Demographic Assessment of Mono(2-ethylhexyl) Phthalate (MEHP) and Monoethyl Phthalate (MEP) Concentrations in Common Bottlenose Dolphins (Tursiops truncatus) From Sarasota Bay, FL, USA

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Abstract

Common bottlenose dolphins (Tursiops truncatus) have previously demonstrated exposure to phthalates. Phthalates and phthalate esters are commonly added to consumer goods to enhance desirable properties. As the amount of plastic marine debris increases, these chemicals can easily leach from these products into the surrounding environment. To evaluate demographic variability in exposure, eight phthalate metabolites were quantified in urine samples collected from free-ranging bottlenose dolphins sampled in Sarasota Bay, FL, USA (2010–2019; n = 51). Approximately 75% of individual dolphins had detectable concentrations of at least one phthalate metabolite. The most frequently detected metabolites were mono(2-ethylhexyl) phthalate (MEHP; n = 28; GM = 4.57 ng/mL; 95% CI = 2.37–8.80; KM mean = 7.95; s.d. = 15.88) and monoethyl phthalate (MEP; GM = 4.51 ng/mL; 95% CI = 2.77–7.34; ROS mean = 2.24; s.d. = 5.58). Urinary concentrations of MEHP and MEP were not significantly different between sex (MEHP p = 0.09; MEP p = 0.22) or age class (i.e., calf/juvenile vs. adult; MEHP p = 0.67; MEP p = 0.13). Additionally, there were no significant group differences in the likelihood of MEHP or MEP detection for any demographic as determined by a Peto-Peto test. Frequency of detection was similar for both metabolites between males and females (MEHP p = 0.10; MEP p = 0.40) as well as between juveniles and adults (MEHP p = 0.50; MEP p = 0.60). These findings suggest ubiquitous exposure risk for both sexes and age classes, warranting further investigation into potential sources and health implications.

Plain Language Summary

Previous studies have detected exposure to phthalates among bottlenose dolphins, demonstrating environmental contamination. Using archived samples from Sarasota Bay bottlenose dolphins (2010–2019), this study evaluated demographic differences in the magnitude and frequency of detection of phthalate metabolites. Unlike exposure patterns for other common environmental contaminants (e.g., polychlorinated biphenyls [PCBs], polybrominated diphenyl ethers [PBDEs]) where adult male dolphins and first-born calves have differentially higher concentrations, evidence from this study suggests equivalent phthalate exposure risk across sexes and age classes. Given phthalate-associated health impacts observed in human studies and the ubiquity of phthalate use, additional research is warranted to better understand sources of exposure and potential implications for bottlenose dolphin health.

1. Introduction

Phthalates are esters; a colorless, odorless chemical compound originating from phthalic acid. They are used as plasticizers, often in conjunction with polyvinyl chloride (PVC) to soften and extend the durability of plastic, or to enhance certain properties of cosmetics, nail polishes, fragrances, sunscreens, building materials, waxes, food packaging, inks, textiles with prints, deodorizers, insecticides, toothbrushes, and car parts (Table 1; NTP-CERHR, 2003, 2000; Shelby, 2006). Phthalates are not chemically bound to the product being modified, and can therefore easily leach into the surrounding environment (Aurela et al., 1999; FDA, 2001).
GeoHealth

Much of the current research on environmental exposure to phthalates has focused on human populations. Humans can be exposed to phthalates intravenously, by means of medical devices and tubing (Green et al., 2005), through inhalation (Fromme et al., 2011), ingestion (Serrano et al., 2014), or dermal absorption (Sugino et al., 2017). Following exposure, phthalate compounds are metabolized through at least two steps; phase I hydrolysis, where the diester chain is cleaved to its respective monoester, followed by phase II conjugation (Choi et al., 2013). Depending on the type of phthalate, further hydroxylation and oxidation may occur (Frederiksen et al., 2007). Resultant metabolites are then excreted through urine and feces (Choi et al., 2013). Urine is regarded as the ideal matrix to study and quantify phthalate metabolites because analytical methods are sensitive enough to detect low concentrations, and it is less susceptible to contamination than other matrices (Calafat & McKee, 2006; Högberg et al., 2008). Phthalates and their metabolites are known to cause mammalian endocrine-disruption (ATSDR, 2017; Casals-Casas & Desvergne, 2011; De Coster & Van Larebeke, 2012; Jobling et al., 1995; Li et al., 2018; Mathieu-Denoncourt et al., 2015; Miodovnik et al., 2011; Stahlhut et al., 2007). These chemicals, and other anthropogenic chemicals that act as endocrine disrupting compounds (EDCs), interfere with normal biological activity of various hormones and their receptors, resulting in altered production, secretion, and transport of hormones (De Coster & Van Larebeke, 2012). In humans, endocrine disruption can lead to reproductive, developmental, and growth impacts (Hauser & Calafat, 2005; Latini, Verrotti, & De Felice, 2004; Lovekamp-Swan & Davis, 2003; Lyche et al., 2009; Matsumoto et al., 2008; Sathyanarayana et al., 2017; Swan et al., 2015); impacts to other higher order mammalian species are not well understood.

Plastic and phthalate usage have risen globally since the mid-twentieth century. Waste associated with this trend has led to a global distribution and subsequent wildlife exposure, including in the marine environment, of phthalates (Net et al., 2015). Phthalates can enter the environment through municipal and domestic sewage and wastewater (ATSDR, 2019; Clara et al., 2010), plastic pollution (Paluselli et al., 2019), agricultural activities (ATSDR, 1995, 2019), or industrial discharge (ATSDR, 2019). Phthalate exposure has been widely reported among multiple species of aquatic fauna including the roach fish (Rutilus rutilus; Valton et al., 2014), American alligator (Alligator mississippiensis; Brock et al., 2016), European eel (Anguilla; Fourgous et al., 2016), Loggerhead sea turtle (Caretta caretta; Savoca et al., 2018), Leatherback sea turtle (Dermochelys coriacea; Savoca et al., 2018), as well as pawns, molluscs, and other fish species (Hu et al., 2016). Additionally, phthalate exposure was recently reported for common bottlenose dolphins (Tursiops truncatus) sampled in Sarasota Bay, FL in 2016 and 2017 (Hart, Beckingham, et al., 2018). Bottlenose dolphins in Sarasota Bay have been the focus of a long-term study to understand life history, behavior, and health (R. S. Wells, 2009; Wells, Tornero, et al., 2005). The high site fidelity of this population offers an opportunity to study local environmental contamination (Kucklick et al., 2011; Wells, Rhinehart, et al., 2004; Yordy et al., 2010). Among these dolphins, Hart, Beckingham, et al. (2018) reported the detection of at least one urinary phthalate metabolite in 71% of sampled individuals (n = 17; Hart, Beckingham, et al., 2018).

### Table 1

**Commonly Used Phthalates and Their Associated Metabolites**

| Diester compound                        | Monoester compound       | Common uses                               |
|-----------------------------------------|--------------------------|-------------------------------------------|
| Butyl benzyl phthalate (BBzP)           | Monobenzyl phthalate (MBzP) | Vinyl tiles, PVC                          |
| di-n-butyl phthalate (DBP)              | Mono-n-butyl phthalate (MBP) | Formulation component, packaging, chemical processing aid |
|                                         | Mono-isobutyl phthalate (MiBP) |                                           |
| di-(2-ethylhexyl) phthalate (DEHP)      | Mono(2-ethylhexyl phthalate (MEHP) | Added to polyvinyl chloride (PVC)         |
|                                         | Mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHHP) |                                           |
|                                         | Mono-(2-ethyl-5-oxo-hexyl) phthalate (MEOHP) |                                           |
| Diethyl phthalate (DEP)                 | Monoethyl phthalate (MEP) | Added to personal care products to enhance fragrance, packaging, insecticide sprays |
| Dimethyl phthalate (DMP)                | Monomethyl phthalate (MMP) | Cosmetics, insect repellents, pharmaceutical products |

*(NTP-CERHR, 2004). *(ATSDR, 2001). *(ATSDR, 2019). *(ATSDR, 1995). *(CDC, 2017).*
Previous studies of the Sarasota bottlenose dolphins revealed exposure to other environmental contaminants such as polychlorinated biphenyls (PCBs; Wells, Tornero, et al., 2005; Yordy et al., 2010), polybrominated diphenyl ethers (PBDEs; Yordy et al., 2010), organochlorine pesticides (OCPs; Yordy et al., 2010), and dichlorodiphenyltrichloroethane and its metabolites (DDT; Houde et al., 2006; Yordy et al., 2010). Due to the physical and chemical properties of these bioaccumulative contaminants, they are stored in the blubber (Balmer et al., 2011; Fair et al., 2007, 2010; Mackintosh et al., 2016; Yordy et al., 2010), and transferred to offspring during gestation and lactation, resulting in differential concentrations between males and females (Cockcroft et al., 1989; Wells, Tornero, et al., 2005; Yordy et al., 2010). Male dolphins do not have a route for offloading the bioaccumulative contaminants, and instead continue to store certain persistent organic pollutants (POPs) throughout their lifetime in an age-dependent manner where the oldest adults have the highest body burdens and youngest calves (excluding the first-born) have the lowest body burdens (Yordy et al., 2010). Additionally, first-born calves often have higher POP body burdens than their mothers (Cockcroft et al., 1989; Wells, Tornero, et al., 2005; Yordy et al., 2010). In contrast, phthalates are not accumulated. They are quickly metabolized and excreted in urine and feces in humans (Wittassek & Angerer, 2008); thus, similar sex- or age-based differences in exposure may not occur among bottlenose dolphins. Though phthalates are not likely to accumulate, continuous environmental phthalate release may result in chronic exposure risks. In fact, dolphins have previously been reported to have higher average MEHP concentrations than human reference populations (Hart, Dziobak, et al., 2020). Sex- and age-based differences in metabolite concentrations have been reported for human populations (Blount et al., 2000; Hartmann et al., 2015; Huang, Tsai, et al., 2015; Silva et al., 2004; Wenzel et al., 2018), but these differences are often attributed to consumer habits and behavior (Parlett et al., 2013; Wenzel et al., 2018).

Phthalate exposure in humans is multimodal. For example, exposure to DEHP often occurs via ingestion of contaminated foods, inhalation of DEHP-bound dust, or as a result of certain medical procedures such as blood transfusions and hemodialysis (ATSDR, 2019). DEP exposure usually occurs via dermal contact with phthalate-containing personal care products (Guo et al., 2014; Guo & Kannan, 2013; Hubinger, 2010; Koniecki et al., 2011). While sources and routes of phthalate exposure for bottlenose dolphins are currently unknown, we suspect exposure mechanisms are different than humans. Phthalate contamination of marine and estuarine environments can occur via sewage or wastewater discharge (ATSDR, 2019; Clara et al., 2010), and urban or agricultural runoff (ATSDR, 2019, 1995). Agricultural practices can also significantly influence environmental phthalate contamination either through the use of phthalate-containing insecticides and pesticides (ATSDR, 2019, 1995), or through phthalate-containing plastic waste (Vox et al., 2016). Finally, plastic and rubber manufacturing and production could also contribute to marine and estuarine contamination. An estimated 5 trillion plastic pieces are floating in the ocean (Erikson et al., 2014), varying in size (i.e., micro-are < 5.00 mm; meso- and macro-are ≥ 5.00 mm; Hidalgo-Ruz et al., 2012). Ingestion of environmental microplastics have been related to relevant MEHP concentrations in large filter feeders (Fossi et al., 2014). As plastics degrade, phthalates can be directly released into the marine environment (Paluselli et al., 2019). For apex predators, Staples et al. (1997) suggested that prey would not likely bioaccumulate phthalates due to the rapid metabolism of higher order organisms. While phthalate-contaminated fish may not be a likely source of exposure, it seems plausible that plastic-contaminated prey could contribute to phthalate exposure, particularly common plasticizers (e.g., DEHP). While trophic transfer of microplastics in wild marine species is poorly understood, evidence of prey-based microplastic transfer and accumulation has been demonstrated in gray seals under human care (Halichoerus grypus; Nelms et al., 2018).

Studies of phthalate exposure in humans have identified sex-specific associations with thyroid cancer (Liu et al., 2020), altered male sexual differentiation and sexual dimorphism (Gray Jr et al., 2012; Kumar et al., 2014; Parks et al., 2000; Radke et al., 2018), as well as early pregnancy loss (Toft et al., 2012); thus, understanding demographic vulnerabilities to phthalate exposure among bottlenose dolphins may help signal differential risk of adverse health impacts. The objective of this study was to understand if bottlenose dolphins in Sarasota Bay, FL have a differential risk of phthalate exposure. Urinary phthalate metabolite concentrations were compared among sex and age classes (i.e., calf or juvenile vs. adult) using samples collected during 2010–2019.
2. Materials and Methods

2.1. Dolphin Population

Dolphins sampled for this study were among the ∼170 long-term residents of the Sarasota Bay area (R. S. Wells, 2009; Wells, Smith, et al., 2014). Studies of dolphins in Sarasota Bay, FL have been conducted since 1970, using various methodologies including photographic identification surveys, tagging and tracking, focal animal behavioral observations, periodic health assessments, and stranding response (R. S. Wells, 2009). Because of these long-term research efforts, approximately 96% of the dolphins using those waters are readily recognizable (Wells, McHugh, et al., 2013). Sixty-nine urine samples were opportunistically collected from 51 individual bottlenose dolphins sampled in Sarasota Bay, Florida during the years 2010–2019; these included samples reported in Hart, Beckingham, et al. (2018) and Hart, Dziobak, et al. (2020). 13 individuals were sampled more than once, so only the most recently obtained sample was used for the analysis herein.

2.2. Sample Collection

Urine samples for this study (2010–2019) were collected during routine health assessments conducted under scientific research permits #522–1785, #15543, and #20455 from the National Oceanic and Atmospheric Administration’s (NOAA) National Marine Fisheries Service (NMFS), and research studies were reviewed and approved annually by Mote Marine Laboratory’s Institutional Animal Care and Use Committee (IA-CUC). Complete urine collection methods are described in R. S. Wells (2009). To summarize, dolphins of interest were encircled in a net, and brought aboard a specially designed veterinary examination vessel where urine sampling was opportunistically conducted. Urine was collected with a Kendall Sovereign Feeding Tube and Urethral Catheter 8Fr/Ch x 22 in (27 mm × 56 cm). Each catheter was coated with sterile surgical lubricant (surgilube) and inserted into the urethra by a trained veterinarian. Urine samples were initially collected into a 50 mL conical centrifuge tube and transferred to a 10 mL cryogenic vial for storage using a 3 mL disposable plastic pipette. Samples were distributed into 5–10 mL aliquots.

Samples collected from 2010 to 2015 were retrieved from the Sarasota Dolphin Research Program's sample archives (Mote Marine Laboratory, Sarasota, FL, USA). Samples collected from 2016 to 2019 were retrieved from the National Institute of Standards and Technology (NIST) Biorepository (Hollings Marine Laboratory, Charleston, SC, USA). Initial urine sample collections (2010–2015) were not matched with field blanks. Starting in 2016, blank samples were made from deionized (DI) water supplied by NIST and prepared using the same methods that were used during sample collections to account for any contamination during collection and transfer (Hart, Beckingham, et al., 2018). Both the urine samples and blanks were frozen in liquid nitrogen for storage and transport, prior to analysis.

2.3. Sample Processing and Urinalysis

Analysis and quantification of bottlenose dolphin urinary phthalate metabolite concentrations were based on a protocol established by the Centers for Disease Control and Prevention (CDC) for humans and methods used for a previous assessment in bottlenose dolphins as detailed in Hart, Beckingham, et al. (2018). Urinary phthalate metabolites are considered the most reliable indicator of recent (3–6 months; Hauser et al., 2004; Teitelbaum et al., 2008) exposure in human populations due to the rapid metabolism of these chemicals once exposed, and because sampling equipment can be contaminated with parent compounds during analysis (Blount et al., 2000; Calafat & McKee, 2006; Frederiksen et al., 2007; Högberg et al., 2008). This broader temporal study includes bottlenose dolphin urinary metabolite concentrations reported for sample years 2016–2017 from Hart, Beckingham, et al. (2018), as well as results from analyses conducted on samples collected during 2010–2015 and 2018–2019; for a total of 51 samples. Briefly, each urine sample (collected 2010–2015; 2018–2019) was screened for eight phthalate metabolites, including monomethyl phthalate (MMP), monoethyl phthalate (MEP), mono(2-ethylhexyl) phthalate (MEHP), mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP), mono-(2-ethyl-5-oxo hexyl) phthalate (MOEHP), monobenzyl phthalate (MBzP), monoisobutyl phthalate (MiBP), and monobutyl phthalate (MBP). Male samples that had excess sperm were centrifuged (1,000 rpm for 10 min) prior to extraction to separate the urine and prevent the solid phase extraction (SPE) cartridge from becoming clogged. Urine samples (1 mL) were spiked with a suite of the following isotopically labeled internal standards: MBP, MiBP, MBzP, MEP, MEHHP, MEHP, MOEHP,
and MMP. After which, they underwent a deglucuronidation step allowing the monoesters to be released from their conjugated forms for extraction (Blount et al., 2000). After deglucuronidation, samples were extracted via SPE (Agilent Bond Elute Nexus), then separated and quantified using high-performance liquid chromatography (HPLC; Agilent 1100; Waters XBridge BEH C18, 2.5 μm, 2.1 × 50 mm analytical column) coupled to a triple quadrupole mass spectrometer (MS; Applied Biosystems Sciex API 4000) with electrospray ionization (ESI negative) interface. Sample integrations were performed using Analyst software (ver 1.5). Prior to the acquisition of sample data, the instrument was calibrated (standard reference material (SRM) 3060: monoester phthalates in acetonitrile); coefficients of determination ($r^2$) for all metabolites were ≥ 0.995.

As reported in Hart, Beckingham, et al. (2018) quality assurance/quality control (QA/QC) samples (reagent blanks, reagent spikes, matrix spikes, SRM 3672 Organic Contaminants in Smokers’ Urine, and field blanks) were processed alongside the urine samples. Reagent blank values were subtracted from the determined concentration value to account for any metabolite contamination resulting from laboratory processes. Available field blank metabolite concentrations were not found to be statistically different from each other by year or by metabolite. Field blank concentrations were averaged for each metabolite and subtracted from urine samples for any contamination due to sample collection materials (e.g., catheters). Acceptable QA/QC criteria for spike (reagent and matrix) and SRM recoveries were 80%–120%. The limit of detection (LOD) was determined for each metabolite and is based on the lowest point of the calibration curve that could be detected on the instrument divided by the volume of sample extracted. In addition, continuing calibration verification standards were run with each batch ($n = 10$) of urine samples to ensure the integrity of the calibration curve.

2.4. Statistical Analysis

Descriptive statistics were used to summarize phthalate metabolite concentrations and the proportion of dolphins with concentrations above the LOD (“detectable concentrations”) (Tables 2–4). Normality of metabolite concentrations was evaluated using a Normal P-Plot and tested using a Shapiro-Wilk test (non-Gaussian if $p < 0.05$), and for concentrations > LOD log normal distributions were confirmed by Shapiro-Wilk tests ($p ≥ 0.05$) following log-transformation. For the full data set, means and standard deviations were calculated using Regression on Order Statistics (ROS) and Kaplan-Meier (K-M) methods for metabolites with greater than 20% of nondetect (i.e., concentrations below the limits of detection; Helsel, 2005; Hites, 2019). Among dolphins with metabolite concentrations above the LOD, geometric means and 95% confidence intervals were calculated. For dolphins sampled more than once, the sample most recently obtained was used for analysis.

Exposure to MEHP and MEP was evaluated by demography (female, male, calf/juvenile, and adult). Calves (dolphins still associated with their mothers), and juveniles (young dolphins recently independent of their mothers), were differentiated from adults on the basis of sexual maturity. Several measures were used to determine maturity status, including age, calving history, pregnancy diagnosis via ultrasonography, and sex hormone concentrations (Wells et al., 1987, 2014). Dolphin ages were determined by either observed birth year, or analysis of dentinal growth layer groups (Hohn et al., 1989). To evaluate demographic differences in MEHP and MEP detection (yes/no) a Peto-Peto test was used to compare the proportion of censored observations (i.e., concentrations below detection limit) between groups (Helsel, 2005, NADA R package). The magnitude of MEHP and MEP detection was compared between demographic groups using a Mann-Whitney U-test (Rian et al., 2020). All statistical analyses were conducted using Statistica (Version 13, Dell Inc., Round Rock, TX) and R (Version 3.2, R Foundation for Statistical Computing, Vienna, Austria) software packages. Statistical significance was evaluated using $\alpha = 0.05$.

3. Results

3.1. Sampled Dolphin Demographics

Of the 51 individuals used for this study, 58.81% were female, with the majority considered to be adult (66.67%; Table 2). Mean age for dolphins sampled was 16.66 (SD = 11.45) years old. Median age was 16 years old (IQR = 6.00–25.00).
### Table 2

**Characteristics of Bottlenose Dolphin Study Sample Used to Determine Phthalate Metabolite Concentrations From Sarasota Bay, Florida, 2010–2019**

| Characteristic (n = 51) | n  | % of total individuals sampled in this study |
|------------------------|----|---------------------------------------------|
| Sampling year          |    |                                             |
| 2010                   | 4  | 7.84                                        |
| 2011                   | 2  | 3.92                                        |
| 2012                   | 3  | 5.88                                        |
| 2013                   | 1  | 1.96                                        |
| 2014                   | 5  | 9.8                                         |
| 2015                   | 5  | 9.8                                         |
| 2016                   | 7  | 13.73                                       |
| 2017                   | 10 | 19.61                                       |
| 2018                   | 6  | 11.76                                       |
| 2019                   | 8  | 15.69                                       |
| Sex                    |    |                                             |
| Female                 | 30 | 58.9                                        |
| Male                   | 21 | 41.18                                       |
| Age class              |    |                                             |
| Juvenile               | 17 | 33.33                                       |
| Adult                  | 34 | 66.67                                       |

# metabolites detected in an individual\(^{a}\) | n  | % of total individuals sampled in the study |
|-----------------------------------------------|----|---------------------------------------------|
| 0                                             | 13 | 25.5                                        |
| 1                                             | 26 | 50.98                                       |
| 2                                             | 8  | 15.69                                       |
| 3                                             | 1  | 1.96                                        |
| 4                                             | 2  | 3.92                                        |
| 5                                             | 1  | 1.96                                        |

\(^{a}\)Frequency determined for dolphins with concentration above the limits of detection.

### 3.2. Detectable Phthalate Metabolite Concentrations

Concentrations above the LOD were quantified for six of the eight metabolites. MiBP was the only metabolite not detected in any dolphin. MMP was detected in one dolphin; however, the percent recovery during

### Table 3

**Detectable Concentrations for Eight Selected Phthalate Metabolites in Bottlenose Dolphins Sampled in Sarasota Bay, Florida, 2010–2019**

| Metabolite | MMP\(^{d}\) | MEP\(^{b}\) | MEHP\(^{c}\) | MEOHP\(^{d}\) | MEHHP\(^{c}\) | MRzP\(^{c}\) | MBP\(^{d}\) | MiBP\(^{d}\) |
|------------|-------------|-------------|-------------|---------------|---------------|-------------|-------------|-------------|
| # Detects  | 1           | 15          | 28          | 4             | 2             | 3           | 4           | 0           |
| % Detect   | 1.96        | 29.41       | 54.90       | 7.84          | 3.92          | 5.88        | 7.84        | 0           |
| Mean (s.d.; ng/mL) | NA\(^e\) | 2.24 (8.58)\(^b\) | 7.95 (15.88)\(^e\) | NA\(^e\) | NA\(^e\) | NA\(^e\) | NA\(^e\) | NA\(^e\) |
| Geometric Mean (ng/mL; 95% CI)\(^c\) | NA\(^e\) | 4.51 (2.77–7.34) | 4.57 (2.37–8.80) | NA\(^e\) | NA\(^e\) | NA\(^e\) | 0.96 (0.52–1.78) | NA\(^c\) |
| Minimum\(^c\) | 0.32       | 1.3         | 0.26        | 0.2           | 76.6          | 6.26        | 0.56        | NA\(^c\) |
| Maximum\(^c\) | 1.13       | 33.4        | 76.6        | 70.0          | 491           | 11.3        | 1.42        | NA\(^c\) |

\(^{a}\)Calculated for all individuals including non-detects via Kaplan-Meier (Helsel, 2005). \(^{b}\)Calculated for all individuals including non-detects via Regression on Order Statistics (Helsel, 2005). \(^{c}\)Calculated for all individuals with concentrations > LOD. \(^{d}\)See limits of detection (Table S1). \(^{e}\)See Table S2 for individual detectable concentrations.
QA/QC was not high enough to reliably calculate the concentration and was excluded from analysis. The range of limits of detection for all samples, including those published in Hart, Beckingham, et al. (2018) are reported in Table S1. Limits of detection utilized a 1 mL urine sample; two urine samples had volumes less than the required 1 mL, so limits of detection were adjusted accordingly (Table S2). Of the six metabolites quantified for this study, at least one phthalate metabolite was detected in 74.51% (N = 38) of individual dolphins sampled. The number of metabolites detected in a single dolphin sample ranged from one to five (Table 2). The most commonly detected metabolites were MEHP (54.90% of individuals; GM = 4.57 ng/mL; 95% CI = 2.37–8.80; K-M mean = 7.95; s.d. = 15.88), and MEP (29.41% of individuals; GM = 4.51 ng/mL; 95% CI = 2.77–7.34; ROS mean = 2.24; s.d. = 5.58; Table 3), while the least commonly detected metabolites were MiBP and MMP (Table 3).

### 3.3. Demographic Differences

Results from the Peto-Peto test revealed no significant group differences in the likelihood of detection of MEHP and MEP, indicated by the proportion of concentrations above the detection limit. For both metabolites, the frequency of detection was similar between males and females (MEP p = 0.40; MEHP p = 0.10), as well as between juveniles and adults (MEP p = 0.60; MEHP p = 0.50; Table 4). Dolphins were also evaluated for sex- and age-related differences in the magnitude of exposure to MEHP and MEP. While not significantly different, the geometric mean concentration for MEHP among dolphins with concentrations above the detection limits was higher in female dolphins (MEHP: females: GM = 6.60 ng/mL; 95% CI = 2.92–14.95 vs. males: GM = 2.36 ng/mL; 95% CI = 0.73–7.64; p = 0.09; Figure 1). Detectable concentrations of MEP were similar between sexes (females: GM = 5.31 ng/mL; 95% CI = 2.82–10.00 vs. males: GM = 3.91 ng/mL; 95% CI = 1.63–9.36; p = 0.22; Figure 1). Similarly, geometric mean concentrations of MEHP and MEP were not significantly different between age classes (MEHP: calves/juveniles: 3.14 ng/mL vs. adults: 5.81 ng/mL; p = 0.67; MEP: calves/juveniles: 5.27 ng/mL vs. adults: 4.26 ng/mL; p = 0.13; Figure 1).

| Demographic parameters | MEHP n (%) above detection limit | MEP n (%) above detection limit |
|------------------------|---------------------------------|---------------------------------|
| Adult (n = 34)         | 17 (50.00)                      | 11 (32.35)                      |
| Calf/juv (n = 17)      | 11 (64.71)                      | 4 (23.53)                       |
| Female (n = 30)        | 18 (60.00)                      | 7 (23.33)                       |
| Male (n = 21)          | 10 (47.62)                      | 8 (38.10)                       |

*pResults from Peto-Peto test.
4. Discussion

4.1. Overall Findings

Of the 51 individual bottlenose dolphins screened for phthalate exposure, ∼75% had detectable concentrations of at least one metabolite. The most frequently detected metabolites were MEHP and MEP, which is consistent with previous studies that report phthalate exposure in marine mammals including the bottlenose dolphin (Baini et al., 2017; Hart et al., 2018, 2020), harbor porpoise (Phocoena phocoena; Rian et al., 2020), fin whale (Balaenoptera physalus; Fossi et al., 2014), Risso’s dolphin (Grampus griseus; Baini et al., 2017), and striped dolphin (Stenella coeruleoalba; Baini et al., 2017). DEHP is predominantly used in PVC products and other plastic items including children's toys and medical devices (ATSDR, 2019; EPA, 1998; Faouzi et al., 1999) and is often metabolized into MEHP. MEP is derived from DEP, which is most commonly used in cosmetic products (FDA, 2013; Hubinger, 2010). DEHP and DEP are both produced in large quantities; global annual DEHP production exceeds 2 million tons per year (Shelby, 2006) and ∼26 million pounds of DEP is produced every year (Kamrin & Mayor, 1991). Among the dolphins sampled, MMP was the least commonly detected metabolite (n = 1), and MiBP was not detected in any urine sample. These findings could be explained by limited commercial use of MiBP and MMP parent compounds. Di-isobutyl phthalate (DiBP), the parent compound for MiBP, is predominantly used as a plasticizer in polyvinyl acetate emulsion adhesives (ATSDR, 2001). Because of its volatility, DiBP is often not the preferred phthalate for products continuously exposed to high temperatures (ATSDR, 2001). Bioavailability of DiBP may be reduced in the marine environment due to degradative properties; 50%–100% may aerobically degrade within 28 days of exposure to marine water (Staples et al., 1997). DiBP is also expected to sorb to suspended particles, thus providing additional removal from the water column (Swann et al., 1983). MMP is the metabolite of dimethyl phthalate (DMP) which is used in solid rocket propellants, safety glasses, rubber coating agents, and insecticides (EPA, 1987). However, human biomonitoring surveys have been unable to calculate population concentrations of MMP since the 2001–2002 sampling year as there were too many samples below the LOD (CDC, 2019). To better understand MEHP and MEP exposure among these dolphins, urinary concentrations, and the proportion of detects were compared between demographic categories. The frequency of MEHP and MEP detection was not significantly different between sex or age class (p > 0.05). Although not statistically significant, geometric mean concentrations for females were higher than males for both MEHP (p = 0.09) and MEP (p = 0.22), suggesting a possible exposure vulnerability for female dolphins. Additional research and larger samples sizes are warranted to verify this difference and understand potential mechanisms. Contrary to other studies of age- and sex-based differential exposure to POPs and other common lipophilic contaminants found in marine waters (Yordy et al., 2010), these findings suggest widespread susceptibility to phthalate exposure. This could be due to the rapid biotransformation of parent compounds through metabolic processes (Staples et al., 1997), thereby preventing bioaccumulation. In humans, age-related differences in phthalate exposure have been attributed to changes in metabolism and biotransformative properties that could potentially slow with age (Reeves et al., 2019). For example, children tend to excrete higher concentrations of oxidized DEHP metabolites (i.e., MEHHP and MEOHP) than MEHP compared to adults (Wittassek & Angerer, 2008). Were this to be occurring in dolphins, we would expect that calves and juveniles would have higher concentrations and a larger proportion of detectable concentrations of MEHHP and MEOHP, while MEHP concentrations would be higher and more commonly detected among adults. Our results, on the other hand, revealed minimal detection of MEHHP and MEOHP among either age class, and no significant differences in MEHP exposure. This could be attributed to differences in exposure source and magnitude, or metabolic differences between the species.

4.2. Significance of Findings

Our findings suggest nondifferential MEHP and MEP exposure among bottlenose dolphins in Sarasota Bay. As such, there may also be a nondifferential vulnerability to phthalate-associated health impacts. Elevated urinary concentrations of MEHP and MEP have previously been associated with reproductive and developmental impacts in humans, such as shorter anogenital distance (MEHP and MEP; Swan, 2008), incomplete testicular descent (MEHP; Main et al., 2006), increased sperm DNA damage (MEHP and MEP; Hauser
et al., 2007), shorter gestational age at birth (MEHP; Latini, De Felice, et al., 2003), increased sex-hormone binding globulin (MEP; Main et al., 2006), and impaired mental and psychomotor developmental indices in males (Kim et al., 2011). While the impact of phthalate exposure on dolphin health is currently unknown, future studies should take a “One Health” approach to identify dolphin health risks based on human health research (Zinsstag et al., 2011). For example, human health studies have found MEHP detected at concentrations between 2 and 8 ng/mL inversely associated with serum testosterone levels (Meeker et al., 2009; Meeker & Ferguson, 2014). Health impacts from exposure to phthalate parent compounds are currently unknown for bottlenose dolphins. Epidemiological investigations using long-term data collected during Sarasota Bay health assessments are currently underway to understand associations between phthalate exposure and indicators of endocrine disruption.

4.3. Strengths and Limitations
To our knowledge, this is the largest evaluation of phthalate exposure in free-ranging bottlenose dolphins. This large sample size enabled demographic examinations of phthalate exposure, a first for any marine mammal species. Conversely, human studies evaluating demographic differences may rely on sample sizes in the hundreds to thousands (CDC, 2019; Huang, Tsai, et al., 2015; Percy et al., 2016; Reeves et al., 2019). Second, this study relied upon well-established CDC analytical methods to screen for phthalate metabolites in urine, a matrix that consistently yields accurate measurements, even at low concentrations (Calafat & McKee, 2006; Frederiksen et al., 2007). Other matrices to detect metabolites include serum, amniotic fluid and breast milk, however these tend to be less sensitive than urine, and are more susceptible to laboratory contamination (Calafat & McKee, 2006; Frederiksen et al., 2007; Höberg et al., 2008; Silva et al., 2004). Long-term monitoring programs and capture-release health assessments conducted in Sarasota Bay were crucial to completing the demographic analyses as they provided essential information that would otherwise be unavailable, such as age and sex of the animals. Capture-release health assessments were also necessary to obtain urine, and the long-term study of Sarasota Bay dolphins allowed for leveraging of archived samples to increase sample size and statistical power.

This study relied on opportunistic sampling of wild bottlenose dolphin urine. Young dolphins were particularly difficult to sample; young dolphins captured for the first time were often returned to the water before they could be sampled due to undesirable and potentially dangerous behavior on deck. This limited our ability to selectively sample dolphins based on desired demographics and resulted in almost twice as many urine samples from adult dolphins as calf/juvenile dolphins. We were also unable to control the time of day urine samples were obtained. Humans can experience diurnal fluctuations in concentrations of certain phthalates (Silva et al., 2004). For instance, in Silva et al. (2004), MEP concentrations were highest during mid-day, while MEHP was highest in the evening. Potential diurnal fluctuations are not yet understood in dolphins. Due to the nature of the health assessments, we were only able to collect single spot samples of urine for each dolphin. Human studies have shown this can be representative of 3–6 months of exposure; however, that has not been confirmed in dolphins (Hauser & Calafat, 2005; C. F. Huang & Wang, 2017; Teitelbaum et al., 2008). As such, further analysis of phthalate metabolites in Sarasota Bay need to be conducted to fully characterize bottlenose dolphin exposure.

5. Conclusions
During 2010–2019, 75% of bottlenose dolphins had detectable urinary concentrations of at least one phthalate metabolite. Our findings suggest that while Sarasota Bay dolphins have comparable exposure to phthalates, female dolphins are potentially at higher risk of phthalate exposure than males. Concentrations for the most commonly detected metabolites (MEHP and MEP) were not significantly different between males and females or between juveniles and adults ($\alpha = 0.05$). Furthermore, there were no significant differences in the proportion of dolphins with detectable concentrations of MEHP or MEP for any demographic group ($\alpha = 0.05$). Overall, this study provides evidence of widespread phthalate exposure among Sarasota Bay bottlenose dolphins. Given the relative abundance of these endocrine disrupting chemicals and evidence of phthalate associated health implications from human studies, further research is warranted to discern exposure source and associated health impacts in bottlenose dolphins. This study strengthens the body of evidence supporting legislation that limits the use of these chemicals and their release into marine
environments. These findings also support and validate legislation focused on reducing plastic pollution, such as bans on single-use plastics, or regulatory measures against the production and consumption of phthalate-containing products.

Conflict of Interest
The authors declare no conflicts of interest relevant to this study.

Data Availability Statement
The data used for this study can be accessed through the 4TU. Research Data international data repository for science, engineering, and design https://doi.org/10.4212/11455782.
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