaster*

3 Williams, Caroline M.[1,2*]; McCue, Marshall D.[3]; Sunny, Nishanth E.[1]; Szejner-Sigal, Andre[1,2];
4 Morgan, Theodore J.[4]; Allison, David B.[5]; Hahn, Daniel A.[1]

5 [1]University of Florida, Gainesville FL 32601

6 [2]University of California, Berkeley CA 94720

7 [3]St. Mary's University, San Antonio TX 78228

8 [4]Kansas State University, Manhattan KS 66506

9 [5]University of Alabama at Birmingham, Birmingham AL 35294

10

11 *Corresponding author: cmw@berkeley.edu

12 **Abstract**

13 Metabolic flexibility is an important component of adaptation to stressful environments,
14 including thermal stress and latitudinal adaptation. A long history of population genetic studies
15 suggest that selection on core metabolic enzymes may shape life histories by altering metabolic
16 flux. However, the direct relationship between selection on thermal stress hardiness and
17 metabolic flux has not previously been tested. We investigated flexibility of nutrient catabolism
18 during cold stress in *Drosophila melanogaster* artificially selected for fast or slow recovery from
19 cold-coma (cold-hardy and -susceptible), specifically testing the hypothesis that stress adaptation
20 increases metabolic turnover. Using ^{13}C-labeled glucose, we first showed that cold-hardy flies
21 more rapidly incorporate ingested carbon into amino acids and newly synthesized glucose,
22 permitting rapid synthesis of proline, a compound shown elsewhere to improve survival of cold
23 stress. Second, using glucose and leucine tracers we showed that cold-hardy flies had higher
24 oxidation rates than cold-susceptible flies before cold exposure, similar oxidation rates during
25 cold exposure, and returned to higher oxidation rates during recovery. Additionally, cold-hardy
26 flies transferred compounds among body pools more rapidly during cold exposure and recovery.
27 Increased metabolic turnover may allow cold-adapted flies to better prepare for, resist, and
28 repair/tolerate cold damage. This work illustrates for the first time differences in nutrient fluxes
29 associated with cold adaptation, suggesting that metabolic costs associated with cold hardiness
30 could invoke resource-based trade-offs that shape life histories.

31 Keywords: chill coma, metabolism, insect, ectotherm, stable isotopes, stress hardiness

32

33 ## Background

34 Temperature fluctuates markedly on daily and seasonal timescales in temperate regions, and for
35 ectotherms these fluctuations can shape organismal performance and fitness [1], predict
36 distributional limits [2], and may even exert greater selective pressure than mean temperatures
37 [3]. Temperature fluctuations challenge energetic homeostasis in ectothermic animals, and
38 thermal adaptation likely involves modulation of the thermal sensitivity of metabolic pathways.
39 Demand for ATP regulates rates of substrate catabolism and under normal conditions energy
40 supply is matched to demand [4]. However, environmental fluctuations can upset the balance
41 between energy supply and demand, and persistent energetic perturbations can result in declines
42 in performance or death [5]. Resistance or tolerance to stressful temperatures requires
43 fortification of cellular structures (for example, modification of cellular membrane composition),
44 a coordinated down-regulation of metabolic processes during the stressful period to permit the
45 maintenance of homeostasis, and subsequent up-regulation of metabolic processes during
46 recovery to repair damage and restore homeostasis. Thus, metabolic flexibility appears to be an
47 important component of stress hardiness and is selected upon during adaptation to stressful
48 environments [6-8]. Population genetic studies have uncovered abundant genetic variation in
49 core metabolic enzymes that suggests that nutrient fluxes are shaped by natural selection during
50 adaptation to stressful environments to alter allocations among life history traits [9], but no
51 studies have directly measured metabolic flux during thermal stress exposure.

52 When temperatures drop below a critical thermal minimum, insects and other ectotherms enter
53 chill coma, a reversible state of cold-induced paralysis during which they cannot pursue activities
54 that enhance fitness like feeding, mating, or oviposition, and are vulnerable to predation [10, 11].
55 Chill coma recovery time has a genetic basis and responds to climatic selection, showing

56 latitudinal clines on multiple continents whereby insects from colder regions recover more
57 quickly [10, 11], and a robust response to artificial selection [12-14]. Chill coma onset occurs
58 due to failure of the neuromuscular system, and ionic and osmotic homeostasis are progressively
59 lost during low temperature exposure [15, 16]. Ionic, osmotic, and metabolic disturbances
60 accumulate with increasing intensity and duration of cold exposure time [14, 17]. Recovery from
61 chill coma requires reestablishment of metabolic homeostasis, recovery of ionic and water
62 balance, and repair of critical cellular components. Increased metabolic flux, with associated
63 increase in the turnover of metabolites, may provide the flexibility to prepare for and recover
64 from cold exposure more rapidly.

65 Given the importance of nutrient flow through central metabolic pathways including glycolysis
66 and the tricarboxylic acid (TCA) cycle as the source of ATP and production of building blocks
67 for many synthesis reactions, adaptation to thermal stress is expected to modify these pathways.
68 Indeed, numerous studies have suggested that latitudinal clines in polymorphisms in loci
69 involved in central metabolism are the product of selection on the relationship between
70 energetics and seasonal adaptation [7, 18]. Furthermore, numerous transcriptomic, proteomic,
71 and metabolomics screens have associated components of central metabolism with either thermal
72 adaptation or thermal acclimation responses [19]. Recent work has shown that metabolic rates
73 are increased in flies that are selected for rapid recovery from chill coma, that these flies have
74 greater ability to suppress their metabolic rates in response to cold exposure, and that they
75 maintain metabolic homeostasis more effectively during cold exposure [14, 20]. Yet, even
76 though this large body of studies presumes that flux through critical pathways of central
77 metabolism are altered by thermal adaptation, these approaches do not directly test whether
78 metabolic flux is associated with thermal adaptation.

79 Here we use carbon stable isotope tracers [21, 22] to investigate how evolution can shape
80 substrate oxidation before, during, and after cold exposure in cold-hardy and cold-susceptible
81 lines of *Drosophila melanogaster*. Our hypothesis is that rapid recovery from cold requires high
82 metabolic turnover, facilitated by faster rates of substrate oxidation and more rapid transfer of
83 carbon skeletons among pools during the course of a cold exposure. We predict cold-hardy flies
84 will have greater metabolic turnover with higher nutrient flux through glycolysis and the TCA
85 cycle.

86 Methods

87 *Experimental design*

88 Experiments were performed on 5-8 day old mated female flies that had been artificially selected
89 for either fast or slow recovery from chill coma (Supporting methods; [23]). These lines have
90 genetically fixed differences in chill coma recovery times, with hardy lines recovering after an
91 average of 6.1 and 5.8 min (replicate lines 1 and 2), compared to 12.4 and 23.7min for
92 susceptible flies [13]. Flies were fed an isotopically labeled tracer (either glucose or leucine), left
93 for a period of time for the tracer to be metabolized and distributed among body pools, then the
94 distribution of the tracer among body pools was measured to infer rates of flux (Fig. S1).
95 Predominant fates of carbon from glucose and leucine tracers are in Fig. S2 and described in
96 Supporting methods. We performed three experiments, with the following aims: 1) measure rates
97 of anabolic processes (glucose incorporation into newly synthesized glucose and specific amino
98 acids), 2) measure glucose catabolism and incorporation into bulk lipid, protein and carbohydrate
99 pools; and 3) measure leucine catabolism and incorporation into bulk lipid, protein and
100 carbohydrate pools.

101 *Experiment 1 – Quantifying anabolism from glucose in cold-hardy versus -susceptible fly lines*

102 Production of amino acids and newly synthesized glucose at 25 °C was measured in cold-hardy
103 and -susceptible flies (n = 4 pools of 20 flies/line, two replicate lines of each selection regime).
104 Flies were fed isotopically labelled U-$^{13}C_6$-glucose tracer and allowed to metabolize the tracer
105 for 4h then were homogenized and incorporation of the tracer into newly synthesized glucose
106 and amino acids was quantified using GC-MS (Supporting Methods).
107

108 *Experiments 2 and 3 – Catabolism and incorporation into body pools in cold-hardy versus –*
109 *susceptible fly lines*

110 We examined glucose (Expt. 2) and leucine (Expt. 3) oxidation and incorporation into body-wide
111 lipid, protein, and carbohydrate pools. We administered tracer (U-$^{13}C_6$-glucose or 1-^{13}C-leucine),
112 and collected samples at the following times (relative to cold exposure):

113 1) **Before**: To measure baseline distributions of labeled tracer in the absence of cold stress and to
114 test whether selection for cold hardiness or susceptibility could have shaped baseline levels of
115 metabolism, vials were immediately flushed with CO_2-free air and flies were held at 25 °C for 3
116 h to collect CO_2, then snap frozen and sent for analysis (described below).

117 2) **During**: To measure how cold stress affected tracer distribution, vials were placed in ice
118 slurry at 0 °C for 0.5 h (sufficient to induce chill coma), flushed with CO_2-free air, held at 0°C
119 for 2.5 h, then snap frozen and sent for analysis. All CO_2 was produced while flies were in chill
120 coma, representing glucose and leucine oxidation during cold exposure.

121 3) **After**: To measure tracer distributions during recovery, vials were placed in an ice slurry at 0
122 °C for 3 h, flushed with CO_2-free air, placed at 25 °C for 3 h, then snap frozen and analyzed.

123 Conditions were similar to the "Before" time point, but flies experienced 3 h of cold exposure
124 between tracer administration and sample collection.

125 Experiments 2 and 3 differed only in the tracer used: glucose for Expt. 2 and leucine for Expt. 3
126 (Fig. S1). Each experiment represents a new set of biological replicates (N = 6 pools of 20
127 [before and after] or 40 [during] flies/line/time point). The increased pool size for "During" time
128 point was necessary to ensure that gas concentrations were sufficiently high for reliable
129 measurements. Any given group of flies was only fed one type of tracer, but had enrichments
130 measured in breath, lipid, carbohydrate and protein pools. We also prepared 3 pools of 20
131 flies/line of control flies (treated identically except fed water instead of tracer) to function as
132 controls for natural ^{13}C enrichment.

133 Samples were snap-frozen and CO_2 was analyzed for tracer enrichments to estimate oxidation
134 rates of each tracer (Supporting methods). Flies were then homogenized and chemically
135 separated into lipid, protein and carbohydrate fractions which were then separately analyzed for
136 tracer enrichments using an elemental analyzer (Supporting Methods).

137 For each experiment, we analyzed differences in tracer enrichments between lines using general
138 linear models with fixed effects of cold exposure time point, selection regime, and compound/
139 body fraction where appropriate, the random effect of selection regime nested within replicate
140 selection line, and precursor pool enrichment as a covariate to control for differences in amount
141 of tracer ingested or body size differences (Supporting Methods). Previous studies have shown
142 conclusively that mass does not differ among these fly lines [14, 20]. We calculated root mean
143 square changes in body tracer enrichments between subsequent time points (Supporting
144 Methods) to explore the movement of tracer among body pools during cold exposure, and

145 compared the magnitude of changes in enrichments over time between hardy and susceptible
146 flies.

147 Results

148 *Experiment 1 – Quantifying anabolism from glucose in cold-hardy versus -susceptible fly lines*

149 There was higher flux of glucose into newly synthesized glucose and amino acids in hardy
150 compared to susceptible flies ($F_{1,16} = 14.5$, $p = 0.002$, Fig. 1). Enrichments differed among
151 compounds ($F_{5,80} = 93.8$, $p < 0.00001$), with glutamate containing the greatest carbon from
152 tracer, then alanine, serine, and proline, followed by glycine, with low but measureable carbon
153 from tracer in gluconeogenically produced glucose. Thus, cold-hardy flies synthesize amino
154 acids and glucose from glucose tracer at a faster rate than cold-susceptible flies.

155 *Experiments 2 and 3 – Catabolism and incorporation into body pools in cold-hardy versus –*
156 *susceptible fly lines*

157 CO_2 production did not differ between the glucose and leucine tracers, so data from Experiments
158 2 and 3 were pooled to analyze patterns in metabolic rates. Cold-hardy flies had greater
159 metabolic plasticity (cold hardiness × cold exposure interaction, $F_{2,135} = 4.7$, $p = 0.011$), having
160 higher metabolic rates than susceptible flies before cold exposure, dropping to similar levels
161 during cold exposure, and rebounding to higher levels during recovery (Fig. 2). Thus, metabolic
162 rates are more plastic in hardy flies.

163 Body enrichments from glucose tracer ingestion were highest in carbohydrates, with lower
164 enrichments in lipids and proteins ($F_{2,138} = 2224.8$, $p < 0.00001$, Fig. 3A-C, Table S2). Hardy
165 flies had higher body enrichments than did susceptible flies ($F_{1,70} = 45.7$, $p < 0.00001$).

166 Enrichments changed over the course of cold exposure differently according to fraction (fraction
167 × cold exposure: $F_{4,138} = 9.6$, $p < 0.00001$). We analyzed each fraction separately to clarify this
168 interaction. Carbohydrate enrichments rose during cold ($p < 0.0001$) and remained elevated
169 during recovery ($p = 0.001$). Lipid enrichment remained constant during cold ($p = 0.670$) and
170 rose during recovery ($p = 0.004$). Protein enrichment rose during cold ($p = 0.007$) then returned
171 to pre-cold levels during recovery ($p = 0.691$). Hardy flies altered their body enrichments more
172 during cold exposure and recovery than susceptible flies (MANOVA cold hardiness × cold
173 exposure: $F_{6,112} = 2.7$, $p = 0.017$), as reflected in greater magnitudes of change in combined
174 enrichments in the three body pools during cold exposure ($F_{1,8} = 5.0$, $p = 0.028$, Table 1).
175 Combined enrichments changed similar magnitudes during cold exposure and recovery, and
176 ended up near the starting point ($F_{1,8} = 5.9$, $p = 0.019$; Table 1).

177 We next examined glucose enrichments in the breath. Breath enrichments are calculated relative
178 to a standard, and represent tracer oxidation relative to other (non-labeled) substrates. It is
179 important to control for enrichments in the precursor pools, which we do using the PC1 of
180 variation in body enrichments. Body enrichments in flies fed a glucose tracer were highly
181 predictive of breath enrichments ($F_{1,71} = 93.5$, $p < 0.00001$) – flies with higher tracer enrichments
182 in their body oxidized more tracer relative to unlabeled substrates. Hardy flies had higher breath
183 enrichments overall than did susceptible flies ($F_{1,36} = 19.4$, $p < 0.001$; Fig. S7A), even controlling
184 for their higher body enrichments (Fig. 4A). There was a nearly statistically significant
185 interaction between cold hardiness × cold exposure that was not retained in the final model ($p =$
186 0.072): the direction of this pattern was towards hardy having greater plasticity in their
187 enrichments over the course of cold exposure and recovery. Breath enrichments resulting from
188 glucose tracer ingestion dropped during cold exposure when body enrichment was included in

189 the model ($F_{2,70} = 8.4$, $p < 0.001$, Fig 4A), largely due to increased enrichment of ^{13}C-labeled
190 substrates in body pools during cold exposure probably resulting from low oxidation rates in the
191 cold. Inspection of raw enrichments showed no drop over time (Fig. S7A), and if the enrichment
192 covariate was not included cold exposure did not alter enrichments ($p = 0.963$). This suggests
193 that reliance on glucose relative to other substrates remains unchanged by cold exposure.

194 Tracer oxidation rates are the product of relative CO_2 enrichments (subject of previous
195 paragraph) and the volume of CO_2 produced, and represent the total amount of tracer oxidized.
196 Glucose tracer oxidation was positively related to body enrichments ($F_{1,72} = 101.2$, $p < 0.00001$),
197 and hardy flies oxidized a greater proportion of the glucose tracer in their body than susceptible
198 flies ($F_{1,37} = 13.0$, $p < 0.001$; Fig. 4B). Absolute glucose oxidation rates dropped strongly during
199 cold exposure (Fig. S7B, $F_{2,70} = 139.1$, $p < 0.00001$), reflecting steep declines in CO_2 production
200 (Fig. 2). Hardy flies oxidized more glucose tracer before cold exposure, less during cold
201 exposure, and more during recovery from cold exposure, particularly when the influence of body
202 enrichments was removed (cold hardiness × cold exposure: $F_{2,70} = 6.2$, $p = 0.003$, Fig. 4B). Thus,
203 hardy flies had higher rates of glucose tracer oxidation in warm conditions, and a greater ability
204 to suppress oxidation rates in the cold.

205 Body enrichments from leucine tracer were affected by the interaction of cold hardiness and cold
206 exposure, and differed between pools ($F_{4,112} = 3.0$, $p = 0.020$; Fig. 3D-F). Carbon from leucine
207 tracer in proteins and carbohydrates dropped during cold and rebounded during recovery, while
208 tracer in lipids rose during cold exposure then dropped during recovery (fraction × cold
209 exposure: $F_{4,112} = 9.0$, $p < 0.0001$). Leucine tracer enrichments in lipids were higher in hardy flies
210 and remained higher throughout the cold exposure, but hardy and susceptible flies did not differ
211 in enrichments in carbohydrates or proteins. Levels of enrichment in carbohydrates and proteins

212 did not differ from each other ($p = 0.332$), but were higher than lipid enrichments ($p < 0.0001$).
213 We analyzed the fractions separately to tease apart interactions. Hardy flies had greater plasticity
214 in leucine allocation to proteins: they had more leucine tracer in their protein pool before cold
215 exposure than susceptible flies, but during cold exposure and recovery hardy and susceptible
216 flies had similar levels of tracer in the protein pool (cold hardiness × cold exposure; $p = 0.001$;
217 Fig. 3F). Hardy flies showed the same pattern of greater plasticity in incorporation of leucine
218 tracer in carbohydrates, but this was statistically non-significant when carbohydrates were
219 analyzed separately ($p = 0.092$, Fig. 3E). Hardy flies did not exhibit greater plasticity in lipid
220 enrichments (cold hardiness × cold exposure; $p = 0.803$, Fig. 3D), but enrichments did rise
221 during cold exposure ($F_{2,72} = 25.2$, $p < 0.0001$), and hardy flies had higher lipid enrichments
222 overall than susceptible flies ($F_{1,72} = 74.2$, $p < 0.0001$).

223 A MANOVA of all three fractions confirmed enrichments from leucine tracer of hardy flies
224 responded differently to cold exposure than did enrichments of susceptible flies (cold hardiness ×
225 cold exposure: $F_{6,102} = 2.2$, $p = 0.049$). Similarly to the glucose tracer experiment, hardy flies
226 experienced greater magnitudes of change in combined enrichments in the three body pools
227 during the cold exposure, indicating that hardy flies changed their tracer distributions more
228 during the course of a cold exposure than did susceptible flies ($F_{1,10} = 3.85$, $p = 0.048$, Table 1).
229 Absolute magnitudes of change in leucine enrichments were 100-fold lower than the
230 corresponding magnitudes for glucose tracer (Table 1), probably because of the generally lower
231 amount of ^{13}C in the flies' bodies (1-^{13}C-leucine tracer was labeled on only one carbon,
232 compared to six labeled carbons for U-$^{13}C_6$-glucose tracer). Thus, similar to the glucose tracer
233 experiment, hardy flies moved tracer between body pools to a greater extent during cold
234 exposure, but in contrast they did not have higher enrichments from leucine overall.

235 In contrast to the glucose tracer experiment, breath enrichments resulting from leucine tracer
236 oxidation dropped sharply during cold exposure ($F_{2,69} = 88.6$, $p < 0.0001$, Fig. 4C). This persisted
237 without including the body enrichment covariate ($p < 0.0001$, Fig. S7C), and suggests that
238 leucine oxidation is particularly cold sensitive relative to glucose oxidation. Similarly to the
239 glucose tracer experiment, body enrichments were positively correlated with breath enrichments
240 from leucine tracer ($F_{1,71} = 30.9$, $p < 0.0001$). Hardy flies had greater plasticity in breath
241 enrichments from leucine tracer than susceptible flies: higher enrichments before cold, dropping
242 to similar breath enrichments during cold, and then higher again during recovery (cold hardiness
243 × cold exposure: $F_{2,69} = 4.4$, $p = 0.016$, Fig.4C).

244 Similar to glucose, leucine oxidation dropped strongly during cold exposure ($F_{2,67} = 399.2$, p <
245 0.0001, Fig. 4D) and was positively related to body enrichments ($F_{1,69} = 38.8$, $p < 0.0001$). The
246 interaction between cold hardiness × cold exposure was marginally non-significant ($p = 0.067$),
247 but the trend was again towards hardy flies had greater plasticity in enrichments over the course
248 of a cold exposure, in agreement with results for glucose oxidation.

249 Discussion

250 Cold-adapted flies – those selected for faster recovery from chill coma – had greater metabolic
251 turnover than cold-susceptible flies. We confirmed our previous findings that cold-hardy flies
252 had higher metabolic rates before cold and greater plasticity in lowering those rates during cold
253 exposure [20], and clearly demonstrated that this pattern resulted from divergence in rates of flux
254 through pathways of protein and carbohydrate oxidation. Cold-hardy flies had higher rates of
255 glucose and leucine oxidation before cold and during recovery compared to cold-susceptible
256 flies. Thus selection for cold hardiness increases metabolic turnover. Respiratory exchange ratios

257 are very close to 1 in both cold-hardy and -susceptible lines at 25 °C, indicating that flies are
258 primarily oxidizing carbohydrate and that CO_2 production is a reasonable proxy for ATP
259 production [20]. Thus, cold-hardy flies likely produce more ATP, providing energy to protect
260 against or repair from cold damage. In addition to producing more ATP, the ability of cold-hardy
261 flies to transfer carbon among body pools more rapidly than cold-susceptible flies over the
262 course of cold exposure and recovery may provide carbon skeletons to synthesize compounds
263 integral to resisting and tolerating cold. This supports our hypothesis that cold adaptation
264 increases metabolic turnover, facilitated by faster rates of substrate oxidation and more rapid
265 transfer of carbon skeletons among pools during the course of a cold exposure.

266 *Baseline levels of metabolic flux before cold exposure*

267 Chill coma recovery times may be reduced in hardy flies as a result of alterations to their
268 physiological starting state, either through altered levels of body constituents that promote cold
269 hardiness such as cryoprotective lipids, carbohydrates or proteins [24], or by an enhanced ability
270 to rapidly synthesize or interconvert such compounds upon sensing cold [25]. Before a cold
271 exposure, cold-adapted flies had higher rates of CO_2 production, glucose and leucine oxidation,
272 and glucose and amino acid biosynthesis, suggesting cold adaptation increases rates of glycolysis
273 and TCA cycle flux. These increased fluxes were associated with increased rates of carbon
274 transfer among lipid, carbohydrate, and protein pools in cold-hardy flies during the onset of cold
275 exposure. Faster nutrient transfer among metabolic pathways results in part from higher rates of
276 anaplerotic and cataplerotic reactions – the influx and removal of four- and five-carbon skeletons
277 from the TCA cycle [26]. The TCA cycle thus forms a vital hub connecting catabolism and
278 anabolism of carbohydrates, proteins, and lipids (Fig. S2), and many studies have shown patterns
279 of allelic polymorphism consistent with seasonal selection on cold and overwintering [7]. High

280 flux through the TCA cycle provides potential for both greater production of ATP and rapid
281 synthesis of compounds integral to resisting or tolerating cold.

282 Lipid membranes are susceptible to cold perturbation, and modulation of membrane lipids is
283 associated with thermal adaptation in ectotherms [19, 27]. Higher basal nutrient flux through the
284 TCA cycle, gluconeogenesis, and pentose phosphate pathway (glyceraldehyde-3-phosphate
285 branch point, Fig. S2) in cold-hardy flies may permit rapid lipid membrane modulation. This is
286 consistent with a previous metabolomic study of the same lines of flies showing elevated levels
287 of a membrane lipid intermediate (phosphocholine) in cold-hardy flies, suggesting higher rates of
288 membrane modification [14].

289 Cryoprotectants like sugars, sugar alcohols, or free amino acids are associated with cold
290 hardiness in ectotherms [19]. High basal nutrient flux may facilitate rapid cryoprotectant
291 production. We observed high enrichments in glutamate compared to other amino acids implying
292 high cataplerotic flux through glutamate dehydrogenase, an intermediate for proline synthesis.
293 Proline is an important cryoprotectant in *D. melanogaster* [28], and we found that flux into
294 proline was higher in hardy flies (Fig. 1). This is in contrast to the results of our previous
295 metabolomic study that found slightly but detectably lower proline levels in hardy compared to
296 susceptible flies, and a drop in proline during the course of cold exposure which was more
297 rapidly restored in hardy flies than susceptible flies during recovery [14]. This observation also
298 provides a caveat for interpreting metabolomics results– higher instantaneous concentrations of a
299 metabolite do not necessarily imply higher rates of flux. Further experiments are required to
300 determine why hardy flies have higher rates of proline production but lower steady state levels.

301 Chill coma recovery time comprises both physiological (ability to stand) and behavioral
302 components (motivation to stand/ vigilance/activity levels), and it is likely that both of these
303 components are targets of selection in natural populations. We have previously shown that the
304 cold hardy lines used in this study have increased activity levels, but that activity represents a
305 small fraction of the metabolic costs of these lines (2.2 %), and are not sufficient to explain the
306 increase in metabolic rate[20]. Additionally, in a set of naturally occurring fly lines, fast recovery
307 from cold is also associated with increased metabolic rates compared to slow recovering lines,
308 while activity does not differ [20]. Together, this suggests that selection on activity is not
309 sufficient to explain the differences in metabolic turnover between hardy and susceptible flies.

310 *Metabolic flux during cold exposure*

311 Cold-adapted flies had greater plasticity in nutrient flux. Despite having higher rates of nutrient
312 flow before cold exposure, nutrient turnover in cold-hardy flies during the cold exposure was the
313 same or lower than in cold-susceptible flies. An increase in tracer enrichments in body pools
314 reflected decreased tracer oxidation in the cold. Metabolic down-regulation during stress occurs
315 in a wide range of organisms [8], and metabolic down-regulation is likely an adaptive response
316 to cold exposure in chill-susceptible insects. Maintaining balance between energy supply and
317 demand is challenging when temperatures change due to differing thermal sensitivities of energy
318 supply and demand pathways. ATP accumulates in cold insects [29, 30], suggesting pathways of
319 energy demand are particularly sensitive to cold-induced disruption.

320 In contrast to glucose, there was no detectable leucine oxidation in the cold. Leucine is broken
321 down to Acetyl-CoA and processed through the TCA cycle. A drop in leucine catabolism in the
322 cold may reflect TCA cycle stalling and failure of anaplerotic reactions. Previous insect studies

323 suggested TCA cycle stalling in the cold, along with increased reliance on glycolysis and
324 gluconeogenesis [25, 31]. Our data agree, with strong evidence for maintenance of glycolysis
325 during cold exposure, and indirect evidence for decreased TCA cycle flux (decreased leucine
326 oxidation). Work with a tracer that directly interrogates TCA cycle flux (e.g. glutamate) is
327 needed to confirm TCA cycle stalling in the cold.

328 *Metabolic flux after cold exposure*

329 During recovery from cold, glucose and leucine oxidation rebounds to near pre-exposure levels,
330 with hardy flies regaining higher rates of CO_2 production and substrate oxidation than
331 susceptible flies. Increased substrate oxidation during recovery enabled cold-hardy flies to
332 transfer carbon among body pools to a greater degree than susceptible flies, with one of the two
333 replicate hardy lines using carbon from U-$^{13}C_6$-glucose to synthesize lipids during recovery.
334 Hardy flies also transferred carbon from the 1-^{13}C-leucine tracer into the lipid pool to a greater
335 degree than susceptible flies across all time points relative to cold exposure. This is consistent
336 with increased substrate oxidation facilitating synthesis of compounds to counteract damage
337 from cold exposure. Lipid membranes are particularly cold sensitive [32] and are a common
338 target of cold lesions [19], so increased lipid synthesis in hardy flies may reflect adaptation to
339 counteract damage to membrane or storage lipids incurred during cold exposure, allowing faster
340 recovery. Previous work showed that these same cold-hardy flies recovered metabolic
341 homeostasis more rapidly after a cold exposure than cold-susceptible flies [14], and higher
342 nutrient flux during recovery may underlie this observation. Increased nutrient flux during
343 recovery may also be associated with low-temperature acclimation [25, 31].

344 *Ecological and evolutionary consequences of cold adaptation*

345 Laboratory selection illustrates what can happen given the segregating variation present in a
346 population, but cannot be taken to imply that this will happen [33]. Here, cold-hardy flies
347 evolved higher metabolic rates that provided a selective advantage in an environment of
348 unlimited nutrients and a regular cold stress exposure in adult flies (to measure chill coma
349 recovery times). However, in natural seasonal environments, nutrient availability declines as cold
350 stress increases with the onset of winter, so natural populations may face resource allocation or
351 acquisition trade-offs that constrain the evolution of energetically expensive cold tolerance
352 strategies [34]. Nonetheless, in nature ectotherms from higher altitudes and latitudes often have
353 higher metabolic rates than similar individuals from lower altitudes and latitudes [35, 36]. This
354 pattern is typically attributed to the need for fast growth and development in environments with a
355 short growing season. We suggest acute low temperature exposure is an alternative selective
356 agent that could also drive the evolution of this phenotype. We also found increased metabolic
357 rates were associated with faster chill coma recovery in naturally occurring fly genotypes [20],
358 further supporting the generality of this phenomenon.

359 Conclusions

360 Maintaining ectotherm performance in fluctuating environments requires metabolic flexibility.
361 Artificial selection to recover quickly from acute cold exposure increased basal rates of
362 metabolic flux in cold hardy flies, enabling cold-adapted flies to move carbon skeletons among
363 body pools more rapidly and thus increasing metabolic turnover. This increase in metabolic flux
364 requires increased nutrient consumption, suggesting that cold-adaptation may incur resource
365 allocation trade-offs that could shape evolution of ectotherm life histories in cold environments.

366 Data Accessibility

367 Data are archived in Dryad (doi:10.5061/dryad.d04s0).

368 Competing Interests

369 We have no competing interests.

370 Authors' contributions

371 CMW conceived of the study, designed and carried out the experiments and statistical analyses,
372 and drafted the manuscript; MDM designed and carried out the breath analyses, interpreted the
373 results, and revised the manuscript; NES designed and carried out the glucose and amino acid
374 analyses, interpreted the results, and revised the manuscript; ASS carried out the experiments;
375 TJM and DBA conceived of the study and revised the manuscript; DAH conceived of the study,
376 designed the experiments, and drafted the manuscript. All authors gave final approval for
377 publication.

378 Acknowledgements

379 L. Castellanos, and J. Kight helped with fly maintenance and biochemistry. S. Kalavalapalli ran
380 GC-MS samples. J. Curtis, Light Stable Isotope Lab at U. Florida processed EA-IRMS samples
381 with G. Browne. A.S. Edison provided comments on the manuscript. Two anonymous reviewers
382 provided extensive feedback that improved the manuscript.

383 Funding

384 This work was funded by NSF IOS-1051890 to DAH and DBA, NSF IOS-1051770 to TJM, and
385 the Florida Agricultural Experiment Station to DAH. DBA received partial support from NIH

386 P30DK056336. The opinions expressed are those of the authors and do not necessarily represent
387 those of the NSF, NIH, or any other organization.

388

389

390 **Figure Captions**

391 Figure 1 - Enrichments of newly synthesized glucose and amino acids above baseline values for
392 cold hardy (white) and susceptible (grey) flies. N = 4 pools of 20 flies/line.

393 Figure 2 – Metabolic rates (estimated by CO_2 production) of cold hardy (white) and susceptible
394 (black) flies. Hardy flies have higher metabolic rates before and after cold exposure, and greater
395 metabolic plasticity. N = 12 pools of 40 (before and after) or 80 (during) females flies/line/time
396 point, values are means ± SEM. Symbols denote replicate lines from experimental evolution
397 experiment.

398 Figure 3 –Enrichments in the lipid, protein and carbohydrate pools of cold-hardy and –
399 susceptible flies before, during and after a 3 h cold exposure, resulting from ingestion of A-C) U-
400 $^{13}C_6$-glucose tracer (Expt. 2); or D-F) 1-^{13}C-leucine tracer (Expt.3).

401 Figure 4 – ^{13}C enrichments from tracer (A-B) or tracer oxidation rates (C-D) before, during and
402 after cold exposure in cold-hardy (white symbols) and -susceptible (black symbols) flies.
403 Symbols denote replicate lines from experimental evolution experiment, N = 6 groups of 40
404 (before and after) or 80 (during) females flies/line/time point. Tracers used were U-$^{13}C_6$-glucose
405 (A & C, Expt. 2) or 1-^{13}C-leucine (B & D, Expt. 3). Data are residuals from a regression of body
406 enrichments on breath enrichments/oxidation rates (see text for details), raw values show
407 concordant patterns and are presented in Fig. S7.

References

[1] Kingsolver, J.G., Ragland, G.J. & Diamond, S.E. 2009 Evolution in a constant environment: thermal fluctuations and thermal sensitivity of laboratory and field populations of *Manduca sexta*. *Evolution* **63**, 537-541. (doi:10.1111/j.1558-5646.2008.00568.x).

[2] Overgaard, J., Kearney, M.R. & Hoffmann, A.A. 2014 Sensitivity to thermal extremes in Australian *Drosophila* implies similar impacts of climate change on the distribution of widespread and tropical species. *Global Change Biology* **20**, 1738-1750. (doi:10.1111/gcb.12521).

[3] Pither, J. 2003 Climate tolerance and interspecific variation in geographic range size. *Proceedings of the Royal Society of London B: Biological Sciences* **270**, 475-481. (doi:10.1098/rspb.2002.2275).

[4] Glazier, D.S. 2015 Is metabolic rate a universal 'pacemaker' for biological processes? *Biological Reviews* **90**, 377-407. (doi:10.1111/brv.12115).

[5] Pörtner, H.O. 2001 Climate change and temperature-dependent biogeography: oxygen limitation of thermal tolerance in animals. *Die Naturwissenschaften* **88**, 137-146.

[6] Storz, J.F. & Wheat, C.W. 2010 Integrating evolutionary and functional approaches to infer adaptation at specific loci. *Evolution* **64**, 2489-2509. (doi:10.1111/j.1558-5646.2010.01044.x).

[7] Eanes, W.F. 2011 Molecular population genetics and selection in the glycolytic pathway. *The Journal of experimental biology* **214**, 165-171. (doi:10.1242/jeb.046458).

[8] Storey, K.B. & Storey, J.M. 1990 Metabolic rate depression and biochemical adaptation in anaerobiosis, hibernation and estivation. *The Quarterly review of biology* **65**, 145-174.

[9] Marden, J.H. 2013 Nature's inordinate fondness for metabolic enzymes: why metabolic enzyme loci are so frequently targets of selection. *Molecular Ecology* **22**, 5743-5764. (doi:10.1111/mec.12534).

[10] Sinclair, B.J., Williams, C.M. & Terblanche, J.S. 2012 Variation in thermal performance among insect populations. *Physiological and Biochemical Zoology* **85**, 594-606. (doi:10.1086/665388).

[11] Gibert, P., Moreteau, B., Pétavy, G., Karan, D. & David, J.R. 2001 Chill-coma tolerance, a major climatic adaptation among *Drosophila* species *Evolution* **55**, 1063-1068.

[12] Anderson, A.R., Hoffmann, A.A. & McKechnie, S.W. 2005 Response to selection for rapid chill-coma recovery in *Drosophila melanogaster*: physiology and life-history traits. *Genetics Research* **85**, 15-22. (doi:doi:10.1017/S0016672304007281).

[13] Bertoli, C.I., Scannapieco, A.C., Sambucetti, P. & Norry, F.M. 2010 Direct and correlated responses to chill-coma recovery selection in *Drosophila buzzatii*. *Entomologia Experimentalis et Applicata* **134**, 154-159. (doi:10.1111/j.1570-7458.2009.00948.x).

[14] Williams, C.M., Watanabe, M., Guarracino, M.R., Ferraro, M.B., Edison, A.S., Morgan, T.J., Boroujerdi, A.F.B. & Hahn, D.A. 2014 Cold adaptation shapes the robustness of metabolic networks in *Drosophila melanogaster*. *Evolution* **68**, 3505-3523. (doi:10.1111/evo.12541).

[15] Findsen, A., Andersen, J.L., Calderon, S. & Overgaard, J. 2013 Rapid cold hardening improves recovery of ion homeostasis and chill coma recovery in the migratory locust *Locusta migratoria*. *The Journal of experimental biology* **216**, 1630-1637. (doi:10.1242/jeb.081141).

[16] MacMillan, H.A., Andersen, J.L., Loeschcke, V. & Overgaard, J. 2015 Sodium distribution predicts the chill tolerance of *Drosophila melanogaster* raised in different thermal conditions.

American Journal of Physiology - Regulatory, Integrative and Comparative Physiology **308**, R823-R831. (doi:10.1152/ajpregu.00465.2014).

[17] MacMillan, H.A. & Sinclair, B.J. 2011 The role of the gut in insect chilling injury: cold-induced disruption of osmoregulation in the fall field cricket, *Gryllus pennsylvanicus*. *The Journal of experimental biology* **214**, 726-734. (doi:10.1242/jeb.051540).

[18] Lavington, E., Cogni, R., Kuczynski, C., Koury, S., Behrman, E.L., O'Brien, K.R., Schmidt, P.S. & Eanes, W.F. 2014 A small system--high-resolution study of metabolic adaptation in the central metabolic pathway to temperate climates in *Drosophila melanogaster*. *Mol Biol Evol* **31**, 2032-2041. (doi:10.1093/molbev/msu146).

[19] Hayward, S.A.L., Manso, B. & Cossins, A.R. 2014 Molecular basis of chill resistance adaptations in poikilothermic animals. *The Journal of experimental biology* **217**, 6-15. (doi:10.1242/jeb.096537).

[20] Williams, C.M., Szejner-Sigal, A., Morgan, T.J., Edison, A.S., Allison, D.B. & Hahn, D.A. 2016 Adaptation to low temperature exposure increases metabolic rates independently of growth rates. *Integrative and comparative biology* **56**, 62-72. (doi:10.1093/icb/icw009).

[21] Welch, K.C., Jr., Peronnet, F., Hatch, K.A., Voigt, C.C. & McCue, M.D. 2015 Carbon stable-isotope tracking in breath for comparative studies of fuel use. *Ann N Y Acad Sci*. (doi:10.1111/nyas.12737).

[22] McCue, M.D. & Welch, K.C., Jr. in press ^{13}C-Breath testing in animals: Theory, applications, and future directions. *J Comp Physiol B*. (doi:10.1007/s00360-015-0950-4).

[23] Gerken, A.R., Mackay, T.F.C. & Morgan, T.J. 2016 Artificial selection on chill-coma recovery time in *Drosophila melanogaster*: Direct and correlated responses to selection. *Journal of Thermal Biology* **59**, 77-85. (doi:http://dx.doi.org/10.1016/j.jtherbio.2016.04.004).

[24] Lee, R.E. 2010 A primer on insect cold tolerance. In *Insect Low Temperature Biology* (eds. D.L. Denlinger & R.E. Lee Jr), p. 3. New York, Cambridge University Press.

[25] Overgaard, J., Malmendal, A., Sørensen, J.G., Bundy, J.G., Loeschcke, V., Nielsen, N.C. & Holmstrup, M. 2007 Metabolomic profiling of rapid cold hardening and cold shock in *Drosophila melanogaster*. *J Insect Physiol* **53**, 1218.

[26] Owen, O.E., Kalhan, S.C. & Hanson, R.W. 2002 The key role of anaplerosis and cataplerosis for citric acid cycle function. *Journal of Biological Chemistry* **277**, 30409-30412. (doi:10.1074/jbc.R200006200).

[27] Overgaard, J., Sørensen, J.G., Petersen, S.O., Loeschcke, V. & Holmstrup, M. 2006 Reorganization of membrane lipids during fast and slow cold hardening in *Drosophila melanogaster*. *Physiological Entomology* **31**, 328-335. (doi:10.1111/j.1365-3032.2006.00522.x).

[28] Koštál, V., Šimek, P., Zahradníčková, H., Cimlová, J. & Štětina, T. 2012 Conversion of the chill susceptible fruit fly larva (*Drosophila melanogaster*) to a freeze tolerant organism. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 3270-3274. (doi:10.1073/pnas.1119986109).

[29] Colinet, H. 2011 Disruption of ATP homeostasis during chronic cold stress and recovery in the chill susceptible beetle (*Alphitobius diaperinus*). *Comparative Biochemistry and Physiology: Part A, Molecular & Integrative Physiology* **160**, 63-67. (doi:10.1016/j.cbpa.2011.05.003).

[30] Coulson, S.J., Fisher, J. & Bale, J.S. 1992 A ^{31}P NMR investigation of the energy charge of the housefly *Musca domestica* (Diptera: Muscidae) during rapid cold hardening and cold shock. *Cryo-Letters* **13**, 183-192.

[31] Colinet, H., Larvor, V., Laparie, M. & Renault, D. 2012 Exploring the plastic response to cold acclimation through metabolomics. *Functional Ecology* **26**, 711-722. (doi:10.1111/j.1365-2435.2012.01985.x).

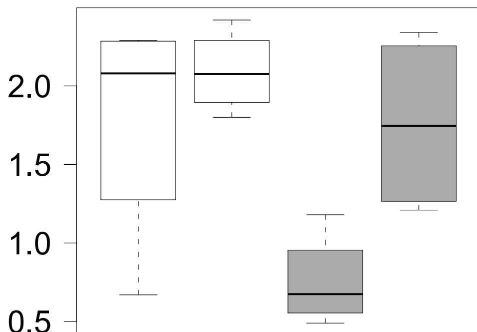
[32] Campos, P.S., Quartin, V., Ramalho, J.C. & Nunes, M.A. 2003 Electrolyte leakage and lipid degradation account for cold sensitivity in leaves of *Coffea* sp. plants. *Journal of plant physiology* **160**, 283-292.

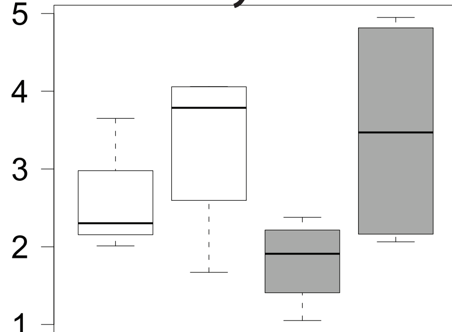
[33] Gibbs, A.G. 1999 Laboratory selection for the comparative physiologist. *The Journal of experimental biology* **202**, 2709-2718.

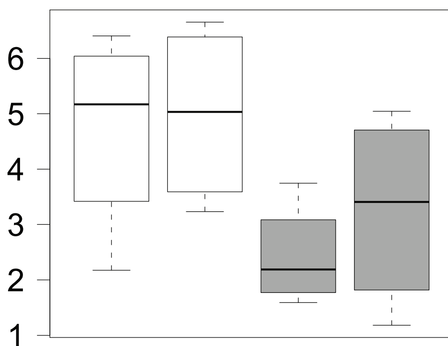
[34] Angilletta Jr, M.J., Wilson, R.S., Navas, C.A. & James, R.S. 2003 Tradeoffs and the evolution of thermal reaction norms. *Trends in Ecology & Evolution* **18**, 234-240. (doi:http://dx.doi.org/10.1016/S0169-5347(03)00087-9).

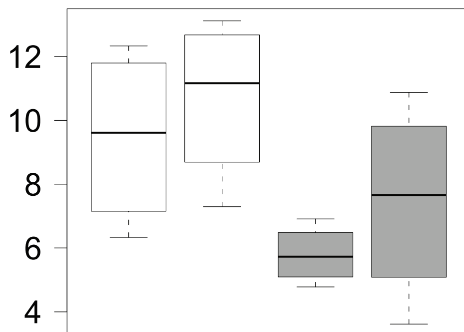
[35] Addo-Bediako, A., Chown, S.L. & Gaston, K.J. 2002 Metabolic cold adaptation in insects: a large-scale perspective. *Functional Ecology* **16**, 332-338. (doi:10.1046/j.1365-2435.2002.00634.x).

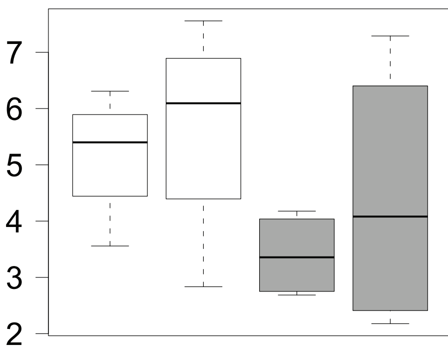
[36] Conover, D.O., Duffy, T.A. & Hice, L.A. 2009 The covariance between genetic and environmental influences across ecological gradients. *Annals of the New York Academy of Sciences* **1168**, 100-129. (doi:10.1111/j.1749-6632.2009.04575.x).

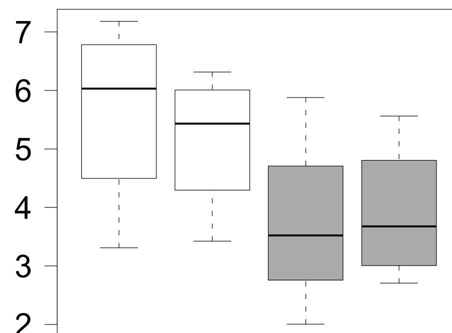










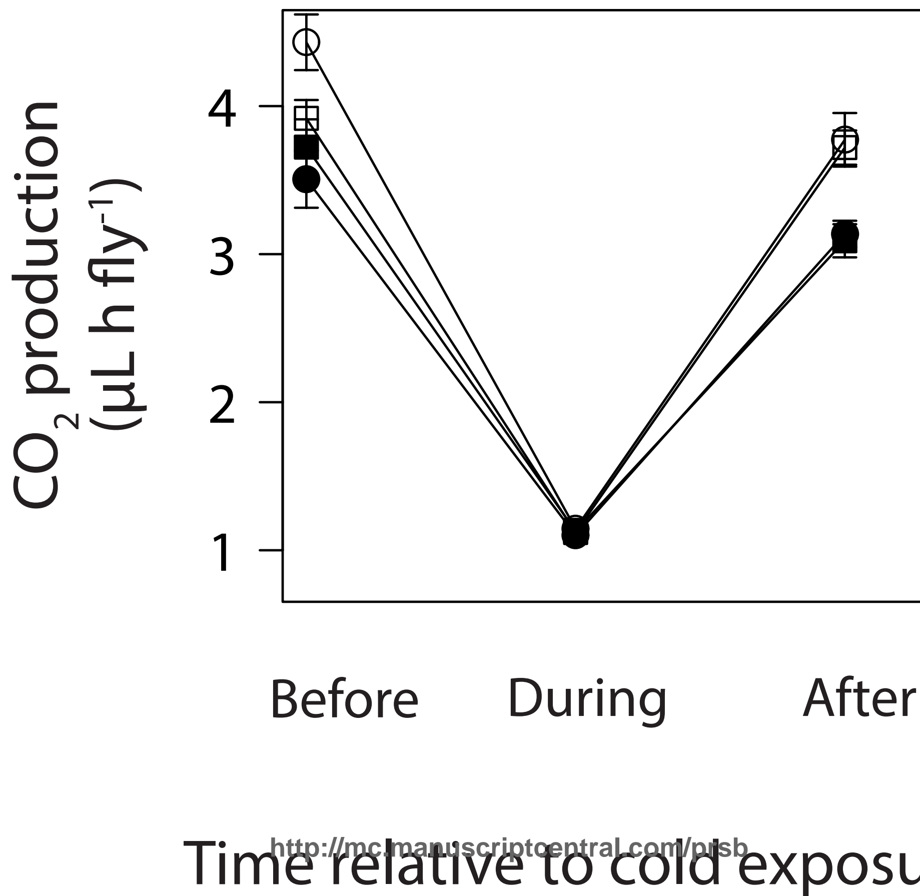


Enrichment from glucose tracer (APE)

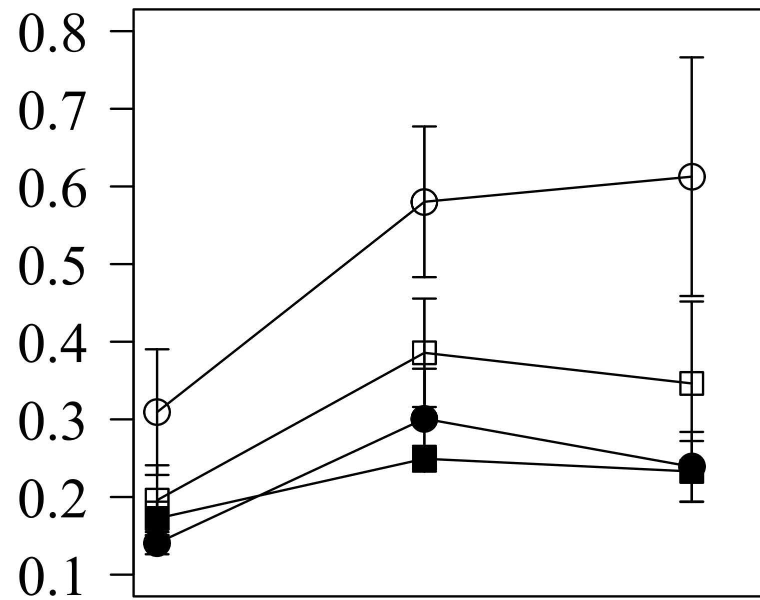
1 2 1 2
Hardy Susceptible

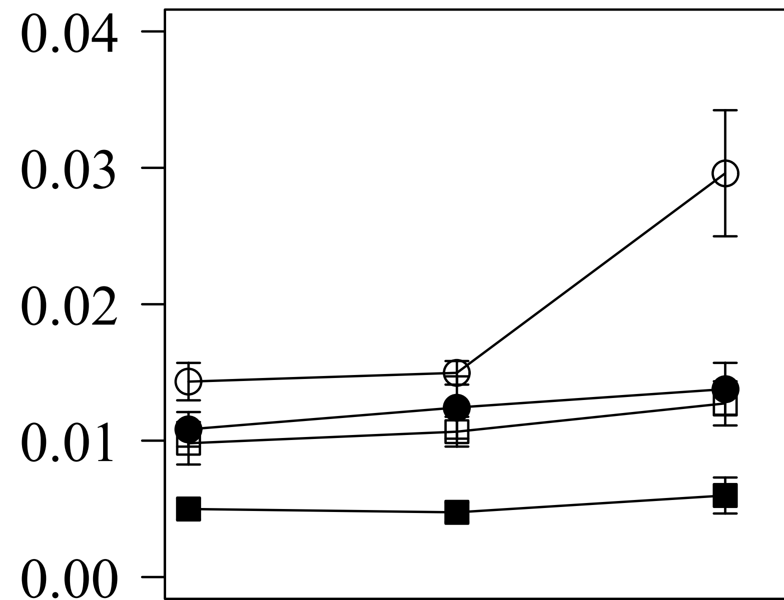
1 2 1 2
Hardy Susceptible

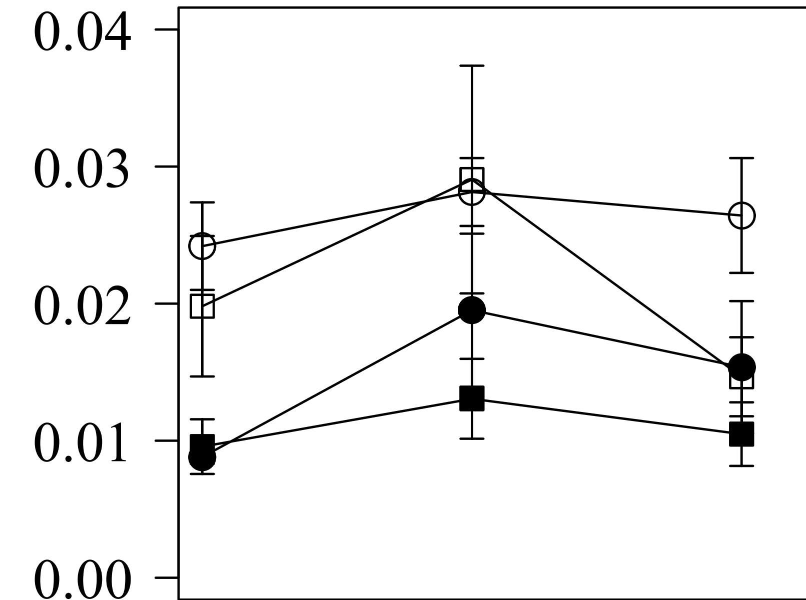
Cold hardiness

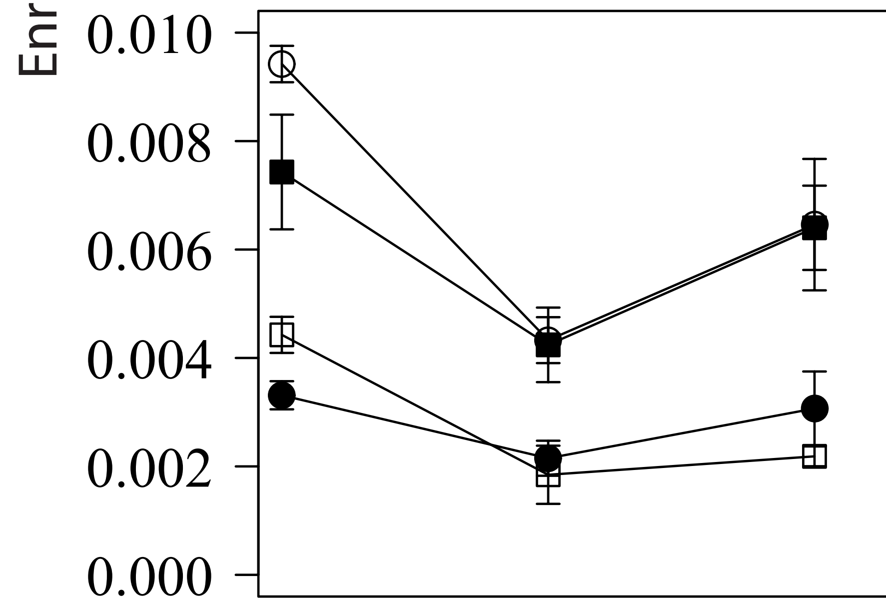


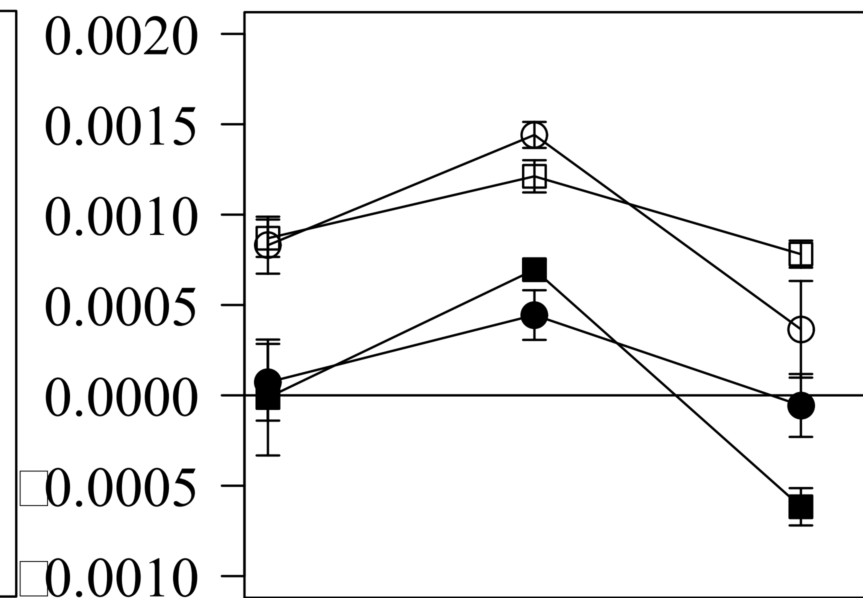
4
3
2
1
CO_2 production
(µL h fly^{-1})
Before
During
After
Time relative to cold exposure

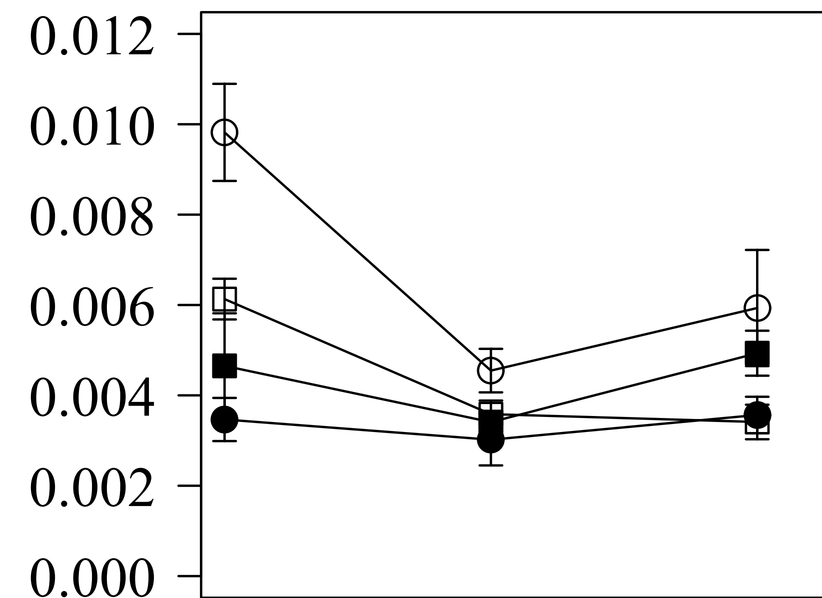












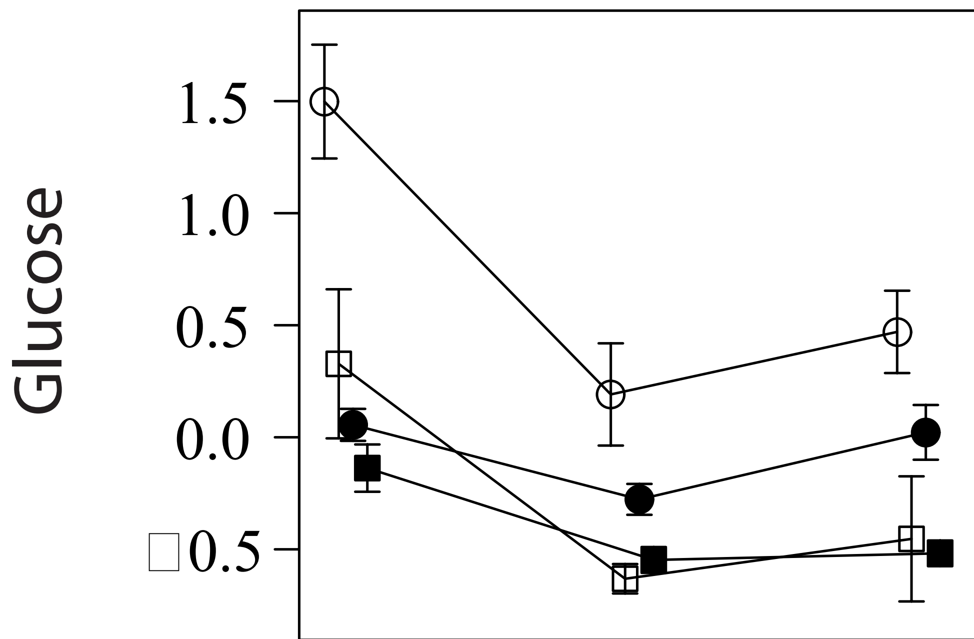
Time relative to cold exposure

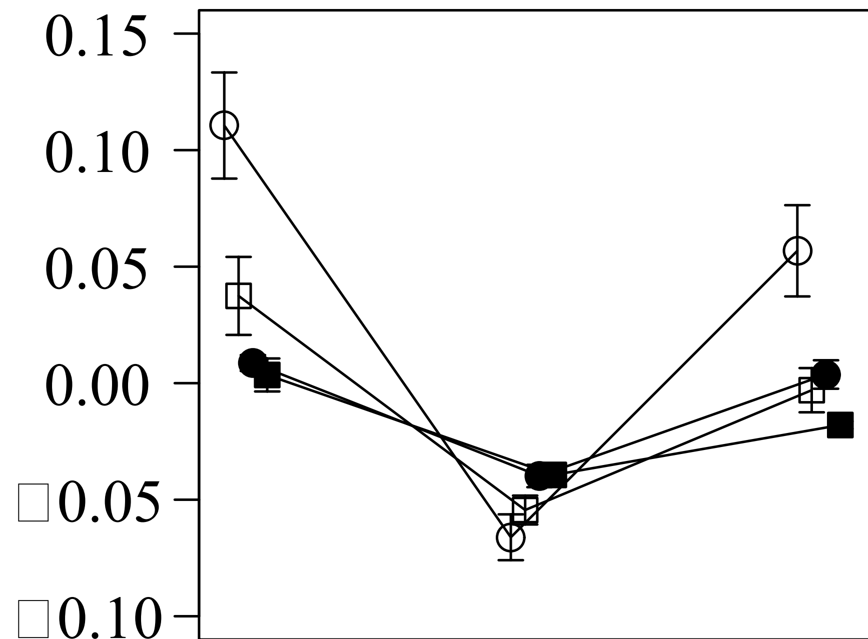
A

Breath enrichment residuals

B

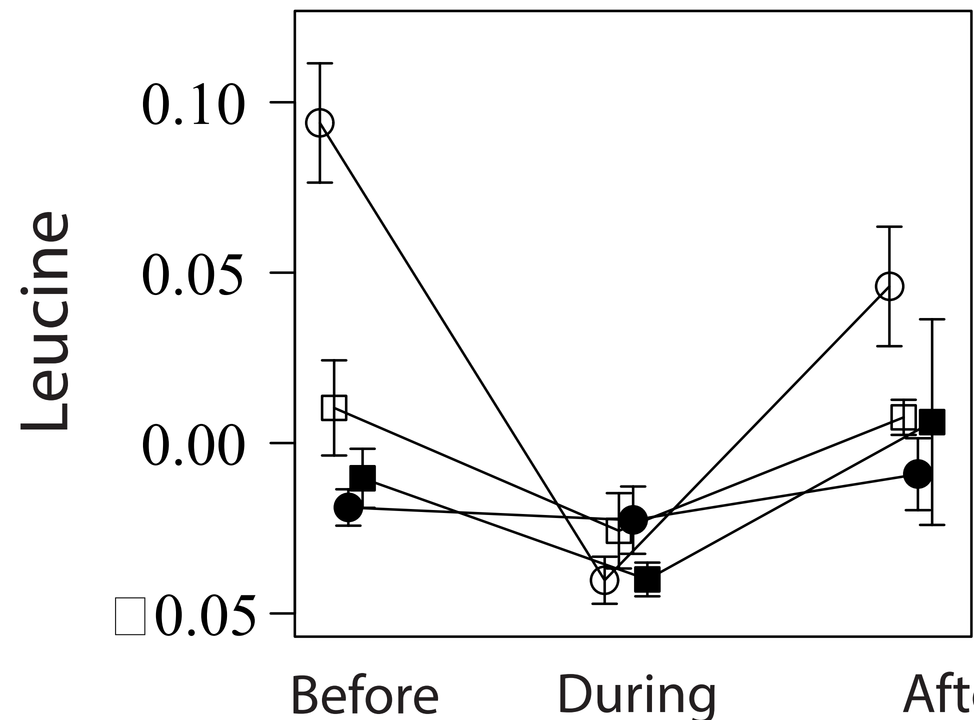
Tracer oxidation rate residuals

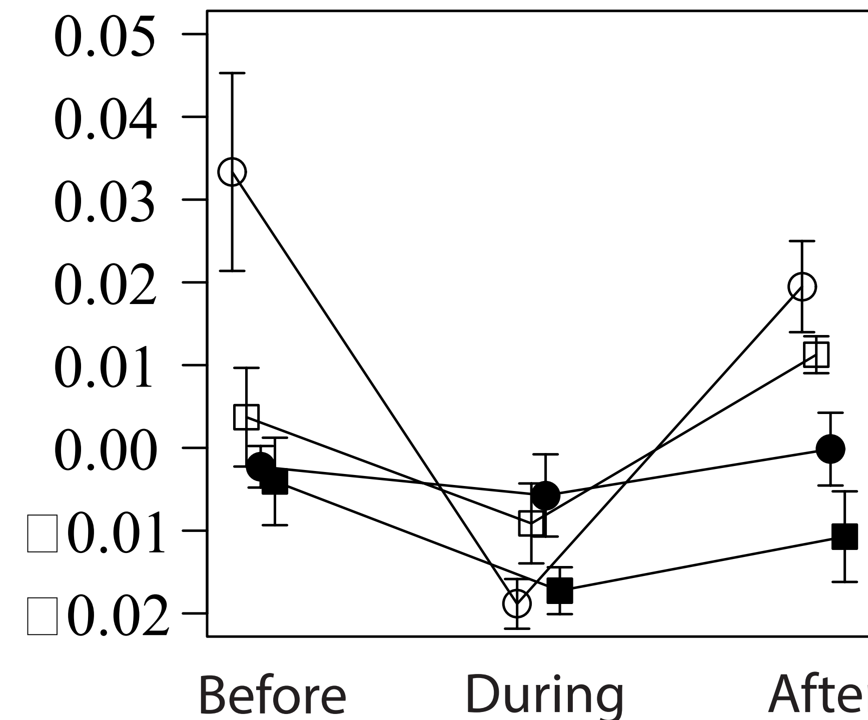




C

D





Time relative to cold exposure