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Plasticity in behavioural responses and resistance to temperature stress in *Musca domestica*

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1 Plasticity in behavioural responses and resistance to temperature
2 stress in *Musca domestica*

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13

14 **ABSTRACT**

15 Organisms can respond and cope with stressful environments in a number of ways including
16 behavioural, morphological and physiological adjustments. To understand the role of behavioural
17 traits in thermal adaptations we compared heat resistance, locomotor (walking and flying) activity,
18 flight performance and morphology of three European populations of *Musca domestica* (Diptera:
19 Muscidae) originating from different thermal conditions (Spain, Switzerland and Denmark) at
20 benign and stressful high temperatures. Spanish flies showed greater heat resistance than Swiss and
21 Danish flies. Similarly, at the stressful high temperature Spanish flies flew longest and Danish
22 shortest. Neither body size nor wing loading affected flight performance, although flies with
23 narrower wings flew longer (wing shape effect). Swiss flies were most active in terms of locomotor
24 activity at the benign temperature, whereas the Spanish flies were able to stay active longer at the
25 stressful temperature. Population differences in behavioural traits and heat resistance were obtained
26 using flies held for several generations in a laboratory common garden setting; therefore we suggest
27 that exposure to and avoidance of high temperatures under natural conditions has been an important
28 selective agent causing the suggested adaptive differentiation between the populations.

29

30 **Keywords:** Flight, heat resistance, insects, locomotor activity, morphology, thermal adaptation.

31

32

33

34

35 INTRODUCTION

36 Temperature is one of the most important abiotic factors affecting the behaviour and physiology of
37 ectotherm organisms (Hochachka & Somero 2002). Typically an increase in temperature causes an
38 increase in metabolism and consequently also in behavioural traits, such as activity, boldness,
39 aggressiveness and exploration, due to suggested connection between metabolism and the
40 expression of behaviour (Biro & Stamps 2008; Briffa, Bridger, & Biro 2013; Careau, Thomas,
41 Humphries, & Reale 2008; Houston 2010; Huey, Hertz, & Sinervo 2003). Behavioural adjustments
42 can also help organisms to reduce the energetic costs associated with environmental stress and
43 critically mediate the interaction of ectothermic animals with temperature (Stevenson 1985). At
44 stressful high temperatures behavioural traits, such as flight and mating behavior, seems to be
45 negatively affected by heat stress well before survival is affected (Krebs & Thompson 2006; Patton
46 & Krebs 2001), and it has been argued that animals respond strongly in behavioural traits
47 (Hoffmann, Sørensen, & Loeschcke 2003).

48 Despite the clear importance of temperature for the behaviour of ectotherms, our
49 knowledge about the particular effects of stressful temperatures on behavioural traits and how
50 behavioural traits evolve during thermal stress is weak (Sih 2013; Wingfield 2013). Many species
51 occur along latitudinal or altitudinal gradients and have adapted physiologically and
52 morphologically to local thermal conditions (Bahrndorff, Holmstrup, Petersen, & Loeschcke 2006;
53 Bubliy, Riihimaa, Norry, & Loeschcke 2002; Dahlgaard, Hasson, & Loeschcke 2001; Gibert, Huey,
54 & Gilchrist 2001; Karl, Sørensen, Loeschcke, & Fischer 2009), so the evolution of behavioural
55 traits will also vary along environmental gradients. This is supported by studies comparing
56 populations and species from different thermal environments with respect to behaviour and thermal
57 resistance. Field observations of adult *Drosophila subobscura* suggest that extrinsic temperature
58 variation induces changes in thermoregulatory behaviour and body temperature and therefore likely

is an important driver of evolutionary adaptation (Huey & Pascual 2009). Dahlgaard, Hasson & Loeschcke (2001) compared mating behaviour in high- and lowland populations of *D. buzzatii* and found population differentiation in oviposition activity and heat knock down resistance. Fasolo & Krebs (2004) found similar results showing that the upper critical temperatures where mating behaviour ceased differed between even closely related *Drosophila* species and populations. Results for butterflies have also shown intraspecific variation in flight at suboptimal conditions (Berwaerts & Van Dyck 2004). In insects, heat resistance often differs between life stages, which has been suggested to be linked to the mobility of the life stage (Bowler & Terblanche 2008; Marais & Chown 2008). For example, the immobile life stages (eggs and pupae) of *D. buzzatii* show higher resistance limits than the mobile life stages (larvae and adults) (Krebs & Loeschcke 1995). All these results suggest that extreme temperatures are important selective agents also for behavioural traits and the physiology and morphology underlying behavioural performance, but also highlight that there is only a limited number of studies that have addressed evolutionary differences in behavioural traits in relation to thermal adaptation.

In this study we investigated heat resistance in terms of survival after heat stress, flight distance, and locomotor activity (walking, jumping and short flying attempts), i.e. multiple aspects involving behaviour and contributing to fitness, of *Musca domestica* (Diptera: Muscidae) from thermally different environments (Denmark, Switzerland, Spain). Flight and locomotor activity are integrated parts of insect behaviour and thus likely involved in thermal adaptation. Flight behaviour is the more efficient alternative when faced with high temperature stress because it allows for a fast retreat over even long distances. Whether this translates into thermal adaptation in flight physiology depends on the frequency of exposure to extreme temperatures (Kingsolver & Gomulkiewicz 2003), gene flow and the extent to which behavioural adjustment is involved in avoiding heat stress. Locomotor activity, on the other hand, is likely to evolve to a greater extent in

83 response to biotic factors such as population density and mating activity (Bahrndorff et al. 2012;
84 Schou et al. 2013), although it also allows quick movement to nearby microhabitats with a more
85 favourable microclimate. To determine if behavioural plasticity in these mobility traits has evolved
86 we compared performance at stressful and benign temperatures. We expected that the Spanish
87 population originating from the warmest environment would show adaptation to critically high
88 temperatures and therefore also be able to sustain flight and activity for the longest time at
89 stressfully high temperatures. At benign temperatures, we expected that the population from Spain
90 would show lower or similar activity and flight distance compared to that of the populations from
91 Denmark and Switzerland. We also measured morphological traits that are often affected by
92 temperature and which relate to flight capacity: wing size and shape and hind tibia length, which
93 can be considered proxies for body size. We expected that larger flies could fly longer due to higher
94 absolute energy reserves (Kaufmann et al. 2013). We also expected wing size and shape as well as
95 the wing loading (body size relative to wing size) to affect flight performance differentially at
96 benign vs. stressful test temperatures, either directly due to differences in lift per stroke or indirectly
97 as part of a flight syndrome correlated with other traits important for flight. In warm environments
98 the flight muscle output is high and hence there are few constraints regarding lift production per
99 wing stroke (Starmer & Wolf 1989). Therefore, flies with relatively narrow wings (low wing aspect
100 ratio) and large body size should fly longer because of the energetic advantage of large body size
101 (Kaufmann, Reim, & Blanckenhorn 2013) and higher speed and manoeuvrability of narrow wings
102 (Starmer & Wolf 1989).

103

104 **MATERIALS AND METHODS**

105 *Fly populations and rearing protocol*

106 The fly populations used were all collected from the field in the summer and autumn
107 of 2011 and had been kept in the laboratory for 4-8 generations before being used. The Danish
108 population of adult flies was collected at a pig farm in June 2011 from Djursland (56.38°N,
109 10.24°E); the Swiss population was collected at a pig farm near the University of Zürich-Irchel
110 (47.40°N, 8.55°E); and the Spanish population at Centre de Recerca en Sanitat Animal, Barcelona
111 (41.30°N, 2.05°E) in October 2011. All populations were founded from at least 100 individuals and
112 kept at densities of approximately 500 individuals per population in the laboratory. Flies were fed
113 sugar, milk powder and water *ad libitum*. Larvae were reared in a medium consisting of wheat bran
114 (24.6%), alfalfa (12.3%), yeast (0.6%), malted sugar (0.9%), tap water (61.6%) and the larval
115 medium was gently stirred daily to facilitate gas exchange until the pupal stage. Newly laid eggs
116 were transferred to larger containers for development. Populations were maintained under
117 laboratory conditions at 24°C.

118

119 ***Heat resistance***

120 Heat resistance of adult flies was established by exposing flies to 41, 42, 43, 44 and
121 45°C and subsequently scoring survival. Flies were transferred to glass vials equipped with foam
122 stoppers and exposed to the temperature treatments for 120 minutes in temperature controlled water
123 baths. The exposure time was chosen to keep temperature treatments ecologically relevant (rather
124 than choosing a shorter exposure time at higher temperatures). Ten individuals per vial and five
125 vials per treatment were tested per temperature, sex and population. After exposure individuals
126 were allowed to recover for 24 h at 24°C before mortality was scored. Individuals that were able to
127 walk in a coordinated fashion after gentle stimulation with a fine brush were considered survivors.
128 The age of the tested flies was 5 days $\pm$ 24 hours.

129

130 ***Locomotor activity***

131 Locomotor activity of the flies, comprising primarily walking but also jumping and
132 brief flight in the vial, was scored using a Locomotor Activity Monitor (LAM) (TriKinetics,
133 Waltham, USA) and measured at 24°C and 43°C. Flies were transferred in groups of 5 to glass vials
134 and placed in the LAM. Each vial was 6.5 cm long and 2.5 cm wide and was positioned
135 horizontally in the LAM, with each end being sealed with foam stoppers allowing gas exchange.
136 Flies were provided with food during the experimental period, whereby a 1.5 ml Eppendorf tube
137 containing a sugar solution (80g/l) was placed inside the foam stopper and was sealed with cotton
138 (for further details see Bahrndorff et al. 2012). During fly monitoring, vials were placed in a
139 temperature cabinet (Binder, Tuttlingen, Germany). Light was provided using a LED light source
140 emitting 70 lux at the level of the flies. Parchment paper in front of the vials was used to ensure
141 equal light intensity in all vials. The light program followed a photoperiod of 16:8 h (L:D) (16 h of
142 light from 07.00 h to 23.00 h followed by 8 h of complete darkness (0 lux) from 23.00 h to 07.00 h).
143 We only measured male locomotor activity to increase the number of replicates and since the fitness
144 effects are easiest to interpret in this sex (Bahrndorff et al. 2012). We tested 5 males per vial and 10
145 replicate vials per temperature treatment and population. Under natural conditions flies frequently
146 occur in very high densities so we believe that testing multiple individuals in a vial provides a
147 realistic measure of locomotor activity and avoids effects of social isolation that typically reduce
148 activity. Each temperature treatment (24°C and 43°C) was carried out independently. The age of the
149 tested flies was 6 days $\pm$ 24 hours.

150

151 ***Flight performance***

152 The flight mills were constructed according to Rowley et al. (1968) and were used to
153 estimate flight performance (distance) for flies at two temperatures, 24°C and 41.5°C. The flight
154 mills were cylindrical (15x6 cm in diameter and height) and equipped with two thin 5 cm long
155 arms, where a thin nylon thread of 1-2 cm was fixed at the tip. A fly was attached to the nylon
156 thread with a minimal drop of heated wax and glue. Based on a pilot experiment it was found that
157 no higher temperatures could be used due to the use of wax to fix the flies. Flies used were 1 to 9
158 days of age, non-virgins, and from one of 7 experimental generations. Live flies were mounted on
159 one arm of the flight mills and a dead fly was mounted on the second arm as counterbalance. The
160 flight was recorded by registering the number of revolutions in 5-sec intervals (Briegel, Knusel, &
161 Timmermann 2001). Flight trials were set up daily at 9.00 a.m. and ran for 22 h until the next
162 morning under constant light. A computer-aided automated system recorded the total length of
163 flight over this period. 8 flight mills were used simultaneously during each run (i.e. temporal block).
164 Trials with less than 20 meters of recorded flight activity were rejected as it is likely that flies were
165 poorly mounted on the arm or had damaged wings (Kaufmann & Briegel 2004).

166

167 ***Wing measurements***

168 Right and left hind tibiae and wings were dissected from each individual used in the
169 flight experiment and glued on a paper sheet. They were then photographed under 10x
170 magnification using a camera (Leica DFC 490) connected to a computer. Digital measurements of
171 hind tibia length and wing vein landmark positions were extracted with the software tpsDig version
172 2.14 (<http://life.bio.sunysb.edu/morph/>). We obtained tibia length, wing loading and wing aspect
173 ratio (as defined below) averaged from the right and left measurements. Wing length was measured
174 as the distance from the tip of proximal inner arc inwards from landmark 1 l to M_{1+2} , and width as

175 the distance $R_1 - M_3 + Cu_1$ as specified in Alves & Belo (2002). Hind tibia length is a good proxy for
176 overall structural size, whereas wing loading refers to body mass relative to the size or area of the
177 wing and was here taken as hind tibia length divided by wing length. Wing aspect ratio is a simple
178 measure of wing shape taken as the wing length divided by the width.

179

180 *Statistical analysis*

181 All analyses were performed with generalized linear (mixed) models, GL(M)Ms. We
182 used restricted error maximum likelihood (REML) available in the lmer() framework of the “lme4”
183 package implemented in R (R Development Core Team 2011). We performed analysis of deviance
184 on the full models to obtain estimates of the significance of the fixed factors, calculated with type-II
185 Wald F or χ^2 -tests following the suggestions of Bolker et al. (2009). Residual degrees of freedom
186 for the F -tests were adjusted according to Kenward & Roger (1997). Data were checked for
187 normality with Shapiro-Wilk tests and the residuals inspected visually. Data transformation was
188 used as specified below. We used Bonferroni corrections for multiple comparisons to assess
189 statistical significant effects (at the $P < 0.05$ significance level).

190 Survival after heat shock was analyzed with a GLM with binomial errors and logit
191 link. We included temperature, population and sex as fixed effects. Analysis of deviance was
192 conducted with type-II likelihood ratio χ^2 -tests.

193 Locomotor activity of the three populations was analyzed by summing the counts of
194 activity over 14h after removing the first two hours to allow acclimation to the new environment.
195 Temperature and population were included as fixed effects. Data were $\log_{10}$ -transformed to
196 homogenise the variance and improve normality.

197 Flight performance was taken as the overall summed flight distance over 14h for each
198 individual. Sample sizes ranged from 23 (Danish females at 24°C) to 31 (Danish males at 41.5°C).
199 We included temperature, population, sex and their interactions as fixed effects of interest. We also
200 added climate chamber (we had to change climate chambers during the experiment) as a fixed
201 blocking effect and flight mill as a random effect. Furthermore, fly age was included in all analyses
202 and additionally tibia length, wing loading or wing aspect ratio in respective separate analyses as
203 covariates to investigate their effect on flight distance. The dependent variable flight distance was
204 $\log_{10}$ -transformed prior to analysis.

205

206 **RESULTS**

207 Temperature data from climate stations close to the collection sites show that the
208 northernmost Danish site displays the lowest recorded temperature, lowest average low
209 temperature, and lowest average temperature (Table 1). The southernmost Spanish site exhibits the
210 highest average temperature and highest average high temperature. The Swiss site, located 569 m
211 a.s.l., shows intermediate values except for highest recorded temperature.

212

213 ***Heat resistance***

214 There was a significant effect of temperature on heat resistance ($\chi^2_2 = 913.19, P <$
215 0.001 ; Table 2; Fig. 1a-b). Spanish flies showed higher survival rates than Swiss and Danish flies at
216 high temperatures ($\chi^2_2 = 38.81, P < 0.001$). Male flies showed significantly lower survival rates than
217 female flies ($\chi^2_1 = 15.01, P < 0.001$; Fig. 1a-b). There was a marginally significant interaction
218 between temperature and population ($\chi^2_6 = 12.75, P = 0.047$), as Spanish flies could better endure

219 higher temperatures. When testing survival rates at each temperature there was a significant
220 population effect ($P < 0.05$ after Bonferroni correction) in males at 43°C ($\chi^2_2 = 11.43, P < 0.001$)
221 and at 44°C ($\chi^2_2 = 16.67, P < 0.001$). In females a significant difference was found at 43°C ($\chi^2_2 =$
222 10.62, $P = 0.005$). There was a significant interaction between temperature and sex because females
223 could better endure higher temperatures ($\chi^2_3 = 9.94, P = 0.019$).

224

225 *Locomotor activity*

226 Locomotor activity varied substantially between temperature treatments ($F_{1,54} =$
227 356.61, $P < 0.001$) and populations ($F_{2,54} = 6.80, P = 0.002$) (Fig. 2; Table 3). At 24°C locomotor
228 activity was highest in the Swiss population during the course of the day (temporal pattern not
229 shown), followed by the Spanish and Danish populations. At 43°C all populations showed a rapid
230 decrease in activity over time (not shown), and after 9 hours at 43°C the activity of the Danish flies
231 was reduced to 5% of their initial activity, whereas it was reduced to 17% and 23% for the Swiss
232 and Spanish populations, respectively. Generally the Spanish flies sustained higher activity levels at
233 this temperature compared to the Swiss and Danish flies, resulting in a significant population by
234 temperature interaction ($F_{2,54} = 4.21, P = 0.020$). Locomotor activity also differed between
235 populations at each temperature tested, which gave significant population effects ($P < 0.05$ after
236 Bonferroni correction) at both 24°C ($F_{2,27} = 9.81, P = 0.001$) and 43°C ($F_{2,27} = 3.68, P = 0.039$).

237

238 *Flight performance*

239 Temperature ($F_{1,299} = 25.68, P < 0.001$) and population ($F_{2,300} = 8.61, P < 0.001$)
240 significantly affected flight distance (Table 3). The flies flew shorter distances at 41.5°C, with the

241 Spanish (female) flies flying longest and the Danish (males) shortest (Fig. 3). The Spanish (female)
 242 flies also flew longest at 24°C, but here the Swiss (female) flies flew shortest, producing the
 243 significant temperature by population interaction ($F_{2,300} = 4.70, P = 0.010$) and a marginally non-
 244 significant sex difference ($F_{1,303} = 2.92, P = 0.089$). Individual comparisons for each sex at each
 245 temperature gave a significant population effect ($P < 0.05$ after Bonferroni correction) in males at
 246 41.5°C ($F_{2,71} = 6.10, P = 0.004$). In females a significant population effect was also found at 41.5°C
 247 ($F_{2,67} = 7.42, P = 0.001$) as well as a marginally non-significant effect at 24°C ($F_{2,67} = 2.47, P =$
 248 0.093). There was a significant effect of the covariate age ($F_{1,298} = 11.15, P < 0.001$; older flies flew
 249 longer; slope estimate = 0.06 ± 0.018 S.E.) and a marginally non-significant effect of wing aspect
 250 ratio ($F_{1,301} = 3.44, P = 0.065$; a tendency for longer flight of flies with more slender wings; slope
 251 estimate = 1.35 ± 0.72), but not hind tibia length ($F_{1,297} = 0.39, P = 0.532$, slope estimate = $0.17 \pm$
 252 0.28 S.E.) or wing loading ($F_{1,299} = 0.55, P = 0.446$; slope estimate = 2.02 ± 2.69). The population
 253 effect diminished when correcting for wing aspect ratio, becoming marginally non-significant ($F_{2,$
 254 $298 = 2.84, P = 0.060$).

255 To further investigate the importance of morphological traits on flight performance we
 256 tested for differences between populations, sex and their interaction (with experimental generation
 257 as a random effect). Spanish flies were the smallest followed by the Danish and the Swiss flies
 258 being the largest, males being larger than females (Fig. S1a). Population differences were similar
 259 for wing loading but females had the largest values for this trait (Fig. S1b). For wing aspect ratio,
 260 Spanish flies had narrower wings compared to Swiss and Danish flies, which were similarly shaped.
 261 Furthermore, males had narrower wings than females and showed a larger difference between
 262 populations than females (Fig. S1c). Population and sex effects were highly significant ($P < 0.001$)
 263 for all three traits in spite of the lack of an effect on flight performance of body size and wing
 264 loading (Table S1). The population by sex interactions were significant for wing aspect ratio ($F_{2,307}$

265 = 9.51, $P < 0.001$), but not for body size ($F_{2,305} = 1.47$, $P = 0.232$) and wing loading ($F_{2,307} = 0.17$, P
266 = 0.847). The similar results obtained with hind tibia length and wing loading can be partly
267 explained by a significant positive correlation of these two covariates (Pearson's $r = 0.48$, $P <$
268 0.001). Wing loading and wing aspect ratio were also correlated (Pearson's $r = 0.40$, $P < 0.001$), but
269 not hind tibia length and wing aspect ratio (Pearson's $r = -0.09$, $P = 0.108$).

270

271 **DISCUSSION**

272 We compared three populations of *M. domestica* originating from different thermal
273 habitats with respect to heat resistance and two behavioural traits (flight and locomotor activity,
274 plus associated wing morphology) measured at both benign and stressful temperatures. The results
275 suggest adaptive differentiation in heat resistance and behavioural traits between populations. The
276 Spanish adult flies survived best at excessively hot temperatures and Danish flies survived worst.
277 These results are comparable to what has been found in other insect species (*Drosophila*:
278 Hoffmann, Shirriffs, & Scott 2005; butterflies: Karl, Janowitz, & Fischer 2008). Similarly, Spanish
279 flies flew the longest and Danish flies the shortest at high temperatures, which was also evident for
280 locomotor activity. These patterns were in contrast to those seen at benign temperatures, giving rise
281 to significant interactions and thus differences in behavioural plasticity among the populations.
282 Similar to these results, a few other studies have found differences between populations and the
283 sexes in flight at suboptimal conditions (Fasolo & Krebs 2004; Merckx, Karlsson, & Van Dyck
284 2006). The morphological parameters body size and wing loading did not affect flight distance,
285 although there was a tendency for flies with narrower wings to fly longer (marginally non-
286 significant effect of wing aspect ratio). The fact that Spanish flies showed the highest heat
287 resistance and were able to sustain flight and locomotor activity for a longer period at a stressful
288 high temperature agrees with the high temperatures recorded at the site of origin (Table 1). Taken

289 together, and in accordance with other work (e.g. Krebs & Thompson 2006; Fasolo & Krebs 2004),
290 our study suggests that exposure to and avoidance of stressful high temperatures under natural
291 conditions has led to adaptive differences between populations, and that warm adapted populations
292 have evolved behavioural phenotypes that may confer an advantage under very high temperatures.

293 Studies have suggested that many organisms use regulatory behaviours (e.g. habitat
294 selection and/or thermoregulation) to buffer or even modify the selective environment that they
295 actually experience (Huey, Hertz, & Sinervo 2003). Avoidance behaviour may therefore be selected
296 under natural conditions and thus lead to genetic differentiation in behavioural traits. Our study
297 suggests genetic population differentiation, both in heat resistance and behavioural traits, despite the
298 flies' high dispersal capacity (and hence gene flow), which should neutralize local genetic
299 differentiation (Sultan & Spencer 2002). Therefore, even in a highly plastic behavioural trait like
300 insect flight, our data indicate that underlying physiological adjustments are important for escaping
301 high temperatures. These results are in agreement with Huey & Pascual (2009) who found that
302 thermoregulatory behaviour only partially compensated for ambient temperature variation in *D.*
303 *melanogaster* because flies in the field were active at temperatures that are very different from their
304 preferred body temperature. Fasolo & Krebs (2004) also found that male *Drosophila mojavensis*
305 sustained courtship behaviour even at temperatures close to their upper thermal limit. These
306 examples clearly suggest that flies cannot afford to operate strictly at their preferred body
307 temperatures, but have to remain active over a broad thermal range to maximize their fitness.
308 Furthermore, under many circumstances the available microclimates may simply not allow for any
309 appreciable behavioural thermoregulation (Gibbs, Perkins, & Markow 2003; Kearney, Shine, &
310 Porter 2009).

311 Patterns of flying and locomotor activity differed between benign and stressful
312 temperatures. Populations showed clear differences in locomotor activity at benign conditions,

313 being highest for Swiss flies, whereas flight distance did not vary substantially among the
314 populations at benign conditions. Locomotor activity has been found to be correlated with fitness
315 related activities, such as mating success (Cobb, Connolly, & Burnet 1987; Kyriacou 1981;
316 Partridge, Ewing, & Chandler 1987; Wisco, Matles, & Berrigan 1997). The large population
317 differences in locomotor activity at benign temperatures could therefore relate to the importance of
318 mating behaviour in combination with possible population specific densities at mating sites as well
319 as different optimal temperatures for the populations tested. However, mating behaviour likely
320 plays a minor role at extreme temperatures as all resources are then allocated to sustaining vital
321 processes or avoidance of the stressful environment.

322 At very high temperatures both flight and locomotor activity differed between
323 populations as Spanish male flies sustained locomotor activity for longer time at 43°C compared to
324 the Danish and Swiss flies. Our results are in agreement with findings in *Drosophila* showing
325 genetic differentiation between populations or genotypes differing in thermal sensitivities for
326 locomotor activity at high temperatures (Kjærsgaard et al. 2010; Latimer, Wilson, & Chenoweth
327 2011). Contrary to locomotor activity of flies, flight likely plays a major role in avoiding stressful
328 conditions. In *D. melanogaster*, heat shock hardening prior to flight at high ambient temperatures
329 increases the likelihood of reaching a food resource in the field (Loeschcke & Hoffmann 2007), but
330 it also increases the survival probability after exposure to even more severe heat stress. This
331 suggests that some aspects of flight capacity and survival after exposure to high temperature are
332 shared and that flight indeed plays a role in thermal adaptation. We found population differences in
333 flight distance at 41.5°C, which is well below temperatures at which mortality increased markedly
334 in the heat resistance assay. Hence selection likely occurs already at lower, sublethal temperatures
335 (Krebs & Thompson 2006), which should be taken into account in comparative studies (Huey
336 1982).

Wing loading has been hypothesized to be involved in adaptation of flight to the thermal environment encountered. In colder environments larger wing size relative to body size should evolve to counteract lower wing beat frequency by producing more lift per stroke (e.g. Starmer & Wolf 1989; Frazier et al. 2008). The results of our study did not support this hypothesis because wing loading (hind tibia length/wing length) did not affect flight capacity in our flight mills, although low temperatures were not tested here. This is in agreement with a field study on *D. melanogaster*, where selection on adult size, wing length and thorax length, were found to be partly independent of temperature (Hoffmann et al. 2007). Body size (hind tibia length) also did not affect flight distance, contrary to a recent finding in the yellow dung fly (*Scathophaga stercoraria*) where larger flies flew longer, more than compensating for their larger absolute energy demands (Kaufmann, Reim, & Blanckenhorn 2013). As expected from the temperature-size rule, the phenomenon that ectotherms usually get smaller as developmental temperature increases (Atkinson 1994; Walters & Hassall 2006), the Spanish flies were the smallest in terms of hind tibia length (Fig. S1a). However, unexpectedly the Swiss flies were larger than the Danish flies, although altitudinal differences could confound the comparison as the Swiss flies were collected at a higher site approximately 570 meters a.s.l. Nevertheless, annual monthly mean temperatures in Zurich, Switzerland, still exceed those observed at the Danish collection site (Table 1). Strong differentiation in body size among *M. domestica* populations has been found in the United States largely independent of latitude (Bryant 1977). This indicates that local biotic and abiotic factors can affect body size independent of flight performance. A trend for wing aspect ratio (wing length/width) to affect flight was found, however (Table 3): flies with greater wing aspect ratio (narrower wings) flew longer on average in our experiment, suggesting that wing shape is generally important for flight performance. Danish flies had the broadest wings relative to their length (Fig. S1c) presumably producing more lift per stroke (Starmer & Wolf 1989). The Spanish flies had the

361 narrowest wings, presumably promoting manoeuvrability while broader wings may improve
362 acceleration (Wootton 1981). This population difference in wing shape explains why the population
363 effect diminished when correcting for wing aspect ratio. However, we cannot be certain that wing
364 aspect ratio is the target of selection or whether some other correlated trait caused this effect on
365 flight performance.

366 Altogether the results of this study suggest adaptive differentiation for heat resistance,
367 flight and locomotor activity and associated wing morphology in populations of *M. domestica*
368 originating from different thermal habitats in Europe. Most notably, locomotor and flight behaviour
369 seem to have evolved under different selection pressures, which is perhaps not surprising as they
370 serve different purposes at different biological scales. At benign temperatures, behavioural
371 differentiation is evident primarily in locomotor activity, likely due to ecological factors such as
372 mating behaviour and population density, whereas flight is more responsive to stressful high
373 temperatures.

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519 **Figure 1:** Proportional survival (mean $\pm$ S.E., n = 5) after heat stress for three populations of *Musca*
520 *domestica*, male (a) and female (b). Flies were exposed to the temperature treatments for 120
521 minutes and survival (walk in a coordinated fashion) was estimated after 24 h of recovery at 24°C.

524 **Figure 2:** Male locomotor activity per fly plotted for three populations of *Musca domestica* under
525 benign (24°C) and stressful conditions (43°C) (mean $\pm$ S.E., n= 10). Activity was measured for 14
526 hours under light conditions.

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534 **Figure 3:** Flight distance plotted for three populations of *Musca domestica* under benign (24°C)
535 and stressful conditions (41.5°C) (mean $\pm$ S.E.). Male (a) and female (b) flight was measured for 14
536 hours under light conditions. Sample sizes ranged from 23 to 31 between treatments.

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542 **Tables**

543 Table 1: Yearly temperature parameters at the collection sites from nearest weather stations
544 (<http://www.weatherbase.com/weather>): Aarhus (56.18°N, 10.37°E), Zürich (47.23°N, 8.34°E) and
545 Barcelona (41.17°N, 2.04°E).

Site	Aarhus	Zürich	Barcelona
	(Denmark)	(Switzerland)	(Spain)
Average temperature (°C) ^a	7	9	15
Average high temperature (°C) ^b	11	12	20
Average low temperature (°C) ^b	3	6	11
Highest recorded temperature (°C) ^c	33	35	33
Lowest recorded temperature (°C) ^c	-24	-20	-7

546 ^a The mean of the daily (24 h) temperature, ^b The mean of the highest or lowest temperature recorded daily (24 h), ^c
547 Highest or lowest temperature recorded at the site.

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554 **Table 2:** Analysis of deviance for heat shock survival
 555 with likelihood-ratio χ^2 -tests.

Source	LR χ^2	df	P ($> \chi^2$)
Temperature	913.19	3	< 0.001
Population	38.81	2	< 0.001
Sex	15.01	1	< 0.001
Temperature $\times$ Population	12.75	6	0.047
Temperature $\times$ Sex	9.94	3	0.019
Population $\times$ Sex	1.29	2	0.524
Temperature $\times$ Population $\times$ Sex	1.32	6	0.970

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567 **Table 3:** Analysis of deviance for locomotor activity (only 24°C) and flight distance
568 with type-II Wald F-tests. The covariates hind tibia length, wing loading and wing
569 aspect ratio were tested for their effect on flight by including each respective covariate
570 in the model in separate analyses.

Trait	Source	F	df	Residual df	P (> F)
Locomotor activity	Temperature	356.61	1	54	< 0.001
	Population	6.80	2	54	0.002
	Temp:Pop	4.21	2	54	0.020
Flight distance	Temperature	25.68	1	299	< 0.001
	Population	8.61	2	300	< 0.001
	Sex	2.92	1	303	0.089
	Temp:Pop	4.70	2	300	0.010
	Temp:Sex	1.78	1	302	0.183
	Pop:Sex	0.37	2	302	0.694
	Temp:Pop:Sex	0.40	2	300	0.667
	Age	11.15	1	298	< 0.001
	Hind tibia length	0.39	1	297	0.532
	Wing loading	0.56	1	299	0.455
	Wing aspect ratio	3.44	1	301	0.065

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576 **Supplemental material**

577 Figure S1: Mean ($\pm$ S.E.) (a) hind tibia length, (b) wing loading and (c) wing aspect ratio in male
578 and female flies for the Danish (DK), Swiss (CH) and Spanish (SP) population of *Musca domestica*.

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587 Fig. S1a

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Fig. S1b

Fig. S1c

611 Table S1. Analysis of deviance for the morphological traits with type-II
612 Wald *F*-tests.

Trait	Source	<i>F</i>	df	Residual df	<i>P</i> (> <i>F</i>)
Hind tibia length	Population	102.07	2	305	< 0.001
	Sex	44.99	1	305	< 0.001
	Pop:Sex	1.47	2	305	0.23
Wing loading	Population	36.25	2	306	< 0.001
	Sex	815.68	1	307	< 0.001
	Pop:Sex	0.17	2	307	0.847
Wing aspect ratio	Population	105.49	2	306	< 0.001
	Sex	189.31	1	307	< 0.001
	Pop:Sex	9.51	2	307	< 0.001

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