Original Article

Reproductive and Neurobehavioral Effects of Clothianidin Administered to Mice in the Diet

Toyohito Tanaka

Department of Environmental Health and Toxicology, Tokyo Metropolitan Institute of Public Health, Tokyo, Japan

Clothianidin was given in the diet to provide levels of 0% (control), 0.003%, 0.006%, and 0.012% from 5 weeks of age of the F_0 generation to 11 weeks of age of the F_1 generation in mice. Selected reproductive and neurobehavioral parameters were measured. In exploratory behavior in the F_0 generation, average time of movement, number of rearing, and rearing time of adult males increased significantly in a dose-related manner. There was no adverse effect of clothianidin on litter size, litter weight, or sex ratio at birth. The average body weight of male and female offspring was increased significantly in a dose-related manner during the early lactation period. With respect to behavioral developmental parameters, swimming head angle at postnatal day (PND) 7 of male offspring was accelerated significantly in a dose-related manner. Negative geotaxis at PND 7 of female offspring was accelerated significantly in a dose-related manner. For movement activity of exploratory behavior in the F_1 generation, number of rearing of female offspring increased significantly in a dose-related manner. Movement time of adult males increased significantly in a dose-related manner. The dose levels of clothianidin in the present study produced several adverse effects in neurobehavioral parameters in mice. Nevertheless, it would appear that the levels of the actual dietary intake of clothianidin are unlikely to produce adverse effects in humans. *Birth Defects Res (Part B)* 0:1–9, 2012. © 2012 Wiley Periodicals, Inc.

Key words: ***behavioral development; clothianidin; exploratory behavior; mice; movement activity; neonicotinoid insecticide; reproductive toxicity; spontaneous behavior***

INTRODUCTION

Clothianidin, (*E*)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine, is a neonicotinoid insecticide available as water-soluble powders, wettable powders, granules, and dusts. It is a broad-spectrum systemic insecticide and effective on insect pest species such as hemipterans, thysanopterans, orthopterans, coleopterans, lepidopterans, dipterans, hymenopterans, and isopterans (Uneme et al., 2006). Clothianidin is registered for agricultural use on various fruits, vegetables, forage, and grain crops. Its permitted residue levels are shown in Table 1. Clothianidin is used for expelling termites in housing. The acceptable daily intake (ADI) for humans has been set at 0.097 g/kg body weight (bw) in Japan (Japan Food Safety Commission, 2008).

In toxicological studies of clothianidin (Sumitomo Chemical Takeda Agro Company, Ltd., 2003), its oral and dermal LD_{50} in rats exceeded 5000 mg/kg and 2000 mg/kg, respectively. The oral LD_{50} in male and female mice was 389 mg/kg and 465 mg/kg, respectively. In 90-day oral toxicity studies, the nonobserved adverse effect level (NOAEL) was 500 ppm in male and female rats (approximately 27.9 mg/kg/day and 34.0 mg/kg/day, respectively), and the corresponding value in male and female dogs was 650 ppm (approximately 19.3 mg/kg/day and 21.2 mg/kg/day, respectively). In a chronic and carcinogenic study in rats, it showed no carcinogenic effects, and the NOAELs in male and female rats were 500 ppm (approximately 27.4 mg/kg/day) and 150 ppm (approximately 9.7 mg/kg/day), respectively. It produced no carcinogenic effects in mice, and the NOAEL in male and female mice was 350 ppm (approximately 47.2 mg/kg/day and 65.1 mg/kg/day, respectively).

With respect to reproductive and developmental toxicity studies (Sumitomo Chemical Takeda Agro Company, Ltd., 2003), clothianidin showed no teratogenicity after administration on days 6 to 19 of gestation in rats (10–125 mg/kg bw/day) and the NOAELs were 10 mg/kg/day in maternal effects and 125 mg/kg/day in fetal effects. It also showed no teratogenicity in administration on days 6 to 28 of gestation in rabbits (10–100 mg/kg bw/day) and the NOAEL was 25 mg/kg/day in maternal and fetal effects. In a two-generation toxicity study in rats, clothianidin produced no adverse effects in reproduction, and its NOAELs in male and female rats were

Correspondence to: Toyohito Tanaka, Department of Environmental Health and Toxicology, Tokyo Metropolitan Institute of Public Health, 3-24-1, Hyakunincho, Shinjuku-ku, Tokyo 169-0073, Japan. E-mail: t-tanaka@poohlover.net

Received 16 August 2011; Accepted 17 October 2011

Published online in Wiley Online Library (wileyonlinelibrary.com/journal/bdrb) DOI: 10.1002/bdrb.20349

Table 1
Summary of the Permitted Residue Levels of Clothianidin in Japan (Food Hygienic Society of Japan, 2008)

		ppm
Cereal grains	Unpolished rice	0.7
	Other grain crops	0.02
Legumes	Soybeans	0.1
	Peanuts	0.02
	Other legumes	0.3
Fruits	Drupes	0.3–5
	Citrus fruits	1–2
	Pome fruits	1
	Tropical fruits	0.02–1
	Berry fruits	0.02–0.7
Vegetables	Cruciferous vegetables	0.02–5
	Potatoes	0.02–0.25
	Cucurbitaceous vegetables	0.02–2
	Composite vegetables	0.02–20
	Fungi	0.02
	Dropwort vegetables	0.02–5
	Solanaceous vegetables	1–3
	Liliaceous vegetables	0.02–15
	Other vegetables	0.02–2
Oil seeds	Oil seeds	0.01–0.02
Nuts	Nuts	0.02
	Cocoa beans	0.02
	Coffee beans	0.04
Tea leaves	Tea leaves	50
Meats	Cattle	0.02
	Goat	0.02
	Hog	0.02
	Horse	0.02
	Sheep	0.02
	Poultry	0.02

150 ppm in parental effects (approximately 9.8 mg/kg/day and 11.5 mg/kg/day, respectively), and 150 ppm in offspring effects (approximately 10.7 mg/kg/day and 12.2 mg/kg/day, respectively).

In neurotoxicity studies in rats (EPA, 2003), clothianidin (>100 mg/kg) decreased arousal, motor, and locomotor activities in the acute neurotoxicity study. In a subchronic neurotoxicity study, it had no adverse effects in neurotoxicity, and its NOAELs in male and female rats were 60.0 mg/kg/day and 71.0 mg/kg/day, respectively. It produced no adverse effects in the developmental neurotoxicity study, and its NOAELs were 42.9 mg/kg/day in maternal effects and 12.9 mg/kg/day in offspring effects. Nevertheless, few neurobehavioral toxicity studies of clothianidin were carried out excluding the above report. However, clothianidin produced several adverse effects on reproductive and neurobehavioral parameters in maternal exposure study (Tanaka, 2011). Therefore, the present study was designed to evaluate the reproductive and neurobehavioral effects of clothianidin exposure in mice throughout different generations.

MATERIALS AND METHODS

Materials

Clothianidin was obtained from Wako Pure Chemical Industries, Tokyo, Japan (Lot no. KWK2749). The chemical characterization was "clothianidin standard for residual pesticide analysis," and the purity was >99.0%. The chemical stability of clothianidin is >12 months at 25°C and >6 months at 40°C (Uneme et al., 2006).

Animals and Maintenance

Male and female mice (Crlj: CD1, 4 weeks of age) were purchased from Charles River Japan, Kanagawa, Japan. They were housed individually in polycarbonate solid-floored cages with wood flakes, and kept in a temperature-controlled room maintained at 25 ± 1°C at a relative humidity of 50 ± 5% on a 12-hr light/dark cycle. They were given control or experimental diets and water ad libitum.

Experimental Design

Clothianidin was administered in the diet to 60 mice (10/sex/group) at dietary levels of 0.003%, 0.006%, and 0.012%, from 5 weeks of age of the F_0 generation to 11 weeks of age of the F_1 generation. The control group (20 mice: 10/sex) was given the basal diets (CE-2; Nihon Clea, Tokyo, Japan) for the corresponding time period. Experimental diets were prepared bimonthly (twice) in our laboratory. After mixing clothianidin with the powdered diet, pellets were formed and fed to mice. The stability of clothianidin was not tested in the pellets during the experimental period. The homogeneity of the test compound was ensured by the preparation procedures of the experimental diets in our laboratory when previously measured by HPLC. The concentration and homogeneity of the test compound in the diet was not tested during the experimental period. Individual food intake of mice was measured during the following five periods: preconception (from 5 weeks of age to mating); mating (5 days); gestation (14 days); lactation (from birth to weaning); and F_1 generation (4–11 weeks of age). Individual food intake (g/kg bw/day) was calculated as (the difference in feed weight between the previous and present week excepting the fallen lumps weight in cage)/(average bw between the previous and present week)/days.

Reproductive Procedure

Mice from the F_0 generation were 5 weeks of age at the start of the study. They were weighed individually on experimental days 0, 2, 4, 7, 14, 21, 28, and 30 during the preconception period. At 9 weeks of age, each female was paired with one male from the same treatment group for 5 days. The females were examined for mating by appearance of the vaginal plug twice a day (morning and evening). The males were separated from females after 5 days. The females were then allowed to carry their litters to term, deliver, and rear all of their offspring. The dams were weighed individually during the gestation (every day on weekdays) and lactation periods (once a week). The dams were examined for delivery at three times a day (morning, early afternoon, and evening).

At birth, live and dead offspring were counted and litter size, litter weight, and sex ratio (male/female) of live offspring were measured on postnatal day (PND) 0. Litter was not culled and all offspring were examined for test procedure. The offspring were individually weighed on

PNDs 0, 4, 7, 14, and 21 during the lactation period. The survival indices were calculated as follows: (live offspring at each period)/(live and dead offspring at birth) × 100 (%). The offspring were weaned when they were 4 weeks of age. One male and one female were randomly selected to continue treatment from each litter. Mice were weighed every week from 4 weeks to 11 weeks of age after weaning.

Neurobehavioral Procedure

The functional and behavioral developmental parameters were measured and scored for all individual offspring during the lactation period in the F_1 generation (Tanaka et al., 1992), and analyzed on score frequencies (Tanaka, 1995). All offspring were examined for behavioral development during the lactation period. The measured parameters were as detailed below.

(1) *Surface righting* on PNDs 4 and 7 (Fox, 1965; Pantaleoni et al., 1988). The offspring were placed on their backs on a smooth surface, and the time required to right themselves to a position where all four limbs touched the surface was recorded. The following scoring system was employed for successful righting: 2 = righting within 1 sec; 1 = righting in >1 sec but within 2 sec; 0 = righting in >2 sec.
(2) *Negative geotaxis* on PNDs 4 and 7 (Fox, 1965; Altman and Sudarshan, 1975; Pantaleoni et al., 1988). The offspring were placed in a head-down position on a plane inclined at 30° and the time required to reorient to a head-up position was recorded. The plane was made of plywood covered with sandpaper (fine grade). The following scoring system was employed: 0 = no response within 60 sec; 1 = response within 60 sec; 2 = response within 30 sec.
(3) *Cliff avoidance* on PND 7 (Fox, 1965; Altman and Sudarshan, 1975; Pantaleoni et al., 1988). The offspring were placed on a platform elevated 10 cm above a tabletop. Forelimbs and snout were positioned such that the edge of the platform passed just behind an imaginary line drawn between the eye orbits. The following scoring system was employed: 0 = no response within 20 sec; 1 = avoided backwards within 20 sec; 2 = avoiding with turn.
(4) *Swimming behavior* on PNDs 7 and 14 (Fox, 1965; Pantaleoni et al., 1988). The offspring were placed in a tank with water temperature maintained at 23 ± 1°C. Swimming behavior was rated for direction (3 = straight; 2 = circling; 1 = floating) and head angle (4 = ears out of water; 3 = ears half out of water; 2 = nose and top of head out of water; 1 = unable to hold head up). Limb movement was rated as 1 = all four limbs used and 2 = only hindlimbs used.
(5) *Olfactory orientation* on PND 14 (Altman and Sudarshan, 1975; Barlow et al., 1978; Meyer and Hansen, 1980). The offspring were placed into an arm connecting two compartments. One compartment was covered with "home" wood flakes (i.e., from their cages) and the other was covered with fresh wood flakes. The time required to enter the compartment with the home wood flakes was recorded. Olfactory orientation was scored for time taken (3 = entered the home wood flakes compartment within 30 sec; 2 = entered within 60 sec; 1 = entered within 90 sec; 0 = no response within 90 sec) and for route (1 = entered the home wood flakes compartment via the fresh wood flakes compartment; 2 = entered the home wood flakes compartment directly).

The exploratory behavior of mice was measured in the animal movement analysis system SCANET CV-40 (Melquest, Toyama, Japan) on distance (DT) mode at 8 weeks of age in the F_0 generation, and at 3 and 8 weeks of age in the F_1 generation. The system consisted of a rectangular cage (300 × 202 × 205 mm) made from acrylate resins with two crossing sensor frames of 72 units of detectors of near-infrared photosensors for measuring spontaneous motor activity (Mikami et al., 2002). The behavioral parameters were recorded for 10 min on all mice at 8 weeks of age in the F_0 and F_1 generations, and on one male and one female mouse selected randomly from each litter at 3 weeks of age in the F_1 generation. The measurement parameters on DT mode were as follows: total distance (cm), number of horizontal activities, movement time (s), number of rearing, rearing time (s), average time of movement (s), average speed (cm/s), average time of rearing (s), and number of defecations.

Each mouse performed one trial per day for 3 days in a Biel-type multiple-T water maze adapted for mice at 7 weeks of age in the F_1 generation (Biel, 1940; Kitatani et al., 1988). The water temperature was maintained at 20 ± 1°C. The time taken and number of errors were measured from the start to finish for a maximum of 120 sec. If the time taken was >120 sec, it was recorded as 120 sec (Kitatani et al., 1988).

The spontaneous behavior of mice was measured in the animal movement analyzing system SCANET CV-40 (Melquest) on DT mode from 9 weeks to 10 weeks of age in the F_1 generation. The behavioral parameters were measured on all mice for 120 min at an interval of 10 min after 10 min latency. The measurement parameters were the same as those for exploratory behavior except for the number of defecations.

Statistical Analyses

Food intake, litter size, litter weight, and bw were assessed with Bonferroni's multiple comparison test after the analysis of variance or the Kruskal–Wallis test. Sex ratio and survival were assessed with the chi square test or Fisher's exact test of frequency analysis. Behavioral developmental data were assessed with the chi square test (multi) or the Mantel–Haenszel test (multi) of frequency analysis. Exploratory behavior data were assessed with the Steel–Dwass test of nonparametric methods (Martin and Bateson, 1990). In spontaneous behavior data, the longitudinal pattern was assessed with the profile analysis in the parallelism hypothesis test, and variables at each point of time were assessed with the Steel–Dwass test within each treatment group. Multiple-T water maze performance data were assessed with the Sign–Wilcoxon test for trials and assessed with the Shirley–Williams test as compared to controls. Dose–response effects were assessed with the Jonckheere test for ordered alternatives or the cumulative chi square test (multi) for frequency data.

Guidelines

The design of the present study was based on the guidelines issued by ICH (2005) and OECD (2007) adapted for mice. In our institute, mice are usually employed in reproductive and neurobehavioral toxicity studies to conserve resources (chemicals, diets, space) and background data for mice are sufficient for the evaluation of neurobehavioral effects (Tanaka, 2004). We conducted the present study in accordance with the guidelines set by the Science Council of Japan (2006). Animal experiments conformed with the Japanese "Act on Welfare and Management of Animals" (Act No. 105 of 1 October 1973, revised on 22 June 2005), Notice No. 88 of 28 April 2006 of the Ministry of the Environment of Japan "Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain," and Notification of 1 June 2006 of the Ministry of Health, Labour and Welfare of Japan "Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Research Institutions under the jurisdiction of the Ministry of Health, Labour and Welfare."

RESULTS

Intake of Food and Chemicals

There was no difference ($p < 0.05$) related to the treatment of clothianidin on the average intake of food during any period. The average intake of chemicals was increased consistently in a dose-related manner (Table 2).

F_0 Generation

The average bw of male and female mice showed no difference ($p < 0.05$) with respect to treatment during the preconception period. In movement activity of exploratory behavior at 8 weeks of age, average time of movement, number of rearing, and rearing time of males increased significantly in a dose-related manner ($p = 0.041$, 0.034, and 0.047, respectively) in the trend test (Fig. 1). Other variables showed no adverse effects of clothianidin in either sex. One dam each of the middle- and high-dose groups aborted during the gestation period (Table 3). The average bw of dams showed no difference ($p < 0.05$) related to the treatment during the gestation and lactation periods.

F_1 Generation

At birth, no difference ($p < 0.05$) related to the treatment was observed in litter size, litter weight, or sex ratio (Table 3). The average bw of male offspring was increased significantly in the low-dose group at PND 7 and in the middle-dose group at PNDs 4 and 7 (Table 4), and it showed a significantly dose-related increase at PNDs 4 and 7 ($p < 0.01$ and 0.05, respectively) during the lactation period in the trend test. The average bw of female offspring was increased significantly in the low- and middle-dose group at PNDs 4 and 7, and it showed a significantly dose-related increase at PNDs 4 and 7 ($p < 0.001$ and 0.05, respectively) during the lactation period in the trend test. The survival indices showed no difference ($p < 0.05$) related to the treatment during the lactation period in either sex (Table 5).

With respect to the behavioral developmental parameters, the development of swimming head angle was delayed significantly in the middle-dose group in male offspring at PND 7 (Fig. 2), and this effect was significantly dose related ($p < 0.01$). The time taken for olfactory

Table 2
Average Daily Food and Chemical Intake of Mice Administered Clothianidin in a Two-Generation Toxicity Study

	Dose level (%)			
	0	0.003	0.006	0.012
Food intake (g/kg/day)				
F_0 generation				
Preconception				
Male	150.6 ± 8.28	145.6 ± 14.24	149.0 ± 10.96	154.0 ± 15.15
Female	165.9 ± 15.49	165.6 ± 19.14	166.1 ± 12.98	177.2 ± 22.95
Mating	132.6 ± 17.75	127.9 ± 13.83	140.0 ± 14.08	144.3 ± 15.6
Gestation	150.5 ± 14.79	149.7 ± 21.72	147.0 ± 10.66	152.8 ± 21.33
Lactation	525.3 ± 61.47	507.1 ± 36.43	510.1 ± 44.25	502.2 ± 54.84
F_1 generation				
Male	160.5 ± 11.01	149.3 ± 7.41	150.1 ± 7.39	159.0 ± 8.50
Female	182.2 ± 19.45	181.8 ± 14.68	185.2 ± 19.74	183.2 ± 27.46
Chemical intake (mg/kg/day)				
F_0 generation				
Preconception				
Male	-	4.37 ± 0.427	8.94 ± 0.657	18.48 ± 1.818
Female	-	4.97 ± 0.574	9.97 ± 0.779	21.26 ± 2.754
Mating	-	3.84 ± 0.415	8.40 ± 0.845	17.31 ± 1.872
Gestation	-	4.49 ± 0.652	8.82 ± 0.640	18.34 ± 2.559
Lactation	-	15.21 ± 1.093	30.60 ± 2.655	60.26 ± 6.581
F_1 generation				
Male	-	4.50 ± 0.222	9.00 ± 0.444	19.08 ± 1.020
Female	-	5.45 ± 0.440	11.11 ± 1.184	21.99 ± 3.295

Each value represents daily intake during each period (mean ± SD).

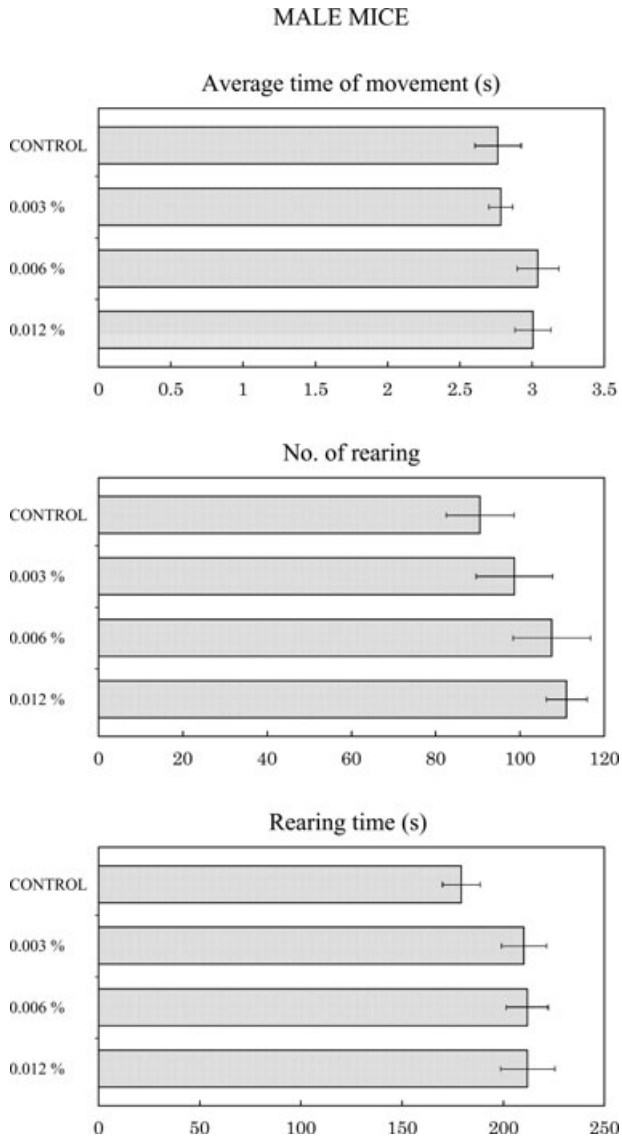

Fig. 1. Movement activity of exploratory behavior at 8 weeks of age of F_0-generation male mice in a two-generation toxicity study of clothianidin administered to mice. Each value represents the mean ± SE. Each variable showed significantly dose-related manners in a trend test ($p < 0.05$).

orientation at PND 14 was accelerated significantly in the middle-dose groups in male offspring (Fig. 2). In female offspring, surface righting at PND 4 was accelerated significantly in the low-dose group, and the development of swimming head angle was accelerated significantly in the low- and middle-dose groups at PND 7 (Fig. 2). Negative geotaxis was accelerated significantly in the low-dose group (Fig. 2), and this effect was significantly dose-related ($p < 0.01$). The route of olfactory orientation at PND 14 was delayed significantly in the middle-dose groups (Fig. 2). Other variables showed no difference ($p < 0.05$) related to the treatment in either sex.

For the movement activity of exploratory behavior at 3 weeks of age, the number of rearing of female offspring increased significantly in a dose-related manner ($p = 0.042$) in the trend test (Fig. 3). Other variables showed no adverse effects of clothianidin in either sex. After weaning, there was no difference ($p < 0.05$) related to the treatment with clothianidin on multiple-T water maze performance at 7 weeks of age in either sex. In the movement activity of exploratory behavior at 8 weeks of age, the movement time (s) of males increased significantly in a dose-related manner ($p = 0.045$) in the trend test (Fig. 4), and the average time of rearing (s) of males decreased in the middle-dose group (Fig. 4). Other variables showed no adverse effects of clothianidin in either sex.

For movement activity of spontaneous behavior from 9 weeks to 10 weeks of age in the F_1 generation, all variables measured in each sex were parallel in a longitudinal pattern, except for the movement time of females. In males, the number of horizontal activities showed significantly inactive at 40 min in the low-dose group (Fig. 5). In females, the average speed (cm/s) showed significantly inactive at 70 min, and the rearing time (s) showed significantly inactive at 50 min in the middle-dose group (Fig. 5). The average bw of male and female mice showed no difference ($p < 0.05$) related to the treatment after weaning.

DISCUSSION

In the present study, clothianidin produced some significant effects on reproductive and neurobehavioral parameters. Although there is no significant effect in the high-dose group, the average bw of male and female offspring showed a slightly dose-related acceleration during the early lactation period in the trend test. Survival indices during the lactation period showed no significant difference between control and treatment groups, and the average litter size during the lactation period for each group was similar. There was no difference related to treatment with clothianidin on average food intake of dams during the gestation and lactation periods. The average bw of dams showed no difference related to treatment during the gestation and lactation periods. These changes in offspring weight may have been caused by clothianidin treatment because offspring consumed similar nourishment in each group. Tanaka (2011) reported that clothianidin treatment accelerated offspring bw during the lactation period in maternal exposure study. It seems, therefore, that clothianidin treatment slightly accelerated offspring physical growth during the lactation period, and those effects showed reproducible.

For the behavioral developmental parameters, swimming head angle in male offspring showed a significant tendency to be accelerated in treatment groups at PND 7, though there is no significant effect in the high-dose group. It therefore seems that the dose levels of clothianidin produced influences on swimming head angle, indicative of development of the sense of equilibrium, in male offspring during the early lactation period. Although there is no significant effect in the high-dose group, negative geotaxis in female offspring showed a significant tendency to be accelerated in treatment groups at PND 7. It therefore seems that the dose levels of clothianidin produced influences on negative geotaxis, indicative of development of the coordinated movement, sense

Table 3
Summary of Data of Litters at Birth in a Two-Generation Toxicity Study of Clothianidin Administered to Mice

	Dose level (%)			
	0	0.003	0.006	0.012
No. of females examined	10	10	10	10
No. of pregnant females	10	10	10	10
No. of litters	10	10	9	9
No. of live offspring	122	122	119	119
No. of dead offspring	4	1	0	1
Average litter size	12.2 ± 2.20	12.2 ± 1.48	13.2 ± 1.39	13.2 ± 1.79
Average litter weight (g)	19.12 ± 3.127	19.59 ± 2.625	21.31 ± 2.183	20.48 ± 2.479
Sex ratio (male/female)	0.94 (59/63)	1.07 (63/59)	1.09 (62/57)	0.83 (54/65)
Average sex ratio (male %)	49.3 ± 12.62	51.6 ± 16.83	51.6 ± 10.36	46.0 ± 19.21

Each value represents the mean ± SD.

Table 4
Summary of Average Body Weight (g) of Offspring during the Lactation Period in F_1 Generation Mice in a Two-Generation Toxicity Study of Clothianidin Administered to Mice

	Dose levels (%)			
	0	0.003	0.006	0.012
Male offspring				
PND 0	1.59 ± 0.117	1.62 ± 0.134	1.62 ± 0.219	1.57 ± 0.149
PND 4	2.82 ± 0.424	2.95 ± 0.292	3.04 ± 0.457***	2.95 ± 0.339
PND 7	4.17 ± 0.561	4.41 ± 0.391*	4.40 ± 0.675**	4.37 ± 0.545
PND 14	6.01 ± 0.706	6.18 ± 0.594	5.97 ± 0.820	6.14 ± 0.855
PND 21	9.80 ± 2.008	10.44 ± 1.610	9.87 ± 2.328	9.68 ± 2.591
Female offspring				
PND 0	1.54 ± 0.114	1.59 ± 0.132	1.60 ± 0.179	1.53 ± 0.153
PND 4	2.66 ± 0.335	2.95± 0.345***	3.02 ± 0.395***	2.87 ± 0.416*
PND 7	3.98 ± 0.539	4.39 ± 0.509***	4.43 ± 0.589***	4.22 ± 0.603
PND 14	5.84 ± 0.743	6.04 ± 0.788	5.90 ± 0.678	5.97 ± 0.812
PND 21	9.34 ± 1.987	10.31 ± 1.922	9.89 ± 2.051	9.18 ± 2.311

Each value represents the mean ± SD. Significantly different from controls: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table 5
Summary of Number of Offspring and Survival Index (%) during the Lactation Period in F_1 Generation Mice in a Two-Generation Toxicity Study of Clothianidin Administered to Mice

	Dose levels (%)			
	0	0.003	0.006	0.012
Male offspring				
PND 0	59 (98.3)	63 (100.0)	62 (100.0)	54 (100.0)
PND 4	59 (98.3)	63 (100.0)	62 (100.0)	52 (96.3)
PND 7	58 (96.6)	63 (100.0)	62 (100.0)	52 (96.3)
PND 14	58 (96.6)	63 (100.0)	62 (100.0)	52 (96.3)
PND 21	57 (95.0)	63 (100.0)	62 (100.0)	52 (96.3)
Female offspring				
PND 0	63 (95.5)	59 (98.3)	57 (100.0)	65 (98.5)
PND 4	63 (95.5)	59 (98.3)	56 (98.2)	64 (97.0)
PND 7	63 (95.5)	59 (98.3)	56 (98.2)	64 (97.0)
PND 14	62 (93.9)	59 (98.3)	56 (98.2)	63 (95.5)
PND 21	61 (92.4)	58 (96.7)	56 (98.2)	63 (95.5)

Each value represents number of offspring: survival index (%) in parentheses.

of equilibrium, and muscular power, in female offspring during the early lactation period. Although there is no significant effect in the high-dose group, time taken for olfactory orientation at PND 14 in male offspring, and surface righting at PND 4 and swimming head angle at PND 7 in female offspring showed significant accelerations in the middle-dose group. These influences may have been caused by increased bws in treatment groups because the bw of male and female offspring showed a significantly dose-related acceleration during the early lactation period. Nevertheless, Tanaka (2004) reported that behavioral developments during the lactation period were not influenced by offspring weight under natural conditions. It therefore seems that the dose levels of clothianidin produced influences on these variables of behavioral development in male and female offspring during the early lactation period. Tanaka (2011) reported that clothianidin treatment accelerated surface righting of behavioral development in female offspring during the early lactation period in maternal exposure study. Although there is no significant effect in the high-dose group, swimming direction and time taken for olfactory orientation in female offspring showed significant accelerations in the middle-dose (0.006%) group in the previous study (Tanaka, 2011). It seems that the differences are not simply statistical noise

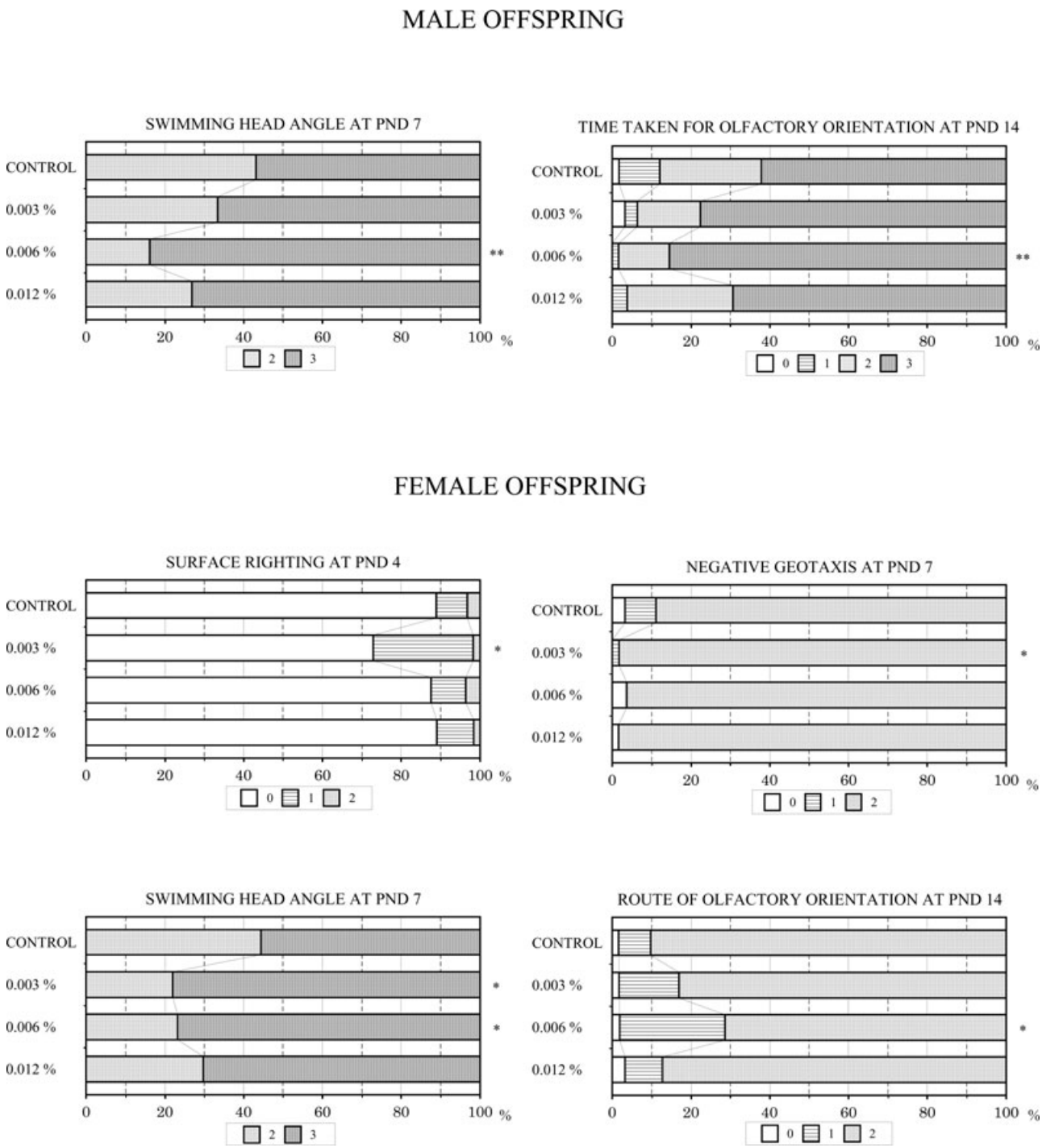

Fig. 2. Score frequencies for behavioral development in the lactation period in a two-generation toxicity study of clothianidin administered to mice. Significantly different from controls: $^{*}p < 0.05$, $^{**}p < 0.01$. Swimming head angle of male offspring and negative geotaxis of female offspring showed significantly dose-related manners in a trend test ($p < 0.01$).

because these effects on similar variables of behavioral development were found in the separate studies. These results suggest that clothianidin treatment slightly accelerated behavioral development of offspring during the early lactation period.

For exploratory behavior in the F_0 generation, the average time of movement, number of rearing, and rearing time showed significant tendencies to be increased in treatment groups of adult males. The number of rearing of female offspring showed a significant tendency to be increased in treatment groups for exploratory behavior in the F_1 generation. Tanaka (2011) reported that clothianidin treatment accelerated average speed of exploratory behavior in male offspring in maternal exposure study.

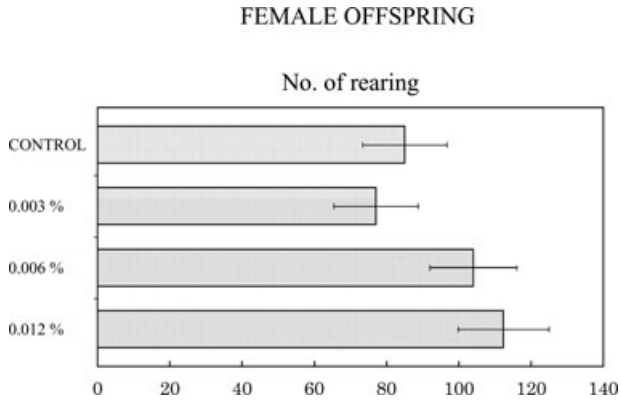

Fig. 3. Movement activity of exploratory behavior at 3 weeks of age of F_1-generation female mice in a two-generation toxicity study of clothianidin administered to mice. Each value represents the mean ± SE. Number of rearing showed significantly dose-related manners in a trend test ($p < 0.05$).

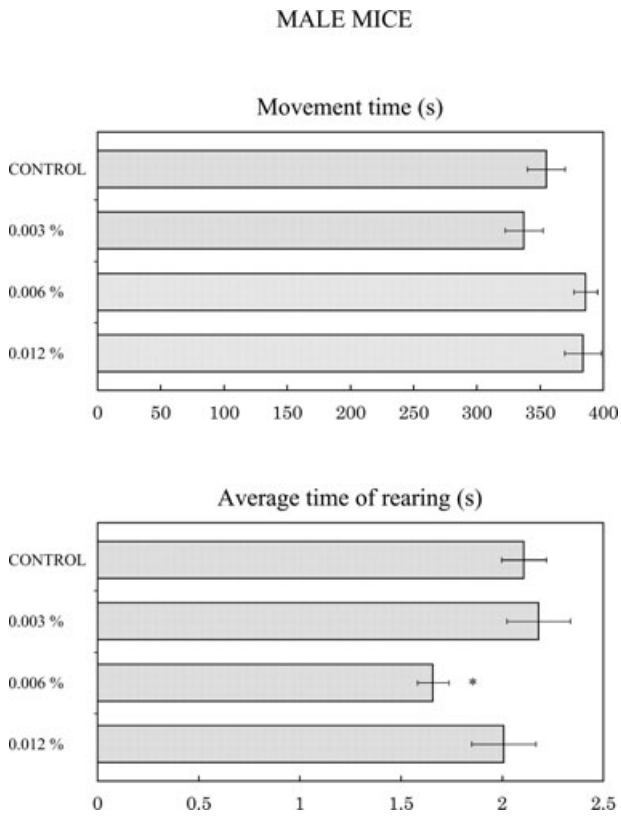

Fig. 4. Movement activity of exploratory behavior at 8 weeks of age of F_1-generation male mice in a two-generation toxicity study of clothianidin administered to mice. Each value represents the mean ± SE. Significantly different from controls: $*p < 0.05$. Movement time (s) showed significantly dose-related manners in a trend test ($p < 0.05$).

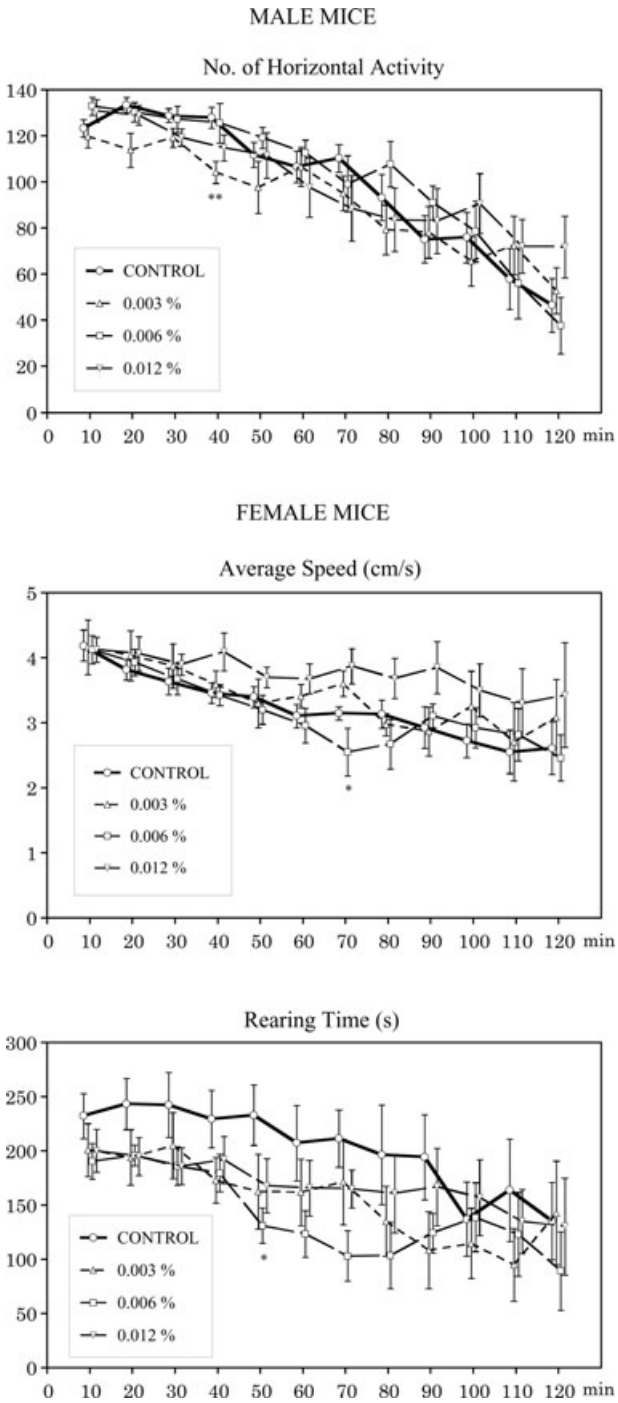

Fig. 5. Movement activity of spontaneous behavior from 9 to 10 weeks of age of F_1-generation mice in a two-generation toxicity study of clothianidin administered to mice. Each value represents the mean ± SE. Significantly different from controls: $*p < 0.05$, $**p < 0.01$.

These results suggest that clothianidin treatment accelerated exploratory behavior of offspring during the lactation period. In adult males, the movement time showed a significant tendency to be increased in treatment groups for exploratory behavior in the F_1 generation. Although there is no significant effect in the high-dose group, average time of rearing of males was decreased in the middle-dose group. In the previous study (Tanaka, 2011), same variable of females was decreased in the middle-dose (0.006%) group. The effects of clothianidin on the central nervous system may cause these changes on movement activities because clothianidin was observed to be

concentrated in the brain after oral and intravenous administration in rats (Yokota et al., 2003).

For spontaneous behavior of the F_1 generation, the number of horizontal activities in males showed significantly inactive on 40 min in the low-dose group. The average speed and rearing time in females showed significantly inactive in the middle-dose group on 70 min and 50 min, respectively. It therefore seems that the middle dose of clothianidin may produce lower activities for spontaneous behavior in adult female mice of the F_1 generation. Nevertheless, Tanaka (2011) reported that the middle dose (0.006%) of clothianidin produced more activities for spontaneous behavior in adult male mice of the F_1 generation in the previous study. These results of the present study were inconsistent with that of the previous study because clothianidin treatment was stopped at weaning in the previous study.

The dose levels of clothianidin in the present study produced several adverse effects on neurobehavioral parameters in mice. The dose of clothianidin produced significantly adverse effects on exploratory behavior in the F_0- and F_1-generation mice. It therefore seems that sensitivity for clothianidin is dependent on sex in mice because clothianidin treatment produced different effects on several variables of neurobehavioral development. Because several similar variables of neurobehavioral parameters were affected in the middle-dose (0.006%) group in the present and previous (Tanaka, 2011) studies, the dose of clothianidin may be effective to neurobehavioral development in mice. The middle-dose level (equivalent to 9–33 mg/kg bw/day) was based on the current ADI of clothianidin (0.097 mg/kg bw). Therefore, these results suggest needing to reappraise the ADI of clothianidin. Nevertheless, the estimated daily intake of clothianidin is presumed to be much lower, approximately 3.91 μg/kg bw/day in the national average, 6.65 μg/kg bw/day in infants (1–6 years of age), 3.38 μg/kg bw/day in pregnant women, and 4.05 μg/kg bw/day in senior citizens (>65 years of age) in Japan (Japan Food Safety Commission, 2008). It would therefore appear that the levels of the actual dietary intake of clothianidin are unlikely to produce adverse effects in humans.

REFERENCES

Altman J, Sudarshan K. 1975. Postnatal development of locomotion in the laboratory rat. Anim Behav 23:896–920.

Barlow SM, Knight AF, Sullivan FM. 1978. Delay in postnatal growth and development of offspring produced by maternal restraint stress during pregnancy in the rat. Teratology 18:211–218.

Biel WC. 1940. Early age differences in maze performance in the albino rat. J Genet Psychol 56:439–453.

Environmental Protection Agency (EPA). 2003. Pesticide fact sheet: clothianidin. Available at: http://www.epa.gov/opprd001/factsheets/clothianidin.pdf. United States Environmental Protection Agency. Accessed 1 July 2009.

Food Hygienic Society of Japan. 2008. Specifications and standards for foods, food additives, etc. Under the Food Sanitation Law (abstracts). J Food Hyg Soc Jpn 49:J20–J175. (in Japanese).

Fox WM. 1965. Reflex-ontogeny and behavioural development of the mouse. Anim Behav 13:234–241.

International Conference on Harmonization of Technical Requirement for Registration of Pharmaceuticals for Human Use (ICH). 2005. Detection for toxicity on reproduction for medicinal products & toxicity to male fertility. ICH Tripartite Guideline S5(R2). Available at: http://www.ich.org/LOB/media/MEDIA498.pdf. Accessed 1 July 2009.

Japan Food Safety Commission. 2008. Pesticide evaluation book: clothianidin. 3rd ed. Available at: http://www.mhlw.go.jp/shingi/2008/12/dl/s1209-9d_0001.pdf. Japan Food Safety Commission. (in Japanese). Accessed 10 January 2009.

Kitatani T, Akaike M, Takayama K, Kobayashi T. 1988. Teratological study of cefodizime sodium in mice. Intravenous administration during period of organogenesis. J Toxicol Sci 13(Suppl I):191–214. (in Japanese).

Martin P, Bateson P. 1990. Measuring behaviour: an introductory guide. Tokyo: Tokai University Press. p 102–122.

Meyer O, Hansen E. 1980. Behavioural and developmental effects of butylated hydroxytoluene dosed rats in utero and in lactation period. Toxicology 16:247–258.

Mikami Y, Toda M, Watanabe M, Nakamura M, Toyama Y, Kawakami Y. 2002. A simple and reliable behavioral analysis of locomotor function after spinal cord injury in mice. J Neurosurgery 97:142–147.

Organization for Economic Cooperation and Development (OECD). 2007. Developmental neurotoxicity study. OECD Guideline for Testing Chemicals 426. Available at: http://titania.sourceoecd.org/vl=1469022/cl=13/nw=1/rpsv/cgi-bin/fulltextew.pl?prpsv=/ij/ oecd-journals/1607310x/v1n4/s27/p1.idx. Accessed 1 July 2009.

Pantaleoni G, Fanini D, Sponta AM, Palumbo G, Goirgi R, Adams PM. 1988. Effects of maternal exposure to polychlorobiphenyls (PCBs) on F_1 generation behavior in the rat. Fundam Appl Toxicol 11:440–449.

The Science Council of Japan. 2006. Guidelines for proper conduct of animal experiments. The Science Council of Japan. Available at: http://www.scj.go.jp/ja/info/kohyo/pdf/kohyo-20-k16-2e.pdf. Accessed 10 January 2007.

Sumitomo Chemical Takeda Agro Company, Ltd. 2003. Technical information: summary of toxicity studies with clothianidin. J Pest Sci 28:167–173. (in Japanese).

Tanaka T. 1995. Reproductive and neurobehavioral effects of imazalil administered to mice. Reprod Toxicol 9:281–288.

Tanaka T. 2004. The relationships between litter size, offspring weight, and behavioral development in laboratory mice *Mus musculus*. Mammal Study 29:147–153.

Tanaka T. 2011. Effects of maternal clothianidin exposure on behavioral development in F_1-generation mice. Toxicol Ind Health. in press. (DOI: 10.1177/0748233711422726)

Tanaka T, Takahashi O, Oishi S. 1992. Reproductive and neurobehavioural effects in three-generation toxicity study of piperonyl butoxide administered to mice. Food Chem Toxicol 30:1015–1019.

Uneme H, Konobe M, Akayama A, Yokota T, Mizuta K. 2006. Discovery and development of a novel insecticide 'clothianidin'. Available at: https://www.sumitomo-chem.co.jp/cgi-bin/research/list_e.cgi. Sumitomo Chemical Co., Ltd., Tokyo. (in Japanese). Accessed 10 January 2009.

Yokota T, Mikata K, Nagasaku H, Ohta K. 2003. Abortion, tissue distribution, excretion, and metabolism of clothianidin in rats. J Agric Food Chem 51:7066–7072.