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

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Abstract

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

Introduction

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

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

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

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

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

Materials and Methods

Fly species

For this study, all experiments were performed using three different fly species (Table 1). We used the highly inbred laboratory strain *Drosophila melanogaster* w1118, which was kept at 18°C on standard food (400 g of 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, 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 were *Musca domestica* wild type ITA1 collected in Italy, Altavilla Silentia in 2013 (Y. Wu and L. Beukeboom, GELIFES, The Netherlands) and *Ceratitis capitata* wild type Egypt II (IAEA). The *Musca* strain was reared at room temperature (RT) (22±2°C) on food composed by 500 g of wheat bran, 75 g of wheat flour, 60 g of milk 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). Adult *Musca* flies were provided with sugar water. *Ceratitis* were kept at 28°C, 55 ± 5% RH on a diet composed 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, 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 1:3 mixture of sugar and yeast extract.

Treatment of experimental groups

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

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

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

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

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

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

Data collection

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

Wing shape was analyzed using landmark-based geometric morphometric methods[40,41]. We digitized 11 anatomically homologous landmarks on wings of the three species (Fig. 1). To provide a better coverage of the wing surface of *Ceratitis* and *Musca* wings, we included two additional landmarks (12 and 13). The landmarks were the following (nomenclature is given after[42]): 1, branching point of veins R_1 and R_S (base of R_{2+3} and R_{4+5}); 2, branching point of veins R_{2+3} and R_{4+5}; 3, intersection of veins C and R_1; 4, intersection of vein R_{4+5} and crossvein *r-m* (anterior crossvein); 5, intersection of crossvein *r-m* and vein M_{1+2}; 6, intersection of vein M_{1+2} and crossvein *i-m* (posterior crossvein); 7, intersection of crossvein *i-m* and vein M_{3+4}; 8, intersection of M_{3+4} and the wing margin; 9, intersection of veins C and R_{2+3}; 10, intersection of veins C and R_{4+5}; 11, meeting point of the anal part of the wing and the alula; 12, intersection of veins CuA_2 and A_1+CuA_2 (*Ceratitis*) or the tip of vein A_2 (*Musca*): 13, intersection of A_1+CuA_2 and the wing margin (*Ceratitis*) or the tip of vein A_1+CuA_2 (*Musca*).

Procrustes superimposition and growth trajectories

Wing images were digitized using tpsUtil[43,44] and tpsDig2[45,44] in order to obtain raw *x* and *y* landmark coordinates. Using superimposition methods, it is possible to register landmarks of a sample to a common

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

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

Interspecies comparison

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

Intraspecies comparison: sexual dimorphism, temperature and density effects

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

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

Results

Wing shape variation in dipteran species

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

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

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

Sexual dimorphism in growth trajectories

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

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

Sexual dimorphism in wing shape

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

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

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

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

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[25]. 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_{2+3}, R_{4+5}, 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_{2+3} 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

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

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

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

Discussion

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

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

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

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

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

Sexual dimorphism in size and shape relationships

Variation in size is often known to entail changes in shape[27]. Therefore, we tested whether a similar trend was present for wings of the three studied dipteran species. We compared growth trajectories between sexes, which supported the presence of SShD in the three species (Fig. 3, Table 3). In *Drosophila*, we found a clear contribution of the allometric component to the shape difference between males and females (Fig. 4). For instance, a shift of CuA1 along the wing margin described by[25], we could only detect when the allometric 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[64]. 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)[65]. Since the BMP signaling (e.g.
378 [66–68]) and JNK pathway are crucial regulators of wing size in *D. melanogaster*[69], 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[25].
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[27]. 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)[27], 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[27] 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

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

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

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

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

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

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

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

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

Author Contributions

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

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Data availability

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

Competing Interests

The authors declare that they have no competing interests.

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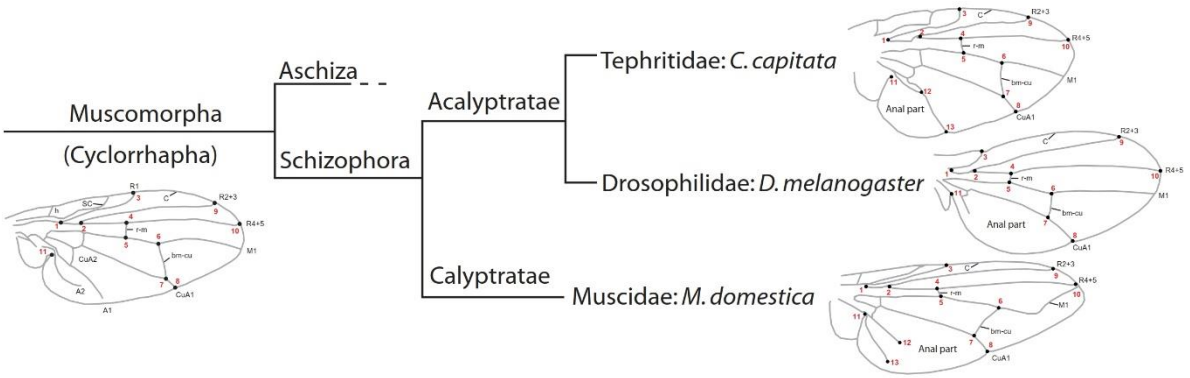
683

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-madial-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

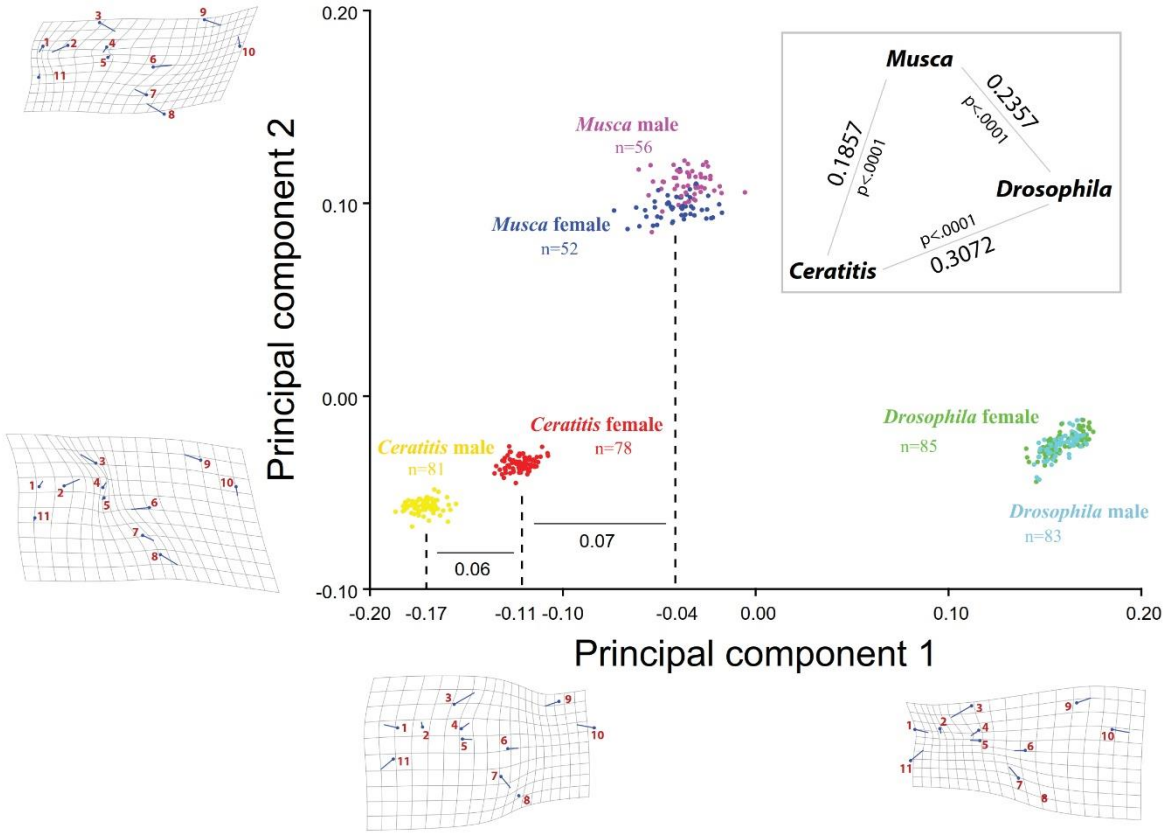


Fig. 2 Wing shape variation between *D. melanogaster*, *C. capitata* and *M. domestica* after size correction

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

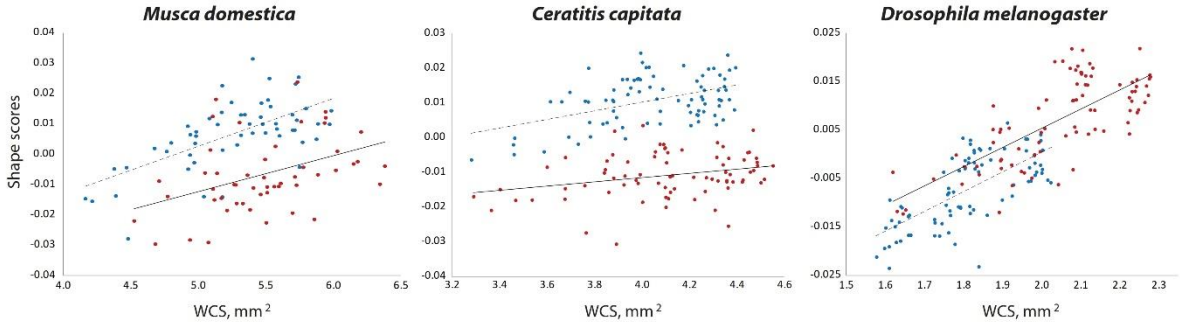
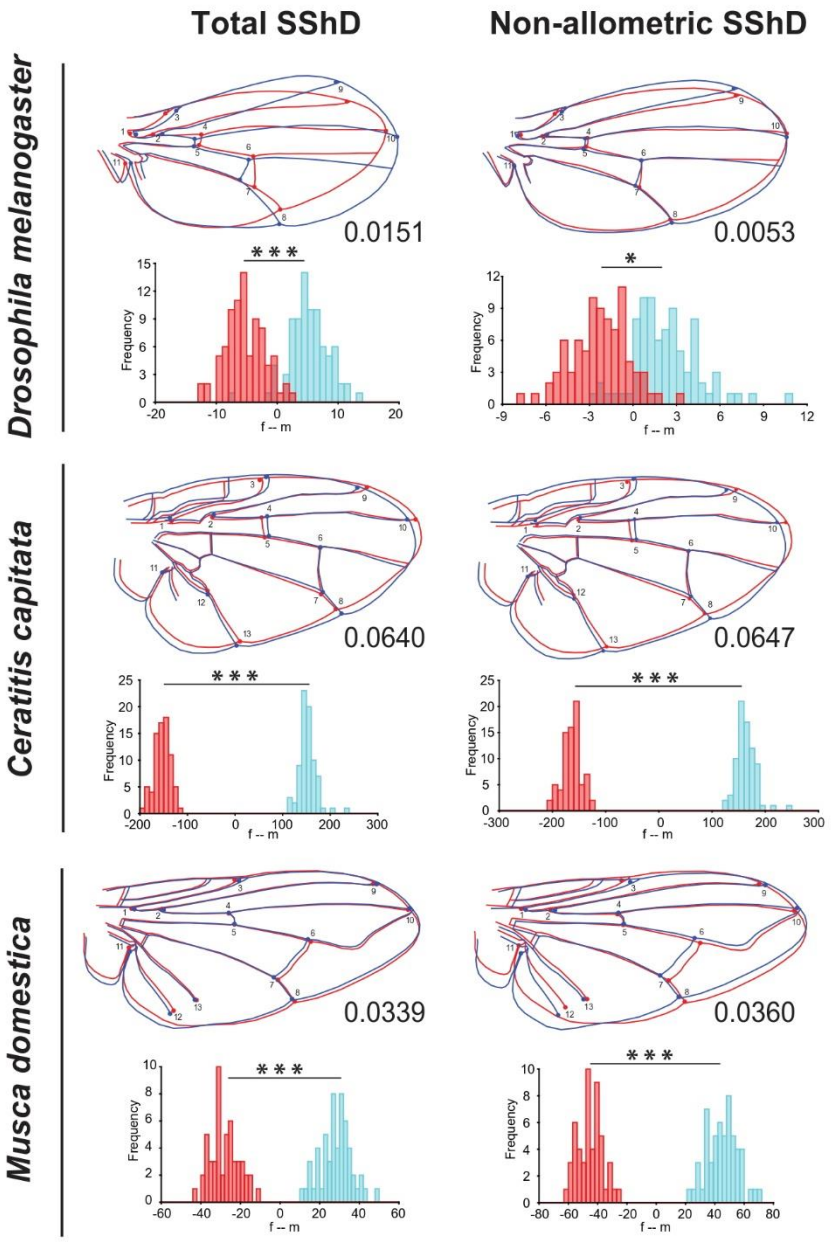


Fig. 3 Growth trajectories for *Drosophila*, *Ceratitis* and *Musca*

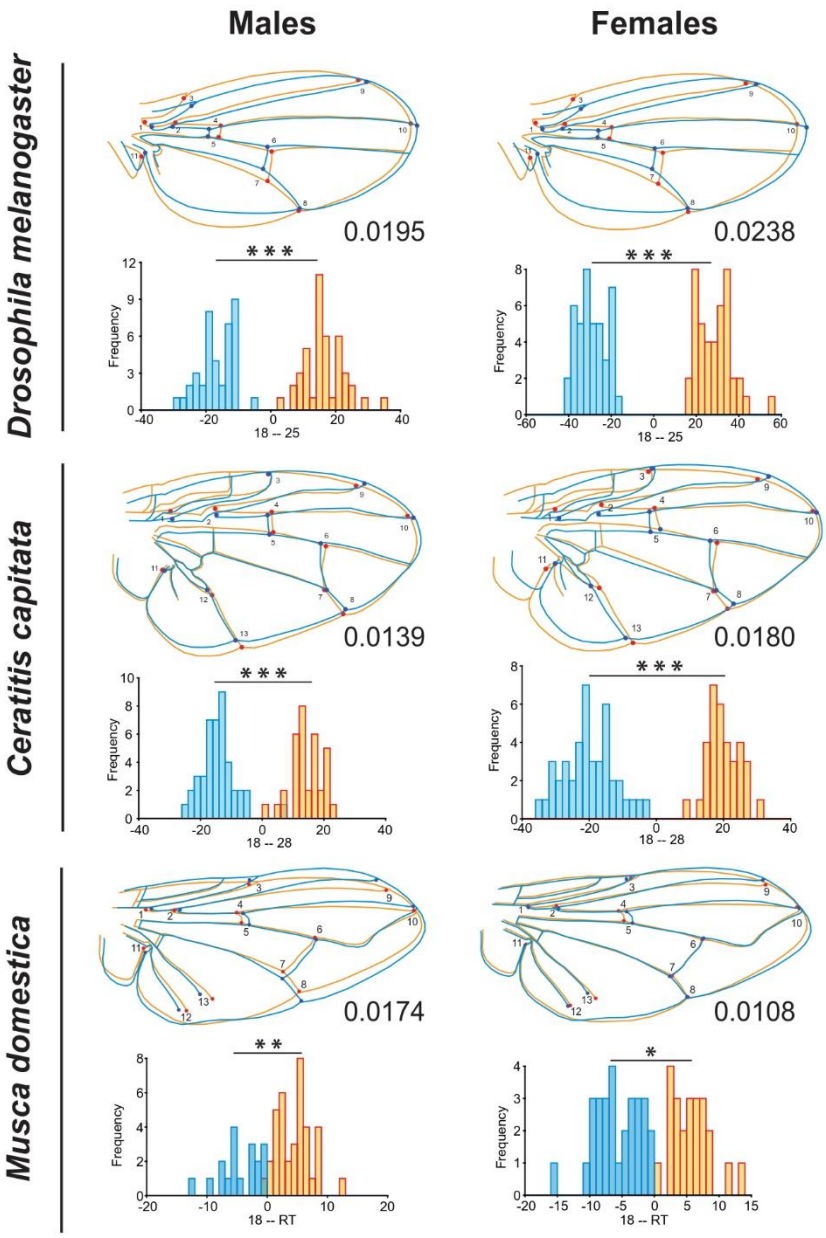
The growth trajectories are shown with shape scores as a function of WCS for males (blue dots and dashed line) and females (red dots and solid line) in the three species.



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.

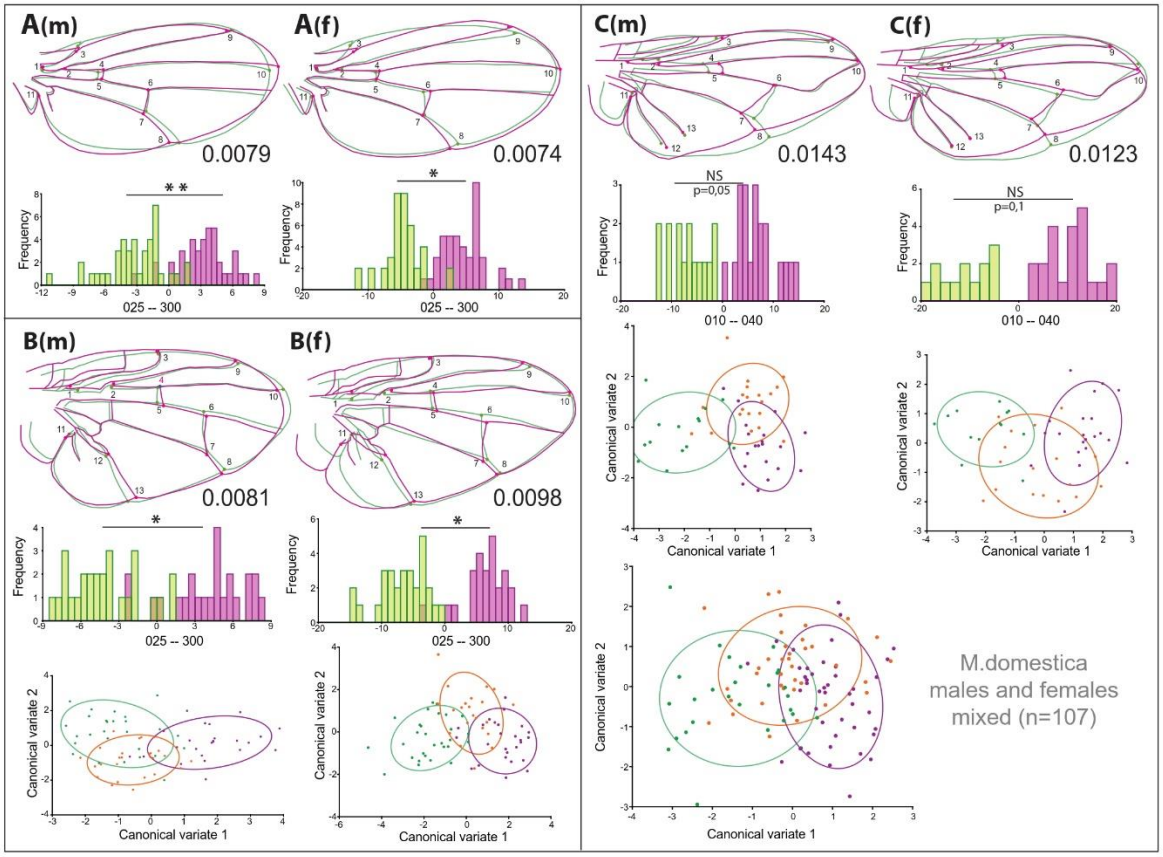
712



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.

Species	Strain	Sex	N	Size range (WCS^{1-11}, mm)	Median (IQR) (WCS^{1-11}, mm)
Musca domestica	Italian strain 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)
Ceratitis capitata	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)
Drosophila melanogaster	w^{1118}	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
Musca domestica	All	107	4.71	<0.0001
	Males	55	7.70	0.0008
	Females	52	3.86	0.061†
Ceratitis capitata	All	159	4.03	<0.0001
	Males	81	7.00	0.0003
	Females	78	5.04	0.0025
Drosophila melanogaster	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_{slope}	$p_{intercept}$
Musca domestica	WCS	0.001941	18.934	<0.0001	
	sex	0.006725	65.598	<0.0001	<0.0001
	WCS:sex	0.000079	0.771	0.382	
Ceratitis capitata	WCS	0.00025	6.379	0.01260	
	sex	0.01928	497.782	<0.0001	<0.0001
	WCS:sex	0.00012	3.213	0.07500	
Drosophila melanogaster	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
Drosophila melanogaster	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
Ceratitis capitata	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
Musca domestica	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