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EFFECTS OF PETROLEUM ON MINK APPLIED AS A MODEL FOR REPRODUCTIVE SUCCESS IN SEA OTTERS

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ABSTRACT: Ranch-reared mink (*Mustela vison*) were used as a model in an experimental trial to investigate the potential effects of exposure to two petroleum products on sea otters (*Enhydra lutris*). Mink were exposed either dermally on one occasion 60 days prior to breeding or via low level contamination of their diets daily from 60 days prior to breeding (January 1994) until weaning of kits (June 1994). For dermal exposure, we placed mink in either a slick of Alaskan North Slope crude oil ($n = 24$) or bunker C fuel oil ($n = 24$) on sea water or sea water alone ($n = 10$) for 1 min. For dietary exposure, we fed mink rations containing 500 ppm of either Alaskan North Slope crude oil ($n = 24$) or bunker C fuel oil ($n = 24$; control, $n = 15$). The number of liveborn kits did not differ significantly among mink exposed dermally (5.0 kits/female for crude oil and 6.5 kits/female for bunker C fuel oil) and unexposed controls (5.3 kits/female). However, only 2.3 and 0.7 kits were produced per female for those exposed through the diet to crude oil and bunker C fuel oil, respectively. Females with reduced reproductive success had no clinical signs of toxicosis or behavioral abnormalities. In addition, kits of females exposed through the diet had poor survival to weaning. Once mature, kits born to females exposed to bunker C fuel oil in the diet had significantly reduced reproductive success (3.4 kits/female) although their only exposure to the petroleum products was in utero or during nursing. Therefore, it is possible that sea otter populations consuming contaminated food sources or colonizing previously oiled habitats will have reduced reproductive success.

Key words: Contaminants, *Enhydra lutris*, experimental study, mink, *Mustela vison*, oil, petroleum, reproduction, sea otters.

INTRODUCTION

While the immediate effects of crude oil in the sea otter's (*Enhydra lutris*) environment have been illustrated by the Exxon Valdez incident in Alaska (USA) in March 1989, potential effects of petroleum product exposure on the reproductive performance of sea otters have been more difficult to evaluate from field observations. Survival of sea otter pups in oiled areas of Prince William Sound (Alaska) was lower than in non-oiled areas between 1990 and 1993 (Monnett and Rotterman, 1992; Ballachey et al., 1994). In addition, the overall survival of pups in both oiled areas and areas not directly oiled was lower after the spill in 1990–91 than in 1992–93 (Ballachey et al., 1994). However, by comparing ratios of adult sea otters to pups in oiled and non-oiled areas of Prince William Sound, researchers concluded that the production of pups by adult female sea otters was not diminished by oil exposure (Bodkin and Udevitz, 1992). Unfortunately, it is very difficult to assess reproductive outcomes in individual animals in large-scale field studies without the use of radio telemetry for detailed monitoring. Therefore, it is not certain whether the reproductive rates in adult females from oiled and non-oiled environments were similar because oiling had no effect, or whether some other factors were compensating to keep the population at carrying capacity. For example, females not exposed to oil in the environment could have exploited reproductive territories of females which died from toxicity or were no longer reproductively active due to toxic effects of exposure. The results of these field studies

and the limited data on the reproductive effects of sublethal doses of petroleum products on mammals, prompted the present investigation.

Because it is very difficult to accurately assess the reproductive effects of petroleum products on numerous sea otters in a field situation and such an evaluation depends upon an environmental accident to occur with all research protocols and permits in place, we designed a clinical trial using mink as an experimental animal model for sea otters. Characteristics of mink (*Mustela vison*) which enhance its usefulness as an experimental model for the sea otter include: mustelid family member; high metabolic rate; intense grooming behavior; semi-aquatic nature; utilization of a diverse group of prey items; and known susceptibility to a variety of environmental contaminants (Wren, 1991). In addition, the mink model has been utilized by Natural Resource Damage Assessment researchers investigating the effects of the Exxon Valdez incident on reproduction (Bickham et al., 1998).

The objectives of this study were to evaluate the reproductive effects of two petroleum products in females via an acute dermal exposure to environmental oil and in a second group of females via chronic, low-level contamination of their food. We hypothesized that the exposure of females only through their food would simulate the recolonization of previously oiled environments by sea otters which had not been directly exposed to petroleum products. Alaskan North Slope crude oil (ANS crude oil) and bunker C fuel oil were chosen as exposure products because they are commonly transported or used as fuel in the Pacific.

MATERIALS AND METHODS

Standard, ranch-type mink were randomly selected from their age-class in the commercial breeding colony for use in these experimental trials; all animal housing, husbandry, and treatment was approved by and in compliance with the United States Department of Agriculture (Pullman, Washington, USA) and our animal health and welfare protocol for humane animal use. Mink were individually housed in wire mesh cages with attached solid-wall nest boxes in sheltered, open-air enclosures. The randomized clinical trials consisted of groups of mink dermally exposed to either ANS crude oil or bunker C fuel oil, groups exposed through the diet to both petroleum products, and a control group for each trial. The investigation into the reproductive impairment was part of a large-scale study investigating the multi-systemic effects of petroleum product exposure in mink as a model for sea otters. The dermal trial initially included 58 mink, 24 of which were exposed to crude oil in sea water, 24 of which were exposed to the fuel oil in sea water, and 10 controls which were exposed to sea water alone. Sample size selection was based on detecting a 45% difference between mean litter sizes of affected and unaffected groups (Vetstat Statistical Software, Davis, California, USA). All animals moved freely for 1 min in the oil/sea water (or sea water alone) solution (500 ml oil to 4 L sea water which resulted in an oil slick of approximately 1.5 cm in depth). The mink were exposed 60 days prior to breeding to allow adequate blood, fur, and skin samples to be collected bimonthly prior to breeding. In order to simulate environmental exposure, the oil was not removed from the dermally exposed mink. Instead the oil weathered on the mink and was ingested by the mink during grooming. The total number of females bred was reduced to 33 ($n = 8$, crude oil; $n = 15$, fuel oil; $n = 10$, control) due to the attrition from the toxic effects of acute exposure ($n = 11$ due to crude oil; $n = 7$ due to fuel oil) and sampling protocols for the multi-systemic study which required euthanasia and harvesting of organs at specified intervals throughout the study ($n = 1$ at 1 wk, $n = 1$ at 2 wk, $n = 3$ at 6 wk after crude oil exposure; $n = 1$ at 1 wk, $n = 1$ at 6 wk after fuel oil exposure). Once bred, females were not subjected to sampling protocols because we hypothesized that the stress of disturbance might affect reproductive outcomes.

The ingestion trial initially consisted of 63 mink which we randomly allocated to treatment groups: 24 were fed ANS crude oil, 24 were fed bunker C fuel oil, and 15 were controls. To the diet prepared for the commercial mink production colony, we added either ANS crude oil or bunker C fuel oil to a final concentration of 500 ppm. This concentration was selected to simulate the level of exposure that might result from sea otters ingesting contaminated invertebrates in a previously oiled environment (Hartung, 1995). Diets were prepared every 4 wk, and the feed was frozen in aliquots amounting to a 2 day supply and thawed as

needed. According to protocols established by the United States Environmental Protection Agency (Corvallis, Oregon, USA), dietary exposure began 60 days prior to breeding and continued through weaning of the resulting kits (Ringer et al., 1992). Mink were fed *ad libitum* throughout the exposure period. As in the dermal trial, females were not subjected to sampling protocols after breeding, and the number of females eligible for breeding was reduced to 59 (n = 22, crude oil; n = 22, fuel oil; n = 15, control) due to attrition (n = 1, crude oil; n = 1, fuel oil) and the sampling requirements of the large-scale, multi-systemic study which required euthanasia (n = 1 at 6 wk after initiation of the crude oil diet; n = 1 at 1 wk after the initiation of the fuel oil diet).

Females were bred in March 1994, according to standard mink husbandry practices (Joergensen, 1985) using male mink which had proven to be successful breeders. Briefly, female mink were placed with males on 2 consecutive days and then again 1 wk later for 2 consecutive days. Female mink were observed daily for signs of whelping. Once whelping commenced, females were observed throughout the day, and the number of liveborn and stillborn kits in the litter was recorded by the colony manager. When observing reproductive outcomes, the colony manager was unaware of treatment status. Reproductive outcomes measured included number of females bred, gestation length, number of females whelping, number of liveborn kits, number of stillborn kits, number of liveborn kits surviving 21 days, and number of liveborn kits surviving to weaning. Crossfostering of kits post-whelping was not practiced. Kits were weaned at 6 wk of age, and dams were humanely sacrificed at weaning for postmortem examination. Conception in females which bred but did not whelp was determined by evidence of uterine implantation sites on gross pathological examination. To confirm gross observations of implantation sites in non-gravid mink, histologic sections of ovaries, salpynx, and uterus were examined blindly from six mink that bred and did not whelp but were suspected to have been pregnant based on gross examination. Sections from three positive controls (bred and whelped) and three negative controls (never bred) were also evaluated similarly. Evidence for implantation sites, based on data from mink, included changes in mucosal contour or thickness, presence of hemosiderin-laden macrophages in the mucosal stroma, and hyalinization of mucosal and mural arteries (Backlin and Bergman, 1995; McEntee, 1990).

Kruskall Wallis one-way analysis of variance (KWANOVA) and pairwise comparison procedures (BMDP Statistical Software, Los Angeles, California, USA) were used to test for differences among means of gestation length, number of liveborn kits, number of kits born dead, number of liveborn kits surviving 21 days, and number of liveborn kits surviving to weaning. We combined ingestion and dermal control groups (n = 25) because pairwise comparisons indicated no difference ($P > 0.10$) in mean number of liveborn kits per female bred, mean number of liveborn kits surviving 21 days, and mean number of liveborn kits surviving to weaning among the two groups. We used chi-square contingency tables (Epi Info, Version 6, Atlanta, Georgia, USA) to compare the proportions of eligible females which bred among exposure groups and the proportions of breeding females which whelped among exposure groups. To determine the representativeness of our study population, we compared the average number of liveborn kits per litter in the overall breeding mink colony (n = 2,200 breeding females) to those of the controls.

To assess the possible carryover effects of petroleum exposure on the second generation, 83 female kits which were produced from this trial were bred in March 1995 to unexposed males from the colony which had sired offspring in the previous year. These included only 11, 12, and five female kits from the dermal exposure ANS crude oil, ingestion exposure ANS crude oil, and ingestion exposure bunker C fuel oil groups, respectively, because these numbers represented the total number of female kits surviving in these groups. Forty female kits were randomly selected for breeding from dams in the control group, and 15 female kits from the dermal exposure bunker C fuel oil group were randomly selected also. Reproductive outcomes measured included number of females bred, number of females whelping, number of liveborn kits, number of kits born dead, and number of liveborn kits surviving to weaning. KWANOVA and chi-square analysis were used to test for differences in means and proportions, respectively, as previously described.

RESULTS

The significant results of this reproductive trial in mink exposed to petroleum products are summarized in Table 1. Fewer females exposed to bunker C fuel oil through their diets whelped than did the other females ($P < 0.05$). On gross pathologic examination, all ingestion exposure females (both ANS crude oil and bunker C fuel oil groups) which had bred but not

TABLE 1. The effects of experimental petroleum product exposure on the reproductive performance of mink.

Exposure group	Females exposed (*n*)	Females surviving to breeding (*n*)	Eligible females bred (*n*)	Females whelping (*n*)	Liveborn kits per female (mean)	Kits surviving 21 days per female whelping (mean)	Kits surviving to weaning per female whelping (mean)
Control	25	25	25	22A[a]	5.3A[b]	4.9A[b]	4.6A[b]
Dermal-crude oil	24	8	8	8A	5.0A,C	4.3A,B	4.3A,B
Dermal-bunker C	24	15	15	15A	6.5A	5.1A	5.0A
Ingestion-crude oil	24	22	22	17A	2.3B,C	1.9B	1.7B
Ingestion-bunker C	24	22	20	7B	0.7B	1.8A,B	1.8A,B

[a] Proportions of females whelping represented in this column differ ($P < 0.01$, chi-square contingency tables) if they do not share a common letter.

[b] Means within a column that do not share a common letter differ ($P < 0.05$, KWANOVA and pairwise comparisons).

whelped showed evidence of past pregnancy and implantation. Evidence of implantation was confirmed histologically in sections from five of the six mink examined.

Compared with the controls ($\bar{x} = 5.3$ liveborn kits), the average number of liveborn kits per breeding female was reduced ($P < 0.05$) by about 57% and 87%, respectively, for the mink exposed to ANS crude oil and bunker C fuel oil in their diets. While both ingestion exposure groups had statistically lower means ($P < 0.05$) than those of the control group and mink exposed dermally to bunker C fuel oil (5.3 and 6.5 liveborn kits/female bred, respectively), the dermal exposure ANS crude oil group mean (5.0 liveborn kits/female bred) did not differ significantly ($P > 0.10$) from the ingestion ANS crude oil group (2.3 liveborn kits/female bred). The average number of kits surviving to 21 days of age per litter (1.9) and the average number of kits surviving to weaning per litter (1.7) were lower ($P < 0.05$) for the mink exposed to ANS crude oil through their diets than those of the control group and dermal bunker C fuel oil group (4.9 and 5.1 kits surviving 21 days/litter and 4.6 and 5.0 kits surviving to weaning/litter, respectively). While the average number of kits surviving 21 days (1.8) and the average number surviving to weaning (1.8) for the mink exposed to bunker C fuel oil in their diets were low, they did not differ significantly from the other means because of the low number of females whelping in this group ($n = 7$) and in the dermal crude oil group ($n = 8$). The mean number of liveborn kits per female bred, number of kits surviving to 21 days of age, and number of kits surviving to weaning did not differ ($P > 0.10$) among the control and dermal exposure groups. No statistical difference ($P > 0.10$) was observed among mean gestation lengths and average number of stillborn kits per litter for any of the exposure groups. Control group dams (4.9 kits/litter at 21 days of age, $n = 22$ whelping females) had almost identical number of kits per whelping female as in the mink colony (4.9 kits/litter, $n = 1182$ whelping females). This comparison provides evidence that our trial control group was representative of other females in the colony. In addition, body weights among exposure groups did not differ from the controls at the completion of the trial or from the average weight of similarly aged females in the mink colony ($P > 0.10$).

The significant results of the reproductive trial in the offspring of mink exposed to petroleum products are summarized in Table 2. The proportions of females bred and the proportions of females whelping did not differ among exposure groups ($P > 0.10$). Compared with the control group (6.4 kits/female bred) and dermal exposure ANS crude oil group (6.4 kits/female bred), the average number of liveborn kits

TABLE 2. The reproductive performance of offspring of mink experimentally exposed to petroleum products.

Exposure group of dam	Females eligible for breeding (*n*)	Females bred (*n*)	Females whelping (*n*)	Liveborn kits per female bred (mean)	Kits surviving to weaning (mean)
Control	40	40	38	6.4A[a]	5.6A[a]
Dermal-crude oiil	11	11	11	6.4A	5.1A
Dermal-bunker C	15	15	14	5.0A,B	4.2A
Ingestion-crude oil	12	11	9	4.4A,B	4.7A
Ingestion-bunker C	5	5	4	3.4B	3.0A

[a] Means within a column that do not share a common letter differ ($P < 0.05$, KWANOVA and pairwise comparisons).

per female bred was about 46% lower for the mink exposed to bunker C fuel oil in their diets (3.4 kits/female bred; $P < 0.05$). The means of this variable for the control, dermal exposure, and ingestion exposure ANS crude oil groups did not differ. No difference ($P > 0.10$) in the mean number of kits surviving to weaning among the exposure groups was observed.

DISCUSSION

We attempted to select a level of petroleum product exposure for the ingestion trial which would be similar to the polycyclic aromatic hydrocarbon (PAH) concentrations found in invertebrates after an oil spill incident. Using data collected on total PAH concentrations in mussel tissue after the Exxon Valdez incident, Hartung (1995) estimated total oil concentrations in mussels from some areas of the spill path to exceed 1,250 ppm in 1989 and to remain close to that value in 1990. Selection of the 500 ppm total oil dietary concentration was intended to simulate the PAH level detected in the mussels more than a year after the Exxon Valdez oil spill. The mink were fed this diet for less than 6 mo, so we believe that the selected petroleum concentration and duration of exposure were appropriate for the trial. Although the mink were fed *ad libitum,* the average daily consumption of 130 g of feed per mink resulted in a total daily dose of approximately 0.065 g/kg body weight of mink, which is 15 to 150 times lower than previously published studies investigating the effects of petroleum products on mammals (Leighton, 1990; Hartung, 1995). Even four years after the Exxon Valdez oil spill, Boehm et al. (1996) detected weathered oil in contaminated mussel beds which would result in a daily sea otter consumption of 0.019 g/kg body weight. While the oils used in this trial were not intentionally weathered, the dose was conservatively based on detectable PAH concentrations in prey species. Future investigations may concentrate on the differences between similar concentrations of PAHs in diets which contain weathered versus not intentionally weathered petroleum products.

Because delayed implantation is a characteristic of both mink and sea otter reproductive systems (Kenyon, 1969; Joergensen, 1985), we hypothesized that the length of gestation would be protracted for mink which had experienced a significant stressor such as petroleum product exposure. Instead we found that mink exposed through their diet to petroleum products, especially bunker C fuel oil, bred and became pregnant at a normal rate, but were less successful maintaining their pregnancies, produced fewer kits per female, and had decreased survival among liveborn kits. Histologic confirmation of implantation in five of the six animals examined which bred but did not whelp strengthens these conclusions. Conception in the sixth animal was suspected due to the gross evidence of a fluid distended uterus with dark focal areas suggestive of implantation sites. It is possible that these implantation

sites were not included in the sections examined histologically.

Reproductive failure post implantation in most species can have a variety of infectious and noninfectious causes including toxic exposures. Toxins causing reproductive failure often have their effect on the endocrine system or through interference with critical compounds in metabolic pathways, such as retinol. Ingestion of one PAH, benzo[a]pyrene, resulted in distribution to the liver, lungs, kidneys, and gonads of rats (Yamazaki and Kakiuchi, 1989) and has been shown to reduce reproductive success in both rats (Rigdon and Rennels, 1964) and mice (Mackenzie and Angevine, 1981). Subsequent studies by our laboratory are attempting to elucidate the mechanism of action on the reproductive system with special attention being paid to the possibility of PAH binding and endocrine disruption at the aryl hydrocarbon receptor.

The reduced number of kits produced per breeding female in the offspring of mink exposed to bunker C fuel oil through their diets was a very interesting finding. These female offspring were only exposed to the petroleum product transplacentally or via milk from the exposed dam. The female offspring had been weaned from their exposed dams and therefore had not even been indirectly exposed to petroleum products for more than 9 mo before breeding and 11 mo before whelping. The lack of statistical difference ($P > 0.10$) in the proportion of bred females which whelped suggests a reduced dose effect or a potential difference in the pathogenesis of reproductive impairment between the directly exposed group and the offspring, since females directly exposed to bunker C fuel oil in their diets whelped less frequently than the other females directly exposed and controls ($P < 0.05$). Reproductive failure due to genotoxicity seems more plausible in the offspring (Bickham et al., 1998) given the lack of direct exposure and thus implausibility of direct toxic effects. Because both generations were bred to males which were known to have sired many litters, were given ample opportunity to breed, and were managed by the same husbandry and breeding staff, study design bias appears to be an unlikely explanation for the difference in reproductive outcomes between generations. Continued investigation into the potential hormonal and genetic etiologies of reproductive impairment across generations is warranted.

Unfortunately, it was not possible to directly assess the levels of PAH in the tissues of mink exposed in this study or their offspring using gas chromatography coupled with a mass spectrometry (levels not detectable; data not shown). Polycyclic aromatic hydrocarbons are extremely difficult to detect in mammalian tissues at exposure levels relevant to environmental contamination. While this large-scale study was successful in developing a rapid, inexpensive technique for detecting PAH residues on mammalian hair (Mazet et al., 1997), initial efforts to improve detection limits of existing techniques and develop new techniques for detecting PAHs and their metabolites in blood and liver were not successful. A promising recombinant cell bioassay for quantifying exposure has now been optimized, and analysis of PAH in mink sera, as well as the induction of cytochrome P450 in liver, is in progress to further the utility of this mink model for sea otters (Ziccardi et al., 2000).

Results of this study may help to explain the reduced survival of sea otter pups in oiled areas of Prince William Sound that occurred after the Exxon Valdez incident (Ballachey et al., 1994). At the very least, the association between oil exposure and reduced survival in sea otters has been strengthened. Without such clinical trials, it is very difficult to establish and confirm causal relationships.

Our findings also indicate that the reproductive success of sea otters exposed to petroleum products through their diet and/or for long periods of time may be reduced. Even if not exposed directly to environmental oil pollution, individuals re-

colonizing previously oiled environments may be affected. Therefore, sea otter populations may be affected both directly due to the mechanical and toxic properties of oil and indirectly through reduced reproduction and survival of pups. This information becomes more important when considering a population which is below carrying capacity, such as the threatened southern sea otter (*Enhydra lutris nereis*) population of California.

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