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# EFFECTS OF PRUDHOE BAY CRUDE OIL ON HATCHING SUCCESS AND ASSOCIATED CHANGES IN PIPPING MUSCLES IN EMBRYOS OF DOMESTIC CHICKENS (*GALLUS GALLUS*)

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ABSTRACT: Fertile white leghorn chicken eggs were exposed to 0, 1, 2, 4, 6, 8 or 16 μl of Prudhoe Bay Crude oil (PBCO) on day 9 of incubation. The effects of oil on pipping and hatching success, body weight gain after hatching, serum creatine kinase levels, and pathological changes in organ systems were assessed in embryos that had survived acute toxic effects and were alive on day 18 of incubation. Exposure to oil greatly reduced pipping and hatching success. Severe edema and hemorrhage in the pipping muscle, multifocal subcapsular hepatic necrosis, marked depletion of lymphocytes in the bursa of Fabricius with infiltration by heterophils, and occasional dorso-caudal subcutaneous edema were observed in treated embryos. Pipping muscles were heavier in oil-exposed embryos. Embryos exposed to 4 μl of PBCO had significantly reduced gain in body weight post-hatching. Serum creatine kinase levels were significantly elevated in the oil-exposed embryos only at the time of hatching. There was no evidence that exposure to oil caused degenerative changes in pipping muscle cells.

*Key words:* Chicken, embryo, Prudhoe Bay crude oil, toxicity, pipping muscle, hatching, creatine kinase, experimental study.

## INTRODUCTION

The toxicity of crude petroleum oil to embryos of birds' eggs exposed during incubation is well known (Leighton, 1993). Spilled oil floats on marine or fresh water and is readily absorbed by the feathers of swimming or diving birds. Sublethal external contamination of nesting birds can result in transfer of oil to the shell of the eggs during laying or incubation in sufficient quantities to cause overt toxicity and teratogenicity in embryos of various avian species (Hoffman, 1978, 1979 a, b; King and Lefever, 1979; Albers, 1980; Albers and Gay, 1982; Lewis and Malecki, 1984; Couillard and Leighton, 1990a). In previous studies, Couillard and Leighton (1989, 1990a, b) reported mortality and lesions in chicken embryos up to day eighteen of incubation. Complete hatching failure of embryos that were alive on the eighteenth day of incubation was observed in a subsequent experiment in this laboratory intended to assess survival of hatchlings from oil contaminated eggs (F. A. Leighton, unpubl.). We therefore designed an experiment to assess the effect of microliter (μl) quantities of Prudhoe Bay crude oil (PBCO) on late embryonic events and on pipping and hatching success, and to test the hypothesis that failure to hatch is associated with degenerative changes in pipping muscle cells.

## MATERIALS AND METHODS

Two experiments were conducted. In both experiments, fertile white leghorn chicken (*Gallus gallus*) eggs were shipped from Keystone Hatchery, Niverville, Manitoba, Canada, within 2 days of collection. On arrival, eggs were kept at 4 C and turned twice daily for 2 days. Eggs then were removed from the cold room and allowed to stand for 4 hr at 24 C before being randomly set on trays in an incubator (Humidaire, New Madison, Ohio, USA), preset at a temperature of 37.5 C and 50 to 55% relative humidity. The eggs were turned automatically once per hour for 18 days of incubation, at which time the automatic turner was stopped, holding trays were kept horizontal and the relative humidity was increased to 65%—all to facilitate hatching. Infertile eggs and embryos with retarded development were identified by candling and removed on days 6 and 9 of incubation, prior to application of PBCO.

In experiment 1, the viable embryos on the ninth day of incubation were randomly divided into six treatment groups of 53, 48, 47, 48, 48,

TABLE 1. Effect of Prudhoe Bay crude oil on the survival of chicken embryos and associated pathological changes, Experiment 1. Oil was applied on eggshell on Day 9 of incubation.

| | Dose of Prudhoe Bay crude oil per egg (μl) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 4 | 8 | 16 |
| Alive on day 9[a] | 53 | 48 | 47 | 48 | 48 | 48 |
| Alive on day 18[b] | 52[c] | 39 | 33 | 32 | 26 | 21 |
| Pipped | 51 (98)[d] | 37 (95) | 26 (79) | 25 (78) | 18 (69) | 5 (24) |
| Hatched | 46 (88)[e] | 36 (92) | 25 (76) | 19 (59)[f] | 1 (4)[f] | 0 (0)[f] |
| Pipped but died | 0 (0) | 1 (3) | 1 (3) | 6 (19) | 17 (65) | 5 (24) |
| Unpipped | 1 (2) | 2 (5) | 7 (21) | 7 (22) | 8 (31) | 16 (79) |

[a] Embryos alive prior to exposure to oil.
[b] Total number of embryos that survived acute toxicity and were alive on day 18.
[c] One embryo was euthanized on day 13 of incubation.
[d] Number of embryos (percent of those alive on day 18).
[e] Five embryos were euthanized immediately after pipping to serve as controls.
[f] Significantly ($P < 0.002$) different from the controls, by a chi square test.

48 eggs each. Each received 0, 1, 2, 4, 8 or 16 μl of PBCO, respectively (Table 1). Oil was applied externally on the eggshell with a 10μl Drummond microdispenser pipet (Model 210, Drummond Scientific Company, Broomall, Pennsylvania, USA) at a single location overlying a prominent chorio-allantoic membrane blood vessel. The oil was allowed to spread freely on the eggshell before re-incubation.

Eggs were candled to check for mortality on days 13 and 18 of incubation. Dead embryos were removed from each group and the numbers recorded. Five embryos were randomly identified from the control group and were euthanized using carbon dioxide as soon as each pipped. Shell and yolk sacs were removed and the embryos were fixed whole in 10% phosphate-buffered formalin (Lillie and Fullmer, 1976). These five embryos served as control embryos for histology. Pipping and hatching activities in each group were monitored and recorded at 4-hr intervals. Embryos that never pipped and those that pipped but died without hatching were removed from the eggshell on day 22 of incubation. These embryos were examined grossly, yolk sacs were removed and whole embryos were fixed in 10% buffered formalin. Fixed portions of liver, heart, spleen, kidney, lung, pipping muscle (*musculus complexus*), and bursa of Fabricius were trimmed and embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E) for light microscopy. Von Kossa's stain was used on liver sections to detect mineralization (Lillie and Fullmer, 1976).

Hatchlings from each group were transferred to separate compartments of a brooder (Petersime Brood-unit, Petersime Incubator Company, Gettysburg, Ohio). Chicks were provided with chick and duck starter (Federated Cooperative, Saskatoon, Saskatchewan, Canada) and clean water ad libitum. Weights of each chick were taken once every week for 3 wk.

In Experiment 2, the eggs were divided into two groups of 60 (control) and 274 (oil-exposed). The same procedures and conditions were followed as in Experiment 1 up to day 9 of incubation. Fertile eggs in the larger group were treated externally with 6 μl of PBCO on day 9 of incubation. Eggs were candled to remove and record dead embryos on days 13 and 18 of incubation. Ten embryos from each group were randomly selected and euthanized using carbon dioxide on day 18 of incubation, at pipping, at hatching, and 5 days post-hatching. Each embryo was weighed and blood was obtained by heart puncture for evaluation of serum creatine kinase (CK). The CK was assayed by a modified Oliver-Rosalki method (Dart CK (CPK)-NAC. 1988. Coulter No. 7546880. Coulter Electronic Inc., Hialeah, Florida, USA). Pipping muscle, gastrocnemius muscle, breast muscle, liver, and heart were fixed in Karnovsky's fixative (Karnovsky, 1965) for histopathology. Wet weights of pipping muscle and heart from each embryo were determined prior to fixation.

Differences in treatment effects for pipping and hatching rates were analyzed by a chi-square test. Dependence of total embryo mortality on dose of oil was assessed by regression analysis. Data for weight gains post-hatching were compared by a one way analysis of variance (ANOVA). Wet weights of pipping muscle, ratios of pipping muscle to body weight, and serum creatine kinase were compared by unpaired *t*-tests (Statworks Program, Calabasas, California, USA). The results were considered significant at $P \leq 0.05$.

TABLE 2. Pathological changes in chicken embryos exposed to Prudhoe Bay crude oil (PPCO), Experiment 1.

| | Dose of PBCO (μl) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 4 | 8 | 16 |
| Pathological changes | | | | | | |
| Hepatic necrosis | 0 (6)[a] | 1 (3) | 1 (5) | 5 (15) | 7 (24) | 6 (19) |
| Lymphoid atrophy in bursa of Fabricius | 1 (6) | 1 (3) | 2 (5) | 5 (15) | 5 (24) | 3 (19) |
| Subcutaneous edema | 0 (6) | 2 (3) | 0 (5) | 5 (15) | 0 (24) | 0 (19) |
| Malposition | 1 (6) | 0 (3) | 3 (5) | 3 (15) | 3 (24) | 1 (19) |

[a] Number of embryos affected (number examined)

## RESULTS

In Experiment 1, continuous embryo mortality was noted throughout the period after oil exposure, including during the hatching period in embryos known to have been alive on the eighteenth day of incubation. Mortality increased with increasing dose of PBCO (Table 1). The $r^2$ for regression analysis of total mortality to dose was 0.71 ($P = 0.022$). Hatchability and pipping rates decreased with increasing dose of PBCO (Table 1).

A small proportion of embryos that never pipped were malpositioned with the head at the narrow end of the eggshell (Table 2). Some embryos that never hatched had grossly visible, irregular pale zones at the margins of the liver which histologically corresponded to subcapsular multifocal to locally extensive areas of hepatic necrosis with mineralisation (Table 2). Large fluid-filled subcutaneous vesicles on the right dorso-caudal aspect were observed in 5% and 16% of embryos dosed with 1 μ and 2 μl of PBCO, respectively. The bursa of Fabricius from treated embryos had markedly depleted lymphoid tissue in the plicae with infolding of the lining epithelium and infiltration of interstitium by moderate numbers of heterophils (Table 2). Embryos that died within the eggshell had advanced autolysis which hampered assessment of lesions.

Weight gain in chicks during 3 wk posthatching could only be assessed in chicks from groups that received 0 to 4 μl PBCO (Table 3). It was slightly but significantly lower in chicks from eggs exposed to 4 μl of PBCO.

In Experiment 2, marked edema and hemorrhage in and around the pipping muscle from oil-exposed embryos was noted (Fig. 1a, b). Based on histological examination, these pipping muscles had only very sparse, multifocal segmental fragmentation and occasional vacuolation of mus-

TABLE 3. Body weights (g) of white leghorn chicks that hatched after exposure to Prudhoe Bay crude oil (PBCO) on day 9 of incubation, Experiment 1.

| | Dose of PBCO (μl) | | | |
|---|---|---|---|---|
| | 0 | 1 | 2 | 4 |
| Number successfully hatched | 46 | 36 | 23 | 19 |
| Stage of development | | | | |
| Week 1 | 46.4 ± 0.5[a] | 45.7 ± 0.7 | 44.0 ± 0.8 | 42.6 ± 0.9 |
| Week 2 | 103.2 ± 1.4 | 97.4 ± 1.5 | 96.9 ± 1.7 | 88.8 ± 3.0 |
| Week 3 | 172.7 ± 2.6 | 165.1 ± 2.6 | 171.8 ± 3.0 | 153.5 ± 3.6 |
| Mean weight gain (g) | 126.1 | 119.4 | 127.8 | 110.9[b] |
| Relative weight gain (%) | 100 | 95 | 101 | 88 |

[a] Mean ± SE.

[b] significantly ($P < 0.05$) different from control.

FIGURE 1. Pipping muscle. A control embryo is shown in Figure 1a; note less prominent pairs of pipping muscles with scant edema fluid. An oil-exposed embryo is shown in Figure 1b; note swollen dark pipping muscles with gelatinous material between the two muscle pairs and subcutaneous tissue. Scale is in mm.

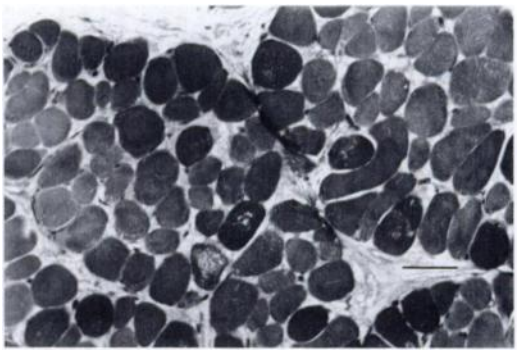

FIGURE 2. A cross section of 1b; occasional vacuolation of the sarcoplasm of muscle fibers is shown. H&E. Bar = 100 μm.

cle fibers (Fig. 2). No edema or hemorrhage were evident in breast and gastrocnemius muscles or heart, or in any muscle of control embryos. Mean pipping muscle wet weight and relative muscle weight (muscle weight/total body weight) were significantly ($P < 0.05$) higher in oil-exposed embryos than in the control group during pipping (Table 4). The mean relative weight of pipping muscle was significantly ($P < 0.05$) greater in oiled groups than in controls on day 18 of incubation, at pipping, at hatching, and at 5 days post-hatching (Table 4). Levels of serum CK were elevated in oil-treated embryos only at hatching (Table 5). The range of CK values was very wide in both groups. Subcapsular hepatic necrosis with mineralization observed in oil-exposed embryos in experiment 1 was also present in treated embryos in experiment 2 (Table 4). The lesions were still observable at the termination of the experiment, 5 days post-hatching.

## DISCUSSION

Exposure of chicken embryos to PBCO caused dose-related mortality throughout the period from the ninth day of incubation to hatching. Between 6 and 44% of total mortality in oil-exposed embryos occurred after day 18 of incubation, associated with failure to pip or to crack the shell post-pipping to enter the climax stage of hatching. A dose of PBCO greater than 1 μl caused significant ($P < 0.002$) reductions in total hatching success (chi-square test). This is in agreement with earlier reports about crude petroleum oil embryotoxicity (Lewis and Malecki, 1984). The low hatchability in oil-exposed chicken embryos that were alive on day 18 of incubation was caused in large part by mortality associated with failure to pip and with increased mortality after pipping. The highest mortality after pipping was in embryos exposed to 8 μl of PBCO, while 16 μl caused the highest percentage of unpipped, dead-in-shell embryos (Table 1).

Subcapsular hepatic necrosis observed in some embryos exposed to PBCO is in agreement with previous reports (Couillard and Leighton, 1989, 1990a, b) and still was evident 5 days post-hatching (Table 3).

The hatching process entails a series of complex and coordinated, embryological, biological, and mechanical events which must occur at the proper place and time during embryonic life for successful emergence from the shell (Oppenheim, 1973). Pre-hatching activities in chickens commence around day 16 of incubation with appearance of a behavioral pattern characterized by smooth, coordinated tonic movements (Oppenheim, 1973). For suc-

TABLE 4. Effect of Prudhoe Bay crude oil (PBCO) on pipping muscle wet weight (g) at various stages of chicken embryo development, Experiment 2. Embryos were exposed to PBCO on day 9 of incubation.

| Stage of development | PBCO (µl) | Number tested | Mean body weight (g) | Mean pipping muscle wet wt(g) | Mean relative pipping m.wt (g/g)[a] | Hepatic necrosis[b] |
|---|---|---|---|---|---|---|
| Day 18 | 0 | 10 | 29.1 ± 0.4[c] | 0.377 ± 0.010 | 0.013 ± 0.000 | 0 |
| | 6 | 10 | 28.2 ± 0.7 | 0.432 ± 0.020 | 0.015 ± 0.002 | 4 |
| At pipping | 0 | 10 | 38.4 ± 0.6 | 0.561 ± 0.040 | 0.015 ± 0.001 | 0 |
| | 6 | 10 | 37.8 ± 1.5 | 0.903 ± 0.080[d] | 0.024 ± 0.002[d] | 4 |
| Pipped/died | 0 | 10 | 38.4 ± 0.6 | 0.561 ± 0.040 | 0.015 ± 0.001 | 0 |
| | 6 | 10 | 39.1 ± 1.0 | 1.321 ± 0.060[d] | 0.034 ± 0.001 | 3 |
| At hatching | 0 | 10 | 39.6 ± 1.0 | 0.503 ± 0.030 | 0.013 ± 0.001 | 0 |
| | 6 | 10 | 38.7 ± 0.6 | 0.726 ± 0.010 | 0.019 ± 0.003[d] | 2 |
| 5 day post-hatching | 0 | 10 | 57.3 ± 2.7 | 0.167 ± 0.010 | 0.003 ± 0.000 | 0 |
| | 6 | 10 | 50.7 ± 1.5 | 0.183 ± 0.010 | 0.004 ± 0.000[d] | 1 |

[a] Muscle weight/total body weight.
[b] Number of embryos examined that had hepatic necrosis.
[c] Mean ± SE.
[d] Significantly ($P < 0.05$) different from control, $t$ test.

cessful hatching, an embryo positions the head towards the air cell (large end of the eggshell), tucks the head under the right wing, penetrates shell membranes to initiate pulmonary respiration, pips and cracks the egg shell, and ultimately enters climax phase which ends with emergence from the shell (Hamilton, and Willier, 1952; Oppenheim, 1973). Membrane penetration, pipping, and cracking of the egg shell during climax are achieved by the egg tooth on the beak and the pipping muscle (Brooks and Garrett, 1970). These two embryonic structures usually are at their maximum sizes between days 17 and 20 of incubation, just prior to pipping (Brooks and Garrett, 1970). Therefore, malposition of the head at the narrow end of eggshell or damage or dysfunction of the pipping muscle or egg tooth may impair pipping, initiation of pulmonary respiration, cracking of the eggshell and subsequent hatching.

TABLE 5. Serum creatine kinase concentration (IU/l) in chicken embryos and hatchlings exposed to Prudhoe Bay crude oil (PBCO) on day 9 of incubation, Experiment 2.

| | Number tested | Dose of PBCO (µl) | |
|---|---|---|---|
| | | 0 | 6 |
| Stage of development | | | |
| Day 18 | 10 | 3,043 ± 710[a] | 4,048 ± 744 |
| At pipping | 10 | 2,315 ± 690 | 4,717 ± 920 |
| At hatching | 10 | 1,692 ± 281 | 5,704 ± 1,862[b] |
| At 5 days post-hatching | 10 | 3,243 ± 811 | 1,924 ± 310 |

[a] Mean ± SE.
[b] Significantly ($P < 0.05$) different from the control group, $t$ test.

Severe edema and hemorrhage in the pipping muscle and overlying subcutaneous tissues were observed in oil-treated embryos that were alive on day 18 of incubation but which failed to hatch. The increase in wet weight of pipping muscles in oil-exposed embryos was attributable to this edema and hemorrhage. During normal hatching, the pipping muscle greatly enlarges from day 17 to 20 of incubation with mild physiological edema which cushions the muscle when pipping and cracking the eggshell (Rigdon et al., 1968; Ramachandran et al., 1969; Klicka and Kaspar, 1970). Necrosis in the pipping muscle of chicks has been associated with failure of otherwise normal embryos to hatch (Rigdon et al., 1968). The marked edema with hemorrhage, observed in oil-exposed embryos in this experiment, are evidence that this physiological process of enlargement and activity of the pipping muscle

was pathologically exaggerated in embryos exposed to oil.

Creatine kinase was elevated in oil-exposed embryos at hatching. However, there was wide variation in CK values within groups. Increases in serum CK levels commonly are associated with muscle injury (Anderson et al., 1976). However, the minimal muscle fiber degeneration observed histologically in these chicks at hatching may not have been sufficient to account for elevated CK values. Exercise and stress cause increases in serum CK levels in turkeys (*Meleagris gallopavo*) and lambs (*Ovis aries*) (Tripp and Schmitz, 1982; Boyd, 1983). Therefore, stress associated with increased but ineffective exertion of pipping muscles during hatching may explain the significant ($P < 0.05$) increase in CK levels noted only at hatching.

With the present study, we provide further evidence of the extreme toxicity of petroleum oil to avian embryos. High rates of mortality can occur during the hatching period in embryos that survive exposure to oil earlier in incubation. Our data do not support the hypothesis that failure to hatch is associated with degenerative changes in pipping muscle cells. The pathogenesis of failure to hatch in oil-exposed embryos remains unknown.

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