Sublethal Effects of the Polycyclic Aromatic Hydrocarbon, Naphthalene, on Japanese Quail and Mallard Duck Hatchlings

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INTRODUCTION: The overall objective of the study was to understand how polycyclic aromatic hydrocarbons (PAHs) contribute to the adverse effects observed in birds exposed to petroleum (e.g., Coon and Dieter, 1981; Holmes, 1990; Stubblefield et al., 1995a; Jessup and Leighton, 1996; Newman, S.H. 1998). A wide range of petroleum products contain several hundred parts per million (ppm) of parent and alkylated naphthalene and phenanthrene (Stubblefield et al, 1995b; Wang et al., 2003). Naphthalene, a two-ringed polycyclic aromatic hydrocarbon (PAH), is present at relatively high concentrations found in a wide variety of petroleum products (Wang et al., 2003). For example, naphthalene has been reported at 261 mg/kg oil in fresh Alaska North Slope crude oil (Wang et al., 2003); 620 mg/kg in Exxon Valdez crude oil (Lee et al., 2005), 820 mg/kg in diesel fuel (Wang et al., 2003) and 1200 mg/kg in North Sea crude oil (Lee et al, 2005). Naphthalene has been detected in tissues of mallard ducks orally exposed to South Louisiana crude oil for 14 days (Lawler et al., 1978) and has been hypothesized to contribute to some of the toxic responses exhibited by birds exposed to oil (Leighton, 1993). These sublethal effects associated with petroleum exposure include hemolytic anemia, reduced growth, impaired reproduction, and renal tubular necrosis (Leighton, 1993; Fry et al., 1985, Coon and Dieter, 1981; Holmes et al., 1978; Szaro et al., 1978; Grau et al., 1977). Additionally, damage to and inflammation of the gastrointestinal tracts of birds ingesting oil has been observed, as well as more systemic effects on the immune system (Hartung et al., 1966; Briggs et al., 1996).

Unfortunately, the toxicity profile of naphthalene in birds is not well characterized and this severely limits ecological risk assessment for this receptor group (Douben, 2003; Kapustka, 2004). Previously we observed adverse effects of naphthalene on body weights and kidney weights of adult Japanese quail following chronic (14 wk) exposure (Klasing, 2006). The purpose of this study was to extend these observations to growing quail chicks and mallard ducklings.

METHODS:

Animals and Experimental Design: A total of 168 hatchling Japanese quail from the University of California, Davis, Avian Science colony (University of California, Davis, CA, USA) were selected from an initial population of 200 chicks so that initial body weights were as similar as possible. Hatchlings were housed in brooder batteries with woven wire floors and provided turkey starter (Purina Mills, St. Louis, MO, USA) and tap water for ad libitum consumption. They were provided 16 hr light each day and were observed daily for signs of health. Cages were cleaned weekly and waterers were

cleaned daily. Experimental diets consisted of ground turkey starter with 0, 50, 100, 200, 400, or 800 mg/kg of naphthalene (Concentrations measured on the last of each experiment are shown in Appendix 1). Four pens were randomly assigned to each treatment level. Due to the volatile nature of naphthalene, feed was added to each pen every day and extra feed was stored in airtight plastic bags and refrigerated. Bird and feed weights were taken on days 3, 6, 10 and 14. On day 14, birds were fasted for three hrs and then bled from their vena cava. Blood from all chicks in a pen was pooled. Birds were killed with CO_2 and organs (liver, spleen, intestines, kidney, thymus and bursa) were carefully dissected free of facia as part of an autopsy to determine signs of gross pathology. Pooled tissues from each pen were blotted free of blood and weighed. Tissues from one bird randomly selected from each pen were placed in buffered formalin (pH 7.0) for histological evaluation. Intestinal segments (1.5 cm in length) were taken from the mid-point of the duodenum, flushed with saline to remove digesta and preserved in formalin.

One day old mallard ducklings were obtained from a commercial supplier. A total of 90 ducklings were selected from a population of 120 so that initial body weights were as similar a possible. Husbandry and experimental design were identical as described for quail with the exception that each of the 3 pens contained 5 individuals.

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. Plasma concentrations of the acute phase protein lysozyme were assayed by measuring the rate of lysis of the bacteria *Micrococcus lysodeikticus* as indicated by a decrease in opacity measured with a microplate reader.

Histopathology. Fixed organ samples were embedded with paraffin, sectioned, mounted and stained with hematoxylin-eosin by a commercial laboratory (Idexx Laboratories, West Sacramento, CA). Three sections per tissue were evaluated for histopathology including presence of necrosis, inflammation, cellularity, and presence of inclusion bodies. Severity of necrosis or inflammation was scored from 0 to 4. Leukocytic infiltration and inclusion bodies were quantified using Image-Pro-Plus analysis software for the PC (Media Cybernetics, Del Mar, CA).

Duodenal samples were 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. 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 using JMP (SAS Inc, Carey NC). Qualitative data from autopsy and from histopathology were analyzed by Chi-square using JMP.

RESULTS:

Growth and Intake. There was not an effect of naphthalene at any level on the rate of growth, feed intake, or conversion of feed into body weight gain of quail chicks (Table 1) or mallard ducklings (Table 2).

Table 1. Effect of Naphthalene on Gain, Feed Intake & Efficiency of Feed Conversionof Japanese Quail (day 14).

Level (mg/kg)	Gain (g/c/d)	Intake (g/c/d)	Efficiency*
0	3.26	6.66	0.49
50	3.29	6.44	0.51
100	3.29	6.57	0.5
200	3.32	6.78	0.49
400	3.27	6.54	0.5
800	3.24	6.61	0.49
SEM	1.4	3.6	0.03
P value	0.99	0.85	0.79

*Grams of gain in body weight per gram of feed consumed

Table 2. Effect of Naphthalene on Gain, Feed Intake & Efficiency of Feed Conversion of Mallard ducklings (day 14).

Level (mg/kg)	Gain (g/c/d)	Intake (g/c/d)	Efficiency (gain/feed)
0	16.9	35.5	0.51

50	15.2	31.1	0.49
100	15.7	32.5	0.48
200	16.9	29.7	0.57
400	16.5	36.5	0.46
800	14.8	34.9	0.43
SEM	2.4	3.1	0.08
P value	0.93	0.64	0.84

*Grams of gain in body weight per gram of feed consumed

Organ weights and gross pathology. There was not an effect of naphthalene at any level on organ sizes relative to bodyweight of quail chicks (Table 3) or mallard ducklings (Table 4). At autopsy, there were no indications of gross pathology of any organ in Japanese quail chicks. Similarly, gross pathology was not detected in mallard ducklings with the exception of a low frequency of congestion of the duodenal mucosa in ducks fed naphthalene but not those fed the control diet. However, this congestion was mild and, even at the highest naphthalene level fed, was found in only 2 of the 15 ducklings in that treatment Table 5).

Table 3. Effect of naphthalene on relative organ weights (g/100g body weight) of Japanese quail chicks.

Level (mg/kg)	Liver	Bursa	Spleen	Kidney
0	2.66	0.09	0.087	0.12
50	3.00	0.1	0.086	0.14
100	2.66	0.11	0.082	0.14
200	2.48	0.10	0.091	0.14
400	2.53	0.09	0.076	0.12
800	2.53	0.09	0.068	0.11
SEM	0.19	0.009	0.006	0.017
P value	0.48	0.77	0.13	0.52

Table 4. Effect of naphthalene on relative organ weights (g/100g body weight) of mallard ducklings.

Level (mg/kg)	Liver	Bursa	Spleen	Kidney
0	4.6	0.15	0.14	0.14
50	5.4	0.16	0.10	0.14
100	4.5	0.17	0.12	0.16
200	3.3	0.17	0.10	0.13
400	4.8	0.14	0.18	0.17
800	3.5	0.16	0.09	0.14
SEM	0.6	0.01	0.03	0.07
P value	0.21	0.78	0.24	0.83

Table 5. Effect of naphthalene on congestion of the duodenal mucosa of mallard ducklings.

Level (mg/kg)	Number of birds (n=15)
0	0
50	1
100	0
200	2
400	1
800	2

Treatment differences were not significant (P=0.24)

Blood characteristics. In Japanese quail chicks, naphthalene at 800 mg/kg significantly decreased the hematocrit levels relative to control chicks (Table 6). Relative to chicks fed the control diet, naphthalene at 400 or 800 mg/kg significantly decreased hemoglobin concentrations.

There was a significant treatment effect on the hematocrit and hemoglobin levels of mallard ducklings (Table 7). Hematocrit level differed between the 100 and 400 mg/kg groups; however, there was no clear dose response relationship in this parameter. Hemoglobin concentrations generally decreased with increasing dietary naphthalene levels and the groups consuming 400 or 800 mg/kg had significantly lower levels than the control group.

In Japanese quail chicks, naphthalene at 100, 200 or 400 mg/kg significantly decreased the relative numbers of heterophils (avian equivalent of neutrophils) compared to control chicks (Table 8). Compared to chicks fed the control diet, naphthalene at 200 or 400 mg/kg significantly decreased the number of lymphocytes. Monocytes, eosinophils and basophils were not affected by naphthalene.

In mallard ducklings, naphthalene did not influence the relative number of any white blood cell (Table 9). Plasma chemistry values and acute phase protein concentrations were not affected (P>0.10) by naphthalene in either species (Tables 10 and 11).

Table 6. Effect of naphthalene on the hematocrit and hemoglobin levels of Japanese quail chicks.

Level (mg/kg)	Hematocrit (%)	Hemoglobin (g/dl)
0	36.7^{AB}	6.5^{B}
50	37.3^{AB}	6.4^{BC}
100	37.0^{AB}	6.6^{B}
200	35.7^{BC}	6.0^{AB}
400	38.0^{A}	5.5^{A}
800	34.3^{C}	5.9^{AC}
SEM	0.64	0.2
P value	0.02	0.03

Table 7. Effect of naphthalene on the hematocrit and hemoglobin levels of mallard ducklings.

Level (mg/kg)	Hematocrit (%)	Hemoglobin (g/dl)
0	46^{AB}	6.9^{B}
50	51^{AB}	6.7^{ABC}
100	54^{A}	6.4^{ABC}
200	52^{AB}	6.8^{BC}
400	31^{B}	5.5^{A}
800	41^{AB}	5.9^{AC}
SEM	4.6	0.2
P value	0.04	0.04

Table 8. Effect of naphthalene on white blood cell numbers in the blood of Japanese quail chicks.

Level	Heterophils	Lymphocytes	Monocytes	Eosinophils	Basophils
0	44.3^{B}	39.0^{A}	7	6.3	3.3
50	44.3^{B}	40.3^{A}	5.7	5.3	4.3
100	57.0^{A}	31.7^{AB}	4.7	3.7	3
200	64.7^{A}	17.3^{C}	6.3	7.3	4.3
400	65.3^{A}	20.3^{BC}	4.7	7.3	2.3
800	53.7^{AB}	32.0^{A}	5.7	5.3	3.3
SEM	4	3.8	1.74	1.4	1.1
P value	0.008	0.004	0.91	0.48	0.8

Table 9. Effect of naphthalene on white blood cell numbers in the blood of mallard ducklings.

Level	Heterophils	Lymphocytes	Monocytes	Eosinophils	Basophils
0	37.9	41.6	9.3	6.2	4.9
50	37.5	42.9	9.1	5.8	4.6
100	39.5	42	7.4	6.7	4.4
200	39.4	41.2	8.8	5.8	4.8
400	37	42.3	8.6	6.7	5.4
800	38.2	43.4	7.7	6.1	4.6
SEM	2.9	3.1	1.3	1.8	1.3
P value	0.44	0.91	0.18	0.79	0.81

Table 10. Plasma chemistry and acute phase protein concentrations in Japanese quail averaged across all treatments.

Serum protein (g/dl)	3.7 $\pm$ 0.2
Triglycerides (mg/L)	79 $\pm$ 6.6
ALT (IU/L)	12.8 $\pm$ 2.0
LD (IU/L)	422 $\pm$ 21
AST (mg/L)	501 $\pm$ 28
Uric acid (mg/L)	8.5 $\pm$ 1.3
Albumin (mg/L)	1.9 $\pm$ 0.4
Haptoglobin (ug/dl)	2.4 $\pm$ 0.3
Lysozyme (mg/ml)	13.3 $\pm$ 2.5

There were no significant (P>0.10) treatment effects on these parameters.

Table 11. Plasma chemistry and acute phase protein concentrations in mallard ducklings averaged across all treatments.

Plasma protein (g/dl)	4.8 ± 0.3
Triglycerides (mg/L)	46 ± 5.8
ALT (IU/L)	27.5 ± 6.9
AST (IU/L)	35 ± 5.3
Na (mmol/L)	451 ± 5.7
K (mmol/L)	5.9 ± 1.2
Cl (mmol/L)	102 ± 1.4
Ca (mmol/L)	8.5 ± 0.2
iPhoshpate (mg/dl)	4.7 ± 0.3
Uric acid (mg/L)	8.5 ± 1.3
Albumin (g/dl)	2.0 ± 0.3
Haptoglobin (ug/dl)	3.8 ± 0.4
Lysozyme (mg/ml)	9.6 ± 0.9

There were no significant (P>0.10) treatment effects on these parameters.

Morphometry of duodenal mucosa. Japanese quail chicks fed 800 mg/kg naphthaline had shorter villi and thicker lamina propria than chicks fed the control diet (Table 12). The lamina propria of chicks fed 800 mg/kg naphthaline contained a greater number of leukocytes than that of control chicks. The number of intra-epithelial lymphocytes was also increased in the chicks fed 800 mg/kg compared to control chicks.

Naphthalene affected the duodenal mucosa of mallard chicks in a manner similar to that in Japanese quail (Table 13). Ducklings fed 800 mg/kg naphthalene had shorter villi with more intra-epithelial lymphocytes and had lamina propria with more leukocytes compared to controls. Ducklings fed 200 mg/kg naphthalene also had shorter villi than controls.

Table 12. Effect of naphthalene on the histology of the duodenal mucosa of Japanese quail chicks.

Level	lamina propria thickness (µm)	villus height (µm)	villus width (µm)	crypt depth (µm)	intra-epithelial lymphocytes (#/villi)	lamina propria leukocytes (#/villi)
0	22^{AB}	221^{C}	37	45	11^{AB}	21^{A}
50	23^{AB}	215^{C}	35	43	9^{A}	25^{AB}
100	26^{AB}	233^{C}	34	45	11^{AB}	20^{A}
200	20^{A}	220^{C}	39	47	16^{AB}	31^{AB}
400	24^{AB}	195^{AB}	33	41	16^{AB}	28^{A}
800	30^{B}	180^{A}	39	39	21^{B}	39^{B}
SEM	2.1	7.8	3.1	2.9	3.1	4.2
P value	0.05	0.02	0.41	0.11	0.04	0.04

Table 13. Effect of naphthalene on the histology of the duodenal mucosa of mallard ducklings.

Level	lamina propria thickness (µm)	villus height (µm)	villus width (µm)	crypt depth (µm)	intra-epithelial lymphocytes (#/villi)	lamina propria leukocytes (#/villi)
0	126	844^{B}	219	106	18^{A}	39
50	134	825^{AB}	209	112	16^{A}	34
100	120	849^{B}	233	100	15^{A}	34
200	138	813^{A}	218	96	19^{AB}	38
400	102	821^{AB}	227	108	22^{AB}	42
800	111	806^{A}	235	101	28^{B}	44
SEM	4.6	8.6	7.6	3.9	2.8	3.5
P value	0.08	0.03	0.14	0.37	0.04	0.3

Histopathology. Naphthalene level did not significant significantly (P>0.2) affect the histology of any tissue in Japanese quail chicks. The kidney frequently displayed interstitial nephritis characterized by heterophil infiltration and infrequent signs of necrosis; however, this pathology was found in similar frequency in all treatment groups (P=0.46).

Ducklings fed 800 mg/kg naphthalene, but not those fed lower levels, had significant multifocal necrosis of thymic lobules (Table 14). In some individuals, thymic vessels showed multifocal perivascular cuffing with lymphocyte infiltration; however there was not a significant effect of treatment on this pathology (P=0.22).

Table 14. Effect of naphthalene on the incidence and severity of thymic pathology in ducklings.

Level (mg/kg)	Necrosis index*	lymphocytes (#/10^2 µm)
0	0.3^{A}	5.7
50	0.2^{A}	3.2
100	0.4^{A}	4.7
200	0.5^{AB}	4.3
400	0.4^{A}	7.9
800	1.8^{B}	9.2

*Scale: 0 equals no observable necrosis through 4 equals severe necroses.

DISCUSSION.

Japanese quail and mallard ducks are model avian species that are frequently used in studies of ecotoxicology because of their well known husbandry parameters, relatively quick life cycle and easy availability. Both species grow very rapidly post-hatch and are vulnerable to toxicological influences on development and maturation.

Naphthalene, even at the highest level, did not impact growth rate or food intake of either species during the 14 day exposure period. The lack of effect on food intake indicates that changes in other parameters were a direct action of naphthalene and not an indirect effect of changed nutrition. Naphthalene did not have any observable effect on organ sizes or gross pathology. This observation, together with a lack of effect on food intake, suggests that the levels used in these experiments were well below acutely toxic levels.

Naphthalene appeared to affect the homeostasis of circulating white and red blood cells. Hemoglobin concentrations were decreased by feeding naphthalene to either species. White blood cells of quail were more sensitive to naphthalene than those of ducks. Naphthalene caused an increase in heterophils and a corresponding decrease in lymphocytes. Such an increase in the ratio of heterophils to lymphocyte is indicative of inflammation (Koutsos and Klasing, 2001). Similarly, chronic inflammation causes anemia. Thus, it is possible that the observed changes in blood cells were secondary to inflammation in the intestine and the thymus.

The intestinal epithelium was very sensitive to naphthalene as evidenced by shortening of villi, thickening of the lamina propria, and infiltration of leucocytes. All of these

changes are indicative of inflammation. Given that naphthalene exposure occurred via the feed, it is likely that it was having a direct inflammatory effect on the intestinal epithelium; although an indirect effect via impaired defenses against the intestinal microbiota cannot be ruled out.

Besides the intestines, the only organ that was histologically influenced by naphthalene was the thymus. The thymus is a site of generation of T lymphocyte diversity, which is critical for cellular immunity. The first two weeks after hatching is the time of maximal thymic activity and the thymus is among the most metabolically and developmentally active tissues in the body. Thus, it is not surprising that the thymus was especially sensitive to naphthalene. Given the importance of T cell development early in life for immunity in adulthood, T lymphocyte functions may be a sensitive sentinel for long term toxic effects of naphthalene.

The lowest observable effect levels of naphthalene in growing quail, growing ducklings, and adult reproducing quail (Klasing et al., 2007) are shown in Table 15.

Table 15. Dietary naphthalene concentrations that significantly affected various parameters in quail and ducks.

	Dietary level (mg/kg)		
Parameter	Growing ducks	Growing quail	Reproducing quail
Body weight			200
Feed intake			200
Kidney weight			100
Hematocrit		800	200
Hemoglobin	400	400	
Blood leukocytes		100	
Intestinal pathology	200	400	200
Thymic pathology	800		

Missing values indicates that there were no significant effects ($P<0.05$).

In general, hatchling quail and ducks were similarly affected by naphthalene. A two week study in hatchling quail was similarly efficacious as a 14 week study in reproducing adults for determining the lowest observable effect level for naphthalene.

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Appendix 1

Naphthalene concentrations (mg/kg) in experimental diet.

	Naphthalene measured at end of experiment	
Amount Naphthalene added	Quail Experiment	Duck Experiment
0	BRL	BRL
50	22	20
100	40	38
200	111	97
400	268	269
800	640	541

BRL = Below Reporting Limit (<10 mg/kg)