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Differences in Purine Metabolite Concentrations in the Diet of Managed and Free-Ranging Common Bottlenose Dolphins (*Tursiops truncatus*)

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Abstract

Ammonium urate nephrolithiasis has been reported in common bottlenose dolphins (*Tursiops truncatus*) managed under human care but rarely occurs in free-ranging dolphins. In terrestrial mammals, including human beings, consumption of purine-rich seafood may predispose to urate urolith formation because purines are metabolized and excreted in urine as urate ions. Dolphins consume a piscivorous diet, but the purine content of their diet is unknown. Free-ranging dolphins consume live, temperate-water fish, whereas managed dolphins consume frozen, stored, and thawed cold-water species that dolphins would probably not encounter in the wild. Purine metabolite concentrations vary with species and cold storage methods, so the purine intake of managed and free-ranging dolphins may differ. The concentrations of eight purine metabolites were measured in fresh frozen fish species commonly consumed by free-ranging dolphins and in seven frozen, stored, and thawed fish and squid species commonly consumed by managed dolphins. Total purine content was calculated for two model diets typically consumed by managed dolphins and a model diet reported to be consumed by bottlenose dolphins in Sarasota Bay, Florida. Total and individual purine metabolite concentrations differed significantly ($p < 0.05$) among individual species and among model diets. The mean total purine content of model managed dolphin diets was twice that in the model free-ranging dolphin diet. Inosine and IMP were measured because they can convert to hypoxanthine during frozen storage. Hypoxanthine concentrations were higher relative to inosine and IMP in managed species after frozen storage than in unstored free-ranging species ($p < 0.05$). These differences may explain the higher prevalence of ammonium urate nephrolithiasis in some managed dolphins compared to free-ranging dolphins and implies that the purine intake of some managed dolphins can be decreased by altering the proportions of species fed. Further research is needed, however, to determine whether such a change prevents ammonium urate nephrolith formation in dolphins.

Key Words: diet, kidney stones, ammonium urate nephroliths, IMP, inosine, hypoxanthine, purines, bottlenose dolphins, *Tursiops truncatus*

Introduction

Common bottlenose dolphins (*Tursiops truncatus*, hereafter referred to as dolphins) managed under human care are reported to develop ammonium urate nephroliths. One facility reports a prevalence of 35% among dolphins in their population, whereas nephroliths have rarely been found in free-ranging dolphins (Smith et al., 2013). As is true for other mammals, including human beings, nephroliths may either be an incidental finding in clinically asymptomatic managed dolphins or the cause of renal compromise secondary to urinary tract obstruction (Venn-Watson et al., 2010a, 2010b; Scales et al., 2012; Schmitt & Sur, 2012; Smith et al., 2013). Nephrolith development is more common in older managed dolphins, with a mean age of 25 y reported in one study; however, sex has not been associated (Venn-Watson et al., 2010a; Smith et al., 2013).

Ammonium urate crystals are more likely to form and then aggregate into stones when the urinary concentration of ammonium and urate ions increases at a given urine pH (Marshall & Robertson, 1976; Werness et al., 1985; Osborne et al., 1995; Moran, 2003). Urinary ammonium ion concentrations increase when ammonium ions are excreted by the kidney in response to acidosis (Halperin et al., 1990; Curthoys & Watford, 1995). We have suggested in a related report that a decreased dietary cation-anion difference (DCAD) may predispose to ammonium urate nephrolith development in managed dolphins by encouraging ammonium ion excretion (Ardente et al., 2017). In a similar fashion, purine-rich diets can promote urate urolith formation by increasing urate ion concentrations in urine because dietary purines are metabolized to uric acid, which is then excreted in urine (Ho et al., 1979).

Seafood and animal organ meat, like liver, contain more purines than other foods, and the diet of dolphins consists primarily of whole fish (Clifford & Story, 1976; Choi et al., 2004). Purine concentrations in fish filleted for human consumption have been reported to vary among fish species and with *post-mortem* handling (Lou et al., 2001; Aubourg et al., 2007). In particular, concentrations of inosine 5'-monophosphate (IMP), inosine, hypoxanthine, and xanthine vary greatly in cold-stored fish with species, storage temperature, and storage duration (Fraser et al., 1967; Lou, 1998; Aubourg et al., 2005; Digre et al., 2011). Free-ranging dolphins consume a wide variety of live, temperate-water fish and invertebrates, whereas dolphins under human care consume several frozen, stored, and thawed commercially available cold-water fish and squid species that some dolphin species would likely not encounter in the wild (Barros & Odell, 1990; McCabe et al., 2010; Venn-Watson et al., 2013; Wells et al., 2013). Thus, the purine composition of the diet of some managed dolphins may differ both in the relative proportions of purine metabolites and in total purine content when compared with the diet of free-ranging dolphins.

To our knowledge, there are no reports of either the total purine or individual purine metabolite concentrations of whole fish species consumed by dolphins. Furthermore, most studies of the purine content of a food have only measured the concentrations of the four nucleobases—(1) adenine, (2) guanine, (3) hypoxanthine, and (4) xanthine—when determining whether a food is rich, moderate, or low in purines (Clifford & Story, 1976; Lou, 1998; Choi et al., 2005). Other purines, however, like IMP, inosine, and adenine 5'-monophosphate (AMP), have been shown to be absorbed by rats, and then metabolized to uric acid and excreted in urine (Ho et al., 1979; Savaiano et al., 1980). If purine absorption and metabolism in dolphins is similar to that in rats, these other purines, which are not normally measured in foods, could affect urine urate concentrations in dolphins. Measuring individual purines may also be important because not all purines affect uric acid excretion to the same degree. For example, adenine and hypoxanthine affect the amount of uric acid excreted by human beings more than guanine and xanthine (Clifford et al., 1976).

Thus, the purpose of this study was to determine the individual concentrations of a wider range of purine metabolites than the four nucleobases in species commonly consumed by dolphins to determine whether differences in individual or total purine concentrations in model dolphin diets could explain the higher prevalence of nephrolithiasis in managed dolphin populations compared to free-ranging dolphins. We hypothesized that individual and total concentrations of purines would vary among fish species, that model managed dolphin diets would contain more purines than the model free-ranging dolphin diet, and that hypoxanthine concentrations would increase relative to inosine and IMP in frozen stored managed diet species when compared to fresh frozen free-ranging diet species.

Methods

Sample Collection and Processing

Fish and squid samples were collected by the Chicago Zoological Society's Sarasota Dolphin Research Program with approvals from the Mote Marine Laboratory and University of Florida (UF) Institutional Animal Care and Use Committees.

Samples of eight fish species commonly consumed by free-ranging dolphins residing in Sarasota Bay, Florida (*free-ranging species*; McCabe et al., 2010; Wells et al., 2013; Table 1) and six fish species and one squid species commonly consumed by managed dolphins (*managed species*; Table 1) were obtained and processed using methods that have been described previously (Ardente et al., 2017). Free-ranging fish species were caught in and around Sarasota Bay. Fish were humanely euthanized by immersion in sea water containing 500 ppm tricaine methanesulfonate (MS 222, Western Chemical, Ferndale, WA, USA) and then transported in dry ice to the College of Veterinary

Table 1. Composition of model free-ranging and managed common bottlenose dolphin diets*

Diet species	% "as fed" weight	% Mcal ME†
Free-ranging diet		
Pinfish (*Lagodon rhomboides*)	23.2	27.1
Gulf toadfish (*Opsanus beta*)	38.8	24.0
Sheepshead (*Archosargus probatocephalus*)	10.2	9.2
Spot (*Leiostomus xanthurus*)	5.9	11.4
Pigfish (*Orthopristis chrysoptera*)	1.4	1.6
Striped mullet (*Mugil cephalus*)	12.4	19.3
Ladyfish (*Elops saurus*)	3.4	2.9
Spotted sea trout (*Cynoscion nebulosus*)	3.5	2.6
Managed diets		
Managed diet #1		
Icelandic capelin (*Mallotus villosus*)	60	54.0
Pacific herring (*Clupea pallasii*)	20	31.9
Pacific mackerel (*Scomber japonicus*)	10	8.7
West coast Loligo squid (*Loligo opalescens*)	10	5.4
Managed diet #2		
Canadian capelin (*Mallotus villosus*)	60	47.4
Atlantic herring (*Clupea harengus*)	10	15.2
Pacific herring (*Clupea pallasii*)	10	17.2
Pacific mackerel (*Scomber japonicus*)	10	9.4
Pacific sardine (*Sardinops sagax*)	10	10.8

*Table is amended from Ardente et al. (2017); fish and squid energy content can also be found in this reference.
†Mcal = megacalorie (1 Mcal = 1,000 kilocalories); ME = metabolizable energy

Medicine (CVM), Clinical Nutrition Laboratory, UF in Gainesville where fish were stored at -80º C until further processing. Commercially available fish and squid species were supplied by two facilities that care for bottlenose dolphins. Fish and squid had been caught during one commercial fishing season and frozen stored at -20º C. These lots of fish and squid were tested for spoilage by the management facilities and then shipped overnight on dry ice to the UF laboratory where they were stored at -20º C. The total frozen storage time was 6 to 9 mo, which is typical for fish consumed by managed dolphins at these facilities.

Free-ranging fish species were thawed the minimum amount needed to allow grinding, whereas managed diet fish species were thawed more completely to mimic the standard operating procedure of one bottlenose dolphin facility. Free-ranging fish species were air thawed in a temperature-controlled cold room (11 to 12º C) for approximately 1 h until fish thawed to a firm, slightly malleable texture. In accordance with standard operating procedures from one dolphin management facility, managed diet species wrapped in plastic were air thawed for about 20 h in a cold room (11 to 12º C) and then were removed from their plastic bags and rinsed with cold tap water (approximately 16º C).

Five samples of each species were individually ground and analyzed. A minimum of 300 g of ground fish was needed to perform all nutrient analyses on every sample. At least two individual fish or squid were included in each sample, but the number of individual fish or squid included in each sample varied depending on the size of the species so that each sample of smaller species contained more individuals than samples of large species. Ground samples were homogenized and stored at -80º C until purine analysis was performed. All analyses were performed by a blinded researcher.

Sample Purine Extraction

Each fish sample purine extraction was performed as previously described (Ardente et al., 2016). Briefly, 2 g of fish or squid sample was homogenized with 20 mL of ultra-pure water and 500 µL of the internal standard solution, using sonication, heating, and cooling. Extract supernatant was filtered, and then equal amounts of the filtrate- and HPLC- (high-performance liquid chromatography) grade hexanes were combined and centrifuged (6,500 rpm, 7 min, 20º C). The bottom layer (4 mL) was transferred to a new tube; HPLC-grade methanol (4 mL), acetone (4 mL), and 10% formic acid in water (80 µL) were added; and the sample was centrifuged (18,100 rpm, 17 min, 15° C). Five aliquots of 1,500 µL each were pipetted into five separate 5 mL microcentrifuge tubes for standard addition quantification using the first aliquot as a blank, and subsequent aliquots as 2×, 4×, 6×, and 8× increasing concentrations. Microcentrifuge tubes were then mixed and centrifuged (3,000 rpm, 5 min, room temperature) before drying samples down under a gentle stream of nitrogen gas (35° C). The dried samples were reconstituted with 500 µL of 10 mM NH4CH3CO2, mixed, centrifuged (4,000 rpm, 15 min), and transferred to 2 mL LC vials for LC-MS/MS (liquid chromatography with tandem mass spectrometry) analysis.

Purine Analysis

Adenine, guanine, hypoxanthine, xanthine, uric acid, AMP, IMP, and inosine were separated and quantified using LC-MS/MS as previously described (Ardente et al., 2016). Separation was achieved on a Phenomenex Luna PFP(2) column (150 mm × 3.0 mm, 5 µm) under a gradient elution with mobile phase A as 0.1% acetic acid and mobile phase B as methanol. The flow rate was 500 µL/min with an injection volume of 10 µL. Purine concentrations were calculated relative to the metabolizable energy (ME) content of the fish and squid species. For each species, protein and fat content were measured as previously described, and ME density was calculated using Atwater factors (Ardente & Hill, 2015; Ardente et al., 2017). The total purine content was calculated both as the sum of all eight metabolites and of the four traditionally measured nucleobases: (1) adenine, (2) guanine, (3) hypoxanthine, and (4) xanthine.

Model Dolphin Diets

The individual and total purine content of three model diets was calculated by multiplying the purine content of each individual species (mmol/Mcal) by the fraction of ME that each species provided to the total ME of each of three model diets (Table 1). These model diets consisted of a model free-ranging dolphin diet based on the proportions of fish species reported to be consumed by dolphins in Sarasota Bay and two model managed dolphin diets that are fed to dolphins under human care (Wells et al., 2013; Ardente et al., 2017). The ME provided by each species to these model diets was determined by multiplying the measured ME density of each species by the proportional weight of each species in each diet as previously described (Ardente et al., 2017).

Statistical Analysis

Values are reported as means ± 1 standard deviation. Comparisons among fish and diets were performed with statistical software (*SAS® System for Windows 9.4*, SAS Institute Inc., Cary, NC, USA). The distributions of nutrient concentrations within species were assessed for normality visually and using the Shapiro-Wilk test. Concentrations that were not normally distributed or with widely different variances were log transformed before being compared. Individual purine metabolite concentrations were compared among fish species nested within either managed or free-ranging groups using a general linear model design (*SAS* procedure *GLIMMIX*). Multiple comparisons were performed with a Tukey-Kramer correction. Linear estimates utilizing the least square means were used to compare individual and total purine contents among model diets (*SAS* procedure *LSMESTIMATE*).

The primary endpoint was to determine whether there was a 50% increase in the concentration of hypoxanthine and other purines during frozen storage. Based on previous reports of the variability in concentrations of hypoxanthine and other purines in filleted fish during storage, comparing five samples of each species gave an 80% power to detect a 50% change in hypoxanthine concentration with a type I error of 0.05 (Lou, 1998; Piñeiro-Sotelo et al., 2002; Kabacoff, 2012).

Results

Purine metabolite concentrations differed significantly ($p \leq 0.05$) among individual species (Table 2). Concentrations of adenine, uric acid, hypoxanthine, xanthine, AMP, and inosine were greater ($p \leq 0.05$) in managed diet species than free-ranging diet species, whereas IMP concentrations were greater ($p \leq 0.05$) in free-ranging diet species than managed diet species. Adenine, uric acid, IMP, and AMP concentrations were present in very small to negligible amounts in almost all species, with a few exceptions. Ladyfish, in particular, contained at least twice the IMP concentration compared with all other species. Guanine and inosine were present in high concentrations in all species, except for Loligo squid and toadfish,

Table 2. Purine metabolite concentrations and ratio* in fish and squid species consumed by managed and free-ranging common bottlenose dolphins

Species	Adenine†	Guanine	Uric acid†	HXA†	Xanthine†	AMP†	IMP‡	Inosine†	Total#	
	mmol/Mcal ME									IMP+INO:HXA‡
Free-ranging diet£										
Pinfish	0.002 ± 0.001[d,e]	1.5 ± 0.1[a]	0.01 ± 0.004[a,b]	0.5 ± 0.05[b]	0.2 ± 0.03[d,e]	0.08 ± 0.009[a,b,c]	0.1 ± 0.03[c,d]	2.2 ± 0.1[b,c]	4.9 ± 0.3[c,d]	3.9 ± 0.6[a]
Gulf toadfish	0.1 ± 0.01[a,b]	0.1 ± 0.04[b]	0.04 ± 0.009[a]	2.1 ± 0.1[a]	0.1 ± 0.01[e]	0.01 ± 0.007[d,e]	0.007 ± 0.003[c,d]	1.2 ± 0.3[c]	3.9 ± 0.2[c,d]	0.6 ± 0.4[d,e]
Sheepshead	0.03 ± 0.003[c]	1.6 ± 0.09[a]	0.001 ± 0.0009[b]	0.5 ± 0.02[b]	0.1 ± 0.01[e,f]	0.07 ± 0.01[a,b,c,d]	0.3 ± 0.03[b]	1.5 ± 0.09[c]	4.3 ± 0.1[c,d]	3.5 ± 0.7[a]
Spot	0.01 ± 0.002[c]	0.8 ± 0.04[a]	0.0004 ± 0.0002[b]	0.2 ± 0.01[c]	0.04 ± 0.002[f]	0.04 ± 0.01[b,c,d,e]	0.2 ± 0.06[b,c]	0.9 ± 0.08[c]	2.4 ± 0.1[d,e]	6.0 ± 1.1[a]
Pigfish	0.04 ± 0.005[c]	1.8 ± 0.2[a]	0.01 ± 0.007[a,b]	0.3 ± 0.06[b,c]	0.2 ± 0.04[d,e]	0.03 ± 0.01[c,d,e]	0.06 ± 0.02[c,d]	2.2 ± 0.1[b,c]	5.0 ± 0.4[c,d]	6.8 ± 2.2[a]
Mullet	0.01 ± 0.001[c,d]	0.9 ± 0.1[a]	0.001 ± 0.0005[b]	0.3 ± 0.05[b,c]	0.1 ± 0.01[e,f]	0.002 ± 0.0006[e]	0.01 ± 0.008[c,d]	1.3 ± 0.1[c]	2.7 ± 0.4[d,e]	4.2 ± 0.6[a]
Ladyfish	0.01 ± 0.002[c,d]	2.4 ± 0.1[a]	0.005 ± 0.001[a,b]	0.9 ± 0.1[b]	0.2 ± 0.02[d,e]	0.03 ± 0.01[c,d,e]	0.7 ± 0.07[a]	2.7 ± 0.5[a,b,c]	7.2 ± 0.8[a,b,c]	3.5 ± 1.0[a,b]
Spotted sea trout	0.02 ± 0.001[c]	1.9 ± 0.08[a]	0.002 ± 0.00[b]	0.6 ± 0.09[b]	0.09 ± 0.01[e,f]	0.02 ± 0.002[d,e]	0.2 ± 0.05[b,c]	2.8 ± 0.1[a,b]	5.8 ± 0.2[b,c]	5.0 ± 1.7[a]
All species	0.04 + 0.00	1.5 + 0.05	0.02 + 0.00	0.8 + 0.03	0.2 + 0.01	0.05 + 0.00	0.2 + 0.02	1.9 + 0.1	4.6 + 0.1	4.2+2.1
Managed diet										
Icelandic capelin	0.09 ± 0.02[b,c]	2.5 ± 0.2[a]	0.003 ± 0.001[a,b]	2.0 ± 0.2[a]	1.6 ± 0.1[b]	0.02 ± 0.004[d,e]	0.1 ± 0.02[c,d]	2.0 ± 0.09[c]	8.5 ± 0.7[a,b]	1.1 ± 0.2[c,d]
Canadian capelin	0.20 ± 0.03[a]	1.4 ± 0.2[a]	0.003 ± 0.003[a,b]	2.7 ± 0.2[a]	3.7 ± 0.4[a]	0.01 ± 0.003[d,e]	0.0002 ± 0.0002[d]	2.2 ± 0.1[b,c]	10.5 ± 0.9[a]	0.8 ± 0.1[c,d]
Pacific herring	0.001 ± 0.001[e]	1.3 ± 0.05[a]	BLD¥	0.8 ± 0.03[b]	0.2 ± 0.02[d,e]	0.0006 ± 0.0004[e]	0.0008 ± 0.0003[d]	1.5 ± 0.1[c]	3.9 ± 0.1[c,d]	1.7 ± 0.3[b,c]
Atlantic herring	0.0004 ± 0.0002[e]	1.4 ± 0.08[a]	0.003 ± 0.002[a,b]	1.0 ± 0.04[b]	0.2 ± 0.02[d,e]	0.002 ± 0.001[e]	0.003 ± 0.00[d]	1.6 ± 0.1[c]	4.4 ± 0.2[c,d]	1.4 ± 0.2[c]
Pacific mackerel	0.03 + 0.002[c]	2.7 + 0.4[a]	0.004 ± 0.0008[a,b]	1.4 + 0.2[a,b]	1.3 ± +0.1[b,c]	0.1 ± 0.01[a]	0.02 + 0.006[c,d]	4.4 + 0.2[a]	10.1 + 0.6[a]	3.3 ± 1.3[a,b,c]
Pacific sardine	0.002 ± 0.0008[d,e]	2.1 ± 0.2[a]	0.01 ± 0.007[a,b]	0.7 ± 0.06[b]	0.5 ± 0.06[c,d]	0.06 ± 0.01[b,c,d]	0.03 ± 0.01[c,d]	2.6 ± 0.2[b,c]	6.1 ± 0.5[a,b,c]	3.3 ± 0.4[a,b]
Loligo squid	0.002 ± 0.0007[d,e]	0.01 ± 0.008[b]	0.01 ± 0.004[a,b]	2.6 ± 0.1[a]	0.8 ± 0.05[c]	0.1 ± 0.009[a,b]	0.03±0.01[c,d]	0.7 ± 0.04[c]	4.4 ± 0.2[c,d]	0.3 ± 0.0[e]
All species	0.06 ± 0.01	1.7 ± 0.09	0.01 ± 0.00	1.7 ± 0.08	1.2 ± 0.01	0.07 ± 0.00	0.03 ± 0.00	2.1 ± 0.1	6.9 ± 0.2	1.7 ± 1.2

*Values are means ± 1 standard deviation for each species ($n = 5$) or ± 1 standard error for all species within each diet group ($n = 40$ for all free-ranging diet species; $n = 35$ for all managed diet species). HXA = hypoxanthine, AMP = adenine monophosphate, IMP = inosine monophosphate, and INO = inosine.

[a, b, c, d, e, f, g] Values with different superscripts within a column are significantly different ($p \leq 0.05$).

£Free-ranging diet species are listed in order of greatest to least contribution to the total energy content of the diet.

†Managed diet species contain more purine metabolite than free-ranging diet species ($p \leq 0.05$).

‡Free-ranging diet species contain more IMP and had a greater ratio of IMP+INO to HXA than managed diet species ($p \leq 0.05$).

¥BLD = below limit of detection

#Total purine content obtained by summing the concentrations of all eight measured metabolites.

Table 3. Purine metabolite* concentrations and relevant ratio for model diets consumed by managed and free-ranging dolphins

Purine metabolite (mmol/Mcal)	Managed model diet #1	Managed model diet #2	Free-ranging model diet
AMP	0.04 ± 0.00[a]	0.04 ± 0.00[a]	0.05 ± 0.00[a]
IMP	0.07 ± 0.02[a]	0.01 ± 0.00[b]	0.15 ± 0.01[c]
Adenine	0.06 ± 0.01[a]	0.14 ± 0.02[b]	0.05 ± 0.00[a]
Guanine	2.01 ± 0.16[a]	1.63 ± 0.11[a]	1.09 ± 0.05[b]
Inosine	1.98 ± 0.06[a, b]	2.19 ± 0.08[a]	1.61 ± 0.10[b]
HXA	1.70 ± 0.13[a]	1.92 ± 0.15[a]	0.91 ± 0.05[b]
Xanthine	1.14 ± 0.08[a]	2.06 ± 0.22[b]	0.14 ± 0.01[c]
Uric acid	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.02 ± 0.00[b]
IMP+Inosine: HXA	1.42 ± 0.09[a]	1.53 ± 0.07[a]	3.36 ± 0.12[b]
Total purines, 4 metabolites[†]	4.92 ± 0.35[a]	5.74 ± 0.41[a]	2.20 ± 0.09[b]
Total purines, 8 metabolites[‡]	7.02 ± 0.38[a]	7.98 ± 0.46[a]	4.03 ± 0.13[b]

*Values are means ± standard error. Abbreviations for purine metabolites: HXA = hypoxanthine, AMP = adenine monophosphate, and IMP = inosine monophosphate.

[a, b, c]Purine concentrations with different superscripts across rows are different among model diets ($p \leq 0.05$).

[†]Total purine content obtained by summing the content of Adenine+Guanine+HXA+Xanthine

[‡]Total purine content obtained by summing the content of all eight metabolites measured herein

which contained almost no guanine. Icelandic capelin, Canadian capelin, and Loligo squid among managed diet species and Gulf toadfish of free-ranging species contained the most hypoxanthine. The sum of IMP and inosine relative to hypoxanthine concentrations differed ($p \leq 0.05$) among free-ranging and managed diet species (Table 2). On average, free-ranging species had a 2.5 fold greater ($p < 0.05$) ratio of IMP and inosine to hypoxanthine, except for Gulf toadfish, which had a lower ratio than all other free-ranging fish because of its greater hypoxanthine concentration.

The total purine content was greater ($p \leq 0.05$) for managed diet species when compared with free-ranging diet species, but also varied among individual fish species ($p \leq 0.05$) within each group (Table 2). Among managed diet species, total purine content was greatest in Canadian capelin and Pacific mackerel, and three-fold more than in herring and Loligo squid, which contained the least purines. Of the free-ranging diet species, the total purine content of ladyfish was 2.7 to 3 times greater than the total purine content of spot and mullet.

The mean total purine content of model diets was approximately 1.25 to 1.5 times greater when the total included eight rather than four purine metabolites (Table 3). The mean concentration of a total of eight purine metabolites in the managed model diets (7.02 and 7.98 mmol/Mcal ME, respectively) was approximately twice that in the free-ranging model diet (4.03 mmol/Mcal ME; $p \leq 0.0001$; Table 3). The two managed model diets had similar individual purine metabolite contents except that managed model diet #1 contained more IMP and less adenine and xanthine than managed model diet #2. The free-ranging model diet contained more IMP but less guanine, inosine, hypoxanthine, and xanthine than the managed model diets. The sum of IMP and inosine relative to hypoxanthine concentrations was 2.2 to 2.4 times greater for the free-ranging model diet when compared with managed model diets ($p \leq 0.0001$; Table 3).

Discussion

To our knowledge, this study is the first to quantify eight purine metabolites in a wide range of fish species consumed by dolphins and to estimate the difference in total purine intake of managed and free-ranging dolphins. Our results suggest that managed dolphins consuming diets similar to these two model diets would consume almost twice the concentration of purines compared to the diet of free-ranging dolphins in Sarasota Bay. This may explain why some managed dolphins may be more predisposed to ammonium urate nephrolithiasis than free-ranging dolphins.

We have previously shown that the managed model diets likely have a more negative DCAD than the free-ranging model diet, depending on relative intestinal mineral absorption (Ardente et al., 2017). In other mammals, consumption of a diet with a more negative DCAD results in the excretion of more protons and ammonium ions in urine (Ender & Dishington, 1970; Kealy et al., 1993; Block, 1994; Remer & Manz, 1995). Ammonium urate will have a tendency to precipitate in urine when the product of ammonium and urate ions exceeds the solubility product constant of ammonium urate (Marshall & Robertson, 1976). Above this constant, crystal formation and aggregation may be impeded by inhibitors, ionic forces, and urine flow; however, crystals can form spontaneously if urine becomes even more concentrated. Factors that affect the formation product at which ammonium urate precipitates in human urine include urine dilution, pH, and the presence of a nidus of ammonium urate (Bowyer et al., 1979). Thus, in managed dolphins, increased ammonium ions from a more negative DCAD diet and increased urate ions from consumption of a higher purine diet may together increase the risk of ammonium urate precipitation in urine and explain why the prevalence of ammonium urate nephroliths in some managed dolphins may be higher than in free-ranging dolphins.

It may be possible to reduce the uricogenic potential of managed dolphin diets by feeding species that have a lower total purine content. The purine content was determined relative to ME because the quantity of fish consumed each day is determined by the energy dolphins need to maintain body condition (Ardente & Hill, 2015; Ardente et al., 2017). Fish with the highest ME density (e.g., mullet, spot, and herring) contained the least purines relative to ME and could be substituted for species in the managed dolphin diet that are less energy dense and contain more purines.

Nevertheless, the uricogenic potential of dietary purines in dolphins is unknown. Many mammals are able to convert uric acid to allantoin with the enzyme uricase, primarily in the liver. In rats, for example, dietary hypoxanthine and xanthine result in greater allantoin concentrations in the urine, but uric acid concentrations remain unchanged (Brulé et al., 1988). On the other hand, human beings are unable to convert uric acid to allantoin because they lack functional uricase and, therefore, excrete more urinary uric acid when fed a diet supplemented with adenine, hypoxanthine, AMP, and IMP, but not when fed a diet supplemented with guanine (Clifford et al., 1976; Brulé et al., 1992). The purine metabolic pathway of dolphins has not been investigated; therefore, it is unknown how efficiently dolphins absorb, metabolize, or excrete purine metabolites; how efficiently allantoin is synthesized from uric acid; what role their reniculated kidney may play; and how long it may take for stones to develop and cause clinical disease (Cave & Aumonier, 1962). Thus, the relative contribution of each purine metabolite to the production of urinary uric acid in urine is unknown, and any one of the metabolites may be important in ammonium urate stone development in dolphins.

Inosine is not typically measured in food but was present on average in greater concentrations than any of the other purine metabolites. The gastrointestinal epithelial absorption of inosine has been reported to be saturable in rats because luminal inosine concentrations increased with increasing oral doses of AMP. Thus, very high concentrations of dietary inosine may be inconsequential, but inosine is readily converted to hypoxanthine during frozen storage, so the uricogenic potential of inosine should not be discounted (Salati et al., 1984).

Guanine concentrations were also greater than most other metabolites in most species, probably because metallic scales are rich in guanine (Sumner, 1944; Choi et al., 2004). Gulf toadfish and Loligo squid, which lack metallic scales, were the only two species which contained lower amounts of guanine. Guanine is not very uricogenic in people, but if it is absorbed from the dolphins' gastrointestinal tract and converted to uric acid, Loligo squid or toadfish could be fed to dolphins in greater proportions to decrease the total guanine content and uricogenic potential of the diet.

As expected, handling and frozen storage appeared to affect concentrations of purine metabolites in the managed diet species compared with the free-ranging diet species (Aubourg et al., 2007). Concentrations of IMP have been reported to be greater in fresh fish and to decrease over time, degrading to inosine and then hypoxanthine during chilled storage (Ryder, 1985). In this study, concentrations of IMP were very small in both groups of fish, either because there was little in the first place or because both groups had undergone some degree of freezing and thawing. IMP concentrations, however, were much less in managed species which had undergone frozen storage at -20° C and then were completely thawed when compared to free-ranging fish which were immediately frozen at -80° C and minimally thawed.

Concentrations of both inosine and hypoxanthine were substantial, but concentrations of hypoxanthine in managed diet species were on average twice that in free-ranging diet species, whereas concentrations of inosine were only slightly greater among managed species than free-ranging species. This finding suggests that purine metabolite degradation had progressed further in frozen stored species than in freshly frozen fish. Ongoing purine metabolite degradation during frozen storage may impact the uricogenic potential of the fish because hypoxanthine has been shown to be more uricogenic in people when compared with IMP and inosine (Clifford et al., 1976).

Among managed diet species, Canadian and Icelandic capelin contained twice as much hypoxanthine as Atlantic and Pacific herring and mullet. Thus, it may be possible to decrease urinary uric acid excretion in managed dolphins by replacing capelin, which often represents a large proportion of the managed diet, with herring. Storing fish for less than 6 mo may also reduce hypoxanthine intake. Even if all of the inosine in free-ranging fish is converted to hypoxanthine during frozen storage, the hypoxanthine concentration should not exceed the combined concentrations of inosine and hypoxanthine. When the sum of inosine and hypoxanthine concentrations are compared among free-ranging fish species, it appears that substituting mullet for capelin would reduce the concentration of hypoxanthine and uricogenic potential of the diet.

There were two species in which purine metabolite concentrations did not follow the same pattern as that observed in other species within their group. Ladyfish had at least twice the IMP content of all other species. They fought vigorously when caught and died more rapidly compared to other free-ranging species—often these fish appeared dead before being euthanized. During supramaximal anaerobic activity, an additional ATP and AMP can be generated from two adenosine diphosphate molecules, whereupon AMP is broken down to IMP, inosine, and eventually hypoxanthine (Voet & Voet, 2011). Vigorous muscle movements in the ladyfish may have utilized more ATP, therefore generating more IMP. Rapid freezing may then have prevented further metabolism. Gulf toadfish also differed because it contained hypoxanthine at a concentration greater than any other free-ranging species and comparable to that contained in capelin, the managed diet species with the greatest hypoxanthine content. Toadfish were the only fish caught in crab pots by local commercial crabbers. The fish were then transported live back to shore in 18.9-liter buckets of sea water, where they were processed like the other fish. It is possible that the stress of being contained in a bucket of water with no supplemental dissolved oxygen resulted in hypoxemia, increased utilization of ATP, and generation of more hypoxanthine (Marklund et al., 2000).

This study has some limitations. All fish and squid species were pooled and ground for analysis, and there was an inherent heterogeneity of the samples of some species because whole intact fish, composed of diverse tissues, were ground. This was particularly true for some of the smaller, bonier species like pinfish, where it was challenging to ensure ground sample was well-homogenized. Furthermore, free-ranging species varied in size and sex based on availability, whereas commercially caught fish species were sorted for uniform size and sex. This may have led to more variability among free-ranging species compared to managed species. Seasonal variations in protein and fat have been reported to occur in other species. All species in this study, from both diets, were caught during one season—during the required commercial season for managed species and during the summer months for free-ranging species; therefore, seasonal and regional variations not accounted for in this study may affect protein and possibly purine content (Henderson et al., 1984; Nunes et al., 1992; Vollenweider et al., 2011). For the managed diet species, the duration of frozen storage was fixed at 6 to 9 mo to represent the typical duration that fish fed to dolphins are stored prior to feeding. Frozen storage has been well-documented to affect the nutrient content of fish, so it is possible that storage times less than 6 mo or greater than 9 mo may have yielded different results (Ackman et al., 1969).

The percentages of each species included in the model diets may not be representative of the diets consumed by all managed or free-ranging dolphins. The relative proportions, storage, and handling of fish and squid varies within and among facilities, depending on the requirements of individual dolphins and management preferences. The free-ranging model diet was based on published information from one population of free-ranging inshore dolphins residing in the Sarasota Bay region of Florida. The species consumed by these and other dolphin populations changes with geographical location, season, habitat (inshore vs pelagic), individual prey preferences, age, sex, reproductive state, and overall health.

Direct comparisons of the model diets also assumes that any dolphin, whether free-ranging or under human care, is consuming the same total number of calories in a day. In reality, there is great variation in the energy needs of any individual or group of dolphins depending on activity levels, water temperature, and life stage. Preliminary data suggest that free-ranging dolphins may have higher energy requirements than managed dolphins. An average 160-kg free-ranging dolphin in Sarasota Bay has been reported to have an average daily energy requirement ranging from approximately 16 Mcal/d in the winter to 22 Mcal/d in the summer (Costa et al., 2013). Among dolphins under human care at one facility, however, nonpregnant, nonlactating adults have been reported to consume approximately 8.5 to 12 Mcal/d, and growing male and female dolphins to consume approximately 8.5 to 16 Mcal/d (Reddy et al., 1994). Purine intake is ultimately affected by the amount and type of food consumed at one feeding and over the course of 1 d. Free-ranging dolphins, therefore, may be consuming, metabolizing, and excreting more purines than some managed dolphins, even when the free-ranging diet contains less purines than the managed diets on an equal caloric basis. Variations in facility fish storage, processing methods, and supplementation practices, including vitamin and mineral supplementation or hydration practices, may also mitigate the effects of purine intake and DCAD on urolith formation in managed dolphins.

In conclusion, the individual purine metabolites differ significantly between the fish and squid species fed to dolphins under human care and the fish species consumed by free-ranging dolphins. Additionally, the managed model diets have a greater total purine content than the free-ranging model diet. The differences in both individual and total purine concentrations may contribute to the development of ammonium urate nephrolithiasis in managed dolphins; therefore, it is theoretically possible to decrease the purine content of the diet by altering the species and proportions of species fed to managed dolphins. Further investigation is necessary to determine the uricogenic potential of individual purine metabolites in dolphins and whether consumption of a lower total purine content diet, similar to the free-ranging diet examined in this study, would discourage ammonium urate stone formation in managed dolphins.

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