Sex may influence environmental diphenhydramine accumulation in Round Stingrays

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Abstract

Despite the amount of treated wastewater discharged into the Southern California Bight, few studies have examined pharmaceutical compounds in local biota. The Round Stingray (*Urobatis halleri*) was selected as a representative elasmobranch species to perform an exploratory study on environmental pharmaceutical exposure. Archived liver samples of males and females from juvenile to adult size classes from several locations (n = 53) were examined for 18 pharmaceutical and illicit drug compounds using isotope-dilution LC-MS/MS. Very few compounds were detected in stingray livers, with diphenhydramine as the only pharmaceutical above quantitation limits. Only stingrays collected from the urban site (mainland California) had detectable levels of diphenhydramine compared to no detections in reference stingrays (offshore island). Sex and sampling location substantially influenced both detection rate and concentrations. Our results suggest that aspects of species' ecology and physiology should be considered for future studies investigating pharmaceutical exposure in elasmobranchs.

Keywords: diphenhydramine, elasmobranch, accumulation, sex differences, marine

Introduction

Pharmaceuticals and personal care products are increasingly being documented in aquatic environments where wastewater is released from sewage treatment plants (Caliman & Gavrilescu 2009). In particular, many studies focus on "closed" or "linear" systems such as rivers, where water samples can be collected at discrete distances from the point of release. This affords opportunities to thoroughly examine the extent of wastewater plumes (Conley et al. 2008, Acuña et al. 2015) and related effluent discharge to concentrations in local biota (Brooks et al. 2005). However, quantifying the wastewater plume in marine systems is much more difficult due to the potential non-linear relationship between treatment plants and mixing of released treated water bodies that are affected by currents, tides and storms. Thus, determining potential exposure of local biota to pharmaceuticals in an open system is challenging.

While animals utilizing habitats adjacent to outfalls are exposed to pharmaceuticals (Daughton & Ternes 1999), and particularly so in effluent-dominated and dependent systems (Brooks et al. 2006), documenting exposure in other species not directly associated with outfall pipes is necessary to understand the extent to which human activity may influence local biota. In highly populated areas, such as Southern California, anthropogenic influence on the local marine environment could be quite large due to the sheer volume of wastewater released daily. Since pharmaceuticals can negatively affect normal physiological functioning in a variety of ways (Jobling et al. 1998, Gagné et al. 2006, Valenti et al. 2012, Weinberger & Klaper 2014), large inputs of wastewater from major population centers could adversely affect wildlife (reviewed by Huerta et al. 2012).

Southern California, USA, is a densely populated area with four major wastewater treatment plants servicing the Los Angeles to San Diego area. These plants release > 1.1 billion

57 gallons of treated water to the marine environment every day[1], representing a potentially large
58 human fingerprint with respect to pharmaceuticals and other down the drain chemicals. Indeed,
59 endocrine disruption is documented in Hornyhead Tubot (*Pleuronichthys verticalis*) sampled
60 from sites near outfall pipes compared to reference sites (Baker et al. 2009, Vidal-Dorsch et al.
61 2013). Despite high wastewater inputs to the marine environment, few studies have focused on
62 pharmaceutical influence in other local marine species, such as elasmobranchs (sharks, skates,
63 and rays). Elasmobranchs have characteristics that make them vulnerable to anthropogenic
64 contaminant accumulation (Fisk et al. 2002, Lyons et al. 2013, Beaudry et al. 2015) such as long-
65 life spans, late maturity, relatively low fecundity, and tendency to occupy higher trophic
66 positions. Traditionally, human impacts on elasmobranchs are studied from a direct interaction
67 perspective, such targeted or incidental capture via fishing (Stevens et al. 2000); however,
68 indirect interactions through exposure to manmade chemicals also be important for the
69 conservation of these species (Lyons 2018). For instance, few studies have quantified
70 pharmaceutical accumulation in elasmobranchs (Gelsleichter & Szabo 2013), despite their
71 overlapping use of coastal marine habitats with humans and their propensity to accumulate other
72 anthropogenic chemicals (Mull et al. 2013, Weijs et al. 2015).
73 Elasmobranchs can be difficult to study due to conservation concerns and their elusive
74 nature, which has limited research in this taxon. However, utilizing elasmobranch species that
75 are abundant, with aspects of their ecology and physiology already known, represent useful
76 models for research into elasmobranch toxicology. The Round Stingray (*Urobatis halleri*) is a
77 bottom dwelling, coastally associated elasmobranch that is easy to sample across a range of size

[1] http://www.lacsd.org/wastewater/wwfacilities/joint_outfall_system_wrp/default.asp; https://www.lacitysan.org/san/faces/home/portal/s-lsh-wwd/s-lsh-wwd-cw/s-lsh-wwd-cw-p?_adf.ctrl-state=4sxxqz6a8_5&_afrLoop=2048493681227692#!; https://www.ocsd.com/home/showdocument?id=18769; https://www.sandiego.gov/mwwd/facilities/ptloma

78 classes and locations. Since the Round Stingray is known to accumulate other anthropogenic
79 contaminants (Lyons et al. 2014), it is an ideal candidate for exploring not only the presence or
80 absence of pharmaceuticals but also factors that could influence exposure such as size, sex, or
81 locality. Therefore, the objectives of this study were two-fold: 1) to determine levels of
82 pharmaceuticals in stingrays collected from a heavily urbanized environment relative to a
83 reference site, and 2) to identify if aspects of ecology or physiology could influence
84 accumulation or exposure. This work is important for understanding the degree to which
85 stingrays, and thus other elasmobranchs, may be exposed to pharmaceuticals in the environment,
86 which will support future work exploring how this may affect elasmobranch health.

87 **Methods**

88 *Study sites*

89 Southern California is a highly urbanized area. As such, the adjacent marine environment
90 receives high anthropogenic inputs from a variety of industrial to residential sources. In contrast,
91 Santa Catalina Island (herein "Catalina"), located 35 km offshore, is a pristine site due to
92 relatively few number of residents and geographical features limiting transport of contaminants
93 from the mainland to the island. For example, a deep channel (> 700 m in depth) separates the
94 island from the mainland and south-to-north water current flow through the Southern California
95 Bight (SCB) reduces mainland influence on the island. Thus, Catalina serves as a useful
96 reference site for studies examining contaminant impacts on local marine biota (Hose et al.
97 1989), including the Round Stingray (Lyons et al. 2014, Sawyna et al. 2017).

98 *Sample collection*

99 Archived liver tissue was obtained from stingrays collected from several areas in the SCB
100 as part of a previous study (Lyons et al. 2014) and ongoing fish abundance surveys conducted by

101 the Cabrillo Marine Aquarium and Vantuna Research Group. Mainland stingrays were captured
102 from three locations (Cabrillo Beach, Seal Beach, San Diego Bay), which represented our urban
103 sites separated by ~24 km (CB-SB) and 175 km (SB-SDB), and reference stingrays were
104 sampled from two locations on Catalina Island (Catalina Harbor, windward side, and Two
105 Harbors, leeward side). Catalina collection sites are located ~20 km away from the main
106 population center on the island (Avalon), and therefore, unlikely to experience urban exposure
107 like stingrays from the mainland population. Both males and females across a range of sizes were
108 selected for pharmaceutical analysis to investigate the effect of site, sex, and age on
109 accumulation.
110 *Pharmaceutical analysis*
111 Liver samples (0.5 g wet weight) were homogenized, spiked with deuterated internal
112 standard (50 µL of 2000 ppb solution containing all target analytes) and extracted with 8 mL of a
113 1:1 mixture of 0.1 M aqueous acetic acid and methanol in a 20-mL borosilicate glass vial by
114 gentle end-over-end inversion for 20 min at room temperature (~25°C). Following previously
115 reported methods, samples were transferred to 50 mL polypropylene copolymer round-bottomed
116 centrifuge tubes (Nalgene Co., Nalgene Brand Products, Rochester, NY) and centrifuged at
117 20000 rpm for 45 min at 4 °C. The supernatant was transferred into 18 mL borosilicate glass
118 culture tubes (VWR Scientific), and the solvent was evaporated under N_2 gas at 45 °C in a
119 Turbovap evaporator. Dried samples were reconstituted with 1 mL 95:5 0.1% (v/v) formic
120 acid–MeOH and filtered using Pall Acrodisc hydrophobic Teflon Supor membrane syringe
121 filters (13 mm diameter; 0.2-µm pore size; VWR Scientific, Suwanee, GA) before analysis. Two
122 blank sample spikes and two pairs of matrix spikes were included for quality assurance and
123 control evaluation. Samples were analyzed via isotope-dilution liquid chromatography-tandem

mass spectrometry (LC-MS/MS). Target analytes, chemical vendors, and instrument parameters followed previously reported methods (Ramirez et al. 2007, Du et al. 2012, Bean et al. 2018, Burket et al. 2018).

Results

Morphometrics

From the urban site, a total of 19 males (15-22.4 cm disk width [DW]) and 20 females (15.1-22.4 cm DW) were collected, which spanned juvenile to adult size classes. Among urban males, six were sampled from Cabrillo Beach, twelve from Seal Beach and one from San Diego Bay. For females, two were collected from Cabrillo Beach, twelve from Seal Beach and six from San Diego Bay. Stingrays from the reference site were collected in fewer numbers, but were generally larger than urban stingrays for males (n = 7; 21-25.8 cm DW) and females (n = 7; 13.9-26.4 cm DW).

Pharmaceuticals

Of the 18 targeted compounds (Supplemental Table 1), only diphenhydramine was detected in quantifiable amounts from Seal Beach and Cabrillo Beach stingrays (Supplemental Table 2). No compounds were detectable in stingrays from San Diego Bay (urban site). Norfluoxetine and fluoxetine were also infrequently detected (n = 3 and 2, respectively), but at levels below limits of quantification. Only stingrays (sexes combined) from the urban site had quantifiable (12/39, 31%) or detectable (14/39, 36%) pharmaceuticals, while all samples from the reference site had no detections for any of the screened compounds (0/14, 0%), despite their presumably older age given their larger size (Supplemental Table 2).

Among stingrays from the Seal Beach urban site where comparable numbers of males and females were sampled (n = 12 each), we found indications that sex may influence

147 accumulation. A higher number of males had quantifiable levels of diphenhydramine (8/12,
148 67%) than females (3/12, 25%; **Figure 1A**). Furthermore, only the largest, mature females had
149 quantifiable levels (>21.8 cm DW), while the smallest male with quantifiable levels was a
150 juvenile (15.5 cm DW). Of the stingrays with quantifiable levels, males had both higher and
151 more variable concentrations (1.18 ± 1.8 µg/kg) than females (0.19 ± 0.046 µg/kg), although this
152 was not statistically higher due to the low numbers of females (n = 3; **Figure 1B**).

153 Concentrations appeared to increase in an exponential fashion in males (**Figure 2**),
154 suggesting diphenhydramine accumulates with size, and by, extension, age. Females only had
155 detectable levels at larger sizes that were also lower than comparably sized males, suggesting
156 that sex affects both concentration and accumulation trajectories in Round Stingrays. We found
157 some evidence that accumulation patterns were similar among consecutive months, as males
158 exhibited similar accumulation curves between May and June (**Figure 2**). However, sampling
159 over a wider time period would be needed to confirm if this temporal pattern is stable throughout
160 the year.

161 While sampling effort along the mainland coastline was uneven, we found some
162 indication that location may influence concentrations of diphenhydramine. Of the three urban
163 sampling locations, both male and female stingrays had highest concentrations and the number of
164 quantifiable samples at Seal Beach (**Figure 3**). Stingrays sampled from locations farther away
165 had non-detectable levels of diphenhydramine. For instance, all San Diego stingrays (1 male, 6
166 females) had non-detectable levels, while Cabrillo Beach stingrays had either quantifiable levels
167 or levels under the limit of detection with no stingrays having non-detectable levels. While most
168 of southern California is highly urbanized, this suggests that environmental pharmaceuticals vary
169 along the coastline and vary based on sex and maturity status.

170 **Discussion**

171 By 2050, 70% of the global population will reside in urban areas, and much of this
172 population will be located near coastlines. Southern California, with a population of over 23
173 million people, is home to the largest county in the USA, and two of the top 10 largest cities in
174 the USA. Considering this highly populated region, and therefore the amount of treated
175 wastewater output into the marine environment, the dearth of pharmaceuticals detected in Round
176 Stingrays was unexpected. The low frequency of pharmaceutical detection could be related to
177 aspects of stingray ecology and has implications for the design of future studies examining
178 pharmaceuticals in other elasmobranchs. For example, one reason why we detected so few
179 chemicals could be due to the physical separation of wastewater outfall pipes with preferred
180 stingray habitat. Many of the major water treatment plants (i.e. Los Angeles, Orange County,
181 San Diego) have their wastewater released several kilometers from the coast and at depths of at
182 least 60 m^2. Between the late spring to mid-fall, stingrays aggregate in high numbers close to
183 shore (Hoisington & Lowe 2005), and are likely interacting waters that are more surface-
184 influenced than water at depth where outfall pipes are located. Further, our previous work in an
185 urban estuary of the Gulf of Mexico in Texas identified lower levels of pharmaceuticals in
186 estuarine fish that are primarily benthic, apparently due to decreased waterborne exposure with
187 depth (Du et al. 2016, Scott et al. 2016). Although previous studies have proposed that stingrays
188 move offshore and utilize deeper depths during the winter (Babel 1967), it is unlikely that they
189 are using habitat at the depths of where the outfall pipes occur.

190 Nevertheless, we were able to detect the presence of three pharmaceuticals, with
191 diphenhydramine being the only one quantifiable. Similar to other studies (Ramirez et al. 2009),
192 stingrays sampled from the more urban-influenced site had higher rates of detection and

[2] https://www.ocsd.com/services/regional-sewer-service

193 concentrations than stingrays sampled from an offshore island where human influence is
194 significantly lower. This demonstrates that Catalina Island can serve as a useful reference site for
195 future pharmaceutical, and potentially other "down the drain" chemical, studies in southern
196 California. Interestingly, while our stingrays had low detection rates of pharmaceuticals, two of
197 our male stingrays had liver levels that were substantially greater than maximum values of fish
198 sampled (and measured as whole fish homogenates) downstream from four of five major cities
199 examined by Ramirez et al. (2009), one of which was more than double that of the maximum
200 overall value. However, this could be an artifact of comparing different tissue types (i.e. liver
201 versus whole body homogenates). Nevertheless, Berninger et al. (2011) measured chronic and
202 acute responses of Fathead Minnows (*Pimephales promelas*) to varying water concentrations of
203 diphenhydramine and found that even at low doses, diphenhydramine effected behavior,
204 specifically feeding rate, while higher doses affected growth. While we do not have
205 concentrations of this pharmaceutical from waters where stingrays were sampled, the potential
206 exists for diphenhydramine to exert effects in Round Stingrays like it does in other fishes.
207 Further work should explore the extent to which diphenhydramine, or other pharmaceuticals,
208 influence stingray physiology or behavior and how that may be modulated by exposure to other
209 known anthropogenic chemicals such as mercury (Lyons et al. 2017) and legacy organic
210 contaminants (Lyons et al. 2014), and the implications that has for other elasmobranchs.
211 Our study suggests sex may play a role in influencing diphenhydramine concentrations in
212 stingrays from the urban site. Of significance was the fact that males appeared to bioaccumulate
213 this compound as they grew (i.e. aged), but not females. Such observations are interesting
214 because Wang & Gardinali (2012) calculated bioaccumulation factors for diphenhydramine in
215 Mosquito Fish (*Gambusia holbrooki*). However, the following year, these authors found

diphenhydramine uptake to be modest and depuration to occur in only a matter of days (Wang & Gardinali 2013). In laboratory studies, Nichols et al. (2015) advanced these observations to develop a predictive model of the influence of pH on inhalational exposure and bioconcentration of diphenhydramine in fish. Further, we previously observed trophic dilution for diphenhydramine in an effluent-dependent wadeable stream (Du et al. 2014), an an urban estuary (Du et al 2016) and an effluent-dominated stream influenced by snowmelt (Haddad et al. 2018) , which collectively suggested that inhalational exposure is more important than dietary routes. Since diphenhydramine was detected in males at both smaller sizes and higher concentrations than females, this indicates that aspects of female physiology or ecology lowers their potential to accumulate diphenhydramine. For instance, elasmobranchs are well known to sexually segregate (Klimley 1987) and the Round Stingray is no exception (Jirik & Lowe 2012). In particular, during the summer and fall months female stingrays will seek refuge in salt marshes to gestate, resulting sexual segregation with mature males remain in coastal, open waters. This may lower females' potential exposure to pharmaceuticals through their use of habitats that are distinct from males for at least part of the year.

The hypothesis that habitat highly influences exposure and accumulation is typified by the fact that male stingrays from our reference site had no detectable levels of contaminants, despite their larger size and presumably advanced age over stingrays from the urban site that did have detectable levels. Furthermore, we found variation in detection rates and concentrations of stingrays sampled at different locations along the urban mainland coastline. While we do not know the degree of stingray movement between these locations, it supports previous findings demonstrating that habitat plays an important role in accumulation of pharmaceuticals (Du et al 2016).

One main physiological difference between adult males and females is the latter's ability to maternally transfer contaminants to offspring. Females stingrays are known to transfer other lipophilic contaminants (Lyons & Lowe 2013), and the implication that parental transfer of pharmaceuticals occurs in teleosts (Parrott & Bennie 2009, Galus et al. 2014) suggests that similar phenomenon may occur in elasmobranchs as well. In combination with differential habitat use, females may have had lower levels of diphenhydramine than comparably sized males due to their ability to transfer it to offspring, a depuration pathway unavailable to males. Furthermore, female stingrays exhibit different biochemical responses to aryl hydrocarbon exposure than males, particularly for P450 expression and activity (Lyons et al. 2014). This sex-related difference could also extend to how pharmaceuticals are physiologically handled in adult males and females, particularly because previous *in vitro* studies with Rainbow Trout (*Oncorhynchus mykiss*) indicate limited biotransformation of diphenhydramine (Connors et al. 2013). Future studies should investigate if the ability to metabolize pharmaceutical compounds changes across ontogeny and the interaction this may have with sex.

Future Directions

To our knowledge, this is the first study examining pharmaceuticals in a benthic elasmobranch species. The lack of compounds detected at quantifiable levels in Round Stingrays from a highly urban area indicates that species ecology must be considered in future studies aiming to explore pharmaceutical exposures in elasmobranchs. For instance, if the Round Stingray represents a sentinel for coastally-associated elasmobranchs, then other species might also be exposed to similar or greater concentrations of diphenhydramine and other substances, particularly if surface water discharges of effluents are observed. Larger, pelagic elasmobranchs, however, may be at relatively low risk due to the dilution ability in open water, while deep-water

elasmobranchs that are more bottom-oriented, such as skates, could be at more risk in regions where effluent discharges are released at depth. Since diphenhydramine was measured in stingrays at concentrations higher than fish from more "closed" systems (i.e. rivers), where behavioral effects at low doses were observed, this suggests that further investigation is needed to investigate the physiological effects of diphenhydramine in elasmobranchs.

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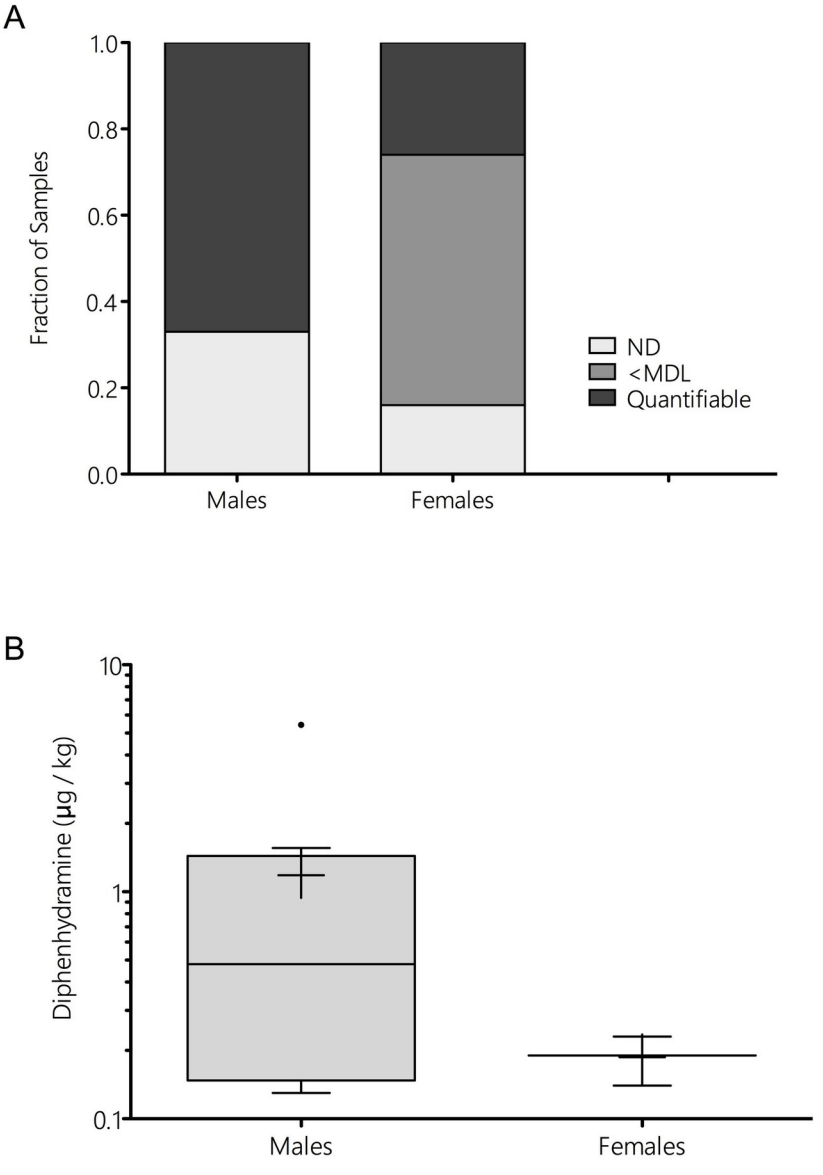
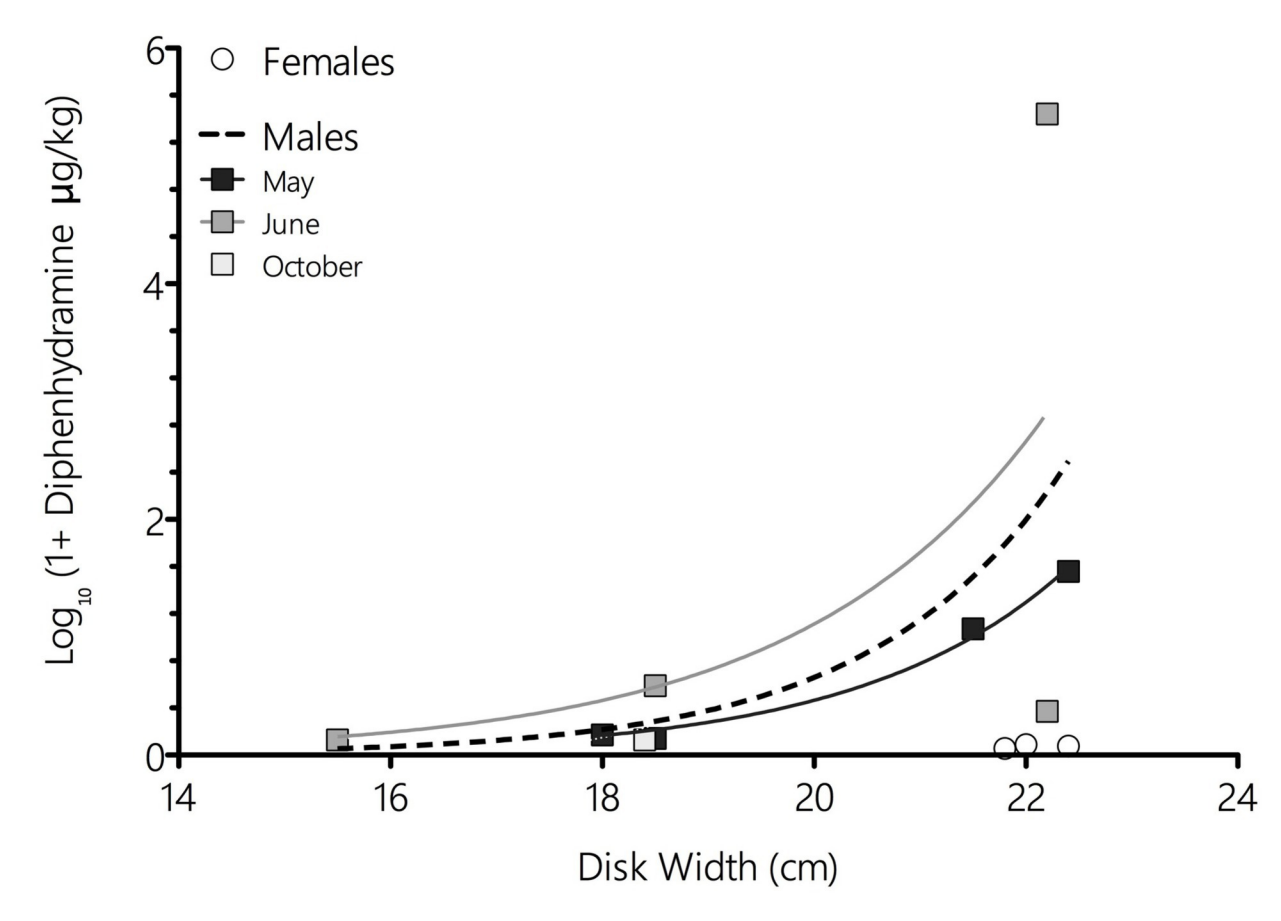
Figure Legends

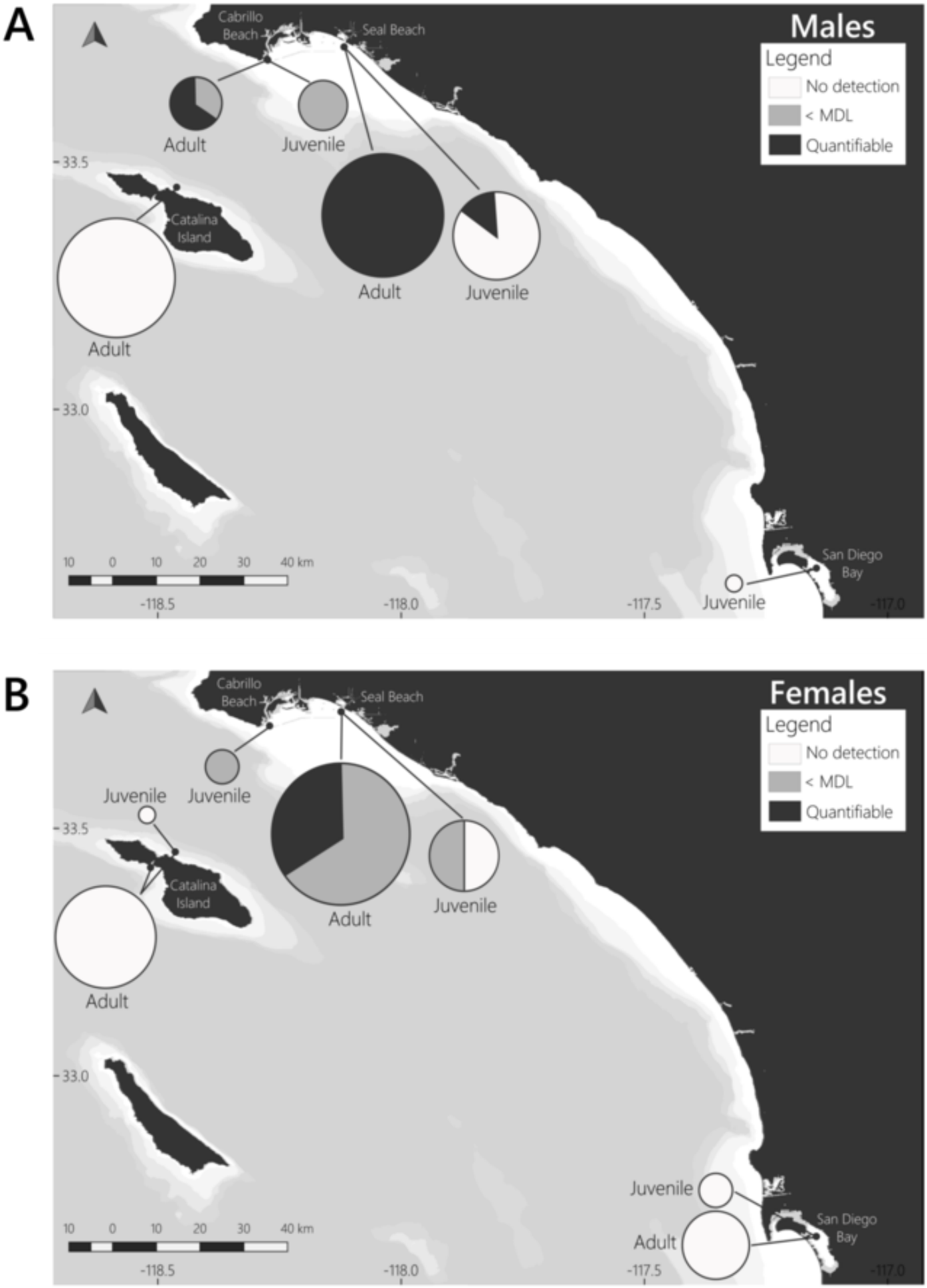
Figure 1. Seal Beach (A) males and females differed in the proportion of samples with quantifiable levels of diphenhydramine (dark grey), samples that were below detection limits ("<MDL"; medium grey) and samples with no detections (light grey). (B) Mean concentrations of diphenhydramine (represented by crosses) were higher males than females, although this difference was not statistically significant due to low numbers of females with quantifiable levels of diphenhydramine.

Figure 2. Diphenhydramine concentrations increased in males (squares) as they grew whereas only the largest females (circles) sampled in this study had quantifiable levels. During June (grey squares, lines) and May (black squares, lines) diphenhydramine levels in males increased in similar patterns. Figure represents all sites combined.

Figure 3. Males (A) and females (B) may vary in their exposure to diphenhydramine either spatially or ontogenetically. Bubble size reflects the relative number of samples at each site by maturity status (adult or juvenile) and by sex, with each bubble proportional Seal Beach adult females (n = 8). Bubbles are colored based on the proportion of stingrays within each grouping that had quantifiable levels (dark grey), levels below detection limits ("<MDL", medium grey), or no detections (light grey).


A
1.0
0.8
0.6
0.4
0.2
0.0
Fraction of Samples
ND
<MDL
Quantifiable
Males
Females
B
10
1
0.1
Diphenhydramine (µg / kg)
Males
Females


Females
Males
May
June
October
Log10 (1+ Diphenhydramine µg/kg)
Disk Width (cm)
0
2
4
6
14
16
18
20
22
24


A
Males
Legend
No detection
< MDL
Quantifiable
Cabrillo Beach
Seal Beach
Adult
Juvenile
Catalina Island
Adult
Juvenile
Adult
33.5
33.0
10 0 10 20 30 40 km
-118.5
-118.0
-117.5
-117.0
San Diego Bay
Juvenile
B
Females
Legend
No detection
< MDL
Quantifiable
Cabrillo Beach
Seal Beach
Juvenile
Juvenile
Catalina Island
Adult
Juvenile
Adult
33.5
33.0
10 0 10 20 30 40 km
-118.5
-118.0
-117.5
-117.0
Juvenile
Adult
San Diego Bay