ECOLOGICAL FACTORS INFLUENCING STRESS IN NORTHERN RIVER OTTERS (*LONTRA CANADENSIS*)

by

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A Thesis

Presented to

The Faculty of Humboldt State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science in

Natural Resources: Wildlife

July, 2011

ABSTRACT

Ecological Factors Influencing Stress in Northern River Otters (*Lontra canadensis*)

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Baseline levels of stress hormones are necessary for maintaining physical homeostasis in mammals. Excessive amounts of stress, however, can cause serious pathologies. Chronic effects of stress include increased susceptibility to infectious diseases due to suppressed functioning of the immune system, diminished growth rates, protein loss, neuron cell death and inhibited reproduction. A myriad of external factors including unhealthy, human-induced environmental conditions as well as naturally occurring fluctuations in resources, habitat attributes, health and sociality have the potential to cause deleterious changes in stress response in mammals. I validated a non-invasive enzyme immunoassay (EIA) for use in river otters (*Lontra canadensis*) and used these techniques to evaluate stress hormone concentrations extracted from feces of northern river otters in coastal northern California. I assessed how stress was correlated with anthropogenic and socioecological factors. Fecal samples were collected from river otter latrines at seven coastal river otter activity centers. I evaluated the relative importance of several ecological variables including location (as a surrogate for river otter activity center contamination level), water turbidity, water temperature variation, diet, parasite presence and scat grouping size (as an index of conspecific interactions or group size) as predictors of cortisol stress hormone concentrations of the river otters using AIC_c and multiple regression. The best fit model included location, turbidity, diet and parasite presence which explained 23% of the variation in stress response observed in this coastal river otter population. When the variables were assigned to one of two *a*

priori models, anthropogenically influenced environmental conditions (location and turbidity) or socioecological variables (diet and parasite presence) and compared using multiple regression, the socioecological variables contributed more than twice as much to variations in stress levels than the anthropogenic variables. These results suggest that a complex combination of human induced and naturally occurring pressures are associated with physiological stress levels in this population of river otters. Indirect measures of river otter population health gained utilizing these techniques can be useful to help establish the ecological status of aquatic ecosystems that may affect many other wildlife species, as well as nearby human communities.

ACKNOWLEDGMENTS

I would like to thank my committee members Drs. Micaela Gunther, Jeff Black, Kristine Brenneman and Matthew Johnson for their support and guidance. Kristin Brzeski, Wendy Pearson, Marlene Wagner and Sara Lowry assisted with fecal sample collection. Dr. Janine Brown and the staff of the Endocrinology Research Laboratory at the Smithsonian Conservation Biology Institute provided training and support in the use of enzyme immunoassay methods. Funding was provided by Humboldt State University Sponsored Programs Foundation, Humboldt State University Office of Graduate and Research Studies, Eureka Rotary Club, Richard J. Guadagno Memorial Scholarship, and Humboldt State University Women's Enrichment Fund. This project was approved under IACUC # 07/08.W.46.A.

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INTRODUCTION

Otter species have long been known to be indicators of environmental health and river otter (*Lontra canadensis*) presence is closely associated with undisturbed and unpolluted habitat (Prenda et al. 2001). The disappearance of these mammals from parts of their range usually indicates habitat loss or degradation, specifically a decline in water quality associated with presence of chemical contaminants (Mason and Madsen 1993, Mason and Macdonald 1993, 1994). Development and aquatic pollution eliminates river otter habitat, destroys their prey base and disrupts reproduction, leading to local extermination of the species (Mason and Macdonald 1993).

Populations of otters declined worldwide from the 1950s to the 1980s (Melquist and Hornocker 1983). During this time, river otters in North America were extirpated from much of the interior of the continent (Mason 1995). The Eurasian otters (*Lutra lutra*) of northwest and central Europe followed a similar pattern of decline (Mason and Macdonald 1986, Foster-Turley et al. 1990). Many declines in Eurasian otter populations have been associated with an increase in the use of dieldrin, a chlorinated hydrocarbon widely used in European (and US) agriculture as an insecticide in the 1960-1970s (Chanin and Jeffries 1978). In the UK, Eurasian otter distribution was found to be negatively correlated to levels of polychlorinated biphenyls (PCBs) in scat (Kruuk and Conroy 1991, Mason et al. 1991). Similar problems have been documented in river otters in the United States. For example, male river otters harvested along the Lower Columbia River in Oregon and Washington were found to have severe physical abnormalities which were likely to influence reproductive success and were correlated with high levels of PCBs and other contaminants (Henny et al. 1996). Ranch raised American mink (*Mustela vison*), a mustelid closely

related to the river otter, have similarly demonstrated extreme sensitivity to PCBs (Aulerich and Ringer 1970). Bioaccumulation of these persistent toxins is especially problematic for river otters due to their aquatic habitat and piscivorous diet.

The cessation of use of these toxic chemicals in the 1980s and subsequent, partial dissipation of these pollutants from the otters' habitat have led to recolonizations and successful reintroductions of otters to many parts of their historic distribution (Melquist and Hornocker 2003). However, the legacy of these disturbances are apparent in areas where otters are still absent.

This study took place in coastal Humboldt County, California, in an area with known dioxin contamination due to historic logging practices (Lappe 2001). Dioxins are biologically active endocrine-disrupting chlorinated hydrocarbons associated with reproductive and developmental defects, altered endocrine activity and other sub-lethal effects in river otters, other mammals, fish, and birds (Wren 1991, Hontela et al. 1992, Jönsson et al. 1993, Leonards et al. 1997, Mohr et al. 2008).

The deleterious effects of environmental perturbations and habitat degradation leading to extirpation of river otter populations have been well documented (Chanin and Jeffries 1978). However, determining the sub-lethal effects of these factors on individual wild animals without handling the animals or utilizing carcasses has not been adequately explored. Recent advances in the detection of biomarkers such as glucocorticoid (GC) stress hormones associated with the endocrine system's hypothalamic pituitary axis (HPA) can be a useful tool to help elucidate these relationships (Wasser et al. 1997). The response of the HPA is one of the primary physical mediators of a stressor experienced by a mammal (Chrousos 1995). When a stress is perceived via the senses, the hypothalamus is stimulated

to release corticotropin-releasing factor, which stimulates the anterior pituitary to release adrenocorticotropic hormone (ACTH) into the bloodstream (Chrousos 1995). ACTH travels to the adrenal glands where GCs are secreted. GCs increase cardiovascular and metabolic functions while depressing non-essential functions (Chrousos 1995, Calcagni and Elenkov 2006). Presence and purpose of GCs have been found to be well preserved across the mammalian class, as well as in birds and fish (Wingfield et al. 1997). These physiological stress biomarkers can be indicative of the health of an individual or a population of animals and are increasingly being used to monitor populations of animals under duress (Sapolsky and Pulsinelli 1985, Wasser et al. 1997, Matteri et al. 2000, Romero and Wikelski 2001). Fecal GCs accurately measure long term stressors often missed by the single point concentrations associated with plasma GC analyses, and repeated fecal sampling of the same individual is possible without causing additional stress (Goymann 2005, Touma and Palme 2005). These techniques are especially useful for studies of free-ranging animals that are difficult to observe and/or capture (Monfort 2003).

Baseline levels of stress hormones, or glucocorticoids (GCs), are necessary for maintaining homeostasis in the body and mobilizing energy (Sapolsky and Pulsinelli 1985). Typical stressors cause adaptive, acute effects which exhibit themselves with subsequent increases in glucocorticoid levels followed by a generally rapid return to baseline levels. Chronic stress and the corresponding maximal GC output can lead to an inability to mount an adaptive response to an acute stressor (Hontela et al.1992, Wingfield and Romero 2001, Reeder et al. 2004). Chronic effects of stress can also include increased susceptibility to infectious diseases due to suppressed functioning of the immune system, diminished growth

rates, protein loss, neuron cell death and inhibited reproduction (Sapolsky and Pulsinelli 1985, Moberg 2000).

Stress can be inherent to the life history traits of a species; for example, northern river otters are routinely challenged by socioecological pressures to maintain their body temperature in an aquatic environment, meet their metabolic needs with a specialized diet, maintain immunological health, and coexist with con-specifics who share their resources. To further complicate matters, river otters are challenged by anthropogenic changes to their environment, making habitat requirements increasingly more difficult to attain. Their aquatic habitats are now commonly impaired by chemical contaminants and increased sedimentation, conditions which could a) directly impair the function of these animals' endocrine systems (Fossi and Marsili 2003) or b) affect the availability of prey and ability to capture prey (Watt 1991, Binkley and Brown 1993) likely leading to changes in physiological stress response.

Non-invasive enzyme immunoassay (EIA) techniques were used, after validation, to investigate the eco-epidemiological relationships between exogenous factors that produce stress in wild river otters and the associated endogenous physiological response. I was particularly interested in the effects of anthropogenically induced increases in aquatic contaminants and water turbidity because these factors represent relatively recent changes to the otters' local habitat. The anthropogenic factors may present a novel challenge to river otters, likely resulting in disturbances to healthy stress response and corresponding high GC levels. River otters are more likely to have evolved with and adapted to variations in water temperature, prey availability, intestinal parasite loads and conspecific interactions;

socioecological factors that have varied consistently over time in comparison to the anthropogenic variables.

METHODS

Study Area

The study area included seven otter activity centers, located in aquatic habitats on or near Humboldt Bay, California. A river otter activity center includes terrestrial latrine sites and nearby resting areas and aquatic foraging habitat (Melquist and Hornocker 1983, Kruuk 1992). Citizen science reports of river otter observations were collected from 2000 - 2008 on the coasts, wetlands and watersheds of Humboldt County, California (Black 2009). These observations were used to assess the general spatial and temporal use of local river otter habitat and to choose activity centers for data collection. Activity centers, from north to south, included Little River, Mad River, Mad River Slough, Arcata Marsh and Wildlife Sanctuary (AMWS), Woodley Island, Elk River and Humboldt Bay National Wildlife Refuge (HBNWR) (Fig. 1). All activity centers were in close proximity to both saltwater and freshwater habitats.

Soil disturbance and contamination caused by logging, road building, and development on local watersheds within the study area have been sources of chemical pollution and excessive water turbidity in Humboldt Bay (Western Hemisphere Shorebird Reserve Network 2007). The greatest source of pollution in the Bay resulted from the use of the fungicides pentachlorophenol (PCP) and tetrachlorophenol (TCP). PCP and TCP are chlorinated hydrocarbons that were utilized as popular wood preservation treatments in the mills of Humboldt County until the 1980s, when their use was banned. PCP and TCP were often applied to lumber without regard for soil contamination and carelessly disposed of, leaving many former mill sites saturated with a persistent toxin (Ecological Rights Foundation 2007).

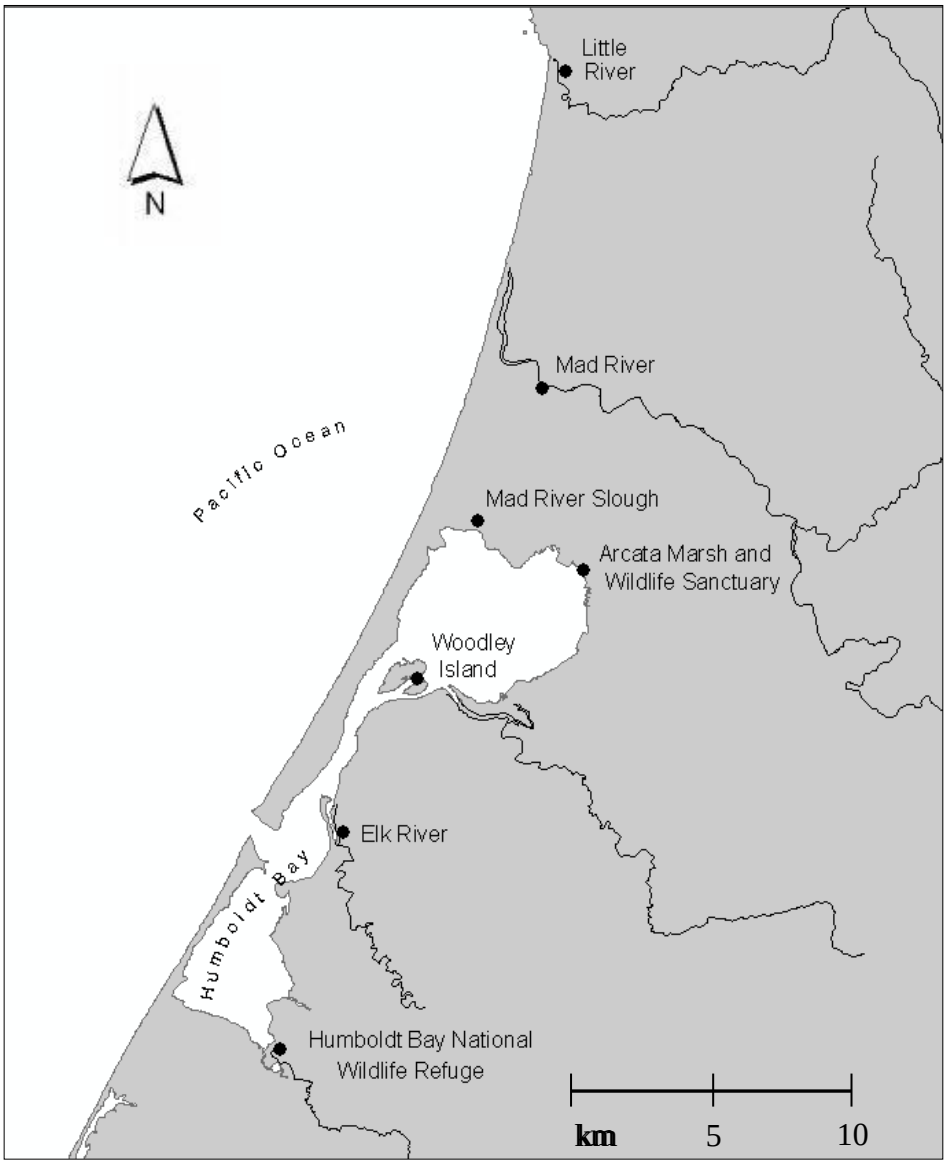


Figure 1. Otter activity centers in coastal Humboldt County, California, utilized for investigation of factors influencing river otter fecal cortisol levels Jun – Nov 2008.

Several of the otter activity centers utilized in this study were located in areas declared as impaired by contaminants and excessive sedimentation (North Coast Regional Water Quality Control Board [NCRWQCB] 2007). Section 303(d) of the federal Clean Water Act requires states to identify water bodies that do not meet water quality standards and are not supporting their beneficial uses (NCRWQCB 2007). Humboldt Bay was 303(d) listed in 2006 as impaired for dioxin equivalents and PCBs. Shellfish from the Bay were found to contain levels of dioxins that exceed the Office of Environmental Health Hazard Assessment (OEHHA) screening levels (State Water Resources Control Board 2009) and exceed California Proposition 65 "no significant risk levels" for carcinogens and chemicals that cause reproductive toxicity if consumed by humans (Lappe 2001, OEHHA 2006). The contamination in the Bay is in close proximity to the otter activity centers at the Mad River Slough, Arcata Marsh and Wildlife Sanctuary, Woodley Island and Elk River and may affect otters utilizing those areas. The Arcata Marsh and Wildlife Sanctuary (AMWS), located in the northeast corner of Humboldt Bay was originally a tidal mudflat and salt marsh, then the site of two lumber mills and an ocean-side landfill which leached toxins into Humboldt Bay (City of Arcata 2007). Since the early 1980's, this area has functioned as a wastewater treatment facility and integrated wildlife sanctuary (City of Arcata 2007). As recently as 2003, soil and groundwater samples from a former mill site on the Mad River were found to be contaminated with PCP (Adams 2005). The Mad River and Elk River activity centers are located within waterway segments which are on California's 303(d) list of waterways impaired for excessive sedimentation. No historic mill sites were known to have been located near the river otter activity center on the Little River, nor was this river listed as impaired for contaminants or turbidity. Sediment samples from Humboldt Bay

National Wildlife Refuge (HBNWR), located in south Humboldt Bay, were found to be very low in dioxin levels (Lappe 2001). In summary, Mad River, Mad River Slough, AMWS, Woodley Island and Elk River served as otter activity centers known or suspected to be contaminated with dioxins and other contaminants while Little River and HBNWR served as non-contaminated reference sites for this study.

Scat Collection, Diet Identification, Parasite Analysis and Scat Grouping Size

I collected river otter scat from the seven otter activity centers ~3x / wk from June - November 2008. Otters are elusive but their scat is easy to identify and is often left at conspicuous latrines above tide line. Latrines are an obvious visual indicator of an otter's activity center. Resting, feeding and territorial scent-marking activities often take place near latrines (Kruuk 1995). Males, single females and females with pups all use activity centers, and repeated use of specific latrines continues for years (Melquist and Hornocker 1983, Kruuk 1992).

Scat collection was restricted to after the breeding season, when females are with pups and female home range is smallest, and before dispersal of young, to help reduce the influence of these potentially confounding factors (Melquist et al. 1981). Scat collection began at sunrise and freshness of scat was determined based on observed extent of fecal desiccation. Extent of fecal desiccation and hormone degradation depends on elapsed time between deposit and collection and weather conditions (Millspaugh and Washburn 2004). Although only "fresh" scats were collected, they were still ranked for apparent freshness and later compared for differences in stress hormone content based on presumed freshness. An "A" was assigned to scat found early in the morning and which did not exhibit any desiccation whereas a "B" rating was given to slightly desiccated scat. A "C" rating was

reserved for scat collected near the end of the field day (but not later than 1:00 PM), showing obvious signs of desiccation but most likely deposited on the same day it was collected. Scat was collected in sterile Whirl-Pak bags (Nasco, Fort Atkinson, Wisconsin) and stored in a cooler with ice while in the field then transferred to a freezer and stored at -20° C until preparation for hormone assays (Wasser et al. 2000).

Diet was determined by presence of prey material in scat at time of collection and confirmed visually after lyophilization of scat material. Prey items in scat were categorized as fish, crab, bird, invertebrate or mixed. Presence of scales indicated fish diet, presence of masticated carapace indicated crab and presence of feathers indicated bird. "Invertebrate" was a broad category containing insects identified by wings and body segments, and included the occasional crayfish (*Astacoidea*). Crab was given its own category based on the size of this prey item and handling time required to capture and consume it in comparison to the prey items categorized as invertebrate. Mixed diet contained more than one prey type.

Presence of parasites was determined at time of scat collection and was indicated by presence of visible adult-form cestode segments. Parasites were identified visually, without the assistance of magnifying equipment. Scat labeled as "parasite present" were heavily infected and therefore it is likely that low level infections were missed. Cestodes could not be identified to generic level due to lack of identifying characteristics (e.g., scolex) found in and collected from scat. Scat grouping size was used as an index for conspecific interactions or potential group size and was defined by the total number of scats found with the collected sample. Several scat found together may indicate a group of otters traveling together or several otters that have recently visited a latrine without

direct interaction. Otters may interact indirectly by investigating foreign scents left at latrines, or by leave scat to communicate their presence to other otters visiting that latrine (Oldham and Black 2009). This index gave the best approximation of these direct or indirect associations between individuals without actually observing defecation by otters.

Collection and Analysis of Water Samples

I accessed turbidity data for several of the activity centers via data collected by the Central and Northern Ocean Observation System (CeNCOOS, http://cencoos.humboldt.edu). For activity areas where turbidity levels were not available from this source, I analyzed water samples 1x / wk, on site, with a handheld turbidimeter, or a water sample was collected in Whirl-Pak bags, cooled to 4°C and processed in a turbidimeter within 24 hrs. All data was recorded in Formazin Turbidity Units (FTUs). To avoid disturbance of sediments, turbidity data were collected prior to other work done at the site. Water samples were collected at a depth of 0.3 m below the water surface, away from the bank and, if possible, at the centroid of flow for streams (Texas Commission on Environmental Quality 2007). Water temperature loggers (HOBO, Bourne, MA, USA) were deployed below low tide level at Little River, Mad River Slough, Woodley Island, Elk River and HBNWR. For each site, weekly average water temperature was calculated. At Mad River and the AMWS, water temperature (0.1ºC ± 0.01 ºC) was taken weekly with a handheld digital thermometer 0.6 m below water surface.

Chemical Contamination

Based on previously collected contamination level data, I categorized Mad River, Mad River Slough, AMWS, Woodley Island and Elk River as activity centers impacted by contamination associated with past logging industry practices, while Little River and HBNWR were categorized as relatively non-contaminated reference sites (Lappe 2001).

Cortisol Enzyme Immunoassay

Enzyme immunoassays were used to determine the concentration of cortisol in fecal samples collected from river otter activity centers. The cortisol EIA used a polyclonal cortisol antibody (R4866, 1:20,000; C. Munro, University of California, Davis), a horseradish-peroxidase (HRP) conjugated cortisol label and cortisol standards (Young et al. 2004). EIA plates (Nunc Maxisorb 96 well, Thermo Scientific, Rochester, NY, USA) were coated with 50 µl cortisol antibody solution (1:8500 dilution) and incubated at 4°C for 12-24 hrs. Two laboratory-prepared cortisol controls were diluted to bind at 30% and 70%. Nine cortisol standards were prepared from a 1000 pg/well cortisol stock serially diluted 2:1. Controls, standards and samples were run in duplicate on each plate. Fifty µl of sample diluted 1:6, controls and standards were pipetted into plate wells and 50 µl of 1:20,000 HRP 2º antigen were added to each well. Plates were then incubated for 2 hr at room temperature to allow competitive binding of sample antibody and HRP conjugated 2º antibody. Plates were rinsed four times with EIA wash solution (Brown et al. 2008) and 50 µl of peroxidase substrate (ABTS) [2,2'-azino-bis(3-ethyl-benzthiazoline-6-sulphonic acid) + 0.5 M H_20_2] for colorimetric detection of HRP binding activity was added to each well. Plates were then incubated at room temperature on a plate shaker for 10-15 min while color developed. Optical density (OD) of each

sample was read by an automated micro-ELISA plate reader using a 450 nm filter when blank plate wells were 0.7-1.0 OD. To control for intra-assay variation, samples were re-analyzed if replicates of controls, standards or samples exceeded 10% coefficient of variation (CV). Inter-assay CVs were calculated for standards and controls and compared to laboratory control monitors (Smithsonian Conservation Biology Institute, Center for Species Survival, Endocrinology Research Laboratory). Samples were re-analyzed if inter-assay CVs exceeded 15%. Samples were re-diluted and re-analyzed if binding was less than 20% or greater than 80%.

Extraction of hormones from scat

Extraction methods followed those of Brown et al. (2008). All scat samples were lyophilized (Labconco Lyophilizer, Kansas City, Missouri, USA), sifted and weighed out to 0.1 ± 0.01 g powdered, dry fecal matter. I combined 4.5 ml 100% ETOH, 0.5 ml distilled H_2O and sample matter in a 16 x 125 mm glass test tube. Samples were vortexed for 10 s, shaken on a commercial rack shaker (Multi-Pulse Vortexer, Glas Col, Terre Haute, Indiana) for 30 min and centrifuged at 2200 rpm for 20 min. The supernatant was then poured off into separate tubes. I again added 4.5 ml ETOH and 0.5 ml distilled H_2O to the centrifuged pellet remaining in the original tube and vortexed for 10 s, shook on a rack shaker for 5 min and centrifuged for 15 min at 2200 rpm. The supernatant was poured off once again into the second set of glass tubes and dried down under forced air. The extract was then re-suspended in 3 ml ETOH, sonicated for 15 min, vortexed for 10 s, dried under forced air, re-suspended again in 1 ml MEOH, sonicated for 15 min, vortexed for 10 s and dried one last time. This final extract was prepared for use in assays

by reconstitution in 1 ml dilution buffer followed by vortexing for 10 s and sonicating for 15 min.

Enzyme immunoassay validation

Before an EIA can be used to determine fecal cortisol levels associated with exogenous stressors, the methods must be validated for the particular species. Validation was completed on captive river otters following the methods of Brown et al. (2008). Validation of these methods for river otters included high performance liquid chromatography (HPLC), sample matrix interference test, adrenocorticotropic hormone (ACTH) challenge test, evaluation of extraction efficiency, and test for parallelism (Touma and Palme 2005, Keay et al. 2005).

High performance liquid chromatography. Cortisol and corticosterone are chemically similar glucocorticoid metabolites associated with mammalian stress responses (Keay et al. 2005). In order to identify which GC metabolite was present in river otter feces, and to evaluate whether or not there was EIA cross-reactivity between metabolites, HPLC was used to separate and identify cortisol and corticosterone fecal GCs. Six 20-µl aliquots of previously extracted hormone samples from ACTH challenge test, with known high levels of cortisol concentration were combined in a 16 x 125 mm glass tube and dried down using forced air. Five ml of 90% ethanol was added to the tube and contents were filtered first using a C18 cartridge (Spice Cartridge, Rainin, Inc., Oakland, CA) followed by a 0.45 µm filter and dried down again. Each sample was then reconstituted in methanol containing 10,000 counts per minute (cpm) / 50 µl of titrated cortisol and corticosterone ^{3}H tracers. The HPLC (Microsorb C-18 Column; Rainen Inc., Woburn, MA) was performed using a 20 - 100% methanol and water gradient at a 1 ml /

min flow rate; 80 1-ml fractions were collected. Subsequently, 100-µl aliquots of each fraction were analyzed on a beta counter (Beckman Coulter, Inc., Brea, California) for ^{3}H radioactivity levels. The remainder of the sample fractions were dried down and reconstituted in 125 µl EIA dilution buffer (0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4, 0.15 M NaCl; pH 7.0). Detection of cortisol, via EIA, was then performed on fractions containing peaks of cortisol and corticosterone radioactivity labels to ensure that the cortisol EIA was specific to cortisol and did not exhibit cross-reactivity with corticosterone.

A random set of samples was then analyzed for both corticosterone concentration with an established radio immunoassay (RIA) and with the cortisol EIA methods detailed below to ensure that both cortisol and corticosterone followed similar response patterns. Utilization of cortisol EIA to determine GC concentrations in mammals is less common than using corticosterone RIAs because cortisol is usually found in lower concentrations than corticosterone in most mammal species. However, RIA utilizes more toxic chemicals that are difficult to dispose of and therefore, cortisol EIA methods were chosen for this study.

Sample matrix interference test. This analysis tested for potential substances in the fecal sample that could have interfered with the EIA, independent of antibody/antigen binding. A sample pool was created using 10 samples with known, low concentrations of cortisol. Aliquots of pooled sample were then spiked with an equal amount of each serially diluted standard (except lowest standard) and analyzed for cortisol concentration (see EIA methodology above for standard formulation). To determine background hormone levels, the original pooled sample (without standard) was also analyzed for cortisol concentration. Percent recovery was determined using the following formulas:

Amount expected = (concentration of standard$_i$ spiked with sample) / 2

Amount observed = (concentration observed from assay results – background)

% recovery = (amount observed / amount expected) * 100

Concentrations of expected and observed cortisol levels were then compared using linear regression.

Adrencorticotropin hormone challenge test. The ACTH challenge test was used to confirm that this river otter species responds to an external stressor with an associated increase in fecal GCs (Keay et al. 2005). Two male river otters from the National Zoological Park in Washington D.C., USA were used in the ACTH challenge test. The river otters were housed together and their food was dyed different colors in order to distinguish each individual's scat (New 1959). Scat from each river otter was collected twice daily, once in the morning and once in the afternoon, for 2 weeks prior to the administration of ACTH. On the morning of the test, the river otters were captured, briefly restrained and injected with 5 IU/kg of synthetic ACTH gel (Wedgewood Pharmacy, Swedesboro, NJ, USA) similar to methodologies described by Touma and Palme (2005). Scat collection continued twice daily for another week following the injections. Cortisol concentrations in collected scat samples were analyzed with EIA to determine whether or not cortisol levels peaked, as expected, after ACTH injection.

Extraction efficiency. Extraction efficiency was calculated to determine the efficiency and consistency of cortisol extraction from fecal matter. Prior to extraction, each sample was spiked with 100 µl 210,000 cpm ^{3}H radio-labeled cortisol. The sample was then extracted as per methods described above. After extraction, 50 µl of the final 1.0-ml extract was aliquoted to a plastic scintillation vial. A comparative control was

made with a 100-µl aliquot of the stock radio-labeled hormone. Three ml of Ultima Gold scintillation fluid (Perkin Elmer, Waltham Massachusetts, USA) was added to sample and control vials. Radioactivity disintegrations per minute (DPMs) in these vials were then determined using a beta counter. Background radiation was determined by analyzing the radioactivity in a scintillation vial containing only scintillation fluid. Percent extraction efficiency was determined using the formula:

[(DPMs observed – background) / (DPMs expected – background)]* 100

where "observed" = sample DPMs, and "expected" = control DPMs

Parallelism. The test for parallelism was performed to determine if the cortisol in scat samples was immunologically similar to the laboratory standard cortisol and could be measured proportionally, i.e., they varied in such a way that a known sample concentration was a constant multiple of the corresponding standard concentration (Brown et al. 2008). Nine standards were created from a neat standard (1000 ng/g) serially diluted 2:1. The sample cortisol was similarly diluted. Percent binding was determined by EIA for all samples and standards. Standard % binding and cortisol concentration were plotted to generate a standard curve to compare with the sample curve to evaluate similarity, i.e., parallelism.

Activity Center Differences

The environmental attributes of otter activity centers used in this study were assumed to be similar due to the proximity of sites to each other, and their location in similar habitat. I assumed their major difference lay in site quality related to contamination level. Therefore I investigated differences in water turbidity, water temperature, diet

composition, parasite presence and scat grouping size among activity centers to determine if these activity centers differed at the activity center scale.

Statistical Analyses

Data were analyzed for normality and non-normal data were either mathematically transformed to near normality or nonparametric tests were performed for analyses. Two outliers with cortisol levels above 10,000 ng/g dry feces were removed from the sample set. These high hormone concentrations were suspected to be the result of a laboratory error. Hormone concentration data were natural log-transformed, and turbidity data were square-root transformed to meet assumptions of normality for analysis. Variables were not found to be multicollinear. The confounding influence of time on cortisol concentration was analyzed and found to be not significant. ANOVA was used to determine if scat freshness affected hormone concentration. Baseline cortisol concentrations were calculated for both the captive individuals and the wild population. Baseline cortisol concentrations were used in lieu of averages in order to represent a typical homeostatic level of stress hormones (Songsasen et al. 2006). Differences between the two captive animal's baseline and the captive vs. wild population baseline were analyzed with 2-sample t-tests. Pearson correlation was used to determine relationships between cortisol concentration and water temperature and cortisol and group size.

I categorized the variables I investigated into 2 *a priori* models; 1) anthropogenically influenced environmental variables: water turbidity and location (used as a surrogate for site contamination), and 2) socioecological variables: water temperature, diet, parasite presence and scat grouping size (used as an index for

conspecific interactions). Akaike's Information Criterion, adjusted for small sample size (AIC_c) was used to determine which model explained more of the variation in fecal cortisol concentration. AIC_c was also used to determine the most precise and parsimonious model comparing all possible combinations of variables. AIC values were calculated using the following formula:

$$AIC_c = -2*\ln(RSS/n)+2*K+(2*K*(K+1))/(n-K-1)$$

where RSS = residual sum of squares, n = number of data points, and K = the number of parameters in the model.

Multiple regression was then performed on the top ranked model to determine how much variation in fecal cortisol concentration could be attributed to the variables contained in the model. To help elucidate the relationship between fecal cortisol concentration and location, and cortisol concentration and diet in the top ranked model, I used the beta coefficients (β) generated from the regression equation associated with this analysis to create predicted, relative cortisol concentrations associated with the activity centers and diet types.

Differences in otter diet and parasites at the seven activity centers were examined with chi-squared analysis. Differences in water temperature, turbidity and group size at the activity centers were analyzed with ANOVA followed by *post-hoc* Tukey-Kramer analyses when appropriate. Statistical significance levels (p) for all analyses were less than or equal to 0.05 except for multiple regression and AIC analyses where significance was less than or equal to 0.10 as suggested by Burnham and Anderson (2008). Means are presented ±1 SE. All statistics were performed with NCSS2004 Statistical Software (Kaysville, Utah, USA).

RESULTS

EIA Validation

The cortisol HPLC exhibited two peaks of glucocorticoid immuno-reactivity from eluates of the river otter fecal sample pool (n=10). These metabolites were eluted between fractions 36 – 47 (Fig. 2). The first metabolite eluate peak, at fraction 39, exhibited high radioactivity (DPM, relating to presence of ^{3}H labeled cortisol) co-eluted with a high concentration of cortisol as determined by cortisol EIA. The slightly less polar peak presented at fraction 45, with high DPMs representing radiolabeled corticosterone but a correspondingly low concentration of cortisol as determined by the cortisol EIA. Thus the EIA properly identified cortisol with little cross reactivity with corticosterone. The sample matrix interference test demonstrated that extraneous substances in the fecal sample matrix did not interfere with the EIA, independent of antibody/antigen binding (R^2 [adjusted for small sample size] = 0.99, Fig. 3).

The ACTH challenge test was performed on two captive otters. Male A exhibited the expected peak in fecal cortisol concentration after administration of the challenge, demonstrating that ACTH, as an acute stressor, initiated adrenal gland activity (Fig. 4a). The response of Male B was inconclusive (Fig. 4b) due to variation in cortisol levels prior to ACTH administration. The two captive animals had similar baseline cortisol concentrations of 258.9 ± 26.2 ng/g and 259.6 ± 24.8 ng/g dry feces respectively (t_{104} = 0.28, p = 0.77). Comparison of corticosterone RIA and cortisol EIA performed on the same sample set exhibited similar immunoreceptivity profiles, demonstrating that both methodologies yielded similar results (Fig. 5). Corticosterone concentration was greater

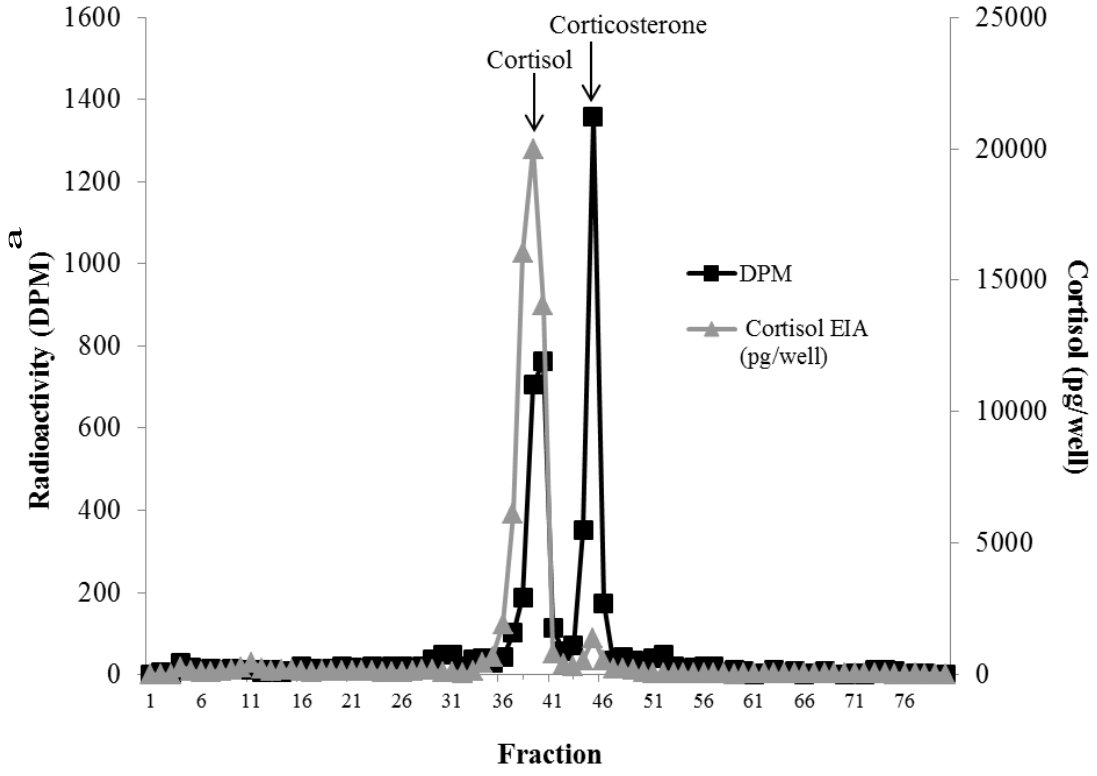


Figure 2. HPLC separation of immunoreactive glucocorticoid metabolites in river otter feces conducted as part of river otter EIA methodology validation at the National Zoological Park.

[a] DPM (disintegrations per minute) is a measure of radioactivity.

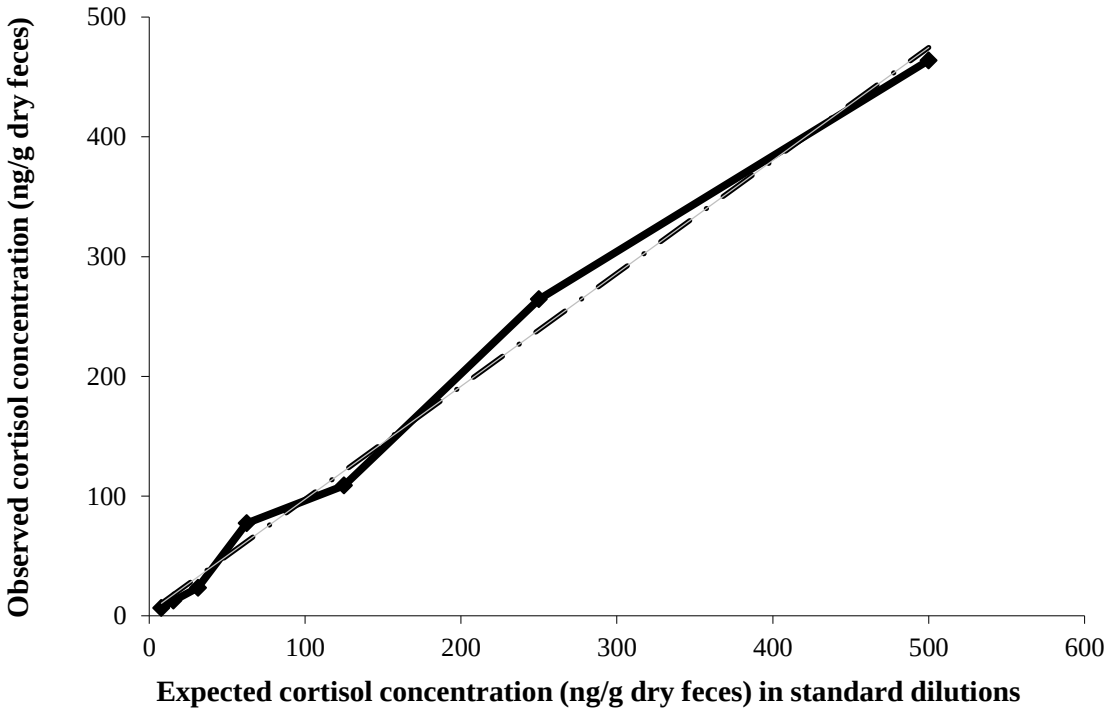


Figure 3. Observed vs. expected cortisol concentration from captive river otter feces subjected to EIA sample matrix interference test as part of an EIA validation at the National Zoological Park, Washington, D.C.

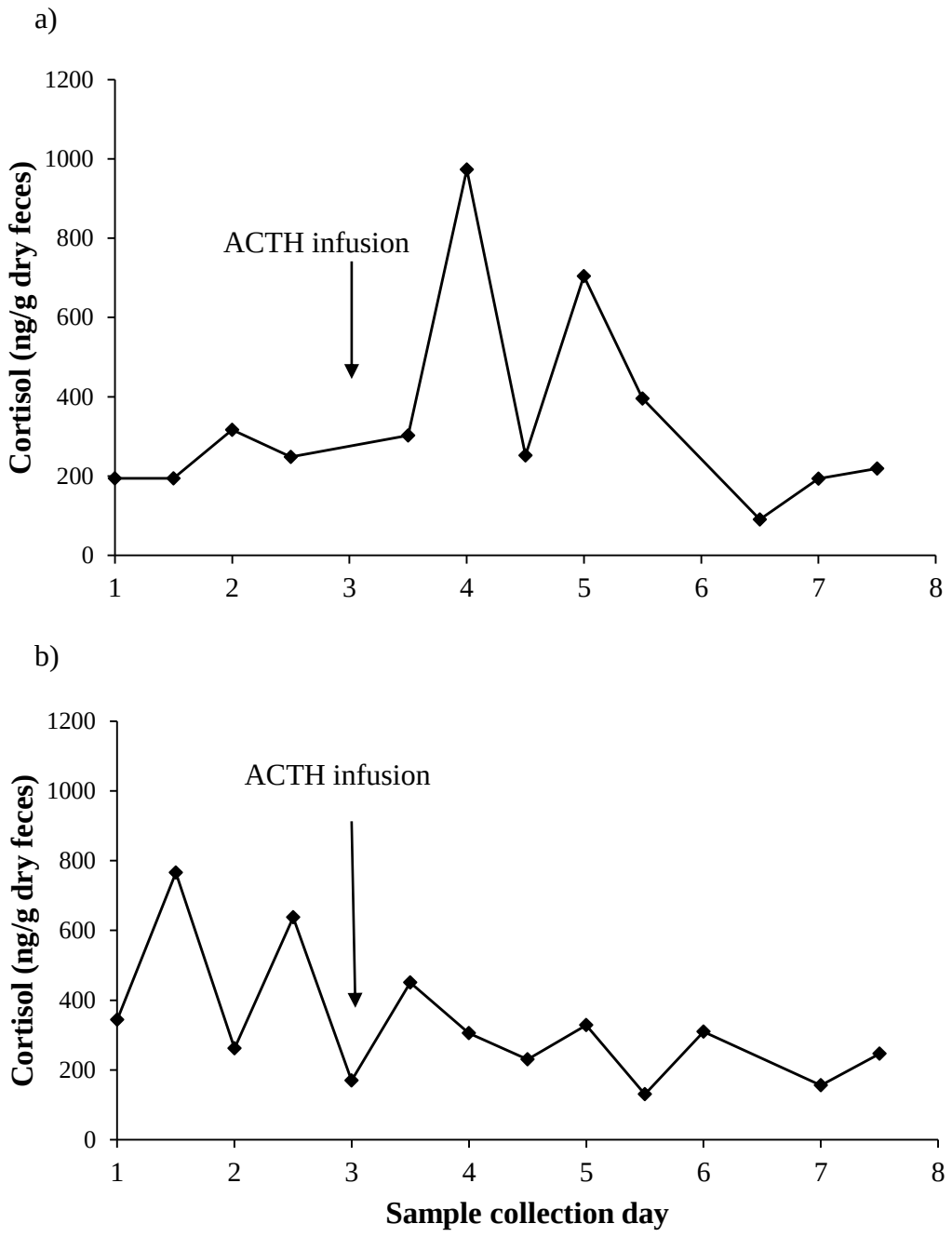


Figure 4. Cortisol in feces of river otter "Male A" (a) and "Male B" (b) before and after administration of exogenous ACTH at the Smithsonian National Zoological Park, Washington D.C.

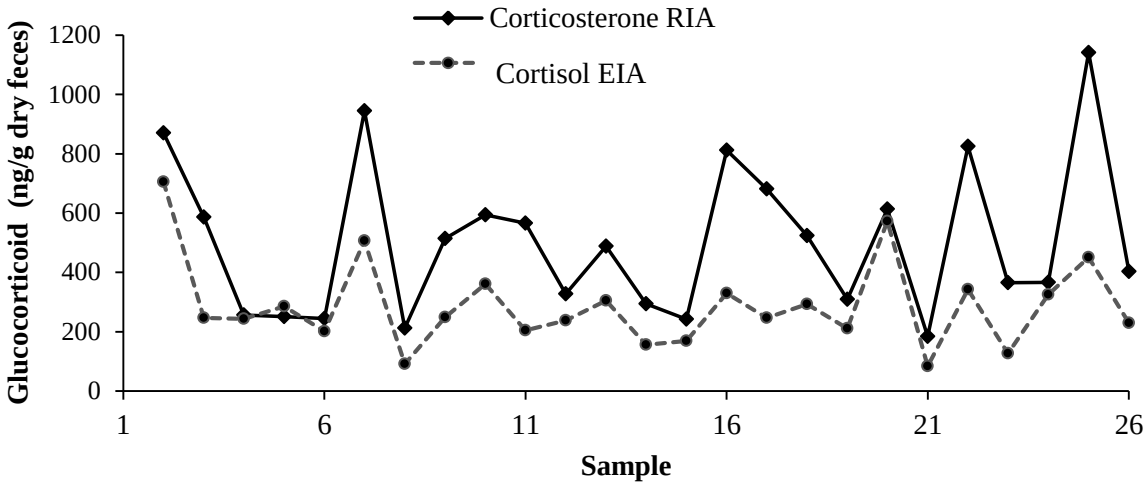


Figure 5. Results of corticosterone RIA and cortisol EIA performed on the same samples of captive river otter scat at the National Zoological Park, Washington D.C., USA.

and exhibited greater variance than cortisol for most samples, but the overall longitudinal pattern was similar to that of cortisol.

Evaluation of extraction efficiency was performed on all samples. Average extraction efficiency was 86% ± 0.003. The test for parallelism demonstrated that the sample hormone was immunologically similar to the standard hormone and could be measured proportionally (Fig. 6). Average inter-assay control and standard CVs were less than 15% and similar to the laboratory control monitor for cortisol.

Factors Affecting Cortisol Levels in Wild Otters

I analyzed 483 scat samples collected from wild otters. There was no difference in hormone levels of the freshest scats ranked A and those ranked B and C so all samples were pooled for subsequent analyses ($F_{2,545} = 0.25$, $p = 0.78$). Fecal cortisol concentration in the wild population had a baseline hormone level of 832.5 ± 27.6 ng/g, ranged from 8.8 – 6773.5 ng/g and differed significantly when compared to the captive otters' baseline of 259.1 ± 25.4 ng/g ($t_{587} = 4.23$, $p < 0.01$).

Water temperature and group size were not found to contribute parsimoniously to variation in fecal cortisol concentration when AIC was utilized for variable selection and thus these variables were removed from further analyses. The anthropogenic and socioecological models accounted for 7% (adjusted $R^2 = 0.07$) and 17% (adjusted $R^2=0.17$) of the variation in cortisol levels, respectively (Table 1). AIC_c weights were then calculated for 15 models representing all combinations of the variables location, turbidity, diet and parasite presence (Table 2). The most precise and parsimonious model included all four variables ($R^2 = 0.23$, Table 3). Little River and AMWS were associated

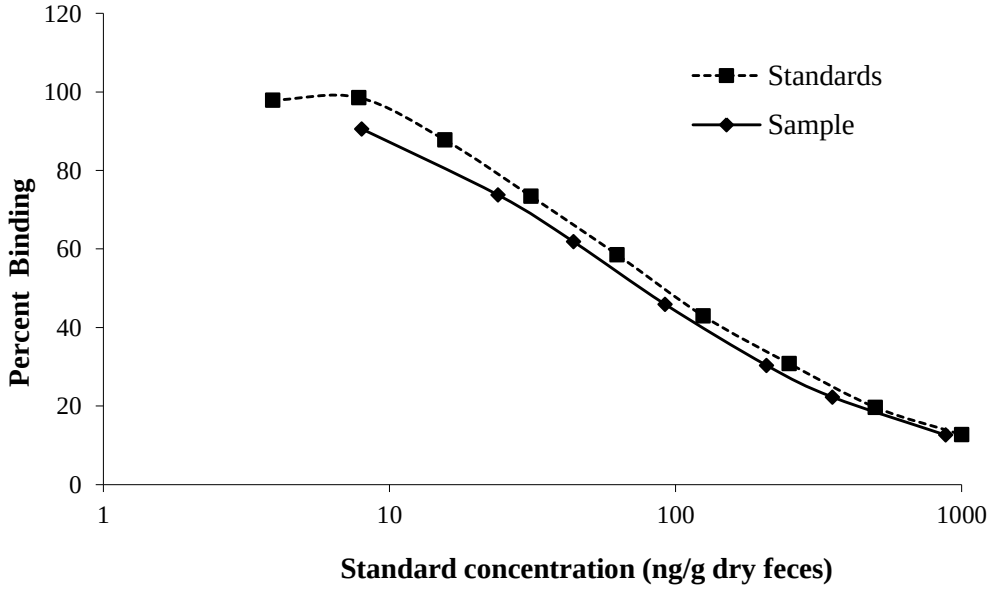


Figure 6. Parallelism for cortisol standard with serially diluted volumes of river otter scat (concentrations natural log transformed) as part of validation of EIA methods at the National Zoological Park, Washington D.C.

Table 1. Model-selection results, based on Akaike's Information Criterion, corrected for small sample size (AIC_c), generated from multiple regression analysis comparing the effects of socioecological vs. anthropogenic variables on river otter fecal cortisol concentration in coastal Humboldt County, California, Jun – Nov 2008.

Model	RSS[a]	K[b]	AICc	ΔAICc	wi[c]	R^2	Adjusted R^2
Socioecological: diet, parasite	255.77	6	-293.25	0.00	1.00	0.18	0.17
Anthropogenic: location, turbidity	286.98	9	-231.55	61.70	0.00	0.08	0.07

[a] Residual sum squared of error
[b] Number of parameters
[c] Akaike weight. Model weights indicate the weight of evidence that each model is the best approximating model.

Table 2. Model-selection results, based on Akaike's Information Criterion, corrected for small sample size (AIC_c), generated from multiple regression analysis of the effects of all possible variable combinations on river otter fecal cortisol concentration in coastal Humboldt County, California, Jun – Nov 2008.

Model	RSS[a]	K[b]	AICc	Δ AICc	w_i[c]
diet, parasite, turbidity, location	240.94	13	-307.4	0.00	0.53*
diet, parasite, location	242.18	12	-307.1	0.36	0.44*
diet, turbidity, location	246.27	11	-301.1	6.3	0.02
diet, location	247.56	11	-298.6	8.8	0.01
diet, turbidity	253.50	6	-297.5	9.9	0.00
diet, parasite	255.78	6	-293.2	14.2	0.00
diet, parasite, turbidity	257.48	7	-288.0	19.5	0.00
parasite, turbidity, location	279.88	10	-241.5	65.9	0.00
parasite, location	282.23	9	-239.6	67.8	0.00
parasite, turbidity	290.44	4	-236.1	71.4	0.00
turbidity, location	287.03	9	-231.5	76.0	0.00
turbidity	297.82	3	-226.0	81.4	0.00
parasite	303.86	3	-216.3	91.1	0.00
diet	261.78	5	-284.1	23.3	0.00
location	289.13	8	-230.0	77.4	0.00
intercept only (null model)	17702.13	1	1738.9	2046.3	0.00

[a] Residual sum squared of error
[b] Number of parameters
[c] Akaike weight. Model weights indicate the weight of evidence that each model is the best approximating model.
* The confidence set of candidate models includes all models with w_i within 10% of the highest ranked model. Only the highest ranked and 2nd ranked model fit these criteria.

Table 3. Multiple regression examining the effects of location, water turbidity, diet, and parasite presence on fecal cortisol concentration ($R^2 = 0.23$, adjusted $R^2 = 0.20$) of river otters in coastal Humboldt County, California, Jun - Nov 2008. Reference category for Location = Arcata Marsh and Wildlife Sanctuary, Diet = Fish, and Parasite = Absent.

Variable	β	β ± 95% confidence interval	SE	*p*
Intercept	6.16	5.81 – 6.51	0.180	<0.001
Location- Little River	-0.530	-0.861 – -0.199	0.168	0.002*
Location- Woodley Island	-0.361	-0.714 – -0.008	0.180	0.045*
Location- Mad River Slough	-0.339	-0.593 – -0.085	0.129	0.009*
Location- Mad River	-0.313	-0.619 – -0.007	0.156	0.045*
Location- Humboldt Bay Refuge	-0.282	-0.499 – -0.066	0.110	0.012*
Location- Elk River	-0.189	-0.391 – 0.013	0.103	0.066*
Turbidity	0.064	-0.012 – 0.139	0.039	0.100*
Diet- Crab	-0.813	-1.02 – -0.608	0.105	<0.001*
Diet- Bird	-0.557	-0.935 – -0.181	0.192	0.004*
Diet- Mixed	0.010	-0.151 – 0.172	0.082	0.900
Diet- Invertebrates	0.163	-0.144 – 0.469	0.156	0.297
Parasite- Present	0.500	-0.199 – -0.801	0.153	0.001*

* variables with significance levels $p \leq 0.10$.

with the lowest and highest predicted fecal cortisol concentrations, respectively (Figure 7). Crab and invertebrate diet categories were associated with the lowest and highest predicted cortisol levels, respectively (Figure 8).

Activity Center Differences

Turbidity varied significantly between locations ($F_{6,475} = 56.3$, $p < 0.001$, Fig. 9a) with Mad River and Little River having the least turbid water and Elk River and AMWS exhibiting the highest average turbidity. Average water temperature only ranged from 13.8 ° C to 17.6 °C between locations over the course of the study but varied significantly between activity centers ($H_{6,476} = 83.7$, $p < 0.001$, Fig. 9b). Composition of prey remains in river otter scat from the seven activity centers differed significantly ($X^2_{24} = 129.8$, $p < 0.001$, Fig. 9c). Scat from Elk River had the greatest percentage of fish when compared to other locations while scat from Little River had the lowest percentage of fish and the greatest percentage of crab found in scat. Parasite presence at the seven activity centers differed significantly between sites ($X^2_{27} = 14.4$, $p = 0.045$, Fig. 9d). There was no sign of parasite infection in scat from Little River or Mad River whereas 8.9 and 11% of the scat at Elk River and AMWS contained parasites, respectively. Scat grouping size was lowest at HBNWR ($\bar{x}$ =2.3 individuals) and the largest average scat grouping at Woodley Island ($\bar{x}$ = 3.1 individuals) and Elk River (3.5 individuals, $H_{6,476} = 17.3$, $p = 0.008$, Fig. 9e.). When these activity center differences were assessed alongside predicted, relative cortisol concentrations at the activity centers, AMWS stood out as the site with the highest predicted cortisol levels

as well as the highest average water turbidity, temperature, and percent fecal parasite presence.

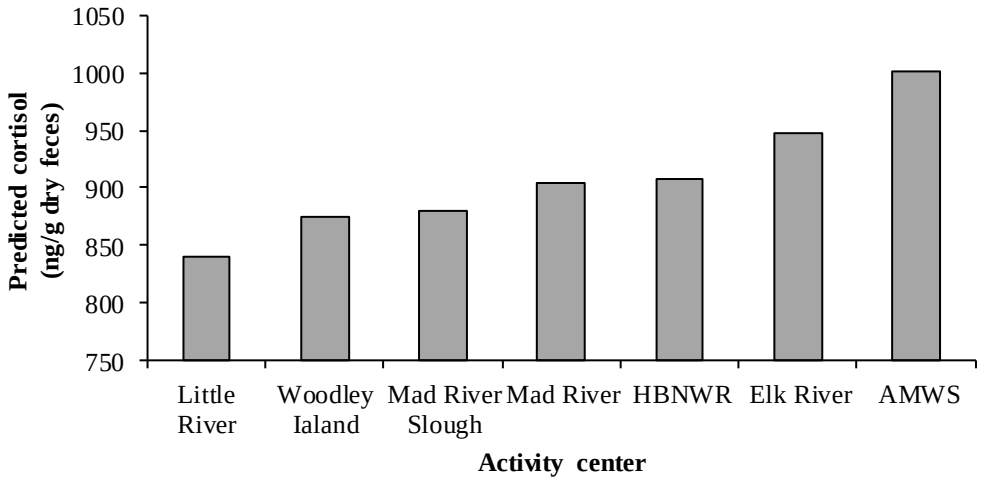


Figure 7. Relative relationship between cortisol concentration and river otter activity centers. Cortisol concentrations are predicted values based on beta coefficients (Table 3) from multiple regression equation. Activity centers are ordered from predicted least to greatest cortisol concentration.

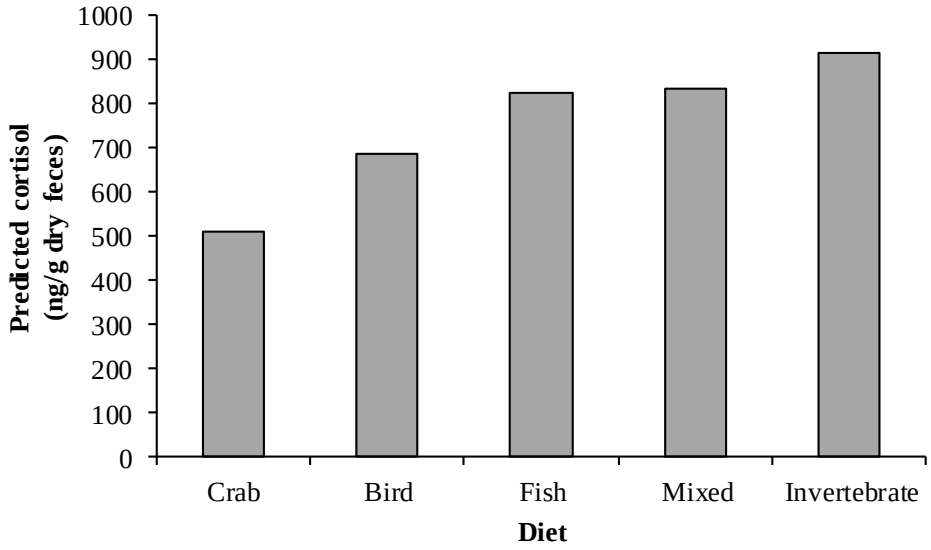


Figure 8. Relative relationship between cortisol concentration and diet composition. Cortisol concentrations are predicted values based on beta coefficients (Table 3) from multiple regression equation. Activity centers are ordered from predicted least to greatest cortisol concentration.

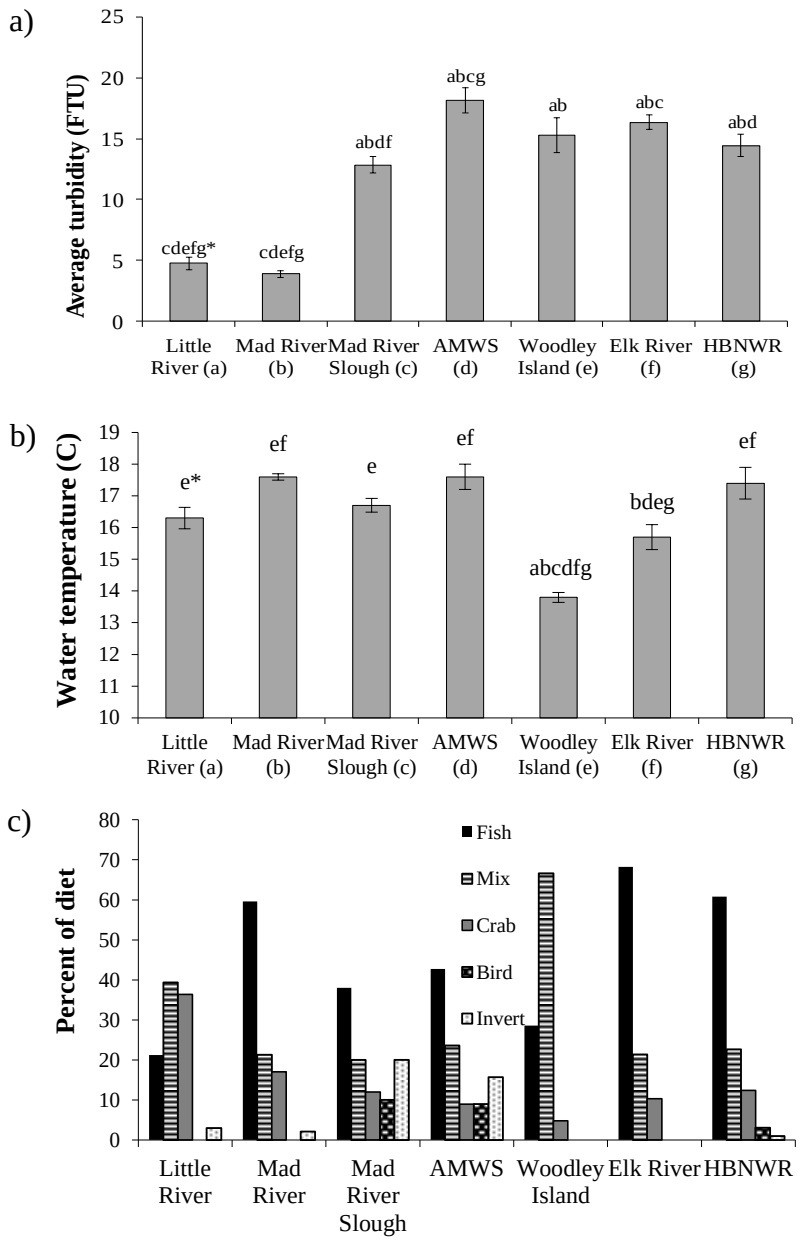
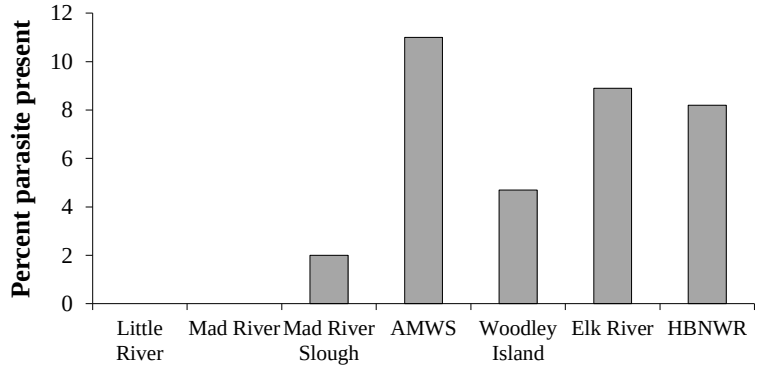


Figure 9. Relationship between location and water turbidity (a), water temperature (b), diet composition (c), percent fecal parasite presence (d) and scat grouping size (e) at seven river otter activity centers in coastal Humboldt County, California, Jun – Nov 2008. Sites are listed geographically from north to south. AMWS = Arcata Marsh and Wildlife Sanctuary, HBNWR = Humboldt Bay National Wildlife Refuge. Sample sizes (n) listed above bars in "e" are the same for figures "a-d". Means presented (±1SE). Letters above bars represent locations that differ significantly from one another at $p \leq 0.05$.

* Pair-wise activity center differences as determined from *post hoc* analysis (Tukey-Kramer).

d)



e)

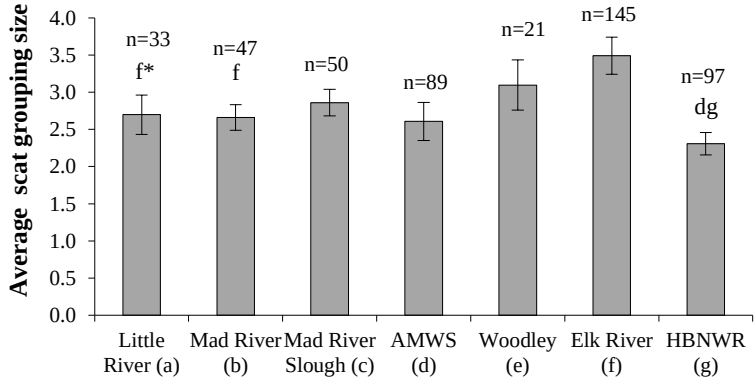


Figure 9 (continued). Relationship between location and water turbidity (a), water temperature (b), diet composition (c), percent fecal parasite presence (d) and scat grouping size (e) at seven river otter activity centers in coastal Humboldt County, California, Jun – Nov 2008. Sites are listed geographically from north to south. AMWS = Arcata Marsh and Wildlife Sanctuary, HBNWR = Humboldt Bay National Wildlife Refuge. Sample sizes (n) listed above bars in "e" are the same for figures "a-d". Means presented (±1SE). Letters above bars represent locations that differ significantly from one another at $p \leq 0.05$.

* Pair-wise activity center differences as determined from *post hoc* analysis (Tukey-Kramer).

DISCUSSION

Validation of Enzyme Immunoassay Methods

Methods for quantifying physiological stress with cortisol EIA in river otters were successfully validated. The results of the HPLC and sample matrix interference tests demonstrated that the cortisol EIA is specific to cortisol and is not influenced by similar glucocorticoid metabolites or other substances in the fecal extract. The two captive otters displayed different responses to the ACTH challenge test. Male A exhibited the expected pattern: low fecal cortisol concentration before the test, a spike in cortisol the afternoon following the test and a subsequent return to lower levels in the following days. Male B displayed several cortisol concentration spikes throughout the duration of the ACTH test, rendering his response inconclusive. Differences between the animals' responses could be attributed to individual differences in sensitivity to ACTH or ability to mount a stress response, interference of other unknown stressors in their captive environment, or an ACTH dosage that was not high enough to stimulate a response in Male B. The variation in GC levels before and after the challenge may indicate that this species is more reactive to their environment than other species subjected to an ACTH challenge, such as cheetahs (*Acinonyx jubatus*) and clouded leopards (*Neofelis nebulosa*), which have demonstrated more consistent GC levels pre- and post-challenge when subjected to this test (Young et al. 2004). These differences could be attributed to the life history and metabolic differences between species. River otters, with their high energetic demands, may benefit more from perceiving and reacting to changes in their environment. In contrast, feline species such as clouded leopards and cheetahs, which spend a large percentage of time resting may benefit from being less reactive to their environment. Despite some variation,

the link between endogenous reactions to ACTH challenges in carnivores has been well documented in general (Wasser et al. 2000). The only other otter species that has been subjected to this test is the sea otter (*Enhydra lutra*), which exhibited the expected positive endogenous adrenal response to exogenous ACTH.

Results of the corticosterone RIA and cortisol EIA comparison demonstrated that either method could be used to quantify glucocorticoid metabolite concentration. Cortisol EIA was chosen because these methods utilize less toxic chemicals and eliminate the need to dispose of radioactive materials. The average extraction efficiency of cortisol from fecal matter was similar to that of other carnivore species (Young et al. 2004) and the results of the parallelism test demonstrated that the sample and the standard were immunologically similar and could be measured proportionally.

Wild river otters had higher baseline fecal cortisol levels than captive otters, results dissimilar to comparative research on other species such as sparrows (*Zonotrichia spp*.) and spider monkeys (*Ateles geoffroyi yucatanensis*) (Marra et al. 1995, Rangel-Negrin et al. 2009). The case for expectations of greater stress response in captive or wild populations can be made for either group. Environmental degradation, variation in prey availability (Saltz and White 1991) breeding routine, acute exercise (Girard and Garland 2002) and several other factors addressed in this study could contribute to stress in a wild population. In captivity, human presence, artificial provisioning of food and habitat attributes, and forced social grouping could be expected to elevate stress levels (Morgan and Tromborg 2007). The higher cortisol levels in this wild population could reflect an actual difference between the wild and captive study groups or could be an artifact of the small captive population sample size or differences in gender ratios. The effects of gender

on cortisol levels in mammals are complex and difficult to predict. Sex related hormones are known to modify the responses of the HPA axis and affect the synthesis, secretion, metabolism and excretion of GCs. The captive animals were male brothers, while the gender structure of the wild population is not known with certainty. A recent genetic analysis of a subset of this population demonstrated that the population is slightly male-biased (Brzeski 2010).

Factors Affecting Stress in Wild Otters

Contrary to my predictions, the socioecological model was more strongly associated with physiological stress response than the anthropogenic model. However, this result does not necessarily mean that water contamination and turbidity do not affect stress hormone levels in river otters. It is possible that these disturbances were not great enough within the study area to pass a threshold where an effect would be detected. Other ways of detecting changes in physical stress response could also be considered. For example, Wada et al. (2009) found that tree swallows (*Tachycineta bicolor*) exposed to mercury exhibited a diminishing ability to mount an acute stress response. However, assessing these acute stress responses requires capturing and handling the animal, methods that may not be appropriate for species such as river otters.

Stress levels of river otters varied among differentially contaminated activity centers. I expected to see a positive correlation between the highly contaminated activity centers and fecal cortisol levels. There is some evidence for this; river otters at AMWS had the highest cortisol levels, while the Little River reference site otters exhibited the lowest cortisol levels. AMWS may be impacted not only by logging industry chemical contaminants, but also by contaminants including endocrine-disrupting pharmaceuticals leaching from a

landfill located on the site. However, hormone levels did not correlate as expected to the low contamination levels at HBNWR and high contamination levels at Mad River Slough and Woodley Island. The variable home range sizes and movement patterns of otters utilizing each activity center (Brzeski 2010) could possibly confound the association between stress levels and site contamination. Otters utilizing the activity centers at Mad River Slough, Woodley Island and HBNWR are known to have traveled to, and likely found prey at, sites with different contamination levels, and therefore the scat collected from these areas may not have been representative of the effects of contamination at those locations alone (Brzeski 2010).

Water turbidity was positively associated with stress hormone levels. Increases in precipitation during the fall and winter months in the Pacific Northwest bring increases in water turbidity as stream sediment is re-suspended and transported downstream. This natural increase in turbidity has been found to be amplified and temporally expanded by forestry practices which destabilize streamside environments and eliminate natural sediment traps (Binkley and Brown 1993). Highly turbid water can decrease the prey capture abilities of river otters (Prigioni et al. 2006). River otters choose prey based on preference, availability and attainability. Fish, when available, are generally their preferred prey (Melquist and Hornocker 1983, Penland and Black 2009). However, river otters may switch to more visible prey, such as crab or birds when water is highly turbid (Somers and Purves 1996). These more visible prey items may be of lesser quality or require more effort to catch and consume, leading to increases in physical stress levels. During the wet season, generally November-April in the study area, water and air temperature drop, and the availability of prey items change. Otters must meet the high energetic costs of their cold water foraging

strategies. Unlike many other aquatic mammals, they do not possess a thick layer of fat for thermal insulation. Instead they rely on a layer of dense fur which must be meticulously maintained in order to properly retain the fur's waterproofing and air trapping functions (Estes 1989, Kruuk 1995). Their cold water aquatic lifestyle, high metabolic rate and low body fat leaves a small margin for dealing with additional stressors that may complicate meeting their daily energy requirements. For these reasons, Kruuk (1995) suggested that otters may live at the edge of meeting their energetic needs. However, the lack of stress response to water temperature fluctuations in this sample population suggests that these otters' thermoregulatory abilities are sufficiently adapted to deal with the temperature variation found in this climate or the variation in temperature was not great enough to elicit a stress response.

Diet was related to variations in physiological stress response; crab and bird diet were associated with significantly lower stress levels than fish or mixed diet, while invertebrate diet was associated with greater stress levels than all other diets. The diet of river otters in this study population was similar to a recent and more thorough investigation into the dietary habits of local river otters and demonstrated that fish make up the majority of the otters' diet followed by crab, bird and insect in order of decreasing importance (Penland and Black 2009). Fish are usually the most common prey item found in river otter scat in the coastal areas of northwestern North America (Toweill 1974, Larsen 1984, Manning 1990, Penland and Black 2009). The health benefits of a diet rich in fish have been well documented and there is evidence that otters prefer fast moving prey such as fish, versus slow moving prey, such as crab, and will only eat dead or slow moving prey when they are hungry (Erlinge 1968, Watt 1991).

The significantly low hormone levels found in scat containing crab do not match these descriptions of prey preference and are contradictory to the aforementioned relationship between turbidity and cortisol levels. One would assume that crab is a low quality food item based on the handling time to process a crab and chance of injury from pincers (Watt 1991, Kruuk 1995). Watt (1991) suggests that otters would benefit energetically by spending time pursuing fish instead of crab. His investigation into the calorific content of *Lutra lutra* prey items found that fish, in general, had greater energy value (kJ/g) than shore crab and the energy gain per unit of time expenditure of shore crab was found to be less than that of fish. It is possible that complicating factors such as gut transit time and GCs from prey items could disrupt the relationship between physiological stress response and the corresponding fecal cortisol levels. These factors could contribute to the low cortisol levels associated with crab diet. Gut transit time influenced by diet and associated differences in steroid reabsorption have been demonstrated to affect hormone levels in humans (Goldin et al. 1981 and 1982, Anderson et al. 1987, Lewis et al. 1997) and potentially in Alaskan brown bears (*Ursus arctos*, von der Ohe et al. 2004). However, these studies investigated comparisons between vegetarian and omnivorous diets and high protein vs. high carbohydrate diets; comparisons which may not be relevant to the highly carnivorous diet of the river otter. The potential to ingest and excrete glucocorticoids from prey items must also be considered. Orally ingested glucocorticoids have been found to be bioavailable in the plasma of several species (Heazelwood 1984, Cooper et al.1996) and a field study found an association between salmon with high levels of cortisol and high levels of cortisol in the feces of brown bears preying upon them (von der Ohe et al. 2004). These associations and mechanisms, however, need further investigation.

The association between high GC levels and insect diet is consistent with studies and observations by Watt (1991) and Kruuk (1995) that suggest that fish diet is more energetically profitable than insect diet. Otters are considered specialized piscivorous predators and are more likely to resort to pursuing less desirable prey items when fish availability decreases (Kruuk 1986, Ruis-Olma et al. 2000). If it is assumed that more energy is expended to catch small prey items in comparison to their energetic reward, insects would be consumed in greater quantities, with more energy expended, when other more important prey items, such as fish, were less available.

My results indicated a strong association between parasite presence and elevated cortisol levels. Information relating to the presence of parasites in river otters and their etiological significance is scarce (Kimber and Kollias 2000). In river otters, endoparasites are more common than ectoparasites and are present in most individuals but are not thought to cause significant clinical disease (Kimber and Kollias 2000). Because it was not possible to identify the species of cestodes found in fecal samples due to lack of identifying characteristics, I do not know if the river otter is a true host of the cestode or if the cestode is an incidental parasite from a prey item (Kimber and Kollias 2000). The literature suggests that most cestodes are incidental (Hoberg et al. 1997, Kimber and Kollias 2000). The directionality of the cause and effect relationship between disease and stress was not possible to elucidate in this field study. A few semi-field studies have shown a specific link between stress and parasites wherein the causative factor was determined to be the parasite, followed by an associated stress response. For example, cliff swallows (*Hirundo pyrrhonota*) have been shown to exhibit a positive relationship between ectoparasite load and stress hormone levels, and high stress response was associated with *Mycoplasma*

infection in house finches (*Carpodacus mexicanus*) (Raouf et al. 2004, Lindstrom et al. 2005). However, other studies have found evidence for a complex association between stress, disease and a primary factor such as dietary insufficiency or environmental contamination, wherein stress was determined to be a factor leading to disease. Disease may follow stress because GCs are known to decrease inflammation and thus suppress the immune response, leading to illness (Chrousos 1995, Rigby and Moret 2000). This relationship was seen in a population of Eurasian otters in Denmark exhibiting an association between PCBs and skin lesions and infections along with decreased levels of hepatic retinal, a form of vitamin A, a nutrient important to embryonic development (Leonards et al. 1997). Additionally, experiments with mink (*Mustela vison*) have demonstrated suppressed cell-mediated immune response when exposed to bleached-kraft pulp mill effluent, a byproduct of paper production (Smits et al. 1996.)

Scat grouping size was not found to be associated with fecal cortisol levels. The methods used to calculate group size were an index of social contact and may not only reflect groups of river otters that travel together but also associations of otters that utilize the same latrines and whose home ranges overlap. These intra- and inter-group associations between individuals may be associated with similar stress responses. Within a group, direct interactions may take place between individuals, inducing a stress response. But individual otters who leave scat at latrines (inter-group associations) may be communicating important messages to other latrine visitors (Oldham and Black 2009). Kruuk (1995) suggests that fecal marking at latrines functions as short term communication to con-specifics, conveying that nearby resources are being depleted and both parties would benefit from resource partitioning; an indirect but important interaction.

Otters, and mustelids in general, are less social than most carnivores (Gittleman 1989), however, there is much variation in group structure and group size within *Lontra canadensis* (Blundell et al. 2002). Black (2009) found that group size in this study area ranges from 1-9 individuals with larger groups seen in summer than in winter. These data reflect similar findings of coastal dwelling river otters in southeast Alaska (Blundell et al. 2002). Differences in river otter sociality may reflect habitat variation, resource availability, foraging strategy or reproductive strategy. For instance, Blundell et al. (2002) found that male river otter group size changed in relation to resource availability. River otters appear to demonstrate dynamic social structures that change to best meet their immediate needs. The lack of stress response to group size could be explained by the minimal variation in group size or it could be due to the plastic nature of this species social strategies. More highly social species often exhibit more rigid social structures, changes to which would probably be more likely to affect stress levels.

Activity Center Differences

Although efforts were made to compare activity centers whose most substantial differences were attributed to contamination; water temperature, turbidity, diet, parasite presence and group size all differed among sites. However, some of these statistical differences may be scrutinized for their biological significance. For example, the greatest average pair-wise discrepancy in scat grouping size among activity centers involved a 1.2 animal difference between Elk River and HBNWR. It is not clear if these differences are great enough to say that locations differed biologically with respect to social dynamics.

Implications

Determination of fecal cortisol levels is a useful non-invasive method to monitor adrenal function in river otters and has the potential to be used as a diagnostic tool to identify populations at risk of stress-related pathologies. Monitoring of stress hormone levels could be utilized to help mitigate the adverse effects of anthropogenic and socioecological deviations in captive and wild populations. The interrelationships between the multifactorial effects of anthropogenic and socioecological factors on stress hormone levels make clarifying cause and effect linkages between these variables difficult. The addition of laboratory studies investigating the underlying mechanisms that may cause endocrine disruption and semi-field studies analyzing a more controlled relationship between external factors and stress response would be a valuable addition to this investigation (Vos et al. 2000). Further research is important to the overall understanding of the health of environmentally sensitive, elusive and wild populations of animals such as river otters. This study did not detect a strong association between stress and aquatic contamination, however, this proxy measure of health may not be affected until a threshold level of contaminant associated disturbance, perhaps not present in the study area, has been surpassed. This study implicates that socioecological factors, including diet and parasites, are associated with stress and therefore could affect health in wild river otters. Aquatic environments should be maintained in a way that minimizes additive stress beyond what this species typically experiences via socioecological pressures.

LITERATURE CITED

Adams, J. 2005. Investigation work plan for former McNamara and Peepe Lumber Mill. Prepared by Weiss Associates *for* California Department of Toxic Substances Control. Emeryville, USA.

Anderson K.E., W. Rosner, M.S. Khan, M.I. New, S.Y. Pang, P.S. Wissel, and A. Kappas. 1987. Diet-hormone interactions: protein/carbohydrate ratio alters reciprocally the plasma levels of testosterone and cortisol and their respective binding globulins in man. Life Science 40:1761–1768.

Aulerich, R.J. and R.K. Ringer. 1970. Some effects of chlorinated hydrocarbon pesticides on mink. American Fur Breeder 43:10-11.

Binkley, D. and T.C. Brown. 1993. Forests as non-point sources of pollution in North America. Water Resources Bulletin 29:729-740.

Black, J. 2009. River otter monitoring by citizen science volunteers in northern California: Social groups and litter size. Northwestern Naturalist 90:130-135.

Blundell, G.M., M. Ben-David and R.T. Bowyer. 2002. Sociality in river otters: cooperative foraging or reproductive strategies? Behavioral Ecology 13:134-141.

Brown, J.A. 2008. Wildlife endocrinology manual. National Zoological Park Conservation and Research Center 1-101.

Burnham, K.P., and D.R. Anderson. 2002. Model selection and multimodel inference: a practical information-theoretic approach. 2nd Edition. Springer-Verlag, New York, New York, USA. 488 pp

Brzeski, K.E. 2010. A non-invasive approach examining North American river otter abundance and sociality. Thesis. Humboldt State University, Arcata, California, USA.

Calcagni, E. and I. Elenkov. 2006. Stress system activity, innate and T helper cytokines, and susceptibility to immune-related disease. Annals of the New York Academy of Sciences 1069:62-77.

Chanin, P.R.F. and D.I. Jeffries. 1978. The decline of the otter *Lutra lutra* in Britain: an analysis of hunting records and discussion of causes. Biological Journal of the Linnean Society 10:305-328.

Chrousos, G.P. 1995. The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. New England Journal of Medicine 322:1351-1362.

City of Arcata. 2007. Arcata Marsh and Wildlife Sanctuary http://www.cityofarcata.com/index.php?option=com_content&task=view&id=20&Itemid=47 (accessed 10/15/2007).

Cooper, A.E., A.J. Gray, J. Collington, H. Seddon, I. Beattie, and C.J. Logan. 1996. Excretion and metabolism of tipredane, a novel glucocorticoid, in the rat, mouse, monkey, and human. Drug Metabolism and Disposition 24:1071–1080.

Ecological Rights Foundation. 2007. Environmental Toxics and Lumber Mill Sites. http://www.ecorights.org/mill_sites/mill_toppage.htm (accessed 9/27/2007).

Erlinge, S. 1968. Food studies on captive otters *Lutra lutra L*. Oikos 19:259-270.

Estes, J.A. 1989. Adaptations for aquatic living by carnivores. In: Carnivore behavior, ecology and evolution. J. L. Gittleman (Ed.) Cornell University Press, New York, New York, USA.

Fossi, M.C. and L.Marsili. 2003. Effects of endocrine disruptors in aquatic mammals. Pure Applied Chemistry. 75:2235-2247.

Foster-Turley, P., S. Macdonald and C. Mason (Eds.) 1990. Otters: an action plan for their Conservation. IUCN/SSC Otter Specialist Group. Gland, Switzerland: IUCN.

Gittleman, J.L. 1989. Carnivore group living: comparative trends. Pp. 183-207 In: Carnivore behavior, ecology, and evolution. Cornell University Press, New York, USA 183–207.

Goldin B.R., H. Adlercreutz, J.T. Dwyer, L. Swenson, J.H.Warram, and S.L. Gorbach. 1981. Effect of diet on excretion of estrogens in pre- and postmenopausal women. Cancer Research 41:3771–3773.

Goldin B.R., H. Adlercreutz, S.L. Gorbach, J.H. Warram, J.T. Dwyer, L. Swenson, and M.N. Woods. 1982. Estrogen excretion patterns and plasma levels in vegetarian and omnivorous women. New England Journal of Medicine 307:1542–1547.

Goymann, W. 2005. Noninvasive monitoring of hormones in bird droppings: physiological validations, sampling, extraction, sex differences, and the influence of diet on hormone metabolite levels. Annals of the New York Academy of Sciences 1046:35-53.

Girard, I. and T. Garland, Jr. 2002. Plasma corticosterone response to acute and chronic voluntary exercise in female house mice. Journal of Applied Physiology 92:1553–1561.

Henny, C.J., R.A. Grove, and O. Hedstrom. 1996. A field evaluation of mink and river otter on the lower Columbia River and the influence of environmental contaminants. Prepared for Lower Columbia River Bi-State Water Quality Program. Forest and Rangeland Ecosystems Science Center, National Biological Service, Corvallis, Oregon, U.S.A.

Hoberg, E.P., J.C. Henny, O.R. Hedstrom and R.A. Grove. 1997. Intestinal helminths of river otters (*Lutra canadensis*) from the Pacific Northwest. Journal of Parasitology 83:105-110.

Hontela, A., J.B. Rasmussen, C. Audet and G. Chevalier. 1992. Impaired cortisol stress response in fish from environments polluted with PAHs, PCBs, and mercury. Archives of Environmental Contamination and Toxicology 22:278-283.

Jönsson, C.J, B.O. Lund, Å. Bergman, and I. Brant.1993. Toxicity of o,p,'-DDD and p,p'-DDD in the adrenal cortex of mink (*Mustela vison*). Ecotoxicology 2:41-53.

Keay, J.M., J. Singh, M.C. Gaunt and T. Kaur. 2005. Fecal glucocorticoids and their metabolites as indicators of stress in various mammalian species: a literature review. Journal of Zoo and Wildlife Medicine 37:234-244.

Kimber, K.R. and G.V. Kollias. 2000. Infectious and parasitic diseases and contaminant related problems of North American river otters (*Lontra canadensis*): A review. Journal of Zoology and Wildlife Medicine 31:452-472.

Kruuk, H. 1986. Wild otters: Predation and Population. Oxford University Press, Oxford, United Kingdom 184-198.

Kruuk, H. 1992. Scent making by otters (*Lutra lutra*) signaling the use of resources. Behavioral Ecology 3:133-140.

Kruuk, H. 1995. Wild otters: Predation and populations. Oxford University Press. New York, USA.

Kruuk, H. and J.W.H. Conroy. 1991. Mortality of Otters (*Lutra lutra*) in Shetland. The Journal of Applied Ecology 28:83-94.

Lappe, M. 2001. Potential and likely environmental and human health risks from off-site movement of chemicals from the Sierra Pacific Industries site at 2293 Samoa Road, Arcata, California.

Larsen, D.N. 1984. Feeding habits of river otters in coastal southeastern Alaska. Journal of Wildlife Management 48:1446-1451.

Leonards, P.E.G., Y. Zierikzee, U.A.T. Brinkman, W.P. Cofino, N.M. van Straalen, and B.V. van Hattum. 1997. The selective dietary accumulation of planar polychlorinated biphenyls in the otter (*Lutra lutra*). Environmental Toxicology and Chemistry 16:1807-1815.

Lewis S.J., K.W. Heaton, R.E. Oakey, and H.H. McGarrigle. 1997. Lower serum oestrogen concentrations associated with faster intestinal transit. British Journal of Cancer 76:395–400.

Lindström, K.M., D.M. Hawley, A.K. Davis and M. Wikelski. 2005. Stress responses and disease in three wintering house finch (*Carpodacus mexicanus*) populations along a latitudinal gradient. General and Comparative Endocrinology 143:231–239.

Manning, T. 1990. Summer feeding habits of river otter (*Lutra canadensis*) on the Mendocino National Forest, California. Northwestern Naturalist 71:38-42.

Marra, P.P., K.T. Lampe and B.L. Tedford. 1995. Plasma corticosterone levels in two species of *Zonatrichia* sparrows under captive and free living conditions. Wilson Bulletin 107:296-305.

Mason, C.F. 1995. Habitat quality, water quality and otter distribution. Hystrix 7:195-207.

Mason, C.F. and S. Macdonald. 1986. Otters: Ecology and conservation. Cambridge University Press, New York, USA.

Mason, C.F. and S.M. MacDonald. 1993. Impacts of organochlorine pesticide residues and PCBs on otters (*Lutra lutra*) in eastern England. The Science of the Total Environment 138:147-160.

Mason, C.F. and S.M. MacDonald. 1994. PCBs and organochlorine pesticide residues in otters (*Lutra lutra*) and in otter spraints from SW England and their likely impact on populations. Science of the Total Environment 144:305-312

Mason, C.F., S.M. Macdonald, H.C. Bland and J. Ratford. 1991. Organochlorine pesticide and PCB contents in otter scats from western Scotland. Water, Air, & Soil Pollution 64:617-626.

Mason, C.F., and A.B. Madsen. 1993. Organochlorine pesticide residues and PCBs in Danish otters (*Lutra lutra*). The Science of the Total Environment 133:73-81.

Matteri, R.L., J.A. Carroll, and C.J. Dyer. 2000. Neuroendocrine responses to stress. Pp. 43–76 In: Moberg G.P. and J.A. Mench (Eds.) The Biology of Animal Stress: Basic Principles and Implications for Animal Welfare. CABI Publishing, Oxon, U.K.

Melquist, W.E. and M. G. Hornocker. 1983. Ecology of river otters in west central Idaho. Wildlife Monographs 83:1–60.

Melquist and Hornocker 2003. North American river otter (*Lontra canadensis).* Pp. 708-731 *in* Feldhamer, G.A., B.C. Thompson, and J.A. Chapman (Eds.) Wild mammals of North America: biology, management, and conservation, 2nd edition edited by. Johns Hopkins University Press, Baltimore, USA.

Melquist, W.E., J.S. Whitman, and M.G. Hornocker. 1981. Resource partitioning and coexistence of sympatric mink and river otter populations. Pp. 187- 220 In: Chapman, J. A and D. Pursley (Eds.), World- wide Furbearer Conference Proceedings, Frostburg, USA.

Millspaugh, J.J. and B.E. Washburn. 2004. Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. General and Comparative Endocrinology 138:189-199.

Moberg, G.P. 2000. Biological response to stress: implication for animal welfare. Pp.1–21 In: Moberg G.P. and J.A. Mench (Eds), The Biology of Animal Stress: Basic Principles and Implications for Animal Welfare. CABI Publishing, Oxon, U.K.

Mohr, F.C., B. Lasley and S. Bursian. 2008. Chronic oral exposure to bunker C fuel oil causes adrenal insufficiency in ranch mink (*Mustela vison*). Archives of Environmental Contamination and Toxicology 54:337-347.

Monfort, S.L. 2003. Non-invasive endocrine measures of reproduction and stress in wild populations. Pp. 146–165 In: Holt, W. V., A. R. Pickard, J. C. Rodger and D. E. Wild (Eds) Reproductive Science and Integrated Conservation . Cambridge, London.

Morgan, K.N. and C.T. Tromborg 2007. Sources of stress in captivity. Applied Animal Behaviour Science 102: 262–302.

New, J.G. 1959. Additional uses of dyes for studying the movements of small mammals. The Journal of Wildlife Management 23: 348-351.

North Coast Regional Water Quality Control Board [NCRWQB]. 2007. The 303(d) list of impaired waterbodies. http://www.waterbodies.ca.gov/tmdl/303d_lists2006approved.html (accessed 10/10/2008).

Office of Environmental Health Hazard Assessment [OEHHA]. 2006. Toxicity criteria database. http://oehha.ca.gov/risk/chemicaldb/index.asp (accessed 7/14/2009).

Oldham, A.R and J.M. Black. 2009. Experimental tests of latrine use and communication by river otters. Northwestern Naturalist 90:207-211.

Penland, T.F. and J.M. Black. 2009. Seasonal variation in river otter diet in coastal northern California. Northwestern Naturalist 90:233-237.

Prenda, J., P. López-Nieves and R. Bravo. 2001. Conservation of otter (*Lutra lutra*) in a Mediterranean area: the importance of habitat quality and temporal variation in water availability. Aquatic Conservation: Marine and Freshwater Ecosystems 11: 343–355.

Prigioni, C., A. Balestrieri, L. Remonti, A. Gargaro and G. Priore. 2006. Diet of the Eurasian otter (*Lutra lutra*) in relation to freshwater habitats and alien fish species in southern Italy. Ethology, Ecology and Evolution 18: 307-320.

Rangel-Negrin, A., J.L. Alfaro, R.A. Valdez, M.C. Romano and J. C. Serio-Silva. 2009. Stress in Yucatan spider monkeys: effects of environmental conditions on fecal cortisol levels in wild and captive populations. Animal Conservation 12:496-502.

Raouf, S.A., L.C. Smith, M.B. Brown, J.C. Wingfield and C.R. Brown. 2004. Glucocorticoid hormone levels increase with group size and parasite load in cliff swallows. Animal Behaviour 71:39-48.

Reeder, D. T.H. Kunz, and E.P. Widmaier. 2004. Baseline and stress-induced glucocorticoids during reproduction in the variable flying fox, *Pteropus hymelanus* (Chiroptera: *Pteropodidae*). Journal of Experimental Zoology 310A:682-690.

Rigby, M.C. and Y. Moret. 2000. Life history trade-offs with immune defenses. Pp. 129-142 In: Evolutionary Biology of Host Parasite Relationships: Theory Meets Reality. Poulin, R., S. Morand and A. Skorping (Eds). Elsevier Science, Amsterdam, The Netherlands.

Romero, L.M. and M. Wikelski. 2001. Corticosterone levels predict survival probabilities of Galapagos marine iguanas during El Nino events. Proceedings of the National Academy of Sciences 98:7366-7370.

Ruis-Olma, J., J.M. Lopez-Martin and S. Palazon. 2000. The influence of fish abundance on the otter (*Lutra lutra*) populations in Iberian Mediterranean habitats. Journal of Zoology 254:325-336.

Saltz, D. and G.C. White. 1991. Urinary cortisol and urea nitrogen responses to winter stress in mule deer. Journal of Wildlife Management 55:1–16.

Sapolsky, R.M. and W.A. Pulsinelli. 1985. Glucocorticoids potentiate ischemic injury to neurons: therapeutic implications. Science 229:1397-1400.

Smits, J.E.G., D.M. Haines, B.R. Blakley, and G.A. Wobeser. 1996. Enhanced antibody responses in mink (*Mustela vison*) exposed to dietary bleach-kraft pulp mill affluent. Environmental Toxicology and Chemistry 15:1166-70.

Somers, M.J. and M.G. Purves. 1996. Trophic overlap between three syntopic semi-aquatic carnivores: Cape clawless otter, spotted-necked otter, and water mongoose. African Journal of Ecology 34:158-166.

Songsasen, N., M. Rodden, J.L. Brown and D.E. Wildt. 2006. Patterns of fecal gonadal hormone metabolites in the maned wolf (*Chrysocyon brachyurus*). Theriogenology 66:1743-1750.

State Water Resources Control Board, Draft Staff Report Revisions of the Clean Water Act 303(d) List of Water Quality Limited Segments. Table 2, pp. 8-9 Sept 2005.

Texas Commission on Environmental Quality. 2007. Surface water quality monitoring procedures, Volume 1. http://www.tceq.state.tx.us/comm_exec/forms_pubs?pubs?rg/rg-415/index.html (accessed 10/17/2007).

Touma, C., and R. Palme. 2005. Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. Annals of the New York Academy of Science 1046:54–74.

Toweil, D.E. 1974. Winter food habits of river otters in western Oregon. Journal of Wildlife Management 38:107-111.

von der Ohe, C.G., S.K. Wasser, K.E. Hunt, and C. Servheen. 2004. Factors associated with fecal glucocorticoids in Alaskan brown bears (*Ursus arctos horribilis*). Physiological and Biochemical Zoology 77:313–320.

Vos, J.G., E. Dybing, H.A. Greim, O. Ladefoged, C. Lambré, J.V. Tarazona, I. Brandt, and A.D. Velthaak. 1997. Health effects of endocrine disrupting chemicals on wildlife, with special attention to the European situation. Critical Reviews in Toxicology 30:71-133.

Wasser, S.K., K. Bevis, G. King and E. Hanson. 1997. Noninvasive physiological measures of disturbance in the northern spotted owl. Conservation Biology 11:1019-1022.

Wasser, S.K., K.E. Hunt, J.L. Brown, K. Cooper, C.M. Crockett, U. Bechert, J.J. Millspaugh, S. Larson and S.L. Monfort. 2000. A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species. General and Comparative Endocrinology 120:260–275.

Watt, J. 1991. Prey selection by coastal otters (*Lutra lutra L.).* Dissertation. University of Aberdeen, Scotland.

Wada, H., D.A. Cristol, F.M.A. McNabb and W.A. Hopkins. 2009. Suppressed adrenocortical responses and thyroid hormone levels in birds near a mercury-contaminated river. Environmental Science and Technology. 43:6031–6038.

Western Hemisphere Shorebird Reserve Network. 2007. Humboldt Bay Complex. http://www.whsrn.org/HumboldtBayComplex/index.html (accessed 10/10/2007).

Wingfield J.C., K. Hunt, C. Breuner, K. Dunlop, G.S. Fowler, L. Freed and L. Lepson. 1997. Environmental stress, field endocrinology and conservation biology. Pp. 95-131 In: Behavioral approaches to conservation in the wild. Clemmons, J.R. and R. Buchholz (Eds). Cambridge University Press, Cambridge, USA.

Wingfield, J.C. and L.M. Romero. 2001. Adrenocortical responses to stress and their modulation in free-living vertebrates. Pp. 211-234 In: Handbook of physiology, section 7: The endocrine system, Vol. 4: Coping with the environment: neural and endocrine mechanisms. McEwen, B.S. and H.M. Goodman (Eds). Oxford University Press, New York, USA.

Wren, C.D. 1991. Cause-effect linkages between chemicals and populations of mink (*Mustela vison*) and otter (*Lutra canadensis*) in the Great Lakes. Journal of Toxicology and Environmental Health 33:549-85.

Young, K.M., S.L. Walker, C. Lanthier, W.T. Waddell, S.L. Monfort and J.L. Brown. 2004. Noninvasive monitoring of adrenalcortical activity in carnivores by fecal glucocorticoid analyses. General and Comparative Endocrinology 137:148-165.