Ontogenetic Dietary Shifts and Bioaccumulation of Diphenhydramine in *Mugil cephalus* from an Urban Estuary

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Abstract

Though bioaccumulation of pharmaceuticals has received attention in inland waters, studies of pharmaceutical bioaccumulation in estuarine and marine systems are limited. Further, an understanding of pharmaceutical bioaccumulation across size classes of organisms displaying ontogenetic feeding shifts is lacking. We selected the striped mullet, *Mugil cephalus*, a euryhaline and eurythermal species that experiences dietary shifts with age, to identify whether a model weak base, diphenhydramine, accumulated in a tidally influenced urban bayou. We further determined whether diphenhydramine accumulation differed among size classes of striped mullet over a two year study period. Stable isotope analysis identified ontogenetic feeding shifts of *M. cephalus* occurred from juveniles to adults. However, bioaccumulation of diphenhydramine did not significantly increase across age classes of *M. cephalus* but did correspond to surface water levels of the pharmaceutical, which suggests inhalational exposure to diphenhydramine was more important for bioaccumulation than dietary exposure in this urban estuary.

Keywords: Urban ecosystems, bioaccumulation factor, bioconcentration, effluent, striped mullet, urbanization

Introduction

Reports of human pharmaceuticals accumulating in aquatic biota from inland surface waters have increased in recent years, particularly from rapidly urbanizing regions (Brooks et al., 2005; Du et al., 2014a; Du et al., 2012; Kolpin et al., 2002; Ramirez et al., 2009; Ramirez et al., 2007). Though there is increasing information for freshwater, there remains a poor understanding of the occurrence, bioaccumulation and risks of human pharmaceuticals in coastal systems (Alvarez et al., 2014; Daughton and Brooks, 2011; Du et al., 2016; Gaw et al., 2014; Jiang et al., 2014; Lazarus et al., 2015; Maruya et al., 2012; Meador et al., 2016). Coastal waters receive freshwater inflows, which are influenced by watershed practices, including discharge from municipal and industrial wastewater treatment plants (WWTPs), and runoff from stormwater in agricultural and urban areas. Instream flows of many freshwater streams in semi-arid regions of the world are dominated by or even dependent on effluent discharge (Brooks et al., 2006). These urban systems likely represent worst-case scenarios for potential ecological effects of pharmaceuticals because effective exposure duration increases with limited dilution of continuous chemicals introduction (Ankley et al., 2007). Such exposure scenarios for consumer chemicals are also critically important to understand in rapidly urbanizing coastal systems (Brooks et al., 2006). In fact, a recent global horizon scanning workshop identified developing an understanding of the bioaccumulation and risk associated with pharmaceuticals and personal care products (PPCPs) in wildlife among the top questions necessary to understand risks of PPCPs in the environment (Boxall et al., 2012; Rudd et

al., 2014). Coastal contamination from urban areas was also identified as a priority research need for marine science (Rudd, 2014).

Our recent research observed accumulation of a calcium channel blocker, diltiazem, in plasma of multiple fish species exceeding human therapeutic plasma doses (Scott et al., 2016). We also identified bioaccumulation of several other pharmaceuticals in fish from four estuaries of the Gulf of Mexico in Texas, USA, with differential land use and urbanization features (Du et al., 2016). Whether these observations extend across life history stages of fish or other aquatic life is poorly understood. Further, influences of dietary exposure on bioaccumulation of ionizable contaminants in species displaying ontogenetic feeding shifts across their life histories are not known. In the current study, we selected the striped mullet, *Mugil cephalus,* to explore whether accumulation of an ionizable pharmaceutical differs among life history stages. Ontogenetic shifts in diet specifically occur in smaller *M. cephalus* (1-100 mm; Akin and Winemiller, 2006; Eggold and Motta, 1992), but have received limited study in larger individuals.

The striped mullet is an estuarine species with a wide distribution in tropical, subtropical, and temporal coastal waters in all major oceans between the latitudes of 42° N and 42° S (Thompson, 1966). In many coastal populations, *M. cephalus* lay eggs near shore in the marine environment where these eggs remain suspended until hatch (Strydom and d'Hotman, 2005). After a month at sea in the surf zone, early juveniles transition to coastal estuaries where juvenile and part of the sub-adult life stages are lived (Hsu et al., 2009; Lawson, 2010) before returning to the ocean as adults to spawn. A euryhaline (Cardona, 2006) and eurythermal teleost (Marais, 1978), *M. cephalus* may

represent a ‘sentinel’ species to monitor environmental changes (Whitfield et al., 2012). Herein, an understanding of exposure and accumulation of most contaminants of emerging concern (CECs), including pharmaceuticals, is unknown as organisms grow, but necessary to reduce uncertainty during environmental hazard and risk assessment.

In the present study, we examined whether a model ionizable weak base, diphenhydramine (DPH), was accumulated by *M. cephalus* from a tidally influenced urban bayou, which receives municipal effluent from Houston, Texas, USA. We then determined whether DPH accumulation differed with size of *M. cephalus* over a two year study period. Stable isotope analysis was employed to identify if ontogenetic feeding shifts of *M. cephalus* occurred with age.

Methods and Materials

Study site

Buffalo Bayou (Figure 1) begins in Fort Bend County, Texas, flows to the Houston Ship Channel, and then on to Galveston Bay, a critically important commercial fishery and port in the Gulf of Mexico. Buffalo Bayou was selected for study because this intensively urbanized watershed is the receiving system for appreciable effluent discharge and stormwater runoff from the City of Houston, Texas, the fourth largest city in the USA. During an initial study, we observed a number of pharmaceuticals and other CECs in the surface waters of Houston (Watkins et al., 2014). We sampled downstream of the 69th

Street WWTP, which is the largest WWTP (~200 Million Gallons Daily) in the US EPA Region 6 states of Texas, New Mexico, Louisiana, Arkansas and Oklahoma.

Field sampling

Surface water and biological samples were collected on two different sampling events in October 2012 and September 2013. September and October are considered by the Texas Commission on Environmental Quality as important periods for monitoring surface water quality because these months represent a time of the year when rainfall, and thus instream dilution, is typically lowest, and subsequent exposure to aquatic contaminants is expected to be highest (TCEQ 2012). Sample collection followed Texas Commission on Environmental Quality methods by boat electrofishing, minnow trapping, and cast netting (TCEQ 2012). Specific boat electrofishing locations within a 200 m radius of the discharge were determined by salinity influences on electrofishing. Fish length and weight were measured on site immediately after anesthetization using MS-222. All samples were transported to the lab on ice and stored at -20 °C until further analyses. During each sampling event, duplicate surface water samples were collected ~50 m downstream of the discharge in 4-L pre-rinsed amber glass bottles, transported on ice to the lab, and stored for less than 48 h at 4 °C in the dark prior to filtration and extraction.

Pharmaceutical analysis in water and fish tissue

Analytical methods for surface water and tissue followed previously reported procedures by our research team (Du et al., 2016; Du et al., 2014a; Du et al., 2012; Du et al., 2014b),

which were adapted from earlier reported methods (Lajeunesse et al., 2008; Ramirez et al., 2007; Vanderford and Snyder, 2006). Information for other pharmaceutical occurrence in water and bioaccumulation for other fish species from Buffalo Bayou can be found elsewhere (Du et al., 2016). Isotope dilution was used to compensate for matrix interference with an isotopically-labeled internal standard for the target analyte (Du et al., 2016; Du et al., 2014a).

All tissue samples were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) following a previously reported method, in which instrumentation parameters, separation strategy, detection of the target analyte, calibration method, and method detection limits (MDLs) were detailed (Du et al., 2012). MDLs for the analyte represented the lowest concentration that was reported with 99% confidence that the concentration was different from zero in a given matrix. One method blank sample and a pair of matrix spikes were also analyzed in each analytical sample batch. Matrix spike samples were spiked with 100 µg/kg of the target analyte. All matrix spike recoveries were within 80-120% of this spiking value.

Stable isotope analysis

Stable isotopes ($\delta^{15}N$, $\delta^{13}C$) were determined in the Stable Isotope Core Laboratory at Baylor University using a dual-inlet gas-source Stable Isotope Mass Spectrometer (Thermo-Electron, Waltham, Massachusetts, USA) and an Elemental Analyzer (Costech, Valencia, CA, USA). Whole biological tissue samples were dried for 24 h at 95 °C in a drying oven and crushed to a fine powder using a mortar and pestle. Dried, crushed

samples were weighed to approximately 1 mg and wrapped in Sn capsules prior to the instrumental analysis. Data was calibrated using internationally recognized standards USGS-40 and USGS-41 with analytical precision of ± 0.02 %. Isotopic ratios are calculated using the following equation:

$$\delta X\ (‰) = (R_{sample}/R_{standard} - 1) \times 1000 \qquad (1)$$

where the heavier isotope X is ^{15}N or ^{13}C, R_{sample} is the ratio of heavy to light isotope in the analyzed sample, and $R_{standard}$ is the ratio of heavy to light isotope in the standards (Jardine et al., 2006).

Statistical analysis

Individual *M. cephalus* were partitioned to size classes from <149 mm (< 12 month old juveniles), 150 – 249 mm (~12-24 month old juveniles), 250 – 349 mm (~2 year old juveniles to adults), or > 350 mm (adults). Because the maximum size of *M. cephalus* at one year is reported to be 148 mm in Texas (McDonough and Wenner, 2003), the smallest size class ended at 149 mm and was considered to be fish < 12 months old. Sexual maturity occurs between 250 to 300 mm for males and between 250 to 350 mm for females (Amuer et al., 2003), so the size class of 250 – 349 mm was intended to encompass late juveniles and early adult *M. cephalus*. The largest size class of > 350 mm was selected to represent more mature adult mullet. ANOVA, Tukeys HSD, and one-way T-tests were performed using JMP Pro (alpha of 0.05; Version 9, SAS institute Inc., Cary, NC, USA) and regression conducted with SigmaPlot 13 (Systat Software, San Jose, CA, USA).

Results and Discussion

Studies of pharmaceutical occurrence, bioaccumulation and risks in estuaries of the United States and other parts of world are rare (Du et al., 2016; Scott et al., 2016; Gaw et al., 2014; Klosterhaus et al., 2013; Lazarus et al., 2015; Meador et al., 2016). In the current study, surface water concentrations of DPH were 15 ng/L and 42 ng/L in 2012 and 2013, respectively. The presence of other pharmaceuticals and CECs detected in fish species from Buffalo Bayou appeared to vary among fish species depending on habitat preferences (Du et al., 2016). In addition to our recent observations in urban estuaries of the Gulf of Mexico (Du et al., 2016; Scott et al., 2016), three other studies, from San Francisco Bay, California (Klosterhaus et al., 2013), the Chesapeake Bay, Maryland (Lazarus et al., 2015) and the Puget Sound, Washington (Meador et al., 2016), have reported DPH occurrence in urban estuaries of the USA. Levels of DPH in surface water from the current study were typically 5 to 35 times higher than those previously reported from other estuaries (Klosterhaus et al., 2013; Lazarus et al., 2015; Meador et al., 2016; Scott et al., 2016). Similarly, DPH accumulation in *M. cephalus* was generally elevated compared to other locations and estuarine species in the USA. Individual *M. cephalus* accumulated DPH ranging from 0.06 to 0.28 μg/kg in 2012 to slightly higher levels (0.29 to 2.01 μg/kg) during 2013 (Table 1).

Compared to other estuarine fish species from our recent research (Du et al., 2016), *M. cephalus* accumulated the highest amount of DPH in whole body homogenates (μg/kg). Our observations of DPH in *M. cephalus* from 2012 were similar to DPH levels in salmon and sculpin from Washington, even though surface water DPH concentrations in Buffalo Bayou were 10-15 times higher than in Puget Sound (Meador et al., 2016). However, during 2013 accumulation of DPH in *M. cephalus* was 1-8 times higher than salmon and sculpin from Puget Sound, and significantly greater ($p > 0.05$) than observations from fall 2012. If human ingestion of pharmaceuticals in fish from Buffalo Bayou is considered (Brooks et al., 2005), exposure to DPH from consumption of *M. cephalus* is well below a typical daily dose of DPH. For example, considering a single fish serving is commonly estimated to be 0.289 kg (USEPA, 1989), ~135,000 kgs of *M. cephalus* would need to be consumed in one meal to equal a DPH dose of 25 mg.

Though previous research partitioned juvenile *M. cephalus* among smaller size classes (20-100 mm) associated with shifts among different prey items with fish growth (Eggold and Motta, 1992), in the present study we examined fish size classes up to 400 mm in length. DPH bioaccumulation in the largest fish (>350 mm) was significantly lower ($p<0.05$) than smaller size classes from the 2012, but not the 2013, sampling event (Figure 2). When field BAFs were calculated (Arnot and Gobas, 2006) for both sampling dates, a consistent decrease in BAFs corresponded to increasing size of *M. cephalus*. For example, the highest BAF (28) was observed in 2013 for smaller fish (<149 mm) while the lowest BAF (3.7) was observed in 2012 for the largest size class (> 350 mm) (Table 1).

Observed DPH accumulation differences in fish may have been influenced by several factors, such as species movement, variable surface water DPH exposure, pH, salinity, and dietary exposure. Because bioaccumulation does not occur instantaneously (Arnot and Gobas, 2006), the home range of this species must be considered. Movement *of M. cephalus* above and below the WWTP discharge likely occurred during the current study due to foraging behavior. *M. cephalus* school and move within an estuary to feed on detritus and available food along the benthos (Whitfield et al., 2012). Additionally, adult striped mullet leave estuaries and return to the open ocean to spawn (Ibanez and Benitez, 2004). *M. cephalus* are considered adults when they are sexually mature enough to reproduce, which is typically greater than 250 mm (Amuer et al., 2003).

In the present study, we examined a heavily influenced urban watershed and sampled surface waters downstream from a large (~200 MGD) discharge. Surface water concentrations of DPH and other contaminants may be expected to generally decrease with distance downstream from a WWTP discharge. As water moves away from the WWTP discharge through Buffalo Bayou toward Galveston Bay, it is increasingly diluted by more saline bay water. For example, dilution was observed in DPH water concentrations a short distance between the effluent discharge (200 ng/L) and surface water (42 ng/L) sampled 50 m downstream in 2013 (Du et al., 2016). However, exposure to DPH also occurs above this discharge due to tidal influence and other upstream effluent sources (Scott et al., 2016). In the present study, surface water sampling efforts were not intended to characterize the range of exposure scenarios *M. cephalus* experience

temporally and spatially across their life cycle. Decreased DPH levels observed in older fish may have resulted from more time spent away from effluent discharge.

DPH levels in striped mullet may also have been influenced by dietary exposure. During the larval phase *M. cephalus* are primarily planktonic feeders (Gisbert et al., 1996; Nash et al., 1974; Zismann et al., 1975), then begin to shift as juveniles (e.g., ~10-20 mm) to feed on small invertebrates and benthic organisms (Blaber and Whitfield, 1977; Whitfield et al., 2012). As striped mullet increase in size they will feed more frequently on detritus and inorganic matter (sand) (Desilva and Wijeyaratne, 1977; Eggold and Motta, 1992). Though we did not examine potential DPH exposure from detritus, partitioning of DPH and other ionizable contaminants deserve additional study. For example, Al-Khazrijy and Boxall (2016) recently noted challenges associated with predicting sediment partitioning behavior of ionizable pharmaceuticals, which do not conform to equilibrium partitioning expectations for nonionizable organic contaminants.

A previous study observed $\delta^{15}N$ signatures of *M. cephalus* from Matagorda Bay, Texas, to increase with increasing fish size, which most likely resulted from ontogenetic dietary shifts with age (Akin and Winemiller, 2006). Though ontogenetic dietary shifts have been studied in juvenile *M. cephalus* (1-100 mm; Eggold and Motta, 1992), ontogenetic dietary shifts from juveniles (100 to 300 mm) and adults (>300 mm) of this species are not well understood. In the present study, we employed stable isotopes ($\delta^{15}N$, $\delta^{13}C$) across various *M. cephalus* size classes, from juvenile to adult, to identify whether potential ontogenetic feeding shifts occurred in larger individuals (Table 1). In 2012, $\delta^{15}N$ significantly increased ($p<0.05$) in larger striped mullet, which suggests a change in

feeding and thus trophic position (Figure 3A). A similar relationship was not observed in 2013 (Figure 3B); however, *M. cephalus* in the largest adult classes, which were electrofished in 2012, were not encountered during collection efforts in the following sampling period (Table 1). Though such differences in $\delta^{15}N$ provide a reasonable surrogate for dietary uptake and assimilation, future studies examining ontogenetic feeding shifts in mullet should confirm observations in the present study using stomach content analysis.

Ontogenetic dietary shifts, similar to those exhibited by the striped mullet, have received previous study for influencing bioaccumulation of nonionizable organic contaminants (e.g., PCBs) and mercury (Beaudry et al., 2015; Kraemer et al., 2012; Szczebak and Taylor, 2011; Corsolini et al., 2007). In the present study we observed significantly lower levels of DPH only in the highest size class during 2012 (Figure 2). Conversely, tissue concentrations of conventional nonionizable organic contaminants that do not experience appreciable biotransformation generally increase with age and trophic position (Newman 2009). In addition, lengths and weights of fish in biomonitoring studies are typically reported as a range and then less regarded compared to tissue concentrations during studies of contaminant accumulation (Waltham et al., 2013). Such a practice is often relevant when lipid normalization is employed for organic contaminants. However, as we previously identified with DPH and other ionizable weak bases, partitioning to fish does not solely occur by hydrophobic mechanisms, which is common for nonionizable organic contaminants (e.g., PCBs, dioxins, furans); thus, lipid normalization of weak bases in aquatic organisms is less relevant for bioaccumulation

studies (Ramirez et al., 2009). In fact, our more recent findings highlighted the importance of comparative pharmacokinetics when attempting to understand bioconcentration of ionizable weak bases in fish. The apparent volume of distribution, an important pharmacokinetic parameter, value for DPH of ~3-8 L/kg in humans is almost identical to fish (Nichols et al., 2015). Though observations from the present study indicate that ontogenetic feeding shifts to higher trophic positions did not significantly increase DPH bioaccumulation by *M. cephalus* (Figure 4), future studies are needed to examine other ionizable organic contaminants in estuarine and marine organisms, including those species characterized with feeding shifts across their life cycle.

Bioconcentration of DPH by freshwater fish increases as pH approaches the pKa value, and uptake occurs rapidly with steady state conditions achieved within 24-96 hrs (Nichols et al., 2015). In the present study, significantly higher tissue levels of DPH were observed *in M. cephalus* collected during 2013 compared to the previous year. Similarly, elevated surface water concentrations were observed in 2013 (42 ng/L) compared to 2012 (15 ng/L) (Table 1). It thus appears possible that increasing DPH levels in tissue correspondingly increased with increasing waterborne exposure. A similar relationship may exist between DPH in surface water and fish tissue from a recent study in Puget Sound, though DPH from grab samples of surface water were lower in the Puget Sound (0.96 – 1.5 ng/L) than the current study. Observations in the present study are in agreement with another recent study that identified DPH and several other ionizable weak bases do not exhibit trophic magnification in a municipal effluent-dependent freshwater system (Du et al., 2014a). Thus, inhalational uptake, compared to diet, appears to be a

more important route of exposure (Du et al., 2014a). Comparative pharmacokinetic studies in estuarine and marine fish are lacking but necessary to understand bioaccumulation dynamics of these CECs and other ionizable contaminants.

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Figure Captions

Figure 1: Buffalo Bayou in Houston, Harris County, Texas, USA. The diamond symbol denotes an effluent discharge from a major waste water treatment plant.

Figure 2: Mean (±SD) concentration of diphenhydramine across size classes of *Mugil cephalus* in Buffalo Bayou, Houston, Texas, USA, for two different sampling years. Numbers in bars represent n for each group. *: $p < 0.05$.

Figure 3: Relationship between $\delta^{15}N$ and length (mm) in *Mugil cephalus* vs the length (mm) during two study years (A = 2012, B = 2013).

Figure 4: Relationship between $\delta^{15}N$ and diphenhydramine (µg/kg) in *Mugil cephalus* during two study years (A = 2012, B = 2013).

Table 1. *Mugil cephalus* mean (±SD) weight (g), $\delta^{13}C$, $\delta^{15}N$, diphenhydramine (DPH) tissue levels and bioaccumulation factors (BAF) by size classes from an urban estuary, Buffalo Bayou, Texas, USA, in 2012 and 2013.

	Size Class (mm)	n	Weight (g)	$\delta^{13}C$	$\delta^{15}N$	DPH (µg kg^{-1})	BAF (L kg^{-1})
2012							
	350 >	4	779.6 ± 203.8	−25.59 ± 2.39	18.07 ± 2.58	0.06 ± 0.00	3.7 ± 0.0
	250 - 349	7	265.8 ± 81.5	−25.75 ± 1.90	15.89 ± 2.24	0.25 ± 0.06	17 ± 4.0
	150 - 249	9	89.9 ± 31.0	−26.09 ± 1.71	15.08 ± 3.10	0.19 ± 0.09	13 ± 6.0
	< 149	5	27.4 ± 5.4	−23.50 ± 2.98	12.98 ± 1.78	0.17 ± 0.07	11 ± 4.5
2013							
	150 - 249	6	111.4 ± 28.9	−26.17 ± 1.65	15.72 ± 1.11	0.73 ± 0.57	17 ± 13
	< 149	9	24.6 ± 7.1	−21.51 ± 2.06	15.01 ± 2.06	1.2 ± 0.51	28 ± 12

Figure 1

Figure 2

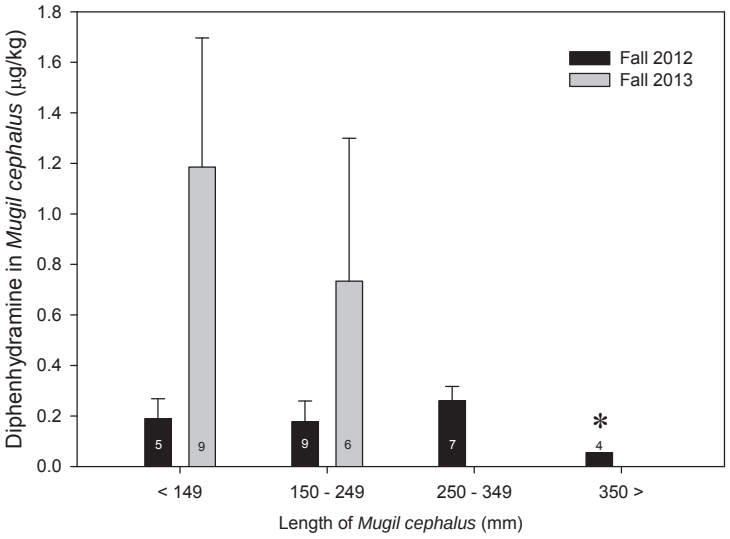

Diphenhydramine in *Mugil cephalus* (µg/kg)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Fall 2012
Fall 2013
5
9
9
6
7
*
4
< 149
150 - 249
250 - 349
350 >
Length of *Mugil cephalus* (mm)

Figure 3

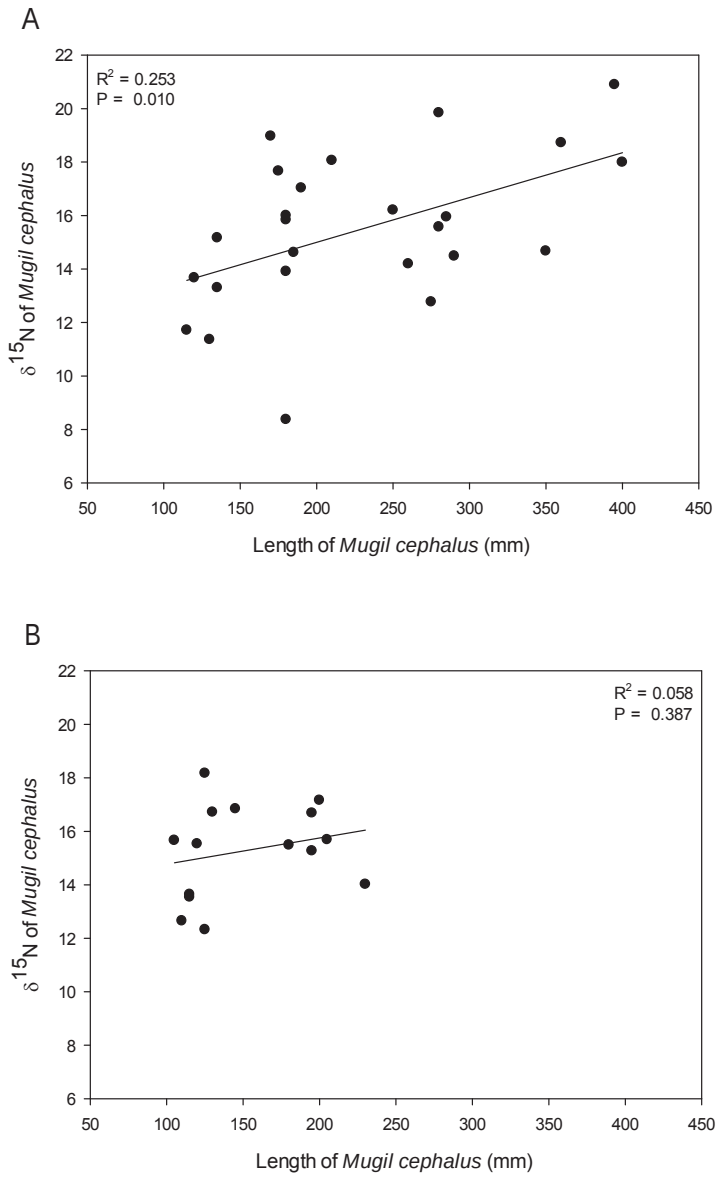
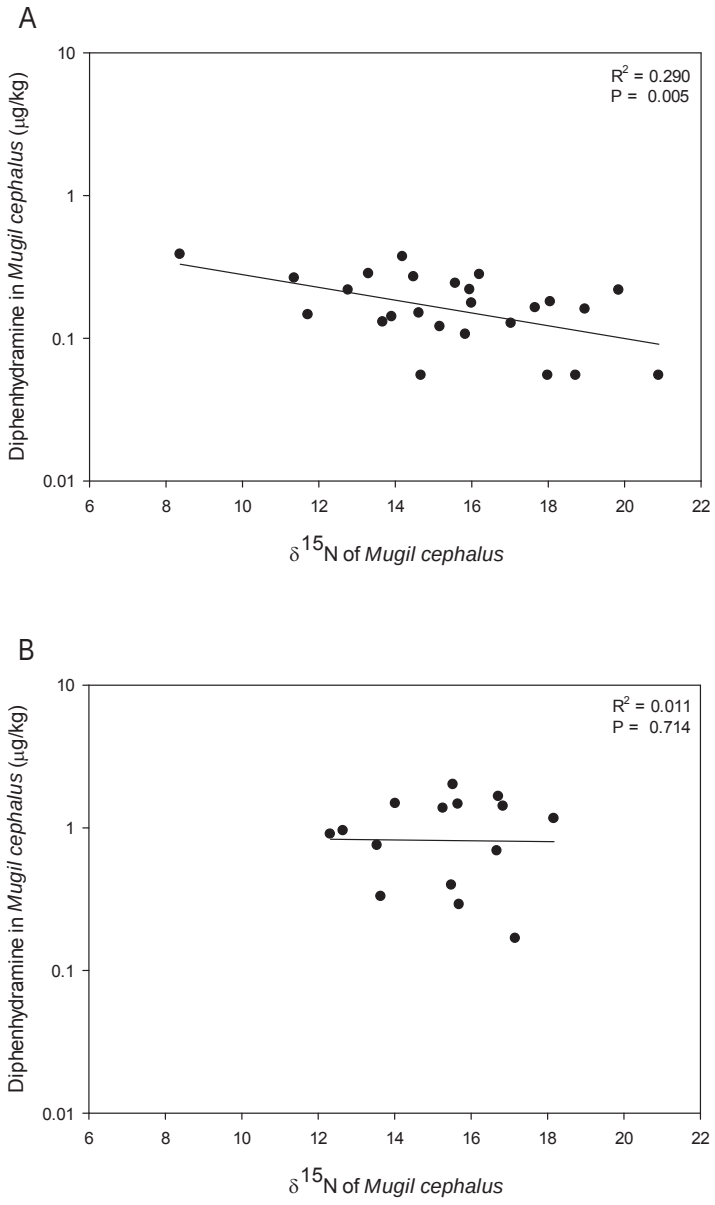

A
R^2 = 0.253
P = 0.010
δ^{15}N of *Mugil cephalus*
Length of *Mugil cephalus* (mm)
B
R^2 = 0.058
P = 0.387
δ^{15}N of *Mugil cephalus*
Length of *Mugil cephalus* (mm)

Figure 4


A
R^2 = 0.290
P = 0.005
Diphenhydramine in *Mugil cephalus* (µg/kg)
10
1
0.1
0.01
6
8
10
12
14
16
18
20
22
δ^{15}N of *Mugil cephalus*
B
R^2 = 0.011
P = 0.714
Diphenhydramine in *Mugil cephalus* (µg/kg)
10
1
0.1
0.01
6
8
10
12
14
16
18
20
22
δ^{15}N of *Mugil cephalus*