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Reproduction Study in Japanese Quail Fed Hexachlorobutadiene for 90 Days

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Reproduction Study in Japanese Quail Fed Hexachlorobutadiene for 90 Days. SCHWETZ, B. A., NORRIS, J. M., KOCIBA, R. J., KEELER, P. A., CORNIER, R. F. AND GEHRING, P. J. (1974). *Toxicol. Appl. Pharmacol.* **30**, 255–265. Adult male and female Japanese quail were fed diets containing 0.3, 3, 10, or 30 ppm of hexachlorobutadiene (HCBD) or 20 ppm of hexachlorobenzene (HCB) for 90 days. HCBD at all dose levels had no effect on body weight, demeanor, food consumption, egg production, the percent fertility and hatchability of eggs, the survival of hatched chicks, and eggshell thickness. The birds showed no evident signs of toxicity during the study. In addition, there were no gross or histopathologic changes evident in the organs or tissues of birds at the termination of the study which could be related to treatment. Ingestion of a diet containing 20 ppm HCB resulted in decreased survival of chicks hatched during the study and an increase in the liver weight of adult birds at the end of the study. No other treatment-related changes were observed.

The use of hexachlorobutadiene (HCBD) as an herbicide, insecticide, fumigant, bactericide, and algaecide has been suggested. Poteryayeva (1966) has reported the death of newborn rats from mothers given a single sc dose of 20 mg/kg HCBD 3 months previously. This observation plus the lack of information on the effect of chronic exposure to this potential environmental contaminant suggested that the effect of HCBD on reproduction should be studied. Thus, the purpose of the present study was to evaluate the effect of HCBD on the reproductive performance of a species of game bird, Japanese quail.

An additional group of Japanese quail was exposed to hexachlorobenzene (HCB) as a positive control. The effect of HCB on reproduction in Japanese quail has been previously reported by Vos *et al.* (1968, 1971).

METHODS

Hexachlorobutadiene and HCB were administered in the diet to adult Japanese quail (Coturnix coturnix japonica) for a period of 90 days. Starting at 2.5 months of age, 6 groups of 16 quail each (3 females and 1 male per cage) received diets containing 0, 0.3,

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3, 10, and 30 ppm HCBD[1] or 20 ppm HCB.[2] A schedule of 14 hr light: 10 hr dark was maintained throughout the study as well as 30 days prior to the beginning of the study. The quail were housed in quail battery breeding cages[3] and were maintained on Purina Layena Game Bird Chow.[4] The birds were leg-banded and randomly allocated to the various treatment groups.

A premix containing 0.1% HCBD or HCB was prepared. Duplicate analyses of these 2 premixes indicated 0.09 and 0.09% HCBD and 0.11 and 0.10% HCB. The diets supplied to the birds ad libitum were prepared weekly from the premix.

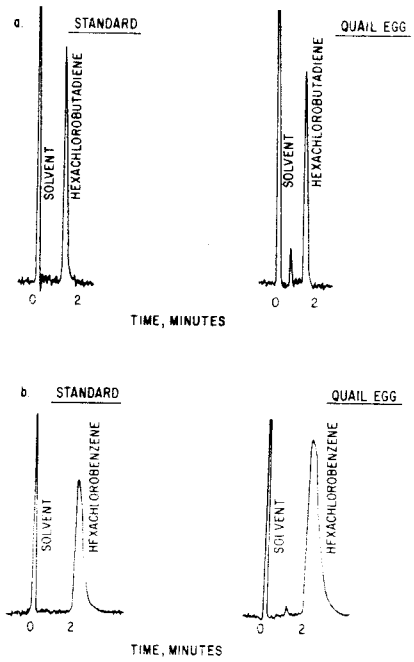

FIG. 1. Hexachlorobutadiene and hexachlorobenzene chromatograms. Conditions: (a) hexachlorobutadiene, 6 ft × 0.125 in. 5% QF-1 on 100/120 Chrom Q, 110°C column, 46 ml/min N_2, 230°C glass-lined injector, 200°C tritium detector on an Aerograph 200 GC; paper speed of 0.5 in./min 8 μl injection at 32×10^{-10} attenuation; standard 25.7 ng HCBD/ml. (b) Hexachlorobenzene, same as described above except for column temperature of 170°C, attenuation of 8×10^{-9}, and a standard containing 418 ng HCB/ml.

Egg production was recorded and calculated as mean egg production per female per day. Body weight was recorded weekly. Food consumption was measured and recorded 3 times weekly. The quail were observed daily for signs of toxicity. During the last month of the experiment, 25 eggs from each group were randomly collected to determine the index for eggshell thickness according to the method of Ratcliffe (1967). The actual shell thickness was also determined as the average of 1 measurement at each pole

[1] 99.00% C_4Cl_6, supplied by The Dow Chemical Co., Midland, MI 48640.
[2] 99.33% C_6Cl_6, supplied by The Dow Chemical Co., Midland, MI 48640.
[3] G.Q.F. Manufacturing Co., Savannah, GA.
[4] Ralston-Purina Co., St. Louis, MO.

and 2 at the equator of each egg. In both cases, thickness was measured with a micrometer to the nearest 0.01 mm. An additional 5 eggs were collected from each group during the last month of the experiment to determine the HCBD or HCB content in the eggs.

Eggs were collected during Days 37–46, 64–73, and 81–90 for incubation to determine the percent fertility and hatchability. Prior to incubation in a Humidair Model 100 incubator–hatcher initiated at the end of each collection period, the eggs were stored in a

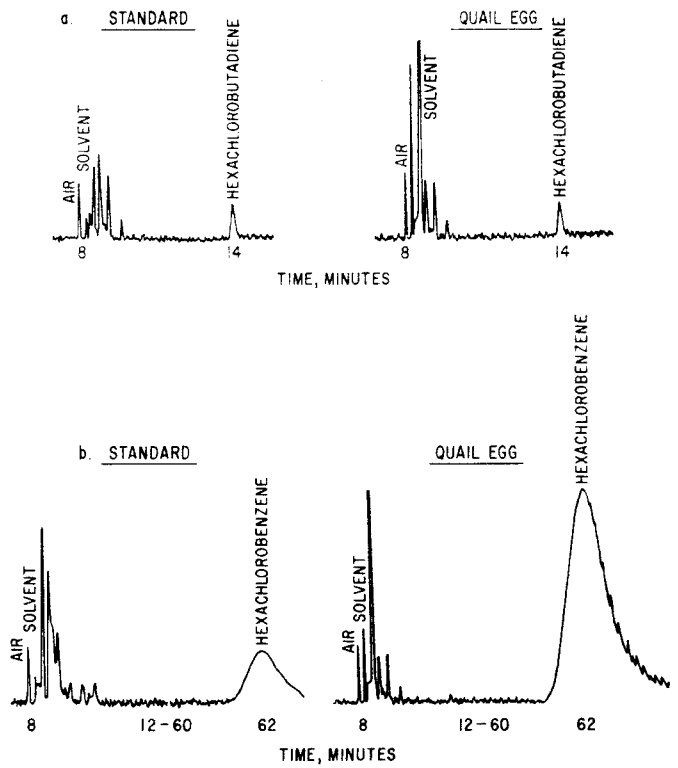

FIG. 2. Validation of hexachlorobutadiene and hexachlorobenzene. Conditions: (a) hexachlorobutadiene, 1000 ft × 0.030 in. id capillary column coated with SP-2401, 170°C column temperature, 205°C glass-lined injector, 282°C electron capture detector Ni^{63}, 43 lb N_2, Aerograph 1400 GC at 4×10^{-10} attenuation, paper speed of 0.5 in./min, injection volume 1 μl. (b) Hexachlorobenzene, same as described above, standard 418 ng HCB/ml, injection volume 1 μl.

refrigerator adjusted to 14 ± 3°C and 70 ± 10% relative humidity. The incubator maintained a 4-hr rotation cycle, a temperature of 99.5 + 0.5°F, and not less than 60% relative humidity (86°F wet bulb). Eggs which failed to hatch were opened to determine if they were infertile or contained dead embryos.

All birds that died or were killed at the termination of the study were subjected to a gross pathologic examination. Selected organs were weighed and/or preserved in buffered 10% formalin for subsequent histopathologic examination.

Analysis of eggs and the dietary premix for concentration of HCBD and HCB was made by a modification of the method of Lacroix and Wang (1971). Samples were ground in a mortar with Celite 545 diatomaceous earth wetted with *n*-hexane. The Celite–sample–hexane mixture was placed in a column overlying a 2- to 3-cm alumina bed, and the hexane eluate was collected. The column was rinsed repeatedly with hexane to remove all of the HCBD or HCB.

Analysis for HCBD and HCB was by gas–liquid chromatography using a 6 ft × 0.125 in. column of 5% QF-1 on 100/120 Chrom Q, 46 ml N_2/min carrier gas, 230°C glass-lined injector, 200°C tritium detector on an Aerograph 200 gas chromatograph. The column temperature was 110°C for HCBD and 170°C for HCB. Under these conditions, the retention time was 1.6 min for HCBD and 2.4 min for HCB. See Fig. 1 for sample chromatograms.

The positive identification of HCBD and HCB in test samples was validated by an additional gas–liquid chromatographic procedure. Test samples and standard solutions containing HCBD or HCB were analyzed using a 1000 ft × 0.030 in. capillary column coated with SP-2401, 170°C column temperature, 205°C glass-lined injector, 282°C electron capture ^{63}Ni detector, and 43 lb N_2 in an Aerograph 1400 gas chromatograph. See Fig. 2 for sample chromatograms.

Statistical analysis of values for body weight, food consumption, egg production, eggshell thickness, and organ weights was made by an analysis of variance. Group means were compared to untreated control values using Dunnett's test (Steel and Torrie, 1960). Evaluation of the percent fertility, percent hatchability, and 7-day chick survival was made by the Fisher exact probability test (Siegel, 1956). The level of significance for all cases was $p < 0.05$.

RESULTS

Incorporation of 0.3, 3, 10, or 30 ppm HCBD or 20 ppm HCB in the diet of Japanese quail for 90 days had no effect on their body weight or food consumption. No treatment-related signs of toxicity or alteration of demeanor were observed at any time throughout the study among birds receiving any dose level of HCBD or HCB. There was no significant variation in egg production within any single treatment group throughout the 90-day study period. In addition, there was no difference in egg production between any HCBD- or HCB-treated groups and the untreated control group of birds at any week during the entire study.

The fertility and hatchability of eggs is indicated in Table 1. Of the eggs collected on Days 37–46, the percent fertility was significantly increased in the group of birds receiving 20 ppm HCB. However, this increased fertility was not seen in the eggs collected on Days 64–73 or 81–90. The only other statistically significant effect was decreased fertility of eggs collected on days 63–73 of birds receiving 0.3 ppm HCBD. However, this effect was not seen in the groups of eggs collected during the earlier or later collection periods, nor was this depressed fertility seen at higher dose levels of HCBD. The percent of fertile eggs which hatched did not deviate from that of the untreated controls among any of the groups of birds receiving either HCBD or HCB at any of the 3 collection periods.

Among the eggs collected on Days 37–46 or 64–73, the 7-day survival of chicks hatched from HCBD-treated birds was not decreased from survival in the untreated control group (Table 2). However, the survival of chicks from eggs collected on Days

81–90 from birds treated with 10 ppm HCBD was significantly lower than survival among untreated control chicks. This decreased survival was not seen among chicks of birds receiving feed containing 30 ppm HCBD, however. The survival of chicks hatched from eggs collected from Japanese quail receiving 20 ppm HCB in the diet was significantly decreased at all 3 times of collection during the study compared to the untreated controls.

TABLE 1

REPRODUCTION RESULTS OF JAPANESE QUAIL MAINTAINED ON DIETS CONTAINING VARYING CONCENTRATIONS OF HEXACHLOROBUTADIENE (HBCD) FOR 90 DAYS

Dietary concentration (ppm)		Eggs collected on Days 37–46			Eggs collected on Days 64–73			Eggs collected on Days 81–90		
		No. set	Percent fertile[a]	Percent hatched[b]	No. set	Percent fertile	Percent hatched	No. set	Percent fertile	Percent hatched
	0.0	101	74	85	106	86	81	103	83	76
HCB[c]	20.0	96	92[d]	83	92	84	90	83	73	81
HBCD	0.3	68	78	84	64	68[d]	77	53	73	78
	3.0	90	78	84	113	90	85	93	86	80
	10.0	100	74	89	86	80	85	84	81	76
	30.0	98	68	89	94	81	76	104	83	75

[a] Percent of set eggs which were fertile.
[b] Percent of fertile eggs which hatched.
[c] One group of quail received HCB in the diet for comparison to the results reported by Vos *et al.* (1968, 1971).
[d] Significantly different from control value by the Fisher Exact Probability Test, $p < 0.05$.

TABLE 2

SURVIVAL OF CHICKS OF JAPANESE QUAIL MAINTAINED ON DIETS CONTAINING VARYING CONCENTRATIONS OF HEXACHLOROBUTADIENE (HCBD) FOR 90 DAYS

Dietary concentration (ppm)		Percent of hatched birds which survived 1 week		
		Eggs collected on Days 37–46	Eggs collected on Days 64–73	Eggs collected on Days 81–90
	0.0	95 (54/57)[a]	88 (53/60)	95 (59/62)
HCB[b]	20.0	78 (52/67)[c]	68 (45/66)[c]	78 (35/45)[c]
HCBD	0.3	98 (42/43)	90 (27/30)	89 (25/28)
	3.0	96 (52/54)	95 (80/84)	90 (56/62)
	10.0	85 (52/61)	92 (48/52)	82 (40/49)[c]
	30.0	91 (53/58)	89 (48/54)	92 (56/61)

[a] Percent (number alive on Day 7/number alive on Day 1).
[b] See footnote *c* in Table 1.
[c] Significantly different from control value by the Fisher Exact Probability Test, $p < 0.05$.

Incorporation of HCBD and HCB in the diet of Japanese quail for 90 days had no effect on the eggshell thickness index or the actual shell thickness. Hexachlorobutadiene and HCB were found within the eggs, however, as indicated in Table 3. Among eggs

TABLE 3

CONCENTRATION OF HEXACHLOROBUTADIENE (HCBD) IN EGGS OF JAPANESE QUAIL MAINTAINED ON DIETS CONTAINING VARYING CONCENTRATIONS OF HCBD FOR 90 DAYS

Dietary concentration (ppm)		Number	ppm of HCBD or HCB in total eggs[a] mean ± SD
	0.0	5	0.00 ± 0.00
HCB[b]	20.0	5	6.20 ± 0.45
HCBD	0.3	5	0.03 ± 0.00
	3.0	5	0.25 ± 0.05
	10.0	5	1.96 ± 0.34
	30.0	5	2.98 ± 0.61

[a] Analysis was performed by gas–liquid chromatography.
[b] See footnote *c* in Table 1.

TABLE 4

GROSS AND HISTOPATHOLOGIC CHANGES IN JAPANESE QUAIL MAINTAINED ON DIETS CONTAINING VARYING CONCENTRATIONS OF HEXACHLOROBUTADIENE (HBCD) FOR 90 DAYS[a]

Dietary concentration (ppm)	HCB, ppm				HCBD, ppm							
	0.0		20.0		0.3		3.0		10.0		30.0	
	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
Gross necropsy examination												
Number of birds	12	4	10	4	10	2	12	4	11	4	12	3
Pale, yellow coloration of liver	7	0	3	0	0	0	6	0	2	0	8	0
Egg bound condition	1	—	0	—	0	—	0	—	0	—	0	—
Peritonitis due to ruptured ova	0	—	1	—	0	—	0	—	0	—	0	—
Bile coloration of liver	2	0	1	0	0	0	0	0	0	0	0	0
Cachexic, moribund condition	0	0	0	0	0	0	0	0	1	0	0	0
Dilated crop	0	0	0	0	1	0	0	0	0	0	0	0
Trauma to cranium–cannibalism	0	0	0	0	0	0	0	0	0	1	0	2
Histopathologic examination												
Number of birds	6	4	6	4	NE[b]	NE	NE	NE	NE	NE	6	3
Liver–fatty metamorphosis, minimal	3	2	6	2							2	1
Liver–fatty metamorphosis, moderate	1	0	0	0							4	0
Liver–fatty metamorphosis, severe	1	0	0	0							0	0
Liver–focal accumulation of mononuclear cells	0	0	0	0							0	1
Focal, granulomatous pneumonitis (aspirated plant material)	1	0	0	0	NE	NE	NE	NE	NE	NE	0	0
Fatty metamorphosis of kidney tubules, moderate	0	1	0	0							0	0

[a] Numbers reported are the number of affected birds.
[b] NE = not examined.

collected during the last 30 days of the study, there was about 0.03 ppm of HCBD in eggs of birds receiving a diet containing 0.3 ppm of HCBD. Approximately 3 ppm of HCBD was found in eggs of quail on a diet containing 30 ppm HCBD. Approximately 6 ppm of HCB was found in eggs of birds receiving 20 ppm HCB in the diet. Neither HCBD or HCB were found in the eggs of control birds. The limit of sensitivity for the analytical procedure was 0.02 ppm of HCBD and 0.05 ppm of HCB. Recovery of HCB from an eggshell and contents spiked with sufficient HCB to give a concentration of 1 ppm was 100 and 99%, respectively. Hexachlorobutadiene recovery at a concentration of 1 ppm was 93% from the shell and 95% from the egg contents. No interfering peaks were encountered in the gas chromatographic analysis of control eggs for either HCBD or HCB. The shell and egg contents were analyzed separately on all the untreated control eggs and on 2 eggs from the 30 ppm-HCBD group. More than 95% of the HCBD found in the 30-ppm eggs was in the egg contents. All other eggs were analyzed for HCBD or HCB as a total egg homogenate.

Nine quail died during the course of the studies; these deaths were not considered to be related to treatment. Gross and histopathologic examination of birds sacrificed at the termination of the 90-day study revealed no changes which were considered related to treatment with HCBD or HCB, as indicated in Table 4. Female birds from both treated and control groups had variable amounts of fat in the liver as indicated by a yellow coloration and fatty metamorphosis of the liver. These alterations in the fat content of the liver and the other pathological changes noted in Table 4 were observed in control and treated birds at essentially the same frequency. Therefore, none of these pathological alterations are considered to be related to treatment.

The absolute organ weights and the organ weights/100 g body weight are presented in Tables 5 and 6. The only statistically significant effect was a decreased weight of the brain of females receiving 10 ppm of HCBD. This effect was not seen at the higher dose level of HCBD. There was an increase in the absolute and relative weight of the liver of females receiving diets containing 20 ppm of HCB. No changes in the absolute or relative weights of the brain, heart, kidney, or testes were detected in male birds receiving either HCB or HCBD. An analysis of variance of the absolute liver weights revealed a significant ratio for treatment, but none of the means were significantly different from the untreated control liver weight by Dunnett's test. There was no effect of treatment on the relative liver weights.

DISCUSSION

Hexachlorobenzene was included in this study as a positive control because Vos had previously reported the effects of this compound on Japanese quail. The effect of a dietary intake of 20 ppm of HCB on reproduction in Japanese quail has been published by Vos *et al.* in a preliminary report (1968) and in a final report (1971). In both studies, the percent of fertile eggs which hatched was significantly decreased while treatment had no effect on the general health of the birds, egg production, or eggshell thickness. At the termination of the study the body weight of females as well as the weight of the heart and liver was significantly increased compared to control values. The relative liver and kidney weights were increased compared to controls. Histopathologic changes were limited to slight damage to the liver and kidneys.

TABLE 5

ORGAN WEIGHTS AND ORGAN:BODY WEIGHT RATIOS OF FEMALE JAPANESE QUAIL MAINTAINED ON DIETS CONTAINING VARYING CONCENTRATIONS OF HEXACHLOROBUTADIENE (HCBD) FOR 90 DAYS

Dietary concentration (ppm)		Number of birds	Terminal body weight (g) (mean ± SD)	Organ weight (g) (mean ± SD)			
				Brain	Heart	Liver	Kidney
	0.0	12	136 ± 11	0.76 ± 0.07	0.98 ± 0.12	4.23 ± 1.10	1.43 ± 0.14
HCB	20.0	10	144 ± 14	0.75 ± 0.08	1.00 ± 0.17	5.37 ± 0.90[a]	1.51 ± 0.23
HCBD	0.3	10	133 ± 14	0.73 ± 0.04	0.99 ± 0.14	3.43 ± 0.33	1.37 ± 0.22
	3.0	12	137 ± 6	0.74 ± 0.04	0.99 ± 0.09	3.67 ± 0.53	1.36 ± 0.12
	10.0	11	135 ± 14	0.68 ± 0.06[a]	0.98 ± 0.12	3.57 ± 0.90	1.29 ± 0.20
	30.0	12	135 ± 13	0.76 ± 0.05	0.93 ± 0.14	3.81 ± 0.70	1.36 ± 0.13
				Organ:body weight ratio (g/100 g body weight, mean ± SD)			
	0.0			0.56 ± 0.07	0.72 ± 0.09	3.10 ± 0.73	1.06 ± 0.12
HCB	20.0			0.53 ± 0.06	0.70 ± 0.08	3.73 ± 0.45[a]	1.05 ± 0.09
HCBD	0.3			0.55 ± 0.05	0.75 ± 0.12	2.59 ± 0.27	1.03 ± 0.14
	3.0			0.54 ± 0.03	0.72 ± 0.07	2.68 ± 0.35	0.99 ± 0.09
	10.0			0.51 ± 0.06	0.73 ± 0.09	2.62 ± 0.54	0.95 ± 0.11
	30.0			0.57 ± 0.07	0.69 ± 0.08	2.87 ± 0.78	1.01 ± 0.14

[a] Significantly different from control by Dunnett's Test, $p < 0.05$.

TABLE 6

ORGAN WEIGHTS AND ORGAN:BODY WEIGHT RATIOS OF MALE JAPANESE QUAIL MAINTAINED ON DIETS CONTAINING VARYING CONCENTRATIONS OF HEXACHLOROBUTADIENE (HCBD) FOR 90 DAYS[a]

Dietary concentration (ppm)		Number of birds	Terminal body weight (g) (mean ± SD)	Organ weight (g) (mean ± SD)				
				Brain	Heart	Liver	Kidney	Testes
	0.0	4	108 ± 12	0.77 ± 0.02	0.99 ± 0.11	1.93 ± 0.37	0.89 ± 0.10	3.67 ± 0.68
HCB	20.0	4	110 ± 4	0.77 ± 0.07	0.90 ± 0.11	2.26 ± 0.41	0.78 ± 0.09	3.82 ± 0.98
HCBD	0.3	2	103 ± 7	0.80 ± 0.00	1.07 ± 0.16	1.42 ± 0.05	0.71 ± 0.30	3.16 ± 0.34
	3.0	4	100 ± 5	0.72 ± 0.07	0.89 ± 0.06	1.55 ± 0.25	0.75 ± 0.05	2.89 ± 0.39
	10.0	4	111 ± 8	0.68 ± 0.05	0.90 ± 0.12	1.63 ± 0.28	0.72 ± 0.08	3.80 ± 0.46
	30.0	3	108 ± 15	0.79 ± 0.05	0.93 ± 0.28	1.79 ± 0.36	0.82 ± 0.20	3.53 ± 0.36
				Organ: body weight ratio (g/100 g body weight, mean ± SD)				
	0.0			0.72 ± 0.07	0.93 ± 0.11	1.80 ± 0.36	0.83 ± 0.12	3.41 ± 0.56
HCB	20.0			0.70 ± 0.05	0.82 ± 0.13	2.05 ± 0.36	0.71 ± 0.08	3.46 ± 0.86
HCBD	0.3			0.78 ± 0.06	1.03 ± 0.08	1.38 ± 0.05	0.68 ± 0.24	3.09 ± 0.54
	3.0			0.72 ± 0.06	0.89 ± 0.08	1.54 ± 0.24	0.75 ± 0.05	2.87 ± 0.26
	10.0			0.61 ± 0.05	0.81 ± 0.05	1.46 ± 0.16	0.65 ± 0.05	3.44 ± 0.56
	30.0			0.74 ± 0.08	0.85 ± 0.16	1.65 ± 0.25	0.75 ± 0.15	3.27 ± 0.14

[a] Evaluation of the absolute liver weights by an analysis of variance revealed a significant F for treatment, but no means were different from the control weight by Dunnett's Test, $p < 0.05$.

In the present study, dietary intake of feed containing 20 ppm HCB for 90 days had no effect on the general health of the birds and did not alter egg production or eggshell thickness, as found previously by Vos. The decreased hatchability of eggs reported by Vos was not found in the present study; however, the 7-day survival of hatched chicks was found to be significantly decreased by 20 ppm of HCB in the diet. The percent hatchability of the control quail in the Vos study was considerably lower than in the present study, 36–47% versus 74–85%. The lower susceptibility of our colony to the effect of HCB may be related to this large difference in reproduction performance between the 2 colonies of birds. The histopathologic changes found in the liver of birds in the present study were similar to those reported by Vos. However, these changes were also found in the control birds in the present study and consequently were not considered to be treatment related. The increase in liver weight observed by Vos *et al.* (1968, 1971) was also found in the present study.

The differences between the 2 studies in certain parameters are probably related to the small number of animals used in each study as well as an expected amount of biological variation which limits the reproducibility of any animal experiment. In addition, differences in sample purity could easily account for the variations between studies.

Ingestion of a diet containing as much as 30 ppm HCBD by male and female Japanese quail for 90 days had essentially no effect on the body weight, demeanor, food consumption, egg production, fertility and hatchability of eggs, survival of hatched chicks, or eggshell thickness. In addition, there were no signs of toxicity observed in the birds and there were no gross or histopathologic changes observed in any of the birds.

The no-effect level of HCB in Japanese quail was found by Vos *et al.* (1971) to be 1 ppm. The no-effect level of HCBD in quail is apparently considerably greater than that of HCB, since the highest dose level in the present study, 30 ppm, had no effect.

The relative sensitivity of rats to HCBD and HCB appears to be in the direction opposite to the relative sensitivity of quail to the 2 compounds.[5] In a study of the toxicity of HCBD and HCB in which female rats received these compounds in the diet for 30 days (unpublished data), the no-effect level of HCB was 10 mg/kg/day, 50 times the no-effect dose level in quail. The no-effect dose level of HCBD in quail is greater than 5–6 mg/kg/day, at least twice the no-effect dose level for HCBD in rats. These comparisons are based on a 90-day study in quail and a 30-day study in rats and, hence, direct quantitative comparison may not be justified.

The failure of HCBD at dietary levels up to 30 ppm to have an effect on reproduction in quail was not due to its exclusion from the birds' reproductive tracts; the concentration of HCBD in the eggs of quail at the termination of the 90-day study was approximately 0.1–0.2 times the concentration in the diet.

In conclusion, ingestion of hexachlorobutadiene in the diet by Japanese quail for 90 days at concentrations as high as 30 ppm caused no measurable signs of toxicity and had no effect on reproduction.

[5] Parts per million (ppm) in the diet were converted to mg/kg/day as follows:

$$\text{mg/kg/day} = \frac{\text{daily food consumption (g)} \times \text{g\% in the diet}}{\text{body weight (kg)}} \times 1000.$$

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