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1 A 'phenotypic hangover' – the predictive adaptive response and multigenerational effects of
2 altered nutrition on the transcriptome of *Drosophila melanogaster*
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26

27 **Abstract**

28 The Developmental Origins of Health and Disease (DOHaD) hypothesis predicts that early-
29 life environmental exposures can be detrimental to later-life health, and that mismatch
30 between the pre- and postnatal environment may contribute to the growing non-
31 communicable disease (NCD) epidemic. Within this is an increasingly recognised role for
32 epigenetic mechanisms; epigenetic modifications can be influenced by, e.g., nutrition, and
33 can alter gene expression in mothers and offspring. Currently, there are no whole-genome
34 transcriptional studies of response to nutritional alteration. Thus, we sought to explore how
35 nutrition affects the expression of genes involved in epigenetic processes in *Drosophila*
36 *melanogaster.* We manipulated *Drosophila* food macronutrient composition at the F0
37 generation, mismatched F1 offspring back to a standard diet, and analysed the
38 transcriptome of the F0 – F3 generations by RNA-sequencing. At F0, the altered (high
39 protein, low carbohydrate, HPLC) diet increased expression of genes involved in epigenetic
40 processes, with coordinated downregulation of genes involved in immunity,
41 neurotransmission and neurodevelopment, oxidative stress and metabolism. Upon reversion
42 to standard nutrition, mismatched F1 and F2 generations displayed multigenerational
43 inheritance of altered gene expression. By the F3 generation, gene expression had reverted
44 to F0 (matched) levels. These nutritionally-induced gene expression changes demonstrate
45 that dietary alteration can upregulate epigenetic genes, which may influence the expression
46 of genes with broad biological functions. Further, the multigenerational inheritance of the
47 gene expression changes in F1 and F2 mismatched generations suggests a predictive
48 adaptive response (PAR) to maternal nutrition. Our findings may help to understand the

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49 interaction between maternal diet and future offspring health, and have direct implications

50 for the current NCD epidemic.

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52 **Background**
53

54 Exposure to aberrant or harmful environments during development and early life can be
55 detrimental to later life health. From these observations is derived the Developmental
56 Origins of Health and Disease (DOHaD) hypothesis (Barker, 2007, Gluckman and Hanson,
57 2004a, Gluckman et al., 2010, Bruce and Hanson, 2010), which seeks to explain why the
58 period from conception to birth and the first few years of life is critical for determining life-
59 long susceptibility to non-communicable diseases (NCDs). Many NCD phenotypes are
60 thought to be caused by developmental perturbations that are a consequence of altered
61 epigenetic marks (Gluckman and Hanson, 2004a, Heindel, 2006), induced by environmental
62 exposure during critical periods of development (Skinner et al., 2013). Alteration to the
63 epigenome regulates gene expression through DNA methylation, histone and chromatin
64 modifications (Callinan and Feinberg, 2006, Peaston and Whitelaw, 2006), providing plasticity
65 to the genome. Consequently, phenotypes under epigenetic regulation provide a pathway
66 though which the genome can interact with the environment (Holliday and Pugh, 1975). If
67 the epigenetic modifications occur at a time during which they are able to affect the
68 germline, such modifications may also influence development of the offspring (Skinner et al.,
69 2013).
70
71 The interaction between the environment and the epigenome and the resulting phenotypic
72 adaptations, coupled with the growing NCD epidemic, led to the predictive adaptive
73 response (PAR) hypothesis (Gluckman and Hanson, 2004b). This hypothesis states that
74 nature of the predictive adaptive response (PAR) is determined by the degree of mismatch
75 between the pre- and postnatal environments. This mismatch results from the information

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76 that the fetus receives on environmental conditions while *in utero*, to which it will respond
77 adaptively by programming its biology to expect that environment. If the actual postnatal
78 environment matches the prenatal prediction, then the PARs are appropriate and disease risk
79 is low; if they do not match then the PAR is inappropriate, and disease risk is increased. For
80 instance, obesity has a distinct epigenetic profile. This pattern could be established in early
81 life as a response to the maternal, fetal and/or early postnatal environment, and later-life
82 nutritional mismatch could mean that the individual has been programmed inappropriately,
83 leading to an increased risk of obesity and associated diseases later in life (Cordero et al.,
84 2015). This implies that the epigenetic hallmarks of early life exposures may be able to be
85 maintained or 'stored' in such a way as to produce long-lasting effects (Lee, 2015).
86
87 Along with stress, drugs and environmental toxicants, one of the main factors that can cause
88 epigenetic perturbation is nutrition; evidence suggests that early-life nutrition can not only
89 affect the long-term health of the individual, but also of their offspring (Tarry-Adkins and
90 Ozanne, 2011, Langley□Evans, 2015, Lillycrop and Burdge, 2015) potentially through
91 epigenetic mechanisms (Aiken and Ozanne, 2013, Haggarty, 2013, Vickers, 2014a, Vickers,
92 2014b, Vaiserman, 2014). Consistent with the DOHaD hypothesis, strong links exist between
93 both maternal and early life nutrition and cardiovascular disease (Barker, 1997), diabetes and
94 obesity (Uauy et al., 2011) along with asthma and allergy, autoimmune disease, cancer and
95 mental health (Gluckman et al., 2011, Barouki et al., 2012, Hanson and Gluckman, 2015,
96 Balbus et al., 2013). Inappropriate maternal nutrition has been linked to incorrect epigenetic
97 'priming' during fetal or postnatal life (Burdge et al., 2007b, Brudasca and Cucuianu, 2016); in
98 particular, diets high in carbohydrate content (sugar) have been shown to program
99 metabolic status and diabetes (Buescher et al., 2013, Musselman et al., 2011).

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100

101 Many nutrition-related phenotypes have been attributed to changes in epigenetic processes
102 and a classic example of this is that of methyl supplementation, which influences coat colour
103 in agouti mice (Waterland and Jirtle, 2003). Nutrition influences are separated into direct
104 effects and also indirect (offspring) effects. Direct effects can be exemplified by high-fat
105 diets inducing obesity and metabolic syndrome, due to the methylation pattern of particular
106 genes and promoters such as leptin (Milagro et al., 2009) and PPARγ (Fujiki et al., 2009), and
107 the differential DNA methylation detected in metabolic syndrome in both humans and
108 rodents (Luttmer et al., 2013, Sánchez et al., 2015). Indirect effects on offspring metabolic
109 phenotype can occur via maternal diet. For example, high fat diets in utero are known to
110 affect offspring epigenetic patterns and methylation status of particular genes, for example,
111 adiponectin and leptin genes (Masuyama et al., 2016), while a high fat diet during pregnancy
112 and lactation can induce epigenetic modifications and differential expression of the μ-opioid
113 receptor, and corresponding hypomethylation of the promoter regions of the gene, in
114 mouse offspring (Vucetic et al., 2010). Additionally, maternal protein restriction can cause
115 hypomethylation of particular genes involved in metabolic processes in fetus and offspring
116 (Lillycrop et al., 2007, Burdge et al., 2007a, van Straten et al., 2010, Burdge et al., 2004) and
117 can also affect methylation in the developing placenta (Reamon-Buettner et al., 2014). Such
118 epigenetic perturbation is not just limited to fetal and early life; the postnatal period is also
119 susceptible to the epigenetic effects of nutrition. For example, hypermethylation of the pro-
120 opipmelanocortin promoter occurs in over-fed rats (Plagemann et al., 2009), and postnatal
121 folic acid supplementation can lead to hypermethylation of PPARα (Burdge et al., 2009). This
122 sensitivity of the epigenome to the effects of the environment (nutrition) also extends into

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123 adulthood, where epigenetic changes have been observed in response to nutritional changes
124 (Christman et al., 1993, Waterland et al., 2006, Hoile et al., 2013).
125
126 In addition to metabolic genes, altered nutrition appears to have broader genomic
127 consequences. For instance, nutrition can also alter markers of inflammation and oxidative
128 stress (Jacometo et al., 2015); a protein restricted diet in pregnancy leads to an increased
129 susceptibility to oxidative stress in offspring (Langley et al., 1994), while a high carbohydrate
130 diet increases the oxidative stress response (Gregersen et al., 2012). A high fat, high
131 carbohydrate meal can induce oxidative and inflammatory stress as reflected by increased
132 reactive oxygen species (ROS) generation in both normal weight (Aljada et al., 2004) and in
133 obese people (Patel et al., 2007), suggesting that oxidative stress and inflammation are major
134 mechanisms involved in metabolic disorders associated with obesity (Fernández-Sánchez et
135 al., 2011) and can also induce epigenetic changes (Sánchez et al., 2015). Indeed, the stress
136 response is well established to be under epigenetic control in humans (Cencioni et al., 2013).
137 In plants, DNA and histone modifications control gene expression when a plant is under
138 environmental stress (Chinnusamy and Zhu, 2009). In terms of applicability to health, we
139 know that low doses of reactive oxygen species, from calorie restricted or high carbohydrate
140 diets, promote health and life span (Ristow and Schmeisser, 2011). Thus considering the
141 above, along with the PAR hypothesis, it is likely that such biological responses to nutrition
142 reflect the idea that induced epigenetic changes that underpin physiological change, and aid
143 in the adaptation of an individual, and potentially its offspring, to an adverse environment
144 (Gluckman et al., 2005).
145

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146 Nutritional deficiency may also lead to the development of psychopathological behaviour
147 such as antisocial, violent and criminal behaviour (Neugebauer et al., 1999). This may in part
148 be a consequence of the capacity of nutritional deficiency to alter brain development (Liu
149 and Raine, 2006), possibly through epigenetic factors, which may lead to changes in brain
150 structure and function (Liu et al., 2015). Supporting this is the suggestion that macronutrient
151 deficiency can cause changes in epigenetic regulation, which can lead to impaired brain
152 development, signalling molecule imbalance, neurotoxicity and differences in
153 neurotransmission (Liu et al., 2015) which all contribute to psychopathological outcomes.
154 For example, studies into participants from the Dutch Hunger Winter cohort found
155 correlations between prenatal famine exposure and schizophrenia (Susser and Lin, 1992,
156 Susser et al., 1996), along with persistent epigenetic changes at, e.g. the *IGF1* gene (Heijmans
157 et al., 2008).
158
159 Thus, considering that gene-specific studies of altered nutrition have demonstrated broad
160 and diverse genetic and epigenetic consequences, it is pertinent to apply this concept to the
161 whole genome. Nutrition is commonly investigated as an environmental factor that is
162 expected to influence the epigenetic landscape, and there are several examples in the
163 literature of response to altered nutrition being 'inherited' multigenerationally (Burdge et al.,
164 2007b, Jirtle and Skinner, 2007, Carter et al., 2004, Golding, 2004) and transgenerationally (F3
165 and beyond, (Waterland and Jirtle, 2003)). As such, altered gene expression, via epigenetic
166 marks in response to nutrition, coupled with the PAR hypothesis, could be the key to
167 understanding the prevalence of obesity and metabolic syndrome. Here we explore the PAR
168 hypothesis and the ability of nutrition to affect gene expression at a whole-genome level by
169 manipulating the diet of the fruitfly, *Drosophila melanogaster*, to investigate the extent to

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170 which gene expression is changed by differing levels of macronutrients. Previous research
171 has shown that a high-sugar maternal diet can alter the body composition of larval
172 *Drosophila* offspring for at least two generations (Buescher et al., 2013), as well as
173 demonstrating that nutrition is able to influence traits relative to metabolic syndrome,
174 longevity and the immune response (Musselman et al., 2011, Morgan, 2012, Roussou et al.,
175 2016). Considering such traits and responses are often under epigenetic control, we predict
176 that dietary manipulation will have broad consequences for the expression of genes involved
177 in epigenetic processes.
178
179
180 **Methods**
181
182 **Fly husbandry**
183
184 *Drosophila melanogaster* stocks used in this study were wild-type Canton-S flies from the
185 Bloomington Drosophila Stock Center at Indiana University. *Drosophila* were cultured in a
186 dedicated invertebrate laboratory using standard techniques. Briefly, flies were maintained
187 in laboratory incubators at 25C in a P Selecta HOTCOLD-C incubator. Larvae were reared on
188 either a standard low protein high carbohydrate (LPHC, standard laboratory fly food) or a
189 high protein low carbohydrate (HPLC) diet. These differential diets consisted of standard
190 brewer's yeast (Health2000, NZ), sugar (New Zealand Sugar Company, Auckland, New
191 Zealand) and cornmeal (Health2000, NZ), in varying ratios (Table 1). Agar (A7002, Sigma-
192 Aldrich, MO, USA), propionic acid (Thermo Fisher, New Zealand, AJA693) and Nipagin
193 (47889, Sigma-Aldrich, MO, USA, 10% w/v in 100% ethanol) were added in equal amounts.

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194 Gross energy (KJ/g) of both the LPHC and HPLC food types was determined by bomb
195 calorimetry, and total protein content (%) was calculated using the total combustion method
196 (Table 1) by the Institute of Food, Nutrition and Human Health at Massey University,
197 Palmerston North, New Zealand.
198
199 **Nutrition experiments**
200
201 *Drosophila* were manipulated under anaesthesia (CO_2) and initially raised on LPHC (low
202 protein, high carbohydrate, standard fly) food. To enable mating, 50 female flies were
203 segregated within 4 hours of eclosion, and incubated with 10-15 male flies, on LPHC food,
204 for 24 hours. Female flies were separated and incubated on either LPHC or HPLC diet.
205 Female flies laid eggs in their specified food and the F1 offspring from the HPLC diet were
206 either maintained on HPLC, or mismatched onto LPHC diet (Figure 1). The further offspring
207 then remained on those matched or mismatched diets, relative to the F0 generation. Thus
208 the biological mismatch relates to flies from a HPLC (non-standard) dietary background that
209 are mismatched onto a LPHC background.
210
211 At each generation, RNA was extracted from female *Drosophila* using a modified kit protocol.
212 Firstly, 10 female *Drosophila* were ground in 300ml of Trizol reagent (Invitrogen) that had
213 been cooled on dry ice, with a disposable, sterile pestle until homogenised. A further 200μl
214 of Trizol was added and the homogenised tissue was incubated in Trizol reagent for 5 min.
215 200 μl of Chloroform was added and the solution was vigorously mixed via vortex for 15
216 seconds. This solution was incubated on ice for 5 min and centrifuged at 12,000 g at 4°C for
217 10 min to separate the aqueous (containing RNA) and organic phases. The aqueous solution

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218 was removed and added to an equal volume of 70% ethanol and mixed via pipetting. The
219 Qiagen RNeasy Kit (Qiagen, Hilden, Germany) was then used to purify the RNA, according to
220 the manufacturer's instructions. RNA was stored at -80°C until needed.
221
222 **Transcriptomic experiments**
223
224 Two F0 generation replicates from each of the LPHC and HPLC diets and two replicates from
225 the F1, F2 and F3 generations with matched and mismatched diets were prepared (boxed
226 numbers, Figure 1), resulting in 16 total RNA samples submitted to the Otago Genomics and
227 Bioinformatics Facility at the University of Otago (Dunedin, New Zealand) under contract to
228 the New Zealand Genomics Limited for library construction and sequencing. The libraries
229 were prepared using TruSeq stranded mRNA sample preparation kit according to the
230 manufacturer's protocol (Illumina). All libraries were normalised, pooled and pair-end
231 sequenced on 2 lanes of high-output flowcell HiSeq 2500, V3 chemistry (Illumina),
232 generating 100-bp reads. Libraries had an average insert size of ~208bp.
233
234 Transcriptomic output was analysed in CLC Genomics Workbench Version 8.5.1. Reads were
235 aligned to the *Drosophila melanogaster* reference genome (BDGP6) as implemented in CLC
236 Genomics Workbench, and differential gene expression (EDGE test) was calculated between
237 samples using an absolute fold change value of >1.5, and an FDR-corrected p-value of
238 <0.001. Transcriptomic data was validated by Nanostring: samples were submitted to the
239 Otago Genomics and Bioinformatics Facility at the University of Otago (Dunedin, New
240 Zealand) under contract to the New Zealand Genomics Limited for nCounter Custom Gene
241 Expression assays (Nanostring). 100ng total RNA in 5uL total volume was processed using

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242 the standard nCounter XT Total RNA protocol. The Total RNA and the CodeSet were
243 combined with hybridisation buffer and incubated at 65C for 20 hours. The Codeset consists
244 of Reporter and Capture probes that hybridise the target sequence of interest, forming a
245 tripartite complex. Hybridised samples were then processed in batches of 12 using the High
246 Sensitivity Protocol (3 hours). Raw data was exported and QC-checked using Nanostring's
247 nSolver data analysis tool (www.nanostring.com). As per the Nanostring CodeSet design
248 criteria, 25 candidate genes for validation were chosen, including two housekeeping genes
249 incorporated (Mnf and Rpl32, Table 2). Raw data was normalised to the geometric mean of
250 both the positive controls (included in the hybridisation steps) and the nominated
251 housekeeping genes. Normalised Nanostring data was compared to transcriptomic data and
252 the Pearson's correlation coefficient was calculated in R (Team, 2008).
253
254 **Gene ontology (GO) analyses**
255
256 Functional annotation clustering (FAC) was undertaken in the Database for Annotation,
257 Visualization and Integration of Discovery (DAVID) v6.8 (Huang et al., 2009b, Huang et al.,
258 2009a) with the following categories: COG_ONTOLOGY, GOTERM_BP_DIRECT,
259 GOTERM_CC_DIRECT, GOTERM_MF_DIRECT, KEGG_PATHWAY and INTERPRO. Up- and
260 down-regulated genes from the F0 generation were submitted separately to DAVID for FAC,
261 and analysed against a background of genes that were expressed and detectable in this
262 dataset, to identify GO terms that were significantly enriched between the HPLC and LPHC F0
263 generation.
264
265 **Statistical analyses**

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266 Significant differences in mean gene expression between different dietary conditions and
267 generations was calculated via ANOVA and Tukey's posthoc testing, as implemented in R
268 (Team, 2008).
269
270 **Results**
271
272 **RNAseq data**
273 Summary statistics for transcriptomic work is shown in Supplementary file 1. Briefly, each
274 sample yielded between 3164 Mbases and 4286 Mbases (average 4001 Mbases), with an
275 average number of reads of 32,006,648 reads (range 25,310,758 – 34,288,584 reads). The
276 mean quality score was an average of 36 PF (range 35.4 – 35.72 PF).
277
278 **Differential gene expression**
279 Of 17,490 genes annotated in the Drosophila genome and contained within the CLC
280 reference database, 12,424 were expressed and detected in these transcriptomic
281 experiments. Of these 12,424 genes, 2946 were differentially expressed (1074 [8.6%] down-
282 regulated and 1872 [15.1%] up-regulated (Supplementary file 2), with an absolute fold
283 change of >1.5, and an FDR-corrected p-value of <0.001, as determined by EDGE test as
284 implemented in CLC.
285
286 **Gene ontology**
287 DAVID uses a clustering approach to reduce redundancy; GO terms that are similar, are
288 clustered together. Each term within the cluster is given a p-value, and the cluster itself is

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289 given an enrichment score (the geometric mean in –log scale of the individual GO term p-
290 values). An enrichment score of 1.3 is equivalent to a non-log p-value of 0.05.
291
292 Functional annotation clustering of genes that are up-regulated in HPLC vs. LPHC indicated
293 that the dataset was highly enriched for genes that are involved in epigenetic processes such
294 as chromatin binding (ES 10.64), DNA replication (enrichment score, ES, 4.64), chromatin
295 regulation (ES 1.62) and histone binding and phosphorylation (ES 1.60) (Table 3). Conversely,
296 clustering of genes that are downregulated in HPLC vs. LPHC indicated that the dataset was
297 highly enriched for genes that are involved in immunity (ES 11.41), fatty acid metabolism (ES
298 3.49), neurotransmission (ES 3.35) cellular metabolic processes (ES 2.49) and oxidative stress
299 pathways (ES 2.19, Table 4).
300
301 **Validation:**
302 Based on the genes that were upregulated in this study, we selected a panel for 20 genes for
303 transcriptomic data validation. All transcriptomic samples were validated by Nanostring,
304 per-sample r value of 0.81-0.95, whole dataset correlation r of 0.86 (Supplementary file 3).
305
306 **Multigenerational gene expression of genes of interest:**
307
308 To determine whether the pattern of gene expression that we identify as being altered in
309 response to diet is maintained across generations (F0-F3, matched [M] and mismatched
310 [MM]) when the HPLC diet is removed, we analysed the multigenerational expression levels
311 of particular epigenetic genes of interest that were identified as being upregulated in HPLC.
312 From the lists of clustered genes generated by DAVID, the same pattern of expression was

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313 observed for every gene, which showed the intermediate-level maintenance of the
314 upregulation of the genes in the F1 and F2 generation, followed by a reversion to F0
315 (matched) gene expression levels by the F3 generation. Figure 2 displays a selection of
316 indicative graphs which display this effect, with ANOVA significance data listed in Table 3
317 (described in Supplementary file 4) and significant pairwise comparisons as determined by
318 Tukey's posthoc testing (Supplementary file 5) indicated by solid and dashed lines. There is
319 no significant difference between the expression of epigenetic genes when comparing the F0
320 LPHC diet and the F3MM flies, despite an intermediate and significant difference between F1
321 and F2 flies mismatched onto LPHC diets.
322
323 When we consider genes that were significantly downregulated between the F0 flies, we see
324 the same pattern of gene expression, in the opposite direction. Choosing to look at genes
325 that are clustered using DAVID (Table 4) we see that genes that are downregulated in
326 response to diet, remain at a low level in the F1 and F2 mismatched cohorts (F1MM and
327 F2MM) but by F3, their gene expression has regained the same level as the LPHC (F0)
328 generation, despite being lower in the F1 and F2 generations (Figure 3 and Supplementary
329 files 4 and 6). This effect is genome-wide, and applies to every gene tested from the lists
330 generated by DAVID.
331
332 **Discussion**
333
334 The primary goal of this study was to investigate the effect of nutrition on the expression of
335 genes involved in epigenetic processes. We have demonstrated that a high protein, low
336 carbohydrate (HPLC) diet results in genome-wide upregulation of epigenetic genes in the F0

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337 generation, compared to that observed in a standard *Drosophila* low protein, high
338 carbohydrate (LPHC) diet; this effect was so strong that the overwhelming majority of genes
339 that were upregulated in response to diet were involved in epigenetic processes, with very
340 few other classes of genes categorised as significantly upregulated. Classes of genes that
341 were downregulated in response to the HPLC diet, versus LPHC, were broader in scope, and
342 included genes involved in the immune response, cell signalling, oxidative stress,
343 carbohydrate and fatty acid metabolism, and neurotransmission. Thus, this study shows that
344 altering a LPHC diet to a HPLC results in genome-wide upregulation of genes involved in
345 epigenetic processes, with a coordinated down regulation of genes with broad physiological
346 functions. The coordinated nature of the gene expression data we observed imply, firstly,
347 that genes involved in the processes of neurotransmission, oxidative stress, metabolism and
348 immunity appear to be under epigenetic control, and secondly, that this epigenetic control,
349 and altered biological response, is influenced by nutrition.
350
351 In response to the genomic and epigenomic changes observed in the F0 generation, we
352 further questioned if dietary alteration resulted in developmental programming of the
353 biological response to diet; specifically, whether the changes induced by the HPLC diet in the
354 F0 generation persisted beyond F0, upon removal of the HPLC diet. The genomic changes
355 induced in the F0 generation persisted at intermediate levels in mismatched F1 and F2
356 generations, in the absence of the HPLC diet. By the F3 generation, gene expression in the
357 mismatched flies had reverted back to the level observed in the F0 matched generation. We
358 hypothesise, firstly, that this multigenerational inheritance of gene expression, followed by a
359 reversion to matched F0 levels by the time the F3 generation is reached, indicates moderate
360 epigenetic programming in the form of a predictive adaptive response (PAR). We further

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361 hypothesise that the 'correction' of the genomic changes induced by dietary alteration after
362 three generations implies that dietary reversion to match the F0 generation may be able to
363 correct an altered genomic landscape, effectively rescuing an aberrant nutrition-induced
364 phenotype.
365
366 The environment is able to interact with genes through epigenetic mechanisms (Holliday and
367 Pugh, 1975) particularly during development (Gluckman and Hanson, 2004a, Gluckman et al.,
368 2010, Bruce and Hanson, 2010); it is during development, when DNA is replicating at an
369 accelerated rate, that the genome is most sensitive to environmental influences that could
370 induce epigenetic alteration, which may influence gene expression and the development of
371 the animal. Crucially, this could also lead to the alteration of the epigenome of the germ
372 cells (Skinner et al., 2013). Any permanent alteration to the germ cell epigenome (Guerrero-
373 Bosagna et al., 2010) may then be transmitted through the germ line, with adverse
374 phenotypic consequences for offspring (Barker, 2007, Skinner, 2011). For example, adult-
375 onset diseases can be induced through embryonic exposure to environmental toxins,
376 primarily endocrine disruptors (Anway et al., 2006, Newbold et al., 2006, Heindel, 2006,
377 Anway and Skinner, 2006). Thus, if epigenetic modifications do become permanent, these
378 modifications can be inherited by future generations and impact disease susceptibility
379 (Anway et al., 2005, Jirtle and Skinner, 2007).
380
381 A large number of studies report transgenerational inheritance in a range of eukaryotes
382 (reviewed in (Jablonka and Raz, 2009)). Many of these studies, particularly in mammals,
383 report inheritance of the acquired trait over two or three generations. Concordant with work
384 by Jirtle and Skinner (2007), we agree that these effects should not be defined as truly

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385 transgenerational, because, mechanistically, exposure of an F0 gestating female to an
386 environmental stimulus (nutrition, toxicants, stress) also exposes the F1 embryo (Figure 1,
387 (Osborne et al., 2013)). Further, for species that develop *in utero*, parental exposure also
388 exposes the germ cells that will form the F2 generation. Thus traits present in the F2
389 generation should be considered as multigenerational, rather than transgenerational, as they
390 could have been induced by direct environmental exposure through the fetus and the germ
391 line. To reflect this, some studies searching for evidence of true transgenerational
392 inheritance are declining to assay the F1 generation entirely (Xia et al., 2016) due to the fact
393 that transmission to the F1 generation can be indicative of both parental effects and
394 programming (Xia and de Belle, 2016). Thus, here we describe our findings from the F1 and
395 F2 generations as multigenerational inheritance, since the gene expression changes induced
396 by the environment in the parental generation revert to F0 levels after the F2 generation.
397
398 The current NCD epidemic is etiologically very complex but it is thought to be mediated, in
399 part, by developmental aberrations arising from the inheritance of altered epigenetic marks
400 (Gluckman and Hanson, 2004a, Heindel, 2006). Many metabolic phenotypes and gene
401 expression differences are linked to differential epigenetic marks that are nutritionally-
402 induced. For example, a protein-restricted diet during pregnancy causes hypomethylation of
403 the hepatic PPARα (peroxisome proliferator-activated receptor alpha) and glucocorticoid
404 receptor (GR) genes in rats, and promotes the same hypomethylation in the F1 and F2
405 offspring of F0 rats fed a protein-restricted diet during pregnancy, despite the nutritional
406 challenge being only in the F0 generation (Burdge et al., 2007b). Others have reported
407 evidence of embryonic environmental exposure influencing the phenotype of the F1
408 generation (Carter et al., 2004, Golding and Team, 2004, Kang et al., 2002, Klip et al., 2002,

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409 Portha, 2005, Tsui and Wang, 2004), as well as, specifically, maternal nutrition exerting effects
410 on the F1 phenotype (Carter et al., 2004, Golding and Team, 2004). Such research strongly
411 implies that epigenetic effects could be the key to understanding the current epidemic of
412 overweight and obese, and associated metabolic syndromes, particularly if nutrition in the F0
413 generation can induce a PAR to nutrition, as we hypothesise is occurring here. Interestingly,
414 comprehensive studies using animal models that investigated the effect of both protein
415 restricted and energy-rich diets during pregnancy on the phenotype of the offspring showed
416 that offspring born to dams fed these different diets exhibited persistent metabolic changes,
417 similar to those observed in human metabolic disease such as obesity, insulin resistance and
418 hypertension (Lillycrop and Burdge, 2015), indicating an element of developmental
419 programming and a possible PAR. These findings imply that both famine (protein
420 restriction) and energy-rich diets, when mismatched back to adequate nutrition, are similarly
421 detrimental to the metabolic health of offspring, and that it is possibly the mismatch itself
422 between inadequate nutrition and proper nutrition which is leading to metabolic disease
423 phenotypes. This highlights the fact that epigenetic mechanisms play a highly complex role
424 in obesity and metabolic pathways (Xue and Ideraabdullah, 2016, Lillycrop and Burdge, 2011,
425 Lillycrop and Burdge, 2015, Li et al., 2010).
426
427 In addition to the striking expression level changes observed in genes involved in epigenetic
428 processes, one major source of change that we observed in these data are genes involved in
429 oxidative stress. Malnutrition or excess of particular nutrients can cause oxidative damage
430 (Fang et al., 2002). For example, hyperglycaemia, which is an excess of sugar in the blood
431 and one of the hallmarks of diabetes, is linked to a diet that is rich in carbohydrates and fat
432 (Gupta et al., 2013, Yang et al., 2012). A build-up of sugar can lead to tissue damage, and

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433 this can be maintained because of metabolic memory (Sánchez et al., 2015), which itself may
434 induce epigenetic changes and altered gene expression (Ceriello, 2010, El-Osta et al., 2008,
435 Sánchez et al., 2015). Given that increased carbohydrate intake can induce oxidative stress as
436 reflected by increased reactive oxygen species (ROS) generation (Aljada et al., 2004, Patel et
437 al., 2007), our results are consistent with the observation that high carbohydrate diets are
438 implicated in increased oxidative and metabolic stress (e.g. (Langley et al., 1994), and that the
439 genome may be responding adaptively to dietary stressors. We know that oxidative stress
440 responses are often under epigenetic control (Cencioni et al., 2013), and also, that maternal
441 nutritional deficiency in pregnancy can lead to altered methylation and increased oxidative
442 DNA damage in the brains of adult offspring (Langie et al., 2013), which, as well as being
443 directly influenced by nutrition, may predispose to neurological disorders in later life.
444
445 Consistent with, and leading on from this observation, these data display a decrease in the
446 expression of genes involved in neurotransmission and neurodevelopment when exposed to
447 a HPLC diet. There is strong evidence linking oxidative stress to neurodegeneration and
448 neurodegenerative disease, such as Alzheimer's disease (Tabaton and Tamagno, 2007). In
449 addition, it is also clear that an increase the production of ROS, induced by environmental
450 factors, can increase risk of a multitude of neurodegenerative diseases (Migliore and
451 Coppedè, 2009). Thus, it stands to reason that, in these data, nutrition may be impacting on
452 the production of ROS and the expression of genes involved in ROS pathways and those
453 involved in neurotransmission and neurodegeneration.
454
455 In addition to the DOHaD hypothesis, there are also free radical early life theories, which link
456 environmental agents (e.g. diet, heavy metals) with perturbations of gene regulation and

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457 expression (in, for example, the *APP* gene), and the onset of, e.g., Alzheimer's disease (Lahiri
458 et al., 2007). Free radical early life theories also link the necessity for oxygen in histone
459 demethylase action to epigenetic processes in development (Hitchler and Domann, 2007).
460 These theories are supported by the observation that nutrition during pregnancy can induce
461 epigenetic changes that result in altered nervous system development (McGowan et al.,
462 2008) and also offspring cerebral function (Gallagher et al., 2005). Further, nutrient
463 availability during the pre and postnatal periods can lead to long-lasting changes in neuron
464 development (Niculescu et al., 2004), as well as influence the development of
465 psychopathological behaviour (Neugebauer et al., 1999). This is because nutritional deficit
466 may lead to altered brain development (Liu and Raine, 2006), possibly via epigenetic factors
467 that can lead to changes in brain structure and function (Liu et al., 2015). Given that
468 micronutrient availability can heavily influence neurotransmission, due to the fact that the
469 function of the brain is inherently related to its metabolism of nutrients (Liu et al., 2015) in
470 the form of vitamins and minerals that function as co-enzymes in neurotransmission and
471 neurotransmitter metabolism, our gene expression data are supportive of these linkages.
472
473 Thus, through our data, we hypothesise that the genome-wide changes we observe in genes
474 involved in epigenetic pathways could be responsible for the gene expression changes in
475 other, broad, biological process seen in response to diet. The intermediate maintenance of
476 these gene expression changes, even when the HPLC diet is removed, suggests a PAR to diet;
477 the biology of the mismatched flies is programmed to expect a certain type of diet, and is
478 responding adaptively, with altered gene expression in the absence of HPLC, albeit at a
479 slightly lower level. The complete reversion of this in the F3 generation suggests an element
480 of phenotypic rescue, implying that altered nutrition did not affect the germline, and that the

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481 gene expression changes are not fixed transgenerationally, and thus may have the capacity
482 to be corrected over time.
483
484 To date, there have been no genome-wide assessments of the effect of nutrition on total
485 gene expression. This study contributes to our understanding of the myriad ways in which
486 nutrition can influence gene expression, phenotypes and future health outcomes, with
487 relevance to the DOHaD hypothesis and the current NCD epidemic. While our study
488 demonstrates multigenerational inheritance of gene expression values, rather than
489 transgenerational, it is worth noting that the phenotypic effects of gene expression changes,
490 rather than the gene expression itself, can persist and show multigenerational, and
491 potentially transgenerational, inheritance. For example, a low protein diet given to
492 *Drosophila* can increase H3K27me3 through upregulation of the E(z) protein. Interestingly,
493 while the upregulation of the (E)z protein was not detected in the F2 generation, the
494 associated increase in methylation H3K27me3 was in fact detected in the F2 generation (Xia
495 et al., 2016) and the coordinated effect on longevity was also present through to the F2
496 generation. This suggests that while the gene expression and protein level is not inherited
497 *per se*, the effects and/or functions of those genes possibly could be. It is possible that a
498 phenomenon such as this may be present in these data; a permissive state may be achieved,
499 whereby we might not detect gene expression changes inherited to F3 and beyond, but we
500 may see associated genomic conformational or phenotypic changes in F3 and beyond.
501 Further functional studies based on dietary manipulation are required to confirm this. In
502 particular, it will be pertinent to prove causality between an epigenetic alteration and a
503 change in regulation of genes involved in the traits we observe. To do so, we suggest a
504 combination of phenotypic measures such as assessing lifespan, oxidative stress resistance,

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505 and immunity, as well as exploiting mutant *Drosophila* strains for genes of interest, to assess
506 the effect of epigenetic alterations on downstream gene expression and associated
507 phenotypes.
508
509 The mechanisms by which environmental perturbations affect the phenotype of the
510 individual in the next generation are vital to understand. While hints of transgenerational
511 epigenetic inheritance in more natural situations exist (e.g. (Jablonka and Raz, 2009)), more
512 work is required to determine if an evolved mechanism exists for this transmission and its
513 significance for the health, development, and evolution.
514

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527

528 **Data availability**

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529 All data supporting this work is included in the supplementary material.

530

531

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828

829 **Figure Legends**

830

831 Figure 1 – Fly diet experiments. LPHC, low protein, high carbohydrate (standard) diet; HPLC,
832 high protein low carbohydrate diet; F1(M), F2(M) and F3(M), flies maintained on LPHC diet
833 for three generations; F1(MM), F2(MM) and F3(MM), flies that were raised on HPLC in the F0
834 generation, and mismatched back to LPHC at eclosion in the F1 generation, and were
835 maintained for two more generations on the mismatched (LPHC) diet. Boxed numbers
836 denote the number of replicates used for each condition for transcriptomic experiments.

837

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838 Figure 2 – Indicative graphs of gene expression of genes up-regulated in the F0 generation,
839 with significances as determined by ANOVA, between F0-F3 generations on matched and
840 mismatched diets. Pairwise comparisons by Tukey's posthoc testing indicated by solid and
841 dashed lines, as described in the key. Y axis denotes mean expression level from
842 transcriptomic experiments, X axis denotes the dietary condition as per Figure 1. Note
843 differing Y axis scales. Gene names are stated as per their FlyBase gene symbol IDs.
844
845 Figure 3 – Indicative graphs of gene expression of genes down-regulated in the F0
846 generation, with significances as determined by ANOVA, between F0-F3 generations on
847 matched and mismatched diets. Pairwise comparisons by Tukey's posthoc testing indicated
848 by solid and dashed lines, as described in the key. Y axis denotes mean expression level
849 from transcriptomic experiments, X axis denotes the dietary condition as per Figure 1. Note
850 differing Y axis scales. Gene names are stated as per their FlyBase gene symbol IDs.
851
852 **Table Legends**
853
854 Table 1 – Fly diet components and content information
855
856 Table 2 – Codeset design for Nanostring. Gene, gene symbol based on FlyBase
857 nomenclature; NSID, Nanostring internal identifier; Position, region in the target mRNA
858 being probed
859
860 Table 3 – Functional annotation clustering (FAC) as performed in DAVID. Genes that were
861 significantly up-regulated in these data in HPLC compared to LPHC were compared to a

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862 background list of genes that were expressed and detected in these data. Analyses of
863 variance were carried out on expression between all samples in this study (F0-F3 matched
864 and mismatched) with p-values and statistical significances listed.
865
866 Table 4 – Functional annotation clustering (FAC) as performed in DAVID. Genes that were
867 significantly down-regulated in these data in HPLC compared to LPHC were compared to a
868 background list of genes that were expressed and detected in these data. Analyses of
869 variance were carried out on expression between all samples in this study (F0-F3 matched
870 and mismatched) with p-values and statistical significances listed.
871
872 **Supplementary Files**
873
874 Supplementary file 1
875 Supplementary file 1.xlsx
876 Summary statistics from RNA sequencing experiments.
877 All experiments were carried out on the Illumina platform. The libraries were prepared using
878 TruSeq stranded mRNA sample preparation kit according to the manufacturer's protocol
879 (Illumina). All libraries were normalised, pooled and pair-end sequenced on 2 lanes of high-
880 output flowcell HiSeq 2500, V3 chemistry (Illumina), generating 100-bp reads. Libraries had
881 an average insert size of ~208bp.
882
883 Supplementary file 2
884 Supplementary file 2.xlsx

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885 Drosophila genes that are significantly differentially expressed between HPLC and LPHC F0
886 generation.
887 12,424 Drosophila genes were expressed and detected in these transcriptomic experiments.
888 Of these 12,424 genes, 2946 were differentially expressed (1074 [8.6%] down-regulated and
889 1872 [15.1%] up-regulated), with an absolute fold change of >1.5, and an FDR-corrected p-
890 value of <0.001, as determined by EDGE test as implemented in CLC.
891
892 Supplementary file 3
893 Supplementary file 3.xlsx
894 Transcriptomic data validated by Nanostring:
895 100ng total RNA in 5uL total volume was processed using the standard nCounter XT Total
896 RNA protocol. The Codeset consists of Reporter and Capture probes that hybridise the target
897 sequence of interest, forming a tripartite complex. Hybridised samples were processed in
898 batches of 12 using the High Sensitivity Protocol (3 hours). Raw data was exported and QC-
899 checked using Nanostring's nSolver data analysis tool (www.nanostring.com). As per the
900 Nanostring CodeSet design criteria, 25 candidate genes for validation were chosen, with two
901 housekeeping genes incorporated within this amount (Mnf and Rpl32). Raw data was
902 normalised to the geometric mean of both the positive controls (included in the
903 hybridisation steps) and the nominated housekeeping genes. Normalised Nanostring data
904 (expression level) was compared to transcriptomic data (mean expression level) and the
905 Pearson's correlation coefficient was calculated in R
906
907 Supplementary file 4
908 Supplementary file 4.xlsx

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909 Analyses of variance (ANOVA) of F0-F3 matched and mismatched dietary conditions, up and
910 down regulated genes.
911 ANOVAs of genes that have been identified through FAC as being significantly up or
912 downregulated in the F0 generation (in HPLC, vs. LPHC). Df = degrees of freedom, Sum sq =
913 sums of squares, Mean sq = mean squares, F value = ANOVA F value, Pr(>F) = p-value of
914 significance of F, SIgnifcance = significance indicator. Gene name = FlyBase gene symbol.
915
916 Supplementary file 5
917 Supplementary file 5.xlsx
918 Pairwise comparisons/Tukey's posthoc testing of significant ANOVAs of genes that were
919 signficantly upregulated in the F0 generation.
920 These data indicate the significant differences between the means of the different nutritional
921 states (matched/mismatched) and generations. diff = difference, lwr,upr = confidence
922 intervals, p adj = p value adjusted for multiple testing.
923
924 Supplementary file 6
925 Supplementary file 6.xlsx
926 Pairwise comparisons/Tukey's posthoc testing of significant ANOVAs of genes that were
927 signficantly downregulated in the F0 generation.
928 These data indicate the significant differences between the means of the different nutritional
929 states (matched/mismatched) and generations. diff = difference, lwr,upr = confidence
930 intervals, p adj = p value adjusted for multiple testing.
931

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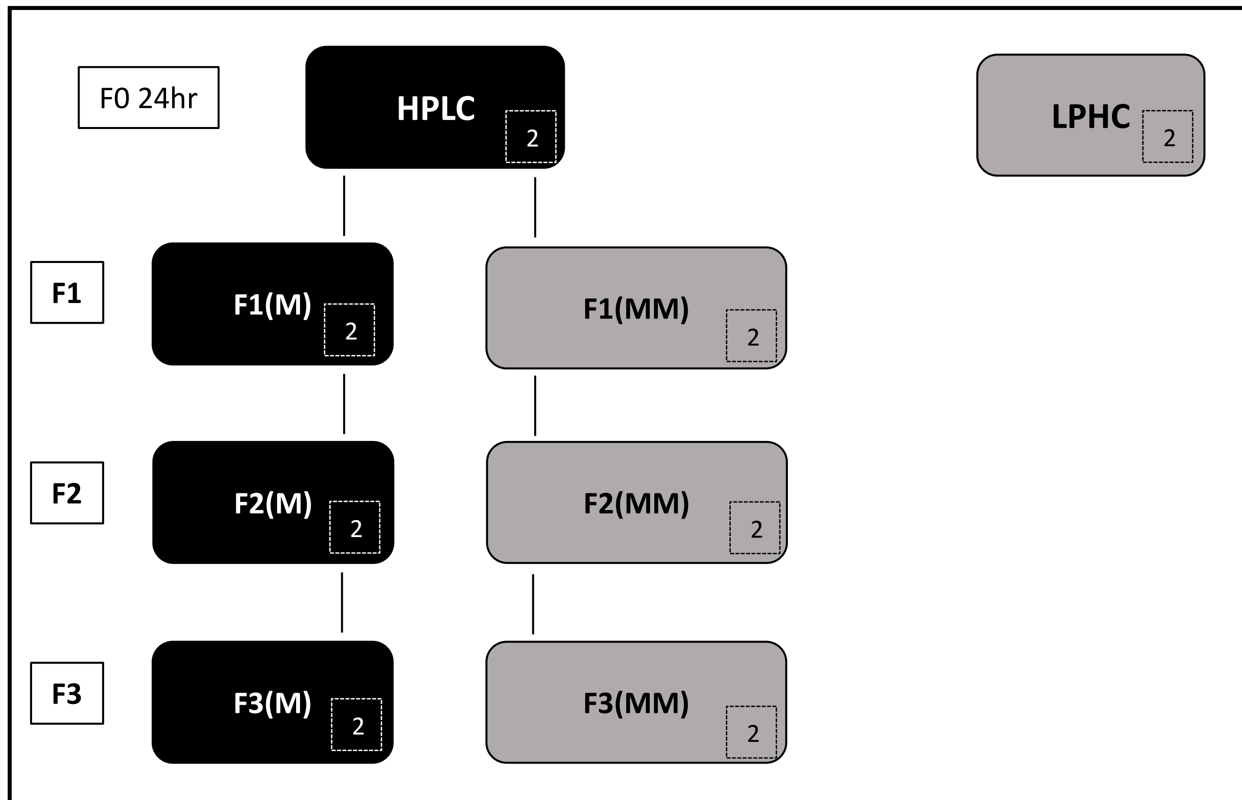

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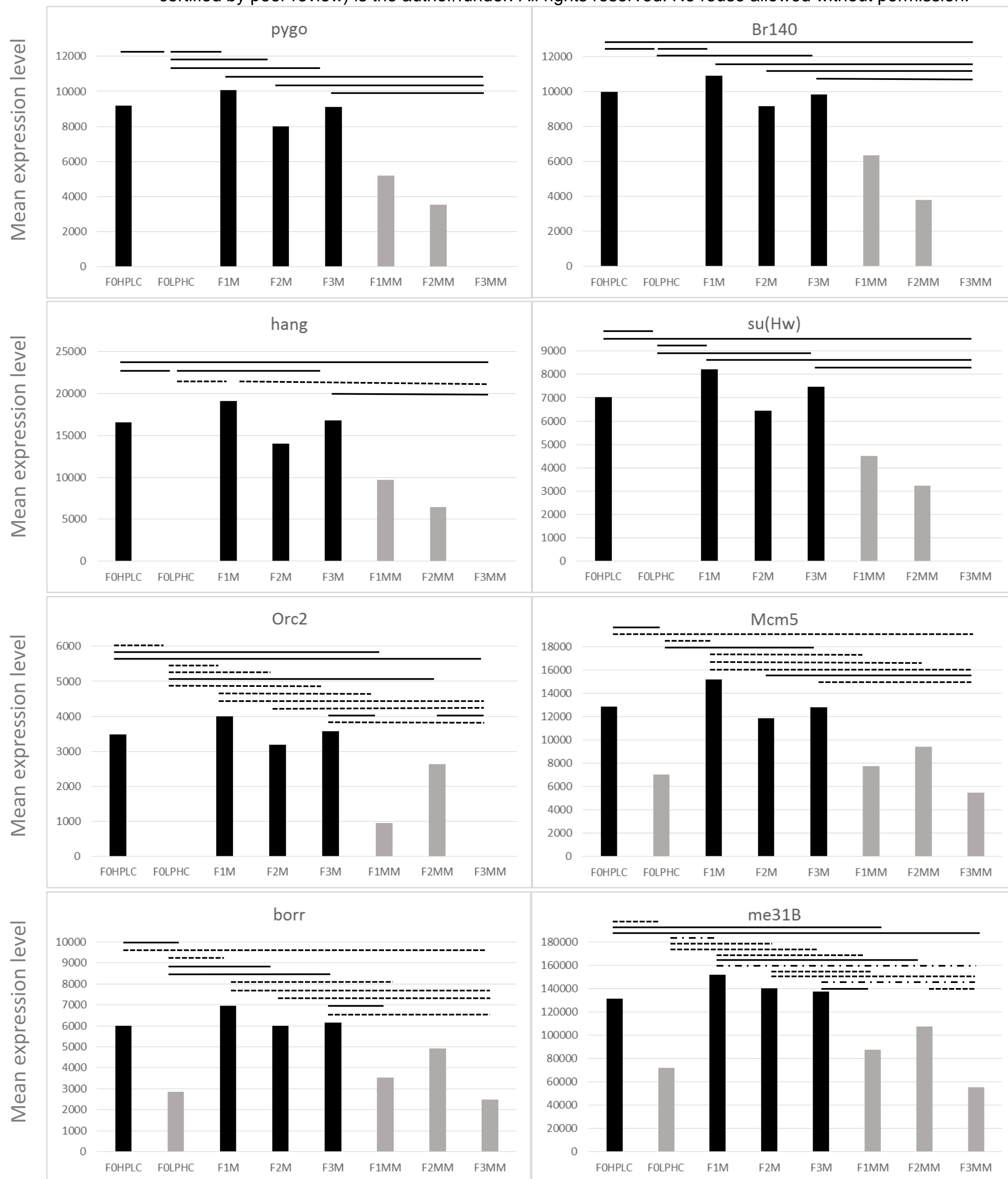

Tukey's posthoc testing significant at p < 0.05 *

Tukey's posthoc testing significant at p < 0.01 **

Tukey's posthoc testing significant at p < 0.001 ***

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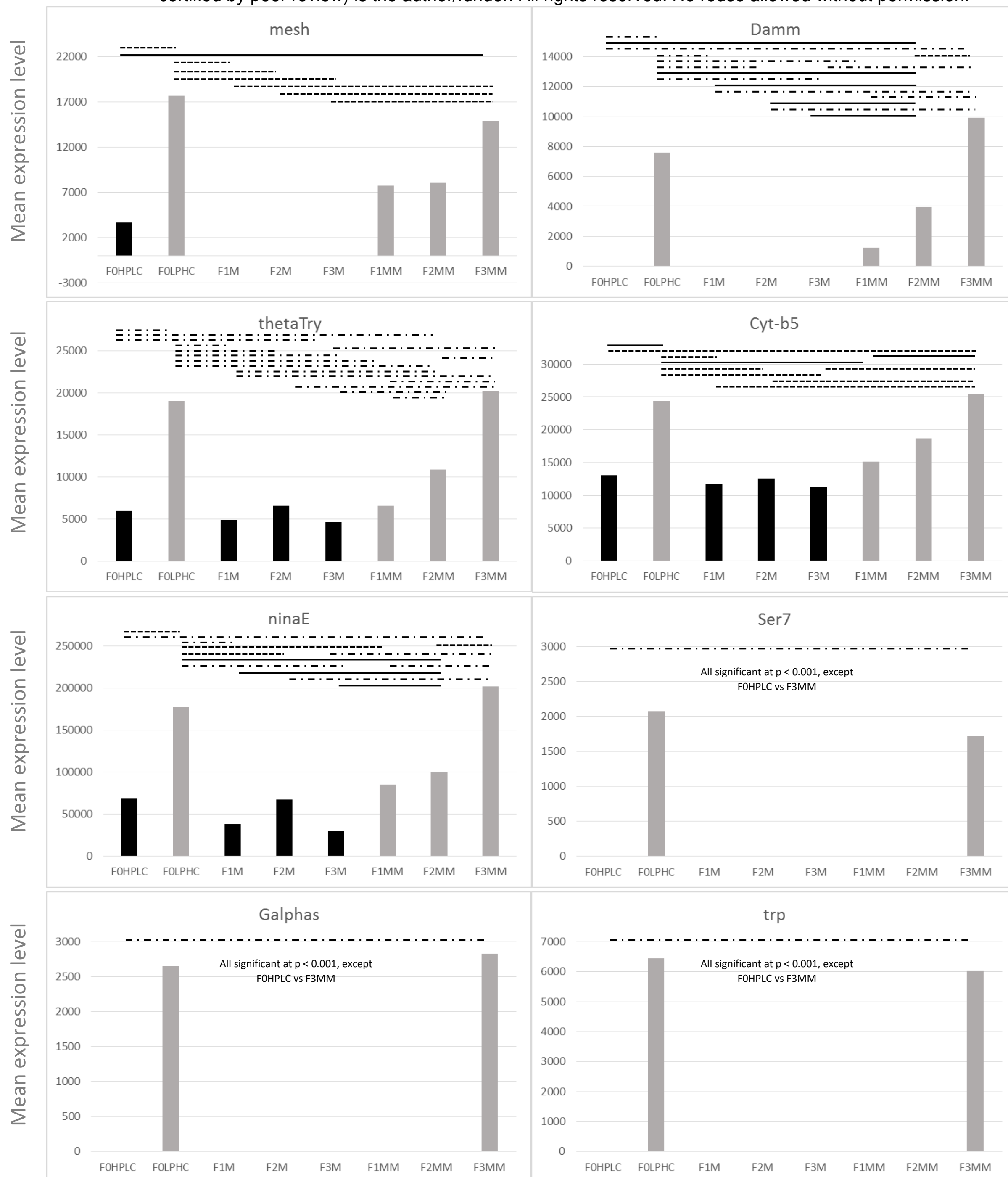

Tukey's posthoc testing significant at p < 0.05 *

Tukey's posthoc testing significant at p < 0.01 **

Tukey's posthoc testing significant at p < 0.001 ***

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Table 1

Component	**High Protein**	**Low protein**
Agar (g)	9	9
Cornmeal (g)	66.7	66.7
Sugar (g)	31.24	46.7
Yeast (g)	148.76	16.7
Propionic acid (ml)	6.6	6.6
Nipagen (ml)	5	5
Water (L)	1	1
Protein (%)	8	5.3
Gross energy (KJ/g)	0.9	2.1
Yeast:sugar ratio	01:00.2	01:02.8

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Table 2

Gene	Accession	Position	NSID
asf1	NM_079439.2	601-700	NM_079439.2:600
Caf1-105	NM_136745.3	1231-1330	NM_136745.3:1230
Def	NM_078948.2	8-107	NM_078948.2:7
E(bx)	NM_167819.2	4681-4780	NM_167819.2:4680
E(Pc)	NM_078974.2	2551-2650	NM_078974.2:2550
E(spl)m4-BFM	NM_079786.1	241-340	NM_079786.1:240
E(spl)m5-HLH	NM_079787.2	396-495	NM_079787.2:395
Fbp1	NM_079341.1	3001-3100	NM_079341.1:3000
Mnf	NM_168444.1	866-965	NM_168444.1:865
Hml	NM_079336.2	7236-7335	NM_079336.2:7235
Hmt4-20	NM_130497.2	3201-3300	NM_130497.2:3200
Inos	NM_058057.4	1026-1125	NM_058057.4:1025
Lip3	NM_057983.3	946-1045	NM_057983.3:945
Lsd-1	NM_170092.2	836-935	NM_170092.2:835
Lsp1beta	NM_057276.3	1351-1450	NM_057276.3:1350
Ocho	NM_080514.1	416-515	NM_080514.1:415
Pc	NM_079475.2	991-1090	NM_079475.2:990
Pgm	NM_079936.2	841-940	NM_079936.2:840
Rpl32	NM_170461.1	342-441	NM_170461.1:341
Sap30	NM_132934.2	216-315	NM_132934.2:215
Su(var)3-3	NM_140937.2	1781-1880	NM_140937.2:1780
Su(var)3-7	NM_079618.2	3516-3615	NM_079618.2:3515
Top2	NM_057412.3	3111-3210	NM_057412.3:3110
Tps1	NM_134983.2	2056-2155	NM_134983.2:2055
Ubx	NM_206497.1	1321-1420	NM_206497.1:1320

Annotation Category	Term	Enrichment score	Gene	FlyBase Gene ID	Function	p-value of ANOVA	Significance of ANOVA
Interpro	Zinc finger	10.64	Bre1	FBgn0086694	Ubiquitinates histone H2B on lysine 120 at most RNA Polymerase II transcribed genes	0.0304	*
			pygo	FBgn0043900	Binds His3 methylated tail to associate with arm as part of Wnt-induced transcription	0.0030	**
			e(y)3	FBgn0087008	PBAF complex, chromatin binding and gene silenecing	0.0596	.
			nej	FBgn0261617	Acetylates histone proteins and regulated gene expression	0.1420	NS
			E(bx)	FBgn0000541	Nucleosome remodelling, chromatin organisation	0.0809	NS
			Pcl	FBgn0003044	Chromatin binding, colocalises with the ESC/E(Z) complex	0.0439	*
Interpro/GOTERM_MF_DIRECT	Zinc finger, DNA binding	4.64	E(var)3-9	FBgn0260243	Chromatin maintenance	0.0014	**
			Br140	FBgn0033155	Enhancer of polycomb-like	0.0027	**
			MTA1-like	FBgn0027951	Chromatin binding, chromosome condensation	0.0665	.
			spn-E	FBgn0003483	Chromatin binding, chromosome condensation	0.0153	*
			hang	FBgn0026575	Response to oxidative stress	0.0031	**
			phol	FBgn0035997	Polycomb group protein recruitment to Polycomb Response Elements	0.0006	***
			Chd1	FBgn0250786	Remodelling and assembly of chromatin	0.0865	.
			Cp190	FBgn0000283	Chromatin binding	0.0165	*
			cg	FBgn0000289	Binds to Polycomb Response Elements	0.0047	**
			su(Hw)	FBgn0003567	Negative regulation of chromatin silencing	0.0058	**
			Chd3	FBgn0023395	Chromatin assembly or disassembly, nucleosome remodelling	0.0027	**
GOTERM_BP/MF/CC_DIRECT, INTERPRO	DNA replication, MCM complex	4.42	Orc1	FBgn0022772	Origin Recognition Complex, DNA replication, chromatin binding	0.0013	**
			Orc4	FBgn0023181	Initiation of DNA replication	0.0136	*
			Orc2	FBgn0015270	DNA replication, chromatin binding	0.0004	***
			Mcm5	FBgn0017577	Chromatin binding, chromosome condensation	0.0008	***
			RecQ4	FBgn0040290	DNA stability, DNA rewinding	0.0047	**
			Mcm10	FBgn0032929	Heterochromatin organisation, chromatin silencing	0.0008	***
			polybromo	FBgn0039227	Chromatin binding	0.0104	*
GOTERM_BP/MF/CC_DIRECT, INTERPRO	Microtublues, kinesin	4.05	cid	FBgn0040477	Histone H3 variant, epigenetic mark for centromere identity	0.0000	***
GOTERM_MF_DIRECT, INTERPRO	Helicase	3.10	XNP	FBgn0039338	Heterochromatin organisation, chromatin silencing	0.0924	.
			me31B	FBgn0004419	Gene silencing by miRNA	0.0001	***
			Mi-2	FBgn0262519	Chromatin binding, nucleosome binding	0.0261	*
INTERPRO	Structural maintenance of chromosomes	2.61	SMC2	FBgn0027783	Chromatin binding, chromosome condensation	0.0020	**
			glu	FBgn0015391	Chromatin binding, chromosome condensation	0.0146	*
			SMC1	FBgn0040283	Chromatin binding	0.0263	*
			SMC3	FBgn0015615	Chromatin binding	0.0129	*
GOTERM_CC/BP_DIRECT	Rb-E2F complex, Myb complex	2.32	mor	FBgn0002783	Brahma associated proteins complex, PBAF complex	0.0100	*
INTERPRO, GOTERM_MF_DIRECT	Chromo domain	1.99	Chro	FBgn0044324	Histone binding, chromosome organisation	0.0098	**
			Chd1	FBgn0250786	Remodelling and assembly of chromatin	0.0865	.
			Mi-2	FBgn0262519	Chromatin binding, nucleosome binding	0.0261	*
			msl-3	FBgn0002775	Methylated histone binding, chromatin binding	0.1760	NS
			Chd3	FBgn0023395	Chromatin assembly or disassembly, nucleosome remodelling	0.0027	**
GOTERM_MF/BP_DIRECT	Nucleosome mobilisation, nucleosome binding	1.76	dre4	FBgn0002183	Chromatin binding, nucleosome binding	0.0021	**
			E(bx)	FBgn0000541	Histone binding, chromatin organisation, chromatin remodelling	0.0809	.
			Su(z)12	FBgn0020887	Polycomb repressive complex 2, histone methyltransferase activity'	0.0391	*
INTERPRO	WD40 repeat	1.67	Hira	FBgn0022786	Chromatin binding	0.0000	***
			Caf1-105	FBgn0033526	Chromatin assembly factor, histone binding	0.0001	***
			wds	FBgn0040066	Histone acetyltransferase activity, chromatin remodelling, Trx complex	0.0032	**
			ebi	FBgn0263933	Chromatin binding	0.0070	**
GOTERM_BP/CC_DIRECT	Histone phosphorylation	1.6	borr	FBgn0032105	Histone phosphorylation, chromatin binding	0.0006	***
			ball	FBgn0027889	Histone threonine kinase activity, histone phosphorylation	0.0009	***
			aurB	FBgn0024227	Chromatin organisation	0.0004	***

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Annotation Category	Term	Enrichment score	Gene	FlyBase Gene ID	Function	p-value of ANOVA	Significance of ANOVA
INTERPRO	Immunoglobulin-like domain/fold	11.42	mesh	FBgn0051004	Ig fold	0.000611	***
			Ppn	FBgn0003137	Ig domain, extracellular matrix structural constituent	0.0101	*
INTERPRO/GO_TERM_BP_DIRECT/GOTERM_MF_DIRECT	Proteolysis, peptidase	6.16	Jon65Ai	FBgn0035667	Serine-type endopeptidase, proteolysis	0.00293	**
			MP1	FBgn0027930	Serine-type endopeptidase, proteolysis	0.00187	**
			thetaTry	FBgn0011555	Digestive enzyme with serine-type peptidase activity	1.68E-08	***
			zetaTry	FBgn0011556	Digestive enzyme with serine-type peptidase activity	0.000488	***
			Decay	FBgn0028381	Cysteine-type endopeptidase activity, caspase	0.00188	**
			Damm	FBgn0033659	Cysteine-type endopeptidase activity, caspase	6.79E-06	***
			alphaTry	FBgn0003863	Serine-type endopeptidase, proteolysis	5.69E-05	***
			Ser7	FBgn0019929	Serine-type endopeptidase, proteolysis	1.51E-12	***
			Ser6	FBgn0011834	Serine-type endopeptidase, proteolysis	5.17E-03	**
			Swim	FBgn0034709	Polysaccharide binding, cysteine-type peptidase activity, proteolysis	1.35E-02	*
			Jon74E	FBgn0023197	Serine-type endopeptidase, proteolysis	1.58E-03	**
			psh	FBgn0030926	Peptidase and serine-type endopeptidase activity, defense response	4.41E-08	***
			Jon65Aiv	FBgn0250815	Serine-type endopeptidase, proteolysis	1.74E-04	***
			Jon65Aiii	FBgn0035665	Serine-type endopeptidase, proteolysis	6.77E-05	***
			Ance-4	FBgn0033366	Peptidase	6.97E-04	***
			Ance	FBgn0012037	peptidyl-dipeptidase activity	6.97E-04	***
			Jon25Bii	FBgn0031654	Serine-type endopeptidase, proteolysis	8.62E-04	***
GOERM_BP_DIRECT/INTERPRO/GOTERM_MF_DIRECT	G-protein coupled receptor signalling, rhodopsin-like	6.02	Rh3	FBgn0003249	G-protein coupled photoreceptor activity	3.73E-03	**
			AkhR	FBgn0025595	Ccarbohydrate, lipid and tryglyceride homeostasis	1.88E-09	***
			Rh2	FBgn0003248	G-protein coupled photoreceptor activity	4.65E-02	*
			Galphas	FBgn0001123	Signal transduction, chemical synaptic transmission	1.07E-11	***
			ninaE	FBgn0002940	G-protein coupled photoreceptor activity, rhodopsin-like	2.80E-05	***
			Ggamma1	FBgn0004921	G-protein, gamma subunit, signal transducer activity	4.16E-05	***
			Gbeta76C	FBgn0004623	G-protein coupled photoreceptor activity	2.71E-02	*
			Rh4	FBgn0003250	G-protein coupled photoreceptor activity	1.83E-03	**
GOTERM_MF_DIRECT/GOTERM_BP_DIRECT	Fatty acid elongation	3.49	eloF	FBgn0037762	Fatty acid elongase	4.60E-05	***
GOTERM_MF_DIRECT	Neuropeptide hormone activity/binding	3.35	Nplp2	FBgn0040813	Neuropeptide signaling, humoral immune response	1.39E-02	*
			Nplp3	FBgn0042201	Neuropeptide hormone activity, neuropeptide signaling	3.45E-01	NS
			Neb-cGP	FBgn0083167	Hormone activity, regulation of growth	3.72E-02	*
GOTERM_MF/CC/BP_DIRECT	Calcium-dependent phospholipid binding	2.72	AnxB9	FBgn0000083	calcium ion binding, calcium-dependent phospholipid binding	7.69E-03	**
GPTERM_MP_DIRECT, INTERPRO, COG_ONTOLOGY	Calcium ion binding, signal transduction, EF-hand domain	2.5	Cals	FBgn0039928	Calcium ion binding, chemical synaptic transmission	2.29E-02	*
			Gel	FBgn0010225	Actin binding, calcium ion binding	1.52E-03	**
			Scp2	FBgn0020907	Calcium ion binding	3.43E-02	*
			Scp1	FBgn0020908	Calcium ion binding	8.74E-02	.
			Mlc2	FBgn0002773	Calcium ion binding	3.33E-04	***
			Zasp52	FBgn0265991	Muscle development	3.91E-08	***
			LpR1	FBgn0066101	Uptake of neutral lipids from circulation, calcium ion binding	4.93E-01	NS
			Mlc1	FBgn0002772	Calcium ion binding, myosin	3.91E-04	***
			up	FBgn0004169	Calcium ion binding, calcium ion homeostatis, muscle morphogenesis	3.26E-04	***
			TpnC73F	FBgn0010424	Calcium ion binding	3.01E-10	***
			Gpo-1	FBgn0022160	Glycerol-3-phosphate dehydrogenase activity, calcium ion binding	0.000209	***
INTERPRO, GOTERM_MF/BP/CC_DIRECT	Fatty acyl-CoA reductase, peroxisome	2.47	wat	FBgn0039620	Long-chain-fatty-acyl-CoA reductase activity, oxidation-reduction process	1.21E-06	***
			Spat	FBgn0014031	Amino transferase activity, glyoxylate catabolic process, proxisome	2.02E-02	*
			Cat	FBgn0000261	Catalase, antioxidant activity and ROS metabolic process	1.15E-02	*
			Uro	FBgn0003961	Urate oxidase activity, oxidation-reduction process, peroxisome	1.96E-02	*
			Acox57D-d	FBgn0034629	acyl-CoA dehydrogenase activity, fatty acid beta-oxidation, peroxisome	4.63E-05	***
GOTERM_MF/BP/CC_DIRECT, INTERPRO	Oxidation-reduction, heme binding, iron ion binding, Cytochrome P450	2.2	Men	FBgn0002719	Malic oxidoreductase, determination of adult lifespan	5.16E-04	***
			Cyp6a23	FBgn0033978	Cytochrome P450, heme and iron ion binding, oxidation-reduction process	4.93E-01	NS
			Cyp4p1	FBgn0015037	Cytochrome P450, heme and iron ion binding, oxidation-reduction process	7.98E-02	.
			Cpr	FBgn0015623	NADPH-hemoprotein reductase activity, oxidation-reduction process	2.27E-04	***
			Cyt-b5-r	FBgn0000406	Heme binding, oxidoreductase activity, lipid metabolic process	2.69E-04	***
			antdh	FBgn0026268	Oxidoreductase activity, oxidation-reduction process	4.85E-02	*
			AOX1	FBgn0267408	Aldehyde oxidase, pyridoxal oxidase activity	5.82E-03	**
			Cyt-b5	FBgn0264294	Cytocrome b5-like heme-binding site	6.47E-04	***
			Cyp313a1	FBgn0038236	Cytochrome P450, heme and iron ion binding, oxidation-reduction process	1.45E-02	*
			mt:CoI	FBgn0013674	Mitochondrial Cytochrome C oxidase, heme and iron binding	7.39E-02	.
			Aldh	FBgn0012036	Aldehyde dehydrogenase, oxidation-reduction	9.39E-03	**
			Cyp4e3	FBgn0015035	Cytochrome P450, heme and iron ion binding, oxidation-reduction process	1.27E-05	***
			hgo	FBgn0040211	Homogentisate 1,2-dioxygenase activity, oxidation-reduction process	4.08E-03	**
			Pdh	FBgn0011693	Retinol and alcohol dehydrogenase activity, oxidation-reduction process	4.75E-03	**
			Desat1	FBgn0086687	Fatty acid desaturase, oxidation-reduction process	2.55E-05	***
			Plod	FBgn0036147	Iron ion binding, oxidoreductase activity, oxidation-reduction process	6.75E-04	***
			Fad2	FBgn0029172	Fatty acid desaturase, oxidation-reduction process, lipid metabolic process	1.15E-03	**

			Gpdh	FBgn0001128	Carbohydrate metabolic process, oxidation-reduction process	6.13E-04	***
GOTERM_BP/CC/MF_DIRECT	Calcium ion channel, transmembrane transport	2.13	trpl	FBgn0005614	Calcium channel activity, calcium ion transport	2.13E-02	*
			trp	FBgn0003861	Plasma membrane cation channel, calcium channel	3.06E-09	***
GOTERM_BP/CC/MF_DIRECT	Voltage-gated potassium channel activity	2.03	Irk3	FBgn0032706	Potassium channel activity, potassium ion transport	3.88E-02	*
KEGG_PATHWAY, INTERPRO, GOTERM_BP/CC/MF_DIRECT	Fatty acid metabolism/biosynthetic process	1.93	ACC	FBgn0033246	Acetyl-CoA carboxylase activity, fatty acid biosybthetic process	3.44E-03	**
			Cyt-b5-r	FBgn0000406	Cytochrome b5-like heme binding, lipid metabolic process	6.47E-04	***
			Desat1	FBgn0086687	Fatty acid desaturase, oxidation-reduction process	2.55E-05	***
			Fad2	FBgn0029172	Fatty acid desaturase, oxidation-reduction process, lipid metabolic process	1.15E-03	**
			Acsl	FBgn0263120	Long-chain fatty acid-CoA ligase activity	1.03E-04	***
INTERPRO, GOTERM_MF_DIRECT	Carbohydrate binding	1.91	Clect27	FBgn0031629	Carbohydrate binding	4.40E-11	***
			lectin-37Db	FBgn0053533	Galactose binding	2.68E-04	***
INTERPRO, GOTERM_MF_DIRECT	Potassium ion transport, oxalate transmembrane transporter activity	1.65	Irk3	FBgn0032706	Potassium channel activity, potassium ion transport	3.88E-02	*
			Irk2	FBgn0039081	Potassium channel activity, potassium ion transport	1.96E-08	***
			Dic1	FBgn0027610	Inorganic phosphate transmembrane transporter activity	3.33E-05	***
GOTERM_MF/BP/CC_DIRECT, INTERPRO	Neurotransmitter-gated ion-channel ligand binding	1.45	Cals	FBgn0039928	Calcium ion binding	2.29E-02	*
GOTERM_MF/BP/CC_DIRECT, INTERPRO, COG_ONTOLOGY	Receptor activity, carboxylesterase, lipid metabolism	1.35	alpha-Est7	FBgn0015575	Carboxylic ester hydrolase activity, lipid storage, determination of lifespan	2.49E-04	***
			ACC	FBgn0033246	Acetyl-CoA carboxylase activity, fatty acid biosybthetic process	3.44E-03	**
			Gs2	FBgn0001145	Glutamate catabolic process, neurotransmitter receptor metabolic process	1.46E-03	**
			Est-6	FBgn0000592	Carboxylic ester hydrolase activity	2.93E-02	*
			AcCoAS	FBgn0012034	Acetyl-CoA ligase activity	3.35E-04	***
GOTERM_MF/BP/CC_DIRECT, INTERPRO	Neurotransmitter transporter	1.32	NAAT1	FBgn0029762	Neurotransmitter transporter activity	2.06E-08	***
			Eaat1	FBgn0026439	Glutamate:sodium symporter activity, determination of adult lifespan	2.06E-02	*

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