Effects of Polycyclic Aromatic Hydrocarbon (PAH) ingestion on Japanese Quail.

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INTRODUCTION:

Petroleum products contain alkylated naphthalene and phenanthrene (PAH), which are persistent environmental contaminants from oil spills. Unfortunately, their toxicity profile in birds is not characterized and this severely limits oil toxicity assessment. To begin to characterize the toxicity of PAHs in birds, Japanese Quail were fed naphthalene during their maturation and reproductive phases and signs of toxicity were examined in adults and their chicks.

Japanese Quail were used as a target species because they are a standard bioassay model species for birds. This is because they have well known husbandry parameters, a quick life cycle, and their genetics have been minimally changed during domestication.

METHODS:

Animals and experimental design: Quail from the UCDavis Avian Science colony were hatched on Sept 28, 2005. The quail used in the experiment were selected at four weeks of age from a two-fold larger population so that initial body weights were as similar as possible. Hatchlings were housed in brooder batteries with woven wire floors and provided fed turkey starter (Purina Mills, St. Louis, MO) and water for ad libitum consumption. They were provided 8 hr light each day and cages were cleaned weekly.

When quail were 6 weeks of age, they were paired with mates, and moved to the experimental room that contained racks holding quail breeding cages. The room was kept at a constant temperature of 25C and day length was set at 18 hr to promote breeding. Each reproductive pair was housed in individual cages that had a shared feeder, automatic waterer and sloped floor to facilitate egg collection. There were four dietary treatment groups, each with 12 replicate pens of breeding pairs: Control, 25 mg/kg naphthalene, 50 mg/kg naphthalene, and 200 mg/kg naphthalene. The naphthalene was added to a nutritionally complete diet (game bird breeder; Purina Mills) to give the indicated levels and mixed in a stainless steel mixer (Hobart; Troy, Ohio) for 15 minutes. Diets were stored in tightly sealed containers and refrigerated. Fresh diet was provided to birds daily. New diets were mixed every 4 weeks. Diet samples were taken over a 30 day period to determine actual naphthalene levels.

At the time that diets were introduced (day 1 of the experimental period) the average body weights were 107 and 123 g for male and females, respectively. Every 4 weeks subsequently, birds were weighed and bled via from the vena cava into heparinized syringes. Birds were observed for abnormal behavior or pathological signs on a daily basis. Egg production was recorded daily. During the 11th week, all eggs were collected

and saved for measuring egg weight and shell thickness. After 14 weeks, breeding birds were killed by CO_2 asphyxia, posted for pathology scoring, and tissues collected and weighed.

Half of the eggs collected during week 11 and all of those during weeks 12-14 were incubated (41 C) until hatching (18d). Eggs that did not hatch were examined to estimate fertility and embryonic mortality. Hatched chicks were raised as described above. Body weight was determined on days 7 and 14. On day 14, chicks were killed and tissue samples taken.

Eggshells, including membranes, were air-dried before measurement of shell thickness. Measurements were taken at 3 points around the circumference of the shell that had the widest diameter using a micrometer.

Hematology. Packed cell volume was determined by the microhematocrit method. Total erythrocyte count and total leukocytes were determined using a hemocytometer. For differential leukocyte count, freshly prepared blood smears were stained with Wright's stain and cell types were identified based on morphology. Hemoglobin concentrations were determined by the cynamethemoglobin method, using Drabkin's solution.

Clinical chemistry. Clinical chemistry parameters were determined using an autoanalyzer (Beckman Instruments, Fullerton , CA), according to the instructions of the manufacturer.

Acute phase proteins. Plasma haptoglobin was measured according to manufacturer instructions, using a commercial kit (Phase Haptoglobin kit, Tridelta Diagnostics, TP801). Plasma α-1 glycoprotein was determined by rocket gel electrophoresis as previously described (1).

Histopathology. Intestinal segments from the mid-point of the duodenum (1.5 cm in length) were excised from hens, flushed with saline to remove digesta, and fixed in 100 g/L buffered formalin (pH 7.0). Fixed intestinal samples were embedded with parafilm sectioned and stained with hematoxylin-eosin by a commercial laboratory (Idexx Laboratories, West Sacramento, CA) and evaluated for the following: thickness of the lamina propria; villous height from the base of the lamina propria to the apex of the villus; villous width at its midpoint; and crypt depth between adjacent villi. Morphometric data were collected on 10 different villi per bird on each of two different serial sections. Measurements were made and analyzed by computer-aided light microscopic analysis at magnifications between 10 and 100X using Image-Pro-Plus analysis software for the PC (Media Cybernetics, Del Mar, CA). The number of leukocytes in 10 villi per slide and the number of leukocytes in the lamina propria underneath and within these 10 villi were enumerated. Assessments were made only on cleanly sectioned and perpendicular villi.

Statistics. Data were checked for homogeneity of variance and then analyzed by ANOVA with Tukey's means comparisons.

RESULTS:

There was no pattern of treatment related mortality. Two hens in the control treatment suffered mechanical injuries due to cage malfunctions during week 11 and were replaced with other birds that had not originally been included in the study but were treated identically. One male on the 25 mg/kg treatment died of undetermined causes during the termination of the experiment.

There was no detectable naphthalene in the control diet. Analyzed naphthalene levels in the diet averaged 29.5, 47.9, and 200.5 for the 25, 50, and 200 mg/kg doses, respectively. There was no evident loss in naphthalene concentrations over 30 days of storage of the 25 and 200 mg/kg diets. However, naphthalene decreased from 70.7 to 28.5 mg/kg between day 0 and day 30 for the 50 mg/kg diet. The reason for this disparity is not known.

After feeding the experimental diets for 14 weeks, the body weights and the rate of body weight gain of both male and female quail were significantly lower for those fed the 200 mg/kg diet than those fed any of the other diets (Tables 1-4). Feed consumption was decreased in groups fed either 200 or 50 mg/kg compared to controls (Table 5). Feed consumption is reported for the combined consumption of the males and the females because the two sexes ate from the same feeder; it was not possible to measure the consumption of the sexes independently.

The number of eggs laid (Table 6), egg weights, and the thickness of egg shells (Table 14) were not affected by naphthalene level. Naphthalene also did not effect the fertility of the breeding pairs, hatchability of the eggs, or the incidence of embryonic mortality (Table 7); although the pairs fed 50 mg/kg or above tended (P=0.07) to have decreased fertility.

Post mortem examination of the quail did not reveal any gross pathology associated with treatment groups. Liver, spleen, testes, and ovary weights were unaffected by dietary naphthalene (Tables 8 & 10). However, there was a significant treatment by sex interaction for kidney weight (Tables 8, 9 & 11), indicating that naphthalene at 50 mg/kg and above increased kidney weights in females, but not in males.

Few of the hematological and clinical chemistry endpoints examined were affected by naphthalene exposure (Tables 12, 13, & 15). The exception was hematocrit level, which was decreased by the highest level of naphthalene.

Ingestion of feed containing 200 mg/kg naphthalene caused duodenal villi to be shorter with increased numbers of intra-epithelial lymphocytes and a tendency (P=0.06) to have higher numbers of leukocytes in the lamina propria.

Exposure of hens to naphthalene did not have an effect on the growth rate, mortality, hemoglobin concentration, or white blood cell numbers of chicks hatched from their

eggs. However, it should be noted that all chicks were fed diets that did not contain naphthalene.

DISCUSSION

Based on measured average daily intakes of the diet, analyzed naphthalene concentrations in the diet and average body weight, the daily naphthalene consumption was 6.2, 9.2, and 37.6 mg per kg body weight for male quail consuming diets with 0, 25, 50, and 200 mg/kg respectively. Calculated daily naphthalene intake for females was 5.2, 7.7, and 31.5 mg per kg body weight, respectively.

Female quail fed naphthalene at 50 mg/kg or above had decreased food consumption. However, the hens did not significantly decrease egg production. The combination of decreased feed intake and continued egg production apparently led to diminished deposition of body weight. Males fed naphthalene at 50 mg/kg or above also decrease food consumption and diminished their deposition of body weight. It is not clear if the decrease in feed consumption was due to systemic toxicity caused by naphthalene (e.g. metabolic derangements or inflammatory disturbances in the digestive tract) or if it was secondary to the volatile nature of this compound. Birds are highly reliant on organoleptic cues in their regulation food intake and naphthalene's effect may not have been the result of systemic toxicity. Future experiments should equalize food consumption between control and treated groups in order to make this distinction.

Naphthalene did not affect the quantity of eggs or the quality of those eggs as indicated by size and shell thickness. The reproductive capacity of the quail was also not affected by naphthalene as indicated by fertility, hatchability, and incidence of chick mortality. There also were no indications of diminished chick size at hatching, birth defects, or growth rates. Although naphthalene is transferred to the egg, especially the yolk (2), reproduction does not appear to be a sensitive indication of naphthalene toxicity compared to direct effects on the adult (see below).

Of the organs weights examined, only kidney weights were affected by naphthalene. In laying chickens chronically fed naphthalene, the kidney accumulates the greatest amount of naphthalene and its metabolites(2). Naphthalene metabolites also accumulate in the kidneys of redhead ducks fed crayfish contaminated with naphthalene (3). Naphthalene is metabolically activated to the reactive intermediates, naphthalene oxide (NO) and naphthoquinones, which cause oxidative stress (4). Naphthalene also forms protein sulfhydryl adducts in several tissues, including the kidney (5). In mice, but not rats, the kidney is especially sensitive to naphthalene, with damage to the cells in the proximal tubules (6). In humans, naphthalene toxicity causes renal failure (7). Thus, it is likely that the enlarged kidneys observed in quail were due to hypertrophic compensation of kidney damage. Further histological studies are needed to verify this.

Naphthalene caused signs of intestinal inflammation when fed at 200 mg/kg diet, including shortening of the villi and infiltration of leukocytes into the epithelium and lamina propria. Given that the digestive epithelium is the first tissue exposed to ingested

naphthalene, this tissue would likely be exposed to the highest effective dose. Evidently the highest concentration of naphthaline examined was sufficiently high to cause local inflammation. However, systemic inflammation was not observed as indicated by normal levels of the acute phase proteins haptoglobin and α-1 glycoprotein. Inflammation of the nasal epithelium has also been observed when rats were chronically exposed to naphthalene by inhalation (8).

Conclusion: A reduction in feed intake was the most sensitive indicator of oral naphthalene exposure when males and females are considered together. The NOAEL and LOAEL using feed intake as the criterion were 25 and 50 mg/kg diet, respectively. However, we can not be certain that this is a true toxicological effect rather than an organoleptic effect. In females, but not males, increased kidney weight gave the same LOAEL or NOAEL as did decreased feed consumption. In males the most sensitive tissue change was intestinal inflammation, which occurred at a LOAEL and NOAEL of 50 and 200 mg/kg diet, respectively.

Table 1. ANOVA for Effect of Naphthalene on Body Weights (P values)

Source	Week 6	Week 10	Week 14
Treatment	0.75	0.34	0.01
Sex	0.00	0.00	0.00
Treatment x sex	0.38	0.48	0.43

Table 2. Effect of Naphthalene on Body Weight at Week 14 (g)

	Male			Female			All		
Treatment	Ave		SD	Ave		SD	Ave		SD
0	117.6	±	2.1	146.4	±	2.4	132[a]	±	1.6
25	120.2	±	2.2	143.9	±	2.1	132[a]	±	1.6
50	115.6	±	2.1	145.9	±	2.1	131[a]	±	1.5
200	112.7	±	2.1	138.6	±	2.1	125[b]	±	1.5

Table 3. ANOVA for Effect of Naphthalene on Change in Body Weight (P values)

Source	Week 6 - 10	Week 6 - 14
Treatment	0.34	0.02
Sex	0.00	0.00
Treatment x sex	0.24	0.69

Table 4. Effect of Naphthalene on Change in Body Weight between Weeks 6-14 (g)

	Male			Female			All		
Treatment	Ave		SD	Ave		SD	Ave		SD
0	10.9	±	2.3	23.4	±	2.5	17.2[a]	±	1.6
25	7.8	±	2.4	10.7	±	2.3	14.4[a]	±	1.6
50	8.5	±	2.3	18.9	±	2.3	13.7[a]	±	1.5
200	5.7	±	2.3	13.6	±	2.3	9.6[b]	±	1.5

Table 5. Effect of Naphthalene on Feed Consumption (g)
Week 11-14 (both sexes combined)

Treatment	Ave		SD
0	1247[ab]	±	126
25	1372[a]	±	67
50	1231[b]	±	79
200	1161[c]	±	87

P = 0.00

Table 6. Effect of Naphthalene on Egg Production
(# eggs laid in 4 wks)

Treatment	Ave		SD
0	24.5	±	4.7
25	24.6	±	8.5
50	24.6	±	4.3
200	21.2	±	7.8

P = 0.51

Table 7. Effect of Naphthalene on Fertility (%) and Embryonic Mortality
Week 11-14

	Fertility			Mortality		
Treatment	Ave		SD	Ave		SD
0	92.8	±	9.9	3.3	±	6.2
25	88.9	±	10.7	8.4	±	8.4
50	72.9	±	24.4	9.1	±	8.7
200	74.6	±	25.4	4.1	±	4.1
P value	0.07			0.21		

Table 8. ANOVA for Effect of Naphthalene on Organ Weights of Adults at Week 14
(P values)

Source	Liver	Spleen	Testes	Kidney
Treatment	0.32	0.67	0.11	0.13
Sex	0.00	0.00	0.00	0.00
Treatment x sex	0.50	0.31		0.01

Table 9. Effect of Naphthalene on Kidney Weights of Adults at Week 14 (g)

	Male			Female		
Treatment	Ave		SD	Ave		SD
0	0.08	±	0.05	0.31	±	0.05
25	0.21	±	0.05	0.30	±	0.05
50	0.26	±	0.05	0.15	±	0.05
200	0.11	±	0.05	0.18	±	0.05

Table 10. ANOVA for Effect of Naphthalene on Organ Weights as % Body Weights
(P values)

Source	Liver	Spleen	Kidney	Testes	Ovary
Treatment	0.58	0.76	0.13	0.29	0.87
Sex	0.00	0.04	0.41	-	-
Treatment x sex	0.53	0.33	.01	-	-

Table 11. Effect of Naphthalene on Relative Kidney Weights (g/100 g BW)

	Male			Female		
Treatment	Ave		SD	Ave		SD
0	0.17[b]	±	0.33	0.21[a]	±	0.39
25	0.18[b]	±	0.35	0.21[a]	±	0.33
50	0.22[a]	±	0.34	0.10[b]	±	0.34
200	0.09[b]	±	0.34	0.13[b]	±	0.34

Table 12. ANOVA for Effect of Naphthalene on Blood Hematocrits (P values)

Source	Week 10	Week 14
Treatment	0.72	0.03
Sex	0.00	0.17
Treatment x sex	0.19	0.78

Table 13. Effect of Naphthalene on Hematocrit at Week 14 (% RBCs)

	Male & Female		
Treatment	Ave		SD
0	52.3[b]	±	3.1
25	45.0[ab]	±	3.0
50	44.8[ab]	±	3.1
200	41.6[a]	±	2.0

Table 14. Effect of Naphthalene on Intestinal Histology of Hens (week 14)

Treatment	lamina propria (µm)	villus height (µm)	villus width (µm)	crypt depth (µm)	intra-epithelial lymphocytes (#/villi)	lamina propria leukocytes (#/villi)
0	69[ab]	458[b]	77	81	15[a]	27
25	55[a]	477[b]	75	75	17[a]	15
50	68[ab]	451[ab]	75	85	22[ab]	32
200	94[b]	411[a]	79	88	29[b]	39
Pooled SD	14	15	4	6	5	6
P value	0.04	0.03	0.51	0.63	0.04	0.06

Table 15. Parameters for which there were no Significant Differences (P>0.10) due to Naphthalene in Adults at 14 Weeks

Hemoglobin (g/dl)	6.3*	±	0.8
Lymphocytes (%)	64	±	7
Heterophils (%)	29	±	9
Serum Protein (g/dl)	3.3	±	0.3
Triglyceride (mg/L)	98*	±	11
ALT (IU/L)	17.2	±	3.8
LD (IU/L)	611*	±	29
AST (mg/L)	459	±	35
Uric acid(mg/L)	6.9*	±	0.5
Albumin (mg/dl)	1.8*	±	0.1
Haptoglobin (ug/dl)	3.2	±	0.6
α1-glycoprotein (U/L)	12.2	±	2.5
Shell thickness (μm)	213	±	30

*Significant effect due to sex

Table 16. Effect of Naphthalene on Chicks (day 14)

Hatching weight (g)	6.3	±	0.8
Weight gain (g)	64	±	7
Mortality (%)	29	±	9
Hemoglobin (g/dl)	459	±	35
Lymphocytes (%)	6.9	±	0.5
Heterophils (%)	1.8	±	0.1

No significant treatment effects (P>0.10)

Table 17. Summary of Parameters Significantly affected by Naphthalene in females.

Parameter	Lowest diet level giving effect (μg/kg diet)	Lowest dose giving effect (mg/kg BW/day)
Final weight	200	31.5
Weight gain	200	31.5
Feed intake	50	7.7
Kidney weight (female)	50	7.7
Hematocrit	200	31.5
Intestinal inflammation	200	31.5

1. Adler, K. L., Peng, P. H., Peng, R. K. & Klasing, K. C. (2001) The kinetics of hemopexin and alpha1-acid glycoprotein levels induced by injection of inflammatory agents in chickens. Avian Dis 45: 289-296.

2. Eisele, G. R. (1985) Naphthalene distribution in tissues of laying pullets, swine, and dairy cattle. Bull Environ Contam Toxicol 34: 549-556.

3. Tarshis, I. B. & Rattner, B. A. (1982) Accumulation of 14C-Naphthalene in the tissues of redhead ducks fed oil-contaminated crayfish. Arch Environ Contam Toxicol 11: 155-159.

4. Stohs, S. J., Ohia, S. & Bagchi, D. (2002) Naphthalene toxicity and antioxidant nutrients. Toxicology 180: 97-105.

5. Tsuruda, L. S., Lame, M. W. & Jones, A. D. (1995) Formation of epoxide and quinone protein adducts in B6C3F1 mice treated with naphthalene, sulfate conjugate of 1,4-dihydroxynaphthalene and 1,4-naphthoquinone. Arch Toxicol 69: 362-367.

6. O'Brien, K. A., Smith, L. L. & Cohen, G. M. (1985) Differences in naphthalene-induced toxicity in the mouse and rat. Chem Biol Interact 55: 109-122.

7. Choudhri, A. N., Pasha, M. J. & Ali, B. (1995) Acute renal failure following naphthalene poisoning. J Pak Med Assoc 45: 331-332.

8. Long, P. H., Herbert, R. A., Peckham, J. C., Grumbein, S. L., Shackelford, C. C. & Abdo, K. (2003) Morphology of nasal lesions in F344/N rats following chronic inhalation exposure to naphthalene vapors. Toxicol Pathol 31: 655-664.