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IMPACTS OF PLASTIC POLLUTION ON A PELAGIC MARINE MAMMAL, THE NORTHERN ELEPHANT SEAL

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By

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DEDICATION

I would like to dedicate this thesis to my family and friends for their unconditional support throughout my life and growing career. To my family, Mom, Dad, Lindsay, Bachan, and Kinako, for weekly video chats reminding me that school and research stresses are just that – At home, Mom still falls asleep watching Friends, Dad still looks forward to weekly grocery ads, Lindsay's still getting better grades than me, Bachan still doesn't know why I've spent the last couple years in Stockton, and Kinako is still a licking machine. To my middle/high school friends for never changing and keeping me grounded through annual secret santas. To my college friend/roommate Jamie for supporting my running away to Monterey and being the Monterey Bay Aquarium's best naturalist. To Lysander for listening to me after long days, stepping up in terms of cooking, dishes, and laundry during crunch time for this thesis, and for following me to Stockton and beyond.

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IMPACTS OF PLASTIC POLLUTION ON A PELAGIC MARINE MAMMAL, THE NORTHERN ELEPHANT SEAL

Abstract

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As plastic pollution increases, top marine predators such as marine mammals are becoming increasingly susceptible to plastic particles and their additives. Plastic particles have been found in gastrointestinal tracts and scat of many marine mammals, and quantifying plastic pollution in those that are pelagic can provide insight into plastic pollution in mesopelagic ecosystems that are just beginning to be analyzed. Adapting well-developed laboratory techniques for microplastic (MP) isolation (i.e. density separation and chemical digestion), I isolated MPs from the scat of the deepest diving pinniped, the northern elephant seal (NES), and found that100% of scat samples (n=11) contained high counts of MPs compared to other pinnipeds. Further, as plastic particles move through the digestive tract and potentially translocate into the circulation, body cells may be exposed to these plastic particles. Nanoplastics (NPs;<1μm) have been shown to affect cell viability and redox homeostasis in fish and human cells, while the lipophilic additives bisphenol-a (BPA) and bisphenol-s (BPS) used in plastics production are known endocrine disruptors. However, the effects of plastics (NPs) and plastics additives (BPA and BPS) have not been well-studied in marine mammals. To assess the direct impacts of NPs on marine mammal cells, I exposed fibroblasts isolated from NES skin (n=6 experiments) to polystyrene NPs of two different sizes (0.05μm and 0.5μm) and concentrations ($3.64x10^9$ and $3.64x10^{10}$ particles/mL) and did not find consistent effects on morphology and viability. Cell

viability, which was quantified by an MTT assay, decreased as a result of NP exposure in two experimental replicates, but these results were not reproducible. I found that NPs did not have consistent effects on the morphology or viability of NES fibroblasts, regardless of their size or concentration. Lastly, I examined the effects of plastic additives on the physiology of marine mammal blubber tissue, the primary energy depot and reservoir of lipophilic pollutants. Precision-cut NES blubber slices were exposed to BPA and BPS, alone and in combination with the lipolytic hormone epinephrine, and I assessed their effects on the blubber transcriptome. I found that while BPA and BPS treatments alone did not have a pronounced effect on gene expression, they altered the expression of several genes associated with lipid homeostasis and adipogenesis. These data suggest that NES likely ingest MPs and maybe physiologically affected by exposure to plastic particles and their associated contaminants.

TABLE OF CONTENTS

LIST OF TABLES

Table

LIST OF FIGURES

Figure

CHAPTER 1: INTRODUCTION

Plastics are ever-present in human lives and the environment; their abundance has increased over 300-fold since their introduction in the 1950s (Alimba & Faggio, 2019). In 2010, 4.8 to 12.7 million metric tons of plastic waste entered our oceans from 192 coastal countries, without taking into account accidental or illegal dumping (Jambeck et al., 2015). As plastics mechanically degrade due to wave action, they often produce microplastics (MPs, <5 mm). MPs are considered a "diverse contaminant suite" due to their composition complexities, including different polymers, shapes, sizes, and additives (Rochman et al., 2019). MP concentrations have been well-documented in surface waters (0-1 m depth) and are prevalent in marine ecosystems globally (Cózar et al., 2014; Eriksen et al., 2014). MPs have been found in organisms throughout marine food webs from plankton to seafood ready for human consumption (Andrady, 2011; Rochman et al., 2015).

As plastics are exposed to sunlight, they can release contaminants that adsorb to MPs and nanoplastics (<1 μm; NPs), so organisms ingesting small plastic particles may be exposed to concentrated chemical cocktails. Plastics and their associated contaminants can bioaccumulate and biomagnify up the food chain, yet their physiological impacts are just beginning to be understood (Corsolini & Sarà, 2017; Stienbarger et al., 2021). Understanding these physiological impacts on an individual level can provide a better idea of how these pollutants may eventually scale up to affect communities and populations. While not as persistent or cumulative as other plastic contaminants, plastic additives are potential endocrine disruptors and have been known to disrupt neurological, cardiovascular, and endocrine functions in mammals (Boucher et al., 2016; da Silva et al., 2019; Jochmanová et al., 2015). Determining the

consequences of plastic pollution in marine environments is challenging but important, and disentangling the impacts of this complex stressor on wildlife is an ongoing management concern (Bucci et al., 2020).

Marine mammals are ideal study systems for monitoring plastic pollution as they are top predators and therefore serve as good indicators of environmental pollution and overall ecosystem health (Bossart, 2006). Marine mammals are both economically and ecologically significant to their environments, with some being considered keystone species. They are also impacted by plastic pollution. Not only are marine mammals often entangled by macroplastics, but they are also likely consuming large quantities of MPs and NPs, either directly during filter-feeding by baleen whales, or indirectly by consumption of prey at higher trophic levels. For example, 55% (n=24/44) of scat samples from fur seals that forage in the northeast Pacific contained a mean of 16.6 MP particles. Further, because of their large fat stores and trophic level, they are also susceptible to lipophilic contaminants. While not a persistent organic pollutant, the plastic additive bisphenol-a (BPA) has been detected in the blood and milk of Baltic grey seals, indicating that these pollutants may be offloaded to pups during nursing (Donohue et al., 2019; Nehring et al., 2018). However, the concentrations or impacts of BPA and BPS in deeper-diving, pelagic pinnipeds have not yet been explored.

Northern elephant seals (NES; *Mirounga angustirostris*) in particular allow us to study plastic pollution in a pelagic marine mammal. They spend 90% of their lives out at sea feeding, but unlike other pelagic mammals, NES return to land twice a year, during which time they are easily accessible for sampling. Adult NES predictably haul out at California's Año Nuevo State Park to breed (January-February) and molt (May-June) every year. NES dive in mesopelagic waters (400-600 m) that have been found to contain high concentrations of MPs, compared to

surface waters, and feed on organisms such as lanternfish that have also been found to contain MPs (Choy et al., 2019; Gassel & Rochman, 2019). NES also forage on squid, and though MPs have not yet been analyzed in squid species that are consumed by NES, they have been detected in squid ready for human consumption, suggesting that they are likely to accumulate in NES prey (Daniel et al., 2021). Analyzing NES provides the unique opportunity to examine the consumption of MPs and NPs by pelagic predators, providing insights into plastic pollution in mesopelagic ecosystems, explore the potential biological impacts of MPs and NPs, and predict how open-ocean marine mammals may be affected by exposure to pollutants used in plastics manufacturing. This study is the first to examine plastic pollution in NES and the first to explore the biological impacts of plastic particles and their contaminants on marine mammals.

Study Objectives

First, I sought to (1) determine and quantify MP presence in scat of NES. I hypothesized that given that NES forage on fishes and at depths found to contain MPs, they likely ingest MPs and NPs, which may be detectable in scat samples left by animals on the beach when they haul out for molting and breeding. I then aimed to (2) assess the direct effects of NP particles on NES cell viability and morphology, and (3) explore the effects of plastic additives on NES blubber gene expression. Ingestion of MP and NP particles and additives used in plastic pollution, such as bisphenol-A (BPA) and bisphenol-S (BPS), may lead to their absorption via the digestive system and processing in the liver, causing direct effects on NES cell and tissue physiology. Due to the challenges of conducting contaminant exposure experiments in whole animals, we used *in vitro* systems (culture of skin-derived fibroblasts and blubber tissue explants) to enable carefully controlled plastic and contaminant exposure experiments. Because previous studies have found that smaller plastic particles (NPs) may be more toxic than MPs, I hypothesized that exposure of NES cells to NPs in culture would decrease their viability and alter cellular morphology. I also

hypothesized that exposure of blubber tissue to BPA and BPS would alter the expression of genes associated with lipid metabolism that may have downstream impacts on NES fitness. NES are likely exposed to MPs, NPs, and their associated contaminants via their diets on myctophid fishes. Together, the results of this study can provide insights into biological consequences of anthropogenic pollution in marine ecosystems and can inspire individual and corporate change in everyday plastic usage.

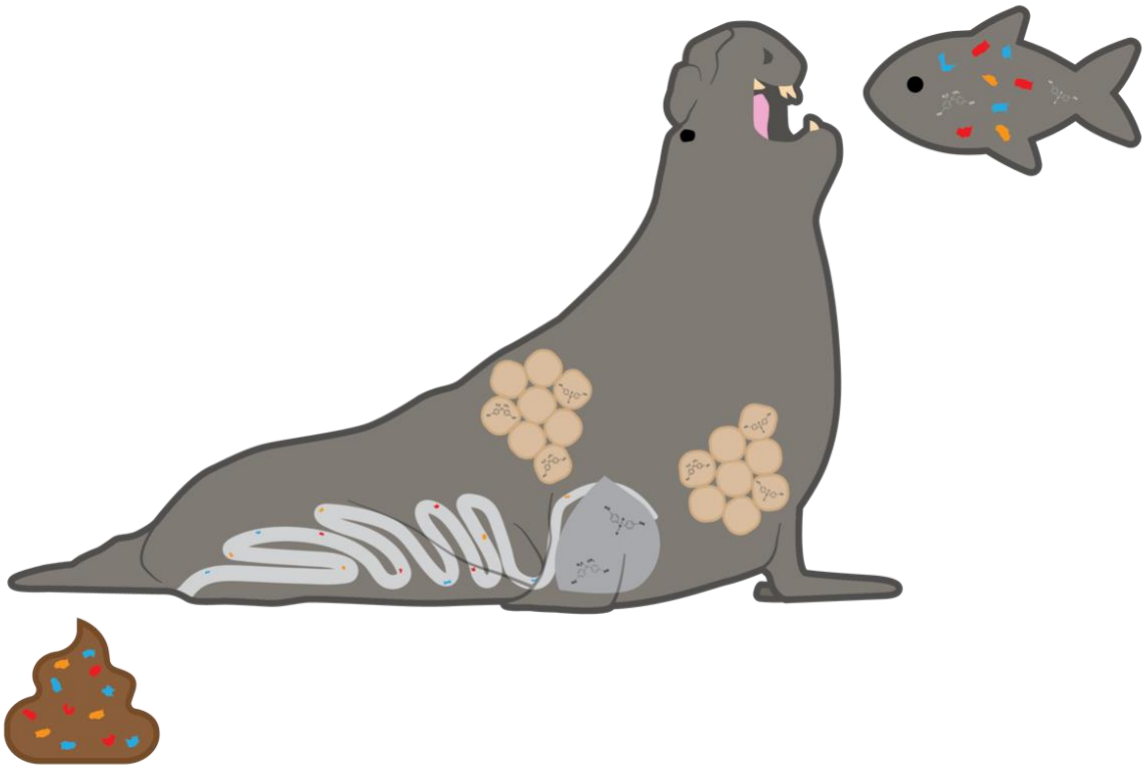

Figure 1. Schematic of MP and additive movement through NES. Tan circles are adipose tissue. Chemical structures are BPA and BPS. Small colorful fragments represent MPs and NPs.

CHAPTER 2: EVIDENCE OF MICROPLASTIC INGESTION BY NES

Introduction

MP concentrations in surface waters have been well-characterized (Andrady, 2011). As MPs persist in marine ecosystems, they are prone to biofouling (accumulating microorganisms such as algae), causing them to become denser and sink further down the water column over time, such that the highest concentrations of plastic waste are deposited on the seafloor (Kaiser et al., 2017). Plastic polymers of different densities also float at different depths in the water column, but there have been few studies that quantify MPs at depth in marine ecosystems due to the cost of sampling deeper waters. However, MPs have been found in waters ranging from 0-1000 m that overlap with marine mammal foraging depths. Marine mammals have also been found to contain MPs. In a study examining gastrointestinal tracts of stranded and deceased marine mammals of 10 species, MP particles were found in 100% of individuals sampled (n=50), indicating that MPs can persist within digestive systems (Nelms, Barnett, et al., 2019). As it is not possible to sample from the digestive tracts of live marine mammals, scat samples are often used to determine MP concentrations that have recently passed through the digestive system (Garcia-Garin et al., 2020; Nelms, Parry, et al., 2019).

MPs have been found in scat of many pinniped species (e.g., harbor seals, gray seals, fur seals, sea lions) but have never been examined in NES, which forage in mesopelagic, rather than coastal ecosystems (Zantis et al., 2021). NES forage at depths of up to 800 m and feed primarily on myctophid fishes, such as Pacific lanternfish, of which 33% have been found to contain MPs (mean = 13.5 particles/fish) (Lusher et al., 2016; Wagner et al., 2017). NES travel from rookeries on the California coast to the Aleutian Islands in Alaska (males) and the northwest

Pacific Ocean near the Pacific Garbage Patch (females) to forage, and utilize a variety of foraging strategies. While many NES males forage closer to the continental shelf and within the benthic zone, females forage near seamounts and the mesopelagic zone (Adachi et al. 2021). Therefore, exposure to MP pollution may differ by sex and diet.

Confirming plastic presence in NES would indicate that MPs are moving through the mesopelagic food web and can provide information on MP concentrations in less-studied offshore ecosystems at depth (300 - 800 m). Before investigating the potential physiological impacts of plastic pollution (Chapters 3 and 4), MP particle presence was first examined in NES scat to determine if NES are ingesting plastics. Scat samples were collected at two coastal rookeries during two different haulouts: Año Nuevo State Park (adult female and juvenile molt) and Point Reyes National Seashore (subadult and adult male molt).

Methods

Sample collection. NES often defecate when they first haul out upon returning to their rookeries for breeding and molting. During the spring of 2020 and 2021, NES fecal samples left on the beach by juvenile and adult female and male NES were collected from Año Nuevo State Park (n=8 scat, likely from juveniles and adult females) and Point Reyes National Seashore (n=3 scat, from subadult and adult males), respectively (Figure 2). The sample size was limited by accessibility to the animals and the low amount of scat present on the beach as animals haul out for several weeks. Fecal samples were collected from the beach into 50 mL Falcon tubes using sterile tongue depressors. Samples were sealed and immediately frozen on-site using either liquid nitrogen or dry ice. Environmental blanks (sand, n=5) were collected from the same sites as fecal samples and frozen as described above. Samples were divided into 9-12 g subsamples upon return to the laboratory and stored in Petri dishes at -80ºC before further processing.

Figure 2. Sampling locations at NES rookeries on the California coast. n refers to the number of scat samples collected at each site.

Isolating MPs from fecal samples. MP isolation was performed following modified published protocols (Figure 3) (Donohue et al., 2019; Garcia-Garin et al., 2020; Hudak & Sette, 2019). Density separation was performed by placing each semi-thawed subsample into a glass beaker with approximately 600 mL of 30% (w/v) NaCl. Subsamples were stirred with magnetic stir bars at high speed for ~15 minutes before they were left to separate overnight without stirring. Once solutions were well-separated, a serological pipette was used to transfer the top fraction containing synthetic material to new beakers for chemical digestion to remove organic material remaining after density separation. Samples were digested using 30% (w/v) KOH added to 1x sample volume and stirred on hot plates at 60ºC for 4 h to 7 days.

Once solutions appeared clear and no longer contained any visible organic material, they were poured through a vacuum filtration system onto fiberglass filters (1 μm particle retention 9.0 cm diameter, VWR, USA). Due to the large volumes of these solutions, subsamples were collected on multiple filters (1-9 filters per subsample). Filters were dried and stored in individual Petri dishes sealed with Parafilm until imaging. Laboratory blanks were processed using the same MP isolation techniques with MilliQ water.

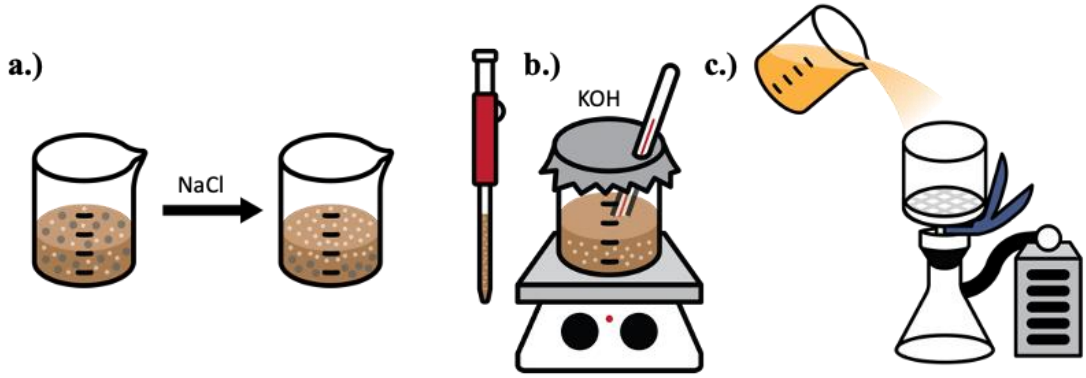

Figure 3. MP isolation workflow. (a) Density separation with 30% NaCl solution and serological pipette transfer of separated solution to a new beaker. (b) Chemical digestion using 15% KOH solution at 60ºC. (c) Vacuum filtration onto fiberglass filters.

Quantifying MP concentrations. MP particles on filters were imaged as follows. First, filters were divided into quadrants (Figure 4). Then, the area of the field of view at 10X magnification on a dissecting microscope was calculated. Photos were taken of one quarter of each of three quadrants of a filter, covering 33% of the area. Quadrants and sections within filters were randomly selected for imaging and MP analysis (Figure 4).

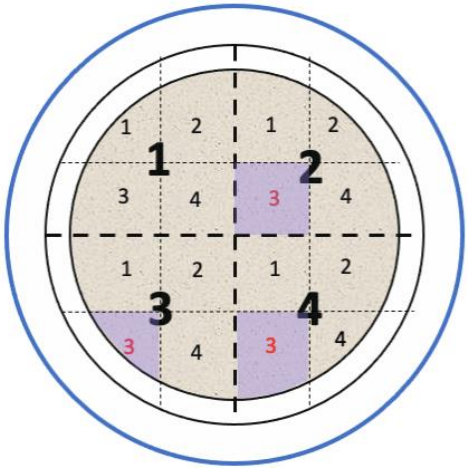

Figure 4. Schematic showing quadrants and sections of quadrants of filters that were numbered for random selection for imaging and MP analysis. For example, if 1 and 3 were randomly generated, photos would be taken in the shaded purple sections.

Statistical analyses. To obtain MP concentrations per filter, the MP counts on each filter were multiplied by 3. Total particle counts obtained per filter were combined with filters from the same subsample. Concentrations were then calculated to determine the mean MP counts per gram of subsample. Subsample concentrations were combined to obtain MP concentrations per whole fecal sample. Lab blank concentrations were averaged and subtracted from scat and environmental concentrations to correct for laboratory contamination. Environmental blanks were then averaged per location and subtracted from sample concentrations accordingly.

Shapiro-Wilk's and the Flinger Killeen tests were used to determine normality and equal variances of MP counts in scat from the two locations, respectively. Our data were not normally distributed, so an unpaired two-sided Wilcoxon signed rank sum test was used to compare MP abundance in fecal samples between different sampling locations. To satisfy the equal variance assumption, data were inversely transformed. All analyses were conducted using R (v4.1.0) in R-studio (v1.4.1106).

Results

MP concentrations in laboratory and environmental blank samples were extremely low (mean (± s.d.) for lab blanks: 0.006 ± 0.008 particles/g; environmental blanks: 0.17 ± 0.13 particles/g). One hundred percent (11/11) of scat samples were found to contain MPs with the mean (± s.d.) normalized concentration of 0.67 ± 1.28 particles/g (wet weight). Unlike previous protocols, sieves were not used to isolate MPs as they did not seem to increase yield during method development and MPs found tended to be either small particles (<1mm) or fibers. MP particle concentrations differed significantly between scat collected at Año Nuevo (n=8, 1.02 ± 1.70 particles/g) and Point Reyes (n=3, 0.31 ± 0.33 particles/g; W = 3780, p < 0.0001; 95% CI: -2.02, -0.40) (Figure 5), however there were no significant differences in MP concentrations between environmental blanks collected from Año Nuevo (0.192 ± 0.118) and Point Reyes (p=0.3213).

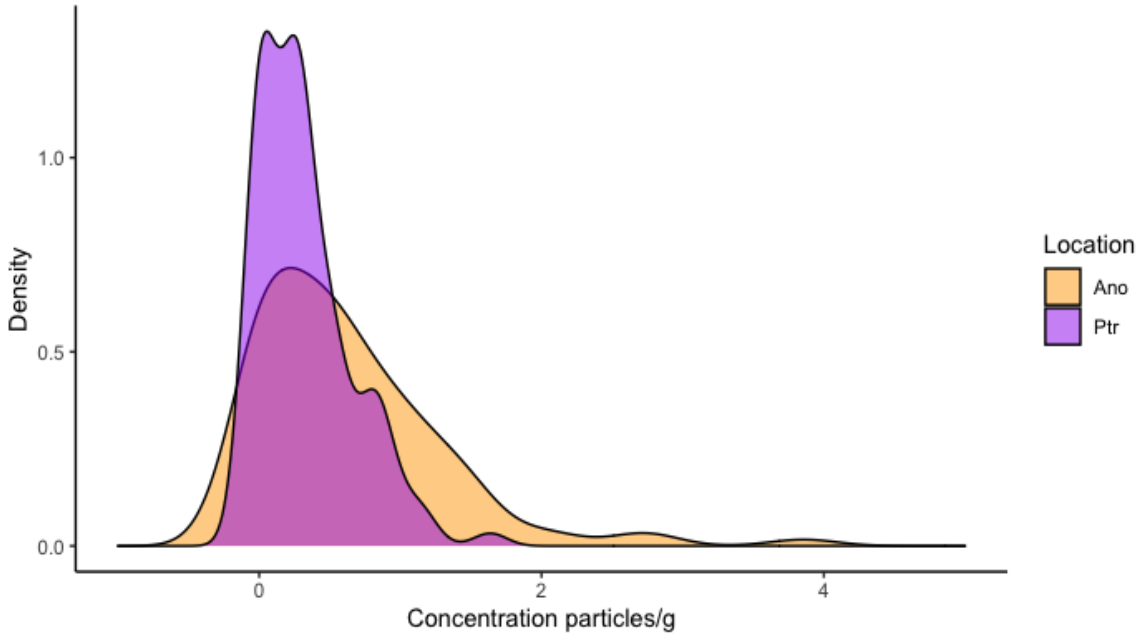

Figure 5. Density plot of MP concentrations in scat collected at Año Nuevo State Park (Ano, orange, n = 8) and Point Reyes National Seashore (Ptr, purple, n = 3).

Table 1
Summary of Reported MP Particle Counts in Scat of Pinnipeds; Adapted from Zantis et al., (2021). Neither Laboratory nor Environmental Blanks Included in Particle Counts. ANNU: Año Nuevo State Park, PTNR: Point Reyes National Seashore.

Species	Sample size	% samples with MPs	Mean particle count +/- SD	Reference
Otariidae				
Antarctic fur Seal	154	100	1.13 ± 0.43	Erikson and Burton, 2003
Phocidae				
Grey seal	26	48	0.87 ± 1.09	Nelms et al., 2018
Harbor seal	32	6	0.06 ± 0.25*	Hudak and Sette, 2019
Northern elephant seal				
ANNU	3	100	9.06 ± 14.0	This study
PTNR	8		3.71 ± 3.89	

*Indicates microfibers were not included in means

Discussion

This study aimed to determine whether MP particles can be detected in the scat of a deep-diving, pelagic marine mammal, suggesting exposure to and potential ingestion of MPs at sea. MPs were present at low concentrations (0.67 ± 1.28 particles/g, n = 11 samples), but above background environmental concentrations (0.17 ± 0.13 particles/g) in NES scat samples collected at two coastal rookeries. However, unadjusted particle counts (not corrected by environmental and lab blanks) were relatively high compared to other pinnipeds (Table 1). This suggests that NES are consuming MPs while foraging offshore and that MP concentrations in the digestive tract are likely much higher when animals are feeding, rather than fasting. Differences in MP concentrations in scat collected at different rookeries, Año Nuevo State Park (1.01 ± 1.80 particles/g) and Point Reyes National Seashore (0.30 ± 0.32 particles/g), are likely due to

differences in foraging behavior between adult males and adult females and juveniles, which were present on beaches during scat collection at Point Reyes and Año Nuevo, respectively.

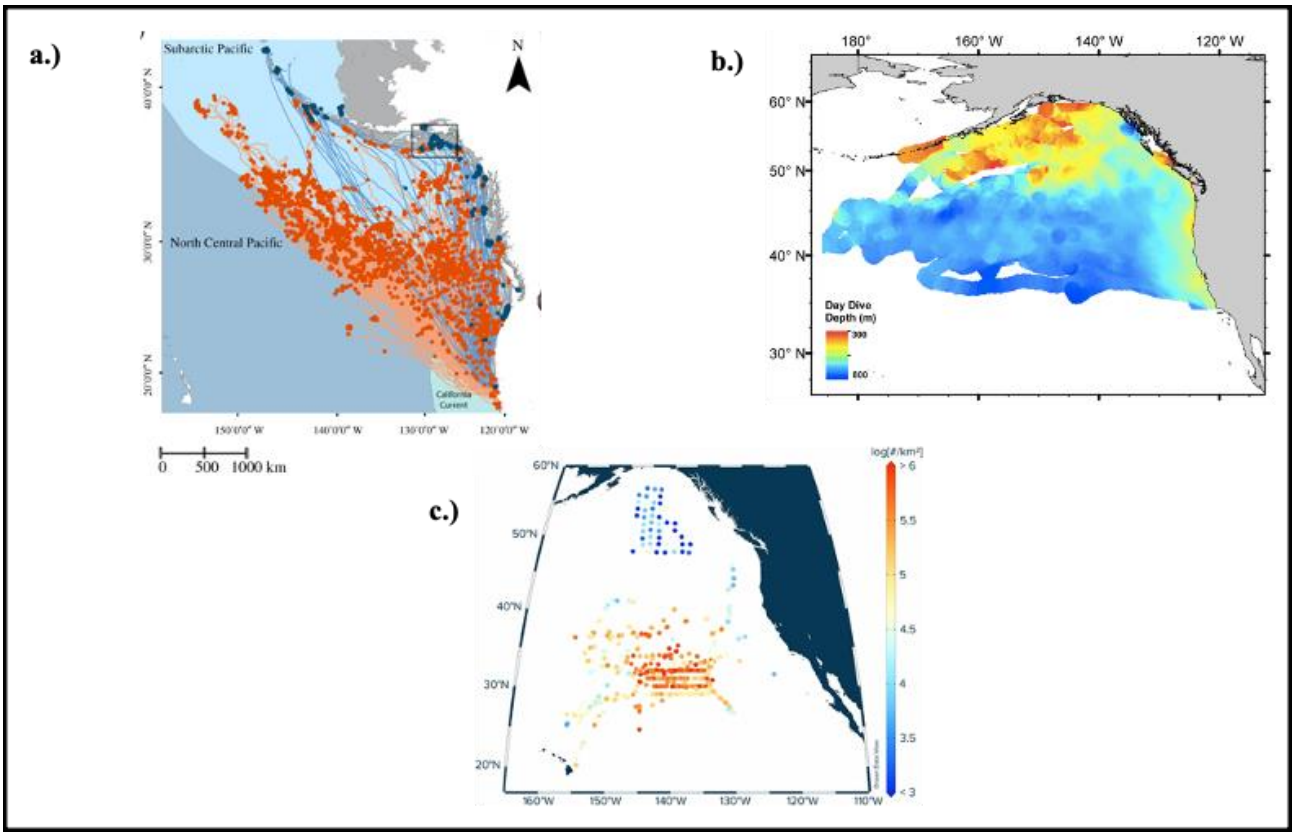

Figure 6. Maps of male and female foraging locations and MP surface concentrations. (a) Map showing foraging locations of NES adult males (blue; n=39) and females (red; n=178); from Kienle et al., 2022. (b) Map showing NES adult female (n=95) foraging locations and depths; from Robinson et al., 2012. (c) Map showing surface microplastic concentrations; from Egger et al., 2020.

Sampling of scat from different age classes at different rookeries complicated our analyses. Año Nuevo State Park and Point Reyes National Seashore are both located on the California coast, and MP concentrations have not been well-characterized in either location. The only studies that have measured MP concentrations along the Northern California coast have been conducted nearshore at the Santa Cruz Boardwalk (3.21 particles/m^3 seawater) and further

offshore in the Greater Farallones and Cordell Bank (1.12 particles/m^3 seawater) (Kashiwabara et al., 2021, Box et al., 2019). Differences in MP concentrations between scat collected at each rookery may be caused by differences in environmental MP concentrations at each location. However, MP consumption is most likely to occur while foraging at sea, although some may occur via incidental ingestion of MP-containing seawater while animals are traveling between foraging grounds and rookeries. While environmental blanks were used to normalize MP concentrations in scat collected at each rookery, further studies should increase sample sizes and sample equal numbers of scats from different sexes within the same rookery.

Scat samples were collected from beaches during haul out periods during which either adult and subadult males (Point Reyes National Seashore) or adult females and juveniles (Año Nuevo State Park) were present. Female and male NES may be exposed to different MP concentrations as a result of distinct foraging behaviors. Female and male NES are also known to forage in different locations and at different depths, which may alter their level of exposure to MP pollution. Female NES travel west and forage in the North Central Pacific, while males travel further north along the U.S. West Coast and forage along the Aleutian Islands (Figure 6a) (Le Boeuf et al., 2000). MP presence has been recorded in surface waters of known foraging locations for both adult male and female NES (Figure 6c). Males primarily forage along the Alaskan coast, where MP concentrations are relatively low, and while uncommon, they are more likely than females to risk killer whale predation and partake in benthic foraging to feed on larger demersal fish(Adachi et al. 2021). Ocean sediment is believed to have the highest MP concentrations due to marine snow accumulation, however, demersal fish have not been found to have higher concentrations of MPs compared to pelagic fish (Lusher et al., 2013). Females forage more frequently on myctophid fishes and do not typically forage in the benthos, however,

they forage in regions closer to the Pacific Garbage Patch (Figure 6c)(Adachi et al. 2021). Female and male NES primarily forage on lanternfish, other mesopelagic fishes, and squid, which have been found to contain MPs (Daniel et al., 2021).

The MP concentrations in NES scat likely represent minimum concentrations remaining in the gut after cessation of feeding. As NES travel back to their rookeries at Año Nuevo and Point Reyes from foraging grounds, they likely defecate before their arrival, removing most MPs from the digesting tract by the time they arrive on the beach. However, gut transit time from ingestion to excretion is unknown in this species (Nelms, Barnett, et al., 2019). Transit time in gray seals has been recorded at 88 h (Nelms et al. 2018). NES, which are more than double in size, likely have longer gut transit times, but they still stop foraging several days before arriving back at the rookery. It is also possible that some MPs may adhere to the gut wall and remain in the digestive tract, rather than be excreted, and that NPs, which are not detectable by the methods used in this study, may be small enough to translocate through gut tissue into the circulation (Shen et al., 2019).

Next, we plan to characterize specific plastic polymers detected in NES fecal samples using Fourier-transformed infrared spectroscopy. Briefly, particles isolated from NES scat will be examined across a spectral library to determine polymer types. MPs may be denser polymers (polytetrathatlate, polycarbonate, polyamide), as those have been found deeper in the water column (Choy et al., 2019). This will allow me to identify potential sources of plastics that may be contributing to MP accumulation in NES prey and foraging locations. Identifying the types of products that may be responsible for plastic pollution in pelagic ecosystems could inform personal and industry standards for plastic production, usage, recycling, and disposal.

Despite a small sample size, which was limited by accessibility to the animals and the small amount of scat present on the beach as animals haul out over several weeks, this study is the first to report evidence of MP ingestion by NES. As MP concentrations have only recently begun to be measured at depth, this study contributes preliminary information about MP presence in offshore mesopelagic ecosystems and their transfer through food webs (Davidson et al., 2012; Lusher et al., 2016). Confirming ingestion of MPs by NES warrants further investigation of the impacts of plastic exposure on marine mammal physiology.

CHAPTER 3: DIRECT EFFECT OF NANOPLASTICS ON NES CELLS

Introduction

As MPs (1 μm to<5 mm) mechanically degrade due to wave action in ocean ecosystems, they produce NPs (<1 μm), which are estimated to be more toxic than MPs (Bergmann 2015). MPs and NPs have been shown to accumulate in digestive tracts and gills of fish larvae. However, because of their small size, NPs are more likely to translocate through the gut wall and come into contact with cells throughout the organism and have been found within muscle and liver tissue in fishes (Clark et al., 2022). Consumption of fishes containing plastics by marine mammals can cause bioaccumulation, indicating that higher trophic level animals, such as marine mammals, may be more likely to be exposed compared to lower trophic level animals and their environments (Chae et al., 2018).

MPs and NPs may impact cellular function in several ways. They have been shown to reduce viability in mammalian cells in a concentration- and size-dependent manner, with smaller NPs having higher levels of toxicity (Xu 2003; Chen et al. 2017; Banerjee and Shelver 2021). NPs may reduce cell viability mechanically, via membrane disruption, or physiologically, by inducing oxidative stress or immune response (Banerjee & Shelver, 2021). However, the physiological effects of NP particles on marine mammals, which are highly resistant to oxidative stress (Del Águila-Vargas et al., 2020), have not yet been explored.

Cell culture provides a system for ecotoxicological studies in organisms that cannot be ethically or practically done *in vivo*. Cell culture techniques have been used in the field of marine mammal ecotoxicology, providing context for our study (Weijs & Zaccaroni, 2016). Cell culture techniques enabled us to conduct direct exposure experiments of NES cells to NPs of

varying sizes and concentrations and examination of their effects on cell morphology and viability.

To determine the isolated effects of plastic particles on cell function, virgin plastics were used in this study. Virgin plastics are plastics that have not been recycled or additionally processed from initial production, so the effects of plastic particles can be analyzed without the presence of additional additives. Specifically, we used virgin PS beads of varying sizes, which have been used in many other ecotoxicological studies (Yong et al., 2020).

Methods

Sample collection and preparation. Skin biopsies were collected from adult female NES (n=3) at Año Nuevo State Park in Spring 2021. All procedures were approved by Sonoma State University IACUC and NMFS Permit #19108. Animals were sampled after molting, a time of rapid skin synthesis and activity. Seals were immobilized with an intramuscular injection of ~1mg/kg tiletamine-zolazepam HCl and sedated with intravenous injections of 0.25-1 mg/kg ketamine. Skin biopsies were collected from the posterior flank of each individual lateral to its spine. Samples were rinsed Dulbecco's Phosphate-Buffered Saline (DPBS, Gibco) and placed in DMEM with 1X antimycotic/antibiotic (A/A, Gibco) on ice until returned to the laboratory.

Fibroblast isolation from tissue. Fur was removed from skin samples, and the remaining tissue was cut into several ~5 mm explants. Two explants were placed into each well of a 6-well plate containing 2.5 mL DMEM with 10% FetalClone III Serum (FC3, Cytiva Life Sciences) and 1X A/A (complete media). Explants were cultured at 37°C with 5.0% CO_2. Culture media was changed and fibroblast growth was evaluated by microscopy every 2 d. Once fibroblasts migrated from skin explants (~7 d), explants were removed and fibroblasts were allowed to grow to 90% confluence.

Cells were detached and counted before cryopreservation. Briefly, cells were rinsed with 2 mL DPBS followed by incubation with 500 μL of 0.25% trypsin-EDTA (VWR) at 37°C for 5 min. After cells were detached from culture dishes, they were diluted with 4.5 mL DPBS and spun at 200xg for 7 min. Cell pellets were resuspended in DMEM and counted using the 0.4% trypan blue exclusion method on a hemocytometer; viability was also confirmed using trypan blue. Cells were spun down and resuspended in freezing media (10% DMSO in FC3) at concentrations of 2-5x10^6 cells/mL. Cells were frozen in a cooling block before being transferred to -80°C for storage.

Nanoplastic exposures. Frozen cells were diluted with DMEM, spun at 200 x g for 7 minutes, and the cell pellet was resuspended in complete media. Cells were seeded into 6-cm culture dishes in a total volume of 5 mL.

Fibroblasts were co-cultured with NP particles using a protocol modified from Espinosa et al., (2017). After reaching 90% confluence, cells were detached, counted, and diluted in media lacking antibiotics (DMEM with 10% FC3). Cell suspension (200 μL) was plated in 96-well plates at a cell density of 1 x 10^5 per well. After 24-48h of incubation (or until cells reached ~60% confluence), PS beads of either 0.05 μm or 0.50 μm diameter were added to treatment wells at concentrations of either 1:10 (2.5μg/mL, 3.64x10^{10} particles/mL) or 1:100 (0.25μg/mL , 3.64x10^9 particles/mL) for 24h (Figure 7) (PolySciences, Microbead Kit #3). Previous studies have used concentrations of PS beads ranging from 0 μg/mL to100 mg/mL (Hwang et al. 2019; Poma et al. 2019). The microbeads used in this experiment were supplied in a 2.5% w/v stock solution necessitating dilute experimental concentrations compared to previous studies. These concentrations, however, may be closer environmental concentrations (<0.1 μg/mL) (Xiao et al. 2022). Several dilutions were tested to gauge different orders of magnitude. Cells were exposed

to NPs for 24h before beads were removed. As a positive control for decreased cell viability and the MTT assay, H_2O_2 was added to cells at 5 mM or 10 mM final concentration for 2h. Cells cultured in media without H_2O_2 or NPs served as a negative control. Negative control treatments were conducted using 6 replicate wells, while NP and H_2O_2 exposures were performed in 3 replicate wells. All wells were rinsed 3x with DPBS to remove residual NPs or H_2O_2 remaining after treatments, which was verified by microscopy.

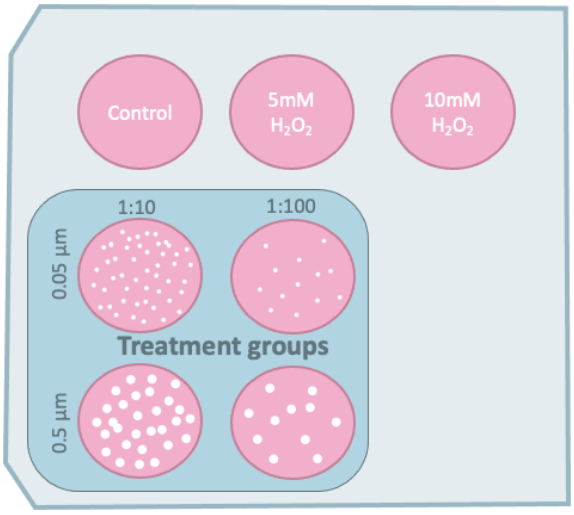

Figure 7. Schematic overview of the NP exposure experiment. Cells were cultured in media alone (negative control), with 5 mM or 10 mM H_2O_2 (positive control), or one of two dilutions (1:10, 1:100) of PS particles of either two sizes (0.05 µm or 0.50 µm). Controls were run in 6 replicate wells and H_2O_2 and NP treatments were run in 3 replicate wells in 96-well plates.

MTT assay. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay (Cayman, US) was used to determine cell viability following NP exposures. Cells were incubated for 4 h with MTT reagent, followed by 16-18 h with a crystal-dissolving solution at 37ºC. Absorbance was measured at 570 nm using a spectrophotometer. Absorbance values are positively correlated with cell viability.

Cell imaging. After sterilization with 100% ethanol, glass coverslips were placed in 12-well plates and incubated with 1 μg/mL fibronectin (FN, VWR) in PBS for 1 hour, followed by 2 rinses with DPBS. Fibroblasts were seeded at $0.1x10^6$ cells/well on FN-coated coverslips in a 12-well plate. Once they reached 50-60% confluence (~2 days), cells were exposed to 0.05 μm PS beads at 1:10 dilution as described above. After 24 h exposure, cells were rinsed and fixed in 4% paraformaldehyde for 15 minutes (VWR), rinsed in PBS, permeabilized with 0.1% Triton-X (VWR), stained with Alexa Fluor™ 568 phalloidin (Invitrogen), and mounted with SlowFade™ Gold Antifade Mountant with DAPI (Invitrogen). Cells were imaged using a Zeiss Axioscope fluorescence microscope at 40X magnification using RFP and CFP filters to visualize nuclei and the actin cytoskeleton.

Statistical analyses. Exploratory analyses to find absorbance means, standard deviations, and % coefficient of variation (CV) were conducted using R-studio. All analyses were completed with 6 experimental runs, and data represent inter- and intra-assay means and variability. Analyses were also conducted on individual experimental runs before analyzing them in combination.

Results

NES fibroblast viability was not significantly impacted by NP presence, regardless of particle size or concentration (Figure 8). In two replicate experiments, cell viability appeared to decrease with decreasing particle size and increasing concentration, but the trend was not consistent. To determine whether residual NPs may have affected MTT results, by potentially altering absorbance of the MTT reagent, negative control experiments were conducted using NPs in the absence of cells. Negative controls did not yield absorbance values above background,

indicating that residual NPs did not impact absorbance values. However, H_2O_2 consistently decreased cell viability in a concentration-dependent manner (Figure 8).

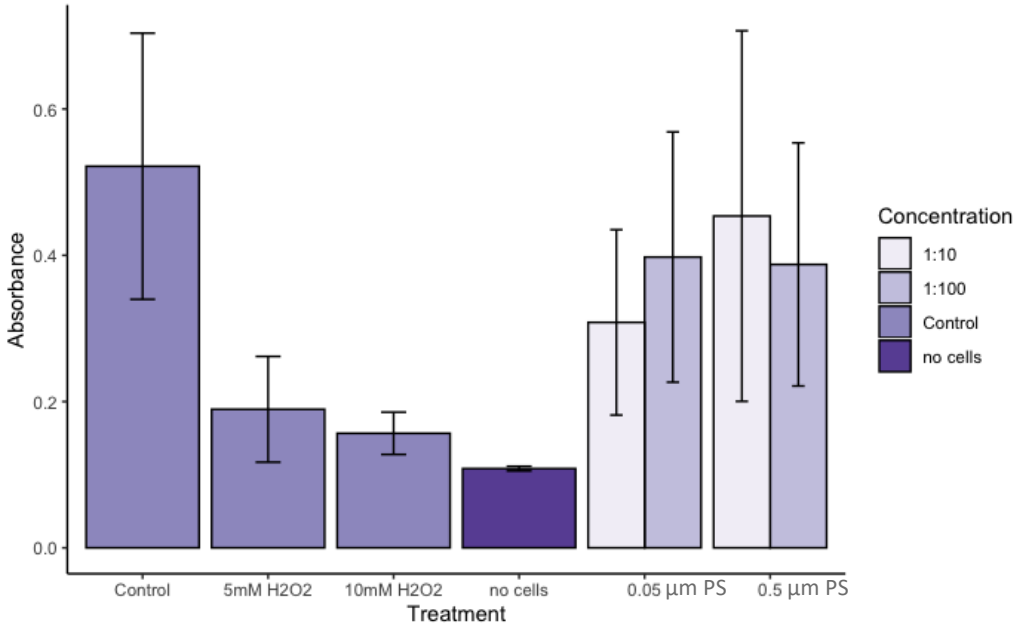

Figure 8. MTT assay results. Average MTT assay absorbance (cell viability) across control treatments and PS NP sizes and concentrations in six experimental runs. Error bars represent standard deviation indicating inter- and intra-assay variability.

NP exposure did not visibly affect cytoskeletal and nuclear morphology of NES fibroblasts (Figure 9).

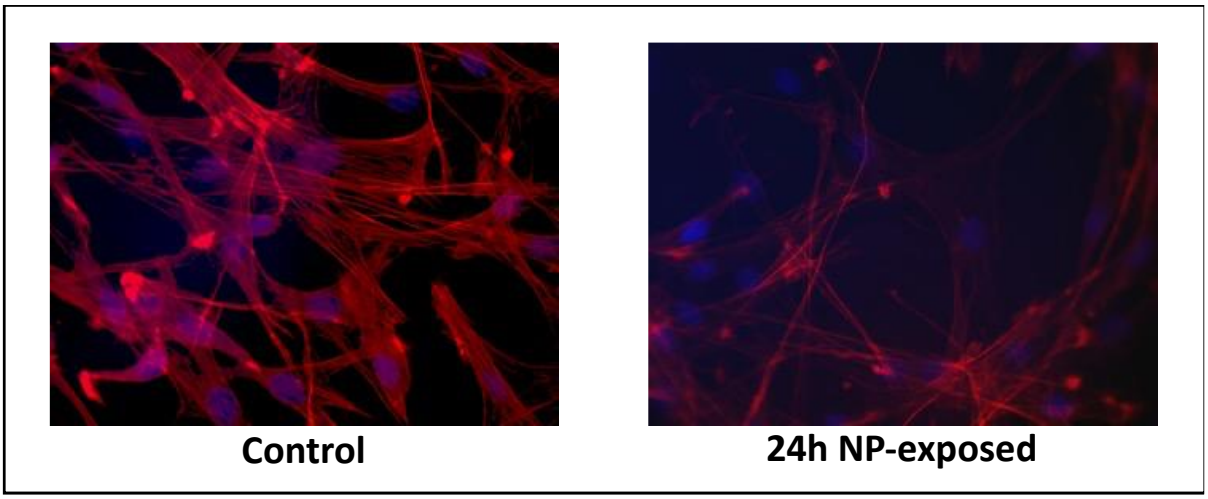

Figure 9. NES fibroblast morphology. NES fibroblasts were cultured for 24 h in control media (left) and media containing 0.05 μm PS beads at 1:10 dilution (right). Actin was stained with phalloidin (red), while nuclei were stained with DAPI (blue).

Discussion

To investigate how marine mammal cells may be affected by exposure to NP particles, NES skin-derived fibroblasts were co-cultured with virgin PS NPs of two different sizes and concentrations. Our results suggest that NES fibroblast morphology and viability were not significantly impacted by NPs of these sizes and concentrations.

Two experiments in our study showed potentially decreased cell viability as a result of NP exposure. However, subsequent experiments, which had high inter- and intra-assay variability, did not show consistent effects of NPs of any size or concentration on cell viability, potentially due to the lack of contact between particles and cells as discussed above. Several other concentrations (dilutions 1:5 and 1:1000) and particle sizes (0.2 μm and 1 μm) were also attempted but these also did not yield consistent results (data not shown). My results were consistent with other studies using fish head kidney leucocytes and human fibroblasts that showed that NP exposure did not significantly affect cell viability, as measured using the MTT assay (Espinosa et al., 2018; Poma et al., 2019). Variability may be attributed to inconsistent cell

seeding density between wells, the small number of cells used in each experiment, and the ability of cells to take up the MTT reagent and release formazan (Ghasemi et al., 2021). Additionally, NES cells may be resilient to stressors. Compared to human cells, NES muscle cells have shown increased antioxidant response to heavy metals (Del Águila-Vargas et al., 2020), suggesting that they may be similarly resilient to other stressors such as NPs. Previous studies have found that NP exposure affects redox homeostasis in fish. Future studies can further investigate the effects of NPs on redox homeostasis by measuring reactive oxygen species levels in NP-exposed NES cells (Espinosa et al., 2018).

Our results align with previous research suggesting that virgin NPs may not have significant effects on cells, especially when compared to naturally weathered plastics. MPs and NPs in natural environments mechanically and photodegrade, resulting in asymmetry that may have more significant effects on cell health than laboratory-produced plastics (Revel et al., 2021). Photodegraded particles may adsorb and subsequently leach persistent organic pollutants. Future studies can be designed to expose NES cells to weathered NPs and other polymer types and shapes, such as microfibers, which are more prevalent in marine ecosystems than microspheres and may have more significant effects on cell health (Kutralam-Muniasamy et al., 2020).

NES fibroblast morphology did not appear to be visibly affected by 24 h of co-culture with NPs. Morphological changes due to NPs have been observed in other studies, which reported endocytosis of NPs by cells (Liu et al., 2021). Our results may be due to the low density and hydrophobicity of NPs, which floated at the surface of cell culture media and likely did not come into direct contact with adherent cells at the bottom of wells. In future experiments, cells can be grown in suspension with NPs that may be neutrally buoyant in media to increase the likelihood of cell-plastic interaction. Alternatively, cells can be grown on glass coverslips,

which can then be floated at the surface of culture media containing NPs. Future experiments can also use florescent beads to determine whether cells are internalizing NPs (and if so, their subcellular localization) by microscopy.

Skin-derived fibroblasts, while easy to isolate and culture, may not be the most biologically relevant system in which to assess the effects of NPs on marine mammal health. As the primary route for NP exposure is via ingestion and absorption by the digestive tract, enterocytes may be more appropriate for this type of study but are difficult to isolate from marine mammals. Peripheral blood mononuclear cells (PBMCs), which include endocytic cells that may internalize NPs, may be a more feasible system in which to assess NP effects. In human PBMCs, exposure has been shown to induce pro-inflammatory cytokines IL-6 and TNF-α (Hwang et al., 2019; Suzuki et al., 2013).

Another limitation of our study was its small sample size. Since biopsies were obtained from only several individuals, cells isolated from multiple animals were pooled into a single stock to maximize cell density for freezing, and aliquots of the same stock were used for replicate experiments. Therefore, true biological replicates were not available. Additionally, NP concentrations were on the low end for viability analyses due to dilute stocks provided by the manufacturer, so future studies can test the effects of higher NP concentrations. Further studies should also be conducted using cells isolated from different animals of varying age classes and both sexes to generalize experimental results for the species.

This is the first study to use a cell culture system to examine the effects of NPs on marine mammal cells. Using virgin PS allowed us to focus on the effects of plastic particles themselves without the confound of absorbed or leached contaminants. While our data showed that NES

fibroblast morphology and cell viability were not consistently affected by exposure to NPs, more research is necessary to explore the effects of PS and other plastics on NES cellular physiology.

CHAPTER 4: EFFECTS OF THE PLASTIC ADDITIVES, BISPHENOL-A AND S ON THE MARINE MAMMAL BLUBBER TRANSCRIPTOME

Introduction

BPA and BPS are lipophilic additives used in the commercial manufacturing of polycarbonate (PC) and low-density polyethylene. They are commonly found in household items such as water bottles, paper receipts, and canned food liners (U.S. Environmental Protection Agency). While recent research has suggested that BPA and BPS may act as endocrine-disrupting chemicals and obesogens, the mechanisms responsible for their downstream effects are currently not well understood (Jacobson et al., 2019; Rubin et al., 2019). The chemical structure of BPA is similar to estradiol and it can bind to nuclear receptors estrogen receptors alpha and beta, ERα and ERβ (Gould et al., 1998), which are involved in reproductive functions and glucose and lipid homeostasis in adipose tissue (Cahua-Pablo et al., 2016). However, BPA and BPS have relatively low binding affinities to these receptors compared to estradiol, suggesting that their biological effects may be mediated by other types of receptors (Kuiper et al., 1998). Nuclear receptor estrogen-related receptor gamma (ESRRG), an orphan receptor that alters expression of ER-target genes in muscle and adipose, among other tissues, has been shown to have high affinity for BPA (Ahmed et al., 2021). ESRRG plays a significant role in energy metabolism as it stimulates mitochondrial biogenesis and oxidative metabolism in muscle and adipocyte browning in adipose tissue (Fan et al., 2018; Müller et al., 2020). In zebrafish, BPS had a potent antagonistic effect on thyroid hormone receptor beta (TRβ) and altered TRβ expression levels (Lu et al., 2018). Together, these data suggest that bisphenol compounds may impact metabolism by altering nuclear hormone receptor pathways.

BPA and BPS may act as obesogens by impacting lipid homeostasis in adipose tissue. Specifically, adipocyte exposure to BPA and BPS may alter insulin signaling, lipolysis, glucose uptake, and adipokine release (Figure 10)(Ahmed et al., 2021). BPA administration to rats *in vivo* impaired insulin signaling (D'Cruz et al., 2012). In humans, metabolic dysfunction caused by chronic bisphenol exposure may ultimately lead to obesity and type-2 diabetes. BPA concentrations in human urine are positively correlated with prevalence of type-2 diabetes and obesity (Trasande et al., 2012). In other animals that have naturally large lipid stores, such as marine mammals, BPA and BPS exposure may impact the ability to mobilize stored fats during reproduction, migration, and other activities (Bahelka et al., 2021).

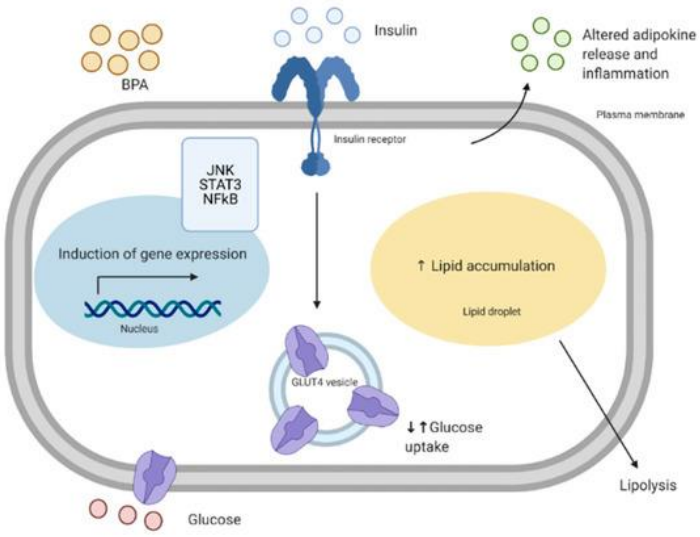

Figure 10. Metabolic processes that are impacted by BPA and BPS in adipocytes (Ahmed et al., 2021).

The primary source of BPA in marine environments is runoff from wastewater treatment plants and landfill sites into coastal waters. However, while offshore sites have lower concentrations of BPA than coastal waters, myctophid fish offshore have been found to have

higher levels of BPA compared to their environment (Kang et al., 2007; Rochman et al., 2013). Although BPA and BPS are not known to be persistent within organisms, they have been detected at higher trophic levels. For example, grey seals have shown evidence of exposure to phenol derivatives, including BPA, via their fish-rich diet. Therefore, fish-eating marine mammals may be chronically exposed to bisphenols, the toxicity of which may increase with trophic levels (Naveira et al., 2021; Nehring et al., 2017, 2018). While bisphenol levels have not been measured in NES, this species may be exposed to BPA and BPS via their main prey source, myctophid fishes, which have measurable levels of BPA (Gassel and Rochman 2019). To a lesser extent, NES may also be exposed to BPA and BPS as a result of the MPs they incidentally ingest, as evidenced in Chapter 2. MPs may act both as a source (e.g., leaching from PC) and a potential sink (e.g., adsorbed to polyamide, PA, and polyurethane, PU particles) for BPA, as PC, PA, and PU are some of the most commonly found polymers at NES foraging depths (Choy et al. 2019; Liu et al. 2019). NES spend the majority of their lives foraging at sea to build fat stores that they rely on while fasting during reproduction and molting on land. After consumption, NES are acutely exposed to BPA and BPS before they are metabolized in the liver, however, in this process, contaminants may reach and affect other, potentially more sensitive tissues (Debier et al., 2006). However, the effects of BPA and BPS on the physiology of NES or other marine mammals are currently unknown. An *ex vivo* approach of precision-cut adipose tissue slices (PCATS, Debier et al., 2020) was used to test how BPA and BPS impact marine mammal blubber function. PCATS were exposed to environmentally relevant concentrations of either BPA or BPS alone or in combination with the lipolytic stress hormone epinephrine (BPA+epinephrine, BPS+epinephrine) to determine how these potential obesogens may impact the ability of NES to mobilize lipids in response to endocrine signals. I then profiled

transcriptome responses of PCATS to these treatments to determine how BPA and BPS affected blubber tissue on a molecular level.

Methods

Sample collection and preparation. Blubber samples were collected in the summer of 2019 from weaned NES pups (n=3) at Año Nuevo State Park in California. Blubber was collected using 6-mm biopsy punches (Miltex Integra, USA). Biopsies were stored in DMEM with antibiotic/antimycotic on ice until returned to the laboratory.

Explant exposures. Once in the lab, blubber biopsies were precision-cut into 1 mm slices that were approximately 15 cells thick, as described in Debier et al., (2020). Tissue slices from each pup were cultured in five treatments: standard tissue culture media for 48 h, BPA (1 nM), BPS (1 nM) for 48 h, epinephrine (100 nM), epinephrine and BPA (epinephrine+BPA, 100 nM epinephrine added after 36 h BPA exposure), or epinephrine and BPS (epinephrine+BPS, 100 nM epinephrine added after 36 h BPS exposure). After the exposures, tissue slices were rinsed in PBS, frozen in 2.0 mL bead tubes with 2.8 mm stainless steel beads (VWR, USA), and stored at -80◦C.

RNA isolation. Explants were homogenized in 1 mL of Qiazol (Qiagen, USA) in a Bullet Blender Storm 24 instrument (Next Advance, USA) for two minutes at power 12. To further disrupt tissue homogenates, samples were sheared using a 21-gauge needle. Total RNA was isolated using the RNeasy Lipid Mini Kit (Qiagen, USA) with an on-column DNase I digest. RNA quantity was estimated using the Qubit 3.0 fluorometer (RNA High Sensitivity Assay, Life Technologies, USA), and RNA integrity was assessed using 2100 Bioanalyzer (RNA 6000 Pico Assay, Agilent Technologies, USA). The mean (± SD) RNA integrity number (RIN; ranging from 1-10) for all samples was 8.7 ± 0.3.

RNA sequencing. mRNA enrichment from total RNA, cDNA synthesis, strand-specific library preparation and fragmentation, and Illumina paired-end 150 bp sequencing were conducted by Novogene Corporation, Inc. (Beijing, China).

Transcriptome assembly and analysis. Transcriptome assembly, annotation, and transcript abundance estimation were performed using the Bridges High-Performance Computing Cluster at the Pittsburgh Supercomputing Center using Extreme Science and Engineering Discovery Environment (XSEDE) (Towns et al. 2014) allocation TG-IBN150010. Sequence quality was assessed using FastQC v 0.11.2 The transcriptome was assembled *de novo* using Trinity v2.11.0 (Haas et al., 2013). The completeness of the transcriptome assembly was assessed using BUSCO v5.0.0 and the *Mammalia* database (Seppey et al., 2019). Transcripts were annotated by blastx against the UniProt SwissProt database (downloaded on March 8, 2021) using DIAMOND v2.0.7.14 in ultra-sensitive mode with an e-threshold of 0.001 (Buchfink et al., 2021). Differentially expressed transcripts that had no significant blastx hits were manually annotated using blastn (e-value < $1e^{-10}$).

Transcript abundance was estimated using Salmon v1.4.0 (Patro et al., 2017). Differential gene expression analyses were conducted using txtimport and DESeq2 v1.35.0 (Love et al., 2014) in R (v4.1.0) in R-studio (v1.4.1106), with blocking by study subject from which biopsies for PCATS were obtained (design = ~ Subject + Treatment).

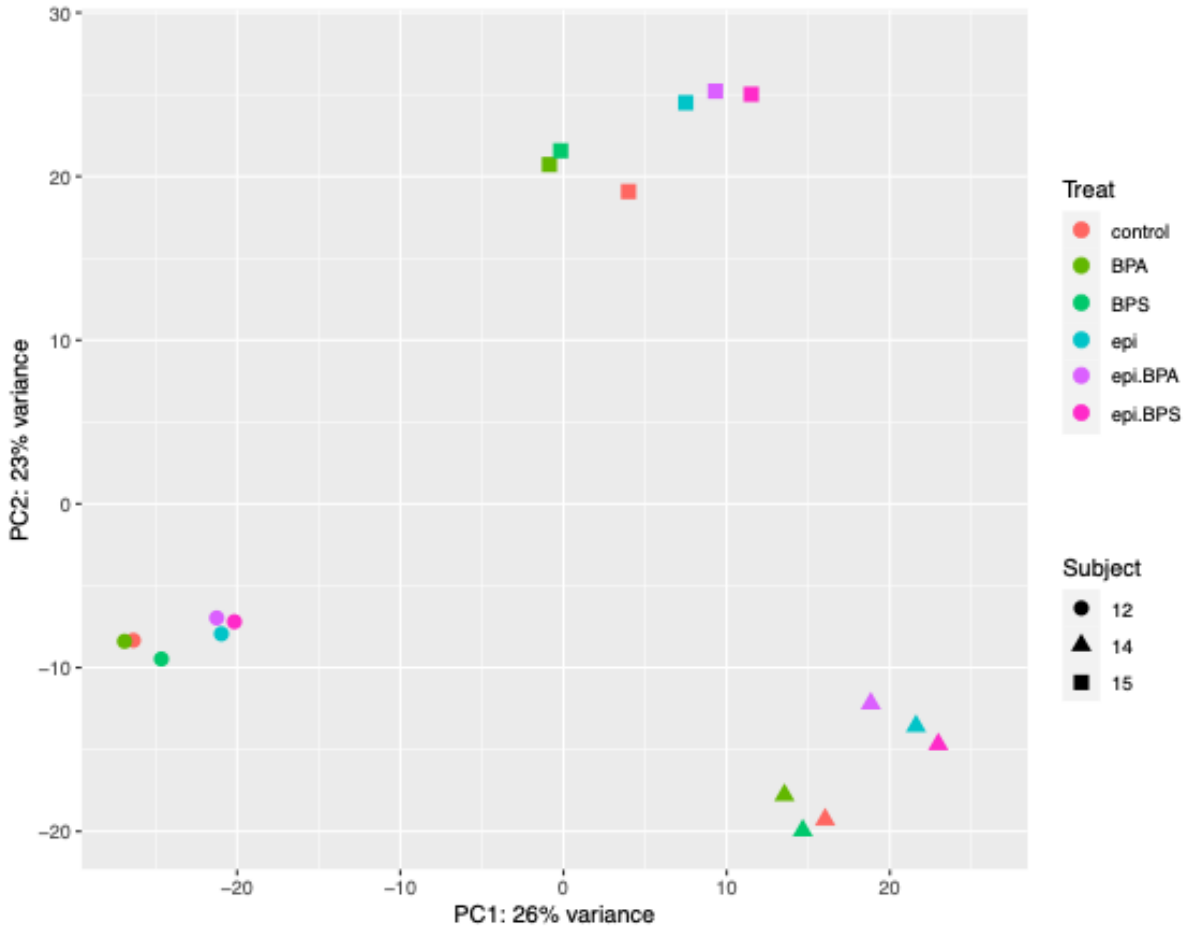

Figure 11. Principal component analysis plot showing sample clustering by study subjects from which PCATS were obtained (shapes), with some sample separation by treatment (colors). For example, epi treatments tended to have higher PC1 and PC2 values.

Results

Transcriptome. The NES PCATS assembly was comprised of 1,413,571 contigs (transcripts) with a median contig length of 423 base pairs. It contained 85.3% of highly conserved genes (Benchmarking Universal Single-Copy Orthologs, BUSCOs) expected to be found in mammalian genomes, suggesting high assembly completeness (Table 2) (Seppey et al., 2019).

Table 2
Transcriptome Completeness Assessment. BUSCO Data Showing the Percentage of Highly Conserved Mammalian Orthologs Present in the Assembled NES Transcriptome.

Total BUSCOs Percentages		
Complete & Duplicated	65.1%	85.3%
Complete & Single Copy	20.2%	
Fragmented	4.7%	
Missing	10%	

Differential gene expression. As evidenced by few DEGs identified between control and BPA- and BPS-treated PCATS, global blubber gene expression was not appreciably altered by exposure to BPA or BPS alone (Tables 3 and 4). However, the DEGs that were identified were associated with several biological processes of interest, which were common to both treatments. BPA altered expression of 5 genes relative to control (4 were upregulated, 1 was downregulated). BPA-regulated genes were associated with adipogenesis (PRKACA), insulin secretion (EIPR1), and obesity and type 2 diabetes (ZBTB7A, CXCL14). CXCL14 was downregulated in BPA-treated PCATS compared to those treated with BPS. BPS altered expression of 3 genes relative to control, which were upregulated (PRKACA, CABLES1, CACNA2D2). Of these, only CACNA2D2, which is associated with insulin secretion, was identified solely in the BPS vs control comparison, and not in any of the others (Figure 12).

Table 3
Counts of Annotated DEGs. DEG Counts in Parentheses Unique to Each Comparison.

Comparison/Gene	Annotated DEGs
BPA vs control	5
BPS vs control	3
Epinephrine + BPA vs epinephrine	1
Epinephrine + BPS vs epinephrine	1
Epinephrine + BPA vs control	31 (6)
Epinephrine + BPS vs control	39 (12)

Table 4
Log Fold Changes in DEGs between Contaminants and Controls and Epinephrine and Contaminants + Epinephrine. Starred (*) genes are Associated with Lipid Metabolism, as Featured in Figure 11.

Comparison/Gene	Log2Fold Change
BPA vs control	
ZBTB7A*	-6.89
CABLES1	3.22
EIPR1*	3.88
PRKACA	4.51
CXCL14	5.79
BPS vs control	
CACNA2D2*	3.00
CABLES1	3.17
PRKACA*	4.76
Epinephrine + BPA vs epinephrine	
BRD3*	4.91
Epinephrine + BPS vs epinephrine	
RBM6*	4.42

To examine whether BPA and BPS alter tissue responses to lipolytic cues, transcriptome profiles were compared between PCATS exposed to epinephrine with those exposed to epinephrine in combination with BPA or BPS. Only two DEGs, one in each comparison, were identified for these treatments. BRD3 was upregulated 4.91-fold in PCATS treated with

epinephrine and BPA compared to those treated with epinephrine alone. RBM6, which is associated with obesity and type 2 diabetes, was upregulated 4.42-fold by epinephrine+BPS relative to epinephrine alone.

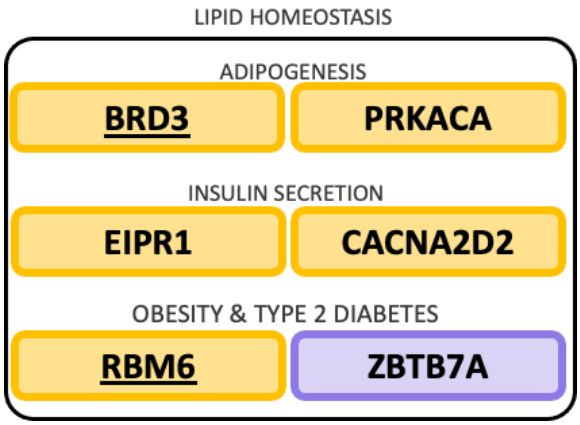

Figure 12. DEGs identified in comparisons between PCATS treatments (epinephrine+BPA vs epinephrine, epinephrine+BPS vs epinephrine, BPA vs control, and BPS vs control) and their known functions. Genes identified in comparisons that included epinephrine (epinephrine+BPA vs epinephrine, epinephrine+BPS vs epinephrine) are underlined. Genes shown in yellow genes were upregulated, while those in purple were downregulated in response to BPA and BPS treatments.

Lastly, transcriptome profiles of PCATS treated with the combination of epinephrine and BPA and BPS with those cultured in control conditions were compared (Table 5). Expression of 49 genes was altered by the combination of epinephrine and BPA compared to control, of which 31 had sequence similarity to genes with known functions in other organisms. Six DEGs were unique to this comparison (i.e., not identified when comparing control to epinephrine or BPA treatments alone), which included ZBTB7C, TBC1D1, and LEP. These genes were associated with adipogenesis (ZBTB7C), insulin-stimulated glucose metabolism (TBC1D1), and regulation of appetite and lipid metabolism (LEP). Expression of 59 genes was altered in PCATS exposed to the combination of epinephrine and BPS relative to control, of which 39 were annotated.

Twelve DEGs identified in this comparison were not altered by epinephrine or BPS treatments alone (relative to control), including ZBTB7C and LEP, which were also affected by the combination of epinephrine and BPA.

Table 5

Log Fold Changes in DEGs with Epinephrine and Control

Epinephrine + BPA vs control Gene	Log2Fold Change
HTR1B	-1.80
LEP*,a	-1.63
ZBTB7C*,a	1.24
PDK4	1.42
AQP3	1.43
PLIN2	1.49
DGAT2	1.56
GFPT2	1.66
PDE8Aa	1.91
NR4A3	2.15
SHISA2	2.19
EDARa	2.21
THSRP	2.34
TDH	2.44
TMEM266	2.79
SSTR2	2.81
TACR1	3.22
HMGCS2	3.42
PRKACA	3.59
SSPO	3.81
LRRC3C	4.76
TBC1D1*,a	4.81
CABLES1	5.01
ARF5a	7.20

Epinephrine + BPS vs control Gene	Log2Fold Change
SPINK5	-1.78
LEP*,a	-1.52
ZBTB7C*,a	1.30
DUSP1a	1.50
PDK4	1.54
PCK1	1.55
PLIN2	1.57
SIK1B	1.57
AQP3	1.58
DGAT2	1.69
SLC43A2a	1.75
GFPT2	1.81
TNCa	1.85
NOG	1.94
NR4A3	2.22
SHISA2	2.27
THRSP	2.33
ADRB3	2.33
PALD1	2.41
CXCL8	2.51
SSTR2	2.56
TDH	2.62
GET1a	2.71
KPNA7a	2.91
TMEM266	3.06
TACR1	3.26
HMGCS2	3.46
NUS1a	3.57
ACOX3	3.57
GPR78a	3.73
FBXO31a	4.08
CABLES1	4.62
LRRC3C	4.96
ARF5a	7.29

* indicates known association with lipid metabolism and [a] indicates differently expressed genes that were uniquely identified in that comparison.

Discussion

In this study, I examined changes in the transcriptome of marine mammal blubber tissue to BPA and BPS treatments to identify potential pathways that may be altered by these contaminants. In addition, as combinatorial stressor exposures are not feasible in whole animal studies, I used the PCATS system to assess the ability of blubber to respond to lipolytic cues after BPA or BPS exposure. BPA and BPS did not have substantial impacts on global blubber gene expression, alone or in combination with epinephrine. However, the few DEGs that I identified were associated with lipid metabolism, suggesting that exposure to bisphenols has the potential to affect lipid homeostasis in marine mammals.

BPA and BPS altered expression of genes associated with adipogenesis (PRKACA), insulin secretion (EIPR1 and CACNA2D2), and type 2 diabetes and obesity (ZBTB7A). In humans, PRKACA mutations may cause Cushing syndrome, which is characterized by morbid weight gain (Zilbermint & Stratakis, 2015). In diabetic mice, BPA exposure increased basal and glucose-simulated insulin secretion in mice, likely to compensate for insulin resistance (Wei et al., 2017). ZBTB7A, which may promote adipogenesis and fat synthesis in adipose and liver tissue by promoting fat synthesis by upregulating fatty acid synthase and the lipogenesis regulator SREBP-1 (Choi et al., 2008; Zhou et al., 2020), was downregulated in NES PCATS as a result of BPA exposure. While contaminants that are thought to be obesogenic should increase expression of genes associated with lipogenesis, this result shows that the effects of bisphenols on adipose function may be more complex than hypothesized. Downregulation of ZBTB7A in blubber in response to BPA may serve to inhibit adipogenesis, a key feature of fasting NES (add REF: Khudyakov et al., 2022 fasting seal paper). Together, these data suggest that BPA and BPS may alter lipid homeostasis in NES, potentially by decreasing adipogenesis, increasing insulin

production, and reducing the insulin resistance and high rates of lipolysis (via upregulation of PRKACA, EIPR1, and CACDA2D2 and downregulation of ZBTB7A) that characterize fasting periods in this species.

To test how BPA and BPS may impact tissue responses to lipolytic stimuli, gene expression was compared between PCATS exposed to epinephrine alone with those that were exposed to contaminants and epinephrine. The addition of BPA and BPS to epinephrine caused upregulation of BRD3 (epinephrine+BPA) and RBM6 (epinephrine+BPS), which are both associated with lipid metabolism in other species. RBM6 was identified as enriched in adipose tissue in a genome-wide association study of humans with obesity and type 2 diabetes, suggesting that it may be involved in lipid accumulation (Fadason et al., 2017). Proteins such as BRD3 in the bromodomain and extraterminal domain family are important coregulators of adipogenesis (Brown et al., 2018). Upregulation of these genes suggests that BPA and BPS may promote adipose tissue expansion, even in the presence of epinephrine, further supporting the hypothesis that bisphenols may interfere with fat mobilization in marine mammals.

Since there were few DEGs in the other comparisons, PCATS treated with epinephrine+BPA and epinephrine+BPS were also compared to control. This comparison enabled us to examine the effects of a combination of stressors (epinephrine, a stress hormone, and anthropogenic pollutants), on marine mammal blubber. The majority of the DEG in this comparison were likely responding to epinephrine treatment, which alters gene expression by activating the transcription factor CREB (Yoon et al., 2021). I identified 6 and 12 DEGs in the epinephrine+BPA vs control and epinephrine+BPS vs control comparisons, respectively, that were not found by comparing control to contaminant or epinephrine treatments alone, suggesting potential downstream interactions between the bisphenols and hormone. These included ZBTBC,

which is in the same protein family as ZBTB7A and is a tumor suppressor that may be upregulated in response to obesity (Zhou et al., 2020). In humans, the adipokine hormone leptin, which is encoded by the LEP gene, acts on the brain to regulate food intake and energy expenditure and on adipose tissue to promote fat oxidation (Caro et al. 1996). LEP was downregulated in both epinephrine+BPA and epinephrine+BPS treatments, indicating that these treatments may also be associated with fat accumulation. These results highlight the utility of the PCATS approach, which enabled us to conduct combinatorial stressor experiments that could not otherwise be conducted *in vivo*.

Several limitations of this study may have reduced our ability to detect genes differentially regulated by BPA and BPS treatments alone. These include low BPA/BPS concentrations, a small sample size, and short exposure times. The BPA and BPS concentrations used in the experiment were similar to measured concentrations of BPA in grey seals, but future studies should measure BPA and BPS in NES to inform experimental concentrations (Nehring et al., 2018). BPA and BPS may impact gene expression in a dose-dependent manner, as has been reported in zebrafish (Sun et al., 2019). Future experiments can include combinations of contaminants, as they would be found in the natural environment, and use higher contaminant doses for treatments. Experimental designs that involve repeated contaminant exposures over longer time periods may more accurately simulate contaminant ingestion during feeding in NES. Additionally, large variability in gene expression between individuals (as seen in the PCA plot) reduced the sensitivity for detecting differences between treatments. Increasing the sample size will increase statistical power for detecting DEGs. Lastly, PCATS were exposed to BPA and BPS for 48 h, which was based on the half-life of ingested BPA reported in other species (Stahlhut et al., 2009) and the duration of culture time during which PCATS maintained high

viability (Debier et al. 2020). However, marine mammals likely experience chronic exposure to contaminants via their diet, suggesting that experiments with longer exposures times may be more biologically relevant, and may result in more pronounced effects on gene expression.

In summary, our PCATS experiments suggest that BPA and BPS exposure may impact metabolic function in marine mammals by altering expression of genes associated with lipid mobilization. Lipid storage and metabolism are vital for NES survival and reproductive success. At sea, NES rely on their ability to accumulate fat stores, so on land, they can rely on mobilizing these fat stores for energy for activities such as mating, molting, giving birth, and nursing offspring. In lactating females, lipid mobilization is especially crucial for transferring maternal fatty acids to developing pups via milk that may reach a lipid content of >50% (Fowler et al., 2018). Environmental BPA and BPS transfer to marine mammals may alter their abilities to complete key life-history stages, such as reproduction, migration, and molting ultimately impacting overall fitness and threatening marine mammal populations.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

Plastic pollution may adversely affect the health and fitness of marine mammals due to their high trophic levels, presence of pollution in foraging grounds, and large fat stores that may accumulate lipophilic contaminants. This study is the first to investigate the presence and impacts of plastic pollution in NES. Unlike other pelagic marine mammals, such as cetaceans, NES haul out on coastal beaches throughout the year and provides an accessible study system in which to investigate the effects of plastic pollution on physiology.

The goals of this thesis were to determine if MPs are present in feces of NES and whether intact NPs and plastic additives affect cell and tissue function. Though MPs have been detected in several marine mammal species (Zantis et al., 2021), my research was the first to explore the effects of NPs on marine mammal cellular physiology. My findings suggest that acute exposure to virgin PS does not significantly alter NES fibroblast morphology or viability, but that more research is necessary to determine whether PS exposure has other sublethal effects and whether other types of plastics affect cellular physiology of marine mammals. This study also indicates that acute exposure to contaminants associated with plastic manufacturing, BPA and BPS, alters the expression of genes involved in lipid metabolism in NES blubber, with potential downstream impacts on fat deposition and mobilization and tissue remodeling during feeding and fasting, respectively, in marine mammals.

My study provides a baseline measurement for plastic ingestion and retention in the digestive tract by NES by measuring MP concentrations in scat of fasting animals. More accurate measurements can be obtained by analyzing the contents of GI tracts of stranded animals post-mortem. Determination of MP presence and trophic transfer within mesopelagic

food webs will require additional sampling and MP analysis from myctophid fish (NES prey) and seawater from NES foraging locations (Saunders et al., 2019).

The types and concentrations of plastics found in marine mammals can then inform future studies of the mechanisms by which NPs impact cellular physiology. My in vitro exposures were the first to examine the effects of virgin PS NPs on NES fibroblast viability. Despite finding that fibroblast morphology and viability were not significantly affected by co-culture with NPs, future studies can now focus on environmentally and biologically relevant exposures. Using weathered particles of known plastic types that have been found in NES scat MPs can provide a better understanding of how NES may be impacted by plastic pollution in their natural environment.

In addition to plastics themselves, the additives used to manufacture plastics, which are present in the environment and have been measured in several fish species, may have physiological effects on marine mammals. I used *ex vivo* experiments to identify genes altered by BPA and BPS in NES blubber. While the contaminants alone did not have substantial impacts on gene expression, the few markers that I identified were associated with lipid homeostasis in other mammals, suggesting that bisphenols may interfere with the ability of NES to mobilize fat stores during fasting periods associated with key life-history stages. Longer exposure times or higher BPA and BPS concentrations in future experiments may help identify additional genes regulated by contaminants in blubber. To determine the impacts that contaminants may have on blubber function during key life-history stages, this experiment should also be conducted using tissue from breeding and molting adult NES of different sexes.

Ultimately, this study was the first to explore plastic pollution in NES and the first to test the effects of NPs in marine mammals. Plastic additives BPA and BPS altered expression of

genes that may interfere with necessary physiological functions in NES, such as fasting on land during key life-history stages. More research is necessary to understand the biological implications of plastic pollution as plastics continue to increase in marine ecosystems.

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