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Structural Equation Modeling and Nested ANOVA: Effects of Lead Exposure on Maternal and Fetal Growth in Rats

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This study provided an assessment of the effects of lead on early growth in rats based on structural equation modeling and nested analysis of variance (ANOVA). Structural equation modeling showed that lead in drinking water (250, 500, or 1000 ppm) had a direct negative effect on body weight and tail length (i.e., growth) in female rats during the first week of exposure. During the following 2 weeks of exposure, high correlation between growth measurements taken over time resulted in reduced early postnatal growth. By the fourth week of exposure, reduced growth was not evident. Mating began after 8 weeks of exposure, and exposure continued during gestation. Decreased fetal body weight was detected when the effects of litter size, intrauterine position, and sex were controlled in a nested ANOVA. Lead exposure did not appear to affect fetal skeletal development, possibly because lead did not alter maternal serum calcium and phosphorus levels. The effect of lead on individual fetal body weight suggests that additional studies are needed to examine the effect of maternal lead exposure on fetal development and early postnatal growth.

INTRODUCTION

Epidemiological studies have shown that lead exposure during pregnancy is associated with reduced growth during infancy (Shukla *et al.*, 1989) and early childhood (Mooty *et al.*, 1975). Analysis of data derived from the National Health and Nutrition Examination Survey (NHANES II) has demonstrated a negative relationship of blood lead with height, chest circumference, and body weight in children (Schwartz *et al.*, 1986). There is supporting evidence that lead will cause growth retardation in rats (Wapnir *et al.*, 1977, 1979; Kimmel *et al.*, 1980; Hammond *et al.*, 1989). However, it has been suggested that lead does not directly act on growth processes, but may indirectly reduce growth by depressing food intake (Hammond *et al.*, 1989). Structural equation modeling may be useful in assessing the mechanisms of lead-related effects on early growth (Buncher *et al.*, 1991).

Although lead in drinking water is associated with retardation of postnatal growth in rats (Kimmel *et al.*, 1980; Hammond *et al.*, 1989), there is evidence that oral lead exposure during gestation will not affect litter weight in rats (Kimmel *et al.*, 1980; Hubermont *et al.*, 1976). However, analysis of variance (ANOVA) which controls for intrauterine position (Barr and Brent, 1970) and sex (Bruce and Norman, 1975) has not been reported in the assessment of lead-related effects on fetal body weight.

The purpose of this study was to provide an assessment of the effects of lead on early growth in rats. The study objectives were to determine (1) if there is

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a period of exposure in which lead directly inhibits growth in rat weanlings, and (2) if maternal lead exposure will reduce individual fetal body weight, skeletal ossification, and crown-to-rump length.

MATERIALS AND METHODS

Lead Exposure and Animals

Prior to mating. Fifty female and 15 male Sprague–Dawley caesarean delivered rats (Charles River, Portage, MI) were obtained at age 21 days and were singly housed in stainless steel wire cages. The housing light cycle was 12 hr light and 12 hr dark under controlled temperature (22–25°C). After 4 days of housing to allow acclimatization, the rats were assigned randomly to treatment groups.

Lead acetate trihydrate was dissolved in deionized water to give drinking water concentrations of 250, 500, or 1000 ppm lead. Acetic acid (0.00125%) was added to aid in dissolution; the control group drinking water was 0.00125% acetic acid in deionized water. These solutions were provided *ad libitum* beginning at Day 25 of age, designated as Day 0 of treatment. Fifteen male rats and 15 female rats were assigned to the control group. Fifteen female rats were assigned to the 500 ppm group. The 250 and 1000 ppm lead treatment groups had 10 female rats each. The male rats were used for breeding purposes only. All measurements (see below) were taken from female rats and their offspring.

A pelleted semipurified defined diet (Purina Mills Test Diet No. 5755, Richmond, IN) was provided *ad libitum,* containing 0.60% calcium and 0.40% phosphorus. The diet was chosen because it is considered nutritionally adequate (i.e., replete) for both growing and pregnant rats according to National Research Council (NRC) guidelines (NRC, 1978), but did not provide the supplementary amounts of nutrients normally found in laboratory rat chow. The same production batch of diet was used throughout the study.

Mating and gestation. After 8 weeks, the female rats were taken when proestrous and mated overnight with males (not treated with lead). Vaginal smears were read on the following morning (at 9 AM). The day of appearance of sperm in the vaginal smears was designated as Day 0 of gestation. The ages and body weights of the sperm-positive dams were recorded after mating. Lead treatment, housing, and feeding conditions remained the same during gestation except that the pregnant females were housed in baskets (with wood chip bedding), beginning on Day 18 of gestation.

Measurements

Prior to mating. Blood was taken by tail section at Week 3 (Day 21) and Week 8 (Day 56) from all rats for blood lead analysis (Brodie and Routh, 1984). The blood samples were collected in 0.1-ml capillary pipets (Environmental Health Associates, Rochester, NY) from incisions made at the base of the tail, after cleaning the tail with a 0.5% disodium ethylenediamine tetraacetate solution and rinsing with deionized water.

Body weight gain (%), tail length gain (%), water intake (ml/week), and food intake (g/week) were measured. An electronic pan balance (Mettler Instruments, Hightstown, NJ) was used to measure food intake and body weights. An adapted 25-cm ruler (1-mm scale) was used to measure rat tail length.

During gestation. Cumulative food intake over a 21-day gestation period was obtained. At Gestation Day 21, blood was taken by tail section from the dams for

lead analysis. The dams were then killed by nitrogen asphyxiation, and about 1.0 ml of blood was immediately collected in heparin-free vacuum tubes by cardiac puncture and centrifuged for serum phosphorus and serum calcium.

Gestation Day 21 fetuses were taken by caesarean section from each dam. Record was made of the litter size, gestation rate (i.e., percentage of mated females which delivered at least one viable fetus), fetal sex, and fetal intrauterine position. After weighing, the fetuses were Alizarin Red S (Sigma Ltd., St. Louis, MO) stained for measurement of mean litter crown-to-rump length and litter skeletal ossification (Aliverti *et al.*, 1979).

Fetal Crown-to-Rump Length and Ossification

Crown-to-rump length was measured in each fetus with a 0.50-mm-graded ruler by means of a caliper measurement from the center of the top of the skull (crown) to the first caudal (tail) vertebra. The mean crown-to-rump length per litter (i.e., litter crown-to-rump length) was calculated.

Ossification was evaluated by counting Alizarin Red-positive ossification sites in the sternum and proximal phalanges of the left forepaw. As reported elsewhere (Ariyuki *et al.*, 1982), there are normally six ossification sites in the sternum and three ossification sites in the proximal phalanges of the forepaw of the nonexposed (control) Day 21 fetal rat. In this study, fetal ossification per litter (i.e., litter ossification) was measured by counting the number of fetuses with six ossification sites in the sternum and three ossification sites in the left forepaw proximal phalanges, then dividing by litter size.

Statistical Analysis

Body weight, tail length, lead intake, food intake, and blood lead. Body weight gain, tail length gain, lead intake, food intake, and blood lead were evaluated by ANOVA with the Duncan–Bonner multiple-range *t* test (SAS, 1985) specific to week (food intake), on Days 2 and 7 and every 7 days until Day 56 (body weight and tail length) or on Days 21 and 56 (blood lead). Gain was expressed as the difference in body weight or tail length from Day 0 relative to body weight or tail length at Day 0. Weekly food intake was mean cumulative intake during each week of treatment.

Structural equation modeling. The hypothesis that lead affects growth (i.e., tail length or body weight gain) through a lead-related effect on food intake was tested with structural equation modeling (Buncher *et al.*, 1991; SAS, 1982). In this study, initial structural equation modeling showed that there was no difference in model outcome based on lead exposure from water consumption (i.e., mg lead/week) compared to the model outcome based on lead exposure according to exposure level (i.e., treatment group).

Fetal body weight. Two ANOVA procedures were used to test the effect of lead on mean fetal body weight. The first was an ANOVA model where litter size was used as a covariate, and the second was a nested ANOVA model where individual fetal body weight variance (fetal intrauterine position and sex) as well as litter size were included. Study groups were analyzed on an ordinal scale (*0*, control; *1*, 250 ppm; *2*, 500 ppm; and *3*, 1000 ppm). The effect of lead on fetal body weight was then assessed with the Duncan–Bonner multiple-range *t* test (SAS, 1985).

For data input into the nested ANOVA, the fetuses were numbered from the ovarian end (upper portion) according to the relative position in which they ap-

peared in the left or right uterine horn. The fetal intrauterine position factor (P) was calculated for each fetus as

$$P = i/(N + 1),$$

where i is the position (either left or right) of the fetus within the uterine horn, beginning at the top of the horn and N is the number of live fetuses per uterine horn.

Three levels of P corresponding to the upper ($P < 0.3$), middle ($P \geq 0.3$ and ≤ 0.7), and lower ($P > 0.7$) positions of the uterine horn, and two corresponding levels (male or female) of the sex variable were used in the nested ANOVA.

Fetal crown-to-rump length and skeletal ossification. The effects of lead on litter crown-to-rump length and litter skeletal ossification were evaluated by ANOVA with the Duncan–Bonner multiple-range t test (SAS, 1985). Effects of sex or position were not incorporated in this analysis because the measurements were taken after the fetuses were combined into corresponding litters prior to processing.

RESULTS

Unless stated otherwise, all comparisons made were to corresponding findings in the control group. The criteria for statistical significance was $P \leq 0.05$.

Effects Prior to Breeding

Lead intake and blood lead levels. Blood lead among the lead exposure groups was less at 8 weeks than that after 3 weeks of exposure (Table 1). Although blood lead decreased, lead intake increased with water consumption in each lead-exposed group. During Week 1, lead intake from drinking water was 0 (±0), 4.5 (±0.29), 20.6 (±1.16), 35.3 (±1.84), and 65.2 (±3.40) mg lead/week in the control, 50, 250, 500, and 1000 ppm groups, respectively. During Week 8, lead intake was 0 (±0), 8.0 (±0.59), 38.9 (±3.87), 67.9 (±2.58), and 115.1 (±6.83) mg lead/week in the control, 50, 250, 500, and 1000 ppm groups, respectively.

Body weight, tail length, and food intake. Lead did not initially have a significant effect on food intake (Table 2). However, by Week 8, food intake was increased in the 500 and 1000 ppm groups.

Body weight gain was reduced in the 250, 500, and 1000 ppm lead exposure groups during the first 3 weeks (Fig. 1). Tail length gain in the 1000 ppm group was

TABLE 1
BLOOD LEAD LEVELS—WEEKS 3 (DAY 21) AND 8 (DAY 56)

Lead exposure group (ppm)	Blood lead levels (μg/dl)	
	Day 21	Day 56
Control	1.6 (0.1)	1.8 (0.1)
250	50.2 (2.4)*	39.8 (3.8)*
500	71.3 (3.1)*	54.5 (3.4)*
1000	100.1 (6.0)*	72.1 (3.1)*

Note. Values are means (SE).

* Significantly ($P \leq 0.05$) different from control based on ANOVA specific to Days 21 and 56 with the Duncan–Bonner multiple-range t test (SAS, 1985).

TABLE 2
WEEKLY FOOD INTAKE (g/WEEK) PRIOR TO MATING

Lead exposure group (ppm)	Week 1	Week 2	Week 3	Week 4
Control	74.6 (2.3)	93.8 (1.7)	100.8 (2.7)	101.3 (3.7)
250	71.7 (2.0)	92.3 (3.0)	100.5 (3.9)	108.5 (3.8)
500	75.5 (2.1)	95.3 (2.0)	106.2 (2.2)	104.4 (2.5)
1000	73.3 (2.7)	97.6 (2.6)	106.6 (3.4)	104.9 (3.7)

Lead exposure group (ppm)	Week 5	Week 6	Week 7	Week 8
Control	103.6 (3.0)	102.7 (3.9)	103.3 (3.8)	100.1 (3.0)
250	107.6 (3.5)	110.6 (5.3)	113.3 (5.1)	108.7 (3.8)
500	110.2 (2.5)	112.6 (5.7)	113.1 (2.5)	114.0 (2.9)*
1000	113.5 (7.7)	117.5 (5.1)*	120.8 (4.1)*	116.2 (5.7)*

Note. Values are means (SE).

* Significantly ($P \leq 0.05$) different from control based on ANOVA specific to week with the Duncan–Bonner multiple-range t test (SAS, 1985).

decreased during the same exposure period (Fig. 2). Although consistent and statistically significant, the growth decrease was not remarkably severe during the first 3 weeks, and no statistically significant decrease in growth was observed after the third week (data not shown).

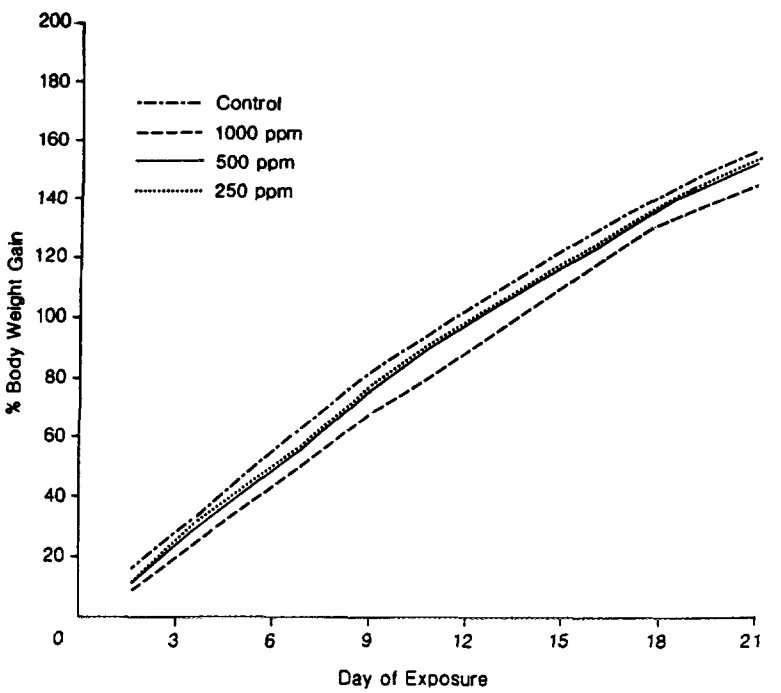

FIG. 1. Body weight in control group rats and in rats exposed to lead in drinking water. Each line connects measured mean body weight gain in each group on Days 2, 7, 14, and 21. Based on ANOVA with the Duncan–Bonner multiple-range t test, body weight gain was reduced ($P \leq 0.05$) in the 250, 500, and 1000 ppm treatment groups on Days 2, 7, 14, and 21.

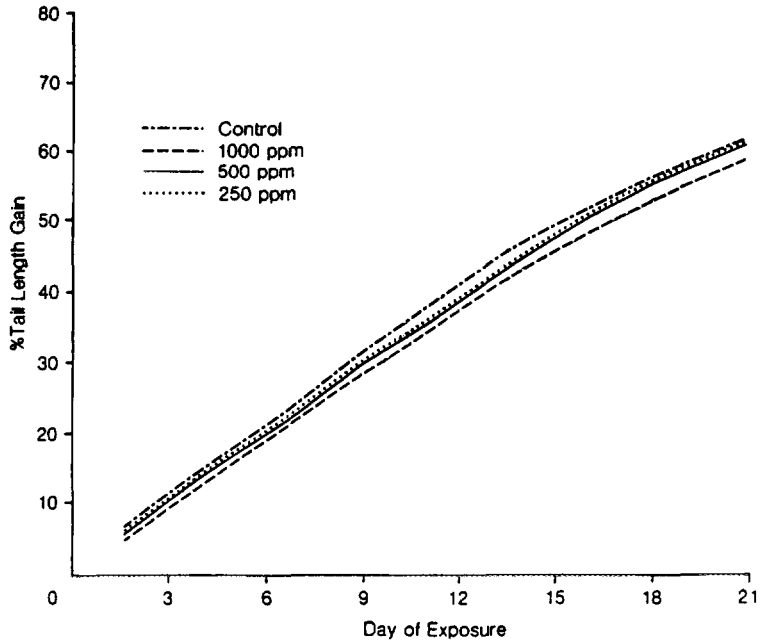

FIG. 2. Tail length in control group rats and in rats exposed to lead in drinking water. Each line connects measured mean tail length gain in each group on Days 2, 7, 14, and 21. Based on ANOVA with the Duncan–Bonner multiple-range t test, tail length gain was reduced ($P \leq 0.05$) in the 1000 ppm treatment group on Days 2, 7, 14, and 21.

Structural equation modeling. Figures 3 and 4 show structural equation models for Weeks 1 to 3, when decreased body weight (250, 500, and 1000 ppm groups) and decreased tail length (1000 ppm group) were observed. The structural equation model regression coefficient (C) was based on regression across the control, 250, 500, and 1000 ppm groups for body weight and across the control and 1000 ppm groups for tail length. A statistically significant lead-related decrease in body weight was observed ($C = -2.76$, $P \leq 0.05$) during the first week only (Fig. 3). Similarly, a statistically significant decrease ($C = -3.25$, $P \leq 0.05$) in tail length gain was evident in the 1000 ppm group during the first week only (Fig. 4). In later weeks, food intake, prior tail length, and prior body weight (not lead exposure) were the most important determinants of growth.

Effects after Mating

Food intake during gestation and number of fetuses per liter. Cumulative food intake during gestation was increased in the 500 and 1000 ppm groups along with litter size (Table 3).

Crown-to-rump length and fetal body weight. Fetal body weights for each study group are shown in Table 4 and are expressed as mean fetal body weight per litter. With litter size controlled in an ANOVA model, lead had no detectable effect on mean fetal body weight. However, with litter size, fetal intrauterine position, and sex controlled in a nested ANOVA model, a statistically significant decrease in fetal body weight was detected in the 250, 500,and 1000 ppm groups. The nested ANOVA accounted for 91% of total variance in fetal body weight. The ANOVA (with litter size only) accounted for only 22% of between-litter variance.

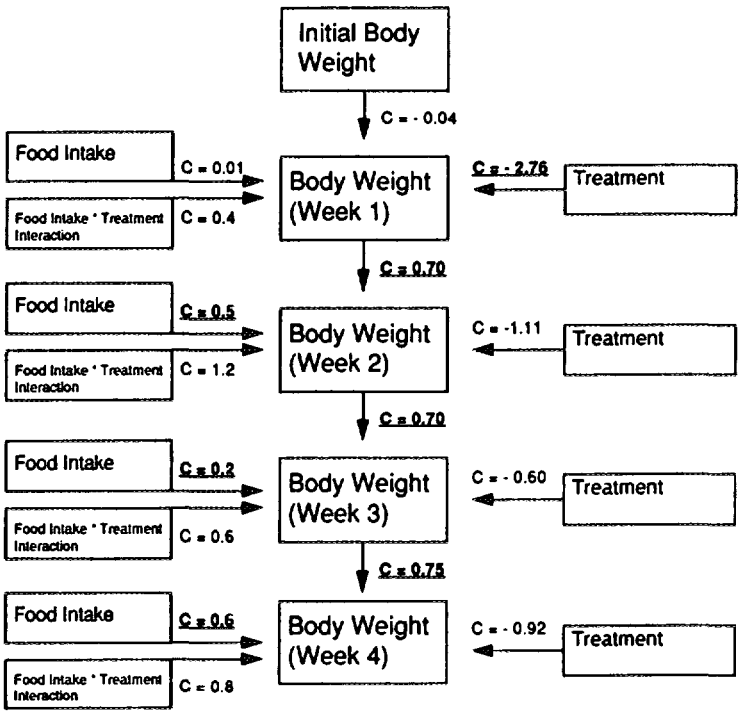

FIG. 3. A structural equation model for body weight. Statistically significant ($P \leq 0.05$) regression coefficients (C) are underlined in bold print. The model is based on body weight gain and food intake in the control group and in groups where statistically significant ($P \leq 0.05$) decreased body weight gain was observed (250, 500, and 1000 ppm groups).

Fetal crown-to-rump length and skeletal ossification. There were no significant lead-related effects on fetal crown-to-rump length or ossification in fetal sternebrae or proximal phalanges (Table 5).

Maternal blood lead, serum calcium, and phosphorus at Day 21 of gestation. Lead did not have a significant effect on maternal serum calcium or phosphorus (Table 6). Blood lead concentrations in the dams at Day 21 of gestation were elevated relative to the blood lead concentrations in the same treatment groups at Week 8 of treatment, prior to mating.

DISCUSSION

Effects on Growth Prior to Mating

Lead and growth: Structural equation modeling. During the first 3 weeks of lead exposure, decreased body weight gain was observed in the *ad libitum*-fed 250, 500, and 1000 ppm groups, and reduced tail length gain was observed in the *ad libitum*-fed 1000 ppm group. However, these effects were not severe and were reversible after Week 3. Although blood levels in the lead-exposed groups were at least about 40 μg/dl, lead-related effects on growth appeared transient.

Additional statistical analyses were used to investigate the transient lead-related effects on growth. Relatively new statistical methods, such as structural equation modeling, can be used to assess the relative importance of numerous factors which may influence a single outcome (Buncher *et al.*, 1991). In this study,

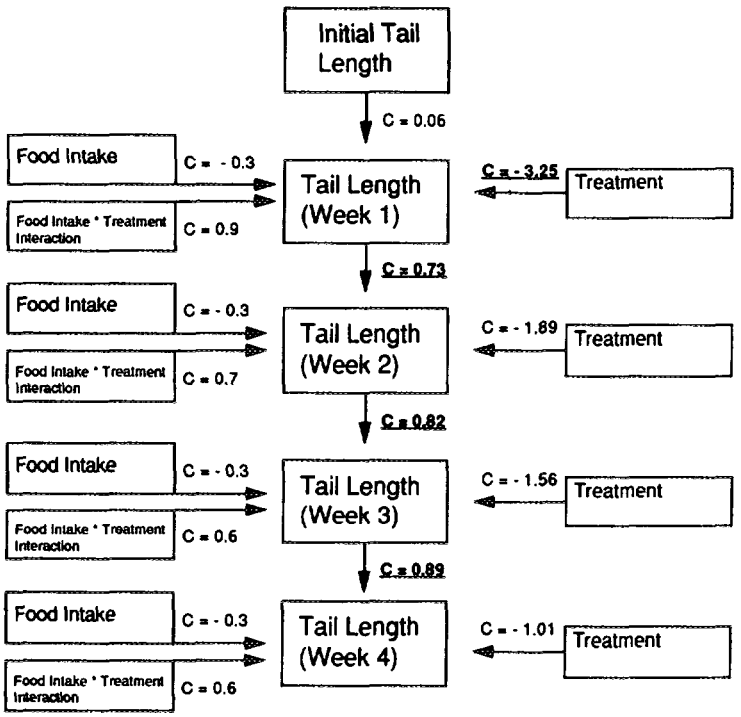

FIG. 4. A structural equation model for tail length. Statistically significant ($P \leq 0.05$) regression coefficients (C) are underlined in bold print. The model is based on tail length gain and food intake from in the control group and in the group where statistically significant ($P \leq 0.05$) decreased tail length gain was observed (1000 ppm group).

structural equation modeling provided a simultaneous analysis of (1) the association of lead treatment with growth (body weight or tail length) and (2) the association between lead intake and food intake.

Structural equation modeling showed that a direct (i.e., independent of food intake) negative effect of lead on growth was evident only during the first week of lead exposure. The most important determinants of subsequent growth in the *ad libitum*-fed rats after 1 week of exposure were prior growth and prior food intake. The lead-related reduction of growth was maintained because of the high correlation between growth indices and food intake over time, not because of a continuous negative effect of lead acting directly on growth.

TABLE 3
GESTATION RATE, FOOD INTAKE, AND LITTER SIZE

Lead exposure group (ppm)	Gestation rate (%)	Cumulative food intake (g)	Litter size
Control	87	352.9 (10.2)	12.6 (0.19)
250	80	351.3 (22.9)	13.5 (0.11)
500	80	406.9 (12.9)*	14.7 (0.09)*
1000	80	427.5 (13.4)*	15.3 (0.13)*

Note. Values are means (SE).

* Significantly ($P \leq 0.05$) different from control based on ANOVA with the Duncan–Bonner multiple-range t test (SAS, 1985).

TABLE 4
FETAL BODY WEIGHT

Lead exposure group (ppm)	Number of litters	Mean fetal body weight (g)
Control	13	5.19 (0.11)
250	8	4.67 (0.18)
500	12	4.91 (0.05)
1000	8	4.89 (0.11)

Note. Values are means (SE); mean fetal body weight per litter in each treatment group.

The greater sensitivity of young animals than of older animals to the toxic effects of lead is widely recognized (Rader *et al.*, 1981; Kostial *et al.*, 1978), and lead-related effects on growth found in children with relatively low blood lead concentrations include reduced growth rates during infancy (Shukla *et al.*, 1989) and reduced statural height (Schwartz *et al.*, 1986). In addition, blood lead levels in the rats in this study decreased with age, indicating that susceptibility to the effects of lead on growth may have been related, in part, to age-dependent changes in blood lead levels.

Lead and food intake. Increased food intake in lead-exposed rats has not been reported in other studies. In this study, the "underweight" lead-exposed rats gradually increased food intake relative to their controls, and regained growth. The lead-exposed *ad libitum*-fed rats followed a pattern analogous to the feeding pattern seen when feed is returned to food-restricted rats. Underweight rats increased food intake when *ad libitum* feeding conditions were restored; increased food intake was evident until body weights returned to normal (Widdowson and McCance, 1963). In lead-exposed rats, similar control processes for body weight maintenance may be triggered when opportunities for weight recovery are provided.

Food mineral composition may alter the effects of lead on growth maintenance processes. A study with lead-exposed weanlings fed a less replete diet with less calcium (0.47%) and phosphorus (0.25%) than the replete diet (0.60% calcium and 0.40% phosphorus) used in this study has shown that reduced food intake was second only to previous weight or tail length as a determinant of reduced body weight and tail length (Hammond *et al.*, 1989). In contrast to the results reported

TABLE 5
FETAL CROWN-TO-RUMP LENGTH AND SKELETAL OSSIFICATION

Lead exposure group (ppm)	Crown-to-rump length (mm)	Ossification (%)[a]	
		Sternebrae	Proximal phalanges (forepaw)
Control	33.2 (0.1)	100 (5.4)	100 (8.3)
250	33.1 (0.2)	97 (5.3)	97 (6.4)
500	33.3 (0.5)	93 (8.4)	90 (9.8)
1000	33.2 (0.7)	90 (11.4)	91 (12.6)

Note. Values are means (SE).

[a] Fetal ossification per litter was measured by counting the number of fetuses per litter with six ossification sites in the sternum or three ossification sites in the forepaw proximal phalanges and then dividing by litter size.

TABLE 6
MATERNAL BLOOD LEAD, SERUM CALCIUM, AND PHOSPHORUS LEVELS AT END OF GESTATION

Lead exposure group (ppm)	Blood lead (μg/dl)	Serum calcium (mg/dl)	Serum phosphorus (mg/dl)
Control	2.4 (0.2)	10.7 (0.3)	6.7 (0.2)
250	61.7 (8.8)*	9.7 (0.3)	6.9 (0.1)
500	79.6 (3.1)*	9.6 (0.4)	6.3 (0.3)
1000	94.2 (3.8)*	9.5 (0.3)	6.2 (0.2)

Note. Values are means (SE).
* Significantly ($P \leq 0.05$) different from control based on ANOVA with the Duncan–Bonner multiple-range *t* test (SAS, 1985).

here, food intake remained depressed during lead exposure and growth did not recover, even though dose group blood lead levels were comparable to those observed in this study.

Effects on Growth after Mating

Food intake and litter size. Dose-dependent increased food intake in the *ad libitum*-fed rats continued after mating in the lead-exposed groups, suggesting a continued compensatory mechanism to maintain nutrient levels during gestation. Increased food intake in the *ad libitum*-fed 500 and 1000 ppm groups may have been related to their larger litter sizes relative to the control group litter size. However, the larger litter sizes in the *ad libitum*-fed 500 and 1000 ppm groups may only reflect group variability, rather than a true lead effect. (Note that litter size was controlled in the following analysis of the effects of lead on fetal body weight.)

Fetal body weight. Before the effect of lead on fetal body weight was examined, factors which are known to affect fetal body weight were tested individually for their influences on fetal body weight. Litter size had a negative effect on fetal body weight in this study and is widely known to have an important impact on between-litter (i.e., between dam) variance in fetal body weight (Jensh *et al.*, 1970; Gad and Weil, 1986). Important within-litter (i.e., among fetuses) factors can also influence fetal body weight. Consistent with other studies (Bruce and Norman, 1975; Barr and Brent, 1970), the male fetuses in this study tended to be heavier than female fetuses, and the heaviest fetuses were generally located in the middle of the uterine horn with the lightest fetuses were found nearest to the ovaries.

When only litter size was used in an ANOVA model to assess the effect of lead on fetal body weight (per litter), no effect on fetal body weight was observed. This finding is consistent with those of other studies with lead-treated pregnant rats (Kimmel *et al.*, 1980). However, when the effect of lead on fetal body weight was tested with a nested ANOVA model which controlled for litter size, intrauterine position, and sex, a statistically significant decrease in fetal body weight was detected in the lead-exposed groups. The nested ANOVA accounted for 91% of total variance in fetal body weight. The ANOVA (with litter size only) accounted for only 22% between-litter variance.

The greatest decrease in fetal body weight was observed in the lowest (250 ppm) dose group. However, analysis of the actual dose response was limited by the

number of litters available in this initial study. True dose–response estimation of the observed overall lead-related decrease in fetal body weight could be refined with additional litters.

Maternal serum calcium and phosphorus, fetal crown-to-rump length, and fetal ossification. Fetal skeletal ossification may have remained normal because maternal serum calcium and phosphorus levels were not affected by lead. Resistance of pregnant rats and their fetuses to changes in maternal calcium and phosphorus has been demonstrated elsewhere; pregnant rats placed on severely calcium- and phosphorus-restricted diets during gestation produced fetuses with normal skeletal calcium and phosphorus levels while maintaining normal maternal serum calcium and phosphorus levels (Verhaeghe *et al.*, 1988).

CONCLUSIONS

This study provided an assessment of the effect of lead exposure on early growth in rats based on structural equation modeling and nested ANOVA. Structural equation modeling showed that reduced growth in lead-exposed rats fed a replete diet was not caused by a continuous adverse lead effect on growth. Lead exposure did not appear to affect fetal skeletal development, possibly because lead did not alter maternal serum calcium and phosphorus levels. However, decreased fetal body weight was detected when the effects of litter size, intrauterine position, and sex were controlled in a nested ANOVA. The effect of lead on individual fetal body weight suggests that additional studies are needed to examine the effect of maternal lead exposure on fetal development and early postnatal growth.

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