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FUEL OIL–INDUCED ADRENAL HYPERTROPHY IN RANCH MINK (*MUSTELA VISON*): EFFECTS OF SEX, FUEL OIL WEATHERING, AND RESPONSE TO ADRENOCORTICOTROPIC HORMONE

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ABSTRACT: Environmental contamination by petroleum hydrocarbons from anthropogenic sources can be a cause of stress for free-ranging wildlife. The response of wildlife to chemical contaminants requires that the hypothalamic–pituitary–adrenal (HPA) axis be precisely regulated to allow for proper glucocorticoid-mediated adaptive responses. Chronic oral exposure to low concentrations of bunker C fuel oil causes the development of adrenal hypertrophy in male ranch mink (*Mustela vison*) without increasing serum or fecal glucocorticoid concentrations. This hypertrophy is an adaptive response to fuel oil-induced adrenal insufficiency. To determine if the same phenomenon occurs in female mink or male mink exposed to artificially weathered fuel oil, female mink were fed 0 ppm (mineral oil) or 420 ppm fuel oil and male mink were exposed to 0 ppm, 420 ppm fuel oil, or 480 ppm artificially weathered fuel oil in the diet for 60–62 days. At the end of the exposure, serum glucocorticoid concentrations were assayed along with body and organ weight measurements. Fecal glucocorticoid concentrations were assayed at time points throughout the exposure. Male mink fed fuel oil or weathered fuel oil and female mink fed fuel oil had adrenal enlargement without any significant increases in the serum or fecal concentration of glucocorticoids, which is consistent with fuel oil–induced adrenal insufficiency. To address the physiological consequences of adrenal insufficiency, fuel oil–exposed male mink were administered an adrenocorticotropic hormone (ACTH) stimulation test. Fuel oil–exposed animals had a smaller incremental increase in serum glucocorticoid concentration after ACTH challenge compared to control animals. Our findings provide further evidence that the HPA axis of fuel oil–exposed animals is compromised and, therefore, not able to respond appropriately to the diverse stressors found in the environment.

Key words: Adrenal insufficiency, bunker C fuel oil, glucocorticoid, *Mustela vison*, petroleum oil, ranch mink, stress response.

INTRODUCTION

The adrenal gland is a primary stress-response organ and the site of the synthesis and secretion of glucocorticoids, which are major mediators of the stress response. This gland is part of the hypothalamic-pituitary-adrenal (HPA) axis, which has an important role in restoring physiological systems to normal homeostasis after stressful events. In addition, glucocorticoids are important in regulating normal metabolism, reproduction, cardiovascular tone, and immunologic defense mechanisms (Sapolsky et al., 2000).

Sources of stress in wildlife are varied and can originate from physical, chemical, and behavioral disturbances. Individual animals or populations of animals may have to react to different stressors simultaneously. Chemical contamination of the environment is considered a common type of stressor to wildlife because many habitats are persistently contaminated with halogenated hydrocarbons, polyaromatic hydrocarbons (PAHs), and heavy metals from human activities (Walker, 2001).

In the coastal marine environment, petroleum oil is an important source of environmental contamination. The source of petroleum hydrocarbons may originate from both marine- and land-based anthropogenic activities in addition to natural seeps. One type of petroleum product that

is often spilled into the marine environment is bunker C fuel oil (fuel oil). This is a refined, heavy oil that is used to power ships and is also transported along marine routes. Compared to other petroleum products, fuel oil has higher concentrations of PAHs (Irwin et al., 1998), which have been shown to be immunotoxins, carcinogens, and endocrine disruptors (Anderson et al., 1974; Santodonato, 1997). Because of its physical and chemical characteristics, it is slow to degrade and may persist in marine environments relatively unchanged for years (Vandermeulen and Singh, 1994; Irwin et al., 1998). The effects on animals from chronic exposure to low concentrations of petroleum hydrocarbons lingering in contaminated environments are not well understood because the physiologic changes tend to be sublethal and do not outwardly appear to have deleterious effects on exposed animals. However, animals living in habitats with chemical contamination may have altered physiological responses to other risk factors, e.g., infectious agents, concurrently present in the same environment.

We have observed that chronic oral exposure to fuel oil causes the development of adrenal hypertrophy in male ranch mink (*Mustela vison*) (Mohr et al., 2008). Mink are important environmental sentinels for toxic chemicals (Basu et al., 2007) and are also used as a surrogate species for sea otters (*Enhydra lutris*) that live in the near-shore marine environment and are at risk for exposure to petroleum hydrocarbon contaminants in the environment. An unexpected finding was that the development of adrenal hypertrophy in fuel oil–exposed mink was not associated with increases in either serum or fecal concentrations of glucocorticoids (Schwartz et al., 2004; Mohr et al., 2008). This would suggest that adrenal hypertrophy is an adaptive response to fuel oil–induced adrenal insufficiency (Hinson and Raven, 2006). One question that warrants further investigation is whether animals that are chronically exposed to low, environmentally relevant concentrations of fuel oil are able to adapt to other environmental stressors by responding with an appropriate release of glucocorticoids. It is important to understand the physiologic changes that impact the health and fitness of wildlife during stressful conditions, and this can best be accomplished by controlled experiments with wildlife surrogates, such as ranch mink.

The objective of this study was to determine the response of fuel oil–exposed and control mink to the model stressor adrenocorticotropic hormone (ACTH) by measuring changes in glucocorticoid concentrations before and after its administration. ACTH is released by the pituitary gland upon stimulation of the hypothalamus by stressors, and it stimulates the synthesis and release of adrenal steroids, e.g., glucocorticoids, into the blood. It also acts as a growth factor for the adrenal gland and is a major regulator of adrenal size. Because our previous fuel oil exposure studies were done with male mink, another objective was to determine if female animals also develop adrenal hypertrophy after chronic fuel oil exposure. In addition, we were interested in determining if oral exposure to artificially weathered fuel oil caused adrenal-function effects similar to those observed for unweathered fuel oil.

MATERIALS AND METHODS

Animals and experimental design

Ranch mink were born and raised at the Experimental Fur Farm at Michigan State University and were housed indoors in individual cages. The protocol for this study was approved by the Michigan State University Institutional Animal Care and Use Committee. In January, a 60–62-day feeding study with fuel oil began with 48 male and 24 female ranch mink, all 8 mo of age at the beginning of the exposure. The animals were weighed and equal numbers of male (18/18) and female (12/12) animals were randomly placed in one of two groups: control and fuel oil exposed. The petroleum product originated from British

Petroleum's Cherry Point Refinery (Blaine, Washington, USA). Fuel oil was mixed into a standard ranch feed at a targeted concentration of 500 ppm. This concentration corresponds to a concentration of petroleum hydrocarbons that would be present in the environment for a prolonged period of time after an oil spill (Mazet et al., 2000). The animals in the control group (0 ppm) were fed an equivalent amount (500 ppm) of mineral oil (Squibb and Sons, Inc., Princeton, New Jersey, USA) mixed into the food. In addition, 12 male mink of similar age were placed in a group that was fed 500 ppm artificially weathered fuel oil. Artificially weathered fuel oil was prepared by adding approximately 400 ml of fuel oil to 3 l of natural seawater. The mixture was stirred uncovered for 10 days at room temperature (Ormseth and Ben-David, 2000). All feed was divided into lots sufficient for 1 day's feeding and stored at −20 C. Samples of feed at the beginning and end of the exposure were analyzed to verify the concentrations of fuel oil and weathered fuel oil added to each group and to assay for organochlorine contaminants. The mean concentrations of fuel oil and weathered fuel oil mixed in the feed were determined to be 420 and 480 ppm, respectively. The feed did not contain any organochlorine contaminants. The animals were not disturbed for the entire exposure period except for one daily feeding. The lights were on a light cycle that followed the natural photoperiod during the exposure period. The mean room temperature was 8.9 C with a range from 4.5 C to 12.2 C. Once every 2 wk metal grates were put underneath the individual cages at 8:30–9:00 AM. Feces were collected from every animal at approximately 6:00 AM and stored frozen (−20 C) for subsequent determination of the fecal cortisol concentration.

At the end of the exposure all mink were anesthetized with an intramuscular (IM) injection of the combination of 22 mg/kg ketamine (Fort Dodge Animal Health, Fort Dodge, Iowa, USA) and 2 mg/kg xylazine (Ben Venue Laboratories, Bedford, Ohio, USA). Whole blood was collected by jugular or anterior vena cava venipuncture from every animal. The serum was used for assay of glucocorticoid concentration. After the initial blood sample was drawn, 12 animals from the fuel oil–exposed group and 12 animals from the control group were injected IM with 50 μg/ml of cortrosyn (Amphastar Pharmaceuticals Rancho Cucamonga, California, USA), an active synthetic subunit of ACTH. In addition, six other animals from both groups were injected (IM) with 0.9% NaCl (saline) and served as non-ACTH stimulated controls. At 90 min after injection of cortrosyn or saline, animals were anesthetized and a second blood sample was collected for determination of the post-ACTH serum glucocorticoid concentration.

After the blood collection, mink from all three groups were euthanized by CO_2 asphyxiation (Beaver, 2001). The animals were weighed and complete necropsies, including body and organ weight measurements, were performed. Tissues (brain, pituitary, thyroid, heart, lung, kidney, urinary bladder, liver, stomach, small intestine, colon, spleen, mesenteric lymph node, adrenal, testis, epididymis, prostate, ovary, uterus) were preserved in 10% buffered formalin and later processed and stained with hematoxylin and eosin for microscopic evaluation. Adrenal cortical cells were observed to have either cytoplasm with fine vacuolation that did not increase the size of the cells or had cytoplasm with excessive vacuolation that rarified the cytosol and increased cell size (clear cells). The extent of coverage by clear cells of the inner two zones of the adrenal cortex (zona fasciculata and reticularis, sites sensitive to the effects of ACTH and responsible for glucocorticoid synthesis) was graded as either minimal (approximately <33%) or extensive (approximately >33%) coverage.

Glucocorticoid assays

Serum concentrations of glucocorticoids were measured in duplicate by competitive immunoassay (Bayer Diagnostics ADVIA ACS-180 Automated Chemiluminescence System, Norwood, Massachusetts, USA) by a method previously validated by our group (Mohr et al., 2008). The specificity for cortisol, the major glucocorticoid of mink (Aulerich et al., 1999), and corticosterone was 100% and 2.8%, respectively. Fecal glucocorticoid was extracted and measured in duplicate with the use of the same competitive immunoassay platform (Schwartz et al., 2004). Collection of feces to quantify glucocorticoid concentrations allows samples to be obtained without stressing the animal. This is particularly important when multiple samples are taken over time from individual animals.

Statistics

Data for presentation were shown as mean values ±SE. Organ weights were expressed as mean relative weights (percent of body weight). The ACTH-stimulation tests were expressed as delta-glucocorticoid concentrations, which represented changes in glucocor-

TABLE 1. Mean ± (SE) body weights, relative combined adrenal weights, and serum glucocorticoid (GC) concentrations in fuel oil–exposed, weathered fuel oil (WFO) –exposed, and control mink.

	Male			Female	
	Control	Fuel oil	WFO	Control	Fuel oil
Body weight (g)	2,272.0 (33.6)	2,165.0 (55.4)	2,175.0 (57.4)	1,221.0 (43.4)	1,215.0 (43.4)
Adrenal weight (%)	0.0057 (0.00016)	0.0065 (0.00026)[a]	0.0061 (0.00025)[a]	0.0063 (0.00036)	0.0069 (0.00045)[a]
Serum GC (ng/dl)	0.71 (0.22)	0.99 (0.40)	0.57 (0.21)	0.73 (0.30)	0.50 (0.19)

[a] Significant differences ($P<0.05$) between control and fuel oil–exposed groups within each sex.

ticoid concentrations before and after cortrosyn or saline injection. Data were analyzed by a mixed-model analysis of variance with fixed effects for sex, exposure group and time, and random subject effects. Post hoc comparisons among the different groups were based on least-squared means (LSMEANS). Data analysis for the ACTH stimulation tests was performed with a mixed-model ANOVA (proc mixed) in which the systematic effects were the exposure group and pre-/post-saline/ACTH injection (SAS 9.1, SAS Institute, Inc., Cary, North Carolina, USA). Results were considered statistically significant whenever $P<0.05$.

RESULTS

Mean body weights were similar with no significant difference among the male mink exposed to both types of fuel oil and mineral oil (control) and also between the female fuel oil-exposed and control groups ($P=0.34$; Table 1). Male and female mink exposed to either fuel oil or weathered fuel oil (male only) had significantly increased relative weights of the combined adrenal glands compared to the control animals ($P=0.04$). For male mink, the mean relative weights of the adrenals were increased 14% and 7% over the control in the fuel oil– and weathered fuel oil–exposed groups, respectively. For female mink there was a 10% increase in mean relative weight of the adrenal glands in the fuel oil–exposed group compared to the controls. Microscopic examination of the adrenal glands showed extensive accumulation of clear cells in the zona fasciculata and reticularis in 6% of the control, 39% of the fuel oil–exposed, and 42% of the weathered fuel oil–exposed male mink. For female mink extensive coverage of the two inner cortical zones with clear cells was 9% and 33% of the control and fuel oil–exposed animals, respectively. Serum glucocorticoid concentrations were measured from every animal in each group (Table 1). Within each sex no significant differences were found between the control and fuel oil (male: $P=0.78$; female: $P=0.50$) or weathered fuel oil (male: $P=0.33$) groups. In addition, there were no significant differences in serum glucocorticoid concentrations between the fuel oil and weathered fuel oil groups ($P=0.47$).

Fecal glucocorticoid concentrations did not differ significantly between the control and both fuel oil groups over the time course of the exposure for the male ($P=0.55$, Fig. 1) and female ($P=0.08$, Fig. 2) mink. Female mink had significantly less glucocorticoid in their feces compared to the males ($P<0.0001$).

ACTH stimulation tests were performed at the end of the exposure on male mink fed fuel oil or mineral oil (Fig. 3). The change in serum glucocorticoid concentration between pre- and postinjection (delta-glucocorticoid concentration) of either saline or cortrosyn was determined for each animal. Mean delta glucocorticoid concentrations were not significantly different in animals from both the control and fuel oil–exposed groups that received saline injection ($P=0.68$). There was a much larger increase in delta-glucocorticoid concen-

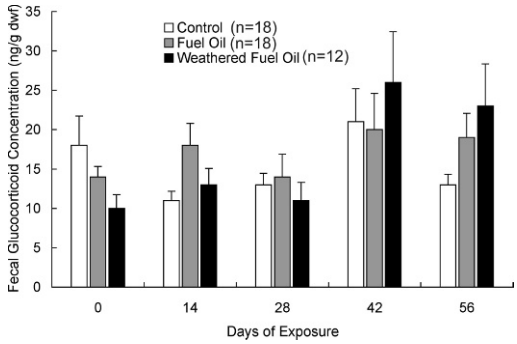

FIGURE 1. Mean ± SE fecal glucocorticoid concentrations in male mink exposed to mineral oil (control), 420 ppm fuel oil, or 480 ppm artificially weathered fuel oil in the diet for 60–62 days. Feces were collected on the stated days of exposure and assayed for glucocorticoids. Fecal glucocorticoid concentrations, reported as nanograms per gram dry weight feces (dwf). There were no significant differences among the groups.

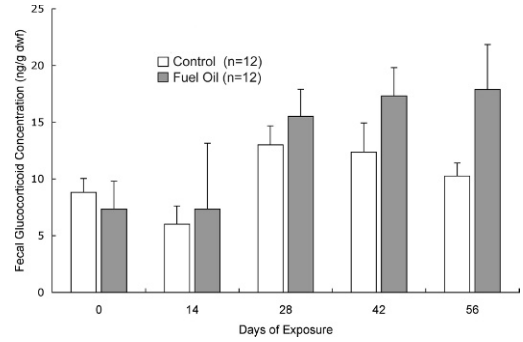

FIGURE 2. Mean ± SE fecal glucocorticoid concentrations in female mink exposed to mineral oil (control) or 420 ppm fuel oil in the diet for 60–62 days. Feces were collected at the stated days of exposure and assayed for glucocorticoids. Fecal glucocorticoid concentrations, reported as nanograms per gram dry weight feces (dwf). There were no significant differences between the groups.

trations in animals from both groups that were injected with cortrosyn (6.5-fold and 3.2-fold change in mean delta-glucocorticoid concentrations of ACTH-injected animals over the mean delta-glucocorticoid concentrations of saline-injected animals for the control and fuel oil–exposed groups, respectively). Importantly, there was a significant difference in delta glucocorticoid concentrations between the control/cortrosyn injected and fuel oil–exposed/cortrosyn injected animals with a 29% decrease in delta glucocorticoid concentration in the fuel oil–exposed group compared to the control group ($P=0.04$).

DISCUSSION

We have previously demonstrated that chronic, oral exposure to an environmentally relevant concentration of bunker C fuel oil causes adrenal hypertrophy in male ranch mink (Mohr et al., 2008). Unique to the study reported here, we have now expanded our observations to include female mink exposed to fuel oil and male mink exposed to artificially weathered fuel oil. In all cases, fuel oil exposure was associated with the enlargement of the adrenal glands at the end of the study. An animal's sex is known to influence responses to xenobiotics in some animals (Burger et al., 2007) because of differences in metabolism (Gochfeld, 2007). Therefore, it is important to determine if the same effects on the adrenal gland can be measured in female animals after exposure to fuel oil. Furthermore, differences in sex effects have relevance for fuel oil toxicity because this petroleum product is known to have endocrine (Mohr et al., 2008) and reproductive effects (Mazet et al., 2001). Similarly, it is important to determine if fuel oil–induced adrenal hypertrophy is also observed in animals exposed to weathered fuel oil because this is the form of fuel oil that will be found in contaminated environments. Our findings indicate that there are no differences in the parameters measured between artificially weathered and fuel oil. Fuel oil is heavily refined, and most volatile and lightweight molecules are removed during the distilling process. Similar changes in composition occur in petroleum products during the weathering process, and therefore, chemical and physical differences between weathered and unweathered fuel oil may be minimal. This is especially true for the

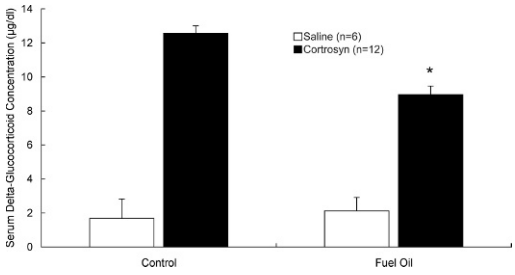

FIGURE 3. Mean ± SE serum delta-glucocorticoid concentrations after adrenocorticotropic hormone stimulation tests in male mink fed either mineral oil (control) or 420 ppm fuel oil in the diet for 60–62 days. Serum glucocorticoid concentrations were measured from each animal before and 90 min after cortrosyn injection. Delta-glucocorticoid concentrations were calculated for each animal and represent the differences in serum glucocorticoid concentrations between pre- and postcortrosyn or saline injection. The asterisk indicates significant differences ($P<0.04$) in delta-glucocorticoid concentrations between the fuel oil–exposed/cortrosyn-injected and control/cortrosyn-injected animals (black boxes).

heavy petroleum oils such as bunker C fuel oil because evaporation rates for this product are very low compared to lighter-weight distillates (Coleman et al., 2003). A limitation in this study is that the fuel oil is artificially weathered in the laboratory; therefore, the petroleum product would not be exposed to the same conditions that would be found in the environment, e.g., temperature, photo, and microbial oxidation. However, the weathering of petroleum oil is considered to be more dependent on the type of petroleum product than on the conditions it is subjected to in the environment (Coleman et al., 2003). Therefore, our findings may have relevance to what would be observed in an exposure with naturally weathered fuel oil.

Serum concentrations of glucocorticoids, measured at the end of the exposure, were not elevated in any group of fuel oil–exposed animals when compared to control animals. Neither were the concentrations of glucocorticoid in the feces, which provides a nonstressful means of measuring hormone concentrations over time. Although not statistically significant, there was a temporal trend suggesting that in both male and female mink the fecal concentrations of glucocorticoids increased in the later part of the exposure with possibly a stimulating effect by both types of fuel oil. We have observed enhancing effects on fecal glucocorticoid concentrations by fuel oil before, but, similarly, the changes were not significant (Mohr et al., 2008).

Even though basal concentrations of glucocorticoids in the serum were not different between the fuel oil–exposed and control groups, we were able to show an important physiologic difference between the two groups. Challenging male mink with cortrosyn revealed that adrenal glands in animals exposed to fuel oil responded to this model stressor by releasing into the serum smaller increments of glucocorticoids compared to the adrenal glands of control mink. This is a unique finding because, typically, adrenal responses to stressors are usually magnified in hypertrophied glands (Berne and Levy, 1993).

The mechanism of the fuel oil–induced adrenal hypertrophy is not known, and we have suggested that it could be an adaptive response to low glucocorticoid output caused by an inhibition of adrenosteroid synthesis by components in the fuel oil (Mohr et al., 2008). This adaptive change maintains basal serum glucocorticoid concentrations within a normal range, which was our observation in this exposure study (Hinson and Raven, 2006). The increased number of fuel oil–exposed animals with excessive vacuolation of the inner two zones of cortical cells of the adrenal gland indicates either that steroid synthesis has decreased, possibly because of fuel oil–induced inhibition of steroidogenesis, as suggested above, or that the adrenal glands are under chronic stimulation from ACTH that is released from the pituitary gland (Hadley and Levine, 2007). In the later case, chronic ACTH stimulation is not associated with an increase in glucocorticoid output, an observation that has

been reported with some forms of chronic stress (Rich and Romero, 2005). In this scenario, fuel oil would be acting as a general stressor to the HPA axis and not a toxicant to the adrenal gland.

It is in challenging the adrenal glands with cortrosyn that the consequences of the effects of fuel oil on adrenal physiology are revealed. A reduction in the increment of glucocorticoid released in the blood after cortrosyn stimulation in fuel oil–exposed animals compared to similarly treated control animals has been called relative adrenal insufficiency (Rothwell et al., 1991), and it indicates that the HPA is not functioning normally. Relative adrenal insufficiency is often observed in sepsis, but may also be seen in other diseases (Arafah, 2006). Our findings suggest that relative adrenal insufficiency may also be initiated by chemical toxicity. The mechanism leading to relative adrenal insufficiency is not known. Most observations of this condition have been made in human beings; however, it has also been described in dogs (Burkitt et al., 2007). We are not aware of any comparable studies in wildlife or in wildlife surrogates, such as ranch mink.

A consequence of chronic exposure to low concentrations of petroleum hydrocarbons to wildlife is that animals would not be able to mount a normal adrenal gland response to additional psychologic, physical, or chemical stressors present in the environment. It remains to be determined if the attenuated responses in glucocorticoid secretion in cortrosyn-stimulated animals that have been exposed to fuel oil translates into physiologic changes attributed to diminished activity of glucocorticoid-sensitive genes. The ability of the adrenal glands to mount an appropriate response to stress can impact a number of physiologic systems, including metabolism, cardiovascular, immunity, and reproduction (Sapolsky et al., 2000). The first three of these systems affect the fitness and survival of the individual, and reproduction can have an impact at the population level. The wide range of physiologic processes influenced by glucocorticoids highlights the importance of considering the effects of low concentrations of environmental contaminants on the stress-response systems of free-ranging animals.

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