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Morphogenetic Consequences of Short-Term Thermal Stress in Short and Long Life House Fly Lines (*Musca Domestica* L.): Geometric Wing Morphometrics

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Abstract—The morphogenetic consequences of exposure to short-term heat stress (STHS) in two housefly strains mass-selected for lifespan were studied based on the assessment of adult wing variability by geometric morphometrics. Significant differences in the size and shape of the wing between the control and impact groups of different genders in the strains were revealed. *Shg* (short lived) and *Lg* (long-lived). The STHS effect manifested in an increase in the size of the wing and a directed change in its shape. The between-group hierarchy of gender and stress-induced differences is expressed in the same way in both strains of flies. The range of linear differences is significantly higher than the gender differences, which, in turn, are higher than the level of stress-induced ones. Instability of imago wing development (*Vm*) in *Shg* was significantly higher than the *Lg* strains, and higher in all groups of females, but in most cases significantly lower in impact groups (taking into account the increase in size, the latter may be associated with the effect of hormesis). It is hypothesized that the directed morphogenetic effects of STHS are based on latent species modifications, the appearance of which in the phenotype is due to stress-induced epigenetic genome rearrangements that cause similar morphological changes in the wing in groups of adult males and females of both strains. The phenotypic plasticity of strains during selection for different lifespans and the changes induced by STHS directly indicate the reality of stress-induced rapid morphogenetic rearrangements under a sharp change in environmental conditions.

Keywords: *Musca domestica*, variability, selected strains, lifespan, heat stress, geometric morphometrics

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INTRODUCTION

The problem of assessing rapid adaptive changes in morphogenesis in natural populations of animals and plants has become extremely relevant in recent decades due to the need to predict the expected changes in the 21st century due to biocenotic crisis phenomena [1, 2] caused by the increased impact of climatogenic and anthropogenic factors on biota [3–5]. For these purposes, various approaches have been proposed in the world, including the use of methods of functional and trait (trait-ecology or trait-based ecology) ecology [6–11]. Experimental assessments of the genetic, epigenetic, and morphogenetic effects of stress-inducing impacts on model natural objects are of key importance for the verification of ecological forecasting methods [12–15]. Of particular interest is not only the possibility of rapid morphogenetic rearrangements in historical characteristic times, but also the phenomenon of transgenerational plasticity (TGP) [16, 17], i.e., the ability to epigenetically inherit the possibility of maintaining the range of modifications previously achieved by parents under certain environmental conditions. Several studies have revealed the manifestation of transgenerational inheritance of stress-induced epigenetic rearrangements of the genome, causing certain morphogenetic changes [18–20]. Registration and visualization of such changes is possible using the methods of geometric morphometrics [21–23], which make it possible to evaluate changes in the shape of objects in the general morphospace, allowing a morphogenetic interpretation of the revealed differences [24, 25].

At the end of the 20th century, in experiments on the effects of severe thermal shock (STS) on the line *Drosophila melanogaster* the most sensitive early stages of ontogenesis were identified, at which similarly directed transpositions of transposable genetic elements (TGEs) occurred, causing certain changes in wing morphogenesis depending on the time of exposure [26, 27]. Later, characteristic movements of TGEs were also established in response to low-dose radiation exposure of Drosophila [28, 29]. In experi-

ments on the selection for low and high survival of inbred lines *Drosophila melanogaster* obtained from individuals of natural populations, it was found [30, 31] that, in contrast to the control, after 18 generations of selection, both the phenotype and the structure of TGE placement changed (*mdg1*, *hobo*, *P*, etc.). Stress-induced transpositions, correlated with morphogenetic rearrangements, were identified for different groups and families of mobile genetic elements on a wide range of stress effects, weak and strong temperature effects, gamma radiation, ethanol vapors, toxic salts of heavy metals, etc. [27, 32]. Heat stress activates the protective system of heat shock factors (HSFs), including the transcription of peptides of the Hsp70 family (Heat shock proteins) [33, 34], which can potentially affect both the acclimation process [35], and on individual development [34].

Nevertheless, on two closely related species of Drosophila, *Drosophila melanogaster* and *D. simulans*, when analyzing the genomic sequences of populations of different latitudes, it was found [36] that the selective forces associated with climatic factors act on the same genes and phenotypes in these sympatric species. The latter indicates the evolutionary-ecological continuity of species adaptations and the role of certain genes in their development. In four species of Drosophila, desert endemics in the south of North America, different resistance of adults to heat stress in different seasons of the year and a high general resistance of young (1-day-old) flies to this factor in comparison with adults of older ages were found [37]. Important results [38] were obtained on a series of genome-wide sequences in assessing associations of the occurrence of SNPs and transposable elements (TEs) in natural populations of *D. melanogaster* in Europe and North America with environmental variables including temperature, humidity, evaporation, wind, daylight, soil type, etc. It was found that from 23 to 51% of the genes that showed significant associations with more than 50 environmental variables differed slightly in different remote populations. At the same time, along with adaptively significant SNPs, ten typical insertions of transposable mobile elements were identified, which also turned out to be associated with environmental variables.

In experiments on the cultivation of fruit flies in a temperature gradient from 12 to 30°C, a directed significant decrease in the size of the wing, as well as changes in its shape, was revealed [39]. With the regression exclusion of the influence of the allometric dependence of the variability of the wing shape on its size, the wing configuration of the experimental flies at extreme temperatures of 12 and 30°C, which cause "moderate" developmental stress, phenotypically approached and differed significantly from that in flies reared at normal temperature conditions (21–25°C). High phenotypic plasticity and stress reactivity of the Drosophila phenotype were also found in the analysis of the combined effect of mutations and development temperature [40]. Previously, another representative of Diptera, the housefly (*Musca domestica*) on the territory of America [41], a high phenotypic plasticity was also revealed in the gradient of habitat conditions in the latitudinal direction. It is shown that the dimensions of the wings of room and field (*M. autumnalis*) flies from the southernmost regions of Brazil become smaller [42], which is associated with an increase in the temperature of development. The latter is due not to the direct effect of temperature, but to a historically long natural selective process that forms genetic changes in local populations. Investigation of the influence of different temperature regimes on the development of houseflies, *Musca domestica* L. [43], revealed a wide range of values (from 20 to 35°C), causing a certain decrease in the survival rate, adult life span, fecundity, and fertility under controlled laboratory conditions. All of this indicates the adequacy of the choice of the housefly as a model object for studying phenotypic plasticity.

In a previous publication [44], we compared the variability of the wistrainsng shape in strains of short- and long-lived houseflies (*Musca domestica*) experimentally obtained after long-term oppositely directed mass selection aimed at early and late reproduction based on geometric morphometrics methods. As a result, significant interline differences in the shape and size of the wing were revealed, as well as gender differences in each strain.

Subsequently, we obtained new experimental material, the results of assessing the effect of short-term heat stress (STHS) on wing morphogenesis in both strains under high-temperature exposure of the same individuals successively at each stage of ontogeny. Comparison of previous and new materials allows using the methods of geometric morphometrics to solve the problem of studying the consequences of a parallel repetitive thermal effect on wing morphogenesis in both strains.

The problem of rapid rearrangements of the morphogenesis of a species under abrupt changes in environmental conditions is of general biological nature, and its solution potentially allows, in the light of the expected climatogenic temperature changes, to get closer to understanding the evolutionary and ecological role of stress-induced phenotypic plasticity, as well as the genetic and/or epigenetic nature of the mobilization reserve of modification variability.

Objective—To study the variability and intragroup diversity of the size and shape of the wing of males and females of short- and long-lived housefly lines (*Musca domestica* L.) in the control group and those simultaneously subjected to STHS at different stages of ontogenesis based on the methods of geometric morphometrics. Particular attention was paid to the assessment of the stability of wing morphogenesis and the ratio of the directions of variability of the selected strains in the general morphospace after repeated heat

stress as an ecological model of extreme stress-inducing temperature fluctuations of the environment.

MATERIALS AND METHODS

Adult houseflies of *Musca domestica* L. derived from a laboratory strain *S*, originating from the strain *Cooper*, were used in the experiments. The source material was kindly provided by Prof. S.A. Roslavtseva (Research Institute of Disinfectology, Moscow). The experimental groups of flies were kept in Capron cages with a metal frame 30 × 30 × 30 cm in size, and dry milk was used as standard food. Fly larvae developed in plastic containers on a medium with moistened bran under standard conditions [45] at room temperature (23–26°C) and illumination with a period of 12 : 12 h [46]. Among the representatives of original strain *S* using selection for early and late reproduction, mass heterogeneous strains were identified *Shg* (short lived) and *Lg* (long-lived), which significantly differed in the average shortest lifespan of adults, 22 and 54 days, respectively. To create a strain of *Shg*, eggs laid in the first two weeks from the day of emergence of adults were selected from the initial strain for three generations. When creating an *Lg* strain, eggs laid no earlier than 25–28 days from the day of emergence of adults were selected. Based on strains *Shg* and *Lg* (respectively 65 and 45 generations of selection for early and late reproduction), control and experimental groups were identified to which selection by the timing of reproduction was not applied in the future.

Short-term exposure to high temperature, STHS (65°C for 10 min), was carried out once at each stage of ontogeny (larvae, pupae, adults), while exposing groups of the same individuals, placing containers with 5-day-old larvae in the substrate, daily puparia, as well as cages with 3-day-old adults in a TC-80-M thermostat. The exposure periods for individual stages of development were chosen in accordance with the previously obtained results of assessing the sensitivity of the housefly to temperature effects in ontogeny [46, 47]. Upon heating, the temperature of the substrate increased by 3–7°C. The exposure of individuals of the stressed groups was carried out in each generation, starting from the first.

The initial number in each group (*ShgC* – control, *ShgS* – stressed; *LgC* – control, *LgS* – stressed) was 50 females and 50 males, selected after leaving the puparia. During the experiment, a tendency to prolongation of development at the puparium stage was noted in the *LgS* group and the opposite trend in the *ShgS* group. At the same time, in short-lived adults, the effect of longer reproduction was noted, accompanied by an increase in fertility, while in the group of long-lived adults under the influence of heat stress, the reproductive period was reduced, but the overall fertility did not change [48].

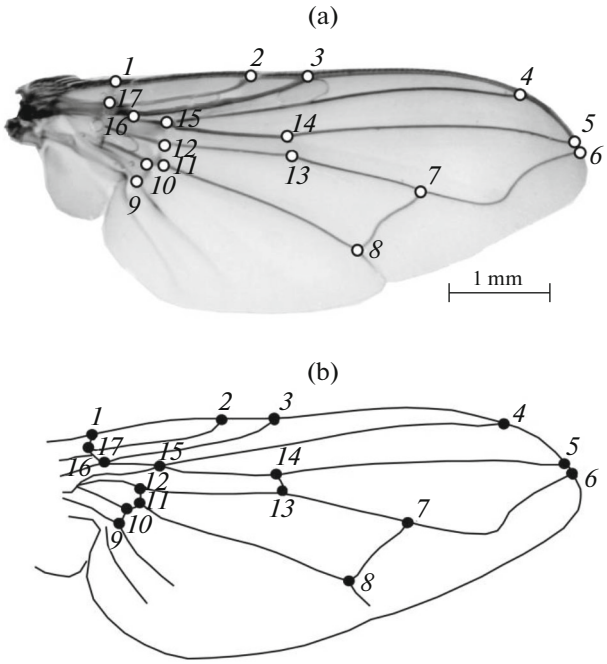

Fig. 1. Localization of landmarks (*1–17*) in the photograph of the right wing (a) of a house fly (*Musca domestica*) and the scheme of their localization on the wing (b) in the form of a dynamic contour model of the configuration – outline.

The wings of adults fixed in solution (3 parts of alcohol + 3 parts of propylene glycol + 1 part of distilled water) or naturally dead were used in the study. The wings were separated with tweezers at the junction with the thorax and placed on glass slides moistened with a fixative solution, and then straightened, covered with coverslips and photographed under the MBS microscope using a UCMOS03100KPA USB camera and ToupView software at 2048 × 1536 pixels.

The geometric morphometrics of the wings was made on the basis of the configuration of 17 landmarks placed on the photographs of the right wings (Fig. 1) using the programs tpsUtil and tpsDig2 created by F.J. Rolf [49, 50].

Studied material in the *Lg* strain amounted to 156 (control groups (*LgC*): 30 of males and 48 of femalesstrain; stress groups (*LgS*), subjected to STHS: 29 of males and 49 of females), and in the strain *Shg* – 144 (control groups (*ShgC*): 17 of males and 42 of females; stress groups (*ShgS*): 42 of males and 43 of females) digitized wings. When indirectly estimating the size of the wings, their centroid size (CS) was used, which was calculated as the square root of the sum of the squared distances from the center of the configuration to each landmark [21]. Considering the potential hierarchy of CS variability factors, the assessment of their contributions, taking into account interactions, was estimated on the basis of a three-way analysis of variance.

Superimposition of landmark configurations was carried out by the method of generalized Procrustes analysis (GPA) based on the least squares method [21] and Procrustes coordinates were calculated, which characterize the variability of the shape of the wings. The assessment of possible wing allometry according to the existing recommendations [22] was performed based on the regression of the first principal component (PC1) on the logarithm of CS. With the help of discriminant and canonical analyzes of Procrustean coordinates, intergroup differences in wing configurations were evaluated.

In order to interpret and evaluate the significance of intergroup variability factors along canonical variables (CV1–CV7), a multivariate three-way analysis of variance, Three-way MANOVA, was performed [51, 52].

By the symmetric matrix of generalized Mahalanobis distances (D) on the basis of cluster analysis (UPGMA) determined the hierarchy of intergroup differences: interstrain, gender, and stress-induced.

The degree of manifestation of sexual dimorphism of CS was assessed by the following formula: $SDM = [(xf/xm) - 1] \times 100$, where xf is the average value of CS in females, and xm, in males [53]. In a multidimensional comparison of the wing shape, the squared generalized Mahalanobis distances were used for this purpose (D^2) with an assessment of their significance levels.

When assessing the level of group instability of morphogenesis (within-group disparity), we used indicator Vm as the volume of the within-group morphospace occupied by the ordinates of the given group [11]. Since, in this case, samples randomly aligned by the number of observations were compared, this indicator makes it possible to obtain comparable characteristics of the dispersion values of the ordinates of individuals in the morphospace: the larger the value of Vm, the less stable the development of individuals proceeds and the dispersion of their ordinates in the morphospace is observed to a greater extent, the expansion of the fan of morphogenetic trajectories [11]. In more favorable conditions, i.e., with minimal stress in the development process, the value of indicator Vm is less than under adverse conditions. Calculation of Vm, i.e., the volume of the morphospace enclosed inside the convex hull [9, 54, 55], constructed from the set of outer edge coordinates of groups of objects, was calculated using the first three canonical variables (CV1–CV3) calculated from the Procrustean coordinates characterizing the variability of the wing shape [11]. The calculation of the volumes of the within-group morphospace was performed in the add-in (add-in) CalculateVolume (author A.G. Kursanov) for Microsoft Office Excel, written on the basis of the built-in MatLab function convhull, which allows you to calculate the volume of the convex hull of a finite set of points (3D convex hull). The hypervolume R program [56] can also be used to calculate the convex hull volume. When estimating the standard error of measurement (Vm ($\pm SE$)) we used the bootstrap technique with random replacement of objects in the sample [57].

The homogeneity of the sample variances was determined using Levene's test for mean values. Pairwise comparisons were made based on Tukey's post-hoc Q-test. The statistrainstical significance of differences in multiple comparisons of samples was assessed using a three-way analysis of variance. The calculations were performed using the TPS [49, 50], PAST4.06 [58], MorphoJ 1.06d [23], and Statistica [52] application packages.

RESULTS AND DISCUSSION

Centroid size of the adult strain wing *Shg* (1843.74 ± 11.77) were generally significantly higher ($t = 5.30$; $p < 0.0001$) than the representatives of strain *Lg* (1759.53 ± 10.72). The results of comparing CS values with standard errors ($\pm SE$) between the control and experimental groups of males and females of two strains of flies are shown in Fig. 2. Since all three factors of variability, line, gender, and exposure to STHS, could have a parallel effect on growth processes, we used a three-way analysis of variance of centroid sizes of the wing between the studied control and impact groups, taking into account their gender. The results of the analysis (Table 1) showed that belonging to the line has the greatest influence on the variability of CS, and the proportion of variance due to interline (mainly genetic) differences was 9% of the total variance. The second place in terms of the effect size (see Table 1) was taken by the factor of STHS the share of dispersion of which was 7%. Gender differences also showed a significant, but smaller effect size (moreover, the proportion of variance due to the sexual dimorphism of flies was 1.2%).

When analyzing the influence of factor interactions on CS, it was found that a significant effect size was manifested only in the interaction of the "line × stress" (L × S) factors, which accounted for 2.3% of the variance. Other factor interactions, including the "L × G × S" variant, were statistically insignificant (see Table 1). It is noteworthy that the effect sizes for interstrain (mainly genetic) differences, although somewhat larger, are comparable in level to those for stress-induced (environmental modification and, probably, epigenetic) differences. According to the system of assessments adopted by Cohen [59], both are above the minimum values, but do not reach the average level. The effect size for gender differences in CS when interstrain data were averaged turned out to be even lower than the accepted minimum level (see Table 1).

In the groups of STHS flies of both strains, a general trend towards an increase in the centroid size of

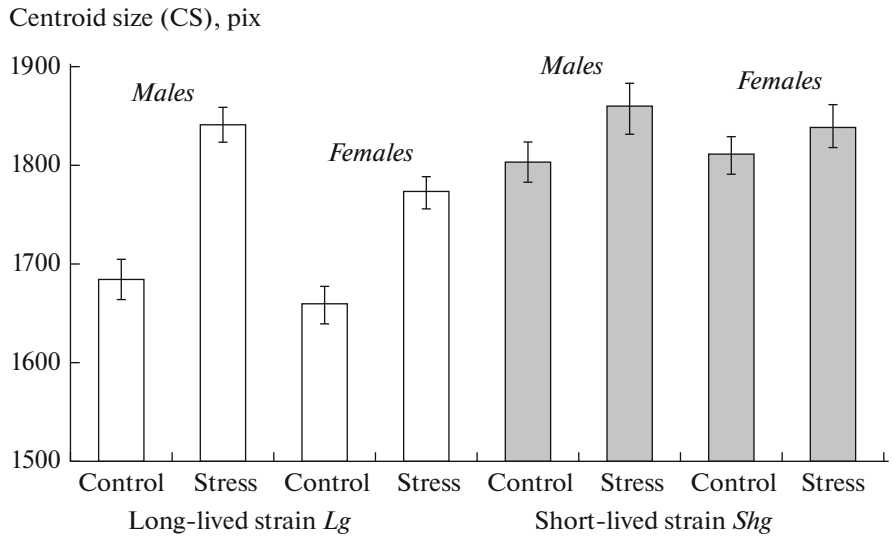

Fig. 2. Comparison of mean centroid sizes CSs (with standard errors ±*SE*) between control and experimental groups of long-lived (*Lg*) and short-lived (*Shg*) housefly strains (*Musca domestica*).

the wing was expressed. Only when comparing the control and impact groups of females of the strain of short-lived (*Shg*) flies the trend of increasing wing size has not been statistically proven. The most contrasting differences in CS values were found in representatives of the strain of long-lived (*Lg*) flies. Since the dimensions of the wings in this line are smaller on average, it can be assumed that in the other line (*Shg*), initially having large CS values, both in control and under stress, the wings approach the largest allowable sizes. Gender differences within the strains in terms of the CS value were not pronounced between the control groups but were manifested in both strains in the stress groups: in females, in both cases, the CS of the wing is smaller than in males. Sexual dimorphism index *SDM* for CS in strain *Lg* in the control group was 0.56, and in the stress group −3.87, while in the strain *Shg*, respectively, 1.24 and 2.87. Consequently, there was a trend towards an increase in gender differences in wing size in stressed flies and a relatively lower growth response of females in response to STHS.

Significant variance, reflecting the interaction of line and stress factors (L × S), demonstrates that growth responses (according to wing size) in control and impact groups manifest themselves differently. Indeed, pairwise comparison of CS values between samples based on Tukey's post hoc Q-test revealed significant differences between the samples of the control and stress groups only in the strain *Lg*: between males, $Q = 8.82$ ($p < 0.001$), between females, $Q = 5.01$ ($p = 0.009$). In strain *Shg* the same pairs of comparison did not show significant differences: between males, $Q = 3.10$ ($p = 0.357$), and between females, $Q = 1.14$ ($p = 0.993$).

Table 1. Results of a three-way analysis of variance of centroid wing sizes (CS) of control and stress (STH) males and females of strains *Shg* and *Lg* with effect size

Source of variability, factor	Sum of squares, SS	Number of degrees of freedom, df	Mean square, MS	F	Significance level, *p*	Effect size, η^2
Line, L	569625	1	569625	33.62	<0.0001	**0.103249**
Gender, G	70893	1	70893	4.18	0.0417	**0.014127**
Stress, S	427812	1	427812	25.25	<0.0001	**0.079590**
L × G	2514	1	2514	0.15	0.7003	0.000508
L × S	140363	1	140363	8.28	0.0043	**0.027589**
G × S	18115	1	18115	1.07	0.3020	0.003648
L × G × S	14	1	14	0.001	0.9774	0.000003
Within-group	4947363	292	16943			
Total	6180301	299				

Significant effect sizes are highlighted in bold.

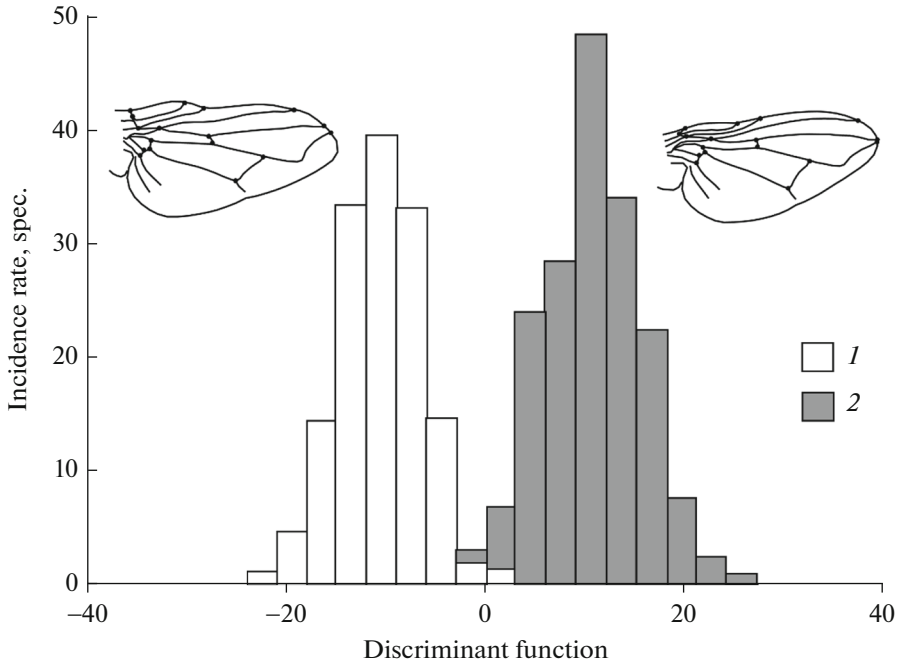

Fig. 3. The results of the discriminant analysis of Procrustean coordinates characterizing the variability of the wing shape of short-lived (*1*, *Shg*) and long-lived (*2*, *Lg*) housefly strains (*Musca domestica*) regardless of gender. The contour configurations of the wings (outlines) correspond to the centroids of the compared strains.

It was interesting to assess whether the wing shape is manifested in representatives of different strains of the housefly, regardless of whether they belong to the control or stress group (STHS). For this purpose, a linear discriminant analysis of the Procrustean coordinates characterizing the variability of the wing shape was carried out on the material combined by experimental groups and gender (Fig. 3). Discriminant analysis revealed significant interline differences in wing configuration (Lambda Wilks' $\Lambda = 0.151$; $D^2 = 4.608$; Hotelling's T^2) = 1781.9; $df_{1.2} = 34, 287$; $F = 47.4$; $p < 0.0001$). The figure shows that the wing of short-lived flies of the strain *Shg* at the base of the costal margin to the intersection with the first radial vein, it has a distinct expansion in the region of the humeral vein, as well as a wider posterior part of the wing plate in the zone of the cubital and anal veins. The probability of correct discrimination and diagnosis of adults of both strains was 98.5% and remained high (96.3%) after cross-validation using the jackknifed estimate procedure. Consequently, stress did not lead to a decrease in the level of interstrain differences that we previously identified between control samples of adults of *Shg* and *Lg* according to the shape of the wing [43].

In this regard, it was interesting to evaluate the manifestation of stable differences in the shape of the wing between the control and stress (STHS) samples: is it possible to distinguish control and impact groups of individuals in a mixed sample of both strains just as almost unmistakably as representatives of different strains? To do this, a discriminant analysis of the Procrustean coordinates between the control and stress groups was carried out without taking into account the belonging of objects to the line and gender. Such a method of comparison is statistically and morphometrically quite justified, since even when comparing communities, it is permissible to use the "taxon-free" method, i.e., combining representatives of different species into generalized samples (removing species "taxonomic boundaries") and pairwise comparison of morphological differences in the appearance of communities [9, 60], for example, in synecological and paleoecological comparisons of fragments of communities and biota. The procedure of linear discriminant analysis actually assumes only a pairwise comparison of samples in all variants, and when comparing different discriminant functions, some correlation is often observed [51, 61], i.e., the axes are not strictly orthogonal, which makes it difficult to use them in multivariate analysis of variance.

The results of this variant of discriminant analysis are shown in Fig. 4: again, the between-group differences were statistically significant (Wilks' $\Lambda = 0.5455$; $D^2 = 3.566$; Hotelling's T^2) = 464.8; $df_{1.2} = 34, 287$; $F = 6.37$; $p < 0.0001$). The probability of correct diagnosis of the adult wing from the control and stress-induced groups was 85.4%, and after cross-validation it was 78.3%, i.e., remained relatively high. The revealed differentiation is great and comparable in level even with typical subspecies differences. It follows from Table 4 that the wings of stress-induced flies

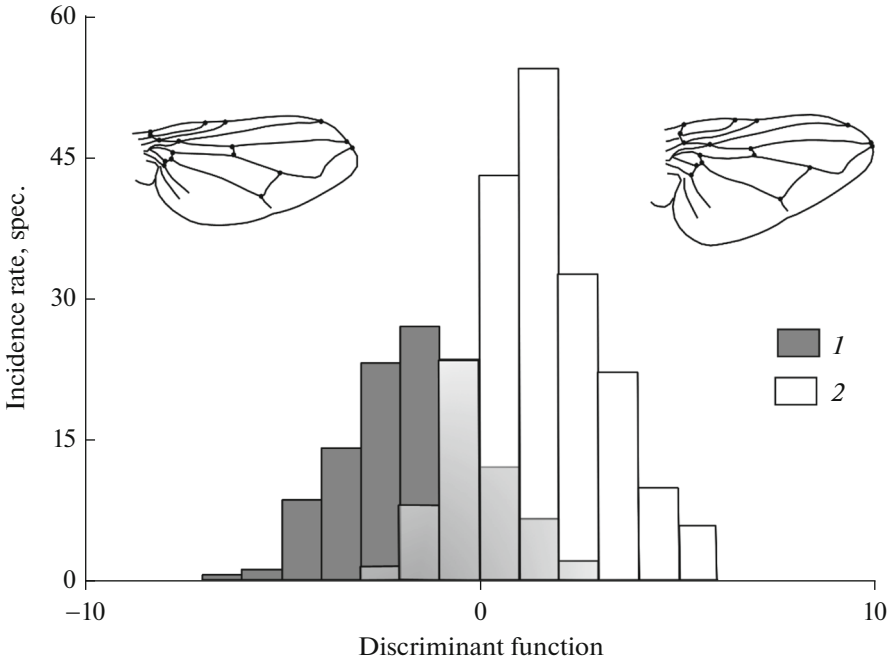

Fig. 4. The results of the discriminant analysis of the Procrustean coordinates characterizing the variability of the wing shape of the control (*1*) and subjected to short-term heat stress at three stages of ontogeny (*2*) of housefly groups (*Musca domestica*) without taking into account their belonging to the line and gender. The contour configurations of the wings (outlines) correspond to the centroids of the compared samples.

in both strains have common structural features, differing only in a pointed apical shape with a widening of the posterior edge of the wing plate in the area of the cubital and anal veins, as well as a relatively increased area of the medial-cubital cell.

Since it was important to evaluate the influence of each of the three mentioned factors on the variability of the shape of the wings, including their possible interactions, i.e., to obtain a complete picture of intergroup differences, we carried out a canonical analysis of the Procrustean coordinates characterizing the variability of the wing shape in samples of males and females of the control and impact groups of both strains of flies (Fig. 5). Since canonical analysis is based on maximizing the ratio of between-group to within-group differences, it makes it possible to estimate the maximum possible differences between sample centroids, taking into account the mutual orientation of their scatter ellipsoids. As a result of calculations, a sequential hierarchy of intergroup variability is orthogonally formed in the morphospace of successive canonical variables [51, 62].

Between-group variability along the first six canonical axes turned out to be highly statistically significant ($p < 0.0001$), and only along the seventh canonical axis the level of significance of differences was only $p = 0.0398$. The ratios of the values of the centroids of the samples, taking into account standard errors, as well as the values of between-group variances and estimates of their significance along the first four canonical variables (CV1–CV4), are given in Table 2. It can be seen that the first four canonical variables characterize about 94.4% of the intergroup variance, i.e., quite fully reflect the basic structure of wing shape variability in housefly lines. Along the first canonical variable (CV1), which accounts for about 63% of the intergroup variance, interlinear differences were clearly manifested (see Fig. 5). The results of the canonical analysis make it possible to estimate the mutual placement of the sample ellipsoids in the general 3D morphospace. Each of the ordinate scatter ellipsoids combines 95% of the within-group variance.

Judging by the signs of the sample centroids (see Table 2), all group line centroids of *Shg* are placed in the region of positive CV1 values, and the strain *Lg*, negative. The second canonical axis (CV2), characterizing 21% of the variance, reflects gender differences. All groups of males along CV2 have negative values of centroids, while all groups of females have positive values. Accordingly, all ellipsoids of female groups are located in the region of positive CV2 values. Differences appeared along the third canonical variable (CV3) (about 6% of the variance) associated with the effect of heat stress on the experimental groups of both strains. The ellipsoids and centroids of all impact groups are shifted in the general morphospace along CV3 in the direction of positive values (see Fig. 5). The arrows in the figure indicate the directions of intergroup differences in the morphospace associated with the factors of "line," "gender," and "stress."

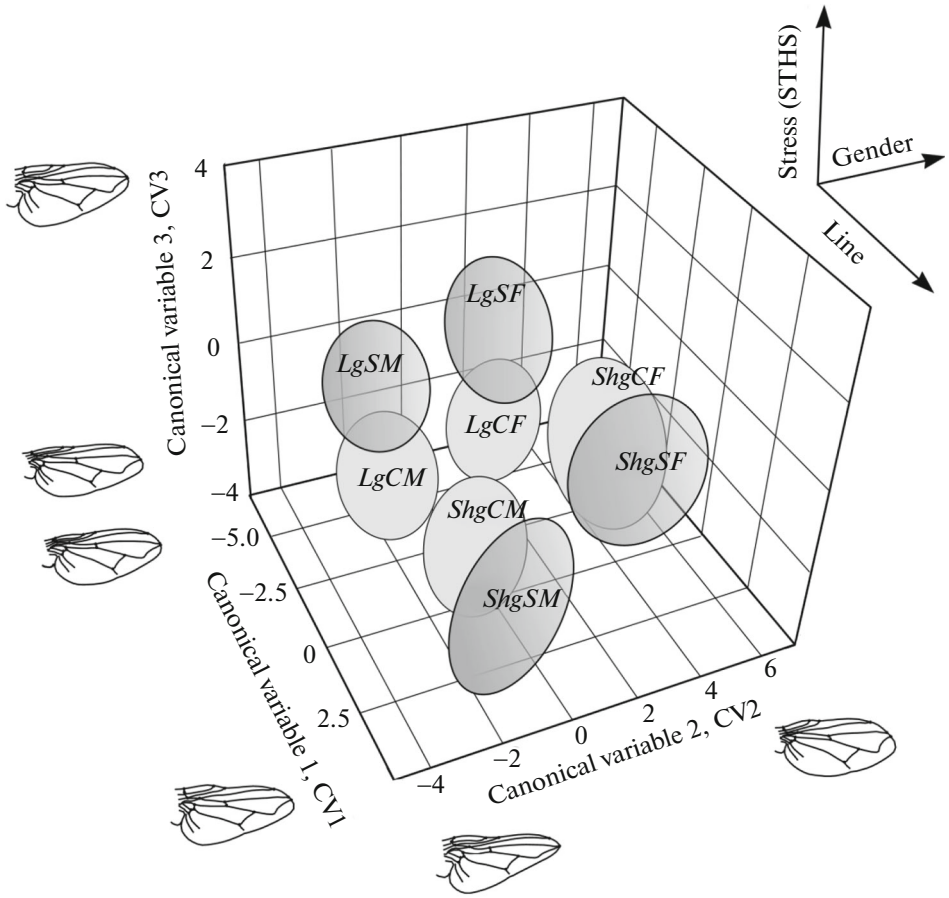

Fig. 5. The results of the canonical analysis of the Procrustean coordinates characterizing the variability of the control wing shape (*C*) and experimental (*S*) groups of males (*M*) and females (*F*) in the lines of long-lived (*Lg*) and short-lived (*Shg*) houseflies (*Musca domestica*). Along the canonical variables (CV1–CV3) there are wing contour configurations (outlines) corresponding to the minimum and maximum values of the axes. The ellipsoids include 95% of the within-group variance of the sample ordinates. Arrows show the directions of intergroup variability of the wing shape in 3D morphospace.

To assess the possible allometry of the wing, a significant relationship between its size and shape, we performed a regression analysis between the values of the logarithmic centroid sizes (lnCSs) and the first principal component (PC1) calculated from the Procrustean coordinates. As a result of calculations, no significant regression relationship between CS and shape was found in any of the studied groups of flies. The proportions of the explained regression ranged from 0.41 to 4.9%, and their significance level varied from $p = 0.659$ to $p = 0.068$. Thus, the between-group differences in the shape of the wings revealed by us are not due to the effects of allometry.

To obtain a quantitative estimate and a more rigorous interpretation of intergroup differences, a three-way multivariate analysis of variance was performed for all seven canonical variables (Tables 3 and 4). Table 3 shows that the combined factorial variance, including interaction options, approximates about 63% of the total variance. If the interstrain differences accounted for about 35.3% of the total variance, then the gender differences accounted for 17.1%, and the stress-induced differences accounted for 3.8%. The proportion of variance characterizing the interaction "line × gender" (L × G) was 2.2%, the variant of the interaction "line × stress" (L × S) was 3.6%, and for the factors "gender x stress" (G × S) it was 1.5%. The triple interaction "line × gender × stress" (L × G × S) was minimally manifested, which accounted for about 1.2%. The unexplained component of the variability was approximately 2.8%. The generalized intergroup differences for each factor and all variants of interactions, including the triple one, turned out to be statistically significant (see Table 4).

Thus, interstrain differences are approximately twice as large as gender differences and are almost an order of magnitude higher than the level of stress-induced differences. The relatively low proportion of variance due to the line × stress (L × S) interaction indicates that both strains of flies exhibit a largely similar morphogenetic response to the stress factor. The insignificant contribution of the interaction of factors

Table 2. The results of the canonical analysis of Procrustean coordinates reflecting the variability of the wing shape of males and females of control and stress (STHS) groups in strains *Shg* and *Lg* houseflies along the first four canonical variables

Line, gender, control/stress	Canonical variable			
	CV1	CV2	CV3	CV4
Sample centroids (±*SE*)				
Shg, males, control	4.078 ± 0.210	−1.395 ± 0.263	−1.827 ± 0.160	0.520 ± 0.246
Shg, females, control	2.115 ± 0.162	2.496 ± 164	−1.329 ± 0.189	0.1690 ± 0.154
Shg, males, stress	3.928 ± 0.157	−1.587 ± 0.135	0.758 ± 0.168	−0.306 ± 0.154
Shg, females, stress	1.791 ± 0.134	1.861 ± 0.115	1.377 ± 0.179	0.448 ± 0.152
Lg, males, control	−1.614 ± 0.178	−2.750 ± 0.142	−0.583 ± 0.128	0.559 ± 0.182
Lg, females, control	−4.144 ± 0.183	0.283 ± 0.138	−0.118 ± 0.152	−1.251 ± 0.144
Lg, males, stress	−0.791 ± 0.147	−1.224 ± 0.217	0.247 ± 0.130	1.636 ± 0.185
Lg, females, stress	−3.951 ± 0.154	0.786 ± 0.117	0.100 ± 0.139	−0.542 ± 0.142
Results of canonical analysis				
Wilks' Λ	0.0040	0.0399	0.1602	0.2977
Eigenvalues	8.9982	3.0159	0.8582	0.66750
Canonical correlation	0.95	0.87	0.68	0.63
Share of dispersion, %	62.73	21.03	5.98	4.65
χ^2	1549.5	903.6	513.7	339.9
Number of degrees of freedom, df	203	168	135	104
F	328.44	110.08	31.32	24.36
Significance level	$p < 0.00001$	$p < 0.00001$	$p < 0.00001$	$p < 0.00001$

gender × stress (G × S) showed that representatives of different genders almost equally reacted to STHS. The dispersion of the interaction of the factors line × gender (L × G) is close in magnitude to the level of dispersion caused by the influence of the stress factor, which indirectly indicates the initial differences formed after selection for different times of reproduction in the morphogenesis of the wing in males and females of different strains.

Using the squared values of the generalized Mahalanobis distances (D^2), we compared the gender differences in the shape of the wing both between the control and between the impact groups of adults (all distances were highly significant, $p < 0.0001$). It turned out that the sexual dimorphism in the shape of the wing in the representatives of each line was somewhat more pronounced between the control samples: control *Shg* – $D^2 = 23.17$, stress *Shg* – $D^2 = 18.66$; control *Lg* – $D^2 = 17.68$, stress *Lg* – $D^2 = 15.11$. Interstrain pairwise comparisons of the same sex groups also revealed large differences between control groups: control males *Shg* and *Lg* – $D^2 = 40.04$, stress males *Shg* and *Lg* – $D^2 = 27.33$; control females *Shg* and *Lg* – $D^2 = 46.15$, impact females *Shg* and *Lg* – $D^2 = 32.74$. Thus, in the control groups, sexual dimorphism in the shape of the wing is more pronounced than in the impact groups; STHS led to some leveling of sex differences in wing morphogenesis.

According to the results of canonical analysis based on the matrix of non-squared generalized Mahalanobis distances (*D*) between the compared samples, we performed a cluster analysis (UPGMA) (Fig. 6) using the Euclidean distance metric (its choice is due to the highest value of the cophenetic correlation coefficient (CCC = 0.95) compared to other metrics). It follows from the figure that the cluster structure hierarchically includes two large clusters, one of which combines all samples of the strain of short-lived flies (*Shg*), and the second, all samples of the strain of long-lived (*Lg*). Each of the clusters is further clearly divided into two hierarchically subordinate subclusters, characterizing, on the one hand, the samples of males and, on the other, females, and the clusters of each sex, into subordinate subclusters of the control and impact groups. Note the relatively high levels of bootstrap support for most cluster nodes, i.e., stability of its structure. The general structure of the cluster reflects the hierarchy of directions of intergroup variability: the highest level of the hierarchy corresponds to interlinear, mainly genetic, differences, the intermediate level characterizes gender differences, and the lowest level, determined by STHS, indicates the level of ecological environmental impact. It is interesting to emphasize that at the cluster branching nodes corresponding to sex and stress-induced differences, the average distances corresponding to the hierarchical levels of sample aggregation were approximately the same for both strains.

Table 3. Results of three-way ANOVA of canonical variables (CV1–CV7) characterizing differences in wing shape variability of control and impact (subjected to STHS) groups of males and females of strains *Shg* and *Lg*

Source of variability, factor	Number of degrees of freedom, df	CV1 (MS)	*p*	CV2 (MS)	*p*	CV3 (MS)	*p*
Line, L	1	**2119.896**	0.0000	77.300	0.0000	1.748	0.1872
Gender, G	1	405.285	0.0000	**643.184**	0.0000	8.824	0.0032
Stress, S	1	1.755	0.1863	5.712	0.0175	**175.783**	0.0000
L × G	1	10.077	0.0017	23.361	0.0000	1.886	0.1707
L × S	1	8.971	0.0030	34.879	0.0000	72.721	0.0000
G × S	1	2.608	0.1074	8.983	0.0030	0.768	0.3817
L × G × S	1	0.790	0.3748	1.581	0.2096	1.813	0.1792
Error	292	1.000		1.000		1.000	
	df	CV4 (MS)	*p*	CV5 (MS)	*p*	CV6 (MS)	*p*
Line, L	1	0.752	0.3865	11.902	0.0006	0.354	0.5522
Gender, G	1	6.671	0.0103	2.004	0.1579	2.565	0.1103
Stress, S	1	52.025	0.0000	1.004	0.3172	2.214	0.1379
L × G	1	22.625	0.0000	**62.018**	0.0000	4.856	0.0283
L × S	1	**82.603**	0.0000	20.027	0.0000	3.061	0.0812
G × S	1	2.131	0.1454	45.651	0.0000	19.557	0.0000
L × G × S	1	9.081	0.0028	1.055	0.3053	**42.832**	0.0000
Error	292	1.000		1.000		1.000	
	df	CV7 (MS)	*p*	Total variance (TV)		Share of variance, %	
Line, L	1	0.061	0.8045	2212.014		35.29	
Gender, G	1	0.078	0.7797	1068.612		17.05	
Stress, S	1	0.003	0.9560	238.495		3.80	
L × G	1	11.542	0.0008	136.364		2.18	
L × S	1	1.955	0.1631	224.218		3.58	
G × S	1	14.197	0.0002	93.894		1.50	
L × G × S	1	**14.995**	0.0001	72.147		1.15	
Error	292	1.000		2044.003		32.61	
Total	299			6268.734		100.00	

MS is the mean sum of squares; *p*—significance level; Bold font indicates the maximum MS values for each canonical variable.

Table 4. Evaluation of the significance of the results of a three-way multivariate analysis of variance (MANOVA) of canonical variables (CV1–CV7) characterizing differences in the variability of the wing shape of control and STHS-treated males and females of strains *Shg* and *Lg*

Source of variability, factor	Wilks' test	Effect size, η^2	F	Number of degrees of freedom of the effect, df_1	Number of degrees of freedom of error, df_2	Significance level, *p*
Line, L	0.11661	0.88339	309.51	7	286	<0.0001
Gender, G	0.21461	0.78539	149.52	7	286	<0.0001
Stress, S	0.55043	0.44957	33.37	7	286	<0.0001
L × G	0.68166	0.31834	19.08	7	286	<0.0001
L × S	0.56565	0.43435	31.37	7	286	<0.0001
G × S	0.75668	0.24332	13.14	7	286	<0.0001
L × G × S	0.80187	0.19813	10.09	7	286	<0.0001

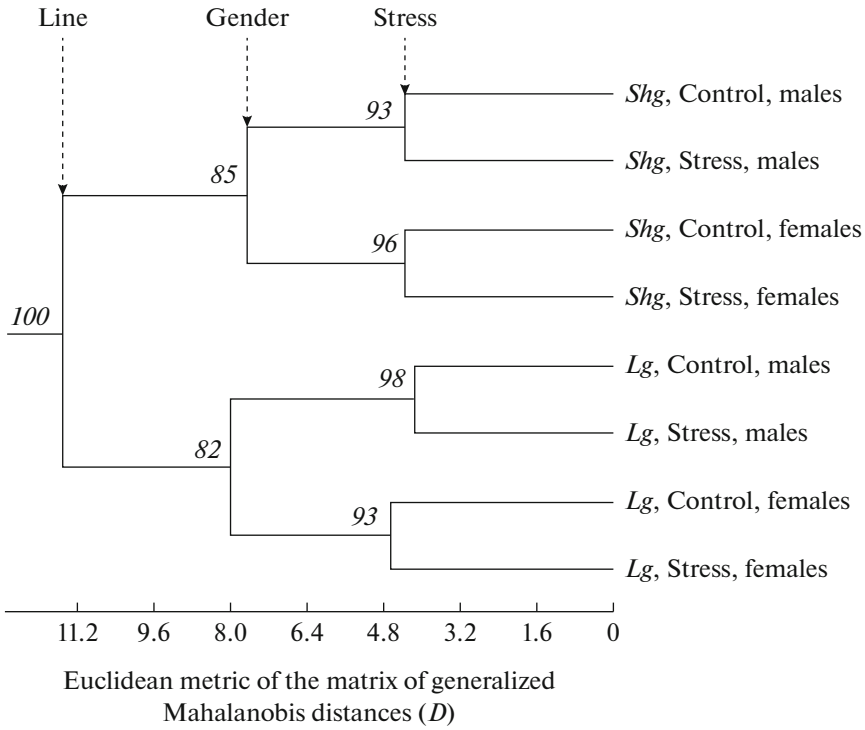

Fig. 6. Cluster analysis (UPGMA) of the matrix of generalized Mahalanobis distances (*D*) in the shape of the wing between the control and STHS-affected groups of males and females in short-lived adults (*Shg*) and long-lived (*Lg*) housefly lines (vertical arrows show levels of the hierarchy of between-group differences).

An important aspect of research concerns the assessment of the levels of within-group disparity and developmental stability of control and impact groups of flies. For this purpose, based on the values of the first three canonical variables, we carried out a series of calculations of the volumes of within-group morphospaces (*Vm*) according to samples randomly aligned by the number of observations (Fig. 7). The average volume of strain morphospace of *Shg* (*Vm* = 132.79 ± 0.51) significantly (t = 20.8; $p < 0.00001$) exceeded that of *Lg* (*Vm* = 111.28 ± 0.90), i.e., the wing morphogenesis of short-lived flies is generally less stable than that of long-lived flies. This general trend also manifested itself in pairwise comparison of the values of *Vm* in similar control and stress groups of males and females of both strains (see Fig. 7).

When assessing the ratio of the contributions of between-group differences due to the factors line, gender, and stress, we also applied a three-way analysis of variance of the values of *Vm*. The overall effect was highly statistically significant (F = 135.7; $df_{1,2} = 7.72$; $p < 0.0001$), and the multiple coefficient of determination characterizing the share of explained variance was $R^2 = 0.93$. The line and gender factors, which accounted for more than 80% of the variance, made the main and almost equal contributions to the between-group differences in the variability of the volumes of withn-group morphospaces (Table 5). In this case, the interstrain differences only slightly exceed the gender differences, and the size of the effect in both cases is close to the maximum.

Thus, the interline (mainly genetically determined) differences reflecting the relatively high stability of the development of wing configurations in the *Lg* strain, but increased instability in the formation of wings in representatives of the *Shg* strain, can be considered as the result of the selection for the timing of reproduction. Gender differences, expressed in greater instability in the development of the wings of females of both lines, apparently reflect both the general species trait of phenotypic variability and, probably, the results of selection. It should be emphasized that in all groups of females, the volumes of within-group morphospaces turned out to be significantly larger than in groups of males (see Fig. 7): in the *Shg* strain, between males and females of the control group $Q = 20.18$ ($p = 0.00013$), while in the stress group $Q = 16.35$ ($p = 0.00013$); in strain *Lg* in the control group, $Q = 6.60$ ($p = 0.00052$), in the stress group, $Q = 11.13$ ($p = 0.00013$). The latter reflects the greater instability of wing development in all groups of females compared to the corresponding groups of males.

The STHS factor, which accounted for about 6% of the variance, significantly less influences indicator variability *Vm*, i.e., the instability of the development of the wing configuration largely reflects the similar general reaction of the stress groups in males and females of different strains. At the same time, as noted

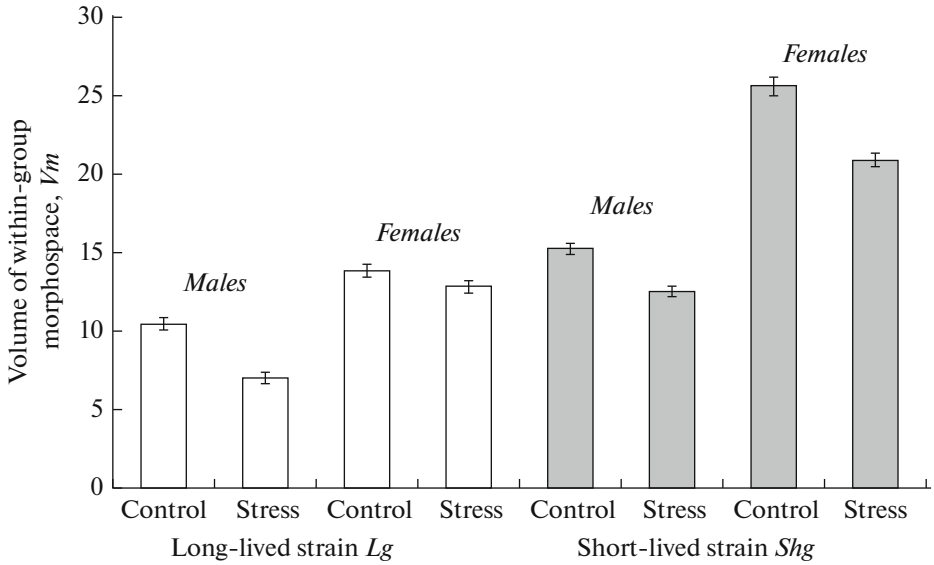

Fig. 7. Comparison of the volumes of the within-group morphospace *Vm* (taking into account standard errors ±*SE*) in the control and stress (STHS) groups of males and females of short-lived (*Shg*) and long-lived (*Lg*) housefly lines (before calculating *Vm* in all initial samples after randomization, the number of observations is equalized: $n = 17$).

above, there is a general trend towards a decrease in the value of *Vm* in all impact groups as compared to their respective control groups (see Fig. 7), but in different strains the differences are expressed somewhat differently. Not surprisingly, the contribution of the line × gender (L × G) interaction is also significant, accounting for about 4% of the variance. It should also be noted that although the proportion of the variance of the interaction of factors line × stress (L × S) was only 0.5%, its contribution is formally close to the minimum level of significance, and the size of the effect exceeds the minimum level accepted by Cohen. The result directly indicates that in both strains, despite the great similarity of the overall decrease in the value of *Vm* in stress groups, sometimes the effect can be expressed in representatives of different strains somewhat weaker or stronger. The effect of the interaction gender × stress (G × S) did not manifest in this case (see Table 5). However, the interaction of three factors line × gender × stress (about 1.4% of the variance) turned out to be significant, and the effect size ($\eta^2 = 0.16$) exceeded the average Cohen difference. This interaction effect reflects the general conse-

Table 5. Three-factor analysis of variance of the volumes of the within-group morphospace (*Vm*) along the first three canonical variables when assessing the differences in the wing shape of the control and stress (STHS) males and females of the strains *Shg* and *Lg*, taking into account the size of the effect and the proportion of the explained variance, %

Source of variability, factor	Sum of squares, SS	Number of degrees of freedom, df	Mean square, MS	F	Significance level, *p*	Effect Size, η^2	Share of dispersion, %
Line, L	1075.17	1	1075.17	427.80	<0.0001	**0.8559**	41.86
Gender, G	1004.01	1	1004.01	399.49	<0.0001	**0.8473**	39.09
Stress, S	154.68	1	154.68	61.55	<0.0001	**0.4609**	6.02
L × G	106.14	1	106.14	42.23	<0.0001	**0.3697**	4.13
L × S	12.47	1	12.47	4.96	0.0290	**0.0645**	0.49
G × S	0.07	1	0.07	0.03	0.8726	0.0004	0.003
L × G × S	35.18	1	35.18	14.00	0.0004	**0.1628**	1.37
Within-group	180.95	72	2.51				7.04
Total	2568.68	79					100.00

Significant effect sizes are highlighted in bold. All groups during the rarefaction procedure are preliminarily randomly aligned according to the minimum number of observations, and the values *Vm* for each sample obtained on the basis of repeated ($n = 10$) bootstrap cycles with random substitution of individuals.

quences of both the selective process and the repeated influence of STHS on the genotypes of males and females, which are reflected in different manifestations of instability in the development of the wings of flies in representatives of the control and stress groups of different sexes in different strains. The highest levels of values of *Vm* appeared in both groups of females of the *Shg* strain (see Fig. 7), which indirectly indicates the incomplete stabilization of wing morphogenesis in females of this strain after the completion of its selection for early reproduction.

Discussing the results, it is necessary to compare them with the conclusions of other authors. In contrast to direct temperature effects, which cause certain modification changes, the relationship between wing size and temperature in geographically distant populations may reflect genetically determined adaptive effects that have arisen historically. In experiments on Drosophila, V. Debat et al. [38], revealed a strong negative dependence of the wing size on temperature: at a high constant temperature of development, the wing had small dimensions. An analysis of the geographical variability of the wing sizes of Drosophila [63] and houseflies [41] also revealed a decrease in wings in more southern latitudes at higher temperatures. Consequently, as in the case of modifications and genetically determined adaptive reactions of different geographic populations, with an increase in environmental temperature, the wing size became smaller. According to our data, in both experimental groups of houseflies, in response to a repeated short-term increase in temperature, the wing size not only did not decrease, but, on the contrary, in most cases increased significantly. The significant effect of the interaction between the line × stress factors, revealed using a three-way ANOVA of the centroid wing size, partially explains the nature of this growth reaction, which proceeds according to the "hormesis" type [64, 65]. Recall that strain *Shg* has generally large wings, and strain *Lg*, have smaller sizes, i.e., growth and development in its individuals are slowed down. In both strains, as already noted, there is a general tendency for the wings to increase in stress (STHS) groups. At the same time, in large flies of strain *Shg* the wing after repeated stress only slightly increases, and in small representatives of *Lg*, increases significantly, which caused a significant interaction effect (L × S). The effect of STHS, apparently, causes an acceleration of growth and development, and to the greatest extent in strain *Lg*.

V. Debat et al. [39], on the example of lines of *D. melanogaster* found that temperature and genomic mutations are able to modify the levels of fluctuating asymmetry (FA) as an indicator of developmental destabilization, and also affect the individual variability of experimental lines. At the same time, it was shown that the value of FA does not directly depend on temperature but is indirectly set by the manifestation of individual variability of groups stimulated by temperature. In a house fly, when assessing developmental stability in terms of *Vm* apparently, a similar picture is observed, i.e., genotypes of males and females show different sensitivity to developmental stress. We did not find an experimental study completely similar to our design on other ectothermic species. However, in the literature there are [64–69] examples of the effect of short-term or moderate-in-degree hypo- and hyperthermic stress exposures, which are often accompanied by the hormesis effect, which manifests itself, among other things, in an increase in size. Therefore, in our case, STHS also causes an increase in size, enhances the stability of development, and leads to a similar morphogenetic change (see Fig. 4) in wings, however, allometric effects are not detected in this case.

We have already noted studies that have proven the manifestation of transgenerational inheritance of stress-induced epigenetic rearrangements of the genome, causing certain morphogenetic changes [15, 18–20]. It is noteworthy that earlier Yu.M. Nikonorov and G.V. Ben'kovskaya [70] found that in both lines of the housefly, the content of transposon copies significantly increases by the adult stage compared to the pupal stage of Hermes in DNA. Transposon Hermes propagation in the fly genome and an increase in its copy number can occur based on the mechanism of transposition without an intermediate episomal form [70]. By analogy with the mechanisms of stress-induced MGE DNA rearrangements identified in Drosophila, which cause certain morphogenetic changes [27], we can expect STHS activation of similar processes of functional rearrangements of the genome and morphogenesis due to transpositions of transposable elements in the housefly. However, along with this version of the explanation, another, in many respects alternative, linking changes in the environment and the genomic response to selection is also possible.

In [71], the frequencies of occurrence of alleles throughout the genome were analyzed using 20 geographically separated populations of *D. melanogaster*. The flies were collected at the beginning and end of the growing season. As a result, reversible parallel seasonal shifts in allele frequencies were established in both North America and Europe, reflecting the general manifestations of seasonal adaptation to a changing environment. It has been shown that seasonal fluctuations in allele polymorphism are complemented by large chromosomal inversions, and there is a correspondence between seasonal and spatial changes in allele frequencies. The authors concluded that fluctuating selection is an important evolutionary force that can influence patterns of genetic variation in the model species. Parallel changes in allele frequencies over the seasons with complete isolation of the compared populations indicate that ecologically stimulated and obviously selectively determined parallel rearrangements of the genetic structure of populations

according to the known mechanism of adaptive polymorphism are possible [72]. Earlier, Academician S.S. Shvarts [73], considering a model of fast directed and reversible changes in the frequencies of morphs in a population, assumed the existence of a special mechanism of homeostatic fluctuations in the genetic structure of populations. It is possible that in the cases considered above, different genotypes of Drosophila with different reproductive potential depending on the conditions of the seasons of the year (for example, threshold effects on their reproductive system of temperature and humidity) could be selected in different seasons. This effect suggests the possibility of triggering a similar selective genotypic mechanism in response to repeated exposures to STHS in a succession of descendants of impact sublines. Accordingly, genotypes that respond to STHS by increasing their size can increase their relative abundance within just a few generations due to greater individual fecundity.

Therefore, there is reason to believe that the efficiency of selection by the time of fly reproduction, which forms genetically differentiated strains, as well as the rapid development of morphogenetic differences between strains and similar unidirectional morphogenetic and growth reactions of impact groups in response to STHS, can be due to both selective mechanisms of rearrangement of the genotypic composition of strains due to genotypes with different reproductive potential under different environmental conditions, as well as stress-induced epigenetic processes, including those caused by MGE transposition, which form stable "long-term modifications" transgenerationally. Further analysis, including genetic and epigenetic, of model experimental groups in combination with morphometric studies can clarify the nature of the mechanisms of rapid stress-induced morphogenetic changes.

CONCLUSIONS

Mass oppositely directed disruptive selection for different periods of fly reproduction was carried out on initially heterogeneous material but having a common origin. Therefore, the revealed parallelisms in changes in the size and shape of the wing in the strains reflect their general potential for possible morphogenetic changes. At the same time, the manifestation of specific morphogenetic reactions in the control and impact groups of flies reflects both epigenetic and genetic changes resulting from directed selection. It should be noted that the results of selection for different life spans [44] generally fit into the species range of reproduction time and longevity of the housefly, which, as is known, varies in natural and laboratory conditions from 2–3 weeks to 2 months and more [74]. In other words, the pool of possible modifications of the original heterogeneous line could well contain potential variants of morphogenesis that arose earlier in the history of the species, on the basis of which the lines of short- and long-lived flies were formed and selected.

Therefore, all of the above leads us to the main hypothesis that the morphogenetic changes that occurred in parallel in both housefly strains after STHS are based on two of the many natural developmental modifications historically developed in the species, which are normally epigenetically regulated and blocked, but can be implemented in a critical situation. Such a natural directional modification, which manifested itself in both strains as a stress-induced rearrangement of wing morphogenesis, caused a complex of similar morphological changes, including an increase in wing size, a unidirectional change in its shape in impact groups, and provided similar processes for increasing their level of developmental stability (with a decrease in *Vm*). In addition, we recall that under the conditions of the experiment with STHS, a tendency was previously noted to prolong development at the puparium stage in the group of stressed long-lived individuals. *Lg* and the reverse trend, in the group of stressed short-lived individuals *Shg* [47]. Both of these tendencies were aimed at normalizing the morphogenesis of stress groups in strain with a general modification. The combination of these phenotypic features can be interpreted as a manifestation of the stress-induced effect of hormesis in impact groups of both sexes. The phenomenon of hormesis has been noted by many researchers under various moderate and short-term stress effects, including the effect of moderate hypo- and hyperthermia, especially on the early stages of insect development [64, 65, 75].

The interstrain differences achieved during selection are very large both in size and in wing shape, despite the relatively small (14–16) number of selection generations. Such rapid and effective changes can also be considered as a result of the fixation in the process of selection of modifications that are pre-existing in the species "wave" due to probable epigenetic rearrangement (according to C. Waddington: accommodation) and transgenerational inheritance of typical morphotypes (according to Schmalhausen: morphoses), on the basis of which strains of short- and long-lived flies were formed. At the same time, the stability of the development of long-lived individuals of the strain *Lg* is higher than short-lived strain flies *Shg*, The morphogenesis of the long-lived flies stabilized relatively quickly (this does not contradict the hypothesis). It remained unclear why a higher level of wing developmental instability was observed and preserved in all groups of females compared to males. It is possible that the modification switching of wing development and its stabilization in males of both strains were carried out "easier" and faster than in females, especially in the strain *Shg*, where the physiological processes of females are tuned for rapid maturation and early reproduction. The established high phenotypic plasticity of strains during selection for different life spans and the changes caused by STHS directly indi-

cate the reality of stress-induced rapid morphogenetic rearrangements of the species under a sharp change in environmental conditions, which allow the housefly to adapt to living in a wide climatic and seasonal range of conditions.

However, the above example of parallel seasonal fluctuations in the genotypic composition of European and American Drosophila populations (see [71]) shows that in the case considered by us, along with probable epigenetic mechanisms, special balanced selective processes of rapid accumulation of certain genotypes, the reproductive potential of which depends on the specific environmental conditions of the environment (temperature, humidity, a complex of seasonal factors).

Further parallel comparative analysis of the genotypic composition and reproductive capabilities of different genotypes, as well as epigenetic profiles of DNA methylation and the placement of transposable elements (TEs) at chromosome sites in representatives of short- and long-lived fly strains, selected according to the time of reproduction, may allow us to test both proposed hypotheses and clarify the emerging ones of evolutionary-ecological issues related to the observed rapid morphogenetic rearrangements.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflicts of interest.

Statement on the welfare of animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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