The cuticle inward barrier in *Drosophila melanogaster* is shaped by mitochondrial and nuclear genotypes and a sex-specific effect of diet

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An important role of the insect cuticle is to prevent wetting (i.e., permeation of water) and also to prevent penetration of potentially harmful substances. This barrier function mainly depends on the hydrophobic cuticle surface composed of lipids including cuticular hydrocarbons (CHCs). We investigated to what extent the cuticle inward barrier function depends on the genotype, comprising mitochondrial and nuclear genes in the fruit fly *Drosophila melanogaster,* and investigated the contribution of interactions between mitochondrial and nuclear genotypes (mito-nuclear interactions) on this function. In addition, we assessed the effects of nutrition and sex on the cuticle barrier function. Based on a dye penetration assay, we find that cuticle barrier function varies across three fly lines that were captured from geographically separated regions in three continents. Testing different combinations of mito-nuclear genotypes, we show that the inward barrier efficiency is modulated by the nuclear and mitochondrial genomes independently. We also find an interaction between diet and sex. Our findings provide new insights into the regulation of cuticle inward barrier function in nature.

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2   **nuclear genotypes and a sex-specific effect of diet**

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12   sex differences, diet, nutrition, lipids

13   **Abstract**

14   An important role of the insect cuticle is to prevent wetting (i.e., permeation of water) and also to

15   prevent penetration of potentially harmful substances. This barrier function mainly depends on

16   the hydrophobic cuticle surface composed of lipids including cuticular hydrocarbons (CHCs).

17   We investigated to what extent the cuticle inward barrier function depends on the genotype,

18   comprising mitochondrial and nuclear genes in the fruit fly *Drosophila melanogaster,* and

19   investigated the contribution of interactions between mitochondrial and nuclear genotypes (mito-

20   nuclear interactions) on this function. In addition, we assessed the effects of nutrition and sex on

21   the cuticle barrier function. Based on a dye penetration assay, we find that cuticle barrier

22   function varies across three fly lines that were captured from geographically separated regions in

23   three continents. Testing different combinations of mito-nuclear genotypes, we show that the

24   inward barrier efficiency is modulated by the nuclear and mitochondrial genomes independently.

25   We also find an interaction between diet and sex. Our findings provide new insights into the

26   regulation of cuticle inward barrier function in nature.

Introduction

28   The insect cuticle plays an important role in maintaining homeostasis by preventing uncontrolled

29   penetration of xenobiotics and water (Hadley,1978; Lockey, 1976; Wang et al., 2016). This

30   barrier function relies mainly on the outer cuticular region composed of the envelope and surface

31   lipids including cuticular hydrocarbons (CHCs) at the cuticle surface (Blomquist et al., 2010;

32   Gibbs, 1995; 2002). In general, CHCs have chain lengths from C23 to C50, and may have double

33   bonds and be branched. The CHC pool is species-specific and shows within-species variation

34   with respect to age, sex and diet (Barbosa et al., 2017;Bonelli et al., 2015; Ishii et al., 2002;

35   Moore et al.,2017;Otte et al., 2015; Rouault et al., 2001). Length variation of CHCs was found in

36   different geographical populations of the fruit fly *Drosophila melanogaster*, where the ratio of

37   two CHC isomers varied with climatic conditions (Ferveur, 1991;1996). More recently, it was

38   reported that the chain length of CHCs correlates with the latitudinal habitats of *D. melanogaster*

39   strains from north (Maine) to south (Florida) along the US east coast paralleling increasing

40   temperatures and desiccation threat (Rajpurohit et al., 2017). Rajpurohit and colleagues also

41   found an association of genomic SNPs with the production and chemical profile of CHCs

42   (Rajpurohit et al., 2017). Consistently, it has been shown that CHC variation in recombinant

43   inbred lines depends on the nuclear genotype in *D. melanogaster* (Dembeck et al., 2015). Based

44   on these works, it is conceivable that CHC chain length is associated with the function of the

45   outward barrier and as a result would affect the desiccation resistance (Rouault et al., 2004).

46   Two findings indicate that also the inward barrier is based on lipids including CHCs. First,

47   penetration of xenobiotics such as the dye Eosin Y is sensitive to lipid solvents (Wang et al.,

48   2016, 2017). Second, cuticle impermeability for water and xenobiotics is disrupted by mutations

49   in genes coding for proteins involved in lipid-based barrier formation (Li et al., 2017; Yu et al.,

50   2017;Zuber et al., 2018). The molecular function of these proteins, including the ABCH

51   transporter Snustorr (Snu) and the extracellular protein Snustorr-Snarlik (Snsl), and their

52   relationship to CHC distribution, however, are yet unexplored. Hence, at least to some extent, the

53   inward and outward barriers share the same molecular constitution.

54   Diet has a significant impact on lipid composition of *D. melanogaster* in general (Brankatschk et

55   al., 2016; Buczkowski et al., 2005; Caravlho et al., 2012; Liang et al., 2000; Martin et al., 2011;

56   Savarit and Ferveur,2002; Wurdack et al., 2015). Food composition, sex and their interactions

57   significantly affected CHC composition and amounts in strains of *D. melanogaster*, maintained

58   on different food types for years (Fedina et al., 2012). Likewise, in the mustard leaf beetle

59   *Pheadon cochleariae*, different food resources (host plants) contribute to variation in CHC

60   profiles, which in turn affects their courtship behaviour (Geiselhardt et al., 2009). The diversity

61   of CHCs is assumed to depend on different metabolic pathways associated with digestion

62   processes and adaptations to specific diets.

63   Mitochondria are important sites of lipid metabolism in the cell, thereby contributing to CHC

64   production. Key steps of this process occur in these organelles. For instance, the bulk of acetyl-

65   CoA required in fatty acid synthesis by the acetyl-CoA carboxylase is produced in the

66   mitochondria (Hardwood et al., 1988; Kennedy, 1962;Wakil et al., 1983). Biosynthesis of haem,

67   a co-factor of cytochrome P-450 enzymes, which modify the long carbon chains in the

68   mitochondria or the smooth endoplasmatic reticulum (ER) (Capdevila et al., 1992), involves

69   enzymes acting in the mitochondria. While the role of mitochondria in lipid metabolism is well

70   understood (Goldin et al., 1968; Mesmin et al., 2016; Scharwey et al., 2013;Tyurinaet al.,2014;

71   Voelke,2004), it is not known whether mitochondrial genetic variation is associated with

72   variation of lipid or CHC synthesis, in turn influencing cuticle barrier function.

73   There are indeed some examples demonstrating the importance of mitochondrial genetic

74   variation for insect ecology, especially in *D. melanogaster* (Ballard et al., 2014; Camus and

75   Dowling, 2018;Dowling, 2014; Wolff et al., 2014). In the case of heat tolerance in *D.*

76   *melanogaster*, for example, Camus et al. (2017)recently showed that variation in this phenotype

77   in Australia was in part associated with mitochondrial single nucleotide polymorphisms (SNPs)

78   that did not change the protein sequence.

79   An issue complicating experimental approaches to assess the contribution by mitochondrial

80   genetic variation on vital body functions is that mitochondrial effects (caused by variation in the

81   mitochondrial genome) on the phenotype can differ across nuclear genomes, across sex and with

82   environmental factors such as diet (Aw et al., 2018; Zhu et al., 2014). It is well known that some

83   organismal dysfunctions associated with mitochondrial mutations are expressed in only some

84   nuclear backgrounds, but not in others (Blumberg et al., 2017,Connallon et al., 2018; Dobler et

85   al., 2014, 2018; Dowling,2014;Kenney et al., 2014; Latorre-Pellicer et al.,2016, 2017; Patel et al.,

86   2016; Reinhardt et al., 2013; Wolff et al., 2014). Several of these effects are sex-specific (e.g.,

87   Immonen et al., 2016). Addition of long-chain fatty acids to the diet has been shown to influence

88   mitochondrial physiology (Holmbeck and Rand,2015; Stanley et al., 2012), and even offset

89   mitochondrial genetic defects (Senyilmaz et al., 2015). Moreover, such nuclear or environmental

90   influences on mitochondrial effects can differ across mitochondrial genotypes (Ballard and

91   Youngson, 2015; Mossman et al.,2016a; 2016b).

92   To detect an effect of genotypic mitochondrial variation on cuticle differentiation requires an

93   advanced experimental protocol in which the effects of mitochondrial variation are isolated from

94   diet, sex and nuclear genome effects. Here, we use a sophisticated experimental design

95   employing *Drosophila* lines with distinct mito-nuclear genotypes reared on different diets. We

96   coupled this with a new *in situ* method that generically measures the cuticle inward barrier

97   function (Wang et al., 2016) and thereby examine the effects of mitochondrial and nuclear

98   genomic variation on the cuticle inward barrier function.

99   This new *in situ* method is based on the ability of the dye Eosin Y to penetrate the cuticle,

100   thereby reflecting the cuticle barrier function in *Drosophila* and other insects (Wangetal., 2016;

101   2017). Penetration of Eosin Y is regionalised, with different body parts taking up the dye at

102   distinct temperatures. Eosin Y staining provides a simple and reliable way to detect cuticle

103   inward barrier properties that are possibly mediated by lipids and CHCs. Here, we analyse the

104   Eosin Y penetration pattern in the wing cuticle that allows fast and efficient assessment of

105   differences in the cuticle inward barrier function caused by the mitochondrial genome, the

106   nuclear genome, sex or diet.

107   **Material and Methods**

108   *Fly line generation and maintenance*

109   We created nine fly lines with different combinations of mitochondrial and nuclear genomes by

110   specific crossing of males and females from three source populations. The three source

111   populations originated from Coffs Harbour, Australia (**A**) (Dowling et al., 2014; Williams et al.,

112   2012), Benin (**B**) (formerly Dahomey), Africa (Clancy, 2008) and Dundas (near Hamilton),

113   Canada (**C**) (MacLellan et al., 2009). According to the "Climate Data for Cities Worldwide"

114   database (https://en.climate-data.org/), Coffs Harbour lies in a zone with a humid subtropical

115   climate (Cfa according to the climate classification of Köppen, average temperature of 18.8°C,

116   1688mm of precipitation), Dundas lies in a warm-summer, humid continental climate (Dfb,

117   average temperature of 8.5°C, 834mm of precipitation) zone and Benin lies in a zone with

118   tropical monsoon climate (Aw, average temperature of 27.4°C, 1320mm of precipitation). We

119   initially crossed 45 virgin females from the source population with the desired mitochondrial

120   genotype to 45 males from the source population with the desired nuclear genotype to create the

121   first generation of each mito-nuclear line. To avoid skewed effects of mito-nuclear combinations

122   due to non-random sampling from each source population, we created each line three times

123   independently (resulting in a total of 27 lines) and kept them separated from each other since

124   then. To generate the second generation (and all subsequent generations) we backcrossed 45

125   virgin female offspring from the line (harbouring the desired mitochondrial genotype due to

126   maternal inheritance of the mitochondria [Birky, 2001]) with 45 males from the source

127 population with the desired nuclear genotype (Figure 1). With this crossing scheme, we removed

128 50% of the remaining nuclear genome from the maternal source population in each generation,

129 leading to theoretical 99.99% removal of the maternal nuclear genome after 17 generations. We

130 continued with the described crossing scheme for another 21 generations (until the experiment

131 started) to avoid selective co-adaptation processes between the nuclear and the mitochondrial

132 genome. We labelled the generated mito-nuclear lines as AA, AB, AC, BA, BB, BC, CA, CB

133 and CC, the first letter denoting the origin of the mitochondrial genome and the second letter

134 denoting the origin of the nuclear genome (hence mito-nuclear lines). We further distinguished

135 the three replicates of each line with a suffix (1 to 3). We kept the mito-nuclear lines and the

136 source populations as 14 day non-overlapping generations at 25°C on a 12:12 hour day-night

137 rhythm. We kept these flies on 7ml standard corn-yeast-sugar medium (corn 90g/l, yeast 40g/l,

138 sugar 100g/l, agar 12g/l, Nipagin 20ml/l, propionic acid 3ml/l) in 25mm vials.

139 All lines we used for the experiment were free of *Wolbachia*. This was confirmed for all lines by

140 diagnostic PCR for *Wolbachia*-specific primers after infected lines were treated with

141 Tetracycline (0.3g/l added to the food) for three generations (Clancy and Hoffmann, 2010). We

142 applied the Tetracycline treatment at least four generations before the start of our experiment.

143 *Food treatment*

144 We used two distinct diets to assess the effect of food composition on the function of the cuticle

145 barrier. The diets were developed by Carvalho et al. (2012) and Brankatschk et al. (2018) and

146 differ in lipid composition while being isocaloric. One food type was plant food (PF, 788 kcal/l).

147 Compared to the standard food, extra malt (80g/l), cold pressed sunflower oil (2ml/l) and treacle

148 (22g/l) were added, while yeast and glucose were removed. The other food type was yeast food

149 (YF, 809 kcal/l). Compared to the standard food, fresh yeast (80g/l) and extra yeast extract (20g/l)

150 were supplied and cornmeal was removed. A detailed description of the recipes for the two food

151 types can be found elsewhere (Brankatschk et al., 2018, Carvalho et al., 2012). In principle, the

152 nutritional value of both types of food is similar. By contrast, their lipid composition differs: the

153 plant food contains longer and more unsaturated lipids that the yeast food. The mito-nuclear lines

154 completed an entire life cycle on either PF food or YF food (i.e., females laid eggs on the

155 designated food type and larvae subsequently developed on this food type) before we collected

156 adult flies for wing staining and measurements (see below). To roughly control larvae density,

157 we used 10 males and 10 females to lay eggs and we standardised egg-laying time to 12 hours

158 for each line. Finally, we checked the egg density roughly by eye to make sure it was

159 approximately equal across all lines. We kept all vials at 25°C on a 12:12 hours day-night

160 rhythm. We collected virgin males and females within six hours of eclosion and kept them in

161 vials (10 flies/vial) separated by sex and line for five days, thereafter we started wing staining

162 and measurements.

163 *Wing staining and measurement*

164 We used 10 flies (20 wings) of each combination (per line/food/sex) for wing staining. We

165 carried out Eosin Y staining according to Wang et al (2016) with a slight modification. Instead of

166 two days old flies in the original protocol, we used five days old flies. We anaesthetized the flies

167 with CO_2, transferred them into a micro-centrifuge tube containing 1 ml of the red dye solution

168 (0,5% Eosin Y (W/V) and 0.1% Triton X-100) and incubated them at 55°C for 30 min. We

169 washed the Eosin Y-stained flies three times with distilled water, isolated wings using tweezers

170 and mounted them in 50% glycerol on glass slides. We collected images using a Leica DMI8

171 microscope with a built-in camera and the software LAS X. For quantification of staining, we

172 converted the images to 8-bit format and recorded the mean grey values that reflect the staining

173 intensity using the Fiji software (Schindelin et al., 2012). We compared the mean grey values in

174 the areas expected to take up Eosin Y and in surrounding areas. In a previous work, we

175 discovered that the Eosin Y staining pattern depended on the genetic background of *D.*

176 *melanogaster* (Wang et al., 2016). If the mean grey values of potentially Eosin Y-positive areas

177   (in the posterior, lower half), were higher than those of potentially Eosin Y-negative areas (in the

178   anterior, upper half), we scored a "presence of staining", otherwise we scored an "absence of

179   staining". We used a semi-quantitative method to classify staining patterns as 'no staining',

180   'front area staining', 'back area staining' or 'front and back area staining'.

181   *Statistical analyses*

182   Prior to data analysis we inspected our data and found that one staining pattern (front patch

183   unstained, back patch stained) occurred in only seven out of 1108 individuals distributed across

184   four of the nine mito-nuclear combinations. We omitted these seven measurements from our

185   analyses and used the remaining 1101 measurements assigned to one of the three staining pattern

186   as ordinate response variable (0, 1, 2 stained spots) for our analysis. We analysed the data with

187   generalised linear mixed models (GLMMs) using the *lme4* package (Bates et al., 2013) in R

188   3.4.2. (R Development Core Team 2017). We started with a full model including the factors diet,

189   sex, mitochondrial and nuclear genome and all their higher-order interactions. We then reduced

190   the model stepwise by excluding factor combinations to improve the Bayesian information

191   criterion (BIC). We used the BIC for model comparison because of the large number of degrees

192   of freedom in our models. Using BIC reduces the chance to have false positive factors in the

193   final model because a high number of degrees of freedom is more penalised than with the AIC

194   (Dziak et al., 2012). We stopped the model reduction when the removal of factor combinations

195   did not increase the explanatory power of the model (the final model). The four linear factors

196   remained in the final model because they were experimentally manipulated and we were *a priori*

197   interested in their effects.

198   The full statistical model included the observed patterns as dependent variable (ordinal data type).

199   Mitochondrial genotype, nuclear genotype, sex and food type, with all their higher-level

200   interactions, were fixed effects of the full model and line was used as random factor to avoid

201   pseudo-replication of data in the analyses. As the response variable was ordinal (see above), we

202 used a Binomial distribution with a log-link error function to analyse the data. We further

203 changed the number of maximal iterations for the model to converge from 1000 to 500000 in the

204 glmer Control to assure model convergence. To find the optimal grouping of the fly lines we ran

205 a principal component analysis (PCA) using SNP analyses of the mitochondrial genomes from

206 all our mito-nuclear lines (unpublished data, RD, DKD, Klaus Reinhardt, Susanne Voigt;

207 GenBank accession PRJNA532313) and the origin of the nuclear genome. In brief we ran PCAs

208 on the frequencies of the three observed staining patterns using 34 SNPs and the nuclear

209 background as explanatory variables. The aim of the PCAs was to see whether and how the 27

210 lines cluster in the plane of the first two principal components. Based on the results from the

211 PCA we grouped the fixed factor mitochondrial genotype to three levels (Figure S1). We

212 grouped the mitochondrial genotypes AA1, AA2, AB1-3 and AC1-3 (hereafter type 'A'), the

213 mitochondrial genotypes BA1-3, BB1-3, BC1-3, CA1 and CA3 (hereafter type 'B') and the

214 mitochondrial genotypes AA3, CA2, CB1-3 and CC1-3 (hereafter type 'C').

215 **Results**

216 We scored the frequency of occurrence of two Eosin Y staining areas at the posterior half of the

217 wing blade of flies from population lines with putatively co-evolved or newly constituted

218 mitochondrial and nuclear genome combinations in order to test barrier efficiency. It should be

219 noted that as these fly lines were derived from natural populations with assumed nucleotide

220 diversity in the mitochondrial and nuclear genomes, and not from isogenic stocks, we did not

221 expect to observe only one staining pattern in flies from a single population. We found variation

222 in the wing-staining pattern across our *D. melanogaster* lines (Figure 2). The frequencies of the

223 wing staining patterns for each of the four fixed factors are visualised in Figure S2.

224 The final model revealed that nuclear (p<0.001, Table 1) and mitochondrial (p=0.002) genotypes,

225 as well as the interaction between sex and food (p<0.001), best explained the frequency of the

226   wing-staining pattern (Table *1*, Figures 3 and 4). Of note, our data do not provide evidence for a

227   significant mito-nuclear interaction effect on the inward barrier function of the cuticle (p=0.141).

228   For different nuclear genotypes, flies with the Australian nuclear genotype generally showed the

229   highest frequency of non-stained wing area (39.94%) and the lowest frequency of both wing

230   areas being stained (43.30%) among all three nuclear genotypes (Figures 4 and S1). Flies with

231   both Benin and Canadian nuclear genotypes showed high frequencies of double stained areas

232   patterns (Benin: 96.26%, Canada: 74.80%) (Figures 4 and S1). The frequency of wing staining

233   pattern with only the rear area being stained was about the same in flies with the Australian and

234   the Canadian nuclear genotype (Australia: 16.76%; Canada: 17.07%) (Figure 4 and S1). This

235   pattern was almost absent in flies with the Benin nuclear genotype (1.07%) (Figures 4 and S1).

236   For different mitochondrial genotypes, the staining frequency for the rear area was similar in all

237   three mitochondrial genotype groups (A: 10.80%, B: 14.96%, C: 7.60) (Figure 4), whereas there

238   was substantial variation in whether no or both wing spots were stained (no staining: A: 19.44%,

239   B: 18.08%, C: 11.85%; staining both areas: A: 69.75%, B: 66.96%, C: 80.55%) (Figure 4).

240   The significant interaction effect between food and sex is underlined by the striking differences

241   of a male-female difference in wing staining pattern for plant food or yeast food (Figure 4).

242   Specifically, most wings of flies reared on plant food showed staining at both posterior areas

243   (female: 234, male: 252), and only a few wings of these flies had non-stained wings (female: 29,

244   male: 34). The frequency of wing staining patterns with only the rear area being stained was

245   lowest in males reared on plant food (Table S1, Figure S2).

246   **Discussion**

247   The physiological and genetic mechanisms involved in the constitution of the inward barrier

248   function of the insect cuticle are largely unexplored. To contribute to our understanding of these

249   mechanisms, we investigated the effects of nuclear and mitochondrial genetic variation, sex and

250   diet and their interactions on the function of the wing cuticle inward barrier. These factors had an

251   effect on cuticle barrier function at different hierarchical levels.

252   *Interactions between sex and diet*

253   According to our statistical model, if we consider sex or diet alone (as main effects), they do not

254   have any significant effect on the frequency of the wing-staining pattern and, by consequence, on

255   the cuticle inward barrier efficiency. Yet, they are involved in a statistically significant

256   interaction, with the effects of each of these factors is contingent on the other. Simply, this

257   suggests an important role of sex on cuticle barrier function that is modulated by diet. Thus, with

258   respect to the cuticle barrier function, we conclude that the responses to food quality between

259   males and females are different. In other words, food utilization for barrier construction and

260   optimization (that may at least partially rely on CHCs) varies between males and females. Indeed,

261   in recent studies it was shown that the impact of the food source on the CHC pattern differed in

262   males and females (Otte, 2015). Thus, it seems that the CHC composition is not only sexually

263   dimorphic with respect to communication (Antony and Jallon, 1982), but also with respect to

264   penetration resistance against xenobiotics.

265   Next, we need to determine sex-specific single nucleotide polymorphisms (SNPs) and identify

266   the CHC composition in males and females of our lines on different food sources to better

267   understand inward barrier function in dependence of diet and sex. These experiments would also

268   allow us to reveal possible differences and similarities between the molecular constitution of the

269   inward and the outward barrier for which rich data is available.

270   Indeed, a difference of the outward barrier between sexes has been observed in *D. melanogaster*.

271   Due to specific modifications of CHC composition after induction of desiccation resistance, the

272   survival rate of females is higher than the survival rate of males under prolonged desiccation

273   conditions (Foley and Telonis-Scott 2011; Stinziano et al., 2015). This effect is independent of

274   starvation. These findings with respect to our results are interesting insofar as they suggest that

275   the outward and inward barriers display independent efficiencies; while, for instance, females are

276   more resistant to water loss than males, resistance to dye penetration is comparable between

277   females and males if we ignore the effect of diet (see below). A simple conclusion is that these

278   two barriers do not rely on CHCs and the respective physical properties alone, but employ

279   distinct and non-analogous factors. Further experiments are needed to unravel the molecular

280   basis of this difference.

281   Likewise, it has previously been demonstrated that diet does control the efficiency of the

282   outward barrier, probably by influencing the amounts and, importantly, the composition of CHCs

283   (Fedina et al., 2012). In line with this work, a recent study reported that the proportion of

284   desaturated CHCs defines the efficiency of the barrier against desiccation (Ferveur et al., 2018).

285   This may be explained by an influence of food metabolites on fatty acid synthesis and, by

286   consequence, on CHC amounts and composition (Fedina et al., 2012; Ferveur 2005; Pavković-

287   Lučić et al., 2016). However, the situation is certainly more complex. The effect of food alone on

288   CHC quality is not sufficient to explain the outward barrier function. Indeed, the effects of food

289   source on desiccation resistance have been repeatedly reported to depend on multiple complex

290   factors including, in addition to lipid metabolism, carbohydrate metabolism and body size

291   (Andersen et al., 2010; Kristensen et al., 2016; Mikkelsen et al., 2010). Moreover, diet has been

292   demonstrated in large-scale studies to have a significant and variable effect on genome-wide

293   gene expression in 20 *D. melanogaster* wild-type strains in various complex traits including

294   puparial adhesion, metamorphosis and central energy metabolic functions (Reed at al., 2010;

295   Reed et al., 2014; Williams et al., 2015). Overall, we therefore conclude that the outward and

296   inward barriers are differentially sensitive to diet.

297   *The nuclear genotype*

298   We observed significant effects of the nuclear genotype on the dye penetration efficiency. In

299   particular, the nuclear genotype of flies from Coffs Harbour with a humid subtropical climate

300   (Cfa according to the climate classification of Köppen) correlates with a wing cuticle inward

301   barrier that is more efficient than in flies with the Dundas (warm-summer, humid continental

302   climate, Dfb) or with the Benin (tropical monsoon climate, Am) nuclear genotype, which

303   correlates with the lowest cuticle inward barrier efficiency. Rough temperature or humidity

304   profiles of the locations (see Materials & Methods) do not explain these correlations arguing that

305   the cuticle inward barrier may not be directly dependent on these factors. In any case, we

306   conclude that the inward barrier function depends largely on varying fly line-specific expression

307   profiles of nuclear genes, some of which presumably are needed for CHC production and

308   deposition including genes coding for Cyp proteins, fatty acid synthases and elongases. Nuclear

309   genetic variations that comply with differences in CHC composition in inbred or geographically

310   separated lines of *D. melanogaster* have been reported to be represented by quantitative trait loci

311   (Foley et al., 2007; Foley and Telonis-Scott 2011) or SNPs (Dembeck et al., 2015; Rajpurohut et

312   al., 2016). The genomic differences between our lines and the associated differences in cuticle

313   permeability are either due to genetic adaptation to the original environmental conditions in

314   Coffs Harbour, Australia, Benin, Africa and Dundas (near Hamilton), Canada, or due to genetic

315   drift within the populations. This remains to be tested.

316   *The mitochondrial genotype*

317   We found a significant effect of the mitochondrial genotype on the wing-staining frequency

318   patterns. Cuticle inward barrier efficiency was similarly reduced in flies with a Dundas

319   mitochondrial genotype. Rough climate profiles of the original locations of our fly populations

320   are insufficient to explain the correlation between the mitochondrial genotype and cuticle inward

321   barrier efficiency. More detailed assessment of the local climate situation and the use of more

322   geographically disjunct fly populations are thus necessary to allow relating cuticle inward barrier

323   efficiency to any climatic factor. A relationship between cuticle inward barrier efficiency,

324   mitochondrial genotype and a climatic factor would, in turn, allow the testing of whether this

325   relationship is in agreement with the *mitochondrial climatic adaptation* hypothesis (Camus et al.,

326   2017). This hypothesis proposes that latitudinal climatic differences shape patterns of standing

327   variation in mitochondrial genotypes across a species distribution, and that these genotypes play

328   a role in determining temperature sensitivity of individuals.

329   In a recent work, no significant effect of the mitochondrial genotype on lipid content was found

330   in *D. melanogaster* lines engineered from laboratory and natural populations (Aw et al., 2017).

331   That study thus indicated that lipid (including CHC) homeostasis is to a large extent independent

332   from the mitochondrial genotype in *D. melanogaster*. Together, we conclude that the function of

333   the inward barrier is sensitive to variation in the mitochondrial genomes of the lines tested. We

334   also conclude that the mitochondrial-dependent inward barrier function is possibly not mediated

335   by CHCs.

336   Interestingly, Aw and colleagues (2017, 2018) did find a significant interaction of the

337   mitochondrial genotype with diet and/or sex on a number of physiological traits including

338   survival and fecundity. According to our data, however, these genotype-by-environment

339   interactions do not seem to play any role with respect to the cuticle inward barrier function.

340   Likewise, we do not detect any interaction between the mitochondrial and the nuclear genomes

341   that would influence dye penetration in our assays. The seeming independence of the inward

342   barrier function from mito-nuclear interaction possibly represents a particular case. Indeed, mito-

343   nuclear interactions have been demonstrated to affect a wide range of biological processes such

344   as developmental time, sex-specific transcription, hypoxia and longevity (Dowling et al., 2010;

345   Mossman et al., 2016a,b, 2017; Rand et al., 2006; Rand et al., 2018). We will need to expand the

346   number of different geographical populations of *D. melanogaster* or refine the quantification of

347   our dye-penetration assay in our analyses to uncover whether any subtle mito-nuclear effects on

348   cuticle barrier function exist.

349 *Conclusions*

350 In summary, in this study we find that Eosin Y penetration through the wing cuticle of mass bred

351 populations from three global populations is variable and that the pattern of variability differs

352 between the lines. Our data indicate that along with CHCs, other cuticle components are

353 sensitive to genetic variation within the nuclear and mitochondrial genomes as well as to sex-diet

354 interactions. Candidates are factors acting in the Snu-Snsl pathway that contribute to the

355 construction of the outermost cuticle layer termed envelope that serves as a physical barrier

356 against penetration and desiccation (Zuber et al., 2018). Nuclear and mitochondrial SNP analyses

357 will help to shed light on this complex trait that is crucial for insect survival. Mitochondrial

358 genes comprising 13 polypeptides of the electron transfer-chain (ETC), 2 rRNAs and 22 tRNAs,

359 are, however, probably not directly involved in cuticle barrier formation (Ballard and Rand, 2005;

360 Burton et al., 2013; Piomboni et al., 2012; St John et al., 2005).

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Tables legend

*Table 1:***ANOVA table for the statistical analysis of the wing pattern frequency.** Analysis of deviance table (type III Wald χ^2 test) for the optimised model. The interaction between sex and food was statistically significant, with both of the two linear terms to be found non-significant. The mitochondrial genotype and the nuclear genotype had also a significant effect on the staining pattern.

736

Table S1: **Wing pattern frequencies.**∘∘ indicates that none of the two areas was stained,∘• indicates that the rear wing area was stained and •• indicates that both areas on the wing were stained. Empty cells indicate that the specific combination of all four factors did not occur in our experiment after recording of the mitochondrial genotypes to five levels based on the sequence data.

742

Figure legends

*Figure 1:***Crossing scheme of fly line generation**. We initially crossed 45 virgin females from population A to 15 males from A, B and C, respectively, to create the first generation. After then, we backcrossed 45 female offspring from the line with 45 males from the desired nuclear genotype. All subsequent generations repeat the back cross scheme. Similarly, we generated mito-nuclear lines as BA, BB, BC, CA, CB and CC by crossing males from the three source populations to females from the populations B and C, respectively.

750

Figure 2: **Eosin Y staining pattern of the fly wing.** As represented by a drawing (A), a proportion of wings does not take up Eosin Y after staining (B). Some wings, by contrast, show

753   staining of a posterior region close to the hinge (C, D). Another set of wings takes up the dye in

754   two posterior regions (E, F).

755

756   *Figure 3*: **Relative frequencies of wing patterns.** The relative occurrence of each wing pattern

757   for each mitochondrial genotype - nuclear genotype -sex-food combination. A, B and C in the

758   first line of the x-axis label are for the mitochondrial genotype. The A, B and C in the second

759   line stand for the nuclear genotype. The third line indicates the sex of the flies m for males and f

760   for females and the forth line label are for plant food (P) or yeast food (Y).

761

762   *Figure 4*: **Nuclear, mitochondrial and interaction of sex and diet on wing pattern**

763   **frequencies.**

764   The relative occurrence of each wing pattern for each mitochondrial genotype, nuclear genotype

765   and sex- food combination, respectively. The interaction between sex and diet affects the number

766   of wings with no staining and with a single spot staining, but not the number of wings with both

767   spots stained. Females (f) on yeast food (Y) show a much higher proportion of single stained

768   wings than males (m) on yeast food and males and females on plant food (P). Males on yeast

769   food have a higher occurrence of unstained wings than females on yeast food and males and

770   females on plant food.

771   In summary, males and females show similar frequencies for double stained area (m: 71.61%, f:

772   72.09%). However, the frequencies between non-stained (m: 21.61%, f: 11.46%) and single

773   stained areas (m: 6.79%, f: 16.45%) showed some difference. Staining patterns between plant

774   food and yeast food were quite different (non-stained: P: 11.07%, Y: 22.56%; single stained: P:

775   3.51%, Y: 20.11%; double stained: P: 85.41%, Y: 57.33%).

776

777

778   *Figure S1*: **Mitochondrial genotype grouping.** The mitochondrial genotypes were grouped

779   based on the result by a PCA. We distinguish three groups. The mitochondrial genotypes AA1,

780   AA2, AB1-3 and AC1-3 were grouped and named type 'A', the mitochondrial genotypes BA1-3,

781   BB1-3, BC1-3, CA1 and CA3 were grouped and named type 'B' ,the mitochondrial genotypes

782   AA3, CA2, CB1-3 and CC1-3 were grouped and named type 'C'.

783

784   *Figure S2*: **Sex and diet linear effects on wing pattern frequencies.** Relative frequencies by

785   sex are: male no staining: 21.61%, rear area stained: 6.79%, both areas stained: 71.61%; female

786   no staining: 11.46%, rear area stained: 16.45%, both stained: 72.09%. Staining frequencies by

787   food: plant food no staining: 11.07%, rear area stained: 3.51%, both areas stained: 85.41%; yeast

788   food no staining: 22.56%, rear area stained: 20.11%, both areas stained: 57.33%.

Figure 1

Crossing scheme of fly line generation.

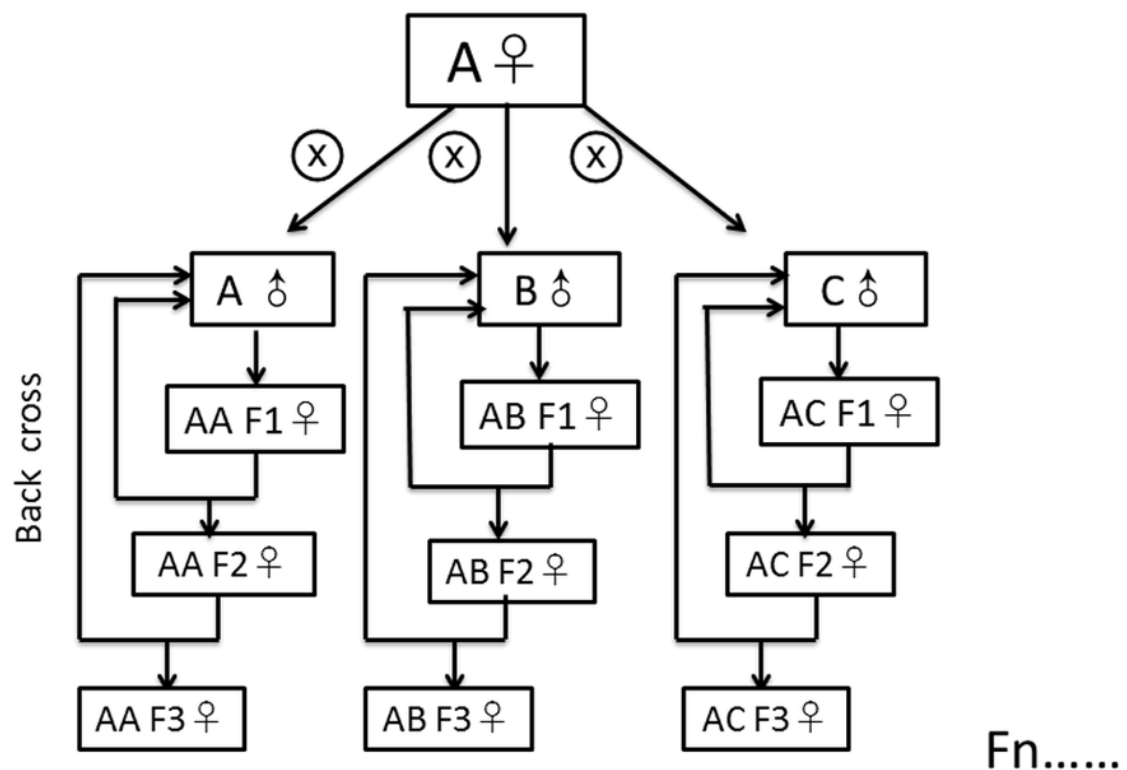
We initially crossed 45 virgin females from population A to 15 males from A, B and C, respectively, to create the first generation. After then, we backcrossed 45 female offspring from the line with 45 males from the desired nuclear genotype. All subsequent generations repeat the back cross scheme. Similarly, we generated mito-nuclear lines as BA, BB, BC, CA, CB and CC by crossing males from the three source populations to females from the populations B and C, respectively.

F1: the first generation   F2: the second generation   F3: the third generation

Figure 2

Eosin Y staining pattern of the fly wing.

As represented by a drawing (A), a proportion of wings does not take up Eosin Y after staining (B). Some wings, by contrast, show staining of a posterior region close to the hinge (C, D). Another set of wings takes up the dye in two posterior regions (E, F).

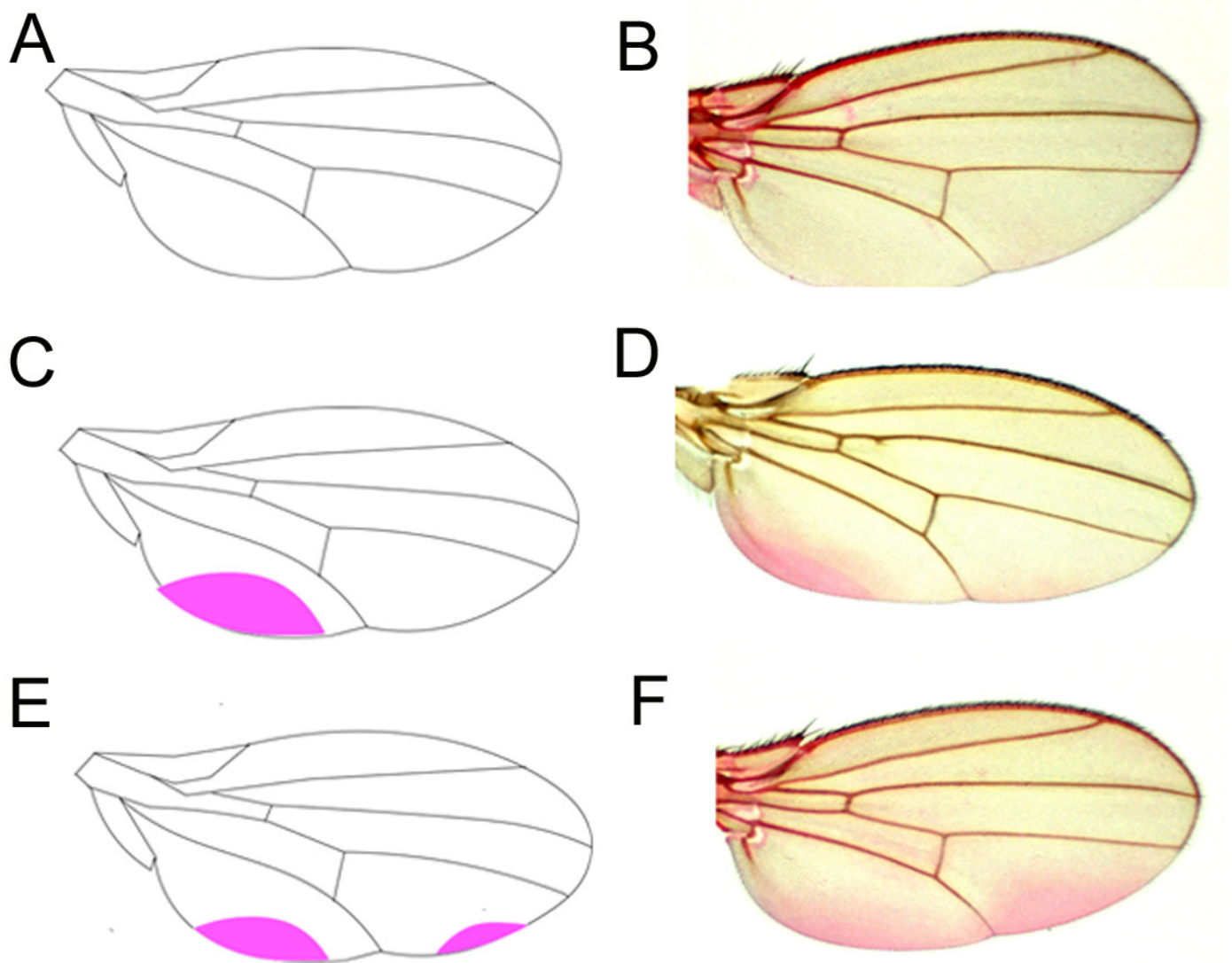



Figure 3

Relative frequencies of wing patterns.

The relative occurrence of each wing pattern for each mitochondrial genotype - nuclear genotype -sex-food combination. A, B and C in the first line of the x-axis label are for the mitochondrial genotype. The A, B and C in the second line stand for the nuclear genotype. The third line indicates the sex of the flies m for males and f for females and the forth line label are for plant food (P) or yeast food (Y).

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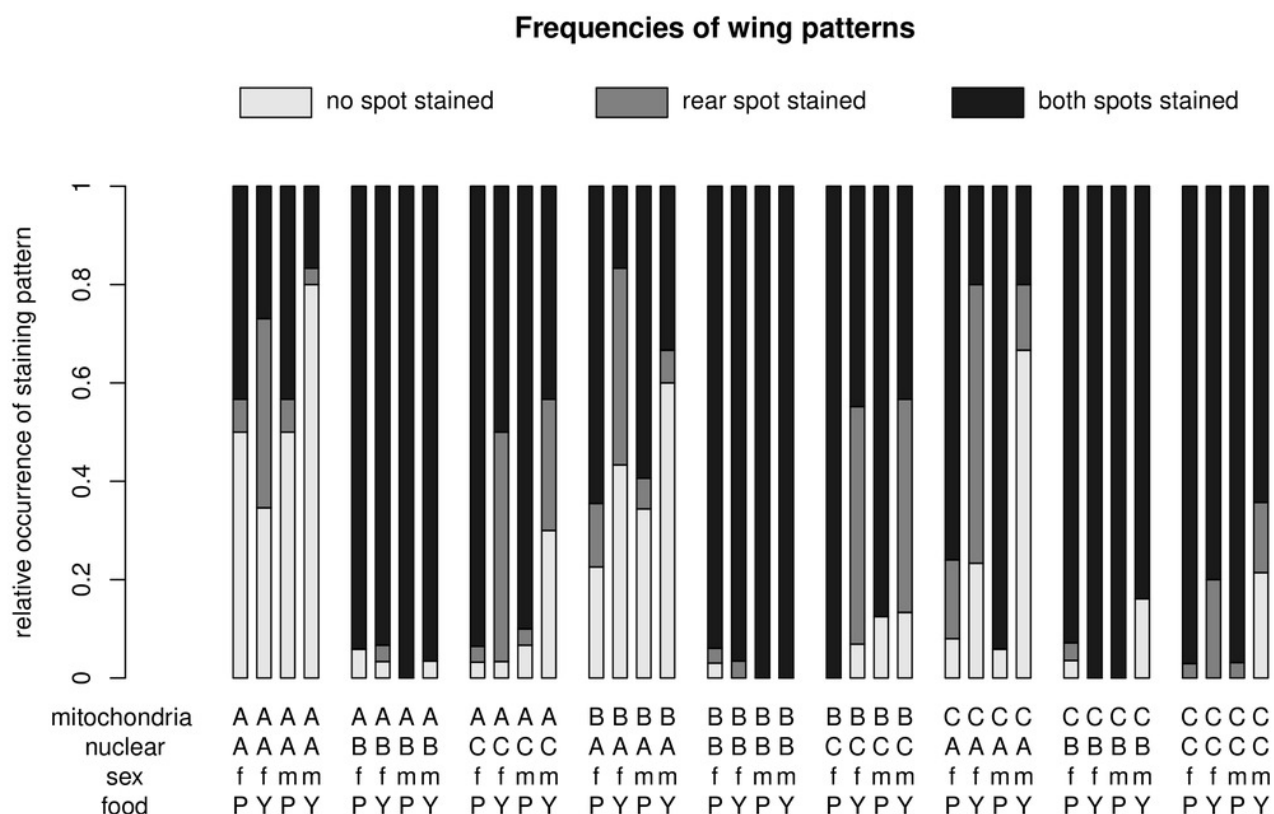
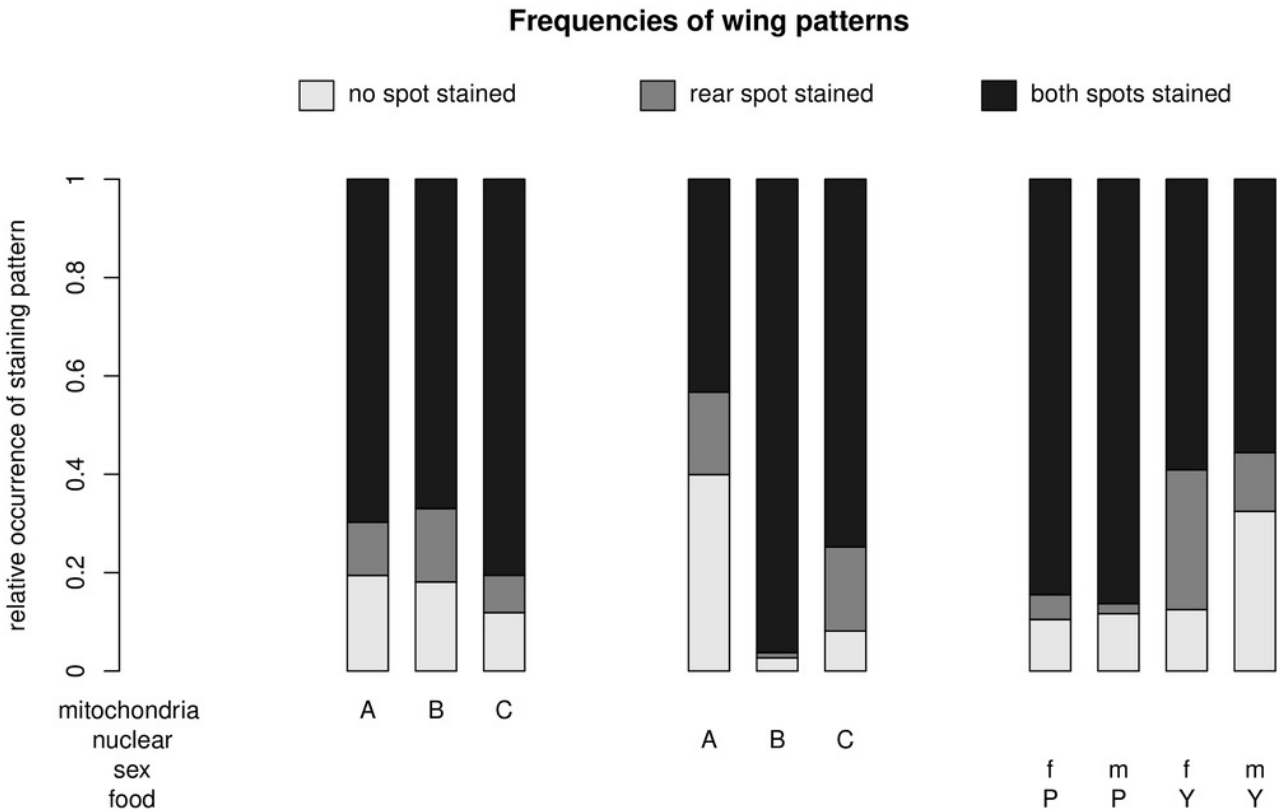


Figure 4

Nuclear, mitochondrial and interaction of sex and diet on wing pattern frequencies.

The relative occurrence of each wing pattern for each mitochondrial genotype, nuclear genotype and sex- food combination, respectively. The interaction between sex and diet affects the number of wings with no staining and with a single spot staining, but not the number of wings with both spots stained. Females (f) on yeast food (Y) show a much higher proportion of single stained wings than males (m) on yeast food and males and females on plant food (P). Males on yeast food have a higher occurrence of unstained wings than females on yeast food and males and females on plant food. In summary, males and females show similar frequencies for double stained area (m: 71.61%, f: 72.09%). However, the frequencies between non-stained (m: 21.61%, f: 11.46%) and single stained areas (m: 6.79%, f: 16.45%) showed some difference. Staining patterns between plant food and yeast food were quite different (non-stained: P: 11.07%, Y: 22.56%; single stained: P: 3.51%, Y: 20.11%; double stained: P: 85.41%, Y: 57.33%).

ANOVA table for the statistical analysis of the wing pattern frequency.

Analysis of deviance table (type III Wald χ^2 test) for the optimised model. The interaction between sex and food was statistically significant, with both of the two linear terms to be found non-significant. The mitochondrial genotype and the nuclear genotype had also a significant effect on the staining pattern.

factor	χ^2	df	p-value
intercept	21.337	1	<0.001
mitochondrial genotype	12.740	2	0.002
nuclear genotype	134.358	2	<0.001
sex	0.161	1	0.688
food	0.646	1	0.422
sex× food	14.146	1	<0.001