

1 **Extensive sexual wing shape dimorphism in *Drosophila melanogaster*, *Ceratitis capitata*, and *Musca***  
2 ***domestica***

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4 Natalia Siomava<sup>1</sup>, Ernst A. Wimmer, Nico Posnien\*

5

6 University Goettingen, Johann-Friedrich-Blumenbach-Institute of Zoology and Anthropology, Göttingen Center  
7 for Molecular Biosciences (GZMB), Department of Developmental Biology, Ernst-Caspari-Haus, Justus-von-  
8 Liebig-Weg 11, 37077 Göttingen, Germany

9

10 <sup>1</sup>current address: Department of Anatomy, Howard University College of Medicine, Washington DC, USA

11

12 \*Corresponding author:

13 [nposnie@gwdg.de](mailto:nposnie@gwdg.de)

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19

20 **Abstract**

21 The ability to powered flight facilitated a great evolutionary success of insects and allowed them to occupy  
22 various ecological niches. In addition to primary tasks, wings are often involved in various premating  
23 behaviors, such as courtship songs and initiation of mating in flight. These specific implications require certain  
24 wing morphology, size, and shape. Although wing properties have been extensively studied in *Drosophila*, a  
25 comprehensive understanding of sexual shape dimorphisms and developmental plasticity in wing morphology  
26 is missing for other Diptera. To acquire this knowledge, we applied geometric morphometrics and analyzed  
27 wing shape in three dipteran species (*Drosophila*, *Ceratitis*, and *Musca*) raised in different environmental  
28 conditions. We extensively studied sexual dimorphism and impact of sex and environment on the adult wing  
29 morphology. We present allometric and non-allometric shape differences between males and females and  
30 show that wing shape is influenced by rearing conditions in a sex dependent manner. We determine common  
31 trends in shape alterations and show that the anterior and posterior crossveins are likely to be plastic regions  
32 changing substantially at different environmental conditions. We discuss our data in the light of vein  
33 development and hypothesize that the observed shape differences might recapitulate different mating  
34 behaviors and flight capabilities.

35

## 36 Introduction

37 Insects represent the only group of arthropods that developed the ability of a powered flight. This adaptation  
38 allowed them to occupy various ecological niches including air and led to a high morphological variation and  
39 great ecological success of the entire class. Flying helps insects to surmount long distances in a relatively short  
40 time, facilitating basic tasks such as finding mating partners and food resources. In modern insects, wings  
41 acquired special significance in other essential processes, e.g. mating and defense. For instance, certain  
42 species use their wings to perform courtship songs, which reflect size and vigor of males and help females to  
43 choose the right mating partner<sup>1,2</sup>. Some insects mate in the air while flying<sup>3</sup>; others initiate the mating process  
44 in flight but always land prior to copulation<sup>4</sup>. These different behaviors together with the intersexual food  
45 competition and various reproductive roles cause a constant selective pressure and result in different kinds of  
46 sexual dimorphisms, including variation in size and shape of insect wings<sup>5,6</sup>.

47 It has been shown that both genetic background and environmental cues contribute to the size variation of  
48 distinct body parts, and wings in particular, across individuals of the same species<sup>7-13</sup>. Variation in growth  
49 rate leads to sexual size dimorphism (SSD), which can be either male- or female-biased, depending on whether  
50 males or females are larger<sup>14-16</sup>. The key component of the SSD is genetically defined in most insects, but the  
51 final body and organ size is determined by environmental changes that occur during development, i.e.  
52 developmental plasticity<sup>17</sup>. Moreover, in response to different rearing conditions, organ size may alter in a sex  
53 specific manner<sup>13,18</sup>. For instance, in female-biased systems, females overgrow males under certain  
54 environmental conditions. It results in disproportionate growth of certain body parts, e.g. wings, and the  
55 whole animal grow to a larger size<sup>19,20</sup>. In this case, wing size changes are likely to be accompanied by shape  
56 changes to assure that the wing remains functional. And indeed, it has been shown that in *Drosophila*  
57 *melanogaster* size and shape of wings are regulated by similar processes during patterning and differentiation  
58 of wing imaginal discs at larval and pupal stages<sup>21-23</sup>. Due to this tight morphological and developmental  
59 coupling, wing size and shape have been considered together for a long time<sup>24</sup>, and research on variation in  
60 shape was inseparably linked to variation in size. At present, however, development of advanced  
61 mathematical methods and geometric morphometrics approaches made it possible to disentangle these two  
62 parameters and analyze size and shape independently<sup>25-29</sup>.

63 Various quantitative techniques were used in order to describe phenotypic variation and possible influence of  
64 rearing conditions on wing shape in *Drosophila* and other insects<sup>30-33</sup>. Although we have an advanced  
65 understanding of sexual shape dimorphism (SShD) in insect wings in general<sup>5,6,14,24</sup>, sexual specificity of the  
66 wing shape changes is still not clear. Thus, we do not know whether shape of male and female wings respond  
67 differently to environmental cues and, if yes, to what extent.

68 We have recently shown that the three dipteran species *Drosophila melanogaster*, *Ceratitis capitata*, and  
69 *Musca domestica* exhibit a clear wing SSD and that the response of wing size to different rearing conditions is  
70 sex dependent<sup>13</sup>. Therefore, these three species represent an excellent model to test whether wing shape  
71 changes in a similar sex dependent manner. In this study, we applied geometric morphometrics to compare

72 and comprehensively describe variation in wing morphology between *Drosophila*, *Ceratitis*, and *Musca*.  
73 Analysis of the total sexual shape dimorphism (SShD) and its non-allometric component revealed that SShD is  
74 not uniform across species and that the reaction of wing shape to different rearing conditions varies between  
75 sexes. We identified two most variable regions, the radio-medial (r-m) and basal-medial-cubital (bm-cu)  
76 crossveins, that changed similarly among the three species in response to various larval densities and  
77 temperature regimes. This finding suggests that these regions may represent developmentally less robust wing  
78 compartments. We discuss our findings in the light of different mating behaviors in *Drosophila* and *Ceratitis*,  
79 which produce courtship songs<sup>2,34</sup>, and *Musca*, which initiates mating by a strike prior to copulation<sup>4</sup>.

80

## 81 **Materials and Methods**

### 82 **Fly species**

83 For this study, all experiments were performed using three different fly species (Table 1). We used the highly  
84 inbred laboratory strain *Drosophila melanogaster* w1118, which was kept at 18°C on standard food (400 g of  
85 malt extract, 400 g of corn flour, 50 g of soy flour, 110 g of sugar beet syrup, 51 g of agar, 90 g of yeast extract,  
86 31.5 ml of propionic acid and 7.5 g of Nipagin dissolved in 40 ml of Ethanol, water up to 5 l). The other two flies  
87 were *Musca domestica* wild type ITA1 collected in Italy, Altavilla Silentia in 2013 (Y. Wu and L. Beukeboom,  
88 GELIFES, The Netherlands) and *Ceratitis capitata* wild type Egypt II (IAEA). The *Musca* strain was reared at  
89 room temperature (RT) (22±2°C) on food composed by 500 g of wheat bran, 75 g of wheat flour, 60 g of milk  
90 powder, 25 g of yeast extract, 872 ml of water and 18.85 ml of Nipagin (2.86 g of Nipagin in 10 ml of Ethanol).  
91 Adult *Musca* flies were provided with sugar water. *Ceratitis* were kept at 28°C, 55 ± 5% RH on a diet composed  
92 by 52.5 g of yeast extract, 52.5 g of carrot powder, 2 g of Sodium benzoate, 1.75 g of agar, 2.25 ml of 32% HCl,  
93 5 ml of Nipagin (2.86 g of Nipagin in 10 ml of Ethanol), water up to 500 ml for larvae. For adult flies, we used a  
94 1:3 mixture of sugar and yeast extract.

### 95 **Treatment of experimental groups**

96 To generate a range of sizes for each species, we applied two environmental factors known to influence the  
97 overall body size – temperature and density. Prior to the experiment, *Drosophila* flies were placed at 25°C for  
98 two days. On the third day, flies were moved from vials into egg-collection chambers and provided with apple-  
99 agar plates. After several hours, we started egg collection by removing apple-agar plates with laid eggs once  
100 per hour. Collected plates were kept at 25°C for 24 h to allow embryonic development to complete. Freshly  
101 hatched first-instar larvae were transferred into 50 ml vials with 15 ml of fly food. Three vials containing 25  
102 larvae each (low density) and three vials with 300 larvae each (high density) were moved to 18°C; the second  
103 set of six vials with the same densities was left at 25°C.

104 *Ceratitis* flies were kept at 28°C and allowed to lay eggs through a net into water. Every hour, eggs were  
105 collected and transferred on the larval food. After 22 h, first-instar larvae were transferred into small Petri  
106 dishes (diameter 55 mm) with 15 ml of the larval food in three densities: 25 (low density), 100 (middle density)

107 or 300 (high density) larvae per plate. Two plates of each density were moved to 18°C. The second set of six  
108 plates was left at 28°C for further development.

109 Eggs of *Musca* were collected for 24 h in the wet larval food at RT. Next day, all hatched larvae were removed  
110 from food, and only larvae hatched within the next hour were transferred into 50 ml vials with 5 g of food.  
111 Collection of larvae was repeated several times to obtain two experimental sets with three replicates of three  
112 experimental densities 10 (low density), 20 (middle density) or 40 (high density) larvae. One set of nine vials  
113 was moved to 18°C, the other was left at RT.

114 After pupation, individuals of *Ceratitis* and *Musca* were collected from food and kept in vials until eclosion with  
115 a wet sponge, which was refreshed every second day.

116 The experimental temperature regimes were chosen for the following reasons. *D. melanogaster* is known to  
117 survive in the range 10 – 33°C, but flies remain fertile at 12 – 30°C with the optimum at 25°C<sup>35</sup>. Reproduction  
118 temperatures in *Ceratitis* range from 14°C to 30°C with the optimum at 28°C<sup>36,37</sup>. Opposite to *Ceratitis*, *Musca*  
119 flies survive at 10 – 35°C<sup>38</sup> with the optimum at approx. 24°C<sup>39</sup>. The low temperature for our experiment was  
120 chose as the one above the survival and fertile minimums for all three species – 18°C. The warm temperature  
121 was aimed to be optimal for each species. During analysis, however, we discovered that RT was likely lower  
122 than optimal for *Musca*<sup>13</sup>. Therefore, results for this species we interpreted with some caution (see below).

### 123 **Data collection**

124 For each combination of temperature and density, we randomly picked at least five flies of each sex in *Musca*  
125 and 10 flies of each sex in *Drosophila* and *Ceratitis*. Both wings were dissected, embedded in *Roti*<sup>®</sup>-Histokitt II  
126 (Roth, Buchenau) on a microscope slide, and photographed under the Leica MZ16 FA stereo microscope with  
127 the Q Imaging Micro Publisher 5.0 RTV Camera.

128 Wing shape was analyzed using landmark-based geometric morphometric methods<sup>40,41</sup>. We digitized 11  
129 anatomically homologous landmarks on wings of the three species (Fig. 1). To provide a better coverage of the  
130 wing surface of *Ceratitis* and *Musca* wings, we included two additional landmarks (12 and 13). The landmarks  
131 were the following (nomenclature is given after<sup>42</sup>): 1, branching point of veins R<sub>1</sub> and R<sub>5</sub> (base of R<sub>2+3</sub> and R<sub>4+5</sub>);  
132 2, branching point of veins R<sub>2+3</sub> and R<sub>4+5</sub>; 3, intersection of veins C and R<sub>1</sub>; 4, intersection of vein R<sub>4+5</sub> and  
133 crossvein *r-m* (anterior crossvein); 5, intersection of crossvein *r-m* and vein M<sub>1+2</sub>; 6, intersection of vein M<sub>1+2</sub>  
134 and crossvein *i-m* (posterior crossvein); 7, intersection of crossvein *i-m* and vein M<sub>3+4</sub>; 8, intersection of M<sub>3+4</sub>  
135 and the wing margin; 9, intersection of veins C and R<sub>2+3</sub>; 10, intersection of veins C and R<sub>4+5</sub>; 11, meeting point  
136 of the anal part of the wing and the alula; 12, intersection of veins CuA<sub>2</sub> and A<sub>1</sub>+CuA<sub>2</sub> (*Ceratitis*) or the tip of  
137 vein A<sub>2</sub> (*Musca*); 13, intersection of A<sub>1</sub>+CuA<sub>2</sub> and the wing margin (*Ceratitis*) or the tip of vein A<sub>1</sub>+CuA<sub>2</sub> (*Musca*).

### 138 **Procrustes superimposition and growth trajectories**

139 Wing images were digitized using tpsUtil<sup>43,44</sup> and tpsDig2<sup>45,44</sup> in order to obtain raw x and y landmark  
140 coordinates. Using superimposition methods, it is possible to register landmarks of a sample to a common

141 coordinate system in three steps: translating all landmark configurations to the same centroid, scaling all  
142 configurations to the same centroid size, and rotating all configurations until the summed squared distances  
143 between the landmarks and their corresponding sample average is a minimum scaling<sup>46,47</sup>. To follow these  
144 three steps, we applied the generalized Procrustes analysis (GPA)<sup>47,48</sup> in MorphoJ 1.05f<sup>49,50</sup>. The wings were  
145 aligned along the R<sub>4+5</sub> vein (landmarks 1 and 10), the mean configuration of landmarks was computed, and  
146 each wing was projected to a linear shape tangent space. The coordinates of the aligned wings were the  
147 Procrustes coordinates. It has been already shown that fly wings exhibit directional asymmetry<sup>51</sup>. Because  
148 asymmetry was not of interest in this study, we averaged coordinates for the right and left wings of each  
149 individual. If only one wing was present, it was used as the mean. The obtained averaged Procrustes  
150 coordinates were further used in analyses as the shape variables.

151 The wing size in this study was quantified as wing centroid size (WCS), computed from raw data of landmarks  
152 (11 for *Drosophila* and 13 for *Ceratitis* and *Musca*) and measured as the square root of the sum of squared  
153 deviations of landmarks around their centroid<sup>26,47,48</sup>. Although the extraction of shape from landmarks in GPA  
154 removes major variation in size, at this step shape data still contain a size component – the allometric shape  
155 variation<sup>52,53</sup>. This variation accounts for shape changes that occur due to increase in size of the organ. For  
156 later analysis, this variation was removed. To determine growth trajectories and characterize morphological  
157 changes in response to wing size, we applied a multivariate regression of the Procrustes coordinates on WCS  
158 pooling among sub-groups of temperature and density. The amount of shape variation was given as a  
159 percentage of the total variation around the sample mean. The percentage numbers were computed to show  
160 the relative importance of allometry for shape variation in each species in general and in two sexes separately.  
161 A permutation test with 10,000 runs<sup>54,55</sup> was applied to test independence between size and shape changes.  
162 Additionally, we computed shape scores according to<sup>56</sup>. These shape scores are the shape variables associated  
163 with the shape changes predicted by the regression model. To visualize the association between size and  
164 shape, we plotted shape scores against WCS. Similarity between trajectories was estimated with the analysis  
165 of covariance (ANCOVA) in R software (*av()* package) with WCS being the explanatory variable<sup>57</sup>.

## 166 **Interspecies comparison**

167 To compare wing shape variation among species, we created a new dataset with all wings pooled together. All  
168 shape comparisons and permutation tests were performed using the MorphoJ software, version 1.05f<sup>49,50</sup>. To  
169 identify and remove the allometric component of the shape variation, we applied a multivariate regression.  
170 For the regression, we used WCS computed from the homologous landmarks, 1 to 11 for each species.  
171 Subsequently, we performed a Principal Component Analysis (PCA) to visualize the non-allometric component  
172 of shape in a scatter plot and visualized morphological differences by thin-plate spline (TPS) deformation  
173 grids<sup>40,47,58,59</sup>. Magnitudes of shape differences between fly wings were computed with the canonical variate  
174 analysis (CVA) and expressed in units of Procrustes distance, which is the square root of the sum of squared  
175 distances between corresponding landmarks. Significance of the results was tested with permutation tests  
176 using 10,000 runs.

177 **Intraspecies comparison: sexual dimorphism, temperature and density effects**

178 Comparison of wing shape within species was performed using 11 landmarks in *Drosophila* and 13 landmarks  
179 in *Musca* and *Ceratitis*. Procrustes ANOVA test performed in MorphoJ 1.05f to test whether there were effects  
180 of sex, rearing temperature, and density on wing shape. We found clear effects of each parameter and,  
181 therefore, continued with more detailed shape analysis.

182 All following analyses were performed using MorphoJ 1.05f. Sexual shape dimorphism (SShD) was estimated  
183 for allometric and non-allometric components of the shape variation together (total SShD) as well as for the  
184 pure shape only (non-allometric component). The size correction was performed for each species by using  
185 residuals of the allometric regression. Magnitudes of the SShD were estimated with the discriminant function  
186 analysis (DFA) and expressed in units of Procrustes distance. DFA identifies shape features that differ at most  
187 between groups relative to within groups and it can only be applied to contrast two experimental groups.  
188 Therefore, we used this method to define SShD (males and females), effects of the rearing temperature (high  
189 and low) in each species and density effect in two *Drosophila* groups (25 and 300 larvae per plate). To better  
190 visualize wing shape changes, we used species specific warped outline drawings with a different scale factor,  
191 mentioned in each figure legend. Additionally, we provide discriminant scores for each DFA. To estimate shape  
192 changes originated from the three rearing densities in *Ceratitis* and *Musca*, we applied canonical variate  
193 analysis (CVA), designed to estimate variation among three or more groups. In addition to CVA, we run a DFA  
194 for the two groups representing the density extremes (the highest and the lowest number of larvae per  
195 plate/vial). For each test, we ran a permutation test with 10,000 random permutations to test for the  
196 significance of the result<sup>54</sup>.

197

198

## 199 **Results**

### 200 **Wing shape variation in dipteran species**

201 To estimate differences in wing shape between *Drosophila*, *Ceratitis* and *Musca*, we digitized 11 landmarks on  
202 anatomically homologous points in all three species. The first ten landmarks marked vein intersections and the  
203 eleventh landmark was placed on the alula opening (Fig. 1). We averaged shape for the left and right wings for  
204 every individual and corrected for evolutionary allometry to exclude size, which is usually the main factor  
205 contributing to the shape variation<sup>60</sup>. Subsequently, we performed a PCA to visualize non-allometric  
206 components of shape in a scatter plot (Fig. 2).

207 With eleven landmarks included in the analysis, we found the largest morphometric distance between *Ceratitis*  
208 and *Drosophila* (Procrustes distance = 0.3072;  $p < 0.0001$ ) (Fig. 2, the inner panel). *Musca* wings were more  
209 similar to *Ceratitis* wings (Procrustes distance = 0.1857;  $p < 0.0001$ ) than to those of *Drosophila* (Procrustes  
210 distance = 0.2357;  $p < 0.0001$ ). The PCA revealed that the first two PCs accounted for almost 98% of the  
211 variation among species. The main shape difference was reflected in PC1 (80.9% of the variation) and  
212 represented the ratio between the proximal and distal parts of the wing. TPS deformation grids showed that  
213 *Ceratitis* wings were broad in the proximal part (landmarks 1-5, 11) and narrow in the distal part (landmarks 6-  
214 10). *Drosophila* wings represented the opposite case with the proximal part being heavily compressed along  
215 the anterior-posterior axis. *Musca* had an intermediate morphology being, however, more similar to *Ceratitis*  
216 (Fig. 2, TPS deformation grids along PC1). Interestingly, along PC1, *Ceratitis* male wings ( $PC1_{\text{mean}} = -0.17$ ) and  
217 *Musca* wings ( $PC1_{\text{mean}} = -0.04$ ) were almost equidistant from female *Ceratitis* wings ( $PC1_{\text{mean}} = -0.11$ ),  
218 demonstrating a strong sexual dimorphism in *Ceratitis*, comparable to the interspecies difference along this  
219 axis (Fig. 2).

220 The second significant PC explained 16% of the variation mainly accounting for the ratio between the length  
221 and width of the whole wing. In *Ceratitis* and *Drosophila*, landmarks 3, 7, and 8 were shifted from the center  
222 towards the margin, increasing the wing width (Fig. 2, TPS deformation grids along PC2). At the same time,  
223 landmarks 2 and 6 were displaced towards the center, resulting in shorter wings. Landmarks 9 and 10  
224 additionally increased these effects and *Ceratitis* and *Drosophila* wings were more compact in comparison with  
225 elongated *Musca* wings. Along the PC2 axis, sexual dimorphism in *Ceratitis* was less pronounced than the  
226 interspecies difference with *Musca*.

227

### 228 **Sexual dimorphism in growth trajectories**

229 In order to estimate the impact of the allometric component on wing shape in the three species, we pooled  
230 flies in sub-groups defined by sex, temperature, or density and performed a multivariate regression of shape  
231 on size<sup>61</sup>. In all three species, we found strong static allometry ( $p < 0.0001$ ; Table 2) but the amount of shape  
232 variation explained by variation in size was similar and relatively small in all species (*Ceratitis*: 4.03%; *Musca*:  
233 4.71%; *Drosophila*: 6.04%; Table 2).

234 When we performed the multiple regressions for males and females separately, we found signatures of static  
235 allometry for both sexes in *Drosophila* and *Ceratitis* ( $p < 0.0001$ ; Table 2 and Fig. 3). In *Musca*, we did not  
236 observe static allometry in females ( $p=0.061$ ) but in males it was highly significant ( $p=0.0008$ ) (Table 2). Sex  
237 specific regressions increased the predicted percentage of shape variation explained by size differences up to  
238 7.7 % (Table 2). An ANCOVA showed that growth trajectories were significantly different between sexes in all  
239 three species at the 5% confidence level (Table 3 and Fig. 3).

240

#### 241 **Sexual dimorphism in wing shape**

242 The PCA and growth trajectory analyses suggested a high level of sexual shape dimorphism (SShD) of wings in  
243 the three studied species (see also<sup>5,62,63</sup>). A Procrustes ANOVA with sex, rearing temperature, or density  
244 chosen as the main effects confirmed this finding (Table 4). Therefore, we first applied a DFA and characterized  
245 the extent of the SShD in *Drosophila*, *Ceratitis* and *Musca*. Subsequently, we split flies by sex into two groups  
246 and examined the effects of temperature and density on shape more closely.

247 The DFA revealed that male and female *Drosophila* wings were significantly different in shape, and both  
248 allometric and non-allometric components contributed to this difference (Fig. 4). The total SShD of *Drosophila*  
249 wings was highly significant ( $p < 0.0001$ ). After size correction, shape difference decreased but remained  
250 significant ( $p=0.01$ ), demonstrating a large impact of wing size on shape in both males and females. Male wings  
251 were broader than female wings, radial veins  $R_{2+3}$ ,  $R_{4+5}$ , and M1 were spread apart, but the length of the wing  
252 was not affected by the size correction. A comparison of the total SShD and non-allometric SShD in *Drosophila*  
253 suggested that the allometric component introduced variation in the wing length and resulted in shorter and  
254 more pointed female wings.

255 In contrast to *Drosophila*, the extent of the total vs. non-allometric wing SShD was very similar in *Ceratitis* and  
256 *Musca* (Fig. 4), suggesting that the allometric component has less impact on the shape in these two species.  
257 Similar to *Drosophila*, *Ceratitis* males had broader wings compared to females. Male wings were also shorter,  
258 mainly due to the contraction of the distal anterior region between landmarks 3-5, 9, and 10. *Ceratitis* wings  
259 were wider both proximally and distally, while *Drosophila* wings were rounded in its distal part only, the  
260 proximal part did not change. These results suggest that the major difference between sexes observed in  
261 *Ceratitis* can be explained by the non-allometric component.

262 In *Musca*, SShD was opposite to the other two flies: male wings were narrower than those of females, but the  
263 anal part of the wing was significantly enlarged. Also, male wings were slightly longer than those of *Musca*  
264 females (Fig. 4).

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## 268 Sexual dimorphism in response to rearing temperatures

269 To further characterize the effects of different rearing temperatures on wing shape in males and females  
270 separately, we first identified and removed the allometric component of the shape variation. In each species,  
271 the DFA analysis clearly assigned wings to one of two rearing temperatures (Fig. 5). In *Ceratitis*, rearing  
272 temperature slightly affected wing shape, but in *Drosophila* the effect was strong. In general, wings of flies  
273 grown at warm temperature (25°C for *Drosophila* and 28°C for *Ceratitis*) were broader than wings of those  
274 grown at 18°C (Fig. 5, orange and blue wing outlines). The highest plasticity and variation in width was  
275 observed in the proximal part of the wing in both species. Another temperature effect was a shortening of  
276 wings at warm temperature. In *Drosophila* and *Ceratitis*, the direction and strength of the shape change was  
277 similar in both sexes. In contrast, shape changes caused by temperature in *Musca* were not that consistent  
278 between sexes. In particular female wings underwent very light changes, while male wings were narrower at  
279 higher rearing temperatures (Fig. 5).

280 In addition to changes in the wing outline in general, we also found displacements of crossveins. A shift of the  
281 radio-medial (r-m) crossvein, defined by the landmarks 4 and 5, was similar in all three species (Fig. 1 and Fig.  
282 5). Interestingly, a shift of this vein was the only significant shape alteration in response to different rearing  
283 temperatures in wings of *Musca* females (Fig. 5). Displacement of the basal-medial-cubital (bm-cu) crossvein,  
284 defined by the landmarks 6 and 7, was variable in the direction and angle. In *Drosophila* wings, at higher  
285 temperature, the entire bm-cu crossvein was shifted towards the margin of the wing. In *Ceratitis*, the landmark  
286 7 did not change the position, but the upper part of the vein (landmark 6) was shifted along the M1 vein. The  
287 bm-cu crossvein was not affected in *Musca* females, but in males the landmarks 7 and 8 were displaced  
288 towards the wing center and resulted in a narrow wing.

289

## 290 Sexual dimorphism in response to different larval densities

291 Density is another powerful factor known to influence wing shape in insects<sup>25</sup>. In order to characterize the  
292 impact of the rearing density on wing shape, we grew flies in high and low densities. For *Ceratitis* and *Musca*,  
293 which are less studied in this respect, we set up intermediate density groups. In the analysis, we first focused  
294 on the two density extremes (the high and the low) because we expected to find there the most pronounced  
295 shape variation (Fig. 6). Later, we included the intermediate groups as well.

296 In *Drosophila*, the high rearing density resulted in elongated, narrowed and more pointed male wings with  
297 R<sub>2+3</sub>, R<sub>4+5</sub>, and M1 veins being close together (Fig. 6A(m)). *Drosophila* female wings responded in the opposite  
298 way: they became more rounded, mainly due to the stretching of the distal part and a shift of the R<sub>2+3</sub> and M1  
299 veins (Fig. 6A(f)). The r-m crossvein was displaced in both sexes, and the dm-cu crossvein was shifted in  
300 parallel with the r-m crossvein in females only (Fig. 6A). Statistical significance of the difference caused by  
301 different densities was confirmed with a permutation test for Procrustes distances run with the DFA

302 ( $p_{\text{male}}=0.004$ ;  $p_{\text{female}}=0.01$ ) and a separate Procrustes ANOVA test ( $F_{\text{male}}=10.22$ ,  $dF_{\text{male}}=18$ ,  $p_{\text{male}}<0.0001$ ;  
303  $F_{\text{female}}=16.23$ ,  $dF_{\text{female}}=18$ ,  $p_{\text{female}}<0.0001$ ) (Fig. 6A, discriminant scores).

304 Unlike *Drosophila*, *Ceratitis* male wings were shorter and slightly broader at the high density. The maximum  
305 change was observed in the proximal anterior region (Fig. 6B(m)). The same region, together with the anal part  
306 of the wing, was enlarged in female wings, but the length remained unaltered (Fig. 6B(f)). Again, we observed  
307 a displacement of the r-m and dm-cu crossveins, which was in the direction similar to *Drosophila*. The  
308 observed changes were statistically significant for the density extremes in both sexes (DFA and CVA  
309 permutation tests for Procrustes distances:  $p_{\text{male}}\leq 0.05$  and  $p_{\text{female}}\leq 0.007$ ; Procrustes ANOVA  $F_{\text{male}}=2.88$ ,  
310  $dF_{\text{male}}=22$ ,  $p_{\text{male}}<0.0001$  and  $F_{\text{female}}=3.62$ ,  $dF_{\text{female}}=22$ ,  $p_{\text{female}}<0.0001$ ) (Fig. 6B, discriminant scores). Wings of flies  
311 raised at intermediate densities exhibited an intermediate wing shape (Fig. 6B, discriminant scores and CVA  
312 scatter plots).

313 *Musca* males and females responded similarly to the high rearing density. The posterior part of the wing was  
314 decreased, the landmark 8 was shifted towards the wing center, the r-m and dm-cu crossveins were displaced  
315 but no variation in the wing length was found (Fig. 6C). In females, the landmark 3 and the whole C vein were  
316 shifted posteriorly and distinguished them from males. Despite the clear wing changes, the observed shape  
317 differences between the extreme rearing densities were not statistically significant in the DFA (permutation  
318 tests:  $p_{\text{male}}=0.05$  and  $p_{\text{female}}=0.1$ ) (Fig. 6C, discriminant scores), while the Procrustes ANOVA test clearly  
319 assigned wings in two distinct groups ( $F_{\text{male}}=1.74$ ,  $dF_{\text{male}}=22$ ,  $p_{\text{male}}=0.01$  and  $F_{\text{female}}=1.71$ ,  $dF_{\text{female}}=22$ ,  
320  $p_{\text{female}}=0.02$ ). Similarly, permutation tests for CVAs gave contradicting results: p-values from permutation tests  
321 for Procrustes distances were statistically insignificant ( $p_{\text{male}}=0.05$  and  $p_{\text{female}}\leq 0.1$ ), while p-values for  
322 Mahalanobis distances were highly significant for both sexes ( $p_{\text{male}}$  and  $p_{\text{female}}<0.0001$ ) (Fig. 6C, discriminant  
323 scores and CVA scatter plots). Because shape changes were similar in males and females in general, with the  
324 only exception of landmark 3, we pooled all wings together to increase the sample size and ran a CVA for 107  
325 flies of three densities (Fig. 6C, bottom CVA scatter plot). This analysis allocated the intermediate density  
326 group between two extremes, while the highest and the lowest density were statistically different in shape  
327 according to the p-values for Procrustes and Mahalanobis distances ( $p=0.03$ ;  $p<0.0001$ ) as well as for the  
328 Procrustes ANOVA test ( $F=2.64$ ,  $dF=22$ ,  $p<0.0001$ ).

329

## 330 Discussion

### 331 SShD in *Drosophila*, *Ceratitis*, and *Musca* wings

332 Besides interspecific differences in wing shape (Fig. 2), we found a clear SShD in all three species. In *Ceratitis*,  
333 variation between males and females in the proximal vs. distal part of the wing was so extensive that it was  
334 even comparable with the interspecific difference, e.g. between *Ceratitis* and *Musca* (Fig. 2, PC1) underpinning  
335 a strong sexual dimorphism, which was later tested and confirmed quantitatively (see Fig. 4). A detailed  
336 analysis of the SShD did not reveal any general trend among three species.

337 In *Drosophila*, we observed a very clear total SShD (Fig. 4). When the allometric component was removed, the  
338 difference became less prominent but still statistically significant. One of our observations was that male wings  
339 were more rounded and  $R_{2+3}$  and  $R_{4+5}$  veins were spread apart unlike in female wings. This observation is in  
340 accordance with previously published data by Bitner-Mathé and Klaczko, 1999<sup>25</sup>. In order to characterize the  
341 shape differences in more detail, we carefully analyzed all landmarks and found that male wings were mainly  
342 wider in their distal part, while the proximal part and vein intersects of this part were rather similar between  
343 sexes.

344 In *Ceratitis*, males could be distinguished from female by the variation in the width of the proximal and distal  
345 wing parts (Fig. 2, PC1 and Fig. 4). We also found an elongation of female wings. The lack of the wing width  
346 variation in our strain contradicts a previously published observation of male wings being shorter and wider  
347 than female<sup>34</sup>. These different conclusions might come from the different definition of the “width”. C.  
348 Churchill-Stanland et al. defined the wing width as a distance from the anterior costal bristle of the wing to the  
349 point of extreme curvature of the second anal cell<sup>34</sup>. Our approach includes the overall wing shape and shows  
350 that by chance the distance chosen by the authors includes a significantly expand anal region. The difference  
351 coming from that region enlarged in male and narrow in females might lead to the initial conclusion of SShD in  
352 the wing width.

353 In *Musca*, the anterior-posterior variation exceeded the proximal-distal variation (Fig. 2, PC2 and Fig. 4). Thus,  
354 *Musca* female wings were wider in the distal region and male wings were wider in the proximal anal region.  
355 The latter was the key difference that allowed to distinguish between sexes (Fig. 4).

356

### 357 Sexual dimorphism in size and shape relationships

358 Variation in size is often known to entail changes in shape<sup>27</sup>. Therefore, we tested whether a similar trend was  
359 present for wings of the three studied dipteran species. We compared growth trajectories between sexes,  
360 which supported the presence of SShD in the three species (Fig. 3, Table 3). In *Drosophila*, we found a clear  
361 contribution of the allometric component to the shape difference between males and females (Fig. 4). For  
362 instance, a shift of CuA1 along the wing margin described by<sup>25</sup>, we could only detect when the allometric  
363 component was included. In general, exclusion of the allometric coefficient decreased the SShD, suggesting

364 that most of the observed shape differences resulted from differences in wing size. Interestingly, in *Ceratitis*  
365 and *Musca*, the impact of the allometric component on wing shape in general was rather weak. Thus, the  
366 variation in the wing length that was explained by the allometric component in *Drosophila* was solely  
367 explained by the non-allometric component in *Ceratitis* and *Musca*.

368 Overall, we found a clear sexual dimorphism in wing shape in three species. A tight connection of the shape  
369 variation and wing size differences in *Drosophila* suggests that the growth regulation, patterning, and  
370 differentiation processes (e.g. vein placement and axis determination) during the larval wing imaginal disc  
371 development may be also tightly linked. These processes, however, may be less connected and independently  
372 regulated in the other two species. A potential mechanism for the link between the increase in wing size and  
373 shape variation may come from a recent analysis of Ethiopian *D. melanogaster* populations that showed that  
374 increased wing size caused by high altitude was accompanied by a loss of buffering against environmental  
375 perturbations<sup>64</sup>. It has recently been shown that the bone morphogenic protein (BMP) signaling pathways is  
376 buffered during embryonic dorsal-ventral axis formation by the action of the BMP-binding protein  
377 Crossveinless-2 and Jun N-terminal kinase (JNK) pathway activator Eiger (Egr)<sup>65</sup>. Since the BMP signaling (e.g.  
378 <sup>66-68</sup>) and JNK pathway are crucial regulators of wing size in *D. melanogaster*<sup>69</sup>, these pathways are excellent  
379 starting points to study a potential connection between developmental buffering mechanisms and the  
380 concerted size and shape control during development.

381

### 382 **Phenotypic plasticity in response to changing environmental conditions**

383 In 1999, Bitner-Mathé and Klaczko used a method of adjusted ellipses for the wing shape analysis in *Drosophila*  
384 and found neither displacement of radial veins nor rounding of wings in response to the rearing temperature<sup>25</sup>.  
385 Based on these findings, they suggested that in this conditions wing shape is more stable than wing size. In this  
386 study, we showed that despite the position of the longitudinal veins on the wing margin was only minorly  
387 affected, a significant difference in wing shape could be seen in *Drosophila* flies grown at different  
388 temperatures (Fig. 5).

389 Previous studies described a stronger response to temperature by the distal part of the wing leading to more  
390 alterations and displacements relative to the proximal part<sup>27</sup>. In contrast to this finding, we detected a high  
391 variation in proximal landmarks (Fig. 5, see landmarks 1, 3 and 11) and only mild changes in distal ones. One  
392 potential explanation for these discrepancies might be the range of rearing temperatures. While Debat et al.  
393 raised *Drosophila* flies at stressful temperatures (12°C and 14°C as the cold temperature and up to 30°C as the  
394 high temperature)<sup>27</sup>, we used intermediate regimes (18°C and 25°C). In their work, they showed the overall  
395 wing shape differences along the first two CVs. The variation in wing shape of flies raised at 12-25°C was  
396 mainly explained by the first CV, while the second CV distinguished the wings of the extreme temperatures  
397 (i.e. 12°C vs. 30°C; see<sup>27</sup> and Fig. 3 in there). It would be interesting to know what exact changes in the  
398 landmark configuration were explained by these two CVs. If the distal part of the wing was more responsive to  
399 stress conditions, which were not covered by our temperature regimes, the variation we saw in proximal

400 landmarks is likely to be somewhat similar to their CV1. We also found pronounced changes in the proximal  
401 part of the wing in *Ceratitis* but not in *Musca* (Fig. 5, see landmarks 1 and 2). In *Musca*, the proximal wing  
402 region was invariable at different rearing temperatures and all variation was restricted to the distal part (Fig.  
403 5).

404 Temperature regimes used in this study for the Italian *Musca* strain might be rather extreme than optimal<sup>13</sup>.  
405 The observed wing shape changes might therefore come from the stress response in *Musca*, similar to shape  
406 changes observed by Debat et al. for *Drosophila* (Fig. 3 in there)<sup>27</sup>. *Drosophila* and *Ceratitis* flies were reared at  
407 favorable temperatures and the pronounced changes in proximal regions observed in these flies might be the  
408 actual response to temperature regimes within the normal stress-free reaction norm.

409 In addition to changes in wing shape along the proximal-distal axis, our shape analysis revealed a high variation  
410 in the positioning of the r-m (landmarks 4 and 5) and bm-cu (landmarks 6 and 7) crossveins that was common  
411 for all three species. Displacement of these veins was found in all temperature and density groups, suggesting  
412 that this region represent a very plastic aspect of wing patterning. Further support for this suggestion comes  
413 from the loss of buffering against environmental perturbations in high altitude Ethiopian *D. melanogaster*  
414 populations that showed an increase in wing size. It has been shown that this decanalization resulted in a  
415 higher level of abnormal wing development with various defects in crossvein development (i.e. incomplete,  
416 missing or additional crossveins)<sup>64</sup>. Wing vein development requires a proper integration of various central  
417 signaling pathways, such as epidermal growth factor receptor (Egfr), Notch, bone morphogenetic protein  
418 (BMP), Hedgehog (Hh) and Wnt signaling (reviewed in <sup>70-72</sup>). A potential link between these pathways and  
419 environmental perturbations has been suggested to be mediated by the heat shock protein Hsp90, since  
420 mutations in *Hsp83*, the gene coding for Hsp90, have been shown to result in various morphological  
421 abnormalities in adult traits, including the formation of additional crossveins<sup>73</sup>. Furthermore, an in-depth  
422 analysis of the effect of varying levels of *Hsp83* expression on wing shape in *D. melanogaster* revealed that  
423 Hsp90 contributes to the buffering of developmental processes against environmental differences<sup>74</sup>. In the  
424 study by Debat et al. (2006), the placement of the r-m and bm-cu crossveins was affected to different degrees  
425 depending on the genetic background of the studied flies. Since crossveins develop later than longitudinal  
426 veins<sup>72</sup> but still use similar signaling pathways (reviewed in <sup>70</sup>), these two vein types probably integrate the  
427 stress response differently either via Hsp90 or additional mechanisms. Additional factors are indeed likely  
428 because Debat et al. stated that Hsp90 alone cannot explain the entire canalization effect and they also  
429 propose the involvement of additional factors<sup>74</sup>. More targeted experimental and molecular studies are  
430 necessary to address the developmental basis of the crossvein plasticity in more detail in insect species other  
431 than *Drosophila*.

432

433 **Potential functional implications of plasticity and sexual dimorphism in wing size and shape for mating**  
434 **behavior**

435 Both size and shape of wings directly influence specific mating behaviors, such as flying or the generation of  
436 mating songs. In many dipteran fly species, male individuals produce species-specific courtship songs by fast  
437 and repetitive wing movements. For instance, Caribbean fruit fly females of *Anastrepha suspensa* judge the  
438 size and vigor of a potential mating partner by the intensity of its courtship song<sup>1,75,76</sup>. Apparently, the intensity  
439 and audibility of these songs directly depend on the wing-beat frequency that the fly can afford for certain  
440 energy costs and wing fragility. In other fly species, such as *M. domestica*, the mating process is initiated  
441 during flight by an attack (“mating strike”) of a male against the back or side of a female. A successful “mating  
442 strike” usually results in the immediate landing and start of copulation<sup>4</sup>.

443 Our morphometrics analysis revealed that *Musca* have wings that are longer and narrower than those of  
444 *Ceratitis* and *Drosophila* (PC2) (Fig. 2). This implies that *Musca* has the highest relative wing span (*b*) among the  
445 studied flies. The wing span is proportional to the moment of inertia<sup>77</sup> and, thus, the moment of inertia should  
446 also be the highest for *Musca* wings. Taking into account that the moment of inertia is inverse to the wing-beat  
447 frequency<sup>77</sup>, we conclude that the wing-beat frequency should be the lowest in *Musca* compared to the other  
448 two species. We also observed male *Musca* wings being more pointed and even slightly elongated compared  
449 to female wings, additionally increasing the moment of inertia and required inertial power. Therefore, in  
450 accordance with the “mating strike” behavior in flight, *Musca* wings may be less suited for buzzing, but their  
451 wing shape might be under selection for better flight performance, that is facilitated by long and narrow  
452 wings<sup>78</sup>.

453 In contrast to *Musca*, *Drosophila* and *Ceratitis* produce courtship songs by buzzing. Usually, their females favor  
454 males with a higher audibility<sup>2,34</sup>. Our study revealed that male wings were wider than female, shorter in case  
455 of *Ceratitis*, and radial veins were more spread apart making wings more compact (Fig. 4). The allometric  
456 component of shape additionally increased this difference in *Drosophila*. The short, wide and rounded wings of  
457 males in these species are likely to displace more air and repeat calling song pulses more quickly than long  
458 narrow wings, and the wing moment of inertia could be low enough to buzz. Interestingly, these flies produce  
459 two different types of wing vibration during the pre-mount courtship: the pulse song and sine song<sup>1,79–82</sup>. The  
460 sine song is a continuous sinusoidal humming generated by small amplitude wing vibrations<sup>83–87</sup>. In *Drosophila*,  
461 its frequency ranges from 110 to 185 Hz<sup>88</sup>, with the median value of approx. 160 Hz<sup>88</sup> or sometimes 130 Hz<sup>89</sup>.  
462 In *Ceratitis*, this frequency is similar to *Drosophila*, 165 Hz<sup>90</sup>. On the other hand, there is a difference in the  
463 pulse songs between these species. In case of *Drosophila*, it is composed of a series of single pulses (one to  
464 three cycles) separated by interpulse intervals. The frequency of these pulses is between 200–280 Hz with the  
465 median value of 240 Hz<sup>91,92</sup>. Instead of pulses, *Ceratitis* use a continuous vibration of wings when a male looks  
466 towards a female and keeps its abdomen bent ventrally<sup>80</sup>. An average frequency of such buzzing is about 350  
467 Hz<sup>1</sup>, which is almost half more frequent. Such high frequency of buzzing would require a lower moment of  
468 inertia, what could be achieved when the wing mass is concentrated near the axis of rotation<sup>93</sup>. Intriguingly,  
469 our shape analysis revealed major differences between *Ceratitis* and *Drosophila* exactly in this region –  
470 variation in the width in proximal vs. distal regions (Fig. 2, PC1 axis). Thus, *Ceratitis* had wider wings in the  
471 proximal part appropriate for high frequency buzzing and in *Drosophila* this part was narrower but perhaps

472 wide enough for low frequency buzzing. Although this hypothesis remains to be tested, it is tempting to  
473 speculate that these shape differences may be linked to the mating behavior of the flies and specific properties  
474 of their courtship songs.

475

#### 476 **Author Contributions**

477 N.P. and E.A.W. conceptualized the research; N.S. performed experiments, conducted data analysis, and  
478 curated data; N.S. was responsible for data visualization; N.S. and N.P. wrote the original draft and N.P., E.A.W.  
479 and N.S. revised the manuscript; N.P. administered the project.

480

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486

#### 487 **Data availability**

488 All data generated for this work is available from the authors upon request.

489

#### 490 **Competing Interests**

491 The authors declare that they have no competing interests.

492

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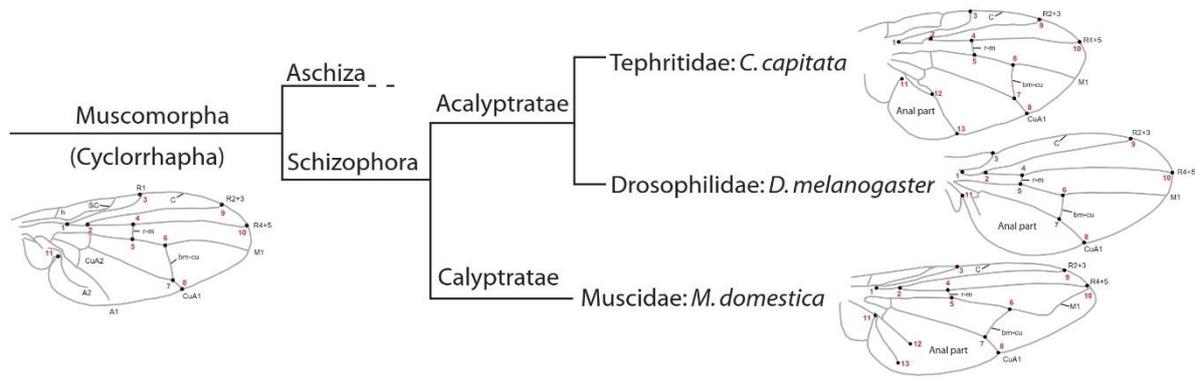
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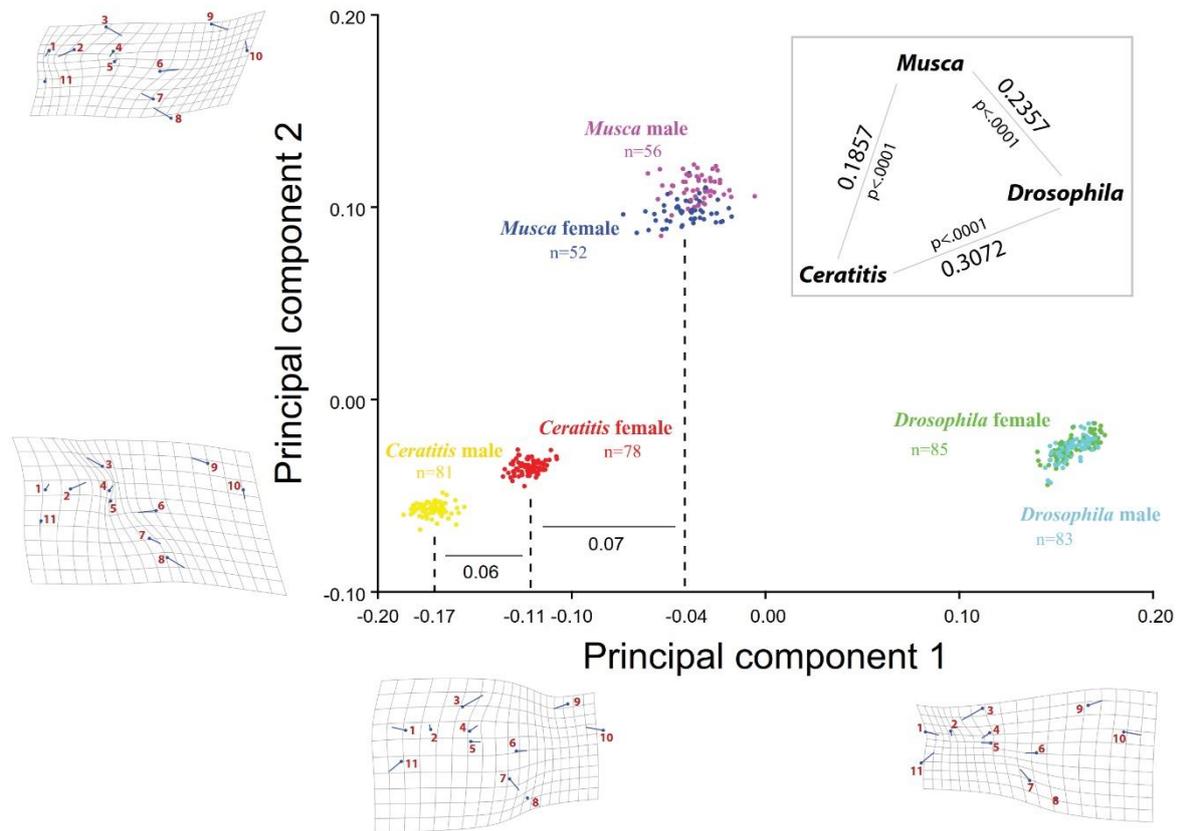
684 **Figures**



685 **Fig. 1 Phylogenetic tree of Muscomorpha with wing outlines and landmarks used for this study**

686 Homologous landmarks 1 to 11 as well as landmarks 12 and 13 in *Ceratitis* and *Musca* are shown as black  
 687 points with the respective number in red. Vein abbreviations: A – anal vein; bm-cu – basal-medial-cubital  
 688 crossvein; CuA – anterior cubital vein; C – costal vein; h – humeral crossvein; M – medial vein; R – radial vein; r-  
 689 m – radio-medial crossvein; SC – subcosta. Branch lengths of the tree do not indicate evolutionary time or  
 690 distance.

691

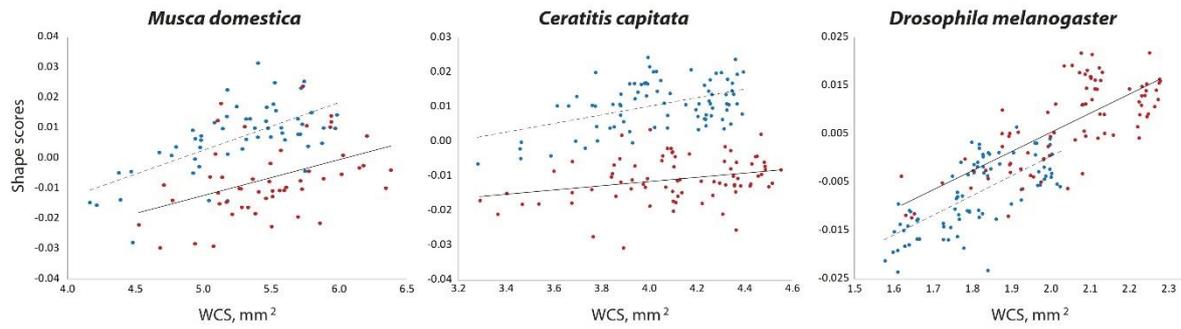


692 **Fig. 2 Wing shape variation between *D. melanogaster*, *C. capitata* and *M. domestica* after size correction**

693 Principal component analysis of shape scatter plot (PC1 and PC2) and associated shape change of the non-  
 694 allometric shape component of side averaged wings. The TPS deformation grids illustrate shape changes  
 695 indicating the relative shifts of landmarks along the axes with the PC scale factor +/-0.2. The dots on the grids  
 696 with the respective landmark number (red) indicate the starting point and the blue lines connect them to the  
 697 final shape. The mean values of shape variance along PC1 and their projections (dashed lines) are shown for  
 698 *Ceratitis* males and females, as well as for *Musca* flies. The inner panel shows morphometric distances  
 699 between wing shapes in the three species and p-values for them.

700

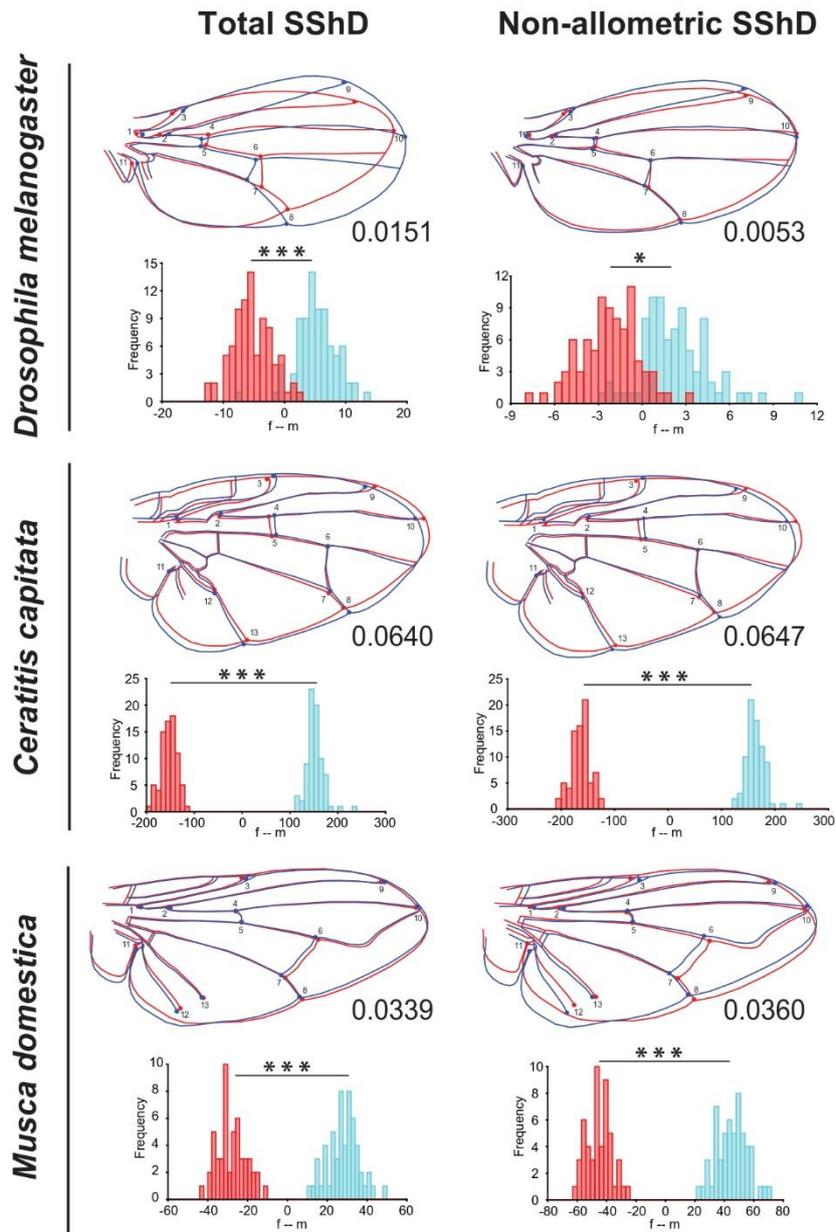
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702 **Fig. 3 Growth trajectories for *Drosophila*, *Ceratitis* and *Musca***

703 The growth trajectories are shown with shape scores as a function of WCS for males (blue dots and dashed  
704 line) and females (red dots and solid line) in the three species.

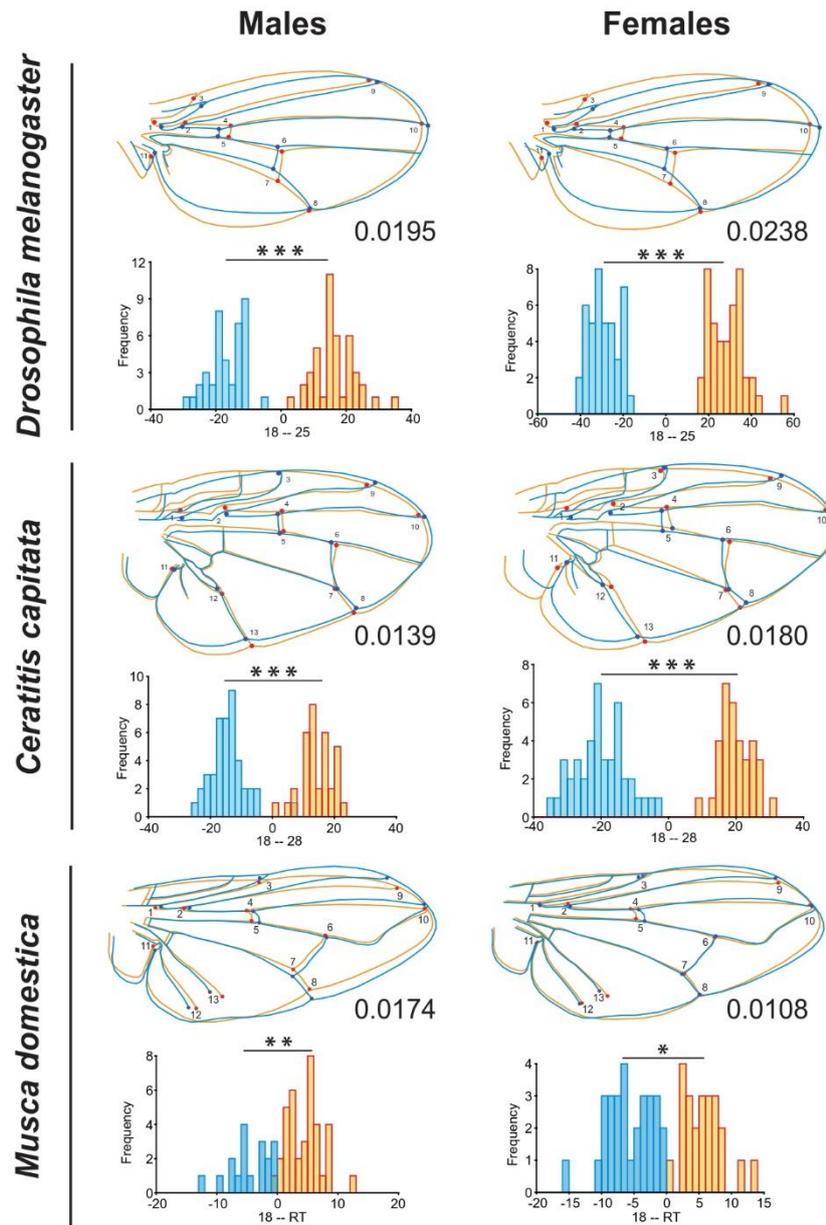
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706 **Fig. 4 Sexual shape dimorphism in wing shape**

707 Total and non-allometric SSHd in *D. melanogaster* (scale factor 9, n=168), *C. capitata* (scale factor 1, n=154)  
 708 and *M. domestica* (scale factors 1, n=107). Wing outlines represent differences between male (dark blue) and  
 709 female (red) average wing shapes. The magnitude of SSHd is indicated in units of Procrustes distance with the  
 710 corresponding p-values (\* p<0.05; \*\*\* p<0.0001). Histograms with the distribution of the discriminant scores  
 711 show shape separation into two distinct groups for each species.

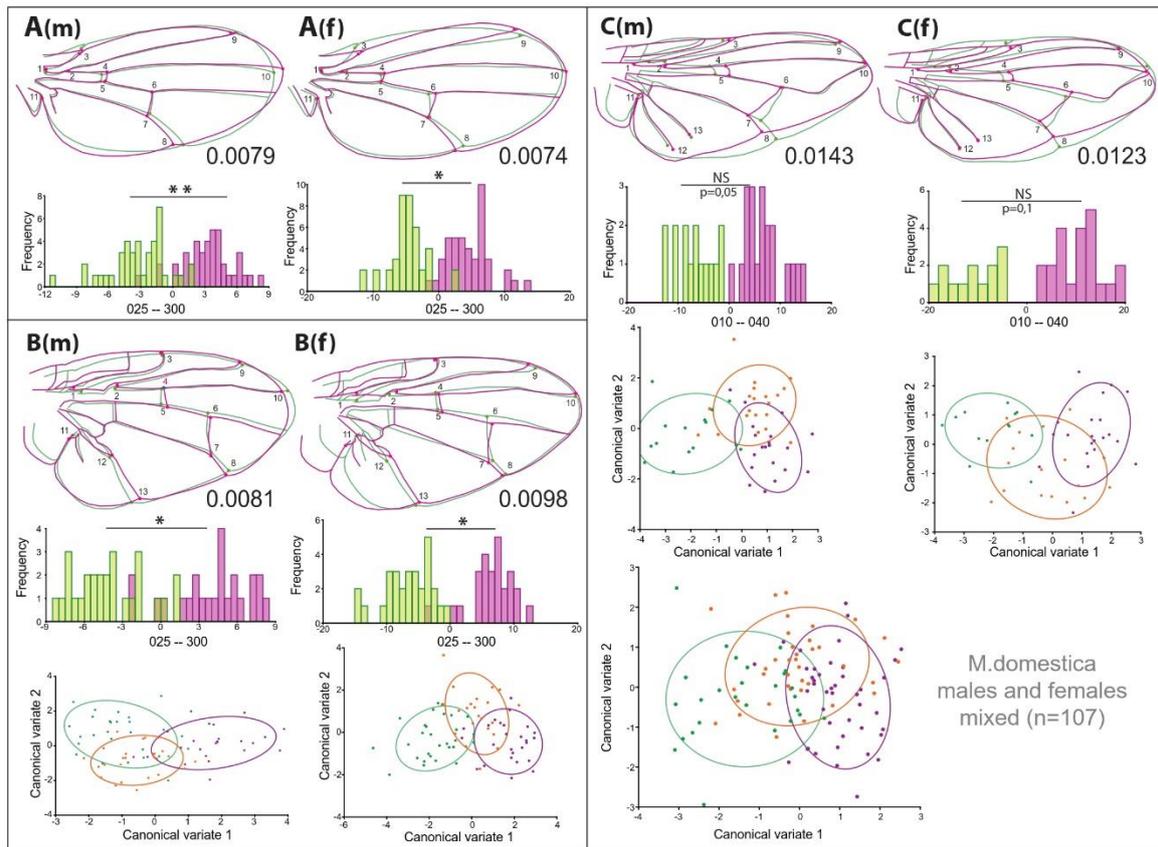
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713 **Fig. 5 Changes of wing shape under conditions of different rearing temperature**

714 Wing shape alterations in response to temperature in *D. melanogaster* (n(males)=83; n(females)=85), *C.*  
 715 *capitata* (n(males)=81; n(females)=78), and *M. domestica* (n(males)=56; n(females)=51). Wing outlines  
 716 represent differences between male cold (light blue) and warm (orange) temperatures with the scale factor 5  
 717 for each species. The magnitude of shape variation is indicated in units of Procrustes distance with the  
 718 corresponding p-values (\* p<0.05; \*\* p<0.001; \*\*\* p<0.0001). Histograms with the distribution of the  
 719 discriminant scores show shape separation into groups and significance of the changes.

720



721 **Fig. 6 Wing shape changes in response to different larval densities**

722 *D. melanogaster* males (a(m)) (n=83) and females (a(f)) (n=85), *C. capitata* males (b(m)) (n=51) and females  
 723 (b(f)) (n=56) and *M. domestica* males (c(m)) (n=36) and females (c(f)) (n=34). Wing outlines represent  
 724 differences between low (green) and high (violet) densities with the scale factor 9 for each species. The  
 725 magnitude of shape variation is indicated in units of Procrustes distance with the corresponding p-values (\*  
 726 p<0.05; \*\* p<0.001; \*\*\* p<0.0001). Histograms with the distribution of the discriminant scores show shape  
 727 separation into groups and significance of the changes. CVA scatter plots show distribution of different density  
 728 groups and the equal frequency ellipses with probability 0.75. The bottom right CVA scatter plot shows  
 729 distribution of *Musca* density groups for all flies together (n=107).

730

731 **Tables**

732 **Table 1 Summary of the fly species used for experiments in the present study, including strain origin and**  
733 **sample size (N)**

734 WCS is shown for landmarks 1 to 11 for each species.

| <b>Species</b>                 | <b>Strain</b>          | <b>Sex</b> | <b>N</b> | <b>Size range<br/>(WCS<sup>1-11</sup>, mm)</b> | <b>Median (IQR)<br/>(WCS<sup>1-11</sup>, mm)</b> |
|--------------------------------|------------------------|------------|----------|--|--|
| <i>Musca domestica</i>         | Italian strain<br>ITA1 | male       | 56       | 3.79 – 5.45                                    | 4.86 (4.53 – 5.10)                               |
|                                |                        | female     | 52       | 4.18 – 5.82                                    | 5.06 (4.75 – 5.34)                               |
| <i>Ceratitis capitata</i>      | Egypt II               | male       | 81       | 2.93 – 3.93                                    | 3.61 (3.46 – 3.82)                               |
|                                |                        | female     | 78       | 2.97 – 4.10                                    | 3.70 (3.56 – 3.93)                               |
| <i>Drosophila melanogaster</i> | w <sup>1118</sup>      | male       | 83       | 1.64 – 2.12                                    | 1.89 (1.81 – 2.00)                               |
|                                |                        | female     | 85       | 1.68 – 2.39                                    | 2.18 (2.01 – 2.25)                               |

735

736

737 **Table 2 Results of the multivariate regression of shape on size for males and females**

738 The shape variation predicted by each regression is shown as a percentage of the total shape variation. † –  
739 non-significant at 5% level.

| Species                        | Sex groups | N   | % Predicted | P-value            |
|--------------------------------|------------|-----|-------------|--------------------|
| <i>Musca domestica</i>         | All        | 107 | 4.71        | <0.0001            |
|                                | Males      | 55  | 7.70        | 0.0008             |
|                                | Females    | 52  | 3.86        | 0.061 <sup>†</sup> |
| <i>Ceratitis capitata</i>      | All        | 159 | 4.03        | <0.0001            |
|                                | Males      | 81  | 7.00        | 0.0003             |
|                                | Females    | 78  | 5.04        | 0.0025             |
| <i>Drosophila melanogaster</i> | All        | 168 | 6.04        | <0.0001            |
|                                | Males      | 83  | 7.04        | <0.0001            |
|                                | Females    | 85  | 7.18        | <0.0001            |

740

741 **Table 3 The effect of WCS, sex, and their interaction on the wing shape scores, tested with**  
742 **ANCOVA**

| Species                        | Effect  | Mean Squares | F       | p <sub>slope</sub> | p <sub>intercept</sub> |
|--------------------------------|---------|--------------|---------|--------------------|------------------------|
| <i>Musca domestica</i>         | WCS     | 0.001941     | 18.934  | <0.0001            |                        |
|                                | sex     | 0.006725     | 65.598  | <0.0001            | <0.0001                |
|                                | WCS:sex | 0.000079     | 0.771   | 0.382              |                        |
| <i>Ceratitis capitata</i>      | WCS     | 0.00025      | 6.379   | 0.01260            |                        |
|                                | sex     | 0.01928      | 497.782 | <0.0001            | <0.0001                |
|                                | WCS:sex | 0.00012      | 3.213   | 0.07500            |                        |
| <i>Drosophila melanogaster</i> | WCS     | 0.013784     | 423.268 | <0.0001            |                        |
|                                | sex     | 0.00066      | 20.257  | <0.0001            | <0.0001                |
|                                | WCS:sex | 0.000001     | 0.038   | 0.846              |                        |

743

744

745 **Table 4 Effects of sex, temperature and density on the wing shape scores, tested with Procrustes ANOVA**

746 Df – degrees of freedom

| Species                        | Effect      | Sums of squares | Mean squares | Df | F      | P      |
|--------------------------------|-------------|-----------------|--------------|----|--------|--------|
| <i>Drosophila melanogaster</i> | sex         | 0.00966851      | 0.0005371396 | 18 | 44.75  | <.0001 |
|                                | temperature | 0.01727365      | 0.0009596474 | 18 | 79.96  | <.0001 |
|                                | density     | 0.00742772      | 0.0004126512 | 18 | 34.38  | <.0001 |
| <i>Ceratitis capitata</i>      | sex         | 0.15685566      | 0.0071298028 | 22 | 467.98 | <.0001 |
|                                | temperature | 0.00340257      | 0.0001546623 | 22 | 10.15  | <.0001 |
|                                | density     | 0.00252489      | 0.0000573839 | 44 | 3.77   | <.0001 |
| <i>Musca domestica</i>         | sex         | 0.02733498      | 0.0012424991 | 22 | 33.41  | <.0001 |
|                                | temperature | 0.00534071      | 0.0002427593 | 22 | 6.53   | <.0001 |
|                                | density     | 0.00298555      | 0.0000678534 | 44 | 1.82   | .0008  |

747