Mate choice and gene expression signatures associated with nutritional adaptation in the medfly (*Ceratitis capitata*)

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Abstract

Evolutionary responses to nutrition are key to understanding host shifts and the potential for reproductive isolation. Previously, experimental evolution was used to describe responses of the medfly (*Ceratitis capitata*) to divergent larval diets. Within 30 generations there was divergence in larval development time, egg to adult survival and adaptation in adult body size. In this study, the gene expression signatures associated with these changes were examined, using mRNA-seq on males following 60 generations of nutritional selection. Variation in gene expression was also validated using qRT-PCR. The results showed observed an over-representation of differential expression in metabolism, oxidative phosphorylation and proteolysis genes. In addition, at generations 60 and 90, we tested whether these evolved patterns (differences in gene expression) were associated with mate choice. We found evidence for assortative mating by diet at generation 60, but not in mating tests within and between replicate lines at generation 90. Hence, there was no consistent evidence for assortative mating by diet, which emphasises the importance of replicate tests of evolutionary responses over time. Overall, the study provides the first genome-wide survey of the putative mechanisms underpinning evolved responses to nutritional adaptation.

Introduction

36 Adaptation to different nutritional ecologies can play key roles in successful expansion into
37 new habitats as well as in reproductive divergence associated with host shifts with the
38 potential to result in reproductive isolation (RI) (Schluter 2000; Coyne and Orr 2004;
39 Rundle and Nosil 2005). For example, in *Rhagoletis* fruit flies (Feder et al. 1994) genetic
40 divergence between populations is associated with host plant shifts. If the host shift occurs
41 in sympatry, this can lead to the subsequent formation of host races (Linn et al. 2003;
42 Nosil et al. 2012; Powell et al. 2014; Soria-Carrasco et al. 2014). This local adaptation and
43 host race formation can have a significant impact on traits associated with fitness on the
44 new host and also, potentially, RI (Feder et al. 1994; Dambroski and Feder 2007; Nosil
45 2007; Nosil et al. 2012). However, much is still unknown concerning the instigation, and
46 underlying mechanisms, of nutritional adaptation (Panhuis et al. 2001; Maan and
47 Seehausen 2011; Butlin et al. 2012; Safran et al. 2013).

48 Mechanistic insights into nutritional adaptation can be gained from next generation
49 sequencing (NGS), which is revolutionizing the study of natural selection (Seehausen et al.
50 2014), adaptation (Elmer and Meyer 2011; Savolainen et al. 2013) and the initial steps of
51 speciation (Nosil 2012). The characterization of patterns of gene expression across
52 genomes offers new opportunities for the study of the putative causal links between the
53 differences in gene expression and adaptive genetic divergence (Pavey et al. 2010). For
54 example, populations of *Drosophila mojavensis* from different regions of the Sonoran
55 desert exhibit premating isolation, mediated by cuticular hydrocarbon profiles (CHCs) and
56 courtship song, based on the cactus host they utilise for reproduction (Etges et al. 2007,
57 2009; Etges 2014). Transcriptomic studies have identified a small suite of metabolic,
58 olfactory and behavioural trait genes whose expression is directly influenced by the host
59 food plant (Matzkin 2012; Rajpurohit et al. 2013; Etges et al. 2015). NGS methods can
60 also be combined with experimental evolution for additional insights into divergence (Rice
61 and Hostert 1993; Kawecki et al. 2012) particularly via '*evolve & resequence'* techniques
62 (Schlötterer et al. 2015). By imposing selection on a particular trait or suites of traits,
63 experimental evolution studies can reveal adaptive responses to selection in real time
64 (Remolina et al. 2012).

65 Here we used mRNA-seq to capture the effects of selection mediated by dietary
66 adaptation on protein-coding gene expression in an experimental evolution framework.
67 Experimental evolution was conducted in the medfly (*Ceratitis capitata*), a global pest of
68 significant agricultural importance, under two divergent larval dietary treatments, to

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69 characterise the responses to nutritional selection (Leftwich et al. 2017) and to describe
70 the underlying changes in expression associated with these responses.

71   The medfly is an extreme generalist and exhibits wide plasticity in host selection,
72 utilization (Levinson et al. 1990; Yuval and Hendrichs 2000) and oviposition (Prokopy et al.
73 1984; Yuval and Hendrichs 2000). Larvae are viable in a wide range of fruits and diets,
74 from both inside and outside of the known host range (Carey 1984; Krainacker et al. 1987;
75 Zucoloto 1993; Nash and Chapman 2014). This highly plastic host choice behaviour is
76 evident in reports that the medfly can infest over 350 different host fruits (Liquido et al.
77 1991). As such, the medfly is of huge economic importance as an agricultural pest. Its
78 ability to exhibit strong plasticity (Carey 1984; Nyamukondiwa et al. 2010) is thought to
79 have facilitated its global invasion (Gasperi et al. 2002; Malacrida et al. 2007). Global
80 populations of medfly vary in a wide range of demographic (Diamantidis et al. 2008a,b,
81 2009, 2011a) and behavioural traits (Briceño et al. 2002, 2007), as well as in genetic
82 structure (Gasperi et al. 2002; Karsten et al. 2015). However, unlike for some Drosophilid
83 species (e.g. Dodd 1989; Rundle et al. 2005), there are as yet no reports of mating
84 isolation between different strains exhibiting different diets (Cayol et al. 2002). In general,
85 mechanisms influencing nutritional plasticity are recognised as key to understanding
86 nutritional adaptation. However, as yet we know little about the underpinning genetic basis
87 and regulatory interactions. This is the key gap investigated in this study.

88   Experimental evolution was practised on two sets of three independent evolutionary
89 replicates, each selected on different larval diets with different nutritional complexity and
90 caloric value (Leftwich et al. 2017). Within 30 generations, this dietary selection led to
91 divergence in larval development time and egg to adult survival and the emergence of
92 local adaptation in adult body size (Leftwich et al. 2017). In the current study, we
93 investigated the evolved gene expression signatures associated with this divergence
94 following 60 generations of selection. We used mRNA-seq to compare gene expression
95 between sexually mature males, following rearing for 2 generations on a common garden
96 diet. To capture tissue specificity, we profiled the Head/Thorax and Abdomen separately.
97 Evolved gene expression patterns were highly replicable, 214 transcripts were identified as
98 differentially expressed (DE) between the dietary treatments. DE was observed in a suite
99 of genes involved in nutrient metabolism, oxidative phosphorylation (OXPHOS), and
100 proteolysis. Nutritional adaptation was associated with replicable but inconsistent
101 differences in mating preferences at generations 60 and 90, suggesting no underlying

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102 assortative mating by diet as a by-product of adaptation to divergent selection on larval
103 diets.

104

105 *Materials and Methods*

106

107 **EXPERIMENTAL EVOLUTION LINES**

108 The experimental evolution lines were derived from the TOLIMAN wild type (originating
109 from Guatemala and reared in the laboratory since 1990; Morrison et al. 2009). For at least
110 two years prior to the start of these experiments the TOLIMAN strain was reared on a
111 wheat bran diet (24% wheat bran, 16% sugar, 8% yeast, 0.6% citric acid, 0.5% sodium
112 benzoate). To initiate the experimental evolution, flies were established on (i) sucrose-
113 based 'ASG' medium (1% agar, 7.4% sugar, 6.7% maize, 4.75% yeast, 2.5% Nipagin
114 (10% in ethanol), 0.2% propionic acid, 684kcal/L) or (ii) 'Starch' (S) medium (1.5% agar,
115 3% starch, 5% yeast, 0.4% propionic acid, 291 kcal/L) (Leftwich et al. 2017). Three
116 independent biological replicates of each of the two regimes were maintained under
117 allopatry. All experiments and culturing was conducted at 25°C, 50% relative humidity, on
118 a 12:12 light dark photoperiod. Adults emerging from each replicate were maintained in
119 groups of approximately 30 males and 30 females in plastic cages (9cm x 9cm x 9cm).
120 Adults from all lines received the same standard adult diet (*ad libitum* access to sucrose-
121 yeast food; 3:1 w/w yeast hydrolysate: sugar paste and water). Each generation,
122 approximately 500 eggs were placed on 100ml of the appropriate diet in a glass bottle.
123 When third instar larvae started to 'jump' from the larval medium, the bottles were laid on
124 sand and pupae allowed to emerge for seven days. Pupae were then sieved from the sand
125 and held in 9mm petri dishes until eclosion of adults began and the next generation was
126 initiated.

127

128 **IDENTIFICATION OF GENE EXPRESSION SIGNATURES OF NUTRITIONAL**
129 **ADAPTATION USING MRNA-SEQ**

130 Eggs were derived from the ASG (A) and Starch (S) experimental evolution lines at
131 generation 60 and reared on a common garden glucose (CG) diet (1.5% agar, 3%
132 glucose, 5% yeast, 0.5% propionic acid) under standard conditions and density for two

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133 generations. Adults emerging from daily collected cohorts of pupae were sex-sorted and

134 reared in single sex cages until seven days post eclosion. 17 - 30 male flies were then

135 flash frozen in liquid N_2 30 minutes after lights on (09:30), in Eppendorf tubes, in groups of

136 10 - 15. These samples were then stored at -80°C until RNA extraction.

137 **RNA EXTRACTION AND MRNA-SEQ**

138 Prior to RNA extraction, males were split into Head/Thorax (HT), and Abdomen (Ab), over

139 dry ice. Total RNA was extracted from samples of 17 - 22 body parts pooled within each

140 replicate of each dietary treatment using the *mir*Vana kit (Ambion), according to the

141 manufacturer's instructions. 5μg (>200ng/μl) of Total RNA from male flies was submitted

142 for sequencing. mRNA-seq was conducted (BaseClear provider) on libraries prepared

143 using the Illumina TruSeq protocol, following polyA selection for mRNA. Sequencing was

144 single-end, 50 cycles on the Illumina HiSeq 2500 platform (Rapid Mode).

145 **BIOINFORMATICS**

146 *Quality control:* Initial quality control was performed on original FASTQ files. FASTQ files

147 were then converted into FASTA format. Reads containing Ns (< 1%) were discarded. The

148 set of redundant reads was collapsed into non-redundant format and the complexity (ratio

149 of non-redundant reads to redundant reads) calculated (Mohorianu et al. 2011, 2017a). To

150 evaluate the extent of the sequencing bias, the per base nucleotide composition was

151 determined. The proportion of reads incident to the reference genome and available

152 annotations was calculated before and after normalization of expression levels, to ensure

153 that the normalization targeted technical inter-sample variability without altering the

154 biological features present (Mohorianu et al. 2017a).

155

156 *Read mapping and normalisation:* The mapping of all reads was done using PaTMaN

157 (Prüfer et al. 2008), full length, with no gaps allowed and with maximum 1 mis-match at

158 any position along the read. For this analysis, we used the Ccap1.1 version of the *Ceratitis*

159 *capitata* genome and curated *C. capitata* transcripts downloaded from NCBI (for rRNAs

160 and protein coding genes). rRNA-reads were excluded from all samples and we then

161 sampled the filtered reads to a fixed total of 29M (the minimum sequencing depth, post

162 filtering), using subsampling (without replacement) (Mohorianu et al. 2017a). Gene

163 expression was calculated as the algebraic sum of the abundances of incident reads. Any

164 remaining variability in the expression matrix was then minimised by using quantile

165　normalisation (Bolstad et al. 2003). The proportion of reads incident to the mRNA
166　annotations corresponding to v1.1 of the *C. capitata* genome was significantly lower than
167　the proportion of genome matching reads. Hence, we used curated *C. capitata* transcripts
168　downloaded from NCBI, which yielded comparable proportion of transcript- and genome-
169　matching reads.

170　*Differential expression analysis:* We first investigated differential expression (DE) between
171　replicates, using a $\log_2$ offset fold change (OFC, with empirically-determined offset of 20
172　(Mohorianu et al. 2017a) to filter out low level noise. Transcripts with a normalized
173　abundance of <100 were excluded. The DE analysis was using a hierarchical approach on
174　two levels (Mohorianu et al. 2017b): (i) body part (tissue) (HT/Ab) and (ii) dietary treatment
175　(A/S). The DE call between treatment types was made on maximal confidence intervals
176　(CIs) and genes with a $\log_2(OFC) > 1$ (corresponding to a 2-fold change difference) were
177　called DE.

178

179　*Annotation and functional description:* To expand the annotation information, DE
180　transcripts were matched to their corresponding *Drosophila melanogaster* homologues
181　using tBLASTx (Altschul et al. 1990). The similarity threshold for matching was alignment
182　length > 50nt, with similarity > 50%. The similarity threshold following matching was
183　alignment length > 50nt, with similarity > 50%. The tissue-specific gene lists resulting from
184　this were tested for functional enrichment using g:Profiler (Reimand et al. 2016) using all
185　*D. melanogaster* genes as the background set.

186

187　**QRT-PCR VALIDATION**

188　Candidates for validation were chosen based on their expression levels (>200 normalized
189　expression) and to cover representative GO categories. Presence plots were used to
190　enhance primer design (i.e. to avoid primers spanning exon/exon boundaries). We first
191　assessed the stability of expression of 3 reference genes (Scolari et al. 2012; Koramutla et
192　al. 2016) in our RNA-seq data. We selected 3 reference genes *(CcRpL13a, CcRpL27, poll)*
193　based on a circular permutation test, high read abundance (> 500,000 reads in each
194　sample, in both body parts) and by assessing the consistency of expression between
195　tissues (|DE| <= 0.0001) and treatments. We then designed primers (using primerBLAST
196　(Ye et al. 2012)) and checked, using PatMaN (Prüfer et al. 2008) for potential multiple

197 matches to other genomic locations (primer details in Table S1). We chose 15 candidate
198 genes of interest (GOIs) for validation.

199   For primer optimisation we used mRNA extracted from whole wild type flies. We
200 removed DNA using TURBO™ DNase (Life Technologies) on 2 μg total RNA. DNase was
201 deactivated using the resin in the TURBO™ DNA-free kit (Life Technologies) and all
202 samples were tested for DNA contamination with no-reverse transcription controls. The
203 QuantiTect Reverse Transcription Kit (Qiagen) was used to reverse transcribe 1 μg total
204 RNA to cDNA, which was then stored at -20 °C. qRT-PCRs were run using a
205 StepOnePlus™ machine (Life Technologies) using iTaq Universal SYBR® Green Supermix
206 with triplicate technical replicates using 10 ng RNA as template in 20 μl reactions in
207 MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode, 0.1 ml (Life Technologies).
208 The qRT-PCR conditions were 95 °C for 30 seconds followed by 40 cycles of 95 °C for 15
209 seconds and 60 °C for 1 minute and data acquisition. Following the qRT-PCR we ran a
210 melt curve analysis (on default settings). All samples showed a single peak on the melt
211 curve. We included a no template control for each GOI on each plate. All primers were
212 used at a final concentration of 5 μM. For primer optimisation (Eurofins MWG Operon) we
213 produced standard curves using at least five 1:5 dilutions of RNA starting at 50 ng cDNA.
214 Samples where no signal was detected during qRT-PCR were given a nominal CT value of
215 40, to allow statistical testing. The difference in $2^{-\Delta CT}$ between treatments was tested using
216 a two-sample t-test for each GOI.

TESTS FOR ASSORTATIVE MATING BY DIET

218 We conducted mate choice tests at generation 60 and 90. Eggs were seeded onto a
219 common garden glucose diet two generations prior to testing (glucose diet: 1.5% agar, 3%
220 glucose, 5% yeast, 0.5% propionic acid). All mating tests were conducted simultaneously.
221 At generation 90, we conducted the same mate choice tests but in addition, examined
222 tests between replicates within each dietary treatment. Flies were sorted by sex within 24
223 hours of eclosion to ensure virginity. Experimental flies were reared in standard 0.8L
224 rearing cages. To enable identification, one male and one female from each population in
225 each mating test were marked with a spot of red paint on the dorsal side of the thorax.
226 Treatments were fully controlled for handling effects and paint marking.

227 *Assortative mating assays:* A multiple-choice design was employed in order to maximise
228 the opportunity for mate choice. Five days post eclosion and 48 hours prior to the mating
229 tests, 25 females of each of the two treatments, and similarly 25 males of each treatment,

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230   were placed into two, single sex, 0.8L rearing cages. The two cages were connected via a

231   sliding door. Both cages were supplied with *ad libitum* 3:1 sugar yeast hydrolysate diet and

232   water. Mating tests were conducted when the flies were 7-8 days post eclosion, with sexes

233   and treatments exactly balanced for age composition. Mating tests were initiated at 09:30,

234   30 minutes after lights on, by slowly raising the sliding door. Mating pairs were gently

235   removed and placed in numbered 1.5ml eppendorf tubes for later identification. Three

236   replicates were conducted on each test day, starting at 30 min intervals. The order in

237   which the replicate pairs were tested was alternated, to control for effects of different start

238   times. Each assay continued until 25 mated pairs had been collected, or until 30 minutes

239   had elapsed. The collection of 25 pairs amounted to half of the total population in the

240   cages. Therefore, any effect of diminishing choice due to removal of flies from the cage

241   was minimized (Casares et al. 1998). At generation 60, four replicates were conducted for

242   each combination of tests. At generation 90, five replicates each of 16-25 staring

243   individuals of each sex per line were tested for the own diet and common garden mating

244   tests. For the within line mating tests, four replicates of 25 starting individuals of each sex

245   were tested for the A v A mating assays, and three replicates of 19-25 individuals of each

246   sex per line were tested for S v S. The identity of both individuals in each mating pair was

247   recorded. As only 50% of matings were sampled, mating pairs were treated as

248   independent (Casares et al. 1998; Coyne et al. 2005) and results were pooled by line

249   replicate prior to further analysis.

250

251   **STATISTICAL ANALYSIS**

252   The number of observed and total possible pairings for each pair type was calculated.

253   These raw data were then analysed using JMATING v1.0 (Carvajal-Rodriguez and Rolan-

254   Alvarez 2006) to calculate descriptive coefficients based on the cross product estimator of

255   isolation (Rolán-Alvarez and Caballero 2000). I_{PSI}, a joint isolation index, varying from -1 to

256   1 (with +1 being total assortative mating, -1 total dissasortative mating and 0 random

257   mating; Coyne et al. (2005)) was used to describe total isolation. The PTI coefficient

258   described positive and negative preferences for mating pairs within each line pair, at each

259   time point. An index of mating asymmetry (IA_{PSI}) captured the difference in frequency

260   between homotypic and heterotypic pairs (Rolán-Alvarez 2004). Values of IA_{PSI} centre

261   around 1 (no asymmetry), with values below one reflecting asymmetry towards the first

262   pair type, and > 1 representing asymmetry towards the second. Finally, we calculated *W*,

263   the cross product estimator of sexual selection, to compare the sexual fitness of males and

264 females (Carvajal-Rodriguez and Rolan-Alvarez 2006) and to give the relative fitness of
265 each treatment in comparison to the fittest treatment within each line pairing. The
266 significance of PSI, PSS, and PTI coefficients was determined as the bootstrap probability
267 of rejecting the null hypothesis of random distribution, after 10,000 iterations of resampling
268 (Rolán-Alvarez and Caballero 2000). When applied to the isolation index (I_{PSI}), the
269 asymmetry index (IA_{PSI}), and the estimator of sexual selection (W), this is the two-tail
270 bootstrap probability that the value is significantly different from one (i.e. random mating,
271 or zero asymmetry). To test for the overall effect of diet in RI as indicated by I_{PSI}, IA_{PSI} or
272 W, probability values for each line replicate comparison were combined using Fisher's sum
273 of logs method, implemented with the 'metap' package in R (Dewey 2016). All other data
274 handling and statistical analysis was conducted in R (R Development Core Team 2015,
275 ver 3.1.1).

276

Results

278 **MRNA-SEQ**

279 *Quality control:* Raw FASTQ files contained between 34.3M and 53.2M reads. Following
280 conversion to FASTA format, we accepted > 99% reads for every sample (Table S2).
281 Other initial checks, such as the nucleotide composition across reads, revealed the
282 expected RNA-seq bias, which was consistent for all samples (Mohorianu et al. 2017a).
283 Next, to optimize the alignment step, the files were transformed from redundant to non-
284 redundant format, yielding between 7.6M and 10.2M unique (non-redundant) reads, with a
285 complexity of between 0.192 and 0.23. The tight range of complexities indicated a reliable
286 sequencing output. The variation in complexities observed between replicates, derived
287 primarily from variation in sequencing depth, suggested that a subsampling normalization
288 would be appropriate.

289 *Genome matching and normalisation of expression levels:* First, rRNA mapping reads
290 were excluded. Next, the resulting reads were subsampled at a fixed total (29M). The non-
291 redundant read count varied between 7M and 8.2M reads, with complexity between 0.235
292 and 0.277, showing that the variation in complexity was reduced following normalization
293 (Table S2). On the normalized data, the proportion of reads matching to the 'Ccap1.1'
294 genome was: redundant 80 to 83%, non-redundant 77%. The proportion of redundant
295 reads incident to the genome was in line with the results observed for the original data,
296 suggesting that the normalization successfully minimised technical variability. The

10

297 proportion of reads matching to Ccap NCBI transcripts was: redundant 75 to 83%, non-
298 redundant 75 to 79% (Table S2). This was similar to the proportion of genome matching
299 reads, a result that was expected as polyA selection was used for the sequencing of the
300 mRNA fragments. As the Ccap NCBI transcript annotations provided a similar proportion
301 of R / NR mapping reads to the reference genome, this dataset was used for the
302 subsequent analysis.

IDENTIFICATION OF DIFFERENTIALLY EXPRESSED PROTEIN-CODING GENES

304 The normalisation provided comparable distributions of expression across samples, with
305 good agreement between replicates throughout (Fig. S1). The amplitude and frequency of
306 DE (Table S3A-D) between tissues (black line, Fig. S2) was greater than for diets
307 treatment (red line, Fig. S2) showing the hierarchical order of levels within the distribution
308 of DE. A total of 359 transcripts showed DE between treatments (Table 1), with 194 being
309 expressed higher in Starch flies, and 161 higher in ASG. These transcripts were matched
310 to *D. melanogaster* homologues using tBLASTx, which yielded a total of 94 matching
311 genes, 29 in expressed in the HT tissue and 65 in Ab. Tests for functional enrichment of
312 this set highlighted 3 enriched GO terms and 1 KEGG pathway, associated with 15 *C.*
313 *capitata* transcripts (Fig. 1).

314

DIFFERENTIAL EXPRESSION IN THE HEAD+THORAX

316 Of the 122 transcripts that yielded a signature of DE in the HT, 43 were expressed at
317 higher levels in ASG flies, and 75 higher in Starch (Table S3A, B). 29 of these were
318 matched to *D. melanogaster* identifiers following tBLASTx searching. Functional
319 enrichment did not return any significant terms. After removal of duplicates present due to
320 isoform variation, 65 DE transcripts remained (Table S3B). Of these, 22 bore informative
321 NCBI predicted annotations and 9 were predicted as uncharacterised loci. As examples
322 from this set, homologues for the *D. melanogaster* serine protease genes γTry and
323 CG34458, as well as cytochrome P450 genes Cyp6a9 and Cyp6a21 were expressed at
324 higher levels in Starch flies. Consistent with this, several transcripts predicted to be serine
325 proteases also showed higher expression in Starch flies (e.g. XM_004517825.1,
326 XM_004517721.1). Homologues of the developmentally associated larval cuticle protein
327 genes Lcp3 and Lcp56Ab1 were expressed at higher levels in ASG flies. The functional
328 significance of the enrichment for serine proteases in Starch and cuticle genes in ASG flies
329 is not yet clear.

DIFFERENTIAL GENE EXPRESSION IN THE ABDOMEN

237 transcripts were DE in abdomen, with 151 expressed higher in Starch flies and 86 higher in ASG (Table S3C, D). Following tBLASTx, 65 *D. melanogaster* identifiers were retrieved. Functional enrichment of these revealed significant overrepresentation for genes involved in the molting cycle (GO-BP:0042303, *P* = 0.0432) and apical constriction (GO:0003383, *P* = 0.0493). Additional enrichment was observed in the molecular function GO term serine-type endopeptidase activity (GO:0004252, *P* = 0.0399) and in genes associated with the oxidative phosphorylation KEGG pathway (ko00190, *P* = 0.0495; Fig. 1). The biological process (BP) 'molting cycle' was associated with 5 *C. capitata* genes. Of these, the majority were expressed at a higher level in Starch flies (Fig. 1). However, a single *C. capitata* gene (LCP5) showed the opposite pattern and was expressed at higher levels in ASG flies. This pattern of LCP5 expression was also seen in the HT for ASG flies. Of the five genes enriched for molting cycle, 3 were also enriched for apical constriction. These genes were all expressed at higher levels in Starch than in ASG flies. For all three of these transcripts there were no *C. capitata* annotations, and hence these genes are referred to by their *D. melanogaster* identifiers in Fig. 1. The majority of genes associated with a functional enrichment were serine-like peptidases. 7 *C. capitata* transcripts were associated with this GO term, and exhibited a bidirectional expression pattern, with 4 expressed at higher levels in Starch than in ASG, and the remaining 3 showing the opposite pattern. The final functional enrichment information came from 3 *C. capitata* transcripts that were associated with the KEGG pathway describing oxidative phosphorylation, these were all expressed at higher levels in Starch flies.

After duplicates were removed, 152 abdomen transcripts showing DE remained (Table S3B). Of these, 53 were associated with *Ceratitis capitata* annotations or predicted annotations and 21 were predicted as uncharacterised *C. capitata* loci. Within this set were 7 genes with *D. melanogaster* homologues that were functionally enriched for serine peptidase activity (up in ASG: *CG3916, CG32808, Ser6*; up in Starch: *lint, CG34458, γTry, Jon65Aiii*). Further to this enrichment, another *D. melanogaster* gene with a serine endopeptidase domain (CG6800) was DE, with higher expression in ASG abdomens. 4 transcripts with predicted annotations as *C. capitata* serine proteases were also called DE (up in ASG: GAMC01001732.1; up in Starch: XM_004517721.1, XM_004517825.1, XM_012306933.1). 5 *C. capita*ta transcripts with *D. melanogaster* homologues showed functional enrichment for the molting cycle (*Cht6, Lcp65Ab1* (*C. capitata* LCP5), *mey, neo, nyo*). Three of these (mey, neo, nyo) also showed enrichment for apical constriction. 3 *C.*

364 *capitata* transcripts with *D. melanogaster* homologues contributed to the enrichment of the
365 oxidative phosphorylation KEGG pathway (*SdhA, Vha100-2, Vha100-4*).

366   Beyond the transcripts described by functional enrichment, there was a wide range
367 of additional *C. capitata* transcripts with *D. melanogaster* homologues showing DE. Of
368 particular interest was a transcription factor associated with behaviour in *D. melanogaster*
369 (*acj6*), which is associated with odorant receptor gene expression, as well as another
370 transcription factor (CG15073). Given the dietary selection imposed, it was also interesting
371 to observe DE in *ppk28*, a gene involved in response to water and osmoregulation in *D.*
372 *melanogaster* (Cameron et al. 2010) and in Ent2, with reported roles in stress / stress
373 signalling and associative learning in *D. melanogaster* (Knight et al. 2010). Predicted
374 annotations of several of the *C. capitata* transcripts lacking *D. melanogaster* homologues
375 was also interesting as within this set there were one odorant receptor (XM_004534136.1
376 (LOC101450382, Odorant receptor 67a-like)) and one odorant binding protein
377 (XM_004521128.2 (Obp99a)) and the *C. capitata* gene CYP4E6 (AF028819.1), a
378 cytochrome P450 associated with the processing of extrinsic and intrinsic metabolites
379 (Danielson et al. 1999).

380 **VALIDATION BY QRT-PCR**

381 Variation in gene expression as determined by mRNA-seq was validated using qRT-PCR
382 (Table S1, S4). There was extremely close agreement between qRT-PCR and mRNA-seq
383 expression. The pattern of gene expression was the same in all replicates of all 15 genes
384 of interest (GOI) tested (Fig. S3-S17; Table S4) except in one case (GAMC01008957.1,
385 AG1HT/SG1HT, Fig. S10). Statistical testing showed significant ($P < 0.05$) differences in 2^-
386 $^{\Delta CT}$ values between ASG and Starch flies in 14 out of 20 tests (Table S4). Within the set of
387 validated GOIs, genes matching *D. melanogaster* homologues, such as SER1
388 (GAMC01003520.1) and TRY7 (GAMC01004081.1) were also validated. Overall, the qRT-
389 PCR experiments provided a strong validation of the bioinformatics analysis of the mRNA-
390 seq data, with 119 out of 120 qRT-PCR tests showing the same direction and/or
391 magnitude as the mRNA-seq.

392 **MATE CHOICE TESTS**
393 At generation 60, homotypic pairs predominated (Fig. 2, Table S5) and S males mated
394 most frequently (Fig. 2, Table S5). However, at generation 90 these patterns were no
395 longer evident. The results are described in more detail, below.

396 *Sexual isolation (PSI), sexual selection (PSS) and total isolation (PTI) indices:* At
397 generation 60, the mating tests for individuals tested on their own diets showed significant
398 deviations from random PTI (Table S6, S7) driven by significant deviations from random
399 PSI (Table S6, S7). There was a clear pattern for homotypic (diet assortative) pairings to
400 exhibit PSI > 1, and heterotypic (diet dissasortative) pairings to show negative values. The
401 resulting PTI was significantly >1 for 'AA' mating type pairs in 2/3 replicates. Also, PTI
402 coefficients for the 'AB' mating pair types were significantly < 1 for all lines (0.44 - 0.52).
403 When tested in the common garden, the patterns were similar. In 2/3 replicates (Table S6,
404 S8) the common garden environment removed any significant effect of treatment on PSI.
405 PSS showed no significant differences from one in 2/3 replicates. PTI was significantly < 1
406 for 'BA' mating types in all line replicates (0.4 - 0.64). Hence at generation 60 there was
407 evidence for significant assortative mating by diet in both the 'on diet' and common garden
408 tests. A competitive advantage of ASG males was evident 'on diet', mediated by sexual
409 selection. The maintenance of the assortative mating pattern in the common garden
410 suggested that genetic adaptation within the lines according to their different environments
411 resulted in the evolution of incipient divergence.

412 At generation 90, mating tests on individuals tested on their own diets again
413 showed significant deviations from random PTI (Table S6, S9) driven by significant
414 deviations from random PSI in a single replicate (Table S9). However, all line replicates
415 showed significant deviations from random PSS (Table S9), indicating no recapture of the
416 assortative mating by diet that was observed at generation 60. This lack of replication of
417 the earlier pattern was also apparent when flies from generation 90 were tested after
418 rearing on a common garden diet, with only a single line showing any significant deviation
419 from random PTI, PSI or PSS (Table S6, S10), and as this effect was not driven by any
420 deviation from random PSI, it was not likely associated with assortative mating by diet. At
421 generation 90 the line replicates were also tested against each other within their dietary
422 regimes, to test directly for potential confounding effects of genetic drift on any sexual
423 isolation observed. Within the ASG regime, significant deviation from random PTI was
424 seen in only one replicate (Table S6, S11) and again was driven by significant deviation in
425 PSS and not PSI, suggesting a lack of assortative mating. In the Starch regime there was
426 no significant deviation from PTI (Table S11).

427 *Analysis of isolation - I_{PSI} - Isolation index:* At generation 60, all lines showed significant
428 assortative mating when tested on their own (X^2 = 27.03, d.f. = 6, P < 0.001) and on the
429 common garden (X^2 = 19.7, d.f. = 6, P = 0.003) diets, (Table S12). However, at generation

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430 90 this signature of assortative mating was not observed in own or common garden diets

431 (Fishers Combined test, X^2 = 5.3, d.f. = 6, P = 0.51). When lines within dietary regimes

432 were tested, there was no significant signature of isolation for the ASG regime (X^2 = 7.5,

433 d.f. = 6, P = 0.26) but a significant isolation index in Starch (X^2 = 19.3, d.f. = 6, P = 0.004).

434 *Asymmetry in matings - IA_{PSI} - Asymmetry index:* IA_{PSI} indicated any asymmetry in the

435 proportion of homo- versus heterotypic matings in the own diet tests. In generation 60,

436 IA_{PSI} was consistently significantly < 1 for heterotypic pairs ('BA' mating type), showing a

437 consistent effect of diet across replicates (Fisher's X^2 = 23.22, d.f. = 6, P < 0.001). The

438 frequency of heterotypic pairs was skewed towards BA type matings, i.e. those comprising

439 S females and ASG males. No significant asymmetry between the proportions of homo-

440 versus heterotypic matings was observed in generation 90.

441 *Divergence in W - Cross product estimator of sexual selection:* In the final analysis we

442 calculated *W,* fixed at one for the fittest individual of each sex. There was no significant

443 difference between treatments in female specific *W* at any generation 60. However, *W* for

444 S males was lower than for ASG males in generation 60 (Fishers X^2 = 24.28, d.f. = 6, P <

445 0.001). This pattern was also recovered in generation 90 (X^2 = 15.75796, d.f. = 6, P =

446 0.015). However, ASG females also had significantly a significantly higher *W* than S

447 females across all replicates.

448

449 *Discussion*

450 We observed evolved differences in the expression patterns of genes involved in

451 metabolism, development and OXPHOS in experimentally evolved populations of the

452 medfly subjected to divergent nutritional selection. These differences underlie the

453 phenotypic signatures of adaptation previously described (Leftwich et al. 2017). However,

454 this adaptation was not associated with any consistent assortative mating by diet between

455 the populations. mRNA-seq successfully captured variation in gene expression in sexually

456 mature males from both selection diets. As all flies were reared on a common garden diet

457 prior to RNA extraction, the measurement of evolved gene expression differences was

458 maximised and proximate responses to diets minimised. The mRNA-seq results for each

459 diet regime and body part were highly repeatable across independent biological replicates.

460 qRT-PCR provided strong support for the genome-wide patterns of gene expression in the

461 mRNA-seq. 214 transcripts showed DE above 2 $\log_2$OFC between treatments. Within this

462 set, 94 transcripts were matched to homologous genes in *D. melanogaster* (44%). The

463   functional description of these homologues, alongside the annotations available for the
464   medfly showed divergence in gene expression patterns between sexually mature adult
465   males from both nutritional regimes.

466   The larval diets which defined the ASG and Starch regimes were highly different in
467   terms of both caloric value and specific nutritional content, giving insight into the likely
468   selection pressures to which each population was challenged. The ASG larval diet
469   contained over twice the Kcal/L in comparison to the Starch. Also, the addition of corn
470   meal to the ASG diet offered additional sources of carbohydrates, proteins, and other
471   dietary nutrients (http://ndb.nal.usda.gov/). This dietary complexity is important as it is the
472   specific nutritional content of diets, rather than caloric content *per se*, that is known to
473   affect life history traits such as lifespan (Mair et al. 2005). The effects of selection on these
474   diets are evidenced by evolved changes in male body mass at sexual maturity (Leftwich et
475   al. 2017).

476   The divergent nutritional selection resulted in a pattern of evolved differences in
477   gene expression. Given the major differences between the diets in the complexity and
478   diversity of carbohydrate content, DE in some form of nutrient metabolism was expected.
479   Indeed, homologues of DE genes in the abdomen tissues of males were functionally
480   enriched for 'Serine-type endopeptidase activity'. The finding that different suites of these
481   peptidases, (putative digestive enzymes; Ross et al. 2003) were upregulated in the
482   different lines could suggest differentiation in digestive strategy as a basis for adaptation to
483   diet. Several Drosophilid systems that exhibit divergence driven by host specialisation
484   show similar, although more extensive, patterns of DE in nutrient related metabolic genes
485   (e.g. Dworkin and Jones 2009; Wurmser et al. 2011; Etges 2014; Etges et al. 2015;
486   Guillén et al. 2015). This effect is predicted on the basis that nutrients available in the
487   larval diet can have large effects on survivorship and also have the potential to drive
488   selection for optimal ability to utilise novel host nutrients. Within the medfly, the impact of
489   larval rearing diet on male adult life history is substantial (e.g. Arita and Kaneshiro 1988;
490   Kaspi et al. 2002). DE in metabolic genes could be associated with phenotypes
491   manifested through effects on body size and nutrient reserves (Davidowitz and Nijhout
492   2004; Edgar 2006), but also through the potential for metabolic programming of adult
493   metabolic traits by larval conditions (Matzkin et al. 2013; May et al. 2015).

494   Interestingly, divergent DE was observed in genes associated with OXPHOS in the
495   Starch regime flies. In the abdominal tissue of flies from a Starch selective background,

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496 three genes with homologues enriching to the oxidative phosphorylation pathway
497 (ko00190) were expressed at higher levels despite being reared on a common garden
498 diet. Genes involved in OXPHOS are expressed in, or interact with the mitochondria
499 which, due to their role as the centres of cellular energy production and rapidly evolving
500 independent genome (mtDNA), are thought to be essential genes in adaptation and
501 speciation (Gershoni et al. 2009; Ballard and Melvin 2010). An important example of this is
502 the lake whitefish species complex (*Coregonus* spp.), in which divergence in OXPHOS
503 gene expression in adult fish shows a tight relationship to the energetic phenotypes
504 represented by two reproductively isolated morphs (Trudel et al. 2001; Derome et al. 2006;
505 St-Cyr et al. 2008; Nolte et al. 2009; Jeukens et al. 2010; Renaut et al. 2010; Evans and
506 Bernatchez 2012). The tissue specific DE seen in OXPHOS genes between males from
507 ASG and Starch backgrounds may be associated with behavioural divergence (courtship
508 activity) in these lines (Leftwich et al. 2017). Starch males showed elevated levels of
509 courtship behaviours related to the expression of pheromones (Leftwich et al. 2017) and
510 the up regulation of energy-related genes in the Starch male abdomen is consistent with
511 this and could reflect the energetically costly process of pheromone biosynthesis (Dicke
512 and Sabelis 1988; Jurenka 2004). The capture of signatures of changes to OXPHOS
513 genes is interesting because it is believed to be important in responses to novel diets
514 through detoxification and altered patterns of host related hormone regulation (Dworkin
515 and Jones 2009; Wurmser et al. 2011).

516   Olfactory and gustatory receptor (OGR) genes are often associated with population
517 divergence by host specialisation (McBride and Arguello 2007; Wurmser et al. 2011; Etges
518 2014) and the establishment of prezygotic barriers during speciation (Smadja and Butlin
519 2009). Such genes mediate chemosensory responses and are associated with host
520 recognition, but also perception of pheromonal communication (Galindo and Smith 2001).
521 Hence it was interesting to observe DE in an OGR gene in this study (OR67a-like), as well
522 as two olfactory binding proteins (OBPs) (Obp19d, Obp99a). Patterns of DE in similar
523 OBPs are seen between allopatric populations of *D. mojavensis* adapted to different host
524 cacti and, in conjunction with other OBPs and behavioural genes, have been suggested to
525 contribute to further population divergence (Etges 2014).

526   Given the previous evidence for nutritional adaptation (Leftwich et al. 2017) the
527 finding that, at 60 generations, there was a consistent pattern of assortative mating by diet
528 across the regimes was an interesting one. However, the tests at generation 90 did not
529 replicate this pattern. In principle the two larval rearing diets provided sufficiently distinct

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530   selective environments to drive the evolution of parallel divergence (Leftwich et al. 2017).
531   However, in this experiment, this did not lead to the establishment of a consistent signal of
532   RI between populations. This may have been expected, as even geographically isolated
533   natural populations have not returned significant RI across a global scale (Cayol et al.
534   2002; Lux et al. 2002). Despite this, significant behavioural differences have been
535   observed between similarly geographically isolated populations, in courtship song (Briceño
536   et al. 2002), and also courtship behaviour (Briceño et al. 2007; Diamantidis et al. 2008b).
537   Further differentiation has been observed between global populations in other life history
538   traits such as growth rate, longevity, and sexual maturation (Diamantidis et al. 2008a,
539   2009), as well as pre-adult traits (Diamantidis et al. 2011b), and resilience to domestication
540   (a potential proxy for an enforced change of host) (Diamantidis et al. 2011a).

541   The variance seen across these traits in global populations of medfly reflects overall
542   genetic diversity of these populations, which is closely linked to the medfly's invasion
543   history (Gasperi et al. 2002; Karsten et al. 2015). Lack of divergence (isolation) between
544   populations that vary globally is likely due to the effects of gene flow between populations
545   (Malacrida et al. 1998, 2007; Karsten et al. 2015), potentially mediated by invasions due to
546   human transport (Wilson et al. 2009). This migration between populations has likely
547   suppressed the capacity for specialisation that can exist, as shown here, and has instead
548   maintained the plasticity exhibited by the medfly as a generalist, in both host and temporal
549   space (Yuval and Hendrichs 2000). Despite this, this study demonstrates that a laboratory
550   population (over 20 years isolated from the wild) maintained sufficient genetic variation to
551   respond to experimentally-imposed divergent selection. Relating this to natural
552   populations, as quarantine measures become increasingly effective and reduce gene flow
553   between global medfly populations (Karsten et al. 2015), the adaptive potential observed
554   here may lead global populations to diverge further.

555

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561   **DATA ARCHIVING**

562 Data are available on the GEO database (Barrett et al. 2013): GSE86029; GSM2291180
563 to GSM2291191.

564

AUTHOR CONTRIBUTIONS

566 W.N., I.M. and T.C. conceived the research; W. N. conducted the experimental work; W.N
567 and I.M. analysed the data; W.N., I.M. and T.C. wrote the paper.

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829   **Supporting Figures and Tables**

830

831   **Figure S1.** Distribution of expression levels before and after normalisation. Left panel
832   represents the raw non-redundant reads, right panel represents the expression levels
833   generated following the application of subsampling normalisation followed by quantile
834   correction. On the X axis are the samples and, on the Y, the log2 expression levels with an
835   offset of +20.

836   **Figure S2.** Hierarchical differential expression (DE) histogram. The frequency distribution
837   of log2OFC DE between sum expression in tissue types (HT/Ab), and in treatment (A/S).
838   The distribution of DE between treatment types, in red, falls below the distribution of DE
839   between tissue types, in black.

840   **Figures S3 - S7.** Validation on RNA-seq analysis using RT-qPCR for genes expresses in
841   both HT and Ab Tissue. Each plot consists of a panel of six figures. The left- and right-
842   hand columns are expression profiles of the candidate gene of interest (GOI). These
843   expression profiles show the algebraic sum of abundances of incident reads at every
844   position of the reference transcript, for each treatment. Expression levels from ASG tissue
845   is presented in the left-hand column, expression levels from the Starch tissue in the right-
846   hand column. Head /Thorax (HT) tissue is presented in the top row of both columns, and in
847   blue. Abdominal (Ab) is presented in the bottom row of both columns, and in red. Line
848   replicates are indicated by different line types. The central column of two figures shows the
849   normalised expression level following analysis (top), and the $2^{-\Delta CT}$ expression level
850   suggested by low throughput validation using RT-qPCR. Again, ASG left, Starch right, HT
851   blue, Ab red.

852   **Figures S8 - S12.** Validation on RNA-seq analysis using RT-qPCR for genes expresses in
853   HT tissue only. Layout of these plots is as described for Fig. S3 – S7.

854   **Figures S13 - S17.** Validation on RNA-seq analysis using RT-qPCR for genes expresses
855   in Ab tissue only. Layout of these plots is as described for Fig. S3 – S7.

856

857   **Table S1. Primers used for RT-qPCR Validation.** Forward (f) and reverse (r) primers
858   used for each GOI in the RT-qPCR validation experiment.

859   **Table S2. Quality control and genome matching of RNA-seq data used in this study.**
860   'fastq2fasta' describes the conversion of sequence data FASTQ format to FASTA format,
861   '%Acc' describes the accuracy of this conversion. 'R2NR' denotes the conversion of the

28

862 FASTA files into non-redundant format (see text), 'NR' = non-redundant, 'R' = redundant,
863 'C' = complexity (see text). 'Bstrp' describes the 29M fixed total subsampling of the data,
864 conducted as a normalisation step (see text).

865 **Table S3a. Differentially expressed (DE) transcripts (full dataset) found in**
866 **Head/Thorax tissue** taken from sexually mature males after 60 generations of
867 experimental evolution on one of two dietary treatments: ASG (AG) or Starch (SG)(see
868 text). Flies were reared for two generations on a common garden diet prior to flash
869 freezing at 7 days post eclosion and subsequent RNA extraction. Transcripts are shown
870 with their NCBI identifier. Normalised expression values are shown for each replicate of
871 each treatment, these are labelled treatment/replicate/sex/tissue (e.g. AG / 1 / M / Ab). DE
872 was called as log2 of the difference between either the highest or lowest (+ 20) normalised
873 expression value of the three treatment replicates, depending on which treatment showed
874 higher expression overall. Also shown are the *Drosophila melanogaster* homologues
875 identified for each gene using tBLASTx (see text). Further to this NCBI annotation
876 information is given.

877 **Table 3b. Differentially expressed (DE) transcripts (dataset with duplicate entries**
878 **removed) found in Head/Thorax tissue** taken from sexually mature males after 60
879 generations of experimental evolution on one of two dietary treatments: ASG (AG) or
880 Starch (SG)(see text). Dataset as described in Table S2a, but where multiple transcripts
881 were present in the NCBI set used to map RNA-seq read to, all reference sequences were
882 aligned to ensure similarity, and duplicates were removed, in these cases the transcript
883 which exhibited the highest DE value is presented as exemplar, and the NCBI id of
884 duplicates is also given.

885 **Table S3c. Differentially expressed (DE) transcripts (full dataset) found in**
886 **Abdominal tissue** taken from sexually mature males after 60 generations of experimental
887 evolution on one of two dietary treatments: ASG (AG) or Starch (SG)(see text). Flies were
888 reared for two generations on a common garden diet prior to flash freezing at 7 days post
889 eclosion and subsequent RNA extraction. Transcripts are shown with their NCBI identifier.
890 Normalised expression values are shown for each replicate of each treatment, these are
891 labelled treatment/replicate/sex/tissue (e.g. AG / 1 / M / Ab). DE was called as log2 of the
892 difference between either the highest or lowest (+ 20) normalised expression value of the
893 three treatment replicates, depending on which treatment showed higher expression
894 overall. Also shown are the *Drosophila melanogaster* homologues identified for each gene
895 using tBLASTx (see text). Further to this NCBI annotation information is given.

29

Table S3d. Differentially expressed (DE) transcripts (dataset with duplicate entries removed) found in Abdominal tissue taken from sexually mature males after 60 generations of experimental evolution on one of two dietary treatments: ASG (AG) or Starch (SG)(see text). Dataset as described in Table S2c, but where multiple transcripts were present in the NCBI set used to map RNA-seq read to, all reference sequences were aligned to ensure similarity, and duplicates were removed, in these cases the transcript which exhibited the highest DE value is presented as exemplar, and the NCBI id of duplicates is also given.

Table S4. Statistical validation of RT-qPCR results. Results of Welch's two sample t test conducted on the 2-ΔCT values of qRT-PCR validation experiments for 15 GOI. Genes were selected to test both tissue specific expression patterns, or organism wide expression. Genes which did not return signal (below detection limit, highlighted in pink) were assigned a CT value of 40 to facilitate statistical testing.

Table S5. Sample size (n) and total recorded matings, also broken down by mating pair type (Female/Male: AA, AB, BA, BA), at generations 60 & 90. Raw frequencies shown. Treatments represent: ASG/ASG - Individuals from the ASG regime, tested on the ASG larval diet, ASG/S - Individuals from the ASG regime, tested after two generations of rearing on the Starch larval diet, S/S - Individuals from the Starch regime, tested on the Starch larval diet, S/ASG - Individuals from the Starch regime, tested after two generations of rearing on the ASG larval diet, ASG/G - Individuals from the ASG regime, tested after two generations of rearing on the common garden (Glucose) larval diet, S/G - Individuals from the Starch regime, tested after two generations of rearing on the common garden (Glucose) larval diet.

Table S6. G statistics for generation 60 and generation 90 mating tests. G statistics calculated within JMATING (see text) to test for deviations from random mating across the whole coefficient dataset. GI = G test of PSI coefficient, GS = G test of PSS coefficient, GT = G test of PTI coefficient.

Table S7. Multiple choice mating tests with all flies reared on their own larval diet, tested at generation 60. Pair sexual isolation coefficient (PSI), pair sexual selection coefficient (PSS), and pair total isolation coefficient (PTI), calculated after Rolan-Alvarez and Caballero (2000), for each line pairing, and possible mating pair type. Coefficient values represent mean coefficient values generated after 10,000 bootstrap resamples from the observed (for PTI) or observed and expected frequencies (for PSI and PSS). Stars represent a significant bootstrap probability of rejecting the null hypothesis, that the

930 coefficient is not different from 1 (no preference, or random mating). Lowercase letters

931 represent diet (ASG = a or Starch = s) and replicate information (1-3). Upper case letters

932 represent 'pair types' (homotypic = AA, BB; heterotypic = AB, BA).

933 **Table S8. Multiple choice mating tests with all flies reared on a common garden**

934 **glucose based larval diet, tested at generation 60.** Pair sexual isolation coefficient

935 (PSI), pair sexual selection coefficient (PSS), and pair total isolation coefficient (PTI),

936 calculated after Rolan-Alvarez and Caballero (2000), for each line pairing, and possible

937 mating pair type. Coefficient values represent mean coefficient values generated after

938 10,000 bootstrap resamples from the observed (for PTI) or observed and expected

939 frequencies (for PSI and PSS). Stars represent a significant bootstrap probability of

940 rejecting the null hypothesis, that the coefficient is not different from 1 (no preference, or

941 random mating). Lowercase letters represent diet (ASG = a, Starch = s, Glucose = g) and

942 replicate information (1-3). Upper case letters represent 'pair types' (homotypic = AA, BB;

943 heterotypic = AB, BA).

944 **Table S9. Multiple choice mating tests with all flies reared on their own larval diet,**

945 **tested at generation 90.** Pair sexual isolation coefficient (PSI), pair sexual selection

946 coefficient (PSS), and pair total isolation coefficient (PTI), calculated after Rolan-Alvarez

947 and Caballero (2000), for each line pairing, and possible mating pair type. Coefficient

948 values represent mean coefficient values generated after 10,000 bootstrap resamples

949 from the observed (for PTI) or observed and expected frequencies (for PSI and PSS).

950 Stars represent a significant bootstrap probability of rejecting the null hypothesis, that the

951 coefficient is not different from 1 (no preference, or random mating). Lowercase letters

952 represent diet (ASG = a or Starch = s) and replicate information (1-3). Upper case letters

953 represent 'pair types' (homotypic = AA, BB; heterotypic = AB, BA).

954 **Table S10. Multiple choice mating tests with all flies reared on a common garden**

955 **glucose based larval diet, tested at generation 90.** Pair sexual isolation coefficient

956 (PSI), pair sexual selection coefficient (PSS), and pair total isolation coefficient (PTI),

957 calculated after Rolan-Alvarez and Caballero (2000), for each line pairing, and possible

958 mating pair type. Coefficient values represent mean coefficient values generated after

959 10,000 bootstrap resamples from the observed (for PTI) or observed and expected

960 frequencies (for PSI and PSS). Stars represent a significant bootstrap probability of

961 rejecting the null hypothesis, that the coefficient is not different from 1 (no preference, or

962 random mating). Lowercase letters represent diet (ASG = a, Starch = s, Glucose = g) and

963   replicate information (1-3). Upper case letters represent 'pair types' (homotypic = AA, BB;

964   heterotypic = AB, BA).

965   **Table S11. Multiple choice mating tests with all flies reared on a common garden**

966   **glucose based larval diet, tested at generation 90 - WITHIN TREATMENT.** Pair sexual

967   isolation coefficient (PSI), pair sexual selection coefficient (PSS), and pair total isolation

968   coefficient (PTI), calculated after Rolan-Alvarez and Caballero (2000), for each line pairing,

969   and possible mating pair type. Coefficient values represent mean coefficient values

970   generated after 10,000 bootstrap resamples from the observed (for PTI) or observed and

971   expected frequencies (for PSI and PSS). Stars represent a significant bootstrap probability

972   of rejecting the null hypothesis, that the coefficient is not different from 1 (no preference, or

973   random mating). Lowercase letters represent diet (ASG = a or Starch = s) and replicate

974   information (1-3). Upper case letters represent 'pair types' (homotypic = AA, BB;

975   heterotypic = AB, BA).

976   **Table S12. I_{PSI} isolation index values (Coyne et al. 2005) for each line pairing after 60**

977   **generations of selection.** Values presented are bootstrap means with standard

978   deviations; P values are the two-tail bootstrap probability that the value is significantly

979   different from one (equivalent to random mating), after 10,000 iterations of resampling.

980

981 **Tables**

982 **Table 1. Differentially expressed (DE) transcripts used for functional enrichment.** DE

983 between treatment types was called on maximal confidence intervals, with genes

984 exhibiting a $\log_2$(OFC) > 1 (corresponding to a 2-fold change difference) classed as DE.

985 From this set tBLASTx (Altschul et al. 1990) was used to retrieve homologues from

986 *Drosophila melanogaster*. These homologues were functionally enriched using g:Profiler

987 (Reimand et al. 2016). The number of transcripts which were retained in the analysis at

988 each stage, and the resulting number of enrichment terms are displayed per tissue.

	Tissue	
	Head/**T**horax	**Ab**domen
Total DE transcripts	**122**	**237**
# DE, Starch > ASG	43	151
# DE, ASG > Starch	75	86
D.melanogaster homologues	29	65
GO/KEGG terms enriched	0	4

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991 **Figure 1.** Differentially expressed transcripts in Abdominal tissue of males after 60
992 generations of adaptation to divergent larval diets. Colour represents row wise Z-score of
993 expression levels normalised by row mean and standard deviation, red represents
994 comparative up regulation, blue represents comparative down regulation. Groupings
995 based on GO enrichment of *D. melanogaster* homologues of medfly DE transcripts are
996 displayed on the left side of the heat map. Gene names are displayed on the right-hand
997 side. ASG samples (AG) are displayed in the 3 left hand columns of the heat map, Starch
998 samples (SG) in the 3 right hand columns, each for replicates 1-3, male abdomen (MAb).

999 **Figure 2.** The number of mating pairs formed in multiple choice mating tests between ASG
1000 and Starch dietary selection lines after 60 & 90 generations of selection. Each plot shows
1001 three replicates, with lowercase letters representing diet (ASG = a or Starch = s) and
1002 replicate information (1-3). Upper case letters represent 'pair types' formed within that
1003 replicate (homotypic = AA, SS; heterotypic = AS, SA). Dark Orange bars represent
1004 homotypic pairings between the ASG males and females. Light orange bars represent
1005 heterotypic pairings composed of a male from ASG, and a female from Starch. Light blue
1006 bars represent the opposite heterotypic pairing, Starch male and ASG female. Dark blue
1007 bars represent homotypic matings between a Starch male and female. A) Flies reared on
1008 their own larval diet, tested at 60 generations. B) Flies reared on a common garden diet for
1009 two generations prior to testing, tested at 60 generations. C) Flies reared on their own
1010 larval diet, tested at 90 generations. D) Flies reared on a common garden diet for two
1011 generations prior to testing, tested at 90 generations. E) Flies from ASG line replicates
1012 tested against other ASG line replicates. Colours represent hetero- and homotypic matings
1013 by the same light / dark pattern as above, but with an individual colour pair for each line
1014 replicate. All flies reared on their own larval diet, tested at 90 generations. F) Flies from
1015 Starch line replicates tested against other Starch line replicates. Colouration as in E. All
1016 flies reared on their own larval diet, tested at 90 generations.

1017
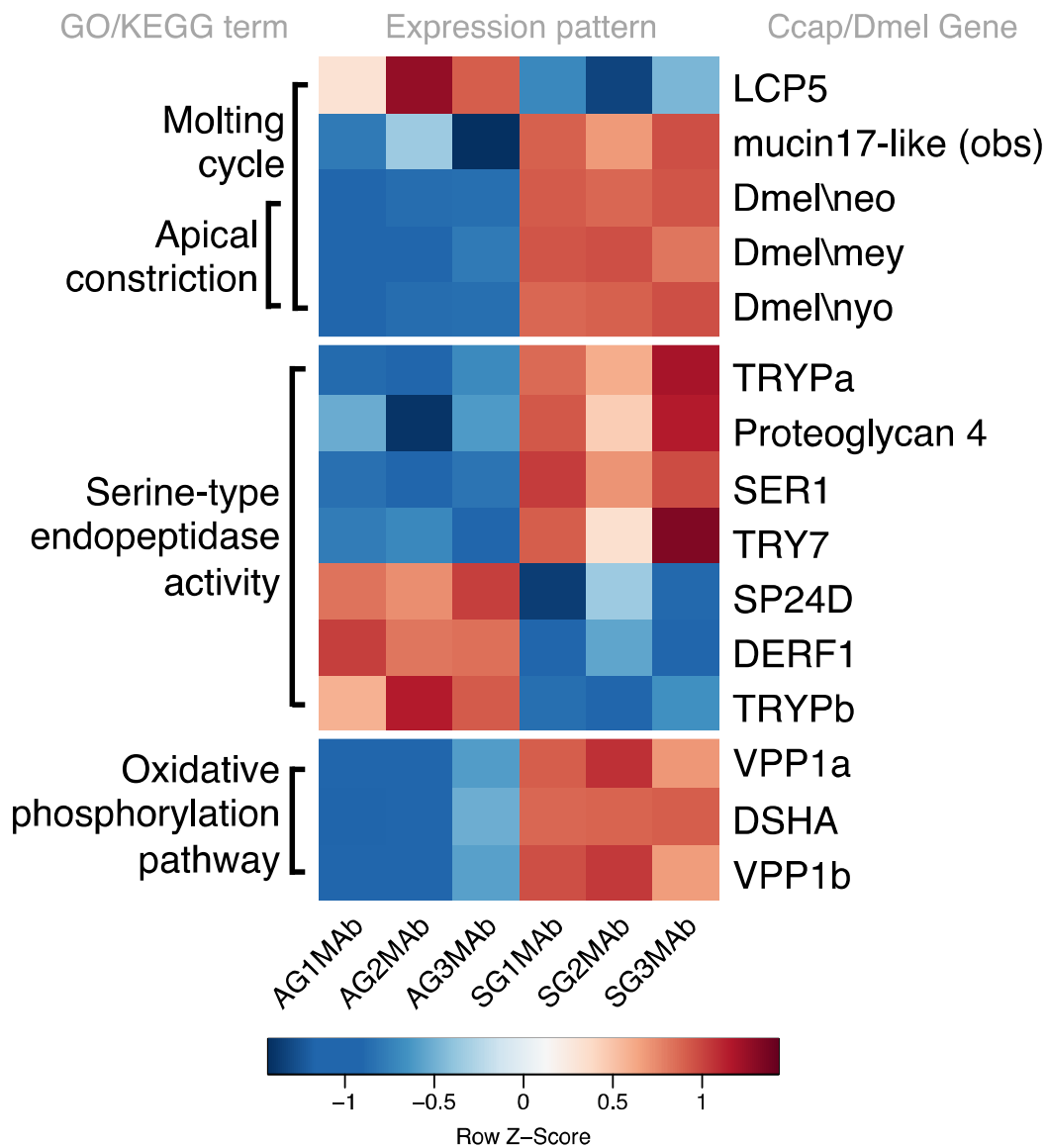
1018 **Figure 1**
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1022 **Figure 2**

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