Potential Synergistic Effects of Temperature and Wolbachia on the Morphology of *Drosophila Melanogaster*

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Abstract. This study investigates the combined effects of Wolbachia infection and temperature on the wing morphology of *Drosophila melanogaster*, focusing on size, shape, and their potential heritability across generations. Utilizing a self-reproducing line of *Drosophila*, F1 flies were reared at four different temperatures (18°C, 25°C, 27.5°C, and 29°C), and wing morphology was analyzed using geometric morphometrics. Significant effects of temperature and Wolbachia infection on both wing size and shape were observed in the F1 generation, with larger wings and cells at lower temperatures and smaller wings at higher temperatures for Wolbachia-infected flies. These findings were consistent with previous studies on thermal plasticity and symbiont-host interactions. However, in the F2 generation, the wing size differences were not retained, while wing shape variation persisted, indicating that temperature and Wolbachia's combined effects on size were not heritable under normalized conditions. The study suggests a more complex inheritance pattern for wing shape, with environmental factors likely playing a key role in both size and shape changes. Future research should explore the molecular mechanisms underlying these interactions, particularly the role of Wolbachia density and gene expression in shaping morphological traits.

Keywords: *Drosophila melanogaster*, wolbachia, temperature, morphology.

1. Introduction

Morphological variations, encompassing physiology, phenology, and morphology, are often the result of complex interactions between genetic factors and environmental influences (Rosenzweig et al., 2008; Hua et al., 2016; Ficetola et al., 2016). Since the foundation of evolutionary biology, the study of morphological variation, including organ size and shape, has been a core theme (Darwin, 1859). *Drosophila melanogaster*, a model organism widely used in genetic and developmental studies, provides an ideal system for investigating these interactions (Tolwinski, 2017). Notably, morphological changes in *Drosophila* not only reflect the influence of genetic factors (Beckingham et al., 2005) but also respond sensitively to environmental changes (Journal of Entomology and Zoology Studies, 2016). Beyond that, biological influences, such as microbial symbionts, have also emerged as significant contributors to morphological development. *Wolbachia*, an intracellular symbiont that is maternally transmitted, infects many arthropod species, including *Drosophila melanogaster*, and exerts considerable influence over host reproductive and morphological traits. (Riegler & Sidhu, 2016). The use of *Drosophila* as a model organism has provided valuable insights into the mechanisms of host-microbe influences at the same time (Martino, 2016). Building on this foundation, this study seeks to explore the synergistic effects of *Wolbachia* infection and temperature variation on the morphological traits of *Drosophila melanogaster*, aiming to uncover their potential interactive mechanisms in morphological regulation.

Drawing from the well-established understanding of how *Drosophila melanogaster* responds to both symbiotic and environmental factors, this study focuses on the wings as the primary morphological trait due to several key reasons. First, *Drosophila* wings are highly plastic, with their size and shape responding rapidly to environmental stimuli, such as temperature changes, making them an ideal model for studying how environmental changes influence evolutionary trajectories (Loh et al., 2008). Additionally, established methodologies for analyzing wing morphology provide reliable and consistent tools, ensuring high reproducibility and accuracy in the results (Gilchrist et al., 2000). Furthermore, homology, both within populations and across species, provides a foundation for precise comparative analyses. The consistent positioning of anatomical landmarks, such as vein

intersections, ensures reliable quantification of morphological variations within populations. Lastly, *Drosophila* wings involve many conserved genes, such as Wnt, that are shared across species, further enhancing their utility as a model system (Komiya & Habas, 2008). The findings offer foundational insights into morphological regulation and may have applications in future studies on other organisms.

The *Drosophila melanogaster* used in this study were a self-reproducing population, ensuring no significant genotypic differences across individuals. This genetic consistency allows the observed morphological variations to be attributed primarily to environmental factors and *Wolbachia* infection status. Eight anatomical landmarks on the wing veins **(Figure 1)** will be used for subsequent shape and size analyses. Geometric morphometric analysis will precisely capture subtle morphological changes, enabling the quantification of shape and size to assess phenotypic plasticity under different environmental conditions. These analyses will be conducted across multiple experimental groups to address the following research questions: Are the left and right wings of *Drosophila* symmetrical? Do temperature and *Wolbachia* infection influence wing size and shape? If so, are these changes heritable?

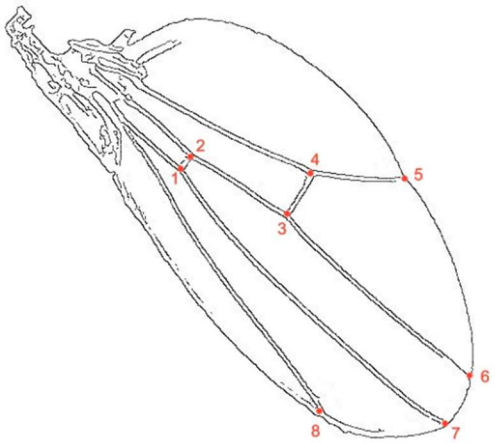

Figure 1. Schematic representation of the left wing of a male *Drosophila melanogaster*, with eight anatomical landmarks indicated. These landmarks, marked in red, represent critical vein intersection points that are key to the subsequent shape and size analysis using geometric morphometrics.

2. Methodology

2.1 Rearing and Maintenance

The Drosophila melanogaster line 799 used in this study was obtained from the Bloomington Drosophila Stock Center (Bloomington Drosophila Stock Center, Indiana University, Bloomington, IN, USA). Flies were reared in the laboratory under standardized conditions, with a 12-hour light/dark cycle at 25°C. The rearing medium comprised 10g of potato starch, 20g of yeast, 3.5g of yeast extract, 15g of dextrose, 6ml of nipagin, 2.5ml of propionic acid, and 550ml of water. Each rearing vial, a standard 5 ml container, was filled with 1.5 ml of food and sealed with a high-density sponge to ensure proper air circulation. To minimize larval competition, 30–40 eggs from F0 individuals were deposited in each vial. During the egg-laying period, F0 adults were maintained at 25°C under identical environmental conditions. A total of over 700 flies were used across all experimental groups, with multiple vials set up per group to account for potential random variation and ensure robust experimental replication.

2.2 Experimental Design and Treatments

For this study, *Drosophila melanogaster* from both *Wolbachia*-infected and uninfected (wild-type) lines were used to assess the combined effects of *Wolbachia* infection and temperature on wing

morphology. The F0 generation was initially maintained at 25°C under controlled laboratory conditions. The F1 generation was then divided into four temperature groups: 29°C, 27.5°C, 25°C, and 18°C. Flies were collected 24 hours after eclosion to ensure complete wing development and avoid any uncertainty related to morphological changes occurring post-eclosion. Additionally, F1 flies used for reproduction to generate the F2 generation exceeded the 24-hour window. To prevent potential effects of reproduction or prolonged adult life on morphology, these F1 flies were excluded from wing dissection and analysis. The F2 generation was reared at 25°C to assess transgenerational effects under consistent temperature conditions.

2.3 Data Collection

Wings from both the left and right sides of each individual were dissected and imaged under a stereomicroscope equipped with a digital camera. High-resolution images (3840x2160 pixels) were captured to ensure clear visualization for subsequent morphometric analysis. ImageJ2 software (Rueden et al., 2017), available through the Neuroimaging Resources website (https://imagej.net/software/imagej2/), was used to identify and mark anatomical landmarks on each wing. These landmarks were then utilized in geometric morphometric analysis to assess shape and size variations across the different experimental conditions.

2.4 Size Variation Analysis

To assess size variation in the wings, centroid size was calculated for each individual. Centroid size, a standard measure of overall wing size, is computed as the square root of the sum of squared distances between all anatomical landmarks and their centroid, providing a reliable estimate of wing size independent of shape (Baab et al., 2012).Centroid size data were collected for all individuals, and a two-way ANOVA was performed to evaluate the main and interactive effects of temperature and *Wolbachia* infection on wing size (Kim, 2014). Additionally, a one-sample t-test was conducted to determine whether the centroid size in each group significantly differed from a reference value or control group.

In order to further understand the causes of the observed size variations, we also examined the size of wing epidermal cells. Previous studies have shown that changes in wing size are primarily due to alterations in cell size rather than cell number (Alpatov, 1930; Masry & Robertson, 1979). A similar approach was applied to the cell size data, using a one-sample t-test to compare the observed values against a predetermined reference, allowing for a more refined assessment of how cellular changes contribute to overall size variation.Additionally, ImageJ2 was used to observe and collect data on cell size changes across experimental groups, allowing for a comprehensive analysis of wing morphology in cellular aspect.

2.5 Shape Procurest Analysis

Wing shape variation was assessed independently of size using Procrustes superimposition.This method aligns anatomical landmarks by eliminating differences in scale, rotation, and position, ensuring accurate shape comparisons between experimental groups (Ross, 2004; Goodall, 1991). Following Procrustes alignment, Principal Component Analysis (PCA) was performed to identify the main axes of shape variation, providing an overview of the major shape differences across the groups. Following PCA, a multivariate analysis of variance (MANOVA) was conducted to assess the combined effects of *Wolbachia* infection and temperature on wing shape, testing the significance of their interaction on morphological variation.

2.6 Left-Right Symmetry Analysis

To investigate potential differences between the left and right wings, both size and shape were analyzed. For size, centroid size was calculated for each wing, and the differences between the left and right wings were computed. The Shapiro-Wilk test was applied to assess the normality of these

differences. Additionally, skewness and kurtosis were calculated to further evaluate the distribution characteristics of the size differences.

For shape, Procrustes distances between the left and right wings were used to quantify shape variation(Rohlf, 1999). Skewness and kurtosis were also analyzed to examine the distribution of shape differences, and the Kolmogorov-Smirnov (KS) test, along with Q-Q plots, was performed to assess whether the shape variation followed a normal distribution.

3. Result

3.1 Left-Right Symmetry Result

The assessment of potential asymmetry between the left and right wings involved both size and shape analyses **(Figure 2)**. Centroid size differences between the left and right wings were calculated, and the Shapiro-Wilk test confirmed that these differences followed a normal distribution ($W = 0.99693$, $p = 0.09381$). The mean difference between the wings was found to be 0.00112, with skewness of -0.033 and kurtosis of 0.339, indicating a relatively symmetrical distribution.

Hedges' g, calculated to compare the centroid size differences to zero, yielded a value of 0.10682, suggesting a negligible effect size. These results indicate no significant directional asymmetry in wing size. Instead, the data support the presence of fluctuating asymmetry, characterized by small, random differences between the left and right wings, without any systematic directional bias (Palmer & Strobeck, 1992).

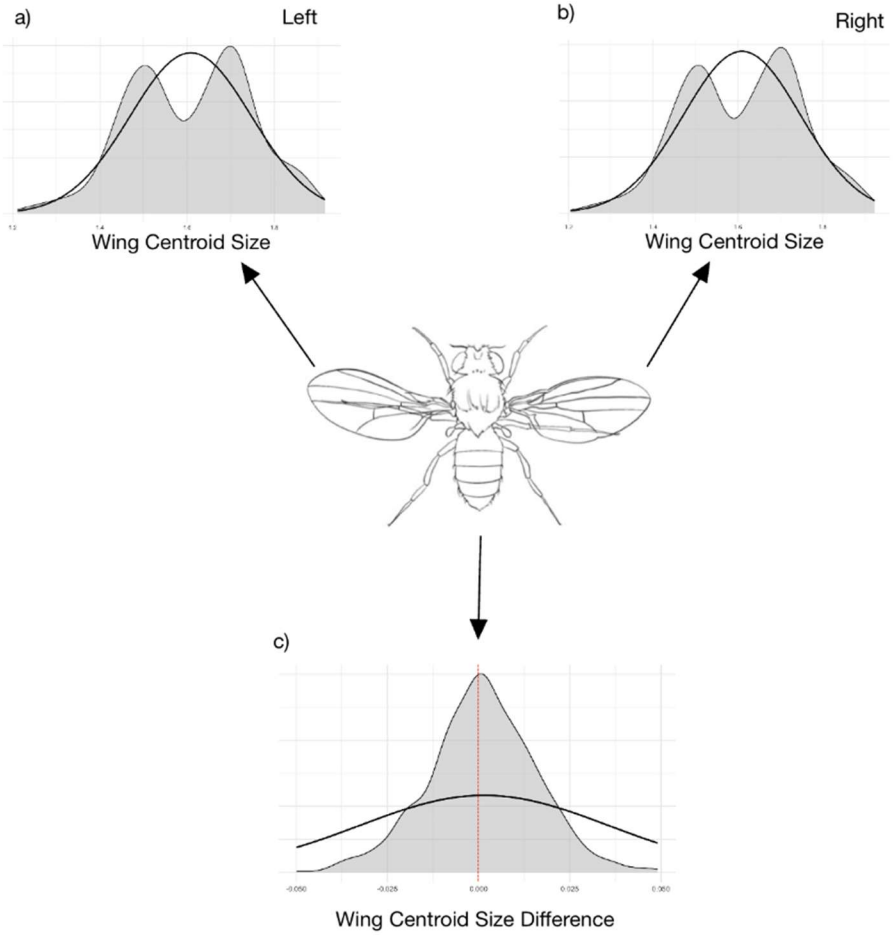

Figure 2. Comparison of Wing Centroid Size and Size Differences. (a)Left wing centroid size distribution. (b)Right wing centroid size distribution. (c) Distribution of differences in centroid size between the right and left wings (right minus left). The red dashed line at 0 represents perfect symmetry.

Shape asymmetry between the left and right wings was assessed using Procrustes distances. The density plot **(Figure 3a)** shows a relatively smooth distribution, with skewness (0.428) and kurtosis (2.366) values indicating slight right-skewness and moderate peakedness, without significant deviations. The Q-Q plot **(Figure 3b)** further explores the normality of the distribution, revealing minor deviations, particularly at the tails. The Kolmogorov-Smirnov (KS) test ($p = 0.01063$) indicated some deviation from normality. However, with a more stringent significance threshold of 0.01, the deviation is minimal. These results suggest that shape differences between the left and right wings are small and random, consistent with fluctuating asymmetry, without evidence of systematic directional asymmetry. Given these findings, subsequent analyses focus solely on the left wing of each individual, as this approach provides a more streamlined assessment while accounting for the observed minor and random shape variations between the left and right wings.

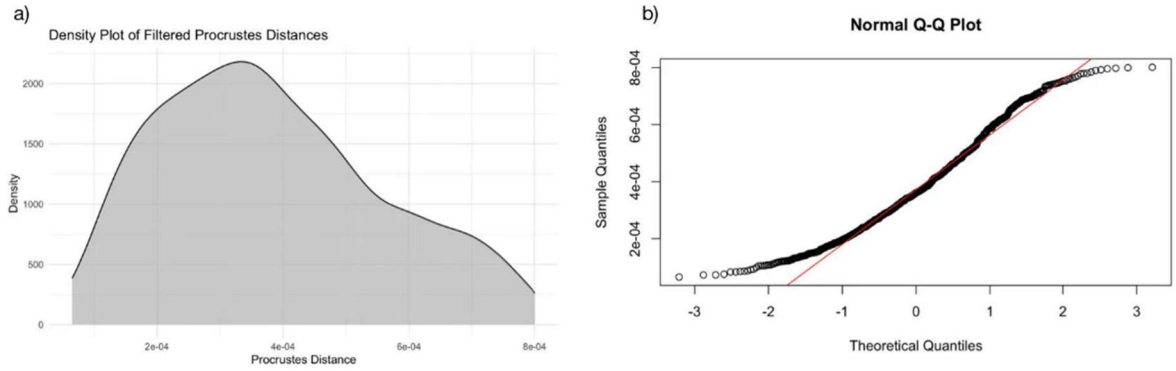

Figure 3. (a)Density plot of the Procrustes distances between left and right wings. (b) Q-Q plot for the Procrustes distances, showing a comparison between the observed distribution of shape differences and the theoretical normal distribution.

3.2 Size Variation Results

Table 1. Results of Size Analysis for F1 and F2 Generations of Drosophila Melanogaster under Different Temperature and Genotype Conditions

Temperature (°C)	Genotype	Welch's t-test Statistics	p-value	95% Confidence Interval	Mean (Wolbachia)	Mean (WT)
F1 Generation						
25°C	Wolbachia vs WT	t = 2.8223, df = 134.94	0.00549	0.0148 to 0.0843	1.648645	1.599086
29°C	Wolbachia vs WT	t = -3.1469, df = 131.59	0.00204	-0.1051 to -0.0240	1.438983	1.503514
18°C	Wolbachia vs WT	t = 3.0819, df = 135.94	0.00249	0.0182 to 0.0832	1.787335	1.736641
27.5°C	Wolbachia vs WT	t = -2.5687, df = 126.04	0.01137	-0.0887 to -0.0115	1.543054	1.593169
F2 Generation						
25°C	Wolbachia vs WT	t = 1.4311, df = 86.82	0.156	-0.0186 to 0.1145	1.638201	1.590289
18°C	Wolbachia vs WT	t = -1.1717, df = 133.43	0.2434	-0.0550 to 0.0141	1.610542	1.630987
29°C	Wolbachia vs WT	t = -0.3742, df = 36.78	0.7104	-0.0868 to 0.0598	1.610645	1.624180

Table 2. ANOVA Results for F1 and F2 Generations

Factor	Df	Sum Sq	Mean Sq	F-value	p-value
F1 Generation					
Genotype	1	0.007	0.007	2.490	0.115
Temperature	3	6.146	2.049	721.317	< 2e-16 ***
Sex	1	5.032	5.032	1772.017	< 2e-16 ***
Genotype	3	0.256	0.085	30.009	< 2e-16 ***
Genotype	1	0.002	0.002	0.541	0.462
Temperature	3	0.017	0.006	1.964	0.118
Genotype:Temperature	3	0.011	0.004	1.332	0.263
Residuals	538	1.528	0.003		
F2 Generation					
Temperature	2	0.029	0.014	1.188	0.306
Genotype	1	0.001	0.001	0.064	0.801
Temperature	2	0.029	0.015	1.209	0.300
Residuals	316	3.829	0.012		

A two-way ANOVA revealed significant effects of temperature and sex on wing size in the F1 generation **(Table 2)**. Temperature had a strong influence on wing size ($F(3, 538) = 721.32$, $p < 2e\text{-}16$), and sex also significantly affected wing size ($F(1, 538) = 1772.02$, $p < 2e\text{-}16$). Additionally, there was a significant interaction between genotype and temperature ($F(3, 538) = 30.01$, $p < 2e\text{-}16$), indicating that the effect of temperature on wing size varied between Wolbachia-infected and uninfected flies. However, interactions between genotype and sex ($F(1, 538) = 0.54$, $p = 0.462$) and temperature and sex ($F(3, 538) = 1.96$, $p = 0.118$) were not statistically significant.

Further analysis using Welch's t-tests was conducted at each temperature level to explore genotype-specific effects. At 25°C, Wolbachia-infected flies had significantly larger wings than wild-type flies (mean: Wolbachia = 1.6486, WT = 1.5991; $t = 2.82$, $p = 0.00549$). In contrast, at 29°C and 27.5°C, Wolbachia-infected flies had significantly smaller wings than wild-type flies (29°C: mean: Wolbachia = 1.4390, WT = 1.5035; $t = -3.15$, $p = 0.00204$; 27.5°C: mean: Wolbachia = 1.5431, WT = 1.5932; $t = -2.57$, $p = 0.01137$). At 18°C, Wolbachia-infected flies again exhibited significantly larger wings than wild-type flies (mean: Wolbachia = 1.7873, WT = 1.7366; $t = 3.08$, $p = 0.00249$) **(Table 1, Figure 4a, 4b, 4c)**.

In the F2 generation, ANOVA results did not reveal significant effects of temperature ($F(2, 316) = 1.19$, $p = 0.306$) or genotype ($F(1, 316) = 0.06$, $p = 0.801$) on wing size. The interaction between temperature and genotype was also non-significant ($F(2, 316) = 1.21$, $p = 0.300$). Consistent with these results, Welch's t-tests for each temperature condition in the F2 generation showed no significant differences in wing size between Wolbachia-infected and wild-type flies (all $p > 0.15$). For instance, at 25°C, the mean wing sizes were 1.6382 (Wolbachia) and 1.5903 (WT), with a non-significant t-value of 1.43 ($p = 0.156$) **(Table 2, Figure 4d, 4e).**

In line with the wing size findings, cell size analysis in the F1 generation revealed significant differences between Wolbachia-infected and wild-type (WT) flies at all temperatures. At 18°C, Wolbachia-infected flies exhibited significantly larger cells than WT flies ($t = 8.5816$, $p = 1.439e\text{-}13$), and a similar pattern was observed at 25°C, with Wolbachia-infected flies having larger cells compared to WT ($t = 11.645$, $p < 2.2e\text{-}16$). Conversely, at higher temperatures, Wolbachia-infected flies showed significantly smaller cells than WT (27.5°C: $t = -15.329$, $p < 2.2e\text{-}16$; 29°C: $t = -20.847$, $p < 2.2e\text{-}16$). These findings mirror the wing size centroid variations, confirming that Wolbachia influences both wing and cell size, with the effect depending on the combined influence of Wolbachia infection and temperature.

In the F2 generation, no significant differences in cell size were observed between Wolbachia-infected and WT flies across any temperature (all p-values > 0.05). This result is consistent with the wing size data in the F2 generation, indicating that the Wolbachia-related cell size differences seen in F1 are not present in F2 under these experimental conditions.

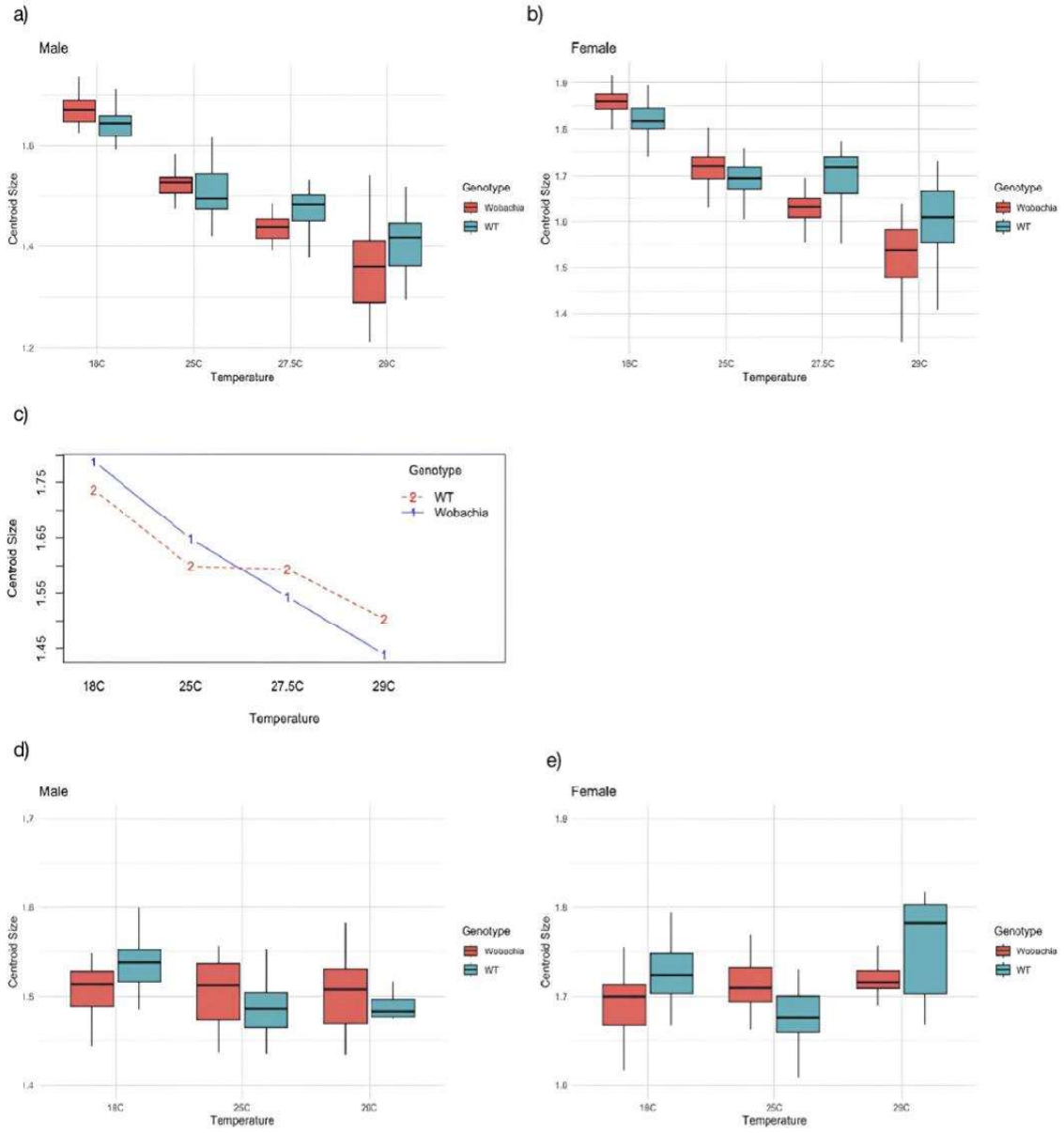

Figure 4. Centroid size variation across different temperatures and genotypes in Drosophila melanogaster. (a) Centroid size of male F1 flies across four temperature conditions (18C, 25℃, 27.5℃, 29℃) for Wolbachia-infected and wild-type genotypes. (b)Centroid size of female F1 flies under the same temperature and genotype conditions. (c) Interaction plot illustrating the effect of genotype and temperature on centroid size in the F1 generation. (d) Centroid size of male F2 flies across temperatures and genotypes. (e) Centroid size of female F2 flies across temperatures and genotypes.

3.3 Shape Variation Result

A multivariate analysis of variance (MANOVA) indicated significant effects of temperature, Wolbachia infection, and sex on wing shape in the F1 generation. Temperature was a primary driver of shape variation ($F = 34.34$, $p < 2.2e\text{-}16$), while Wolbachia infection also introduced distinct

morphological differences between infected and uninfected flies (F = 132.15, p < 2.2e-16). Furthermore, pronounced sexual dimorphism was observed, with males and females displaying significant differences in wing shape (F = 214.57, p < 2.2e-16). There was a notable interaction between temperature and genotype (F = 12.87, p < 2.2e-16), indicating that Wolbachia modifies the temperature-induced morphological changes. The interactions between temperature and sex (F = 3.08, p = 0.0053) and genotype and sex (F = 6.67, p = 0.0014) further contributed to the observed wing shape variation.

These findings are visualized in **Figure 5**, where the Procrustes PCA illustrates the impact of the different factors. In **Figure 5a**, the overall PCA for the F1 generation demonstrates clear separation of shape clusters across different temperature groups, with 18°C and 29°C showing the most pronounced divergence. **Figure 5b** illustrates the combined effects of sex and Wolbachia infection, with distinct clustering between the two genotypes, particularly among females.In **Figure 5c**, the interaction between Wolbachia and temperature becomes apparent, as Wolbachia-infected flies exhibit more pronounced shape shifts under extreme temperature conditions (18°C and 29°C). **Figure 5d** highlights the joint effect of sex and temperature, revealing distinct clustering between males and females within each temperature group, thereby reinforcing the observed sexual dimorphism in wing shape.

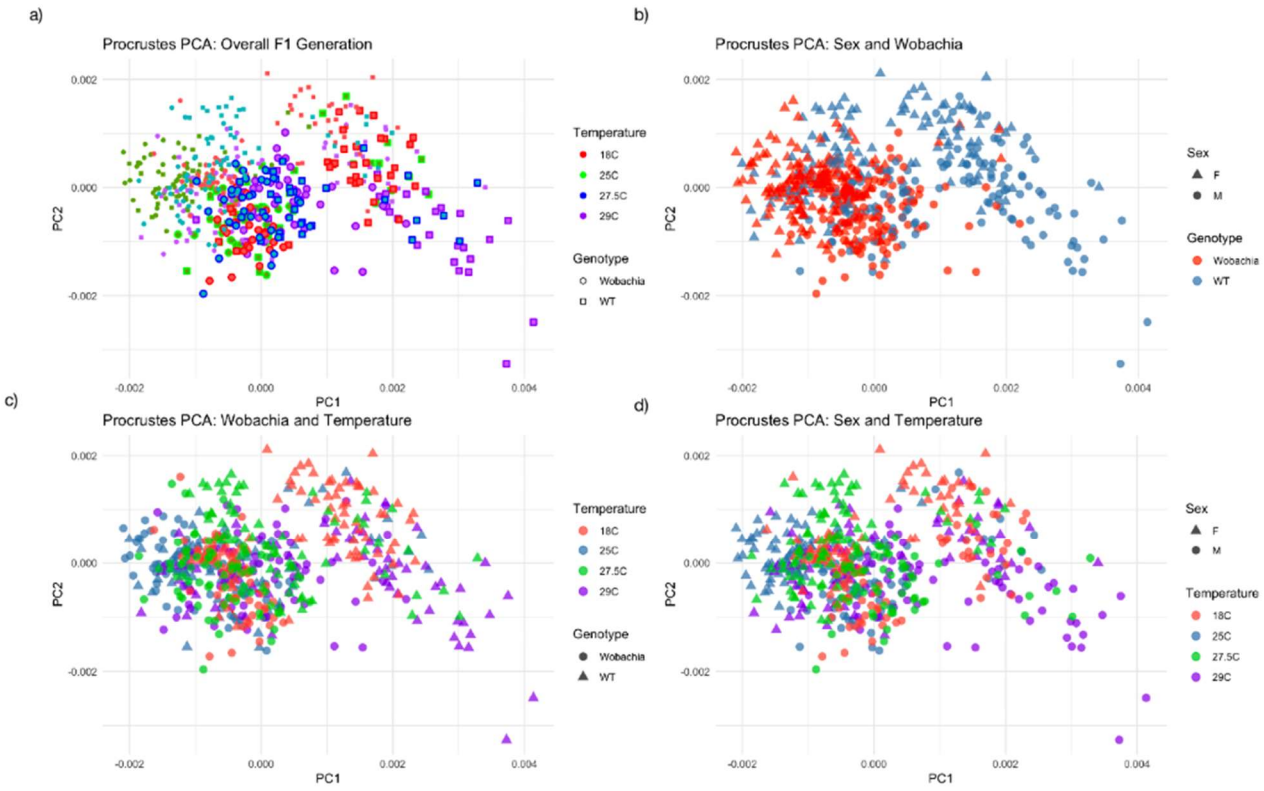

Figure 5. Procrustes PCA of wing shape variation in F1 *Drosophila melanogaster*.(a) Overall shape variation across temperatures (18°C, 25°C, 27.5°C, 29°C), genotypes (Wolbachia-infected, WT), and sexes. Males are outlined.(b) Shape variation by sex and Wolbachia infection.(c) Shape variation by Wolbachia infection and temperature.(d) Shape variation by sex and temperature.

In the F2 generation, wing shape variation was significantly influenced by temperature, Wolbachia infection, and sex, as demonstrated by the results of the multivariate analysis of variance (MANOVA) **(Figure 6)**. Temperature showed a substantial impact on shape (F = 37.31, p < 2.2e-16), while Wolbachia infection also led to considerable shape changes between infected and uninfected flies (F = 220.07, p < 2.2e-16). The analysis further confirmed sexual dimorphism in wing morphology, with

males and females exhibiting distinctly different shapes ($F = 190.28$, $p < 2.2e\text{-}16$).A significant interaction between temperature and genotype ($F = 39.46$, $p < 2.2e\text{-}16$) was observed, indicating that the presence of Wolbachia altered the influence of temperature on wing shape. Moreover, a significant three-way interaction between temperature, genotype, and sex ($F = 5.21$, $p = 0.00039$) highlighted the intricate combined effects of these factors on the overall morphology.

Principal Component Analysis (PCA) of the Procrustes-superimposed coordinates **(Figure 6a–d)** further visualized these differences. Clear clustering by genotype and sex emerged, particularly at extreme temperatures (18°C and 29°C), providing additional support for the observed morphological divergence.

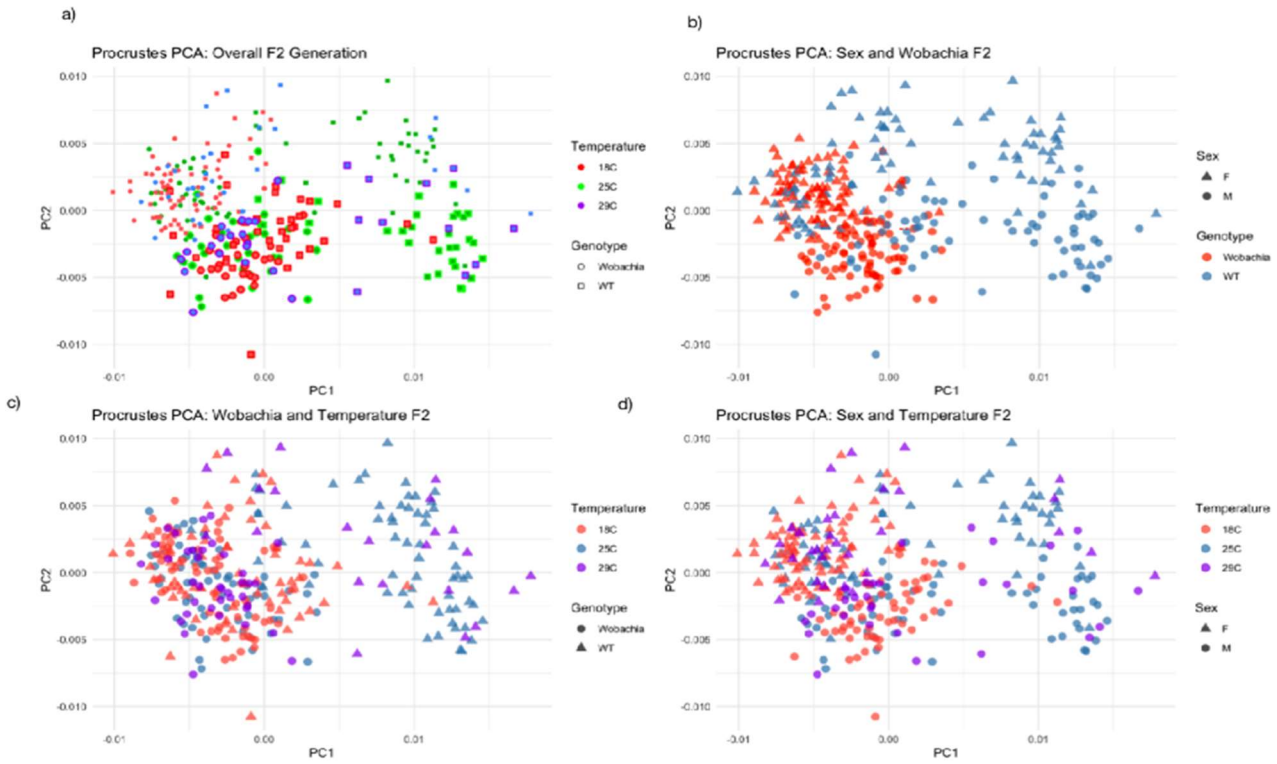

Figure 6. Procrustes PCA of wing shape variation in F2 Drosophila melanogaster.(a)Overall shape variation across temperatures (18°C, 25°C, 29°C), genotypes (Wolbachia-infected, WT), and sexes.(b) Shape variation by sex and Wolbachia infection.(c) Shape variation by Wolbachia infection and temperature.(d) Shape Variation by sex and temperature.

4. Discussion

This study explored the combined effects of *Wolbachia* infection and temperature on the wing morphology of *Drosophila melanogaster*, revealing significant genotype-environment interactions in the F1 generation. Our findings demonstrate that temperature and *Wolbachia* infection jointly influence wing size, cell size, and wing shape, while these effects are diminished in the F2 generation.

4.1 Impact of Size

The significant influence of temperature on wing size aligns with well-documented research on thermal plasticity in *Drosophila*. At lower temperatures (18°C and 25°C), *Wolbachia*-infected flies displayed larger wings compared to their uninfected counterparts, whereas at higher temperatures (27.5°C and 29°C), infected flies had smaller wings. This suggests that *Wolbachia* modifies host responses to temperature, potentially affecting resource allocation and developmental timing, as observed in previous studies where *Wolbachia* influences host development by modifying resource competition and growth rates in other insects (Gavotte et al., 2009).

Our results indicate that variations in wing size are primarily driven by changes in cell size rather than cell number. Consistent patterns were observed in cell size measurements, where *Wolbachia*-infected flies exhibited larger cells at lower temperatures and smaller cells at higher temperatures, closely matching the wing size data. This suggests that *Wolbachia* influences host growth at the cellular level, likely affecting energy allocation. Previous research has shown the importance of cell size in determining overall wing size in insects (Alpatov, 1930; Masry & Robertson, 1979), and our findings support this association.

4.2 Impact of Shape

In our analysis of wing shape, the MANOVA results revealed significant effects of temperature, *Wolbachia* infection, and sex on wing morphology in the F1 generation. Temperature played a critical role, with extreme temperatures (18°C and 29°C) showing the most pronounced shape variation. Additionally, *Wolbachia* infection significantly altered wing shape, with infected flies displaying distinct morphological traits compared to their uninfected counterparts. The interaction between genotype and temperature further demonstrated that *Wolbachia* modifies the host's morphological response to temperature changes, suggesting a complex interplay between environmental conditions and symbiont presence (Guilhot et al., 2020).

Sexual dimorphism was also evident in our findings, with males and females exhibiting distinct wing shapes, further confirming the multifactorial nature of wing morphology regulation (Gidaszewski et al., 2009). Interestingly, the significant interaction between temperature and sex in the F1 generation suggests that males and females may respond differently to temperature variations in the presence of *Wolbachia*, further contributing to the observed morphological diversity.

4.3 Heritability of Wing Shape and Size

In the F2 generation, the wing size differences observed in F1, both in terms of centroid and cell size, were not retained. Neither temperature nor Wolbachia infection had a significant effect on wing size, suggesting that the combined influence of Wolbachia and temperature on size is not heritable under these experimental conditions. This lack of significant variation in the F2 generation highlights the environmental specificity of the Wolbachia-temperature interaction, indicating that these effects diminish across generations when environmental conditions are normalized (i.e., with all F2 flies reared at 25°C). The findings align with previous research that suggests morphological traits can be strongly shaped by environmental factors rather than genetic inheritance alone (Parsons, 1991).

In contrast, wing shape differences persisted in the F2 generation. Significant effects of temperature, Wolbachia infection, and sex were observed, along with key interactions between these factors. Although these results might suggest a heritable component to wing shape, it is equally plausible that the shape differences in F2 are driven by residual effects of early environmental exposure during F1 development. The complexity of environmental stressors, like temperature, which act as evolutionary forces (Hoffmann & Hercus, 2000), complicates the distinction between heritable traits and those shaped by prior environmental conditions. Therefore, while some shape variation persists, the absence of any significant effect on wing size in F2 further supports the idea that environmental conditions play a dominant role in determining these traits.

Overall, our findings suggest that wing shape and size are influenced by environmental conditions and Wolbachia infection in F1, but only wing shape shows continuity into F2. However, it remains uncertain whether this persistence is due to genetic inheritance or lingering environmental effects. Future work focusing on disentangling these factors will be crucial to understanding the heritability of wing traits in Drosophila.

4.4 Future Research Direction

Future research could delve deeper into the molecular mechanisms underlying the interaction between Wolbachia and environmental factors like temperature. A promising area of exploration would be to investigate whether the quantity of Wolbachia within the host correlates with the extent

of morphological changes. By quantifying Wolbachia density across various temperatures and linking these measurements to phenotypic outcomes, researchers could better understand how symbiont load influences host development(Christensen et al., 2019).Additionally, exploring how Wolbachia infection and environmental conditions impact gene expression would provide valuable insights into the molecular pathways involved (Gutzwiller et al., 2015). For example, studies focusing on growth-regulating genes or those involved in cellular metabolism under the dual influence of Wolbachia and temperature could reveal how these factors jointly affect host physiology. This would be especially relevant for identifying potential molecular signatures that drive the observed shape and size variations.

Another key area is to identify the developmental window during which environmental factors exert the strongest influence on morphology. Manipulating temperature at distinct stages—such as embryonic, larval, or pupal phases—and measuring subsequent phenotypic changes could provide valuable insights into when Wolbachia's influence is most critical. Understanding this temporal aspect will enhance our ability to predict and manipulate developmental outcomes. Future research should also explore whether these environmental effects are transgenerational. Tracking phenotypic changes across F1, F2, and F3 generations will determine whether the morphological effects of Wolbachia persist across generations. This approach would also clarify whether epigenetic modifications, such as DNA methylation or histone modifications, contribute to the inheritance of these traits. Such studies would be essential to determine whether temporary environmental exposure or sustained conditions are required to induce stable transgenerational effects.

Finally, integrating advanced molecular techniques such as transcriptomics, proteomics, or CRISPR gene editing could further clarify how Wolbachia manipulates host gene expression. This would deepen our understanding of the symbiotic relationship's role in shaping not only the host's phenotype but also its evolutionary potential in varying environmental contexts.

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