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FLUCTUATING ASYMMETRY OF SHAPE IN RODENTS FROM RADIOACTIVELY CONTAMINATED ENVIRONMENTS AT CHORNOBYL

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Many random errors that occur during individual development can lead to small deviations from perfect symmetry between body sides. Developmental instability is a tendency of the phenotypic value of the trait to deviate from the value expected for an individual with a given genotype and environment. Fluctuating symmetry (FA) can be calculated as variance of the distribution of such differences between the left and right sides, and is a useful index of developmental instability. In this study we addressed the question of whether levels of FA were elevated in the radioactively contaminated populations compared to reference populations of an abundant rodent living in both environments: the yellow – necked mouse (Apodemus flavicollis). We used six populations of these animals from both inside the radioactively contaminated area close to the failed Chornobyl reactor in Ukraine and in reference areas with no apparent contamination by radionuclides. Twenty-four landmarks on the ventral surface of the rodent's skulls were used to calculate the amounts of FA of shape using Procrustes methods. A higher level of FA was documented for the populations in closest proximity to the failed Chornobyl Nuclear Plant. This index was significantly higher in populations from the most contaminated locations in the Chornobyl Exclusion Zone than in the reference areas. On the other hand, populations from the less contaminated areas inside the Chornobyl Exclusion Zone did not express FA values different from those in the reference populations.

Introduction

Random errors, which occur during development, lead to small deviations from perfect symmetry between body sides for bilateral characters. Fluctuating asymmetry (FA) is an estimate of small and non-directional departures from the expected bilateral symmetry [1]. FA provides an estimate of developmental stability, because the two sides of a bilaterally symmetrical organism would share the same underlying genotype, and therefore are expected to be identical in the same environment

[2, 3] FA can be estimated as the variance of a distribution among individuals of the differences between the left and right sides. FA can only be used as a reliable indicator of environmental quality when a substantial number of reference populations with no known contamination are sampled. In this case, it is essential to establish the background level of FA in a species and to know whether FA can vary significantly among reference populations even in the absence of known stressors.

Environmental radiation has imposed a significant amount of stress on populations in the landscapes contaminated after the 1986 Chornobyl meltdown. Several studies conducted in and around the Chornobyl Nuclear Power Plant (ChNPP) have indicated that developmental instability measured as fluctuating asymmetry has significantly increased in plants and animals from the affected populations. Developmental instability was positively correlated with the level of contamination by ^{137}Cs in three different species of plants [4]. In barn swallows, levels of fluctuating asymmetry as well as the frequency of partial albinism have increased near Chornobyl compared to that in the reference areas [5, 6].

Some of these effects have direct influences on individual fitness and could be expressed in lower competitive ability and survival [3]. However, mammalian populations from Chornobyl have not been tested for FA despite the fact that they live in the most contaminated areas around the plant [7]. In the absence of data on humans, small mammal populations may be used as a model for evaluating risks of radioactive contamination in human populations.

Asymmetry has been estimated using many different indices (review in Palmer, 1996) that have different degrees of reliability. Recently a new approach has been applied to the analysis of asymmetry using the linkage between geometric methods and conventional multivariate statistics that is sometimes called the «morphometric synthesis» [1, 8 – 11]. A mixed model ANOVA is an essential part of this approach that allows reliable estimation of the measurement error [12]. To evaluate the level of FA in a population, we used the Procrustes method for quantifying asymmetry of shape [11]. This method uses rotation of the superimposed configurations to achieve the best fit between the opposite sides. Asymmetry is then measured as the deviation between the pairs of the corresponding landmarks on the left versus the right side [11].

Our overall objective is to determine whether asymmetry is elevated in radioactively contaminated populations compared to that in reference populations of the yellow-necked mouse (*Apodemus flavicollis*), which is common around the Chornobyl Nuclear Power Plant as well as in deciduous forests throughout Ukraine. Individual estimates for FA were calculated for each of 13 populations (6 contaminated and 7 reference populations) the shape of skulls. We then tested for differences among populations in the amounts of FA. Finally, we interpret our findings based on the environmental radioactivity estimates for each of the contaminated locations [13].

1. Materials and Methods

1.1. Populations

We collected 843 individuals of *Apodemus flavicollis* from 14 locations in Ukraine. Six of the sites within the Chornobyl Exclusion Zone were used in this study. The first three populations came from the most contaminated 10-km zone in close proximity to the failed reactor. The next three populations were obtained on the western edge of the 30-km Exclusion Zone. Latitude and longitude were recorded for each location using a Geographical Positioning System (GPS). The last group of populations came from the uncontaminated locations along a southwest to northeast transect across Ukraine. Two of the locations from the contaminated area, Gluboke Lake and Tovsty Forest, were sampled in two different years to ensure the reproducibility of our results. Names of the populations, numbers of skulls used in the analysis and the coordinates of their locations are presented in Table 1. To illustrate the degree of radioactive contamination in each of the populations, we used measures collected with thermal luminescent dosimeters (TLDs) as well as the measurements of ^{137}Cs in dry muscle reported from an earlier publication describing frequency distributions of contaminants in the environments around the Chornobyl Nuclear Power Plant [13] Background values for the TLDs were estimated at the International Radioecology Laboratory in Slavutych, Ukraine. Some TLDs were left at Savannah River Ecology Laboratory, Aiken, SC, and USA as additional controls.

1.2. Morphometrics

Our study concentrated on the fluctuating asymmetry (FA) of skulls. Each specimen was cleaned with dermestid beetles and dried. Skulls were leveled on a sand base with the ventral surface up. We took pictures of the ventral surface of each skull with a 35-mm camera using a ring flash that went around the lens to provide even lighting. Two pictures of each skull were taken to account for the effects of placement on the measurement error. Pictures were developed and scanned into individual bitmap files using LS-2000 film scanner. Each picture was given a random name to prevent subjective bias during the subsequent measurement steps [2]. Evolutionary homologous landmarks (N = 24) were chosen on each side of the skull similar to those used in a study with house mice [14]. Landmarks were distributed on the ventral surface of the skull to represent its entire surface. Landmark positions were digitized using a standard software package TPSDIG [15] Landmarks were also independently placed on each of the pictures twice to assess the effects of digitizing on the measurement error. Statistical analyses were conducted using SAS 8,1 software [16].

1.3. Asymmetry of skull shape

We analyzed the shape asymmetry of skulls by superimposing the configurations of landmarks using the Procrustes method [17]. First, landmark configurations of the left sides of the skulls are reflected to their mirror images by subtracting the H X-values from a constant (e.g. 20) to align corresponding landmarks of right and left sides. The centroid size (CS) for each side of each picture for each individual skull was calculated as the square root of the sum of squared distances from a set of landmarks to their centroid [18]. After configurations are scaled to unit CS, a point with average coordinates (centroid) from the right side is given the same coordinates as the centroid from the corresponding left side of the skull. Then, configurations are rotated around their centroid to achieve the best fit. This procedure is included in the software TPSRELW [19]. The output of the Procrustes procedure contains the coordinates of superimposed landmarks. Asymmetry can then be measured as the deviations between the pairs of the corresponding superimposed landmarks.

We used a two-factor mixed-model ANOVA to calculate sums of squares for each of the effects on each X and Y coordinate [2]. Then, we calculated the overall sums of squares for each of the main effects, interaction term and the error by adding the individual sums of squares for each of the effects across the X and Y coordinates [11]. Degrees of freedom for the Procrustes ANOVA were the degrees of freedom for each of the effects multiplied by the number of landmark coordinates minus four. The individual*side interaction was used to test the significance of the main effects. The measurement error was used to test for the significance of the variance component for the individual*side interaction effect that represented a measure of FA.

The measures of FA of shape for each population and the approximate degrees of freedom were calculated according to Palmer [2]. We used multiple F-tests to compare these values and generate appropriate p-values. Then, once more, we applied the sequential Bonferroni procedure to ensure the appropriate table-wide probability of Type I error as in Palmer [2]. The difference between populations was considered statistically significant only when the pairwise p-value was lower then the revised p-value.

1.4. Correlation of the overall asymmetry and ^{137}Cs in the dry muscle tissue

To calculate the measure of asymmetry for each individual, we subtracted the Procrustes aligned coordinates of the landmark configuration of right side from the corresponding coordinates of each individual landmark on the left side of the skull. Then we added the squared differences and calculated the square root of the resulting sum [11]. This distance measure is similar to the mean absolute difference between the left and right side [1], but since it is initially standardized to unit centroid size (CS) during the Procrustes procedure, it is independent of overall size [11]. We used the nonparametric Spearman's correlation (r_S) to assess the degree of association between the individual CS differences, individual Procrustes distances, and the amounts of ^{137}Cs in dry muscle of individual mice.

2. Results

2.1. Departures from normality and measurement error

Normality was examined using Kolmogorov-Smirnov tests of the frequency distribution of the centroid sizes compared to an expected normal distribution. If present, antisymmetry would artificially inflate the levels of FA. The frequency distributions of our data for each population were inspected for the presence of bimodality or unusual outliers. Outliers were traced back to the corresponding individuals and those landmarks were digitized again. Finally, after adjusting the overall error rate to the 0,05 levels, there were no significant deviations from normality as indicated by the Kolmogorov-Smirnov test. Thus, we concluded that there was no evidence of antisymmetry in any of the studied populations.

Measurement error was addressed during the F-tests. We were testing whether our FA estimate was significantly larger than predicted due to error alone. There were three populations (Vyshenky, Tovsty Forest-Forestry, and Ruzhyn) where it was not possible to calculate a reliable estimate of the FA of shape compared to the high measurement error. All of the other measurements of FA of shape were statistically significant ($p \leq 0{,}001$).

2.2. Fluctuating asymmetry

Asymmetry of shape represents a positive correlation of the differences between the coordinates of the optimally aligned landmarks of the superimposed configurations of the left and right sides that results from the Procrustes procedure. All of the calculated FAs of shape were significantly larger than the variance expected due to measurement error ($p \leq 0{,}0001$ (Table 1)). We ranked our populations by their corresponding FA values and performed multiple F-tests with the subsequent Bonferroni correction. All of the populations from the 10-km Exclusion Zone are placed at the top of the table with high FA values, while the reference populations and those from the less contaminated parts of the Exclusion Zone were ranked at the bottom of the table. The FAs of shape in both of the populations from Gluboke Lake and a population from Emerald Camp were significantly greater in FA compared to the rest of the populations but not from each other (Table 1, Fig. 1). The FAs of the samples from the Tovsty Forest population also did not differ from each other.

2.3. Correlation of overall asymmetry and ^{137}Cs in the dry muscle tissue

There was an overall significant correlation between the differences between the landmark coordinates of the Procrustes aligned configurations of the left and right sides and the amounts of intramuscularly ^{137}Cs in the individual mice ($r_S = 0{,}28, p <$

0,001). In addition, there was a significant correlation between the amounts of radiocesium and CS differences ($r_S = 0{,}09, p = 0{,}03$). There were no significant correlations between the Procrustes differences and the amounts of intramuscularly ^{137}Cs or between the amounts of radiocesium and CS differences within any of the individual populations. However, in this analysis, we were unable to separate different types of asymmetry and the measurement error associated with processing our data. These results represent an overall measure of asymmetry assuming a constant amount of measurement error across populations.

Table 1. **Summary of analyses of non-directional asymmetry of shape within and among populations of Apodemus flavicollis from Ukraine. Groups include populations from the 10-km zone around the failed reactor, 30-km zone, and reference populations (Ref). Populations are ranked from the highest (1) to the lowest (13) for the amount of fluctuating asymmetry (FA) in non-directional asymmetry. FA significance is tested against the amount of the experimental error. Results of significant F-test after sequential Bonferroni correction at p < 0,05 for pairwise comparisons of all populations**

Group	Rank	Populations	FA df	FA Values	FA Significance	Significant Pairwise F F-tests
10 km	1	Gluboke Lake 2000	366,00	$1{,}643 \cdot 10^{-07}$	***	4,5,6,7,8,9,10, 11,12,13
10 km	2	Gluboke Lake 1998	69,92	$1{,}513 \cdot 10^{-07}$	***	7,8,9,10,11,12 ,13
10 km	3	Emerald Camp	399,90	$1{,}475 \cdot 10^{-07}$	***	4,6,7,8,9,10,1 1,12,13
Ref	4	Zbarazh Stozhary	368,50	$1{,}043 \cdot 10^{-07}$	***	1,3,11,12,13
Ref	5	Vyshenky	457,10	$9{,}363 \cdot 10^{-08}$	***	1,12,13
Ref	6	Ruzhyn	374,90	$9{,}200 \cdot 10^{-08}$	***	1,3,12,13
Ref	7	Kolochava 1996	427,10	$8{,}786 \cdot 10^{-08}$	***	1,3,12,13
Ref	8	Kolochava 1998	396,70	$7{,}616 \cdot 10^{-08}$	***	1,2,3,12,13
Ref	9	Zbarazh Lysychyntsi	329,90	$7{,}492 \cdot 10^{-08}$	***	1,2,3,12,13
30 km	10	Tovsty Forest 2000	259,10	$7{,}483 \cdot 10^{-08}$	***	1,2,3,12,13
30 km	11	Tovsty Forest 1998	49,83	$4{,}509 \cdot 10^{-08}$	***	1,2,3,4,12,13
30 km	12	Tovsty Forest Forestry	859,60	$3{,}964 \cdot 10^{-08}$	***	1,2,3,4,5,6,7,8 ,9,10
Ref	13	Uzhhorod	735,00	$3{,}920 \cdot 10^{-08}$	***	1,2,3,4,5,6,7,8 ,9,10

***$p \leq 0{,}001$

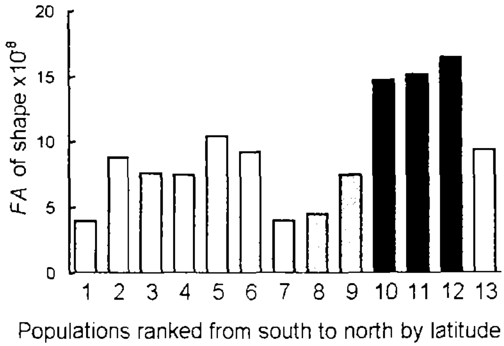


Fig. 1. Values of the fluctuating asymmetry (FA) of shape ranked by the latitude of the location from the southern most (1) to northern most location (13) as in Table 2. Control populations are white, populations from the 30-km zone are gray, and the populations from within the 10 km area from the Chornobyl reactor are black. Significance for each of the values and pairwise differences are presented in Table 1.

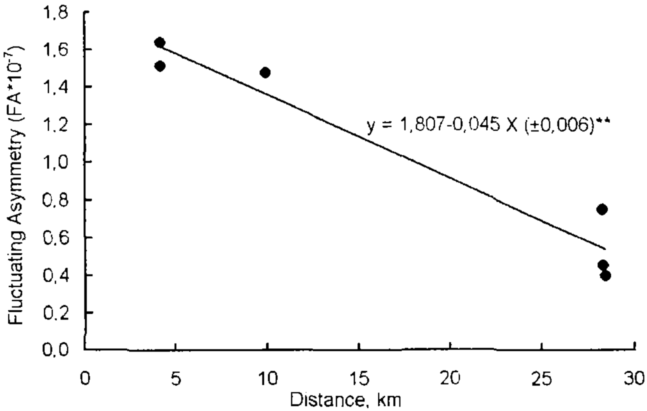
Table 2. **Population locations, sample sizes, numbers of skulls in the analyses, geographical positions, and the environmental dose rates recorded by sets of thermal luminescent dosimeters . Populations are arranged from the southern most at the top to the northern most at the bottom of the table**

Locations	Date	Number of Skulls	Latitude, North	Longitude, East	Group	dose, μR/hr
Uzhgorod	1996	33	48° 44"	22° 07"	reference	n/a
Kolochava	1996	16	48° 26"	22° 37"	reference	n/a
Kolochava	1998	17	48° 32"	23° 38"	reference	n/a
Stozhary	1998	14	49° 39"	25° 43"	reference	n/a
Lysychyntsi	1998	15	49° 42"	26° 11"	reference	n/a
Ruzhyn	1997	20	49° 42"	29° 14"	reference	n/a
Tovsty Forest	1997	11	51° 23"	29° 42"	30-km zone	n/a
Tovsty Forest	2000	12	51° 23"	29° 42"	30-km zone	13,22
Tovsty Forest, Forestry	2000	33	51° 22"	29° 43"	30-km zone	10,7
Emerald Camp	1999	15	51° 20"	30° 09"	10-km zone	29,7
Gluboke Lake	1998	10	51° 27"	30° 04"	10-km zone	n/a
Gluboke Lake	2000	13	51° 27"	30° 04"	10-km zone	414,6
Vyshenky	2000	17	51° 40"	33° 05"	reference	n/a

n/a – not tested

2.4. Correlation of fluctuating asymmetry and the distance from the reactor

There was a significant correlation between the FA of shape values and the distance to the failed reactor for the populations from within the Chornobyl Exclusion Zone (both the 10- and 30-km zones) ($r^2 = 0.94$, $p = 0{,}001$). Regression line for the relationships between FA and distance from the reactor is presented in Fig. 2.



** p ≤ 0,001

Fig. 2. Correlation between the values of fluctuating asymmetry of shape (black circles) and the distance to the Chornobyl reactor ($r^2 = 0{,}94$) Standard error of the slope is given in parentheses.

4. Discussion

Fluctuating asymmetry (FA) refers to the difference between the right and left sides in characters that should otherwise be bilaterally symmetrical. It is likely to be a consequence of epigenetic stress that affects an individual during development. Ionizing radiation should impose significant stress on individual animals in the landscapes contaminated during the 1986 Chornobyl meltdown. Therefore, we predicted that higher values of FA would be observed in animals from the contaminated sites closest to the failed reactor as opposed to the uncontaminated reference sites elsewhere in the Ukraine.

Overall, a higher level of FA was documented for the populations in closest proximity to the failed Chornobyl reactor for the asymmetry of shape. Fluctuating asymmetry of shape was highest in the three populations from the most contaminated locations in the Chornobyl Exclusion Zone (Table 1). Finally, values of FA of shape were highly correlated with the distance from the reactor. However, populations from the less contaminated areas around the failed reactor did not express FA values different from those of the reference populations. Populations that were sampled more than once did not show any difference between years. This indicates that our results were robust and replicable over time.

Differences between the landmark coordinates of the Procrustes aligned configurations of the left and right sides in the individual mice correlated with the amounts of intramuscularly radiocesium overall, but not in any of the individual populations in particular. This effect could be explained assuming the varying contamination levels in different populations from the Chornobyl area [13, 21]. In addition, there might be other contamination agents that could cause the elevated levels of asymmetry, such as radioactive ^{90}Sr that were not accounted for because of the cost of analysis. Lastly, intramuscularly contamination is not the only source of exposure in the affected populations. Animals could also receive a substantial dose of external radiation from their environment that causes most of the developmental instability leading to higher levels of FA.

Although FA tends to increase in the populations exposed to pollution, it should be considered substantial only when the level of FA of stressed population lies significantly above the background level of FA in unstressed populations. The uniqueness of our study is that multiple populations of the same species were sampled in different environments across a large geographical areasouth and north of Chornobyl. There were significant differences between the reference populations in the amounts of FA (Table 1). In addition, despite the differences in geographical location of the sampled populations or their environmental conditions, it appears that FA values were greatest in the populations maximally affected by the radioactive contamination and located closest to the failed reactor (Fig. 2).

Several hypotheses exist to explain the connection between environmental stress and FA [22, 23]. First, an increase in FA may reflect the expression of genetic variation at the phenotypic level due to the incorporation of mutant alleles into the individual genomes [3]. On the other hand, stress resulting from the excessive radiation may increase FA because minor changes in the environmental conditions would have more impact on the phenotype from the exposed population than a population with no known exposure. It is possible that organisms under stress require more energy to perform the same functions as unstressed organisms, including energy spent for repair of the damage inflicted by stress on its own body, as well as energy spent while functioning in the stress-altered environment [24]. Genetic factors may also influence the susceptibility of individuals and populations to the environmental factors creating a genotype-by-environment interaction [3]. In addition, an increase of FA values would be expected in highly inbred populations [25]. Finally, some authors argue that FA is not a good measure of the environmental quality because of the «differential mortality» of animals exposed to the toxic agent [26]. A result of such a selection process, assuming its linearity and constant level, would be that in the locations with higher levels of exposure, a robust subset of individuals would survive and express a lower level of FA. However, the underlying assumptions for this scenario are unlikely to exist outside the laboratory settings.

Our results are consistent with the hypotheses that predict an increase in the levels of FA in populations exposed to the anthropogenic contamination. However, the mechanism for this increase is not clear. It is possible that populations in our study experience high inbreeding ratios. On the other hand, if the differential mortality hypothesis were true for these populations, we would expect the most contaminated populations to have lower FA values than the «moderately» contaminated populations. However, this was not the case (Table 1). On the other hand, the highly skewed distribution of contaminants in the populations from Chornobyl [13] could result in only a few random individuals being killed that live in close proximity to hot spots. Moreover, these individuals may constantly be replaced with migrants from the relatively uncontaminated sites in the area. Even if selective mortality occurs because of the life-threatening doses [21], it is unlikely to result in a significant decrease in overall levels of FA. However, this scenario could occur in some highly polluted and genetically isolated populations.

Asymmetry represents a measure of the developmental instability of a phenotype under given environmental conditions [3]. Asymmetric individuals generally have lower fecundity and poorer survival than the more symmetrical individuals [3]. These differences arise from individuals with lower competitive ability, and higher risks of predation and parasitism compared to their more symmetrical counterparts. Thus, individuals in the contaminated areas around the Chornobyl plant may suffer changes that result in long-term evolutionary consequences. Therefore it is likely, that individuals in the highly contaminated areas would have overall lower average fitness values, which would create population sinks [27]. A failure to detect higher FA values in the populations with low values of contamination indicates that there may be a threshold level of exposure somewhere between 13,2 and 29,7 μR/hr (the difference between the 10-km zone and 30-km zone populations in this study (Table 2)) over which FA can significantly increase above its background level (Table 1, Fig. 2).

In conclusion, FA of skull shape in *A. flavicollis* may represent an indicator of the level of radioactive contamination in the animal's environment. Highly contaminated populations of a small mammal species expressed significantly higher levels of FA calculated as FA of shape. However, FA values of the intermediate populations were not different from the FA values expressed in most of the reference populations from the relatively uncontaminated areas in Ukraine. In addition, we found large differences in the amounts of FA among reference populations from the uncontaminated regions. Higher FA values could indicate that populations from the localities close to the failed Chornobyl reactor must be experiencing significant levels of stress during their development. Finally, these highly contaminated populations may be accumulating mutations that could disrupt normal development in the affected individuals.

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