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2,3,7,8-Tetrachlorodibenzo-p-Dioxin Affects Size and Shape, But Not Asymmetry, of Mandibles in Mice

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Abstract. Fluctuating asymmetry (FA), the random differences between the left and right sides of a bilaterally symmetrical character, is often purported to be a sensitive measure of developmental instability particularly in populations exposed to environmental stressors. As the level of developmental instability increases, often too does the level of FA. In this study we tested the hypothesis that exposure of pregnant mice to low doses of 2,3,7,8-tetrachlordibenzo-p-dioxin (TCDD) would increase the level of FA in the mandibles of their offspring. We used ten landmark coordinates around the mandible to create a single size variable (centroid size) and 20 Procrustes shape variables. These were used to test for effects of dioxin on mandible size and shape and their asymmetries. We found no detectable effect of TCDD on levels of FA in either size or shape of the mandible, but TCDD did produce a significant decrease in mandible size, and a significant effect on the overall shape.

Keywords: TCDD; fluctuating asymmetry; size and shape; mouse mandibles

Introduction

Developmental stability is the ability of an organism to withstand genetic and environmental perturbations encountered during development (Waddington, 1942; Graham et al., 1993). It is considered to be a sensitive indicator of stress in populations, with reduced developmental stability indicating increased environmental stress (Zakharov and Yablokov, 1990; Graham et al., 1993). Most often developmental stability in a population is quantified by fluctuating asymmetry (FA), which is measured by the mean of the absolute differences between left and right sides of a bilateral character (Van Valen, 1962; Palmer, 1994). It has been suggested that FA may be better as an early indicator of environmental quality than more direct fitness estimates (Clarke, 1995). Further, Badyaev et al. (in press) have suggested that the developmental stability of a character, as measured by FA, may be more sensitive to changes in environmental conditions than actual character size.

Fluctuating asymmetry has become an important diagnostic tool in areas such as environmental and conservation biology, and ecotoxicology. A large number of studies, using a wide range of organisms and characters, have shown increased FA from exposure to a variety of environmental and genetic stresses. For example, increased FA due to pollution exposure has been shown in the

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pectoral fins of fish (Valentine et al., 1973), and in the skulls and mandibles of shrews (Pankakoski et al., 1992). Similarly, lead and benzene produced increased FA in *Drosophila* sternopleural bristles (Graham et al., 1993), and Avermectin B1 increased FA in Bush Fly wing vein lengths (Clarke and Ridsdill-Smith, 1990). In contrast, Siegel and Doyle (1975) found no increase in FA in rat long bones (tibia, femur, ulna) after prenatal exposure to audiogenic stress, and the DDT-derived insecticide methoxyclor applied to pregnant mice did not produce a detectable FA increase in the mandibles of their offspring (Leamy et al., 1999). The discrepancies in these findings suggest the need for further FA studies using a variety of organisms, characters and stressors.

2,3,7,8-Tetrachlorodibenzo-p-Dioxin (TCDD) is an environmental contaminant formed during processes such as high temperature combustion and the production of chlorinated phenols (Couture et al., 1990). It is the most acutely toxic member of the polycyclic aromatic hydrocarbons, with widespread distribution, persistency, and the potential for bioaccumulation (Couture et al., 1990). Some of TCDD's most characteristic effects are the production of developmental malformations in the mouse (such as cleft palate, kidney anomalies and hydronephrosis) at doses where there is no obvious maternal or fetal toxicity (Neubert et al., 1973). Dioxin acts in many ways, including the alteration of gene expression (Peters et al., 1999) and modulation of hormone metabolism (Birnbaum, 1995), and produces effects that are often species-, strain-, and tissue-specific (Geyer et al., 1997).

The range of effects produced by TCDD makes it seem reasonable to hypothesize that this toxin should reduce developmental stability. If so, we might expect increased FA in various characters of mice treated with TCDD. This paper describes a study designed to test this hypothesis by comparing mandible size (and shape) asymmetry levels in the offspring of female mice treated/not treated with TCDD. Beyond these asymmetries, mandible size itself, and shape, are analyzed to discover whether they are affected by this toxin.

Materials and methods

Population and data collection

The mice used in this study were derived from two inbred strains, AKR/J and C57BL/6J, obtained from the Jackson Laboratory. All mice were housed in an animal room with temperatures ranging from 20° to 24°C and 12/12 hours of light and darkness, and were fed Purina Rodent Chow *ad libitum*. These strains were crossed in single pair matings to produce 160 F_1s (80 males, 80 females), which were then crossed to produce the experiment population (F_2s). It should be noted that with this breeding design, the F_2 animals measured for FA are genetically variable, although all had an isogenic maternal developmental environment.

When all F_1 mice were of breeding age, they were randomly divided into four groups (two controls and two treatments), and single pair matings were made within each group. Females were checked daily for vaginal plugs, and the presence of a plug designated gestation day 0. Females in both treatment groups received TCDD (via oral gavage) on gestation day 9, chosen because initiation of bone development occurs at this time (Kaufman, 1992). Females in one treatment group (designated T1) received a dose of 1 μg TCDD/kg body weight while the second treatment group (T2) received a lower dose of 0.5 μg TCDD/kg. These levels were chosen because it has been found that doses as low as 1 μg TCDD/kg produced kidney nephrosis in mice (Moore et al., 1973). Two control groups were used, one of which consisted of mice given no treatment (C1). A second control group (C2) was necessitated because the TCDD, obtained from SUPELCO, came dissolved in toluene. The original TCDD concentration was 10 μg/ml, and it was initially diluted to a working concentration of 6 ng/ml by mixing with corn oil. The C2 control group received a dose of toluene-corn oil that replicated the volume of toluene given to mice in the T1 group.

All F_2 offspring of the pregnant F_1 females in both the treatment and control groups were weaned at age 19 days and sexes separated at age

30 days. In order to maximize the number of litters used, approximately five mice per litter were chosen from within each group, resulting in samples of close to 110 mice per group. At 60 days of age they were weighed on a digital scale (to the nearest gram), sacrificed and their skeletons prepared by exposure to dermestid beetles. If necessary, mandibles were then soaked for two hours in a weak ammonium solution to remove any remaining tissue.

The mandibles were separated at the mandibular symphysis and the left and right sides placed under a camera that projected their image onto a computer monitor. Ten readily identifiable points around the mandible (9 peripheral and 1 interior) were chosen for their broad coverage of the structure and for ease of measurement replication (Fig. 1). These points were recorded in millimeters in x, y space using the *Measurement TV* program. After completion of one entire round of measurements, all mandibles were digitized a second time giving two separate estimates of the ten points for both left and right sides.

Size and shape variables

The ten coordinate points were used to create both size and shape variables. A single size measure, centroid size, was calculated for the left and right sides of each mandible (and each repeat measure) by taking the square root of the sum of squared distances between each landmark and the centroid of each mandible (Dryden and Mardia, 1998). The centroid of each side is the point whose coordinates are the means of the x and y coordinates of all 10 landmarks around the mandible.

Shape variables were created using the Procrustes method (Bookstein, 1991; Auffray et al., 1996), which takes the x, y coordinates of the mandibles and eliminates variation in size, position and orientation. These features were removed using four sequential steps that reflect the mandible of one side, and then scale, superimpose and rotate the mandibles to produce an optimal fit between corresponding coordinate points of left and right sides for all individuals

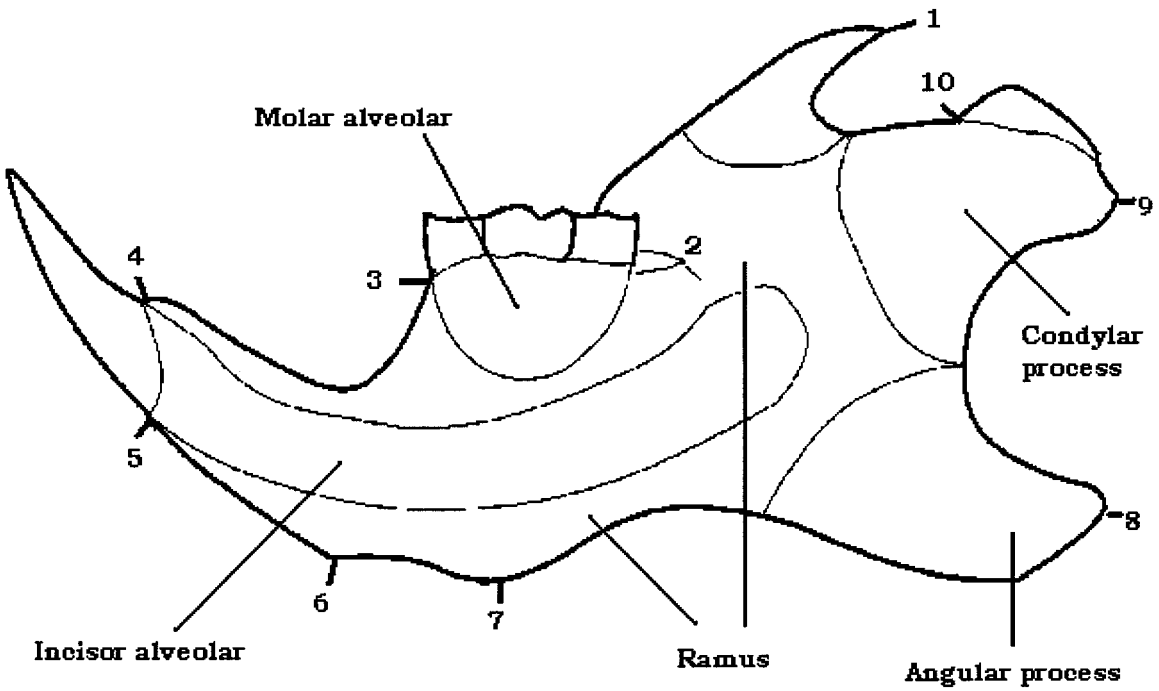

Figure 1. Outline of the medial view of a mouse mandible showing the ten landmark points that were digitized.

(see Klingenberg and McIntyre, 1998). This process created 20 new shape variables (x, y coordinates of 10 points) for both sides of the two repeat measures of each mandible.

It is important when analyzing shape to understand that, as defined in geometric morphometrics, it is a multivariate character (Slice et al., 1996). Consequently, variation at individual coordinates is only useful in explaining changes in the whole shape configuration. In addition, the adjustment for size, position and orientation in the Procrustes method eliminates four degrees of freedom which results in $2k - 4$ shape space dimensions ($20 - 4 = 16$ in this study), where k is the number of landmarks (Klingenberg and McIntyre, 1998). Adjustment for this number of degrees of freedom was made in the Procrustes multivariate analyses described below.

Sources of variation

Prior to the analysis of variance, the distributions of the means of the right and left sides $[(R + L)/2]$ and differences between the two sides $(R - L)$, for both centroid size and the twenty Procrustes shape variables, were examined for normality, skewness and kurtosis. As a result, several mice were eliminated as outliers (Sokal and Rohlf, 1995) and, along with a few broken mandibles, this resulted in a final sample of 409 mice (C1-103, C2-108, T1-98, T2-100).

A mixed model, two-way analysis of variance was used to assess the significance of FA and directional asymmetry (DA) for size and shape (Leamy, 1984; Palmer, 1994). Directional asymmetry occurs when one side of the mandible is consistently larger (Palmer, 1994). A standard ANOVA was used for assessing centroid size (Leamy, 1984; Palmer and Strobeck, 1986) and a modification of this known as the Procrustes ANOVA (Klingenberg and McIntyre, 1998) was used for the shape variables. In the Procrustes ANOVA, the sums of squares were calculated by adding the sums of squares of all twenty shape coordinates (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 16 in this study.

In these ANOVAs, individuals is a random factor that assesses variation among individual mice, and sides is a fixed factor that assesses DA (although it was treated as random in order to calculate its variance component). The individual $\times$ sides interaction assesses FA and the error assesses variation in the replicate measurements (Leamy, 1984; Palmer, 1994). Sex and treatment factors were also included in the model to adjust for their potential effects. Mean squares for individuals and sides were tested over the error mean squares, and mean squares for the interaction were tested over the error. The latter, if significant, indicates that the amount of variation due to FA is greater than that due solely to measurement error, and that the asymmetry analysis may proceed (Palmer, 1994).

Variance components were calculated for the individuals, sides, individuals $\times$ sides, and error factors, and used to estimate the percentage contribution of each to the total variation. The percentage contribution of the error variance to the sum of the four variances, and to the FA variance, provided appropriate estimates of measurement error (Palmer, 1994). Following this preliminary analysis, the means of the two repeat measurements were calculated for each character, and these values used in all subsequent analyses.

Asymmetry variables

For the asymmetry analysis, both signed and unsigned asymmetries of size and shape were calculated. Signed asymmetry is simply measured as the signed difference between the left and right sides $(R - L)$ of an individual, and accounts for both the size and direction of the difference. If the mean of all the individual signed asymmetries in a sample is significantly different from zero, it suggests that DA is present (Van Valen, 1962; Leamy, 1984). To measure FA, any DA present must be adjusted because it can create an artificial increase in the FA value (Palmer, 1994). Unsigned asymmetry, or absolute $R - L$ differences, measures only the magnitude of the difference and ignores the direction. The mean level of unsigned asymmetries in a sample provides a measure of FA (Palmer, 1994).

For centroid size, signed asymmetries were calculated and corrected for DA by subtracting the sample mean of the signed asymmetries (Leamy, 1984). The unsigned asymmetries were then calculated by taking the absolute value of the adjusted signed asymmetries, and their half-normal distribution corrected for using Box-Cox transformations ($[FA + 0.0005]^{0.33}$) (Swaddle et al., 1994). Because shape is multivariate, changing all the negative signs to positive (taking the absolute $R - L$) would have affected associations between landmarks by expressing all the left-right differences in an anterior and dorsal direction (Leamy, pers. comm.). Therefore for the analysis of shape, several steps were taken (Leamy, pers. comm.) that computed the unsigned asymmetry without destroying the multivariate relationship.

Analyses of TCDD effects

Prior to the analysis of any TCDD effects, all variables (body weight, size, shape, and size and shape asymmetries) were tested for additional sources of variation. Centroid size was analyzed using the mean size of the two sides $[(R + L)/2]$ for each individual, whereas shape analysis used the means of the two sides of all 20 shape variables. Sex effects were significant for centroid size and body weight (although not for shape), and were therefore adjusted for all characters by obtaining residuals from an ANOVA (Sokal and Rohlf, 1995). In addition, any scaling effects of size on asymmetry were tested for by their correlation, but no significant scaling was found and therefore none of the variables were adjusted. Litter sizes were essentially the same in all four groups so no adjustments were made for this factor.

Differences between the control and treatment groups were tested for using nested univariate ANOVAs (size characters) and multivariate ANOVAs, or MANOVAs (shape characters). In both the size and shape analyses, the mean squares for the nested factor (litters within treatment group) were used as the error term over which the group mean squares were tested (Sokal and Rohlf, 1995). Significant differences among litters generally indicate the presence of non-genetic maternal effects in organisms such as mice (Falconer and Mackay, 1996). Due to the 4 degrees of freedom that are lost during the Procrustes shape method (Klingenberg and McIntyre, 1998) only 16 of the 20 x and y shape variables were used in all three MANOVAs.

Initially two orthogonal comparisons (C1 vs C2 and T1 vs T2) were made in both the ANOVAs of the size characters and the MANOVAs of the shape characters. No significant differences were found between either the two controls or the two treatments for any of the size or shape characters or their asymmetries, so the groups were pooled to form one control group ($n = 211$) and one treatment group ($n = 198$).

In order to visualize any shape changes produced by TCDD, differences in the shape coordinates between the means of the control and treatment groups at each landmark were graphed directly onto a diagrammatic representation of the mean mandible. These differences, in units of Procrustes shape, were displayed as lines that when drawn from the mean location of each landmark point, represent the magnitude and direction of the change (Klingenberg and McIntyre, 1998).

Results

Table 1 gives the results of the ANOVAs of the repeated measures of size and shape. As can be seen, most of the variation in both size and shape is due to individual differences, especially for centroid size. The significant mean squares for sides indicates that DA is present, although it only contributes less than 2% of the variation in centroid size and less than 3% of the variation in shape. The individual × sides interaction is highly significant for both size and shape, indicating that there is significant FA. FA comprises less than 8% of the total variation in centroid size, but a much higher proportion (nearly 29%) for shape. The error variance comprises only about 5% of the total variation in size, although somewhat more (17%) of the total variation in shape.

The means and standard deviations of centroid size and body weight are shown in Table 2. Cen-

Table 1. Analysis of variance for centroid size (2-factor ANOVA) and shape (Procrustes ANOVA)*

Source	Sums of squares	df	Mean square	Variance component	% Variance
Centroid Size					
Individuals (I)	128.414	404	0.318**	76.00	85.60
Sides (S)	1.203	1	1.203**	1.45	1.65
$I \times S$	7.289	408	0.0179**	6.70	7.64
Error	3.659	818	0.00447	4.47	5.11
Shape					
Individuals (I)	0.822	6464	1.27**	37.53	52.04
Sides (S)	0.016	16	9.85**	1.88	2.60
$I \times S$	0.218	6528	0.334**	20.72	28.73
Error	0.161	13088	0.123	11.99	16.63

* Sums of squares, mean squares, and variance components are in mm^2 for centroid size (variance components $\times 10^3$) and in dimensionless Procrustes units for shape (mean squares $\times 10^4$; variance components $\times 10^5$). The percentage contributions (% Variance) of each variance component to the total variance are also given.
** $P < 0.01$.

troid size differed significantly between the control and treatment groups, being 0.1 mm smaller in the treatment group. There is significant DA of centroid size in both the treatment and control groups, with the left side larger than the right by slightly more than 0.05 mm in both groups, but the level of DA is not significantly different between the two groups. There are no significant differences between the control and treatment groups for either FA of centroid size, or for body weight which averages about 23 grams for mice in both groups.

The Wilks Lambda statistics from the MANOVAs of the shape characters (Table 3) show that there are significant differences among litters between groups for both shape and signed asymmetry of shape. This suggests that the use of the nested factor as the error term in these analyses was appropriate. Using litters as the error term, the treatment and control groups show a significant difference for shape but not for signed or unsigned asymmetry of shape.

Figure 2 depicts the differences in shape between the treatment and control groups. The solid lines indicate the magnitude and direction of the change produced by TCDD at each of the individual shape landmarks. The largest difference in shape is seen at the only interior landmark, located at the rear of the molar tooth row (point 2 in Figure 1). The change in the treatment group compared to the control is about 0.002 shape units in an anterior ($-x$) and dorsal ($+y$) direction. Almost as large are the effects seen in the treatment group at point 3, the anterior end of the molar tooth row, and point 5, the ventral point at which the incisor meets the incisor alveolar (Fig. 1). Point 3 moved in a posterior (0.0006 units) and dorsal direction (0.002 units), while point 5 changed in a posterior (0.001 units) and ventral (0.001 units) direction.

Table 2. Means and standard deviations for centroid size, the signed and unsigned asymmetry of centroid size, and for body weight for mice in the control and treatment groups

	Control		Treatment	
	Mean	Std. dev.	Mean	Std. dev.
Centroid size	14.622	0.281	14.519*	0.282
Signed asymmetry	−0.0544	0.134	−0.0541	0.133
Unsigned asymmetry	0.454	0.112	0.458	0.111
Body weight	23.206	2.129	23.401	2.213

* $P < 0.05$.

Table 3. Wilks Lambda statistics from the multivariate analysis of mandible shape and shape asymmetries

	Groups (df = 16)	Litters (df = 1152)
Shape	2.049*	1.456**
Signed asymmetry	1.150	1.109*
Unsigned asymmetry	1.099	1.024

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

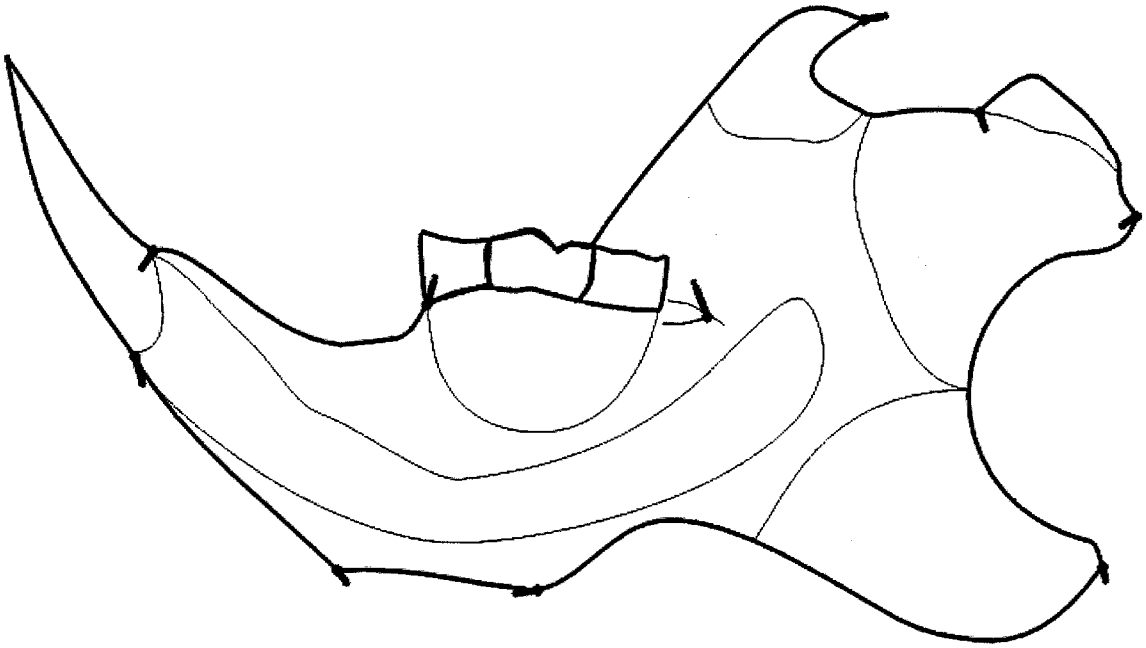

Figure 2. Outline of the medial view of a mouse mandible showing the shape effects of TCDD. The lollipops represent the mean x, y movement (multiplied by 500) of each of the ten landmark points in the treatment group.

Discussion

Increased FA in a population is thought to be a sensitive indicator of reduced developmental stability. We hypothesized that low maternal doses of the toxin TCDD would be sufficient to reduce developmental stability in mouse embryos, and given this, that we would see increased FA in the treatment population. In fact we saw no increase in FA in either the size or shape characters. This was surprising for several reasons, not the least of which is the potent developmental toxicity of TCDD (Birnbaum, 1995). Dioxin activity generally involves it binding to a cytoplasmic receptor (AhR), dimerizing with a second protein (ARNT), and the complex being translocated to the nucleus (Fitzgerald et al., 1996). Once there, it interacts with gene regulatory elements and alters gene expression (Peters et al., 1999). This suggests that TCDD could affect developmental stability by modifying the transcription of genes that control the precision of the pathway for bone development. However, our results indicate that if indeed there were effects on genes for bone development, they were not manifested by a change in developmental stability, at least as assessed by FA.

It is possible that the level of TCDD treatment was too low to affect developmental stability, although previous studies have shown developmental effects in mice produced by quite low maternal doses of this toxin (Couture et al., 1990). Additionally, TCDD is known to have anti-estrogenic properties and because of the role estrogen plays in bone development, it has been suggested that dioxin should affect estrogen-dependent homeostasis (Gierthy et al., 1994). These previous findings suggest that even a low dose of dioxin could eaffect developmental stability. Alternatively, traditional measures of toxicity, such as a lowest observed adverse effect level (LOAEL) for prenatal mortality of 24 μg/kg (Peterson et al., 1993), and the mouse LD_{50} of 200–600 μg/kg (Vanden Heuvel and Lucier, 1993), imply that much higher doses of dioxin may be necessary to elicit a response. This may be particularly so when we consider that a measure such as the LD_{50} is based on the animal treated, whereas in the offspring of treated mice, previous studies have shown that embryos may retain as little as

$0.15 \pm 0.01\%$ of the total dose/gram only 3 days post-dosing (Abbott et al., 1994).

Although FA is considered a means of measuring developmental instability, the characters measured can sometimes confound the expected results. Woods et al. (1999) have suggested that different stressors may produce trait-specific responses in FA. Therefore it is conceivable that FA may have been detected had skeletal characters other than the mandible been chosen. However, given that mouse mandibles have shown increased FA in several other studies (for example Siegal et al., 1977; Leamy, 1984), and that dioxin actually affected the size and shape of the mandibles, it seems unlikely that trait choice is responsible for our negative result.

DA usually is considered to be unsuitable as an indicator of stress (Palmer and Strobeck, 1992), although certain stressors have been known to produce a transition from FA to DA (Graham et al., 1994; Leamy et al., 1999). Although DA was present in mice from both the treatment and control groups in this study, its level was nearly the same in both groups. Thus, TCDD had no apparent effect on this type of asymmetry.

TCDD did have a direct effect on the size of the mandibles, and without any associated change in body weight. Leamy et al. (1999) also found a significant effect for size, but not FA, in offspring of mice treated with methoxyclor. However, whereas methoxyclor increased mandible size (Leamy et al., 1999), the size of mandibles in the mice used in this study decreased with TCDD treatment. Given some of the known mechanisms of action of this toxin, this result is not surprising. TCDD is known to decrease the expression of certain growth factors in many tissues (Abbott and Birnbaum, 1990; Bryant et al., 1997), including Transforming Growth Factor-Beta (TGF-*B*) which is involved in bone development. Although this reduction in growth factor expression due to dioxin has not been studied in developing bone cells, it seems reasonable to expect that its effect would be to reduce the size of mandibles and possibly other bones.

The anti-estrogenic properties of TCDD may also have affected the mandible size. Estrogen is known to modulate bone-cell development (Gray et al., 1987), and affect skeletal growth and adult bone balance (Turner et al., 1990). The anti-estrogenic mechanisms of TCDD are not conclusively known, but hypotheses have been put forward suggesting that TCDD may down-regulate transcription in estrogen-dependent cells (Harris et al., 1990) or deplete intracellular 17*B*-estradiol in a target tissue (Gierthy et al., 1987). Regardless, TCDD's anti-estrogenic mode of action could certainly have played a role in its effect on mandible size.

The magnitude of the difference in centroid size between control and treatment groups is interesting in relation to the effect dioxin appears to have on transcription and target gene expression. Recent studies have identified 34 putative quantitative trait loci (QTLs) for the mean size of ten mandible characters (Leamy et al., 1997) and 12 QTLs for centroid size (Leamy, pers. comm.). A QTL is a segment of chromosome that may house a gene (or genes) affecting quantitative traits (Falconer and Mackay, 1996). Leamy (pers. comm.) found that the average additive genotypic value (half the difference between the two homozygote values) of the QTLs for centroid size was 0.766% of the mean, close to that found here (0.71%) for the difference between the treatment and control groups. This in no way suggests that a single gene is responsible for the dioxin effect, but it is interesting to note that the magnitude of this effect is equivalent to that of a single allelic substitution.

Although all ten landmarks contributed to the significant change in shape, it is noteworthy that the largest effect of the dioxin was seen at the two landmarks bordering the molar tooth row. Previous studies have shown that dioxin affects tooth development in rats (Alaluusua et al., 1993) and mice (Partanen et al., 1998). The former study also showed a significant decrease in rat skull size, although there was a corresponding decrease in body weight. Although the connection is somewhat tenuous, it is possible that the effect dioxin has on developing teeth is associated with the effect it has on the development of the surrounding bone.

Given that dioxin has previously been shown to have an effect on developing teeth, and that the largest effect we saw here was on mandible landmarks in close proximity to the teeth, it would be interesting to measure the teeth of the mice used in this study to test whether dioxin has an associ-

ated effect on mandibles and teeth. It may be that there are genes affecting the development of both teeth and bone that are highly susceptible to the effects of dioxin. In addition, measuring other bone characters (e.g. limb bones) may help determine whether dioxin affects the entire skeleton and not just mandibular bone. This would also provide an opportunity to test for any response in FA. If other characters show size and shape effects without any increase in FA, it would lend support to the idea that character size and shape are more sensitive indicators of dioxin stress than developmental stability.

Dioxin clearly had a negative effect on the mice in this study; however, this effect would not have been detected had FA been the sole character measured. This study therefore highlights the caution that should be taken when drawing conclusions about any change, or lack thereof, found in levels of FA. This is a particularly important consideration for those studies using FA as a tool for monitoring levels and effects of toxins in the environment.

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