MICROPLASTIC EXCHANGE BETWEEN THE AMERICAN HORSESHOE CRAB AND WESTERN HEMISPHERE SHOREBIRDS THROUGH HORSESHOE CRAB EGGS IN JAMAICA BAY, NY

A Final Report of the Tibor T. Polgar Fellowship Program

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ABSTRACT

The possibility of microplastic exchange between the American horseshoe crab (*Limulus polyphemus*) and Western Hemisphere shorebirds through horseshoe crab eggs was examined at Plumb Beach-East in Jamacia Bay, NY. Fertilized and unfertilized horseshoe crab eggs, migratory shorebird fecal pellets, beach sand, and bay water were collected in June 2021 and processed with a hydrogen peroxide (H_2O_2) solution and stained with a Nile Red (NR) dye to identify the presence of microplastics. Using fluorescence microscopy (488 nm) and ImageJ processing, microplastics were counted and sized for each sample. Microplastics were present in all samples, which indicates that microplastics are exchanged between horseshoe crabs and shorebirds through horseshoe crab eggs. Microplastic particles ranged from 0.0026 μm to 13 μm in size. The presence of microplastic particles in unfertilized egg samples indicate that microplastics undergo maternal transfer during oogenesis.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

INTRODUCTION

Microplastics have been of great concern to ecologists and environmental scientists over recent decades because of their increasing presence and rapid emergence into the trophic chain, impacting terrestrial and marine life. The widespread use of plastics as a material for consumer goods has led to a stark increase in the amount of plastic litter that ends up in marine ecosystems, where it is the most abundant type of anthropogenic debris (Thompson 2015). The accumulation of this litter on beaches through trash, runoff, and the direct release of wastewater containing high amounts of microplastics through combined sewer systems, results in the accumulation of microplastics in estuaries and marine ecosystems where they are consumed by marine animals (Andrady 2011). Microplastics that enter the marine ecosystem can either be secondary or primary. Primary microplastics are plastic particles that are produced and used in cosmetic products and textiles, whereas secondary microplastics are created through the degradation of larger plastics through ultraviolet radiation, oxidation, and mechanical forces (Sturm et al. 2021). In the New York area, and specifically Jamaica Bay, the main sources of microplastic introduction are through beaches and Combined Sewer System (CSS) and Waste Water Treatment Plant (WWTP) outfalls that introduce primary and secondary microplastics (Steve 2014; Sturm et al. 2021).

The proliferation of plastics into the trophic chain is of great concern because the accumulation of microplastics in different living organisms is still being researched and discussed. As a result, researchers are still unsure if certain animals are able to sort microplastics out in their digestive tract and pass them or if the microplastics accumulate in their body (Thompson 2015). Microplastics are also a focus for increased study

because they have been shown to absorb chemical pollutants, raising concern over how these chemicals are moving through the food chain and impacting the physiological functions of different marine species (Wardrop et al. 2016).

The impact of microplastics on marine species is especially pertinent when it comes to animals that feed by consuming organisms that are embedded in sediment, which has been known to have a high accumulation of microplastics, especially in shore locations. One of these organisms of great concern is the American horseshoe crab, *Limulus polyphemus*, a marine arthropod that plays a vital role in the ecology of the Hudson River Estuary, especially Jamaica Bay. Horseshoe crabs are a "key species in the estuarine food web, and its eggs provide essential food for a number of migratory shorebirds" (Botton 2009). The American horseshoe crab is classified as a vulnerable species on the IUCN Red List (International Union for the Conservation of Nature 2016), because of over-harvesting and its vulnerability to rising sea levels that reduce the spawning environment. This vulnerable status is especially important because it has a great effect on migratory shorebirds (Botton et al. 2018). The Western Hemisphere shorebirds migrate from the coastal beaches and wetlands of South America to the arctic and subarctic breeding grounds (Botton et al. 1994). Many of these migratory shorebirds have been classified as either being of "greatest conservation concern" to "high conservation concern" in 2016 (U.S. Shorebird Conservation Plan Partnership 2016).

Throughout their migration, the birds land in "staging areas," intermediate stopping points along their flight, so that the birds can feed throughout their long journey (Botton et al. 1994). One of these staging areas is in Jamaica Bay, a portion of the Hudson River Estuary located in New York City (Figure 1) where these migratory birds,

including the Semipalmated Sandpipers (*Calidris pusilla*) and Semipalmated Plovers (*Charadrius semipalmatus*), are known to feed on horseshoe crab eggs (Casper 2020) (Figure 2). This dependence on eggs is widely noted in the other estuaries, such as the Delaware Bay Estuary, where the "abundance of horseshoe crab eggs during the spring shorebird migration contributes to the importance of the Delaware estuary as a feeding ground" (Botton et al. 1994).

Figure 1: Jamaica Bay, New York. The yellow circle indicates the study site, Plumb Beach (Modified from Casper 2020).

Figure 2: Plumb Beach, Jamaica Bay.

Figure 3: Migratory shorebirds feeding at Plumb Beach, Jamaica Bay.

With the growing amount of microplastics in the food web and the importance of this interspecies relationship between horseshoe crabs and shorebirds, the potential transference of microplastics to shorebirds through their consumption of eggs needs to be

studied. The eggs could become contaminated with microplastics, either by maternal transfer, "the process by which the offspring receive a portion of the parent's accumulated trace elements during oogenesis" or through their exposure to the sand and water when they are laid by the female horseshoe crab (Bakker et al. 2017). There is already preliminary evidence that the sand and water of Jamaica Bay does contain microplastics, where WWTP outfalls and CSOs have been identified as point sources (M. Botton, unpublished; Steve 2014). The presence of microplastics in horseshoe crab eggs could help describe another way that microplastics are working their way through the food web.

The connection between horseshoe crabs and migratory shorebirds is an important ecological linkage in the Hudson River Estuary, where both have experienced declines in their abundance over recent years (International Union for the Conservation of Nature 2016; U.S. Shorebird Conservation Plan Partnership 2016). The most abundant spring shorebirds in Jamaica Bay are semipalmated sandpipers and semipalmated plovers, and horseshoe crab eggs are known to be a significant part of their diet from late May through June (Casper 2020). Although there are numerous studies of microplastics in the marine environment, there has not been research into whether microplastics move from horseshoe crabs to shorebirds. If microplastics can be maternally transferred during oogenesis, as hypothesized, then the mobility of microplastics and their impact on the marine environment might be greater than previously thought.

METHODS

Field Methods:

Study Area:

All research was conducted in Jamaica Bay, primarily at Plumb Beach-East (Figure 1) which has a large population of spawning horseshoe crabs and shorebirds from mid-May to early June (Botton et al. 2018). Plumb Beach recently underwent a beach nourishment project on the western section to combat rapid beach erosion (Figure 2). After the beach nourishment project in 2012-2013, the western end of Plumb Beach had significantly finer and more homogenous sediment with less gravel compared to the significantly coarser sediment of Plumb Beach-East (Botton et al. 2018). Necessary permits were provided to Dr. Mark Botton at Fordham University by the New York State Department of Environmental Conservation, New York City Parks Department, and the National Park Service-Gateway National Recreation Area.

Unfertilized egg collection:

In order to determine if microplastics undergo maternal transfer, female horseshoe crabs were randomly selected on the tidal flats of Plumb Beach-East on June 6th, 2021. To collect eggs directly from the female before they have been laid, the genital pores were massaged stimulating the egg release (Figure 4). Additional eggs were collected during the tagging process that was conducted on the same day. Before the tag was inserted, a few eggs were collected from the awl puncture in the prosoma of the female

horseshoe crab. All samples were placed in a glass vial. Eggs were collected from 10 female horseshoe crabs, taking about 20-60 eggs from each individual.

Figure 4: Stimulation of genital pores for egg release.

Fertilized egg collection:

The second group of eggs collected were from the sediments at Plumb Beach-East on June 6th, 2021, where most of the shorebirds were found. Using a metal hand shovel, 10 batches of 20-100 eggs were collected and placed into glass jars.

Water, sediment, and fecal pellet collection:

Ten sediment samples were collected from Plumb Beach-East at the approximate high tide line where most horseshoe crab egg-laying takes place. To obtain water samples, 10 replicate 1 L samples of bay water were collected in a glass bottle and sieved

through a metal 125 μm screen. The residue was then transferred to a glass jar for further processing in the lab.

Shorebird fecal pellets were collected from Jamaica Bay by Emily Casper, a Master's student at Fordham University (Figure 5) (Casper 2020). Fecal pellets were collected at Plumb Beach or Big Egg Marsh, a few km east of Plumb Beach. Based on a DNA analysis of a subsample of 67 fecal pellets, 26 were positively identified as originating from Semipalmated Sandpiper, and 36 from Semipalmated Plover.

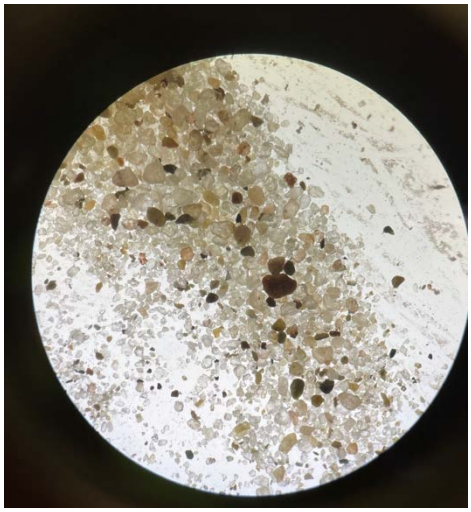

Figure 5: Dried fecal pellet sample.

Laboratory Methods:

Nile Red and microscopy:

In order to determine how microplastics move either through ambient exposure or through maternal transfer or both, Nile Red (NR), a dye that can be used as a "fluorescent vital stain for the detection of intracellular lipid droplets by fluorescence microscopy" (because the lipids component of the microplastics will glow under fluorescence), was

used (Greenspan and Fowler 1985). In order to utilize NR to select for microplastics, a hydrogen peroxide treatment for the digestion of organic material was performed. It has been shown that samples treated with NR after a peroxide bath allow for the greater selectivity of microplastics over organic material because microplastics appear more intense compared to dimmer organic fluorescence (Sturm et al. 2021). NR is a stain that can be used for a variety of microplastics that are commonly found in marine environments including polyethylene and polypropylene (Sturm et al. 2021; Shim et al. 2016). NR staining is most effective for microplastic detection under a wavelength of 450-490 nm and 515-565 nm which falls under the green-yellow wavelength range (Sturm et al. 2021).

To ensure that the Nile Red (0.005 g NR/99 mL acetone + 1 mL 96% Hexanes) stain was effective at staining microplastics, a positive and negative control was run. For the positive control, shavings from a plastic water bottle were treated with NR for 30-60 minutes on a clean slide. Slides were cleaned by placing them in 30% hydrogen peroxide for 60 minutes to remove all organic components. The positive control under the fluorescence microscope was successful, with all the plastic particles fluorescing when viewed under the green (488nm) filter (Figure 6). The negative control was done on Plumb Beach sand that was placed in a muffle furnace at 500 ˚C for 5 hours to combust all organic and plastic particles. The muffle furnace sand was placed on a clean slide and stained with NR for 30-60 minutes. The muffle furnace sand did not fluoresce (Figure 7). The positive and negative control allowed us to determine that the Nile Red methodology was working.

After ensuring that the NR stain was working, each sample was treated with 3-5 drops of Nile Red and left to stain for 30-60 minutes. All samples were covered with aluminum foil to prevent contamination by airborne microplastics. The samples were then viewed under a green fluorescence (488 nm) at 40x magnification. A random 2 by 2 cm area was selected on each watch glass and 20 photos were taken, 5 photos per each of 4 horizontal transects. Images were saved as JPEG files for analysis.

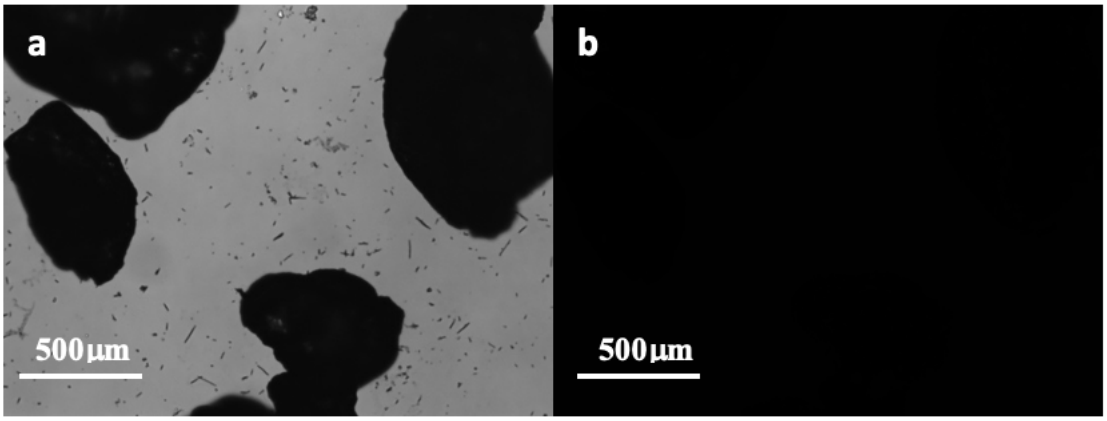

Figure 6: Muffle furnace sand under bright field (a) and green fluorescence (488nm)(b).

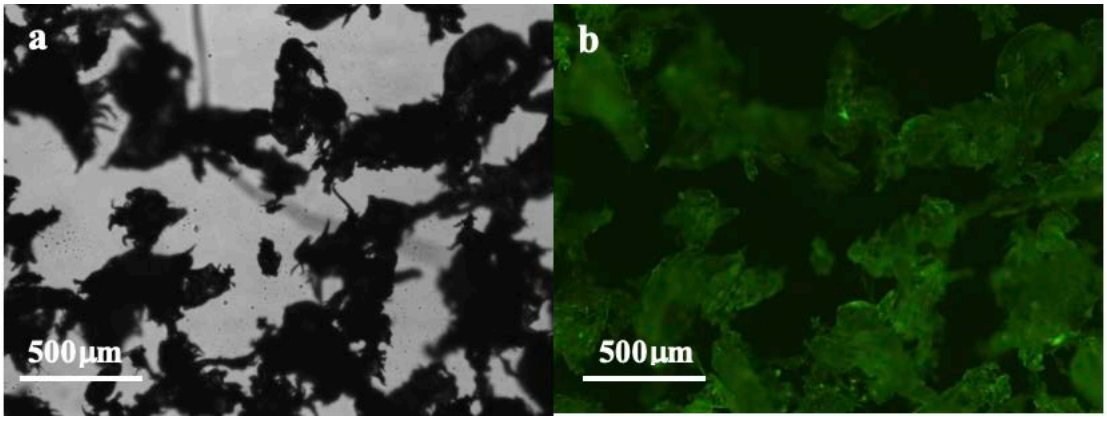

Figure 7: Plastic shavings under bright field (a) and under green fluorescence (488nm)(b).

Egg and fecal pellet processing:

The wet mass of 20 eggs were determined on an analytical balance. To be able to test for microplastics using Nile Red, all organic components were removed using a concentrated H_2O_2 solution (Shim et al. 2016). This was done because lipids in a sample, as well as microplastics, have an affinity for Nile Red. Twenty eggs were placed in a glass test tube and crushed to break the chorion using a glass rod, then each sample was treated with 6 mL of 30% H_2O_2 at 60C for 24 hours, to ensure that all the organic components are dissolved (Masura et al. 2015). The sample was then transferred to a cleaned watch glass that was treated with 30% H_2O_2 for 1 hour to ensure residual organic material was dissolved. The samples were then placed in an oven at 60 ˚C until dry.

Dried fecal pellet samples were weighed on an analytical balance and then placed in a glass test tube and treated with 6 mL of 30% H_2O_2 and placed in a 60 ˚C water bath for 24 hr. The samples were then transferred to a clean watch glass and placed in the 60 ˚C oven until dry.

Water samples:

The remaining particles and liquid were sieved once more on the 125-micron sieve and transferred to a glass test tube and treated with 9 mL of 30% H_2O_2 and placed in the 60 ˚C water bath for 24 hours. All samples were transferred to a clean watch glass and placed in the 60 ˚C oven until dry.

Sediment samples:

A density separation procedure was used to extract microplastics from the sediment. Twenty grams of each sediment sample were weighed out on an analytical balance. Then in a glass beaker, 80 mL of a 73% concentrated NaCl solution was added to the 20 g of sediment and place on a magnetic stirrer for 10 minutes (Miller et al. 2017). The suspended particles were then sieved in a 125-micron sieve and 80 ml of additional NaCl solution was added to the remaining sediment in the beaker and placed on the stirring plate for another 5 minutes before being sieved again. The sample on the sieve was then transferred to a glass test tube and treated with 9 mL of 30% H_2O_2 and placed in the 60 ˚C water bath for 24 hr. The samples were then transferred to a clean watch glass and transferred to the 60 ˚C oven until dry.

ImageJ and data analysis:

Each photo was then processed using ImageJ (https://imagej.nih.gov/ij/). To count the number and size of each fluorescent particle, the size of each image was set to be a 2.25 mm by 1.68 mm area as calibrated by using a stage micrometer under 40x magnification. Then each image was processed with a RenyiEntropy threshold to standardize the processing of each image (Figure 8). The average count and average particle size were outputted using ImageJ and recorded. In order to directly compare each type of sample to one another, the average number of fluorescent particles per 1g of mass was calculated by taking the average microplastic particle count and dividing it by the mass of the sample. To determine if there were significant differences between sample types, an Analysis of Variance (ANOVA) for the means of each sample per

sample type was taken. Differences among means were analyzed using the Tukey HSD multiple comparison test.

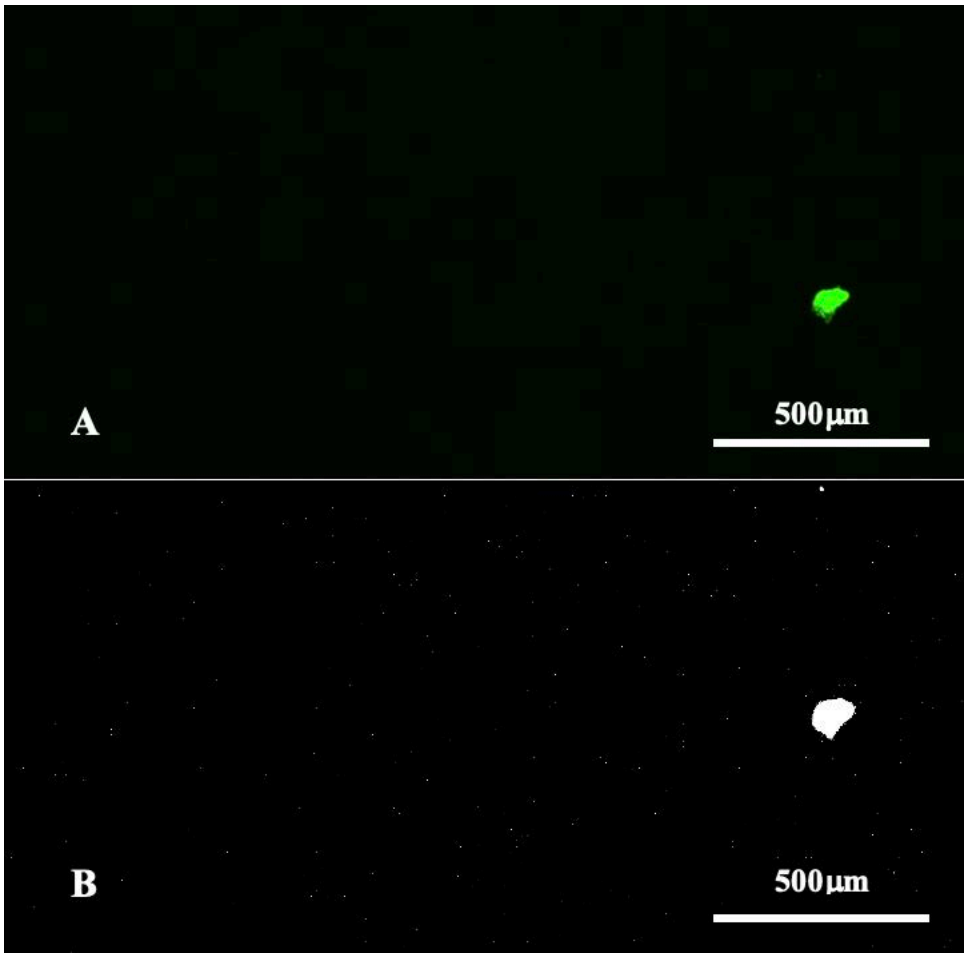

Figure 8: Shorebird fecal pellet sample under green fluorescence (488 nm)(A) and the same sample after being processed with ImageJ (B).

RESULTS

General presence of microplastics:

Microplastic particles were present in all samples (unfertilized and fertilized egg, fecal pellet, sediment, and bay water). The majority of microplastic particles were

irregular edged ovals. Microplastic fibers present in 27% of the photos analyzed for bay water, compared to 4% for fertilized egg samples, 0.5% for unfertilized egg samples, and no fibers observed in the shorebird fecal pellets.

Average microplastic particle size (μm):

Based on the output from ImageJ the average particle size for all samples ranged from 0.00261 μm to 13 μm. There were significant differences in average particle size among groups (ANOVA, $F_{4,45} = 4.38$, $P<0.005$; Table 1). Fertilized egg samples had a significantly larger average particle size of 1.22 μm (Tukey HSD test, $P<0.05$) compared to unfertilized egg ($\bar{x}$ =0.29 μm), fecal pellets ($\bar{x}$ =0.32 μm), beach sand ($\bar{x}$ =0.45 μm), and Bay water ($\bar{x}$ =0.30 μm)(Figure 9).

Table 1: Analysis of Variance for average particle size (μm).

Source	Sum of Squares	Degrees of Freedom	Mean of Squares	F	P
Treatment [between groups]	6.4	4	1.6	4.38	0.0045
Error	16.5	45	0.37		
Total	22.9	49			

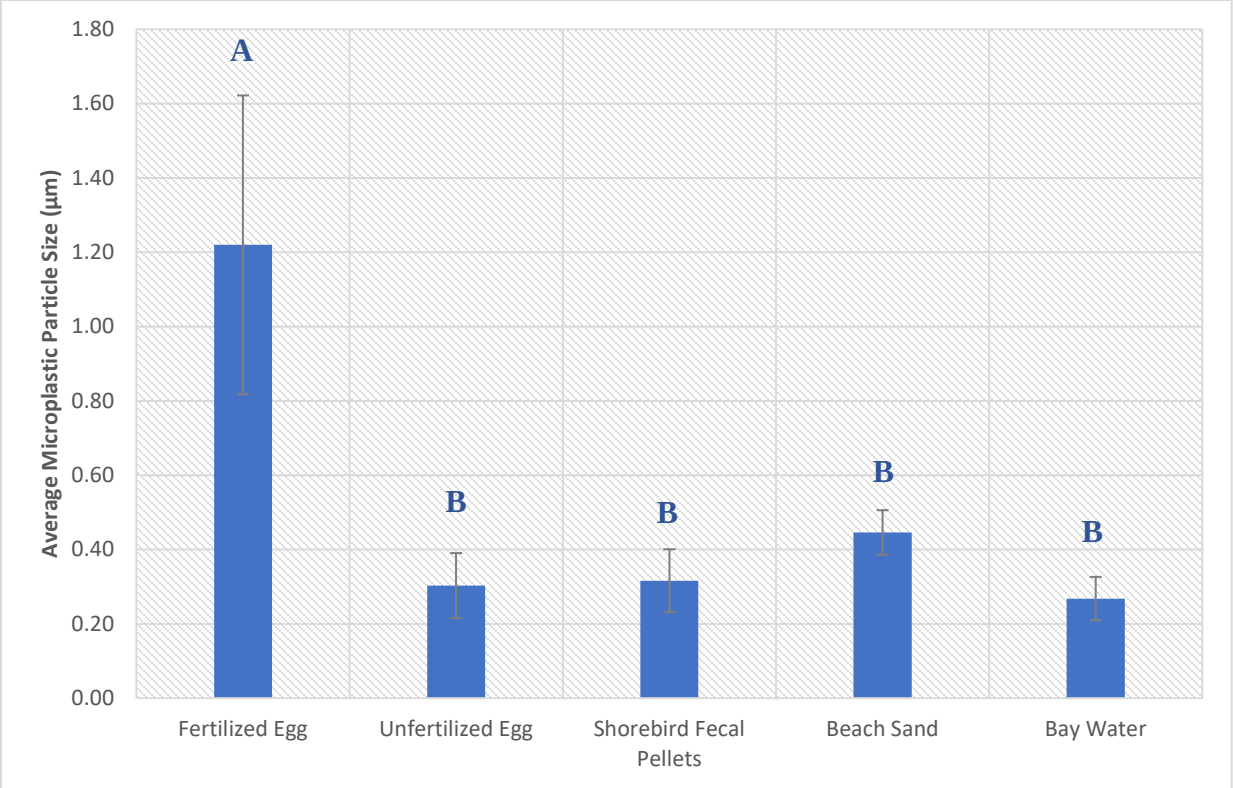

Figure 9: **Average microplastic particle size (µm)(± standard error) for fertilized egg, unfertilized egg, shorebird fecal pellet, beach sand, and bay water. Means with different letters are significantly different (P<0.05).**

Average microplastic particle count per gram:

To be able to compare the average microplastic particle count between samples, all particle count averages were converted to be per gram. The average number of microplastic particles ranged from 0.0024 to 1628.69 particles per gram.

There were significant differences in average particle size among groups (ANOVA, $F_{4,45} = 30.74$, P<0.0001; Table 2). Fertilized egg and unfertilized egg samples were significantly higher in particles per gram compared to shorebird fecal pellets, bay water, and beach sand (Tukey HSD test, P<0.01), with unfertilized eggs containing the highest average number of microplastic particles per gram (Figure 10).

Table 2: Analysis of Variance for average particle count per gram.

Source	Sum of Squares	Degrees of Freedom	Mean of Squares	F	P
Treatment [between groups]	5234129.4	4	1308532.4	30.7	<0.0001
nError	1915778.8	45	42572.9		
Total	7149908.3	49			

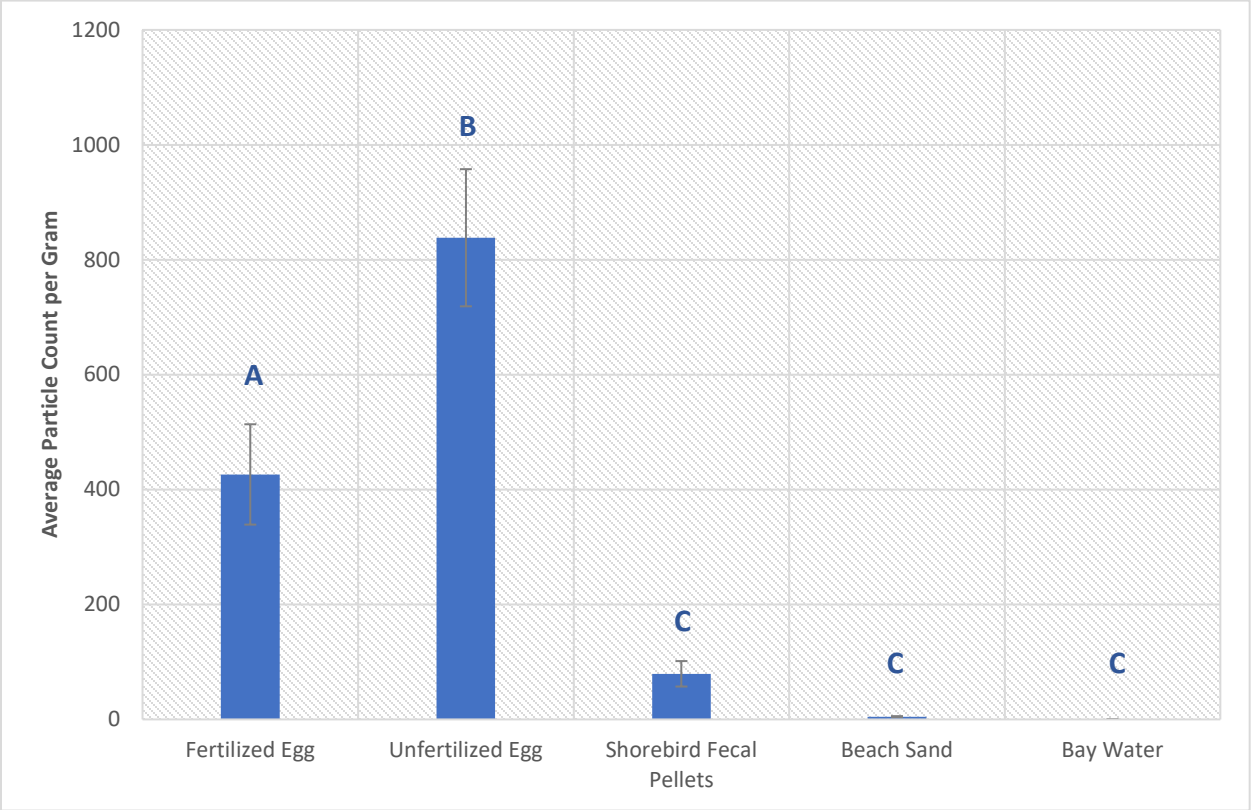

Figure 10: Average particle count per gram (± standard error) for fertilized egg, unfertilized egg, shorebird fecal pellets, beach sand, and bay water. Means with different letters are significantly different (P<0.01). Bay water average particle count per gram is 0.04±0.005.

DISCUSSION

The presence of microplastics in all samples indicates that microplastics are transferred from the American horseshoe crab to Western Hemisphere shorebirds through horseshoe crab eggs. Microplastics are present in fertilized eggs which are consumed by shorebirds along their migration where shorebirds defecated microplastics, indicating that microplastics are being ingested through their food consumption. The microplastics for Jamaica Bay shorebirds during May and June are presumably derived from horseshoe crab eggs, as horseshoe crab eggs are the nearly exclusive food source for migratory shorebirds during their migration (Casper 2020).

The presence of microplastics in unfertilized egg samples indicates that microplastic particles can transfer maternally during oogenesis and that the primary source of microplastics in horseshoe crab eggs comes from the female itself. The maternal transfer of microplastics to the eggs could be a detoxification pathway to offload excess microplastics that have built up in the female's body (Bakker et al. 2017). To determine precisely how microplastics transfer during oogenesis, microplastic concentrations in the somatic tissues of the female horseshoe crabs would need to be taken and in addition the mechanism that microplastics undergo during oogenesis should be researched further.

The significantly higher count of microplastic particles per gram in unfertilized eggs compared to the count of microplastics in fertilized eggs was not anticipated. The chorion of the egg membrane is a relatively impermeable membrane except for small molecules such as H_2O. The only contributing factor that may account for a small percentage of the two-fold difference between egg samples is that the fertilized egg

samples did contain a small number of sediment particles due to the sticky coating the surrounds the eggs after they are laid. This extra mass could skew the averages per mass to be slightly lower. This factor might account for roughly 5% of the difference as the amount of sand per sample was minimal and was not enough to explain the entire difference.

The average microplastic count per gram for the shorebird fecal pellet samples is significantly less than the egg samples ($P<0.01$) but not significantly different from the beach sand or bay water. Although during May and June shorebirds principally consume horseshoe crab eggs during their migration, they are defecating fewer microplastic particles than they are consuming. This could be accounted for through possible bioaccumulation in the gut of the shorebird. Microplastics could in addition accumulate in the gizzard, which mechanically grinds the bird's food before it reaches the stomach (Svihus 2011). Further testing of shorebird gut samples, gizzards, and stomach lining would need to be done to determine if and where microplastics are accumulating if they are not being eliminated through the feces.

The lower average microplastic count per gram for the bay water and beach sand could be due to a confounding of factors. One possible explanation could be that smaller microplastic particles (<125 μm) could have been lost during the sieving process. In addition, the mass of sand containing quartz particles could skew the data to show fewer counts per gram because the mass of the sample is so large (20 g sample) compared to the organic material in eggs (approximately 0.09 g per sample).

The sizing of the microplastic particles and the methodology of how they were separated from the water and sediment compared to the eggs could introduce additional

variation in how many microplastics are being counted. Microbead particles have been recovered at <0.63 μm in beach and bay locations across the U.S. (Kazmiruk et al. 2018). Microbeads were a common source of microplastic to the marine ecosystem due to their use in cosmetic products, but as of 2019, due to the Microbead-Free Waters Act passed in 2015, they have been significantly reduced (U.S. Congress 2015; Sturm et al. 2021; Thompson 2015).

Despite the ban, microbeads are still present in marine ecosystems where the particle sizes of all samples excluding fertilized egg samples fall in this range, and microplastics of this size and smaller could have been lost during the sieving process contributing to the lower average microplastic count per gram. The sieving process for sediment and water samples could have resulted in the loss of microplastics <125 μm, whereas for the egg samples no sieving was required and thus smaller microplastics were able to remain in the sample contributing to the overall count per gram. To determine if the sieving process played a significant role in the data, additional samples would need to be collected using a more particle-conservative sampling process to ensure that smaller microplastics are not lost.

The fertilized egg samples have a significantly larger average microplastic size ($\overline{x}$=1.22 μm) compared to the rest of the samples. The larger particles present in the fertilized samples cannot be fully explained at this time, but despite the larger average microplastic size the size difference may not be biologically important as the size range is fairly close (0.27 to 1.22 μm).

The impact of the microplastic exchange between horseshoe crabs and migratory shorebirds is unknown at this time. The horseshoe crab eggs as a source of microplastics

into the shorebirds could have numerous impacts depending on possible toxins that may be present in the plastic particles, the particles' impact on egg formation, reproduction, and nutrient absorption in shorebirds (Ziccardi et al. 2016; Ribeiro et al. 2019). This study brings to light an important microplastic source for the Western Hemisphere shorebirds, and in additions shows that microplastics can undergo maternal transfer during oogenesis in the American horseshoe crab indicating that the mobility of microplastics is greater than previously understood. This could have significance for understanding how microplastics are working their way through the food web.

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