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SIZE AND FLUCTUATING ASYMMETRY OF MORPHOMETRIC CHARACTERS IN MICE: THEIR ASSOCIATIONS WITH INBREEDING AND *t*-HAPLOTYPE

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Abstract.—Fluctuating asymmetry (FA), a ubiquitous type of asymmetry of bilateral characters, often has been used as a measure of developmental instability in populations. FA is expected to increase in populations subjected to genetic stressors such as inbreeding or environmental stressors such as toxins or parasites, although results have not always been consistent. We tested whether FA in four skeletal size characters and mandible shape was greater in a population of wild-derived mice reared in the laboratory and subjected to one generation of inbreeding ($F = 0.25$) versus that in an outbred group ($F = 0.00$). FA did not significantly differ between the inbred and outbred groups, despite the fact that these two groups differed dramatically in fitness under seminatural population conditions. As far as we know, this is the first study to evaluate the relationship between FA and inbreeding in wild house mice, and our general conclusion is opposite that of earlier work on laboratory inbred strains of mice and their hybrids. Size for two of the characters was significantly less in inbreds than in outbreds, however, and there was a significant difference between inbreds and outbreds in the signed differences of right and left sides in one character (humerus length). Some of the mice in both groups also were heterozygous or homozygous carriers of the *t*-complex. Because mice carrying this chromosome 17 variant are known to have reduced fitness, we also tested whether they had greater FA than mice carrying non-*t*-haplotypes. The overall level of a composite FA index calculated from all four characters was in fact significantly higher in the *t*-bearing mice. These combined results suggest that FA is not a generally sensitive proxy measure for fitness, but can be associated with fitness reductions for certain genetic stressors.

Key words.—Directional asymmetry, fluctuating asymmetry, inbred mice, morphometric size and shape characters, outbred mice, *t*-complex.

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Fluctuating asymmetry (FA), a type of bilateral asymmetry characterized by a normal distribution of right minus left differences about a mean of zero, continues to be thought of as one of our best measures of developmental instability in populations. There is little doubt that the level of FA in various characters often increases if individuals suffer developmental perturbations when subjected to any of a variety of stressors (Zakharov 1989; Parsons 1990). This seems particularly true for environmental stressors such as pollution, various toxins, and parasites (Clarke 1992; Graham 1992), although their effect on FA is not always found (Markow 1995; Leamy et al. 1999; Woods et al. 1999). Similarly, FA sometimes, but not always, responds to genetic stressors such as inbreeding and selection. Thus, inbreeding promotes homozygosity that is expected to render organisms less able to cope with changes in the environment and make them less fit (Lerner 1954). Although inbreds typically show greater variability than outbreds in most characters measured (Falconer and Mackay 1996), they do not always exhibit higher levels of FA in these characters (Clarke 1993). Thus, the relationship between FA and inbreeding appears tenuous (Møller and Swaddle 1997; Vollestad et al. 1999), perhaps because FA reflects the level of genetic coadaptation rather than that of homozygosity per se (Clarke 1993).

The relationship between FA and the fitness of organisms is perhaps even more controversial. Møller (1997) used meta-analysis techniques on a number of studies that suggested FA is negatively correlated with fitness, although these data and the analysis were criticized from several standpoints (see Clarke 1998). Many of these studies found a relationship between FA and sexual selection characteristics such as mating success (Møller 1993; Møller and Pominakowski 1993; Møller and Thornhill 1998), but it turns out that more asymmetrical individuals are not always disadvantaged in the mating process (Markow 1995; Goulson et al. 1999; Bjorksten et al. 2000). Furthermore, there is at least some evidence that character size may be the more important indicator of mating success than the degree of asymmetry (Goulson et al. 1999). Clearly, therefore, additional studies that compare the size and asymmetry of characters to traditional natural selection fitness characteristics such as viability and fecundity are needed if we are to properly sort out the role that FA plays in the overall fitness of organisms.

Meagher et al. (2000) assessed the effects of one generation of inbreeding on the fitness of wild-derived house mice living in seminatural enclosures. Overall, inbred mice had a 58% fitness decline relative to outbreds. The effect was primarily in males where outbred mice produced five times more offspring than inbred mice. These results allowed us to evaluate the association of FA with fitness by comparing FA levels between inbred and outbred mice from this same population (Meagher et al. 2000). In the study reported here, we assessed FA for several morphometric size and shape characters in laboratory-reared mice to test the hypothesis that FA should be greater in the inbreds compared to the outbreds. Some of these mice also were carriers of at least one haplotype of the *t*-complex located on chromosome 17. Because this complex genetic region is known to affect development and viability (Silver 1985), we tested whether the developmental consequences of inheriting a *t*-haplotype were reflected in an increase in FA levels. The use of two different genetic stressors and several characters made it possible to test for differences

between these stressors in their effects on FA in the characters as well as differences among the characters themselves.

Materials and Methods

The Population and Characters

The inbred and outbred house mice (*Mus domesticus*) used in this study were a sample of those reared in the laboratory by Meagher et al. (2000). In that study, wild mice were sampled from two locations, crossed to produce the F_1 generation, and then either mated with siblings or with unrelated individuals to produce inbred ($F = 0.25$) and outbred ($F = 0.00$) progeny. All laboratory mice were housed in cages provided with pine bedding, nesting material, and food and water ad libitum (for additional details, see Meagher et al. 2000). At the time of weaning (about 21 days of age), individual mice were marked for identification by unique ear punches. Ear-punch tissue was immediately frozen for subsequent DNA extraction. All mice were sacrificed when they were quite old (587–878 days of age), and their skeletons were prepared by exposure to dermestid beetles.

During a routine breeding of wild mice from our colony, some showed extreme non-Mendelian transmission of an H-2 (murine major histocompatibility complex) linked microsatellite marker, suggesting the presence of a segregation distortion locus. The H-2 region is tightly linked to the *t*-complex, and subsequent genotyping with *t*-locus-specific oligonuleotide primers in conjunction with the polymerase chain reaction (PCR) verified the presence of a *t*-haplotype within the colony. PCR was subsequently performed to *t*-type all mice used in the present study.

DNA was extracted from frozen ear-punch tissue by digestion with proteinase K (Applied Biosystems, Foster City, CA) followed by ammonium acetate protein precipitation as described in Meagher et al. (2000). DNA was pelleted with isopropanol, washed in 70% ethanol and resuspended in 100 μl TE. Oligonucleotide primers for *t*-genotyping were synthesized according to the published sequences of Schimenti and Hammer (1990).

Genomic DNA template (approximately 200 ng) was amplified in 25-μl reaction cocktails containing: 1.5 U *Taq* polymerase, 0.125 mM of each dNTP, 10 mM Tris-HCl, 2.5 mM MgCl, 50 mM KCl, and 0.2 μM of each primer (forward primer: 5′-GAGTGACCTGCATGCCCACCAGCTGTG-3′; reverse primer: 5′-GAGCTGTGGAGACAGGAAGGGTCAGTG-3′). These primers amplify a region in the distal *t*-complex inversion that contains a 16-bp insertion in *t*-haplotypes but lacks the insertion in wild-type alleles. Amplification reactions were performed in an MJ Research (Waltham, MA) thermal cycler as follows: DNA was denatured for 2 min at 94°C followed by 35 cycles of 30 sec at 94°C, 30 sec at 66°C, and 1 min at 72°C. A 7-min, 72°C extension step proceeded the reaction. PCR products were run for 1 h at 60 W on a 7% standard acrylamide gel and visualized with ethidium bromide on an ultraviolet light box. The DNA from each mouse was subjected to two PCR runs, and no discrepancies between the two genotype scores from these runs were observed for any mouse.

A total of 109 mice were available for the analysis: 56 (27 males, 29 females) from 42 different litters in the outbred group and 53 (24 males, 29 females) from 35 different litters in the inbred group. Litter sizes for these inbred mice (mean = 6.75) were similar ($F = 0.29$, df = 1, 105 $P = 0.59$) to those for the outbreds (mean = 6.88). Similarly, mean ages for the outbreds (719 days) and inbreds (722 days) did not significantly differ ($F = 0.02$, df = 1, 105 $P = 0.88$). Of the 109 mice, 35 possessed at least one *t*-haplotype (+/*t* or *t*/*t*), whereas the remaining 74 were wild-type (+/+). Again, mean ages and litter sizes for mice with *t*-haplotypes (714 days, 6.43) did not significantly differ (age: $F = 0.19$, df = 1, 105 $P = 0.66$; litter size: $F = 2.89$, df = 1, 105 $P = 0.09$) from those for mice with wild haplotypes (724 days, 7.04).

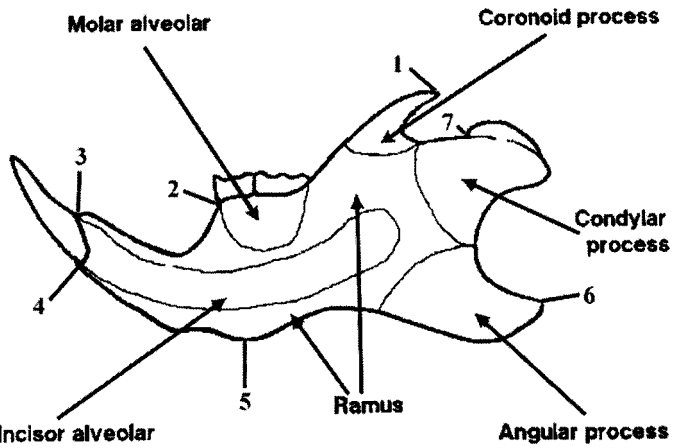

FIG. 1. Outline of a mouse mandible showing the seven points that were digitized.

The characters chosen for measurement included limb bone dimensions as well as a number of mandible dimensions. With regard to the limb measures, total lengths of both left and right sides of the femur (FL), humerus (HL), and tibia (TL) were measured, although not the radio-ulna length because a number of these bones were broken or missing. All measurements were made with a dial caliper to the nearest hundredth of an inch and then converted to millimeters. After an entire round of measurements on all individuals was completed, an additional round was taken so that two measurements for both sides of the three limb characters were available for each mouse.

For measurement of the mandible dimensions, left and right sides of the mandible in each mouse first were separated at the mandibular symphysis and placed under a camera that projected their image onto a computer monitor. Seven points around the periphery of each mandible (Fig. 1), chosen because they represented obvious landmarks, were recorded in millimeters in *x*, *y* space with the Measurement TV program (DataCrunch, San Clemente, CA). Each mandible was digitized twice (all mandibles first were measured and then a second round of measurements was taken that involved repositioning each mandible). As a result, two separate coordinate values of these landmark points were available for both left and right sides of the mandible in each mouse. These landmark points then were used to calculate one overall size measure for both the left and right sides of all mandibles. This measure was the centroid size (CS), defined as the square root of the sum of squared distances between each landmark

of a configuration and its centroid (Dryden and Mardia 1998). The centroid is the point whose *x* and *y* coordinates are the means of the *x* and *y* coordinates of all landmarks.

Mandible shape characters also were created via the Procrustes method (Bookstein 1991; Auffray et al. 1999), which used the *x, y* coordinates of the mandibles and eliminated variation in size, position, and orientation. This was accomplished in four sequential steps that reflected the mandible of one side, and then scaled, superimposed, and rotated the mandibles to produce an optimal fit between corresponding coordinate points of left and right sides for all individuals (see Klingenberg and McIntyre 1998). This process created 14 new shape variables (*x, y* coordinates of the seven landmark points) for both sides of the two repeat measures of each mandible.

As defined in geometric morphometrics, shape is a multivariate character (Bookstein 1991). Consequently, variation at individual coordinates is only useful in explaining changes in the whole shape configuration. Thus, differences in shape between groups cannot be expressed as single values and instead typically are depicted in figures that show the magnitude and direction of the change at each landmark point (Klingenberg and McIntyre 1998). With respect to shape, the adjustment for size, position, and orientation in the Procrustes method described above eliminated four degrees of freedom, resulting in $14 - 4 = 10$ shape space dimensions (see Klingenberg and McIntyre 1998). Appropriate adjustment for this reduction in number of degrees of freedom was made in the multivariate analyses of variance described below.

Preliminary Statistical Analysis

Prior to the analysis of asymmetry, means and differences of left and right sides for each of the replicate measures for all four characters (mandible centroid size and three limb bone lengths) and for the 14 mandible shape characters were examined. Three outliers (Grubbs test, Sokal and Rohlf 1995) in the size characters and four outliers in the shape characters were found, and the relevant characters in these mice were either remeasured or, if appropriate, eliminated. Combined with several of the limb bones or mandibles that were chipped or broken during the skeletonization or measurement process, this resulted in a final sample size that was reduced slightly from the original 109 mice and that varied among the four characters in the inbred/outbred and *t*/wild groups (see below).

A mixed-model, conventional two-way analysis of variance (ANOVA) was used to assess the significance of size FA in each character (Leamy 1984; Palmer 1994), and a modification of this model known as the Procrustes ANOVA (Klingenberg and McIntyre 1998) was used for the shape characters. In the Procrustes ANOVA, the sums of squares were calculated by adding the sums of squares of all 14 shape characters (Klingenberg and McIntyre 1998). Degrees of freedom for the Procrustes ANOVA were obtained by multiplying the degrees of freedom for each factor by the total number of shape dimensions, or 10 in this study. In this model, individuals is a random factor that assesses variation among individual mice, sides is a fixed factor that assesses directional asymmetry (DA, see below), the individuals × sides interaction assesses FA, and the error assesses variation in the replicate measurements (Leamy 1984; Palmer 1994). Mean squares for individuals were tested over the error mean squares, whereas mean squares for sides were tested over the individuals × sides interaction. Mean squares for the interaction were tested over the error and, if significant, indicate that the amount of FA is greater than that due solely to measurement error and thus the asymmetry analysis may proceed (Palmer 1994). Significance for all probabilities generated from the *F*-tests was evaluated using the sequential Bonferroni procedure (Rice 1989).

Beyond testing the significance of the asymmetries and individuals, estimates of the precision of the replicate measurements also were made from the ANOVA. For each of the four size and the multivariate shape characters, variance components were calculated for the three random factors: individuals, the sides × individuals interaction, and error. For shape, this was accomplished by summing all 14 individual variance components for each factor and dividing the total by 10. The magnitude of the error variance relative to that of the sum of these three variances, and especially relative to the individuals × sides interaction (FA) variance, provided appropriate measures of measurement error (Palmer 1994; Leamy 1999).

Once these preliminary analyses were completed, all subsequent analyses made use of the mean of the two repeat measurements for the bone size characters and for the mandible shape characters. Beyond the asymmetries of these characters, it first seemed useful to calculate the mean of the left and right sides for all characters as a measure of their size (limb lengths and mandible) and shape (mandible). These measures for each character then were subjected to the same analyses as the asymmetries (see below) to discover whether they differed between the inbred/outbred and/or *t*/wild groups.

Asymmetry Measures

To obtain measures of DA for the three limb bone characters, mandible centroid size, and the 14 mandible shape characters, right-minus-left side differences were calculated for all individuals. If the means of these *signed* differences between sides significantly differ from zero (using *t*-tests and the sequential Bonferroni procedure to evaluate significance) in any of the inbred/outbred or *t*/wild groups, then DA is assumed to be present (Van Valen 1962). If no DA is present, and the distribution of these signed differences is normal, then variation in these differences represents classical FA (Palmer 1994). Skewness and kurtosis statistics calculated for the signed differences between sides in each of the characters suggested that their distribution was normal and, thus, that there was no apparent antisymmetry, another kind of asymmetry detected by significant platykurtosis (Palmer and Strobeck 1992). The signed differences for each character also were tested via regression to see whether they significantly scaled with the size of the characters (Palmer 1994). All regression coefficients were nonsignificant, suggesting no scaling correction was necessary.

Unsigned, or absolute, differences of the right minus left sides were used to provide measures of FA (Palmer 1994).

For the three limb characters and mandible centroid size, the mean of the right-minus-left differences first was subtracted from the signed difference between the sides to statistically correct for any DA, and then the absolute values of these differences were used to assess FA (Leamy 1984; Hutchison and Cheverud 1995; Leamy et al. 1997). Distributions of FA in these characters, however, were half-normal (Palmer 1994) and were subjected to Box-Cox transformations (Swaddle et al. 1994) of the form $(FA + 0.0005)^{0.33}$ that were successful in achieving normality. FA values for each character also were regressed on the size of the character to test for scaling effects, and as was the case for the signed asymmetries, no significant scaling was detected.

For the mandible shape characters, calculation of unsigned left-minus-right differences presented a difficulty previously explained by Klingenberg et al. (2001). Specifically, changing all the negative signs to positive would have affected associations between landmarks by expressing all the left-minus-right differences in an anterior and dorsal direction (Klingenberg et al. 2001). Changes in signs were therefore made in only one of two equal parts of the space of possible shape changes, the parts being generated by the signs of the inner product between the vectors of left-minus-right differences of each individual and that of the first individual in the dataset (see Klingenberg et al. 2001). Once accomplished, these values provided measures of shape asymmetry as contrasted with the size asymmetry measures generated from the other four characters.

Once all size, DA, and FA values were calculated, they were tested for potential effects due to sex, age, and litter size differences in a linear model, where litter size and age were treated as covariates and sex was treated as a categorical variable. Litter size effects were significant ($P < 0.01$) for all four size characters (regressions of litter size on these characters were positive, varying between 0.06 to 0.13) and sex effects were seen for DA in TL ($P = 0.018$), but age effects were not significant for any of the characters. Therefore, litter size and sex effects were adjusted in these characters by obtaining residuals from a general linear model. The shape and shape asymmetry characters showed no significant effects ($P > 0.05$) in multivariate tests of these three covariates, and therefore needed no adjustment.

Once FA values had been determined for all characters and adjusted for potential effects of covariates as described above, it seemed useful to calculate a composite index of size FA for the four characters (FL, HL, TL, CS). Leung et al. (2000) reviewed the strengths and weaknesses of several such indices; based on their findings, we chose one such index for use. This composite index, designated CFA throughout the analysis, was calculated in each individual by dividing each FA value by the mean FA value for that character in the overall population, and then summing each of these over all four characters. Leung et al. (2000) showed that this index detected differences in FA among groups 85% of the time compared to only 20% using FA of single characters. This index also was clearly superior to multivariate analysis of variance (MANOVA) testing for group differences, but unlike the MANOVA approach, it does assume a common effect for all characters (Leung et al. 2000).

Tests for Breeding and Genotype Differences

The size and asymmetries of FL, HL, TL, and CS were subjected to univariate and multivariate analyses of variance to test for significant differences between inbred and outbred groups and between *t*- and wild genotypes. In these analyses, group, genotype, and the group × genotype interaction all were tested over the factor litters, which was nested within the group × genotype combinations. Litters was included as a factor in these analyses because differences among litters may in part be a reflection of nongenetic maternal effects (Falconer and Mackay 1996). Litter differences themselves were tested over the error. Probabilities of all *F*-tests for each measure (size, DA, FA) of the four characters were evaluated with the sequential Bonferroni procedure (Rice 1989) to ensure an experimentwise error rate of no greater than 5% for each measure. CFA was subjected to its own univariate ANOVA, and probabilities generated in this analysis were not adjusted because this character represented a single, separate index of FA derived from all four characters.

The results of these univariate ANOVAs permitted us to compare the two types of genetic stressors (inbreeding versus *t*-complex), the four characters (FL, HL, TL, CS), and the three measures (size, DA, FA) taken on these characters. This was accomplished in a single ANOVA that tested the significance of each of these three factors (all regarded as fixed) and their first-order interactions. Mean squares for the group and genotype factors from the univariate ANOVAs described above ($12 \times 2 = 24$ total values) were used as the dependent variable in this analysis. For the size and DA measures, the mean square (MS) for each character first was divided by a ratio formed by its associated error MS with that of the error MS for FA in that character to ensure a uniform scaling of these values among all three measures (size, DA, FA). In addition, all scaled MSs were logged, and tests showed that this ensured both normality and homoscedasticity among these values.

The shape, signed shape differences, and unsigned shape differences each were subjected to MANOVAs of the same design as already described. Due to the four degrees of freedom that were lost during the Procrustes procedure, however, each of these three analyses were run with only 10 of the 14 characters (both values at two landmarks omitted). The 10 characters chosen were those for landmark points 1–5, but identical statistical results are produced in such analyses regardless of which two landmarks are omitted (Klingenberg et al. 2001).

RESULTS

Table 1 summarizes the results of the preliminary ANOVA for the limb and mandible centroid size characters and for mandible shape. All five characters show highly significant differences among individuals, and all characters except HL also show significant differences between left and right sides indicative of DA. The highly significant interaction of sides and individuals for all characters suggests that they exhibit significant FA. The extent of this FA varies from about 2% (FL) to more than 5% (CS) of the total variation for the size characters, but more than 16% for the mandible shape character. Measurement error is small, especially for the limb

TABLE 1. The analysis of variance of the three bone length characters (FL, femur length; HL, humerus length; TL, tibia length) and of mandible centroid size (CS) and mandible shape. Mean squares and variance components are in square millimeters $\times 10^4$ for the bone characters and mandible centroid size, but are in dimensionless Procrustes units $\times 10^6$ for mandible shape. The percentage contributions (%) of the interaction and error variances to the total variance also are given.

	Individuals (*I*)		Sides (*S*)		$I \times S$				Error		
	df	MS	df	MS	df	MS	$\sigma^2_{I\times S}$	%	df	σ^2_E	%
FL	92	10800.7**	1	4733.8**	101	144.2**	68.6	2.47	207	7.0	0.25
HL	87	5502.2**	1	10.9	97	155.7**	73.8	5.13	198	8.2	0.57
TL	92	11534.6**	1	1736.5**	99	219.0**	101.0	3.34	205	17.0	0.56
CS	83	59071.4**	1	1537.2**	95	236.4**	94.0	5.93	190	49.4	3.12
Shape	830	673.3**	10	507.0**	950	965.1**	34.4	16.58	1900	28.0	13.51

** $P < 0.01$.

bone characters, where it averages less than 1% of the total variation, but is considerably higher (13.5%) for mandible shape.

Table 2 gives sample sizes, means, and standard errors for the size and signed and unsigned asymmetries of the four bone size characters in the inbred/outbred and *t*/wild mice. The size of all four characters tends to be greater in the outbred mice, with the outbred/inbred differences varying from 0.18 mm (TL) to 0.26 mm (HL). The signed asymmetries of the four characters are positive in sign in seven of the eight cases, indicating that the right side tends to be larger than the left side. After sequential Bonferroni adjustment, however, these right-minus-left differences reach significance only for FL and HL in the inbred mice. HL is distinct in showing a mean positive (but not significant) signed asymmetry in outbreds but a negative signed asymmetry, which is significant, in inbreds. The signed asymmetry for CS is consistent in sign and magnitude in both inbred and outbred groups, although it does not reach significance in either group as it previously did over both groups (Table 1). The mean unsigned asymmetries (FA values) for the three limb characters are higher in the outbred compared with the inbred mice, whereas the opposite is true for FA in CS, but none of the differences appear very large compared to their standard errors. The mean CFA value is greater in outbreds than in inbreds, although again the difference between these values is small.

The means of the three limb bone lengths and mandible centroid size are similar in the *t*- and wild genotypes (Table 2). Significant signed differences between sides, indicating DA, are found for FL, TL, and CS, but only in mice with the wild haplotype. The magnitude of these signed differences for CS is identical for both wild and *t*-mice, however, suggesting that significance was not reached in the *t*-mice because of their lower sample size. Signed differences in TL show the greatest disparity between wild and *t*-mice, but this difference amounts only to 0.047. The mean FA is greater in mice with *t*-haplotypes than in wild mice for three of the four characters (FL, TL, and CS), and this same trend is therefore reflected in the composite FA index (CFA) as well.

Table 3 shows the results of the ANOVA for the size and signed and unsigned differences of sides for each of the four characters. It should first be noted that none of the group $\times$ genotype interactions or litter differences reached significance in the univariate analyses (or in the MANOVAs), so these two factors were eliminated from the analysis. Differences between the inbred and outbred groups are significant for HL and CS, and results from the MANOVA indicated differences between these groups as well over all characters. Significant differences between inbreds and outbreds are also seen for DA in one character (HL), which presumably has also promoted the multivariate significance, but no univariate or multivariate differences between these two groups are seen for FA in the characters. Differences between the *t*- and wild

TABLE 2. Samples sizes (*N*), means, and standard errors (SE) for the size and signed (DA) and unsigned asymmetry (FA) of the four bone characters in the inbred and outbred mice and for wild (+/+) and *t* (+/*t* or *t*/*t*) genotypes. FL, femur length; HL, humerus length; TL, tibia length; CS, mandible centroid size; CFA, composite FA index.

	Outbreds			Inbreds			*t*			Wild		
	N	Mean	SE	*N*	Mean	SE	*N*	Mean	SE	*N*	Mean	SE
FL	52	14.88	0.068	50	14.69	0.078	33	14.75	0.098	69	14.80	0.062
HL	49	11.78	0.048	49	11.52	0.058	32	11.73	0.065	66	11.61	0.049
TL	49	17.36	0.069	51	17.18	0.084	31	17.40	0.095	69	17.21	0.068
CS	51	12.41	0.043	49	12.19	0.063	34	12.31	0.066	66	12.29	0.049
DAFL	52	0.044	0.019	50	0.063**	0.014	33	0.035	0.022	69	0.063**	0.014
DAHL	49	0.034	0.017	49	−0.045*	0.016	32	0.006	0.023	66	−0.011	0.015
DATL	49	0.026	0.023	51	0.034	0.019	31	−0.002	0.029	69	0.045*	0.016
DACS	51	0.043	0.021	49	0.046	0.022	34	0.045	0.030	66	0.045*	0.017
FAFL	52	0.416	0.020	50	0.393	0.019	33	0.425	0.023	69	0.395	0.017
FAHL	49	0.444	0.017	49	0.427	0.018	32	0.434	0.023	66	0.437	0.015
FATL	49	0.462	0.020	51	0.448	0.017	31	0.490	0.021	69	0.439	0.017
FACS	51	0.442	0.022	49	0.477	0.017	34	0.482	0.023	66	0.447	0.017
CFA	42	4.038	0.106	43	3.967	0.089	25	4.271	0.133	60	3.887	0.076

* $P < 0.05$; ** $P < 0.01$.

TABLE 3. The analysis of variance results (all mean squares $\times 10^4$) for the size and signed (DA) and unsigned asymmetries (FA) of the four bone characters and the composite FA index (CFA). Probabilities from MANOVAs for each set of characters are also given. FL, femur length; HL, humerus length; TL, tibia length; CS, mandible centroid size.

	Inbred/Outbred (df = 1)	*t*/Wild (df = 1)	Error (df = 82–99)
FL	10619.6	1156.1	2742.6
HL	14909.5**	1749.5	1392.9
TL	7970.2	7250.2	2940.6
CS	11830.1*	5.4	1449.3
MANOVA *P*	0.014*	0.027*	
DAFL	66.4	152.9	144.4
DAHL	1508.8**	18.2	141.3
DATL	11.3	452.9	211.8
DACS	2.4	0.1	237.8
MANOVA *P*	0.030*	0.706	
FAFL	108.3	179.2	194.6
FAHL	74.6	5.8	151.7
FATL	36.4	549.9	175.4
FACS	380.5	347.4	192.6
MANOVA *P*	0.763	0.027*	
CFA	1755.1	21054.0*	3151.4

* $P < 0.05$, ** $P < 0.01$.

mice are not significant for any of the individual characters, although they reach multivariate significance for the size of the four characters. Perhaps most importantly, the *t*/wild difference reaches significance for the composite FA index, CFA, as well as for the MANOVA test for FA in the four characters ($P = 0.027$). Overall, therefore, the *t*-complex appears to exert effects on both size and FA in the four characters, whereas inbreeding effects are limited to the size of the characters and DA in one character.

The results of the ANOVA of the differences between the two stressors, the four characters, and the three measures of these characters, using the mean squares from Table 3 as previously explained, are given in Table 4. Only the interaction of stressors with characters reaches significance, suggesting that the effects of inbreeding and the *t*-haplotype are not the same among the four characters. Additional analyses were conducted using only DA and FA, and none of these factors in the ANOVA reached significance. This suggests that no differences exist between the magnitude of these two types of asymmetries across the four characters. Similarly, neither characters nor measures reached significance in analyses conducted using only inbreeding or only the *t*-haplotype as the stressor.

Another analysis was run with just size and FA, because these two measures were the major ones of interest. Results of this analysis (Table 4) show significance for the stressor $\times$ character interaction, again confirming the differential effect of the two stressors on the four characters. There also is a significant stressor $\times$ measure interaction, suggesting that inbreeding affects size and FA in the characters differently than does the *t*-haplotype. Main effects of characters and measures also reach significance, but differences between levels of these two factors depend on whether inbreeding or the *t*-haplotype is the stressor.

The results of the MANOVAs for mandible shape and the signed and unsigned mandible shape asymmetries are shown

TABLE 4. The analysis of variance of the two stressors (inbreeding versus *t*-haplotype), the four characters and the measures of these characters (size, DA, and FA) and their interactions using the scaled and logged mean squares from Table 3 (see text for explanation). Analyses were run using size, DA, and FA and only size and FA.

	Size, DA, FA		Size and FA	
Source	df	MS	df	MS
Stressors (S)	1	1.745	1	0.350
Characters (C)	3	1.232	2	1.752*
Measures (M)	2	1.518	2	2.106*
S $\times$ C	3	2.031*	2	2.437*
S $\times$ M	2	1.007	2	1.579*
C $\times$ M	6	1.347	4	1.202
Error	6	0.380	4	0.214

* $P < 0.05$.

in Table 5. Only differences between litters showed significance for mandible shape, so neither inbreeding nor the *t*-complex appear to affect mandible shape or shape asymmetry in these mice. This is in contrast to size and size FA (Table 3) in the four bone characters, for which both inbreeding and the *t*-complex had some effects.

DISCUSSION

Effects of Inbreeding

The primary purpose of this study was to test the hypothesis that inbred mice should have greater FA levels than the outbred mice, and they clearly did not. Even though there were pronounced fitness differences between the inbred ($F = 0.25$) and outbred ($F = 0.00$) groups resulting from only a single generation of inbreeding in the mice used by Meagher et al. (2000), this did not produce detectable effects on either size or shape FA. Insofar as is known, this is the first study to evaluate the association between FA and inbreeding in wild house mice. Our general conclusion that inbreeding did not affect FA levels is opposite to that of earlier work on inbred strains of mice and their hybrids (Leamy 1984). Incidentally, it is extremely unlikely that this result is due to differential early mortality between the inbreds and outbreds, because in the laboratory, survivorship rates did not significantly differ between these two groups (Kaplan-Meier $P = 0.99$; Meagher et al. 2000). Thus, if the inbreds had been more asymmetric in the young mice, they would have had to suffer greater mortality than the outbreds to generate comparable levels of FA in both groups as was found here.

Although it is not known to what extent additional inbreeding might further reduce fitness, greater levels of in-

TABLE 5. *F*-approximations to Wilks' lambda statistics from the multivariate analysis of variance of mandible shape and signed and unsigned shape asymmetries.

	Inbred/ Outbred (df = 10)	*t*/Wild (df = 10)	Interaction (df = 10)	Litters (df = 700)
Shape	1.04	1.16	0.92	1.35**
Signed asymmetry	0.42	1.62	0.89	0.82
Unsigned asymmetry	1.13	0.97	0.72	1.02

** $P < 0.01$.

breeding have been shown to result in increases in FA. Lacy and Horner (1996) discovered FA differences in various skeletal characters between inbred and outbred rats (*Rattus villosissimus*), but only after an apparent threshold was reached after a number of generations of inbreeding. Similarly, Leamy (1984) found that FA for several morphometric characters in mice, including lengths of the humerus, radio-ulna (but not femur or tibia) and mandible, showed FA differences between inbreds and the hybrids produced from crossing these inbreds. All of these mice were basically isogenic (Leamy 1984), however, so the inbreeding level ($F = 1.00$) was not at all comparable to that ($F = 0.25$) seen here. But it will be generally impossible to compare results from wild mice and laboratory inbred strains of mice because the inbred strains have had much of their deleterious recessive load purged during the inbreeding process (Green 1966). Consequently, it is difficult to know if the inbreeding differences in the wild mice ($F = 0.00$ vs. $F = 0.25$) have a greater or lesser fitness impact than the inbreeding differences in the inbred strains ($F = 0.0$ vs. $F = 1.0$). Clarke (1993) has argued that FA increases that sometimes result from inbreeding come more from the breakup of coadapted gene complexes and/or the expression of deleterious recessive alleles than from the decline of heterozygosity. Whatever the case, perhaps more generations of inbreeding in the mice of this study would have produced detectable FA differences.

Beyond the extent of inbreeding, another potential explanation for the failure to detect FA differences between the inbreds and outbreds here involves the choice of the characters for which asymmetry was estimated. Woods et al. (1999) have argued that only characters with relatively low heritabilities but relatively high phenotypic variances may be expected to show consistent increases in FA when subjected to stress (presumably including inbreeding stress). Although the heritabilities of four skeletal characters in this particular population are unknown, heritability estimates for these or comparable characters in laboratory randombred mice have been moderate (about 0.4) in magnitude (Leamy 1974). Further, the coefficients of variation for the size of all four characters (2.9–4.1) suggest that these characters exhibit a fairly low level of variation. The coefficients of variation of inbreds also were not greater than those of the outbreds, a trend opposite to that often found for morphometric characters in mice (Bader 1956; Leamy 1982b). Besides FA measures in the four size characters, however, we assessed FA of shape in the mandible, and this also showed no significant difference between inbreds and outbreds. Multivariate measures of shape asymmetry have rarely been estimated (see Klingenberg et al. 2001), however, so we have little basis for predicting whether this kind of character might be more sensitive than size FA to the effects of inbreeding.

Brother-sister inbreeding did reduce the overall size of the morphometric characters, as has often been found (Leamy 1982a). Thus, all four characters were smaller in inbreds compared with outbreds, two of them (HL and CS) significantly so. This implies that dominance of the genes responsible for these characters was generally in the direction of increased size (Falconer and Mackay 1996), and that increased homozygosity for recessive alleles in the inbreds contributed to their decline in size. This level of inbreeding did not affect mandible shape, however, and this is somewhat surprising given that Klingenberg et al. (2001) showed that there are more genes (quantitative trait loci) for shape than for centroid size in mouse mandibles and that dominance of the genes for shape was significantly more important than those for centroid size. In any event, size rather than FA in morphometric characters such as those used here may be a better indicator of (inbreeding) stress, as sometimes has been proposed on the basis of findings in other experimental studies that have used various stressors (Woods et al. 1999). In addition, larger size may be an advantage in the sexual selection process, at least as measured by mating success (Goulson et al. 1999).

It was interesting that signed differences between left and right sides of one of the four characters, humerus length, significantly differed between inbreds and outbreds. This difference between the two groups very nearly reached the 1% significance level even after sequential Bonferroni correction and apparently was large enough to promote multivariate significance of DA in all four characters as well. Given that the distributions of right-minus-left humerus lengths were normal, it does not appear that this is a spurious result. This change represents a transition from FA in the outbred group to DA in the inbred group, and such transitions have been found before for mandibular characters in mice treated/not treated with methoxychlor (Leamy et al. 1999). Graham et al. (1994) regard transitions from FA to DA as responses to stress, although the conventional view is that FA is our best indicator of stress and developmental stability (Palmer and Strobeck 1992).

Thus, with respect to the inbred and outbred groups, one generation of inbreeding was sufficient to produce a detectable change in the size of the morphometric characters used here, as well as the signed differences in one of these characters, but not the FA of these characters. Based on the large fitness differences seen between inbreds and outbreds living in seminatural enclosures (Meagher et al. 2000), the fact that these two groups of mice had comparable FA levels suggests that FA is not associated with the inbreeding-mediated fitness decline seen in the inbreds. Because the animals in our FA study were the littermates of mice used in the seminatural enclosures, we see no a priori reason to suspect any developmental differences between the mice in this study and the enclosure mice. Moreover, mice used for the FA and enclosure studies were housed together and were only separated well into adulthood, by which time morphological effects produced by genetic stressors should have already been present.

Effects of the t-Haplotype

Unlike the comparison of inbred and outbred mice, FA differences were detectable between the *t*- and wild mice. Also, the FA difference was in the expected direction, with a significantly higher mean CFA value in mice carrying either one or two *t*-alleles. Use of this index was fortunate, because FA differences between *t*- and wild mice did not reach significance for any of the individual characters. In their computer simulations comparing different composite FA indices, Leung et al. (2000) discovered that the composite FA index

we used (their CFA 2) had 50% more power to detect differences in FA among groups than did MANOVA. However, the MANOVA of the FA values for these four characters also showed significance ($P = 0.027$) for *t*/wild differences. (A preliminary MANOVA run for FA in the four characters with the interaction and litter terms included produced results which were not quite significant [$P = 0.053$] presumably in part because its error MS was based on fewer degrees of freedom.) So, in this case, multivariate significance of size FA was seen for the *t*/wild group differences, although the MANOVA results for shape FA in the mandible failed to reach significance for this comparison.

Our observation of increased FA in *t*-bearing mice is additional evidence that +/*t* heterozygotes suffer some fitness decline. This was previously indicated by the lower-than-expected frequencies of *t* in wild populations. Approximately 25% of wild mice carry *t*-haplotypes, which is 50% lower than that predicted by actual segregation distortion rates (reviews in Lenington 1991; Ardlie 1998). Within the 30–40 megabase *t*-chromosomal region, nearly complete linkage is achieved by four large-scale inversions that prevent recombination. Homozygous sterility of *t*/*t* males is speculated to have permitted the accumulation of recessive deleterious mutations within the *t*-complex (Silver 1985), but even partially dominant deleterious mutations might have accumulated by virtue of both their linkage to a locus with increased transmission and the decreased efficiency of purifying selection due to the absence of recombination. Several *t*-specific lethal mutations are known to be distributed over the entire 20-cM length of the *t*-complex (Klein et al. 1984). Their homozygous effect on embryonic lethality is well understood, but it is yet unclear how these or other *t*-linked mutations exert a negative effect on adult heterozygote phenotypes as demonstrated by our finding of increased FA in *t*-bearing mice.

The multivariate significance of the *t*/wild group difference using the size of the four characters was somewhat surprising given that none of the individual characters reached significance in this comparison (Table 3). In addition, the probabilities associated with the mean square for the *t*/wild differences were not particularly low for the four characters (FL = 0.52, HL = 0.27, TL = 0.12, CS = 0.95). However, the coefficients of the estimated canonical vector generated in the MANOVA were highest for HL and TL, characters with the greatest mean differences between the *t*- and wild groups (Table 2), and this vector clearly had enough discriminatory power to detect significant differences between these two groups.

Comparison of Stressors and Characters

Møller (1997) noted that the observed correlations between FA and fitness might be indirect. For example, a stressor might reduce competitive ability, which in turn could cause increased FA, rather than act directly on the level of FA. The lack of a correlation between FA and inbreeding-related fitness observed in this study might be partially explained by the fact that these mice were raised under nonstressful colony conditions. In contrast, the *t*-haplotype could have a direct impact on FA due to its known association with defects during development.

If this is the case, however, we were unable to discover a significant difference in the effects of these two stressors (Table 4). Perhaps one was present but we simply did not have sufficient statistical power in the ANOVA (Table 4) to detect such a difference. The effects of these two stressors did appear to be different among the characters, however, and this can be seen in Table 3, where inbreeding effects tend to be greater than *t*-haplotype effects for FL, HL, and CS, whereas the reverse is true for TL. Similarly, the significant stressor × measure interaction (size and FA measures only) is not particularly surprising given that inbred/outbred differences were seen primarily for the size characters whereas the *t*-haplotype mainly affected FA in these characters (although with some effect on size as well).

Conclusions

These combined results add to the growing literature that suggests the association of FA with fitness is heterogeneous. Although both genetic treatments (inbreeding and *t*) were associated with large fitness differences, they only influenced a small subset of morphometric measures, and FA was not influenced by inbreeding at all. We are unaware of any other studies that have systematically measured lifetime fitness in populations of wild house mice, and our discovery that massive fitness declines associated with inbreeding are not correlated with FA rejects the proposition that inbreeding should cause a decline in developmental stability that will be reflected in increased FA (Møller 1997). FA did appear to be a good indicator of the effects of the *t*-haplotype, however, but size effects also were seen. Given these results, it seems safe to conclude that detection of true fitness differences among groups would be enhanced by measuring both FA and size in a variety of different characters.

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