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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin: its effect on genes for mandible traits in mice

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Abstract

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a powerful toxicant that exerts its effects through the aryl hydrocarbon receptor (AHR) governed by the *Ahr* locus that in mice is located on chromosome 12. We used single marker analyses of the offspring of female mice treated/not treated with TCDD to search for a gene (quantitative trait locus or QTL) on chromosome 12 near the site of the *Ahr* locus to test whether this locus appeared to affect mandible size, shape, and/or asymmetry especially in the treated mice. These mice were sampled from the F_2 generation of an original intercross of two strains (C57BL/6J and AKR/J) known to be divergent in their response to TCDD. A QTL affecting mandible shape was found on chromosome 12, but its effect on mice in the treated and control groups did not differ and it was concluded that this QTL probably was not the *Ahr* locus itself. We also probed a second chromosome (11) and found a QTL whose effects on asymmetry of mandible shape differed in the two environments. These results suggested that the entire genome in these mice should be scanned to search for additional QTLs that might be affected by TCDD to learn more about the potential effects of this powerful toxicant on these genes.

Keywords: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; Epistasis; Mandible size, shape and asymmetry; Mice; QTLs

1. Introduction

The ability of an organism to produce a consistent phenotype in a specific environment is known as developmental stability (Zakharov, 1989). If developmental stability is low in a given organism, then there is a greater chance that the organism will deviate from that phenotype (Palmer, 1994). Developmental stability is sensitive to genetic or environmental stressors such as pollution and toxicants, and is expected to decrease as the level of these stressors increases (Zakharov, 1989; Parsons, 1990). Developmental stability most often has been measured by fluctuating asymmetry (FA), or variation in the random differences between right and left sides of a bilateral character (such as the length of a limb bone). This variation typically is quite subtle, and may be a more sensitive indicator of developmental problems produced by genetic or teratogenic insults than overt kinds of malformations or anomalies (Fraser, 1994).

FA is presumed to be generated from random (non-genetic) developmental perturbations (Palmer, 1994) and thus have a totally environmental basis, but this is still far from clear (Leamy, 1997; Whitlock and Fowler, 1997). The heritability of FA, or the proportion of the total variation in FA that is genetical, typically has been estimated to be quite low (Leamy, 1997). More recently, the genetic basis of FA has been assessed by direct searches for genes (quantitative trait loci or QTLs) that could affect FA. In general, few if any QTLs for FA in mandible characters in mice have been discovered (Leamy et al., 1997, 1998, 2000; Klingenberg et al., 2001), although there is evidence that interactions among two or more QTLs (epistasis) might affect FA in the size of the mandible (Leamy, 2002). With rare exceptions (see McKenzie and Clarke, 1988), however,

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genes for FA in various characters have not been searched for in organisms subjected to an environmental stressor where it is possible that such genes might more easily be discovered.

Toxicants such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are potent environmental stressors. TCDD is formed during high-temperature combustion processes, chemical bleaching of pulp, and the production of chlorinated phenols (Couture et al., 1990). The biological effects of TCDD range from alteration in enzyme activity to cancer and birth defects and the formation of cleft palates in developing mice (Whitlock, 1990; Couture et al., 1990), as well as the development of grossly asymmetric brains in several species of birds (Henshel et al., 1993, 1997; Henshel, 1998). TCDD elicits its effects in biological systems by forming a complex with the aryl hydrocarbon receptor (AHR) that in turn activates various genes such as *Cyp1a1* and *Cyp1a2* that are members of the cytochrome P450 family and play a role in the metabolism of toxicants (Gonzales and Fernandez-Salguro, 1998; Corchero et al., 2001; Nie et al., 2001). In mice, AHR is controlled by genes at the *Ahr* locus located on chromosome 12 about 18 cM (centiMorgans; a unit of map distance) from the centromere (Rowlands and Gusafsson, 1997).

Allen and Leamy (2001) tested for potential effects of TCDD on FA in the size and shape of the mandible in the offspring of female mice derived from an intercross of two strains (C57BL/6J and AKR/J) known to be divergent in their response to TCDD (Poland and Knutson, 1982; Whitlock, 1990). These investigators found that mice whose mothers were treated with TCDD had the same level of FA in the mandible as the control (non-treated) group, and therefore concluded that this toxicant did not reduce developmental stability in the treated mice. On the other hand, the treated mice did significantly differ from the controls in the size and shape of their mandibles, so TCDD apparently had some effect on these kinds of mandible characters (Allen and Leamy, 2001).

In the present study, we made use of these same mice to investigate the genetic basis of the effect of TCDD on the mandibles. Specifically, this study had three aims. One was to test whether the *Ahr* locus itself (or a QTL in this region) on chromosome 12 plays a role in the effect of TCDD on the mandibles. The second aim was to probe a site on another chromosome (number 11) in an effort to locate a QTL affecting mandible size or shape to test whether its effect differed in mice in the two environments. Finally, the third aim was to test for the presence of QTLs affecting mandible FA, especially those that might have a greater effect in the mice exposed to TCDD. We were fortunate in this study to discover QTLs on both chromosomes 11 and 12 that affected the mandible characters, and this also allowed us to test for potential interactions of these two loci (epistasis), and if such epistasis was present, to discover whether it differed between the two environments.

2. Materials and methods

2.1. Mouse population and characters

The mice used in this study were sampled from the F_2 generation of an original intercross of inbred strains AKR/J and C57BL/6J (Allen and Leamy, 2001). These mice were divided into four groups, two of which served as controls and two as treatments. In one control group, the mice were reared from F_1 mothers who received toluene/corn oil (Sigma, St. Louis, MO), the vehicle in which TCDD (Sigma) was administered to the treatment groups. Mice in the second control group did not receive any treatment. Mice in the treatment groups were reared from F_1 mothers who received either 1 μg TCDD/kg or 0.5 μg TCDD/kg via oral gavage. Their body weights ranged from 22 to 32 g, and the dosage volumes ranged from 0.183 to 0.242 ml for the 0.5 μg/kg group, and 0.370–0.530 ml for the 1 μg/kg and toluene control groups. Gavage volumes were not kept constant because a single stock concentration was made of the TCDD to ensure that the dilutions used for dosages in both groups were equivalent. The control animals receiving the vehicle only were given the maximum amount of vehicle equivalent to that for the 1 μg/kg group (0.37–0.53 ml range) so that they could be appropriately compared with both treatment groups.

The dosage levels for TCDD (1 and 0.5 μg/kg) were chosen because it is known that doses as low as 1 μg TCDD/kg can produce kidney nephrosis in mice (Moore et al., 1973), and the intent was to determine if doses in this somewhat low range might exert subtle effects on asymmetry as well (Allen and Leamy, 2001). The F_1 mothers were given their respective treatments on gestation day 9 (GD9, where the presence of the vaginal plug was gestation day 0). GD9 was chosen for dosing because initiation of bone development occurs at that time (Kaufman, 1992).

All of the F_2 mice were weaned at day 19; and on day 30, the sexes were separated. Approximately five mice in each litter were chosen for analysis, and this resulted in total sample sizes of about 100 mice per group. At 60 days of age, the F_2 mice were weighed, sacrificed, a tail clip was taken and stored at -80 °C, and skeletons of all individuals were prepared using dermestid beetles (Allen and Leamy, 2001).

We made use of these mice reared by Allen and Leamy (2001) by first extracting the DNA from the tail clips using the same protocol as Lin et al. (1989). This DNA was then quantified and used to score two microsatellite loci in all F_2 mice. Microsatellites are repeating sequences of bases that occur throughout the

DNA of all chromosomes in mice and whose lengths (number of repeats) differ among the various inbred strains (Mouse Genome Database, 2001). One microsatellite locus, *D12Mit112*, was chosen because it differs in length (is polymorphic) between the C57BL/6J (designated BB) and AKR/J (designated AA) strains, and is located 17.5 cM from the centromere on chromosome 12 (Mouse Genome Database, 2001). This locus is within 0.5 cM of the *Ahr* locus itself, and thus seemed an ideal probe to test the effect of alleles at the *Ahr* locus (or nearby locations on this chromosome). It was expected that the *Ahr* locus would be polymorphic because the C57BL/6 mice are homozygous for the 'responsive' allele (*b1*) and the AKR for the 'non-responsive' allele (*d*), at the *Ahr* locus (Poland et al., 1994), and thus would yield Ahr^{b1}/Ahr^{b1}, Ahr^{b1}/Ahr^{d}, and Ahr^{d}/Ahr^{d} genotypes in the F_2 generation that could be detected by the three genotypes at the *D12Mit112* locus (AA, AB, BB).

We also wished to locate at least one QTL that affected mandible size, shape, or asymmetry in order to test whether its effect differed in the mice exposed/not exposed to the TCDD maternal environment. We therefore made use of a second polymorphic microsatellite, *D11Mit258*, located 65 cM from the centromere on chromosome 11 (Mouse Genome Database, 2001). This marker was chosen because it is in an area where QTLs for mandible size, shape, and asymmetry have previously been found in a population formed from an intercross of the large (LG/J) and small (SM/J) mouse strains (Klingenberg et al., 2001), and this seemed a likely location to probe for a QTL in our population of F_2 mice. Since use of these two microsatellite markers revealed QTLs on both chromosomes 11 and 12 that affected the mandible characters (see results below), no additional markers were used. PCR was completed using the protocol and primers from Research Genetics (Huntsville, AL) and the resulting PCR product was separated using 2.5% agarose gel electrophoresis with ethidium bromide staining for visualization.

Altogether, a total of 387 mice were successfully genotyped for both microsatellites and were available for the analysis. This included 95 mice from 19 different litters in treatment group 1 (1 μg TCDD/kg), 90 mice from 17 different litters in treatment group 2 (0.5 μg TCDD/kg), 101 mice from 18 different litters in control group 1 that was given no treatment, and 101 mice from 20 different litters in control group 2 that received the vehicle only. This total of 387 mice included nearly equal numbers of males and females overall (195/192) and in each of the four groups (treatment groups 1 and 2: 49/46, 45/45; control groups 1 and 2: 47/54, 54/47). The two sexes exhibited differences in the mandible characters that were adjusted for in the analysis (see below).

The mandibles in each mouse were separated into right and left sides, and the image for each side was

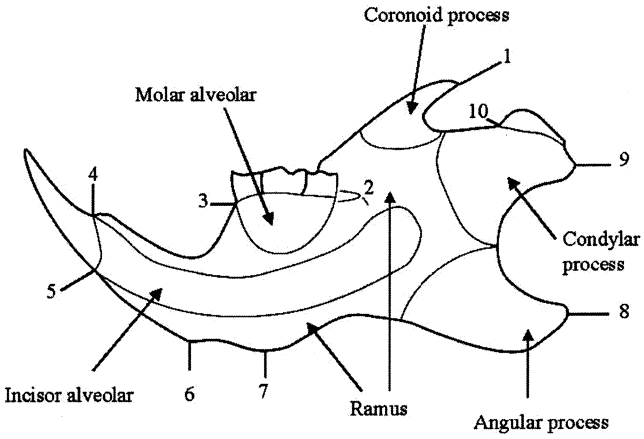

Fig. 1. Outline of the medial view of a mouse mandible showing the landmark points that were digitized.

projected onto a computer screen using a video camera. Ten landmark points on each mandible (Fig. 1) were chosen, and their x,y coordinates were recorded in millimeters using the *Measurement TV* program. To increase the precision of measurements, each mandible was digitized twice, yielding two complete sets of coordinates for both left and right sides of the mandibles in each mouse (Allen and Leamy, 2001). This was accomplished in blind fashion with respect to the groups of mice since each mouse was digitized before it was determined which group it belonged to based on its identification number.

2.2. Size, shape, and asymmetry characters

From the ten digitized points on each side of each mandible, size and shape variables were created in the manner previously explained by Allen and Leamy (2001). Briefly, a single measure of size known as centroid size was calculated by taking the square root of the sum of squared distances between each landmark and the centroid of each side (Dryden and Mardia, 1998). The centroid is the point whose coordinates are the means of the x and y coordinates of all ten landmarks around the mandible. Once the centroid size was calculated for both left and right sides, the mean of the two sides was used as the mandible size character for each mouse. Twenty shape characters (two at each of the ten landmark points) were created for both left and right sides of each mandible, and again the mean of the sides was used for each of these 20 characters. The shape characters were calculated using the Procrustes method (Bookstein, 1991; Auffray et al., 1996; Smith et al., 1997), a mathematical procedure adapted for bilateral characters that has been described in some detail by Klingenberg and McIntyre (1998). Derived in this manner, shape is a multivariate character (Slice et al., 1996) for which differences between various (treatment versus control) groups cannot be expressed

as single values. Instead, they are more appropriately depicted in figures which show the magnitude and direction of the change at each landmark point (Klingenberg and McIntyre, 1998).

Directional and fluctuating asymmetry characters also were created for both mandible size and shape (Allen and Leamy, 2001). Directional asymmetry (DA) occurs when one side of a bilateral character (such as the mandible) is consistently larger than the other side (Palmer, 1994). For centroid size, *signed* differences of the two sides provided a measure of DA whereas FA characters were created by *unsigned* or absolute differences between sides after first correcting for any DA present (Allen and Leamy, 2001). For shape, DA and FA characters were created by equivalent signed and unsigned differences between sides for each of the 20 shape variables (see Klingenberg et al., 2001).

The means of the replicate measures for all size, shape, and size and shape asymmetry characters were calculated for use in all subsequent analyses, but first were tested for potential effects due to sex and litter size. Sex effects were significant for centroid size as was litter size for size and shape, so these characters were adjusted in a linear model that used sex as a classification variable and litter size as a covariable. In addition, any scaling effects of size on the asymmetry characters were tested for by their correlations, but no significant scaling (using the sequential Bonferroni procedure; Rice, 1989) was found and therefore none of the asymmetry characters were adjusted for overall size differences.

2.3. Tests for genotypic, epistatic, and environmental effects

A mixed model ANOVA (Sokal and Rohlf, 1995) was used to test for significance of genotype differences at the markers on chromosomes 11 (C11) and 12 (C12) and environmental (TCDD vs. control) effects on the mandible size characters and their asymmetries. The model for this ANOVA is:

$$Y = \mu + C11 + C12 + E + L + C11C12 + C11E + C12E + C11C12E + \varepsilon,$$

where Y is the dependent character(s) (centroid size and the signed and unsigned asymmetries of centroid size), *C11* and *C12* are fixed effects associated with genotypes on chromosomes 11 and 12 (if either is significant, this would indicate the presence of a QTL), E is a fixed effect that assesses differences among the (treatment and control) environments, L is a random effect associated with differences between litters nested within environments, *C11C12* is an interaction that assesses epistatic effects between the two genotypes, *C11E* and *C12E* are interactions of the genotypes at each marker with the environment (if significant, this would imply that the magnitude of effect of the QTL differs in the TCDD and control environments), *C11C12E* is the interaction of both genotypes (epistasis) with the environment (if significant, this would imply a difference in epistatic effects between environments), and ε is the usual error term. Environmental effects (E) were tested over the nested factors litters (L) whereas all other effects were tested over the error term. Litters was included as a factor in these analyses since differences among litters may in part be a reflection of non-genetic maternal effects (Falconer and Mackay, 1996). Probabilities of all F-tests for each of the three centroid size characters were evaluated with the sequential Bonferroni procedure (Rice, 1989) to ensure that the experimentwise error rate would not exceed 5%. The shape, signed shape differences (DA for shape), and unsigned shape differences (FA for shape) each were subjected to multivariate analyses of variance (MANOVAs) of the same design as the ANOVA already described.

Prior to running the ANOVAs and MANOVAs, we used orthogonal comparisons to test for differences in the size and shape characters between the two treatment groups and between the two control groups. As was found previously by Allen and Leamy (2001), none of these differences were significant ($P > 0.05$). Further, we constructed contrasts to test for differences in genotypic effects (at both putative loci on chromosomes 11 and 12) between the treatment and control pairs, and none of these contrasts reached significance. In all subsequent analyses, therefore, the groups were pooled to form one control group ($N = 202$) and one treatment group ($N = 185$). This pooling increased the number of degrees of freedom for the error mean squares in the analyses of variance, and therefore presumably also increased the statistical power associated with the detection of differences among genotypes.

2.4. QTL effects

The tests for genotype differences as described above are useful only in detecting QTLs linked in fairly close proximity to either of the markers, and give no information about the precise location of these QTLs (Lynch and Walsh, 1998). Nonetheless, if the marker genotypes on either chromosome (C11 or C12) reached significance in the analysis of variance for any of the mandible characters, we calculated the effects of the putative QTL linked to the marker by using the genotypic data at the site of the marker (Fry et al., 1998; Lynch and Walsh, 1998). Also, if QTL effects appeared to be significantly different between environments, these effects were calculated separately for each of the two (TCDD vs. control) environments.

To calculate effects of QTLs linked to either marker, we estimated their additive (a) and dominance (d) genotypic values. The additive genotypic value for a

QTL is one-half the difference in the mean value of the character between the AA and BB homozygotes, and is the principal measure of the effect of the QTL on the character. The dominance genotypic value is the difference between the average value of the character between the heterozygotes and the midpoint of the two homozygotes (Falconer and Mackay, 1996), and estimates the effects of dominance on the character. The a and d values were calculated from multiple regression of the mandible character(s) on additive and dominance index values set at -1, 0, and $+1$, and 0, $+1$, and 0, respectively, for the AA, AB, and BB genotypes. Testing for the significance of these a and d values was done with the use of the standard errors of the regression coefficients.

Multiple regression also was used in a similar fashion to estimate additive and dominance genotypic values for mandible shape. Since shape is a multivariate character, however, the additive and dominance effects are depicted by $\boldsymbol{a}$ and $\boldsymbol{d}$ vectors (each containing 20 shape characters, two at each of the ten landmarks) that indicate both the size and direction of the effect (Klingenberg et al., 2001). The total magnitude of the $\boldsymbol{a}$ and $\boldsymbol{d}$ vectors for each shape QTL was quantified by estimating their length in units of Procrustes distance (Klingenberg et al., 2001). Specifically, the magnitude of additive ($||\boldsymbol{a}||$) and dominance ($||\boldsymbol{d}||$) shape effects were calculated as: $||\boldsymbol{a}|| = (\boldsymbol{a}'\boldsymbol{a})^{0.5}$ and $||\boldsymbol{d}|| = (\boldsymbol{d}'\boldsymbol{d})^{0.5}$ (Klingenberg et al., 2001). In order to test the overall significance of these shape effects, we used multivariate regression of the shape characters on the additive and dominance index values.

While verbal descriptions of additive and dominance effects of a QTL on shape are helpful, graphing the vectors of these effects on landmark points provides a visualization of the overall shape changes that is intuitively much more readily understandable (Klingenberg et al., 2001). We therefore constructed such graphs by drawing lines that connected the point at the mean of each landmark point to the location of the mean plus the additive (or dominance) value for a given QTL This line indicates the magnitude and direction of the shape change at each landmark point (Klingenberg and McIntyre, 1998). Because the QTL effects represented by these vectors were quite subtle, they were multiplied by a factor of 500 in the figures to make them more visible.

3. Results

The means and standard errors of centroid size and the centroid size asymmetries for the three genotypes at the markers on chromosomes 11 and 12 (C11 and C12) for mice in the treatment and control groups are shown in Table 1. As may be seen, the means for centroid size across all genotypes are smaller in the treatment compared with the control group. However, these differences in centroid size between the two groups appear small in all cases. The means of signed differences between sides for centroid size are significantly different from zero in all cases (Table 1), suggesting that there is DA in this character. The signed asymmetries generally are quite similar among the genotypes for C12 in both treatment and control groups, but differ somewhat for C11 especially in the control group. For both C11 and C12, the unsigned asymmetry of centroid size appears generally higher in the treatment compared with the control group, especially for the BB homozygote mice.

Table 2 shows the results of the ANOVA for centroid size and the signed and unsigned asymmetries of centroid size. There is no significant difference in centroid size or the centroid size asymmetries among the three genotypes for either C12 or C11, and thus no evidence that there are QTLs linked to the markers on chromosomes 11 and 12 that are affecting the centroid size characters. Further, the genotype $\times$ environment interaction is not significant for either C12 or C11, nor is there epistasis between the genotypes at these two markers for any of the characters. However, there is a significant group and litter effect for centroid size, suggesting that the mean centroid size differs between treatment and control groups, and among litters nested within these groups.

Table 3 shows F-values approximated from Wilks Lambda statistics generated in the MANOVAs of the shape characters. C11 and C12 are highly significant for shape, indicating that there are QTLs located on both chromosomes that are affecting overall mandible shape. As was the case for centroid size, environment and litter effects are significant for shape as well, but only litter differences are significant for signed shape asymmetries (shape DA). Unsigned shape asymmetries (shape FA) show significant C11 $\times$ environment interaction effects which suggest that C11 is affecting this asymmetry character differently in the treatment and control groups. There is also a significant epistatic interaction between C12 and C11 for unsigned shape asymmetry.

Since QTLs appeared to be present for both shape and (possibly) unsigned asymmetry of shape, we calculated the lengths of their additive and dominance vectors in units of Procrustes distance in the manner previously described. For the QTL on chromosome 12, additive effects for shape, but not unsigned shape asymmetry, are significant (Table 4). Dominance effects are greater than additive effects (overdominance) for shape ($||\boldsymbol{d}||/||\boldsymbol{a}|| = 1.33$), and for unsigned shape asymmetry ($||\boldsymbol{d}||/||\boldsymbol{a}|| = 1.86$) where they reach significance. For the QTL on chromosome 11, the additive, but not dominance, effect for shape again is significant. For unsigned shape asymmetry, neither additive nor dominance effects showed overall significance, but the additive effect for the QTL on chromosome 11 was significantly different

Table 1
Means ± S.E. for centroid size (CENT) and the signed (CS) and unsigned (CU) asymmetry of centroid size for the three different genotypes (AA = AKR/J homozygote, AB = heterozygote, and BB = C57BL/6J homozygote) of putative QTLs on chromosomes 12 (C12) and 11 (C11) for mice in the treatment and control groups

		Treatment			Control		
		AA (42, 38)	AB (93, 91)	BB (50, 56)	AA (45, 50)	AB (106, 112)	BB (52, 40)
C12	CENT	14.534 ± 0.0458	14.516 ± 0.0263	14.468 ± 0.0397	14.616 ± 0.0499	14.629 ± 0.0251	14.606 ± 0.0378
	CS	−0.062 ± 0.0209*	−0.055 ± 0.0132**	−0.055 ± 0.0216*	−0.051 ± 0.0131*	−0.052 ± 0.0184**	−0.056 ± 0.0171**
	CU	0.476 ± 0.0162	0.454 ± 0.0110	0.471 ± 0.0173	0.450 ± 0.0161	0.462 ± 0.0110	0.446 ± 0.0148
C11	CENT	14.543 ± 0.0409	14.510 ± 0.0301	14.479 ± 0.0344	14.627 ± 0.0397	14.610 ± 0.0278	14.640 ± 0.0370
	CS	−0.056 ± 0.0208*	−0.053 ± 0.0142**	−0.063 ± 0.0193**	−0.059 ± 0.0193**	−0.036 ± 0.0119**	−0.091 ± 0.0195**
	CU	0.446 ± 0.0189	0.465 ± 0.0112	0.474 ± 0.0150	0.461 ± 0.0170	0.451 ± 0.0101	0.461 ± 0.0162

The sample size is indicated for each genotype (C12, C11).
* $P < 0.05$;
** $P < 0.01$ in tests of directional asymmetry.

Table 2
Analysis of variance for centroid size and the signed and unsigned asymmetries of centroid size

Source	d.f.	Mean squares		
		Centroid size	Signed asymmetry	Unsigned asymmetry
C12	2	0.0024	0.01562	0.01096
C11	2	0.0498	0.05875	0.01021
Environment (E)	1	0.9432*	0.00182	0.00499
Litters	72	0.1386**	0.01774	0.01512
C12 × E	2	0.0027	0.00060	0.00038
C11 × E	2	0.0503	0.02118	0.00946
C12 × C11	4	0.0523	0.02352	0.01919
C12 × C11 × E	4	0.0446	0.00366	0.00182
Error	297	0.0618	0.01748	0.01156

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

($P = 0.02$) between the two environments. We therefore calculated values for both the treatment and control where it may be seen that the stronger (and significant) additive effect is in the control group. This suggests that TCDD is depressing the additive effects of this QTL on the asymmetry of shape. Dominance effects are higher in the treatment than in the control group, however, but do not reach significance.

Fig. 2 depicts the additive and dominance effects for the QTL on chromosome 12 affecting mandible shape and unsigned shape asymmetry. For mandible shape, additive effects appear most prominent for landmark point 10 (see Fig. 1) on the condylar process which is being pulled in an anterior direction, for point 8 on the angular process which is being shifted in a posterior and ventral direction, and for point 6 which is shifting in a

Table 3
F-values from Wilks Lambda statistics generated in the multivariate analysis of variance of mandible shape and the signed and unsigned shape asymmetries

Source	d.f.	*F*-value		
		Shape	Signed shape asymmetry	Unsigned shape asymmetry
C12	32	2.26**	0.95	1.36
C11	32	3.01**	0.95	0.76
Environment (E)	16	3.46**	1.50	1.39
Litters	1152	1.38**	1.08*	1.07
C12 × E	32	1.09	0.67	1.24
C11 × E	32	0.89	0.88	1.59*
C12 × C11	64	1.11	0.87	1.41*
C12 × C11 × E	64	1.14	1.07	0.82

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

Table 4
Additive ($||a||$) and dominance ($||d||$) magnitudes for mandible shape and the unsigned shape asymmetries

		$\|\|a\|\|$	$\|\|d\|\|$
C12	Shape	0.00513**	0.00681
	Unsigned shape	0.00350	0.00650*
C11	Shape	0.00619**	0.00442
	Unsigned shape (treatment)	0.00309	0.00633
	(control)	0.00447*	0.00401

The additive effect for C11 on unsigned asymmetry of shape was significantly different ($P = 0.02$) in the two environments, and $||a||$ values are shown for the treatment and control environments.
* $P < 0.05$;
** $P < 0.01$.

primarily anterior direction. The dominance effects for this QTL are seen primarily on the three processes as well as for landmark points 2 and 3 which are being shifted in a primarily dorsal direction. For unsigned asymmetry of shape, additive effects appear most prominent for landmark point 8 on the angular process which is being shifted in an anterior and ventral direction and for point 7 which is being pulled in a posterior and ventral direction. Dominance effects are apparent at most landmarks on the mandible.

For the QTL located on chromosome 11 that affects mandible shape (Fig. 3), additive effects are highly prominent for all three processes, where it can be seen that the coronoid and condylar process are shifting posteriorly and the angular process is being shifted anteriorly. Dominance effects for this QTL exhibit a similar pattern except that the coronoid process is shifting in a primarily anterior fashion. Fig. 3 also shows the additive and dominance effects in the treatment and control groups for the QTL on chromosome 11 affecting unsigned asymmetry of shape. For the treatment group, the two largest additive effects of this QTL are seen at landmark points 6 and 10 (condylar process) which are being shifted in a primarily posterior direction whereas in the control group, landmark point 10 is shifting in an anterior direction and there also is a large ventral shift in the angular process. Dominance effects for the QTL on chromosome 11 are quite apparent in the treatment group, with large anterior shifts at points 1 (coronoid process) and 4, a posterior shift in the condylar process (point 10), and a dorsal shift at point 2. In the control group, the magnitude of shifts produced by dominance effects is less, but anterior shifts are clearly visible at points 2 and 8, and there also is a posterior shift at point 7.

4. Discussion

4.1. Chromosome 12 effects

This study had several aims, one of which was to test whether mandible size and shape differences between offspring of mice treated/not treated with TCDD, previously discovered by Allen and Leamy (2001),

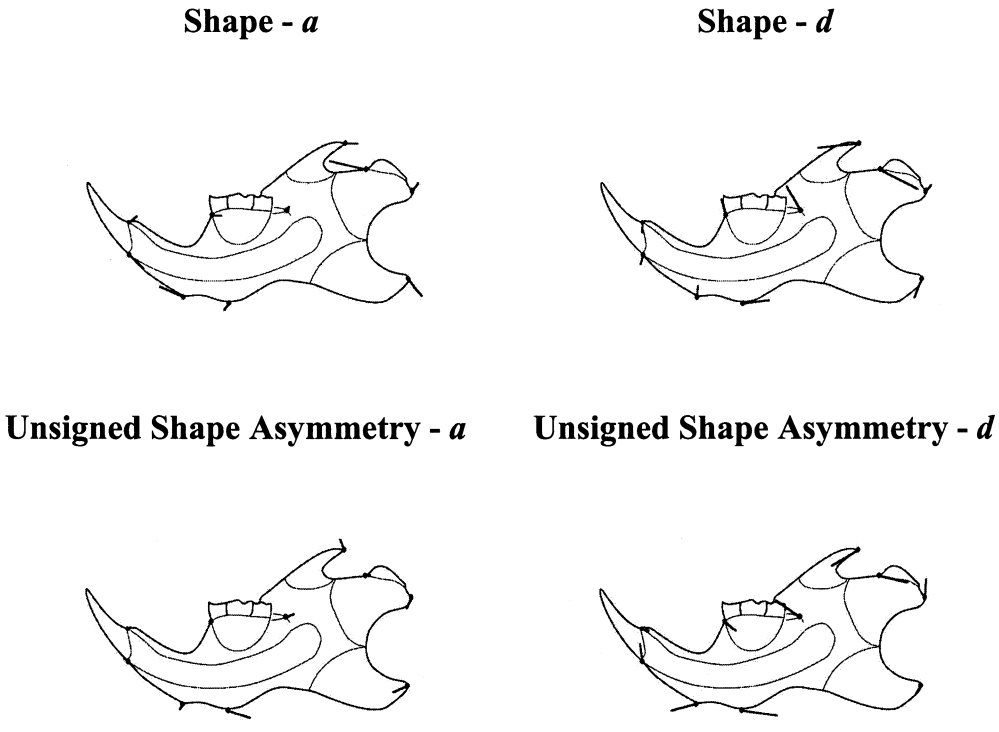

Fig. 2. Effects of the QTL on chromosome 12 on mandible shape and unsigned asymmetry of shape. The lines represent the additive (***a***) and dominance (***d***) effects at each of the ten landmark points. To make the effects easily viewable, they have been multiplied by a factor of 500.

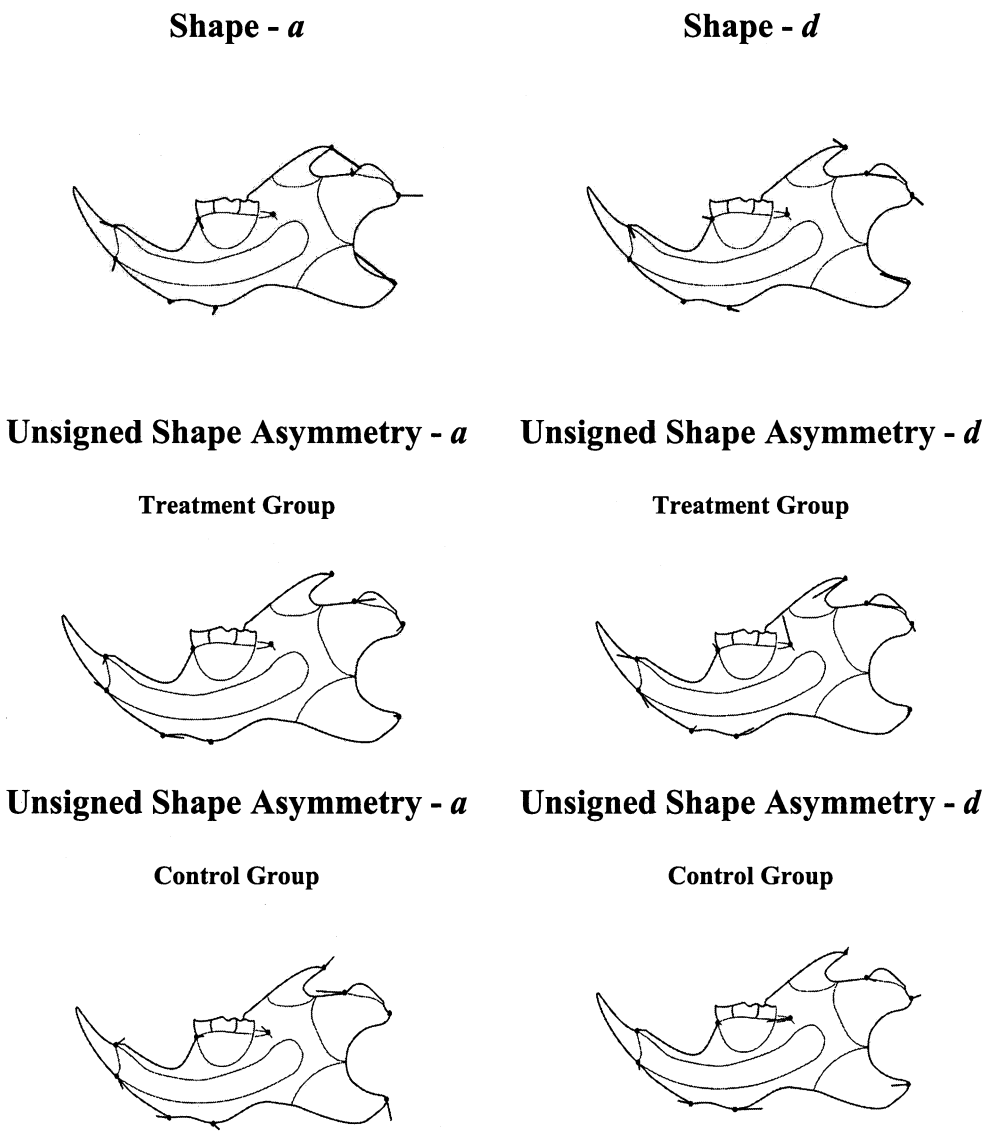

Fig. 3. Additive (***a***) and dominance (***d***) effects (all × 500) of the QTL on chromosome 11 on mandible shape, and for both treatment and control groups for the unsigned asymmetry of shape.

were associated with different genotypes at the *Ahr* locus on chromosome 12. Since TCDD is well known to exert its effects via the aryl hydrocarbon receptor (AHR) governed by the *Ahr* locus (Whitlock, 1990), it seemed reasonable to genotype the F_2 mice to test for an effect at this locus. We used a microsatellite marker extremely closely linked to the *Ahr* locus to genotype the mice, and in fact found evidence for a QTL at or near this site that significantly affects mandible shape, although not mandible size.

The interaction of genotypes at this chromosome 12 marker with the environment was not statistically significant for any of the mandible characters, however, suggesting that this QTL is not acting differently in the treated versus control mice as might be expected if it is the *Ahr* locus itself. Although the possibility that the putative QTL discovered here is in fact the *Ahr* locus cannot be ruled out, it seems more likely that it is some other locus on this chromosome that is acting on mandible shape. Potential candidates for this gene may be either of two QTLs found by Klingenberg et al. (2001) at 27 and 45 cM from the centromere on chromosome 12 that significantly affected mandible shape (but not centroid size) in their mouse population. A full chromosomal scan making use of a number of additional molecular markers, however, would be necessary to better locate the position of our unknown QTL.

The QTL on chromosome 12 exerted its largest (additive) effects on the three processes in the posterior, or ascending ramus, portion of the mandible in a pattern similar to that exhibited by a QTL on chromosome 12 (at 27 cM) discovered by Klingenberg et al. (2001) in their population of mice. On the other hand, the

dominance effects of our QTL on the coronoid, condylar, and angular processes were much more impressive than those for either of the two shape QTLs found on chromosome 12 by Klingenberg et al. (2001). Perhaps more importantly, dominance effects for our QTL also tended to push the two landmark points on either side of the tooth row in a dorsal direction, which was the major shape change caused by TCDD in these mice (Allen and Leamy, 2001; Fig. 2). TCDD is known to affect tooth development in rats (Alaluusua et al., 1993), although we do not know whether the dominance effects of this QTL are reflecting this.

In summary with respect to the QTL on chromosome 12, it appears that the change in mandible size and shape in mice exposed/not exposed to TCDD in utero reported by Allen and Leamy (2001) is most likely not due to effects of alleles at the *Ahr* locus. On the other hand, we have identified a QTL on this chromosome that obviously affects shape of the mandible. This QTL also appears to affect the unsigned asymmetry of mandible shape through an epistatic interaction with a QTL on chromosome 11 (Table 3; also see below). Whatever the true nature of this QTL, its dominance effects tend to be higher than its additive effects for both mandible shape and unsigned shape asymmetry (Table 4). This was also the case for the QTL on chromosome 12 discovered by Klingenberg et al. (2001) that affected mandible shape, although the magnitude of its dominance effects was considerably less.

4.2. Chromosome 11 effects

Beyond testing for the effects of the *Ahr* locus on chromosome 12, we endeavored to find a QTL on chromosome 11 that would affect the mandible characters to discover whether it would do so differently in the two environments and/or whether it would exhibit any epistatic effects. We were perhaps fortunate to find another QTL affecting mandible shape, but we did choose chromosome 11 because Klingenberg et al. (2001) previously found two QTLs on this chromosome that affected mandible centroid size and shape and it therefore seemed to be a logical choice. Whether our unknown QTL showing effects on mandible shape at a marker located 65 cM from the centromere is the same as either of the QTLs (23 and 87 cM) found by Klingenberg et al. (2001) is not known, but again, it is possible. Certainly the $||\boldsymbol{a}||$ value of 0.00619 found for our QTL is between the 0.00403 and 0.00824 values found by Klingenberg et al. (2001) for their two QTLs on chromosome 11. Perhaps more importantly, the pattern of additive shape changes shown by our QTL is reminiscent of that diagrammed for one of the two QTLs (QTL-SH11.2) found by Klingenberg et al. (2001; Fig. 2).

Our most important finding was that the QTL on chromosome 11 exhibited different effects on unsigned shape asymmetries in the two environments (significant C11 × E interaction; Table 3). It will be recalled that the magnitude of the additive shape vector was significantly higher in the control group than in the treatment group, suggesting that TCDD acted somehow to decrease the additive effect of this QTL on unsigned shape asymmetry. On the other hand, the opposite effect was seen for the dominance shape vector where dominance effects were much more obvious in the treatment compared to the control group. This dominance vector did not show significant differences between the two groups, but this may well be due to a reduction of statistical power for tests involving dominance effects (Cheverud, 1996; Klingenberg et al., 2001). In any event, one consequence of the smaller $||\boldsymbol{a}||$ and the larger $||\boldsymbol{d}||$ value in the treatment group is that the QTL exhibited overdominance ($||\boldsymbol{d}||/||\boldsymbol{a}|| = 2.05$) in its effects on unsigned asymmetry of shape in this group (as compared to the control group where $||\boldsymbol{d}||/||\boldsymbol{a}|| = 0.90$).

The presence of a QTL on chromosome 11 whose effect on mandible asymmetry was modified by TCDD suggests that there may be QTLs for FA that are more readily identifiable in stressful environments. Previous investigations searching for QTLs for mandible characters in mice reared in non-stressful laboratory environments have not been particularly successful in identifying such QTLs (for example, Klingenberg et al., 2001). The evidence that the effects of the chromosome 11 QTL on mandible shape FA differed in the TCDD versus control environments also is interesting in view of the fact that mandible shape FA levels themselves did not differ between the mice maternally exposed/not exposed to TCDD. These effects of TCDD on the expression of the chromosome 11 QTL on mandible shape asymmetry are quite subtle, however, and are nowhere near the gross kinds of changes that typically are seen when teratogenic exposures of TCDD are given to mice (Couture et al., 1990) or to birds (Henshel et al., 1993, 1997; Henshel, 1998).

4.3. Epistatic effects

It was interesting to discover significant epistasis between the QTLs on chromosomes 12 and 11 that affected the unsigned asymmetry of mandible shape. Clearly, the effects of these two loci on unsigned shape asymmetry are linked in some way, although there is no evidence that this has anything to do with TCDD since epistatic effects did not differ between the treatment and the control groups. Leamy et al. (2002) found abundant evidence for epistasis affecting unsigned asymmetry of centroid size in mice. Using an approach outlined by Cheverud (2000), they tested all 171 possible pairs of 19 autosomes in the F_2 mice of an original cross of the

Large × Small mouse strains, and found 30 separate instances of epistasis when only two were expected by chance alone. This finding has led Leamy (2002) to suggest that that there may be a large epistatic basis for FA. If so, perhaps we have found further evidence for this, at least for unsigned asymmetry of shape, from the results of our study as well.

4.4. Overall size effects

Might the effects of the QTLs on mandible size, shape, and asymmetry as outlined above be related to an overall change in body size in these mice? To answer this question, this same analysis was repeated with body weight included as a covariate, and although the mean squares in the analyses of variance changed some, the same patterns of significance shown in Tables 3 and 4 held. This suggests that the direct and epistatic effects of these two QTLs on mandible shape and shape asymmetry are not simply correlated responses to overall size changes. Allen and Leamy (2001) previously found that TCDD effects on mandible size and shape in these same mice were independent of body size, so this result is not particularly surprising.

4.5. Conclusions

Our results clearly suggest that we need to use additional markers on chromosome 12 to more conclusively determine whether the QTL we have discovered is the *Ahr* locus. If it is not, as hypothesized from the results of this study, this would suggest that some effects of TCDD, as on mandible shape asymmetry, may be mediated through epigenetic or other pathways rather than only via the AHR receptor. A whole genome scan in these mice would seem particularly worthwhile to see if more QTLs could be uncovered whose direct or epistatic effects on the mandible size, shape, or asymmetry characters differ in the two environments. If such QTLs could be found, this would suggest that TCDD may play a greater role in influencing the effects of various genes throughout the genome than has previously been recognized. Eventual identification of these QTLs would tell us not only the kinds of genes that TCDD is capable of affecting, but also would expand our knowledge of the range of effects that might be expected from this toxicant. Finally, if the level of FA in skeletal structures such as mandibles proves to be altered by the action of TCDD on a variety of QTLs, this would suggest that this powerful toxicant does indeed affect the developmental stability of organisms in the laboratory, and perhaps those in the environment as well.

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