INVESTIGATION OF THE ROLE NUTRITION PLAYS IN THE DEVELOPMENT OF AMMONIUM URATE NEPHROLITHIASIS IN COMMON BOTTLENOSE DOLPHINS, *TURSIOPS TRUNCATUS*

By

AMANDA J. ARDENTE

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2016

To my love and partner, my comic relief and punching bag, my biggest fan and greatest supporter, my comfort and my home
~ Dr. Andrew Smith ~

ACKNOWLEDGMENTS

I have been blessed with an incredibly supportive network of family, friends, and mentors, all of whom have helped to make this journey possible.

I would like to first thank my primary advisor Dr. Richard Hill, for stepping outside of his comfort zone to work with me on this bottlenose dolphin nutrition research. He has taught me the importance of rigorous experimental design and attention to detail in research, but above all, he has encouraged me to slow down, have patience, and allow time for the thought process to develop. I cannot thank Dr. Hill enough for all of the time and energy he has devoted to mentoring me and assisting me with my research, and for his constant encouragement and support, particularly as I neared the end of this long road.

I also would like to thank the University of Florida (UF) Aquatic Animal Health program and Drs. Ruth Francis-Floyd, Iske Larkin, and Michael Walsh, for providing me with this opportunity to further my graduate education. To Dr. Ammon Peck and Mrs. Sally O'Connell in the Office of Graduate Studies and Research at the University of Florida College of Veterinary medicine – I thank you for working to negotiate the logistics of my program and for providing me with the support needed to see my dissertation research through to completion. I am very grateful for the efforts and support of all of these individuals.

Furthermore, I would like to thank each of my committee members, Drs. Michael Walsh, Jesse Gregory, Randall Wells, and Cynthia Smith. Dr. Walsh fostered my interest in nutrition from the very beginning by providing me with opportunities to learn about dolphin management in the field. He also connected me with the professional contacts necessary to make my interest in dolphin related nutrition research a reality.

Dr. Walsh served on my committee and has been one of my biggest advocates – I have benefited greatly from his expertise, encouragement, and enduring optimism. Dr. Gregory introduced me to the topic of food chemistry and taught me analytical methods useful for my future career in nutrition. I am thankful that he was willing to be a part of my program, offering his invaluable knowledge and expertise from the human nutrition and food science perspective. Dr. Wells was instrumental in providing me with opportunities for sample collection with the Chicago Zoological Society Sarasota Dolphin Research Program. Right from the start, he was on-board and committed to assisting me with my dissertation work, and I am grateful for the time he has spent providing me with his expertise both in free-ranging bottlenose dolphin biology and, more generally, in the field of marine mammals. Last, but certainly not least, without the support of Dr. Smith, this very relevant dolphin nutrition research would not have been possible. Every step of the way, Dr. Smith advocated for my involvement in her kidney stone research, offering her time and attention when able to discuss my work and providing me with opportunities to network with others and obtain financial support. Beyond her assistance with my graduate program, Dr. Smith has demonstrated an unwavering belief in my abilities as a future zoo and aquatic animal nutritionist, offering me guidance, encouragement, and support as I continue to pursue my intended career.

In addition to Dr. Smith, I would also like to thank Drs. Sam Ridgway and Stephanie Venn-Watson from the National Marine Mammal Foundation and Dr. Eric Jensen from the United States Navy Marine Mammal Program. These individuals are experts in the field of bottlenose dolphin health and management, and I have been

grateful for and humbled by their belief in me and commitment to supporting this research.

I also would like to recognize the efforts of my honorary mentor, Dr. Timothy Garrett of the UF Southeast Center for Integrated Metabolomics. Dr. Garrett assisted me with the development of the very finicky assays measuring fish and urine purines. Over the course of 3 years, he taught me more than I ever thought I wanted to know about chromatography. He was patient and so gracious with his time, spending countless hours helping to troubleshoot the method – answering my questions at all hours of the days, nights, and even weekends. I cannot thank Dr. Garrett enough for the time and energy he devoted to assisting with my research, and I thoroughly enjoyed being a part of his laboratory team.

Lastly, I must thank James Colee, the statistician from the UF Institute of Food and Agricultural Sciences and Statistics departments. James assisted me with all of the statistical analyses utilized in my data analysis. He was patient with my questions and took the time to explain and teach me various statistical methods. James was an invaluable resource, and I am very appreciative of this time and assistance.

Many individuals at North Carolina State University's College of Veterinary Medicine also touched my life, both personally and professionally, and helped me get to where I am today. In particular, however, I would like to thank Dr. Korinn Saker, my mentor and dear friend. Her belief in me was unfaltering – Dr. Saker created an internship tailored to my interests, and with her guidance, support, and experience I was able to combine my two passions of zoo medicine and nutrition, making this career path a reality. I could not have done this with you, Saker.

Lastly, I would like to thank my parents Chris and Dan Ardente, and my brother Adam. It has been a long road, from Wilson College, through veterinary college, and graduate school - I am so grateful for the love and encouragement they have always shown me and for their belief in my ability to achieve anything I put my mind to. I also could not have achieved this accomplishment without the patient, understanding, and loving support of my partner, Andrew Smith. He helped to pull me through this experience, no doubt – sharing in my successes but also bearing the brunt of my stress and drying my tears of frustration. Andrew encouraged me to push forward and preserve and kept me focused on the end result. Finally, I would like to thank my dog Fancy, for being my running buddy and my source of entertainment when I was tired of reading or writing, and my old girl Maddie, for seeing me through several moves, veterinary school, and this graduate program, for lending her ear when I needed to vent, and for her snuggles when it was time to procrastinate.

TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AMP	Adenosine monophosphate
APRT	Adenine phosphoribosyltransferase
ATP	Adenosine 5’-triphosphate
BBMV	Brush border membrane vesicles
BMR	Basal metabolic rate
Ca	Calcium
CF	Crude fat
Cl	Chloride
CNT	Concentrative nucleotide transporter
CP	Crude protein
CT	Computed tomography
DCAD	Dietary cation-anion difference
DM	Dry matter
DNA	Deoxyribonucleic acid
ENT	Equilibrative nucleoside transporter
FMR	Field metabolic rate
GE	Gross energy
GFR	Glomerular filtration rate
GLUT1	Glucose transporter 1
GMP	Guanosine monophosphate
GTP	Guanosine 5’-triphosphate
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase
HPLC	High-performance liquid chromatography
IACUC	Institutional Animal Care and Use Committee

IMP	Inosine monophosphate
K	Potassium
LLC-PK1	Renal epithelial cell protein kinase transporter 1
Mcal	Megacalorie (1000 kilocalories = 1 Megacalorie)
ME	Metabolizable energy
Mg	Magnesium
MMP	United States Navy Marine Mammal Program
MMPA	Marine Mammal Protection Act
MS/MS	Tandem mass spectrometry
Na	Sodium
NMMF	National Marine Mammal Foundation
P	Phosphorous
PRPP	Phosphoribosyl pyrophosphate
RMR	Resting metabolic rate
RNA	Ribonucleic acid
rSNBT1	Rat sodium-dependent nucleobase transporter 1
S	Sulfur
SLC	Solute carrier family transporter
SNBT	Sodium-dependent nucleobase transporter 1
SRM	Selected reaction monitoring
URAT1	Urate transporter 1
XMP	Xanthosine monophosphate

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

INVESTIGATION OF THE ROLE NUTRITION PLAYS IN THE DEVELOPMENT OF AMMONIUM URATE NEPHROLITHIASIS IN COMMON BOTTLENOSE DOLPHINS, *TURSIOPS TRUNCATUS*

By

Amanda J. Ardente

August 2016

Chair: Richard C. Hill
Major: Veterinary Medical Sciences

Some common bottlenose dolphins (*Tursiops truncatus*) under human care form ammonium urate nephroliths, whereas free-ranging dolphins typically do not. The purine-rich whole fish diet of bottlenose dolphins may influence urate urolith formation in dolphins, as it does in other mammals. Although both groups of dolphins consume a similar diet, free-ranging dolphins consume a variety of live inshore temperate water species, whereas managed dolphins are fed cold water, frozen and thawed species. Macronutrient and total purine content were measured relative to metabolizable energy in fresh frozen samples of eight fish species commonly consumed by free-ranging dolphins and stored frozen samples of seven species (n=5) commonly fed to managed dolphins. Two model managed dolphin diets and a model free-ranging dolphin diet were also generated to compare the total nutrient intake of dolphin populations. Metabolizable energy was calculated using Atwater factors, and dietary cation-anion difference (DCAD) was calculated. Eight purine metabolite concentrations and total purine content were measured for individual fish and squid species and for the model diets using a newly developed assay. Urine purine concentrations, including allantoin

and uric acid, were also measured and compared between free-ranging dolphins and managed dolphins, considering the effect of feeding and presence of nephrolithiasis. Protein, fat, mineral and water contents differed among fish and squid species and between species groups, and total purine content was greater in two model diets typically fed to managed dolphins than the model free-ranging dolphin diet ($p \leq 0.05$). Mean DCAD was more positive for the model free-ranging diet than for both model managed dolphin diets, the extent of which depended on the relative mineral absorptions. Both free-ranging and managed dolphins had urinary allantoin concentrations comparable to other mammals, but a post-prandial increase in uric acid relative to allantoin concentrations in the urine of managed dolphins suggests that the conversion of uric acid to allantoin may be limited after consumption of a large purine-rich fish meal. The differences in nutrient composition of the model managed and free-ranging dolphin diets and the potential limited capacity for purine metabolism in dolphins may promote ammonium urate stone development in dolphins under human care.

CHAPTER 1
INTRODUCTION

Problem Statement

Ammonium urate kidney stones develop in common bottlenose dolphins under human care, *Tursiops truncatus*, but do not typically develop in free-ranging bottlenose dolphins. Kidney stones in managed dolphins may result in azotemia, hydronephrosis, ureteral and urethral obstruction, and renal failure.[1-3] The United States Navy Marine Mammal Program (MMP) and the National Marine Mammal Foundation (NMMF) have reported a prevalence as high as 35% in managed bottlenose dolphins, after thorough investigation using ultrasound and computed tomographic (CT) imaging.[1]

The cause of ammonium urate stones in dolphins under human care is unclear. In other mammals, urate urolith development has been attributed to the nutrient composition of the diet, genetic derangements in purine metabolism, dehydration, and/or underlying disease such as obesity, inflammatory bowel disease, or compromised liver function.[4-8] Thus, there are likely several contributing factors to the development of ammonium urate urolithiasis in dolphins, but this research focused on two potential factors: nutrient differences between the free-ranging dolphin diet and the diet fed to dolphins under human care, and purine metabolic byproducts excreted in the urine of both dolphin populations.

Aims and Objectives

The first aim was to compare the nutrient content of the fish and squid commonly fed to bottlenose dolphins under human care with that of fish commonly consumed by free-ranging bottlenose dolphins. Thus, our first objective was to analyze fish species for gross energy content, proximate analysis, and macromineral content. We

hypothesized that the nutrient content would differ relative to energy among fish species analyzed and between managed and free-ranging dolphin model diets. Our second objective was to determine the purine metabolite concentrations in the fish and squid species. Our hypothesis was that the individual purine metabolite content and total purine content would differ on an energy basis among the fish species and between the representative free-ranging dolphin model diet and the model diets for dolphins under human care.

The second aim of this research was to determine the purine metabolite concentrations present in the urine of free-ranging bottlenose dolphins and dolphins under human care. The first objective was to determine whether uric acid or allantoin is the primary end-product of purine metabolism excreted in the urine of managed and free-ranging bottlenose dolphins. The primary end-product is species-dependent and has not been investigated in bottlenose dolphins. Thus, we hypothesize that both populations of bottlenose dolphins, like most other mammals, will produce primarily allantoin. The second objective was to compare urine purine metabolite concentrations after a meal between free-ranging bottlenose dolphins and dolphins under human care. We hypothesized that both populations of dolphins will excrete similar post-prandial concentrations of urine purine metabolites. The third objective was to investigate how urine purine metabolite concentrations change following a meal in healthy managed bottlenose dolphins. We hypothesized that bottlenose dolphins under human care experience a post-prandial rise in excreted purine metabolites. Finally, the fourth objective was to determine whether urine purine metabolite concentrations differ between fed and unfed bottlenose dolphins under human care with or without kidney

stones. Our hypothesis was that there would be a greater rise in urine purine metabolite concentrations following a meal in dolphins with kidney stones than in those without stones.

CHAPTER 2
LITERATURE REVIEW

Ammonium Urate Stones

Pathophysiology of Stone Formation

Uroliths, or stones in the urinary tract, form when urine becomes supersaturated. Urate and ammonium ions are the two primary solutes contributing to ammonium urate stone formation, and their solubility in urine depends on their individual relative concentrations, as well as the concentration of other solutes in the urine and urine pH. The risk of urate and ammonium ions combining to form crystals increases as the total solute concentration increases; however, the concentration of ammonium ions necessary to produce precipitation decreases from 150 mM to 10 mM when a suspension of ammonium urate is present.[9] The solubility of urate and ammonium decreases as pH decreases, but uric acid stones form below a urine pH of 6.0 and ammonium urate stones form above 6.0. Given enough time and appropriate conditions, crystals will aggregate to form ammonium urate stones.[10-12]

Urate, or uric acid, is produced solely by purine metabolism and found in blood and tissues. Two-thirds of the uric acid produced by the human body is excreted in the urine and the remainder is excreted in the feces.[13] The production and excretion of uric acid is markedly influenced by the diet.[14-16] Humans consuming high purine-containing foods have accumulations of uric acid in the blood and urine, and its solubility is partly pH dependent. Urate is a weak acid with a pKa of 5.4 and is more soluble in the blood and tissues when the pH is approximately 7.4; however, the pH range in urine is dynamic, generally varying from 4.7 to 8.0, so the solubility and subsequent fate of urate

in the urine fluctuates.[16, 17] Alkaline urine promotes tubular reabsorption of soluble urate, whereas acidic urine promotes excretion of insoluble uric acid.[13]

Ammonium ions are released from glutamine by glutaminase and glutamate dehydrogenase in the renal proximal convoluted tubule in response to an acidic urine, which can be a consequence of a high-protein diet. The kidney, therefore, assists in maintaining acid-base balance by buffering acidic urine with ammonium ions.[18, 19] Ammonium ions can also be generated from urea by urease-producing bacteria when a urinary tract infection is present.[20, 21]

Other solutes that are consumed with the diet and contribute to urine supersaturation are sodium, potassium, calcium, magnesium, chloride, sulfate, phosphate, citrate, and oxalate.[10] Thus, the solubility of ammonium ions and urate can be predicted by calculating the total ionic concentrations of these solutes.[22, 23]

The concentration of total solutes in urine is also dependent on the amount of free water excreted by the kidney. The water excreted in the urine can come from water consumed, either by drinking or eating, or generated from nutrient metabolism.[24] Solutes, including dietary protein and sodium, can also influence water excretion.[25] Diets rich in protein result in increased renal blood flow, glomerular hyperfiltration, and increased creatinine and urea excretion, accompanied by increased net water excretion.[26-28] Similarly, excess dietary sodium is excreted in the urine, which causes an osmotic diuresis with an additional loss of free water.[29]

Ammonium urate stone development will be favored in a supersaturated urine given a specific pH range of 6.0 to 7.5, in which ammonium ions tend to combine with urate. The two factors that influence urine pH are dietary protein and mineral content.

Dietary protein causes an increase in net acid excretion by the kidney as sulfate, and to a lesser extent phosphate, found in amino acids are oxidized and organic acids are produced.[30, 31] Mineral concentrations in the urine also influence urine pH based on the relative concentrations of positively charged cations and negatively charged anions.[15, 32] The diet plays a major role in determining these urine electrolyte concentrations. The dietary cation-anion difference (DCAD), calculated as the difference between the sum of the cations (Na^{+} +Ca^{2+} +Mg^{2+} +K^{+}) and the sum of the anions (Cl^{-} +$P^{1.8-}$ +S^{2-}), can indicate the propensity of the diet to form acidic or alkaline urine.[33, 34] The valence for the phosphorous anion at a physiologic pH of 7.4 is 1.8, and the valence for the sulfur anion is 2- because it is released from the oxidation of dietary methionine and cysteine and excreted in the urine as sulfate.[33, 35] As the cation concentration increases in the diet, urine becomes more alkaline; whereas if the dietary anion concentration increases, urine becomes more acidic. Several equations have been used to calculate DCAD, and there does not seem to be consensus as to which equation is most appropriate. Two commonly reported equations that have been shown to have utility in several species are $DCAD_{long}$, which includes all of the aforementioned minerals, and $DCAD_{short}$, which includes only Na, K, Cl, and S.[33, 34, 36] $DCAD_{short}$ and $DCAD_{long}$ do not account, however, for apparent absorptions of the minerals from the gastrointestinal tract and assume each mineral has an equal influence over urine pH. Apparent absorptions for the various minerals are not likely to be equal and are probably species-dependent. For example, absorption coefficients derived from human studies have been used to calculate potential renal acid load (PRAL), or DCAD, in human beings.[37] The coefficients accounting for apparent absorption used in these equations for human beings are 95%

for sodium and chloride, 80% for potassium, 63% for phosphorous (as phosphate), 32% for magnesium, and 25% for calcium. Sulfate (SO_4^{2-}) absorption is based on 75% absorption of sulfur-containing amino acids. DCAD calculated using these absorption coefficients would favor the contribution of dietary anions over cations because of the lower absorption coefficients provided for calcium and magnesium compared to phosphorous and sulfur. Nevertheless, the apparent mineral absorptions differ for pure carnivores like cats. For example, the apparent absorptions of calcium and phosphorous vary depending on the mineral content of the diet; and, the apparent absorption of potassium in cats is 95%, compared to 80% in human beings. Thus, the extent to which minerals are absorbed by the gastrointestinal tract strongly influences any prediction of the effect of dietary mineral content on urine pH.

Stone Occurrence in Mammals

Ammonium urate stones in human beings rarely occur in industrialized countries, with a prevalence of 0.2%, but are endemic in developing countries.[5, 15, 38-48] In these countries, several contributing factors have been identified, including chronic diarrhea leading to volume depletion and electrolyte loss, urinary tract infections, and a nutritionally poor diet.[38] The diet characteristics linked to ammonium urate stone development vary depending upon the country. Some common factors include an acidogenic diet that is low in animal protein, calcium, and phosphorous, and high in cereals.[46] A purine-rich diet is considered another contributing factor for those countries where individuals consume more fish and shellfish.[47] Ammonium urate stone formation as reported in industrialized countries has been associated with inflammatory bowel disease, laxative abuse, obesity, and urease-producing urinary tract infections.[5]

In dogs and cats, ammonium urate is the most common form of urate-based stone. Genetic factors, diet, and underlying diseases contribute most significantly to their development and will be discussed further in the purine metabolism review.[8] Two species of otters, including the Asian small-clawed otter (*Amblonyx cinerea*) and the Eurasian otter (*Lutra lutra*), and two marine mammals, including a northern elephant seal (*Mirounga angustirostris*) and California sea lion (*Zalophus californianus*), have also been documented to have ammonium urate stones.[49-51] Common bottlenose dolphins are the only cetacean in which ammonium urate nephrolithiasis has been diagnosed and thoroughly investigated, but the presence of stones in the Pacific white-sided dolphin (*Lagenorhynchus obliquidens*) were also suspected to be urate-based.[3, 52] The cause of urate stone development in bottlenose dolphins has yet to be determined, but a thorough review of the disease in dolphins will follow.

Purine Biochemistry and Metabolism

Purine sources

Urate, one of the two primary solutes required for ammonium urate stone formation, is produced by purine metabolism. Purines, including nucleotides, nucleosides, and nucleobases, are organic planar, aromatic, heterocyclic molecules consisting of a pyrimidine ring and an imidazole ring (Table 2-1). They have many biologically essential roles, like contributing to the structure of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), adenosine 5'-triphosphate (ATP), and guanosine 5'-triphosphate (GTP).[16]

The nucleotide inosine monophosphate (IMP) is the first purine formed endogenously, either by *de novo* synthesis when in the fed state or by salvage when in the unfed state. *De novo* synthesis begins primarily in the liver with the production of 5-

phosphoribosyl-1-amine from phosphoribosyl pyrophosphate (PRPP) which is catalyzed by glutamine phosphoribosyl aminotransferase.[53] The concentration and availability of PRPP is therefore the rate-limiting factor of *de novo* synthesis.[54] IMP is then produced through a series of subsequent steps involving glycine, glutamine, aspartate, formate, and bicarbonate. IMP forms the nucleotide adenosine monophosphate (AMP) and also guanosine monophosphate (GMP) through an intermediate nucleotide xanthosine monophosphate (XMP) (Figure 2-1). When concentrations of these free nucleotides are elevated, *de novo* synthesis is feedback inhibited.[17, 55]

In the unfed state, nucleotides are produced through an energy-sparing process involving the salvage and recycling of purine nucleobases adenine, guanine, hypoxanthine, and xanthine.[53] The nucleobases are obtained either from the turnover of nucleotides or directly from the diet, and by recycling the nucleobases, the salvage pathway maintains tissue nucleotide pools when substrates for *de novo* synthesis are low.[55] Adenine is recycled directly to AMP by adenine phosphoribosyltransferase (APRT). Similarly, hypoxanthine can be directly recycled to IMP by hypoxanthine-guanine phosphoribosyltransferase (HGPRT). Guanine, however, first must be phosphorylated to its nucleoside guanosine, which is then catalyzed by HGPRT to GMP.[16, 53, 56]

Several factors influence how readily the salvage pathway is utilized for nucleotide synthesis, including species differences, tissue or cell types, and related conditions. For example, red and white blood cells in rabbits, mice, and human beings utilize salvage pathways to produce nucleotides because the cells have low ribose-5-phosphate concentrations, so *de novo* synthesis is not possible.[54] Nucleobases,

particularly hypoxanthine, are also recycled in muscle tissue of human beings. At rest, human beings recycle approximately 95% of the hypoxanthine produced daily either by *de novo* synthesis or nucleotide turnover for inosine and IMP production, while the remaining 5% of hypoxanthine is degraded to xanthine and subsequently excreted.[54, 55] Following high-intensity anaerobic exercise, plasma hypoxanthine concentrations are low while HGPRT activity and IMP concentrations are elevated. Hypoxanthine production increases acutely due to tissue hypoxia. As the muscle adapts and operates more aerobically, hypoxanthine production falls and HGPRT activity increases, indicating that hypoxanthine is being recycled to IMP for additional ATP production.[57, 58] Furthermore, in ruminants, the salvage pathway for nucleotide production is very active in the gastrointestinal tract due to large pools of nucleobases available from bacterial nucleic acid turnover.[54] In the gastrointestinal tract of rats, however, adenine recycling is favored over the other nucleobases and is further affected by feeding status. Unfed rats administered oral doses of individual purine nucleobases absorbed more adenine than guanine, hypoxanthine, and xanthine from their intestinal tract, compared with fed rats.[55]

An exogenous source of nucleotides is the diet. Nucleotides are considered 'conditionally essential' nutrients because they must be included in the diet in some conditions when *de novo* synthesis and salvage are insufficient to maintain nucleobase concentrations.[59] Digestion of nucleic acids occurs rapidly in the gastrointestinal tract: pancreatic nucleases split nucleotides from DNA and RNA, which are then hydrolyzed by nucleosidases and phosphatases into constituent nucleosides and absorbed into the blood by active transport in human beings and rats.[59-61]

Purine transport

Purines may be transported into and out of cells as nucleosides or nucleobases. Cellular transport of nucleosides is active and involves both low- and high-affinity systems.[56, 62] The high-affinity systems involve membrane-bound Na+-coupled nucleoside transporters, called either concentrative nucleoside transporters (CNT) or solute carrier family transporters (SLC). These include CNT1/SLC28A1, CNT2/SLC28A2, and CNT3/SLC28A3.[63] CNT2 and CNT3 actively transport purines into cells and have generalized tissue distributions that are species dependent. For example, CNT2 is not present in the rat kidney but is present in the human kidney. CNT help maintain circulating nucleoside homeostasis. Although most cells can generate purines by de novo synthesis, CNT receptors facilitate nucleoside salvage, thus conserving energy, needed for nucleotide production.[63] The low-affinity nucleoside transport systems involve equilibrative (bidirectional) nucleoside transporters (ENT) and include ENT1/SLC29A1, ENT2/SLC29A2, ENT3/SLC29A3, and ENT4/SLC29A4.[56, 64] These transporters are ubiquitous in all tissues, not only the gastrointestinal tract, and may vary in relative concentrations or membrane locations among tissues. For example, ENT1 operates on the tubular basilar membrane of the rat kidney, whereas CNT operates on the apical membrane; together these two transports can facilitate transepithelial cell transfer of nucleosides.[64] Once a nucleoside is phosphorylated to form a nucleotide, it is trapped within the cell and therefore must either be utilized, dephosphorylated by a 5'-nucleotidase for transport out of the cell, or metabolized to an end product.[16, 62, 65, 66]

Nucleobase transport in mammals is known to involve several species-specific Na+-dependent nucleobase transporters (SNBT). The saturable transporter

rSNBT1/SLC23A4, identified in the small intestine of the rat, permits the cellular uptake and release of guanine, hypoxanthine, and xanthine, but not of adenine.[56] Adenine likely has its own specific intestinal transporter that has not yet been described. In human beings and chimpanzees, the rSNBT1 transporter gene is defective, so nucleobase absorption through enterocytes is questionable. Nevertheless, functional analogs are present in zebrafish, chickens, mice, dogs, and horses. In addition to being species specific, some transporters are location and nucleobase specific. Uric acid is reabsorbed in the renal tubules by specific anion transporters including urate transporter 1, or URAT1/SLC22A12, on the apical surface, glucose transporter 1, or GLUT/SLC2A9, on both the apical and basilar surface; however, species differences for these transporters have not been identified.[67-70] Hypoxanthine utilizes Na+-dependent transporters that are also species and tissue location dependent, including the renal brush-border membrane vesicles (BBMV) in guinea pigs, the intestinal BBMVs in bovine calves, and the renal epithelial cell protein kinase 1 transporter (LLC-PK1) in pigs.[56, 71-73] The BBMVs and LLC-PK1 transporters have a greater affinity for hypoxanthine than the rSNBT1 transporter; nevertheless, as hypoxanthine intake increases, the rate of transport across all transporters increases, until saturation is reached. The bovine calf intestine has greater absorption in the proximal jejunum, whereas the rat intestine has greater absorption and rSNBT1 expression in the distal jejunum. The Na+-dependent transporters within the BBMV presumably are rSNBT1 analogs, thus, depending on the species, transporter analogs may be concentrated variably along the length of the intestine.[56] All these transporters are inhibited by high concentrations of all the nucleobases except adenine and guanine.[56]

Thus, the absorption of dietary purines through the gastrointestinal tract and the concentration of excreted metabolic end-products in the urine depend upon the type of purine being absorbed (nucleoside or nucleobase) and on species-specific transporter differences. For example, when adenosine, AMP, and hypoxanthine were injected intraluminally into the small intestine of rats, all three metabolites degraded within 15 minutes to primarily uric acid, which was recovered from the intestinal contents. Inosine, xanthine, and allantoin were recovered, however, from the intestinal tissue and portal vein as well as the intestinal contents.[61] The effect of orally administered adenine, guanine, hypoxanthine, xanthine, AMP, GMP and IMP was also assessed in human beings, and all metabolites, except for guanine and xanthine, caused an increase in urinary uric acid concentrations.[66]

The concentrations of ingested purines also affect absorption and subsequent excretion. As greater concentrations of purines are ingested, fewer metabolites are absorbed into portal circulation and more remain in the lumen contents, intestinal tissue, and excrement. In contrast, as lower concentrations of purines are ingested, more metabolite absorption occurs with less intestinal retention and excretion. For example, higher doses of oral hypoxanthine administered in rats resulted in less portal vein recovery, greater intestinal recovery, and greater fecal excretion of xanthine.[61] A similar effect was observed with greater doses of oral AMP, with inosine intestinal concentration increasing above all other metabolites. This elevation in intestinal inosine concentrations may signify that inosine transport or catabolism is saturable and may be a rate limiting step in the production of uric acid.[61]

The gut microflora and cellular absorption mechanisms also likely influence the fate of dietary purines once they enter the intestine. A study in rats showed that intravenous administration of purines produced greater metabolite concentrations in tissues than when the purines were given orally. Although limited information is available, it is suspected that gastrointestinal microflora may inhibit the processes that are required for intestinal cellular absorption and transport of purines, including dephosphorylation of nucleotides to nucleosides, potential oxidation of nucleosides to free bases prior to cellular uptake, and both passive diffusion and active carrier-mediated transport across intestinal brush-border cells.[16, 56]

Purine degradation

Purine degradation pathways differ depending upon the nucleotide. AMP is dephosphorylated to the nucleoside adenosine, which is then deaminated to the nucleoside inosine.[55, 74] Inosine can also be produced directly by the dephosphorylation of IMP. Inosine then is further degraded by purine nucleoside phosphorylase (PNP) to hypoxanthine, which is then oxidized by xanthine oxidase to the nucleobase xanthine. Guanine directly degrades to xanthine through deamination. It is at this point then that the pathways are common - xanthine is oxidized once more by xanthine oxidase to produce the waste product uric acid.[16, 56 75]

Purine metabolism occurs in all tissues and involves the action of several essential enzymes to reduce the purine to excretory end products. The extent to which purines are metabolized is based on the tissue location and concentration of degradative enzymes, and is species dependent. For example, dogs and cows have the greatest concentration of xanthine oxidase in the blood and lungs, whereas cats and humans have no xanthine oxidase in blood and the greatest concentration in the liver.[76]

There is also no xanthine oxidase in most human tissues, with the exception of the liver, so hypoxanthine is the final end product of purine metabolism in human tissues. Uric acid, however, is the final metabolic end product of purine metabolism in the human liver because xanthine oxidase is present.[65]

End-products of purine metabolism are excreted primarily in the urine, but also in the feces, and are species-dependent.[55, 56, 61, 77] Uric acid is the primary excretory end-product for human beings, non-human primates, insects, reptiles, and birds.[16] In most other mammals, uric acid is oxidized by urate oxidase, or uricase, in the liver to allantoin, a more soluble end product.[78] Marsupials and monotremes are an exception because, unlike placental mammals, these species have an extra gene for allantoinase that likely makes allantoate from allantoin.[79] In teleost fish, allantoinase converts allantoin to allantoic acid, and in cartilagenous fish and amphibians, allantoic acid is converted to urea as the end product of purine metabolism. The enzyme urease, found in marine invertebrates, is used to make the end product ammonium ion (NH_4^+).[16]

Bottlenose dolphins may be capable of *de novo* synthesis and salvage of nucleotides, with a similar nucleotide degradation pathway to form uric acid like other mammals; however, there have been no studies regarding dolphin purine biosynthesis or metabolism to date. Uric acid concentrations in the urine of free-ranging common bottlenose dolphins and dolphins under human care have been measured (Table 2-2). Compared to dogs and cats with functional uricase, uric acid concentrations in the urine of *fasted* healthy adult dolphins under human care are up to two times greater than uric acid concentrations in the urine of beagle dogs fed a high protein growth diet, and are 70 to 300% greater than uric acid concentrations in the urine of domestic cats.[2, 3, 80-82]

Furthermore, free-ranging dolphins with an unknown fed versus unfed status have urinary uric acid concentrations two to six fold greater than the uric acid concentrations in dog and cat urine.[2] The range of uric acid concentrations (0.114-0.478 mg/mL) in the urine of managed and free-ranging dolphins, however, is more similar to the concentration range (0.250-0.749 mg/mL) reported in the urine of human beings that do not make functional uricase. Allantoin, however, has not been measured in the urine of bottlenose dolphins. Allantoin concentrations in human urine are one-hundredth of the concentrations excreted by other mammals, like cows, sheep, and mice. Thus, it is not possible to conclude whether dolphins excrete primarily uric acid, like human beings, until the presence of allantoin has been confirmed and it has been quantified in the urine of bottlenose dolphins.

Purine relationship to ammonium urate stone development

A purine-rich diet is a risk factor for ammonium urate stone development in human beings residing in countries with fish-based and shellfish-based diets. Greater consumption of purines would lead to greater urinary uric acid concentrations. [47] Nevertheless, ammonium urate stones occur infrequently in human beings. Ammonium urate stones are, however, the most common form of urate-based stone in dogs and cats. Some Dalmatian dogs have an underlying autosomal recessive genetic disorder that causes circulating uric acid concentrations to be 2-4 times greater than in other breeds, making them more sensitive to dietary purine content.[7, 80, 83, 84] Specifically, hepatic conversion of urate to allantoin in some Dalmatians occurs at a significantly lower rate than in non-Dalmatians because of incomplete uric acid oxidation, despite normal concentrations of uricase in the liver.[85, 86] In addition, renal tubules in some Dalmatians reabsorb less urate because of mutations in both SCL22A12 and SLC2A9

transporters, causing elevated urinary uric acid excretion.[87, 88] Therefore, some Dalmatian dogs require a diet low in purine-containing proteins to help prevent stone formation.[8]

Non-Dalmatian dogs and cats can also form ammonium urate stones because of genetic defects, such as portal vascular anomalies, but even without these conditions, consumption of purine-rich food can be a risk factor for increased uric acid excretion and urate stone development.[80, 83, 89-91] Ammonium urate stones also occur in young Egyptian Mau, Birman, and Siamese cat breeds, but a specific underlying genetic or heritable anomaly has not been identified.[92]

Common Bottlenose Dolphins under Human Care

Historical Perspective

The first facility to maintain bottlenose dolphins under human care opened in 1938 and was located just south of St. Augustine, FL. Until the passage of the Marine Mammal Protection Act (MMPA) in 1972, dolphins were routinely captured from the ocean and brought into aquarium-type facilities where they lived out the rest of their lives. The capture of marine mammals was allowed to continue under permits granted by the National Marine Fisheries Service until the late 1980's, after which all capture was prohibited. Following passage of the MMPA in 1972, the standards of care regarding minimum space allotted, water quality, and veterinary care, for animals in an 'oceanarium' setting were established.[93] Today, there are 30 facilities within the U.S. and Canada that are responsible for the care of 474 common bottlenose dolphins.[94] Dolphin nutrition and management within these facilities is based on our knowledge of how this species lives in the wild and how closely we can replicate those living conditions.

Two of the largest populations of bottlenose dolphins cared for by people within the United States are housed by the MMP and SeaWorld, Inc. Since 1962, the MMP has housed and cared for bottlenose dolphins in open ocean enclosures primarily in California. The MMP began in Point Mugu, CA, with wild dolphins obtained from Gulfport, Mississippi, and then migrated to San Diego, CA.[93] Until approximately the late 1980's, the MMP continued to bring dolphins from the Gulf of Mexico region into their population, but then transitioned to breeding MMP dolphins to generate a self-sustaining population of animals.[95] In 1964, SeaWorld opened its first park in San Diego, CA, housing several marine mammal species including common bottlenose dolphins from Florida. Following passage of the MMPA, SeaWorld also initiated breeding programs for their dolphins. The population genetics for SeaWorld dolphins are now managed by the Association of Zoos and Aquariums in order to ensure sustainable genetic diversity within the managed dolphin population.

MMP and SeaWorld dolphins are provided with high-quality veterinary care that includes preventive medicine programs.[93, 96] The daily activities and feeding schedules of the dolphins differ by facility. MMP dolphins are involved in a wide variety of activities, including in-pen training and open-ocean exercises, which vary depending on the individual dolphin and on the needs of the MMP. The feeding schedules then correlate with the training or interaction sessions, so depending on the day's activities, dolphins are fed their total daily diet divided into 3 to 8 meals over the course of the day. For SeaWorld dolphin activities include some combination of training, play, interactive presentations and guest-interactions. The feeding schedule also correlates with a range of activities, but SeaWorld dolphins are fed smaller, more frequent meals (maximum of

20) over the course of a day.[97] Both MMP and SeaWorld dolphins are typically fasted overnight.

Diet of Managed Bottlenose Dolphins

The diet of common bottlenose dolphins under human care commonly consists of whole fish with some squid. The fish species, size of fish, and relative proportions of each species fed depends on the energy density of each species, commercial fish stock availability, caloric needs for life stage, and facility training needs. Fish meals are delivered to dolphins as a positive reward for training and encouraging activity participation; therefore, the total daily diet is split and fed out into several smaller meals in order to avoid satiety and overfeeding. At SeaWorld, for example, caretakers may feed a greater proportion of smaller, leaner fish species to an individual dolphin receiving 20 small meals per day based on its training and activity schedule. The wet weight of fish or squid fed each day is adjusted, sometimes daily, to ensure dolphins are taking in enough calories in their food to maintain normal body weight in adult dolphins and acceptable growth in young dolphins. Alternatively, caretakers working with veterinarians may choose to change the ratio of fattier or leaner fish species in a dolphin's diet in order to accommodate individual dolphin's needs or to compensate for seasonal changes in fish nutrient composition.[98-100]

Managed bottlenose dolphins diets are composed primarily of 2 fish species, capelin (Icelandic and/or Canadian, *Mallotus villosus*) and herring [Pacific (*Clupea pallasi*) and/or Atlantic (*Clupea harengus*)]. In addition, dolphins are fed a smaller percentage of other species, such as mackerel *(Scomber spp*.), Pacific sardine (*Sardinops sagax*), and an invertebrate, west coast Loligo squid (*Loligo opalescens*), depending on the facility. Fish and squid are caught during commercial fishing seasons.

The timeframe of a commercial fishing season is generally set for a specific time of year depending on the location and species being caught. Sometimes the fishing season can include spawning, depending on the fish species. The species fed to the dolphins are prepared for human consumption, so some type of brine solution may be applied prior to sealing the packages and freezing the fish. The composition of the brine solution depends on the commercial fishery but can include saline and/or electrolyte-based solution.[101] The purpose of the brine is to preserve the fish by reducing the water activity, protect the fish from freezer burn during storage, and to enhance palatability of the fish, both in flavor and texture. [102-105]

Following brine application, most species of fish and the squid are frozen in blocks and packaged for frozen storage. Mackerel, however, differs in that it is generally individually quick frozen and then packaged together for frozen storage. Dolphin management facilities will store fish at -20°C typically for up to 1 year, but mackerel and sardine may be stored frozen for a shorter period of time due to a greater histamine content which hastens spoilage.[106]

Enough fish are thawed each day to meet the dietary needs for all dolphins at a facility. Thawing procedures also differ among facilities and involve either the sole use of water, refrigerated air, or a combination of both. For a water thaw, frozen fish are placed directly into large stainless steel sinks. The sinks are then filled with continuously running tap water until the fish blocks can be broken apart into individual fish. During this process, the water is kept below 4°C. For an air thaw, fish are transferred to a refrigerated room (generally kept at 4°C) where they are allowed to thaw over 24 hours. Using a combination of the two thawing methods, fish may undergo a refrigerated air

thaw for 12-24 hours and then be rinsed well under tap water as a final step. Fish are then transported in buckets to the area where individuals or groups of animals are to be fed, and kept on ice in the buckets to maintain a temperature of 4°C or below until the fish are fed to the dolphins.

Nutrient Content of Food Fed to Managed Bottlenose Dolphins

The nutrient content of fish varies widely and depends on species, season, and location. A wide range of gross energy (GE) densities has been documented among and within several of the species commonly fed to managed dolphins, including capelin (4.5-6.7 kcal/g dry matter (DM)), Pacific herring (2.8-7.2 kcal/g DM), Atlantic herring (5.5-6.7 kcal/g DM), Pacific mackerel (*S. japonicus* (4.5-6.0 kcal/g DM), and Loligo squid (5.0-5.4 kcal/g DM) (Table 2-3).[107-110] These differences may be related to seasonal water temperature changes and spawning cycles.[98] For example, capelin undergoes an increase in fat content and a decrease in moisture content prior to spawning and have the greatest energy density when they spawn in March and April.[111]

There are a few published reports describing the nutrient content of the whole fish and squid species commonly fed to bottlenose dolphins (Table 2-3).[108-110, 112, 113] Based on these reports, fish were generally 20-40% dry matter (60-80% water 'as fed'). Furthermore, herring provided approximately 30% more dry matter than capelin and squid, and thus herring provided less water than capelin and squid. The protein and lipid contents varied within species, likely indicating some seasonal variation, and among species. For example, herring had, on average, 75% more fat content relative to dry matter than capelin and striped mullet.

Reports on the macromineral concentrations in capelin, herring, mackerel, and squid also varied within species (Table 2-4), but mineral concentrations were

comparable among analyses, with the exception of a few outliers.[109, 110, 112, 113] For example, reported concentrations of phosphorous in capelin were similar among analyses, ranging from 1.3-2.2% DM, except for one concentration reported by Corse, et al., was comparatively low (0.3% DM). The macromineral concentrations in squid were similar to the mineral concentrations of the fish species, except for lower calcium concentrations in squid compared to fish. The lower Ca content of squid is likely due to squid being an invertebrate species with no bones, whereas all other species reported were teleost fish species, or bony fish species. Slifka, et al. (2013), were the only authors to report the sulfur content of capelin and herring, which were comparable among these two species (1% DM and 1.1% DM, respectively). Squid had a much lower Ca DM content, as would be expected for an invertebrate and a markedly greater Cu content (106-245 ppm), but was otherwise comparable with respect to the other minerals.

Handling and Processing of Fish Fed to Managed Dolphins

The handling and processing that the fish undergo and the time spent in frozen storage can have a significant impact on the concentrations of various nutrients. This handling process begins with the brine application. The mineral content of the fish may be affected by the composition of the brine solution and also whether or not fish are thawed in water. The effect that the brine solution may have on the fish mineral content is strongly suggested by the findings of Slifka, et al. 2013, with sodium and potassium concentrations significantly greater in capelin and herring when compared to the fish species commonly consumed by free-ranging bottlenose dolphins.[113]

Freezing fish has also been well-documented to alter nutrient concentrations. Fish are composed of 60-80% water (Table 2-3). Water forms sharp ice crystals when

frozen that expand into surrounding tissue, causing remaining solutes, like proteins, lipids, carbohydrates, and vitamins, to increase in concentration. This disrupts the muscle fiber structure and causes cells to rupture.[104] Water then leaches out of the tissue at rates dependent on length of frozen storage and temperature fluctuations, resulting in dehydration of the fish tissue over time.[114, 115] The faster the freezing process, such as with individually quick frozen, the smaller the ice crystals will be and thus the less tissue damage and water loss the fish will undergo.[104, 116]

Additionally, lipid oxidation occurs as a consequence of freezing, the degree to which depends on the total fat content and fatty acid composition of the fish.[117-119] Fish with a greater fat content that contains a high percentage of polyunsaturated fats will undergo more rapid oxidation. This oxidation hastens degradation of fat-soluble vitamins and eventually leads to spoiling.[120-122] The degree of fatty acid unsaturation varies among fish species and location, with greater unsaturated fatty acid content in cold water fish species, such as herring and capelin[123, 124]. Therefore capelin and herring are likely to be more susceptible to rapid oxidation during frozen storage and thawing.

The effect of frozen storage on purine metabolite degradation has not been examined, but fish species, chilled storage methods and storage times impact post-mortem purine metabolite concentrations in fish filleted for human consumption. [47, 117, 125-128] For example, salmon filets under refrigeration have baseline IMP concentrations of 5 mmol/kg wet weight tissue and inosine and hypoxanthine concentrations of 0.5-1 mmol/kg wet weight tissue. By day 12 of chilled storage, IMP concentrations decreased to less than 2 mmol/kg and hypoxanthine and inosine concentrations rose to greater

than 2 mmol/kg. Salmon filets were depleted of IMP by day 24 of chilled storage, whereas hypoxanthine and inosine concentrations peaked at 7 mmol/kg and 5 mmol/kg wet weight tissue, respectively.[117] Another study reported hypoxanthine concentrations in Atlantic mackerel stored in ice for 12 days rose from a baseline concentration of 0.7 mmol/kg wet weight tissue to 3 mmol/kg wet weight.[128] Thus, the concentrations of purine metabolites, such as IMP, inosine, and hypoxanthine, in post-mortem fish tissue may vary with species and storage methods, and these differences in total and individual purine metabolite concentrations may affect urine purine and uric acid concentrations.

Finally, the thawing process impacts nutrient content, the extent of which depends on how fish and squid are thawed and the length of time the thaw takes. As the fish and squid undergo an air thaw, thaw-drip loss takes place where the tissue loses both water and denatured proteins.[115, 129] On the other hand, when fish and squid are exposed to running water or placed into a bath of water during thawing, water loss is hastened, taking with it proteins and water-soluble vitamins.[130]

Free-ranging Common Bottlenose Dolphins Residing in Sarasota Bay, Florida

Life History Milestones, Morphologic Characteristics, and Environment

The common bottlenose dolphins residing in Sarasota Bay, Florida, were selected to be the free-ranging control population for this dissertation research. This dolphin population has been observed and studied by biologists and veterinarians since 1970, and more information is known about the life history, environment, breeding activity, energetics, feeding behaviors, and diet of the Sarasota Bay dolphins than any other population of free-ranging bottlenose dolphins.[131, 132] The common bottlenose

dolphins in Sarasota Bay, FL, are an inshore resident population that maintains a tight home range within the shallower waters of the bay all year.[133]

Free-ranging bottlenose dolphins are polymorphic, with variations in size, shape, and body mass based on geographical location and corresponding water temperatures.[134-137] Inshore common bottlenose dolphins like those in Sarasota Bay, FL, are comparatively smaller than their pelagic counterparts with shorter beaks, which are likely adaptations allowing for easier maneuverability in shallow inshore waters.[137, 138] The temperate to subtropical water temperatures of Sarasota Bay fluctuate seasonally, from 13°C in the winter to 35°C in the summer, causing the resident dolphins to lay down up to 38% more blubber in the winter months, increasing their total body mass.[131]

All bottlenose dolphins are also sexually dimorphic with respect to body mass, length, and girth. Generally, female bottlenose dolphins grow continuously for 10 years, then their average weights plateau to between 175-200 kg, their lengths to between 227-250 cm, and their maximum girths to between 135-145 cm, once sexual maturity is reached.[135, 136, 139, 140] Males continue to grow until about 25 years of age or until their body mass is approximately 33-39% greater than females. Males reach an average mature adult body weight of 240-250 kg, a length of 240-275 cm, and a maximum girth of 145-155 cm.[135, 136, 139] Additional specific dimorphic characteristics for the Sarasota Bay male dolphins are greater rostral girth and larger flippers and peduncles, when compared with resident female dolphins.[141]

The age at which females and males reach sexual maturity varies depending on the subpopulation. Generally, females are considered sexually mature between 6-12

years of age, at lengths of approximately 207-235 cm and weights of 150-200 kg.[133, 137, 139, 142, 143] Males are considered sexually mature between 10 and 13 years of age, at lengths of 245 to 260 cm and weights of 200 to 275 kg.[135, 139]

The gestation period of a common bottlenose dolphin lasts 12 months.[135, 144, 145] A cow will give birth to a single calf generally weighing about 18 kg and ranging in length from 84-140 cm. The calf grows rapidly during the first year of its life, weighing between 63 to 87 kg by the time the calf is one year old.[135, 146] The calf nurses for 1-3 years, or longer if the cow permits, and is weaned after reaching an average length of 170-180 cm, according to Barros et al., or a length of 225 cm and weight of 150 kg, according to Wells et al.[147, 148] The calving interval for dolphins is about every 3 to 6 years. During this time, there is a strong bond between mother and calf, and the calf is taught foraging techniques but may also continue to nurse until the next calf is born.[131, 142]

Diet of Free-ranging Bottlenose Dolphins

Free-ranging resident common bottlenose dolphins in Sarasota Bay, FL, consume a whole fish diet.[149] Historically, dolphins were presumed to be opportunistic feeders, foraging behind shrimp boats, stealing fishermen's bait, and taking and consuming any type of fish, seemingly with no preference, depending upon availability.[150-152] Opportunistic feeding may still be a foraging technique used by some dolphins; however, research has demonstrated that bottlenose dolphins in Sarasota Bay have specific foraging and prey selection strategies.

Dolphins in Sarasota Bay divide into fluid groups of 4 to 7 individuals, depending on their sex and reproductive condition.[138, 150] The social groups demonstrate high site fidelity but are dynamic, sharing breeding associations and home ranges within

Sarasota Bay.[133, 138, 150, 153] Dolphins feed either on fish that are available within their home-range year-round or on migratory species that move through that range.[138, 150, 154-157] Bottlenose dolphin foraging behaviors depend upon water depth, bottom substrate, proximity to shore, tide cycles, prey behavior, seasons, and individual preference.[147-149, 154, 155] Within the Sarasota Bay region, the dolphins often swim in shallow flats of depths less than 2 meters, in open bays of depths less than 4 meters, and in channels or passes less than 10 meters deep.[138, 149]

Foraging techniques of bottlenose dolphins in Sarasota Bay include echolocation, acoustic communications, and passive listening.[158, 159] Dolphins foraging in the shallows or seagrass beds of the bay use primarily acoustic communications and passive listening to find prey because seagrass likely creates too much interference for effective echolocation signaling.[158, 160, 161] For example, dolphins may remain within acoustic distance of one another and produce loud, low-frequency sounds to disturb prey from hiding places and alert other dolphins in the area of moving prey.[158] Dolphins use passive listening to positively select for soniferous fish species, including sciaenids, such as the spot croaker (*Leiostomus xanthurus*) and bottom-dwellers, including the Gulf toadfish (*Opsanus beta*).[148, 149, 154, 161162] Toadfish, for example, produce very long, high frequency calls from their shelters or nests, which allows dolphins to seek them out as a primary prey item.[160] Croaker calls can also be heard by dolphins from up to 630 meters away, a distance beyond the range of echolocation. Once the dolphin hears the fish calls, echolocation can then be used to more efficiently survey the wide area and pursue the prey.[160]

The diet of Sarasota Bay resident dolphins is varied, as described by observational studies, stomach content analyses, and prey abundance studies.[132, 149, 161, 162] One study, for example, reported that the stomach contents of 32 dolphins contained 544 prey items representing 36 different taxa, 19 families (including 1 family of cephalopods and 1 of elasmobranchs), 11 genera, and 20 species.[162] Dolphins consume some pelagic species, like striped mullet (*Mugil cephalus*) and lady fish (*Elops saurus*), that school during different times of the year in association with spawning.[149, 162] Mullet school and spawn during the fall months and historically were considered a primary prey species for bottlenose dolphins, likely due to frequent observations of dolphins feeding on jumping mullet. However, several studies have not confirmed positive selection for mullet.[148-150, 161, 163] Lady fish are schooling, nocturnally active fish, so bottlenose dolphins feed on them at night.[149, 164]

Sarasota Bay dolphins also positively select for abundant species, like pinfish (*Lagodon rhomboides*) and sheepshead (*Archosargus probatocephalus*), and soniferous species, like Gulf toadfish, spot croaker, pigfish (*Orthopristis chrysoptera*), and spotted sea trout (*Cynoscion nebulosus*).[161] Seagrass beds are important to free-ranging dolphins because this habitat provides a greater diversity of prey fish species and offers protective shelter to solitary and juvenile fish, as well as to cows with nursing calves.[156, 161] Fish commonly consumed by dolphins range in total length between 50-300 mm, as reported by Barros et al. (1998), and up to 1,027 mm, as reported by McCabe et al. (2010).[148, 149, 161] The smaller fish are juveniles that usually reside in seagrass to seek shelter and find food, consuming primarily algae and grass. Fish then venture into deeper water as they age.[149]

The energy density of the fish species consumed by free-ranging bottlenose dolphins is not well-described overall but has been described for some whole fish commonly consumed by dolphins in Sarasota Bay. Thirteen species, including spotted sea trout, silver perch (*Bidyanus bidyanus*), mullet, pinfish, pigfish, spot croaker, red drum (*Sciaenops ocellatus*), and Gulf toadfish had gross energy densities ranging from 1.0-1.86 kcal/g as fed. Seasonal variations in energy content both among and within these fish species were not significant.[165] Nevertheless, seasonal changes in energy density are likely if the fish is a pelagic species and thus susceptible to atmospheric and thermal changes, and spawning cycles.[111, 118, 166] Whole mullet, for example, has been documented to have an gross energy density of 0.9-1.7 kcal/g and up to 3.76 kcal/g as fed, likely indicating seasonal variation. It is possible, however, that mullet in Sarasota Bay experience less variability due to more stable water temperatures.[107, 167] The effect of stable water temperatures generating less seasonal variability in energy density among bottom-dwelling species, like spot, that swim in deeper water, is reported and was also true for the Sarasota species.[98]

The nutrient concentrations for free-ranging dolphin diet fish species also have rarely been reported, but the dry matter content, protein, fat, and ash contents, and the macromineral content relative to dry matter were described by two reports for striped mullet and by one report for pinfish and pigfish (Table 2-3 and 2-4).[109, 113] Both of these studies, however, report nutrient concentrations on a single sample, so no variability within species can be accounted for. The dry matter content ranged from 25% for pigfish to 36% for mullet; therefore pigfish would provide more moisture ‘as fed’ than mullet or pinfish. Pigfish also provided the greatest protein and least fat content relative

to dry matter among species, whereas pinfish provided the least protein and greatest fat content relative to dry matter among species. The ash concentration relative to dry matter for striped mullet was much greater as reported by Crissey et al. (1994) than by Slifka et al. (2013). The reason for the discrepancy may be due to greater Mg, Na, and K concentrations in mullet reported by Crissey et al., when compared to Slifka et al., contributed to the greater overall ash content. Slifka et al. also measured the sulfur content of the fish species, whereas Crissey et al. did not, but inclusion of sulfur did not impact the total ash content of the mullet. Among fish species, pigfish had the greatest macromineral concentrations.

Slifka et al. further compared the nutrient analyses of the three free-ranging fish species to two fish commonly fed to managed dolphins, capelin and herring. Capelin and pigfish had similar protein and fat contents, whereas herring, mullet, and pinfish had more comparable nutrient concentrations. Calcium and phosphorous concentrations were greater in pinfish, pigfish, and mullet, whereas potassium and sodium concentrations were greater in capelin and herring. Nutrient differences among the free-ranging and managed diet fish species may be due to the fishes' diet, catch season, fat content, body size, bone density, or water quality, temperature, or salinity. Furthermore, the handling, including freezing and thawing, of capelin and herring fed to managed dolphins may also contribute to the variations among species, the extent to which may depend on the fish distributor and dolphin facility.[113]

The study performed by Slifka et al. and Crissey et al. provided baseline information on the nutrient content of a few free-ranging fish species commonly consumed by dolphins in Sarasota Bay. Nevertheless, the few species and small

sample sizes analyzed limits the practical use of this information to alter the diet of dolphins under human care.

Energy Requirements of Bottlenose Dolphins

Free-ranging bottlenose dolphins consume a quantity of fish sufficient to provide the energy required to maintain body condition while supporting basal metabolic functions, thermoregulatory and locomotive costs, and, when applicable, growth, pregnancy, and lactation. A few attempts have been made (Table 2-5) to determine the minimal energy expended per unit time while dolphins are at rest, in an effort to understand the metabolizable energy requirements of dolphins. The BMR of terrestrial mammals has been measured under the assumption that four criteria are met: 1) the animal is mature; 2) the animal is within its thermoneutral zone; 3) the animal is in a post-prandial state; and 4) the animal is at rest.[168] These criteria are difficult to meet for free-ranging or managed dolphins because dolphins are never truly at rest in their aquatic environment.[169]

Williams et al. (2001) measured the oxygen consumption of three post-prandial adult male dolphins under human care exposed to various water temperatures while maintained 'at rest' in a water-filled metabolic chamber.[170] The amount of oxygen consumed by dolphins resting on the surface of the water was temperature-dependent. Oxygen consumption was significantly greater when dolphins were kept for 2-3 hours in water temperatures below 5°C and above 25°C, but consumption (5.8 to 10.1 ml $O_2 \cdot kg^{-1} \cdot min^{-1}$) was stable between those temperatures. This is equivalent to 40 to 70 $kcal \cdot kg^{-1} \cdot day^{-1}$. A similar study performed by Yeates et al. (2008), however, found the low critical water temperature to be slightly higher (7.8 and 10.6°C) and the resting metabolic rate (RMR) measured by oxygen consumption to be substantially lower (3 to

4.4 ml $O_2 \cdot kg^{-1} \cdot min^{-1}$ or 21 to 30 $kcal \cdot kg^{-1} \cdot day^{-1}$).[171] The different values for minimal metabolism obtained by these two studies can be attributed, however, to difference in the method used for maintaining the dolphins and collecting respiratory gases, whether dolphins were fasted versus fed, the age and maturity of the study dolphins, and the body weights of study dolphins. Nevertheless, the values obtained by Williams et al. are also slightly than what would be obtained using the Kleiber equation for a similar size bottlenose dolphin (Table 2-5).

Nevertheless, as soon as dolphins move or are challenged in some way, the BMR is surpassed and energy needs rise. For example, energy requirements increase when bottlenose dolphins are outside of their thermoneutral zone, or the temperature range for which no additional energy is needed, in order to maintain core body temperature, and when moving around for foraging and feeding.[172, 173] Pregnancy and lactation also increase a dolphin's energy requirement – a cow's average daily food intake increases by 52% during the first year of lactation.[144]

To date, however, one research team has reported the field metabolic rate, or FMR. Costa et al. (2013) measured FMR in free-ranging resident dolphins in Sarasota Bay using doubly-labeled water. During the winter months when water temperatures averaged 16.9°C, the FMR was 4.82 ± 0.81 W/kg, or approximately 98 $kcal \cdot kg^{-1} \cdot day^{-1}$. The FMR was much greater (6.79 ± 1.11 W/kg, or approximately 140 $kcal \cdot kg^{-1} \cdot day^{-1}$), however, during summer months when water temperatures averaged 26°C. Thus, Costa et al. estimated that the FMR of dolphins residing in Sarasota Bay was 3.4 to 4.75 times the minimal energy requirements measured by other researchers in managed bottlenose dolphins.[174]

Maintenance energy requirements have also been determined for several populations of dolphins under human care by monitoring caloric intake and body weight responses. Cates et al. (1986) determined that a 150-170 kg dolphin between the ages of 11 and 20 years maintained in the same facility for 7 years requires approximately 80 kcal gross energy·kg^{-1}·day^{-1} in order to maintain its body weight.[175] Individual animal activities and physiologic conditions were not described, but this provides a rough estimate that a 160kg dolphin likely needs approximately 12,800 kcal of gross energy per day to maintain a stable body weight over time.

Estimating caloric requirements, however, based on the gross energy content of the diet may overestimate the dolphin's energy requirements because some of the energy consumed will be lost to the feces and urine. Thus, it is more accurate to estimate maintenance energy requirements based on metabolizable energy, which accounts for the portion of energy provided by the food that is lost to feces and urine.[176] Reddy et al. (1994) calculated the metabolizable energy content of the whole fish fed to 5 male and 11 female dolphins under human care assuming 95% digestibility. The dolphins' maintenance energy requirements were then determined over the course of 3 years based on maintenance of a stable body weight while being fed a consistent fish diet.[177] The dolphins were separated into life stage categories, including subadults, adults, pregnant females, and lactating females. Male and female subadult dolphins required more energy (53-81 kcal/kg body weight) than male and female adult (12+ years of age) dolphins (34-67 kcal/kg). Pregnant females required 36-89 kcal/kg, not increasing intake until just before gestation was reached. Lactating females required the greatest energy intake of 88-153 kcal/kg.[177] Based on Reddy's data, the maintenance

digestible energy requirements for an average 160 kg adult dolphin may be between 5,000 to 10,700 kcal/day. A similar study was performed for another group of dolphins under human care by Kastelein et al. (2002), and dolphins consumed on average 2 to 4% of their body mass (kg) in wet weight of fish, which equates to 6,500 to 18,900 kcal as fed/day for an average daily diet caloric content of 2100 kcal/kg of fish as fed.[178] Thus, the average 160 kg dolphin may consume between 6,700 kcal and 13,000 kcal per day according to Kastelein. The maximum caloric intake was greater for this population of dolphins than what is reported by Reddy, but this may be in part due to the presence of lactating females and ad libitum feeding practices.

Based on the preliminary data from Costa et al. (2013), a 160kg dolphin would consume 15,600 kcal/day in the winter months and up to 22,000 kcal/day in the summer months. Thus, it is likely that managed dolphins in most facilities have a lower maintenance energy requirement than free-ranging dolphins. Reasons for this possible discrepancy include differences in activities performed and the time spent engaging in activity over course of the day, reproductive status, and water temperatures.

Ammonium Urate Nephrolithiasis in Managed Bottlenose Dolphins

Prevalence and Potential Etiologies

Ammonium urate kidney stones develop in common bottlenose dolphins under human care, but are rarely found in free-ranging bottlenose dolphins. The MMP and the NMMF have reported a prevalence as high as 35% in the Navy dolphin population, after thorough investigation using ultrasound and computed tomographic (CT) imaging.[1] The cause of ammonium urate stones in managed dolphins is unclear. In other mammals, urate urolith development has been attributed to the nutrient composition of the diet, genetic derangements in purine metabolism, dehydration, and/or underlying disease

such as obesity, inflammatory bowel disease, or compromised liver function.[4-8] Thus, there are likely several contributing factors to the development of ammonium urate urolithiasis in bottlenose dolphins, but this research focused on two potential factors: nutrient differences between the free-ranging dolphin diet and the diet fed to managed dolphins, and purine metabolic by-products excreted in the urine of both dolphin populations.

Signs, Diagnosis, Sequelae, and Treatment

Bottlenose dolphins under human care that have ammonium urate stone nephrolithiasis may be asymptomatic for an extended period or may have clinical signs including lethargy, inappetance, hematuria, stranguria, and ureteral or urethral obstruction.[3, 179]

Diagnostics performed on dolphins with suspected kidney stones include blood chemistry and urine analyses. Radiographs and ultrasound examination are the first techniques utilized to make a definitive diagnosis, and computed tomography (CT) may be used to make a more complete evaluation. Blood chemistry abnormalities that may worsen with advanced disease and indicate a loss of appropriate renal function include azotemia, anemia, and reduced estimated glomerular filtration rate (GFR), estimated using a serum-based predictive equation of GFR used in human medicine.[3, 180-182] Nevertheless, the mean urine specific gravity was not different between dolphins with and without nephrolithiasis.[3, 183, 184] Other urine analysis findings in dolphins with stone disease include hematuria, urinary pH ~6, hypocitraturia, and urate urinary crystals with advanced disease. [2, 3] The number and size of stones and related collecting duct dilation, hydronephrosis, and hydroureter vary.[1, 2, 179] Nephroliths are commonly found bilaterally but the number of stones may depend on the severity of the disease. Venn-

Watson et al. (2010) classified mild disease as dolphins with 1-19 stones and advanced disease as dolphins with $\geq$ 20 stones.[3]

Several treatment modalities are utilized in order to provide supportive care, prevent further stone formation, and dissolve or break apart existing stones. At the discretion of the veterinary team, oral hydration and medications to treat pain and underlying infections are prescribed. Hydration therapy may be administered either subcutaneously, intravenously, or orally. Oral fluids may be administered by voluntary orogastric intubation in the form of either fresh water, saline, or electrolyte solution. The volume of fluid delivered varies from 1 to 4 L per day, depending on the age and size of the dolphin and the severity of its underlying renal disease.

Prevention and dissolution of ammonium urate nephroliths is reported to be possible at an alkaline pH, both *in vitro* and *in vivo* in human beings and dogs.[185-187] In an *in vitro* study, ammonium urate solubility was affected by both solution pH and buffer ion strength.[186] Ammonium urate was more soluble with a sodium buffer and a pH range of 9-10.8 than with a citrate-phosphate buffer and pH range of 6-8. Increasing the ionic strength of the phosphate buffer to 150 mM improved solubility within that pH range. Nevertheless, no solution completely dissolved the stones and promoting an alkaline urine may predispose to other types of stone formation.[186]

In human beings, treatment with oral potassium citrate, sodium citrate, or sodium bicarbonate (60 mEq per day) increases urine pH, decreases urine ammonium concentrations, increases urine citrate concentrations, and prevents uric acid stone formation.[187-189] Assuming a human being consumes about 2 Mcal ME per day, these treatments would increase DCAD by 30 mEq/Mcal.

In managed dolphins, the effective dose of a urinary alkalinizing agent that would raise the relative cation to anion ratio enough to increase the pH of managed dolphin urine is currently unknown. Ammonium urate stone dissolution has been attempted with oral potassium citrate (60-200 mEq of potassium per day) and sodium bicarbonate (approximately 10 g or 120 mEq of sodium per day); however, neither medication altered serum or urine uric acid concentrations or urine pH over time in the dolphins.[3] The effect of sodium citrate has not been evaluated in dolphins to date. Dolphins under human care consume approximately 8 Mcal/day, so these doses would provide approximately 7.5-25 mEq/Mcal of potassium and 15 mEq/Mcal of sodium, i.e. slightly less than the effective doses taken by human beings relative to energy intake.

Allopurinol is another medication used in human beings and dogs to prevent ammonium urate stone formation.[190, 191] Allopurinol inhibits the xanthine oxidase enzyme that converts xanthine into uric acid; but xanthine stones have formed in dogs and managed dolphins when allopurinol has been used without limiting purine intake.[191] Limiting purine intake would likely be important, therefore, if allopurinol is to be used in dolphins.[2, 3] Thus, urine alkalinizing agents, at the doses previously administered, and allopurinol are not considered successful management tools for management or prevention of ammonium urate nephrolithiasis in managed dolphins.

Surgical intervention methods used in managed bottlenose dolphins with ammonium urate nephrolithiasis have included cystoscopy and ureteroscopy with laser lithotripsy and urethral stent placement while under sedation or general anesthesia.[179, 192] These methods have been effective for removal of existing stones that were causing acute obstruction and post-renal insufficiency.[179, 192] Nevertheless, the potential

underlying causes for ammonium urate stone development, including ammonium ion and uric acid excretion, urine pH, urine supersaturation, etc., persist, so surgical interventions would not help to prevent future stone development.

Risk Factors for Stone Development in Bottlenose Dolphins under Human Care

The cause of ammonium urate nephrolithiasis in managed dolphins has not been determined. Nevertheless, several risk factors for stone formation in other species have been implicated by comparing managed dolphin populations afflicted with stones with free-ranging dolphin populations with no evidence of the disease.

Dietary related factors

The diet of managed bottlenose dolphins differs from the free-ranging dolphin diet, which may play a role in stone development. Free-ranging dolphins consume a great variety of live, temperate water fish and invertebrate species, whereas dolphins under human care are fed a lesser variety of frozen, stored, and thawed cold water fish and squid species. Thus, the nutrient composition is likely to differ between the two diets, with particular respect to water, protein, fat, mineral, and purine contents, as previously mentioned.

Furthermore, the feeding frequency differs between free-ranging and managed dolphins which may play a role. Free-ranging bottlenose dolphins consume fish in smaller, more frequent meals throughout both the day and night, whereas some managed dolphins are fed 3-10 meals over the duration of an 8-9 hour day.[82, 132, 193, 194] Meal size and frequency, therefore, is also likely a risk factor. Bolus fish meals consumed by managed dolphins may provide a large dose of protein and purines that must be digested and metabolized over a shorter time period.

The whole fish diet of dolphins is rich in purines and sulfate-containing amino acids.[60, 195] As the protein is metabolized, sulfur is oxidized to sulfate and purines are degraded to their byproducts. Additionally, oxidized protein produces urea which is converted into ammonium and excreted in the urine.[16, 196] In human beings, these processes precipitate a decrease in urinary pH and result in hypercalciuria, hyperuricosuria, and hypocitraturia.[197-199] Furthermore, the total quantity of cations and anions consumed by dolphins with their fish meal may impact urine pH as the ions are metabolized and excreted. The relative proportions of these ions in the diet may be impacted by fish species and the relative proportions of species fed, and post-mortem handling methods.[113] In other mammals, urine will tend to be more acidic if the relative proportion of anions consumed with the diet is greater than cations.[15, 32]

In response to acidosis, urinary citrate is one of the buffers excreted or reabsorbed by the kidney in order stabilize the pH of the urine or blood, respectively.[200] Human beings consuming high protein and purine diets and those with renal tubular acidosis, renal hypercalciuria, idiopathic nephrolithiasis, gastrointestinal malabsorption, chronic metabolic acidosis, and insulin resistance tend to have low concentrations of urinary citrate. These conditions generate an overall acidosis to which the human body responds by increasing tubular reabsorption of citrate by renal cortical m-aconitase activity via the Na^+-citrate co-transporter, in an effort to regulate blood pH.[197, 201]

Managed bottlenose dolphins experience hypocitraturia like human beings, but free-ranging bottlenose dolphins do not. The urine citrate concentrations reported in fed and unfed managed dolphins were comparable (2±1 mg/g creatinine), whereas reported urine citrate concentrations in free-ranging dolphins were much greater (150±28 mg/g

creatinine).[2] Smith et al. (2014) confirmed the comparable urinary citrate concentrations among unfed and fed managed dolphins, reporting no rise in the post-prandial urine citrate concentrations in fed managed dolphins.[82] The reason why managed dolphins excrete much lower urinary citrate concentrations compared with free-ranging dolphins may in part be a consequence of bolus-style feeding in managed dolphins, compared with the smaller, more frequent meals consumed by free-ranging dolphins. Feeding a large bolus of high-protein and high-purine fish provides managed dolphins with a large dietary acid load that must be metabolized and excreted. This may induce an overall acute metabolic acidosis, which then the kidney must compensate for by reabsorbing urinary citrate in order to buffer the blood pH.[2, 82]

Larger and less frequent meals fed to managed dolphins may also increase urinary ammonium and uric acid concentrations, comparatively more so than is true for free-ranging dolphins consuming smaller, more frequent meals.[82] Bottlenose dolphins under human care excrete increased concentrations of urinary ammonium ions in response to a fish meal and subsequent dietary acid load.[82] The post-prandial rise in urine ammonium results in a more alkaline urine, rather than acidic urine, due to the ions' buffering capacity.[15, 82] The quantity of ammonium excreted in the urine of post-prandial managed dolphins is much greater than all other excreted cations, like sodium and potassium, which results in an elevated ammonium urate supersaturation index. It is likely, therefore, that urate in the urine of managed dolphins would more readily bind to ammonium and form an insoluble complex, as occurs in human beings, rather than binding with sodium or potassium forming more soluble complexes.[202] Elevated urinary

ammonium and uric acid concentrations are both found in human beings with ammonium urate stones.[5, 82]

A post-prandial alkaline tide occurs in dolphins following consumption of a fish meal, when release of acid into the stomach causes a temporary systemic alkalosis which results in excretion of a more alkaline urine (pH 6.15) when compared to the unfed urine pH (pH 5.94).[82] This post-prandial alkaline tide should increase ammonium urate solubility. Thus, free-ranging dolphins consuming smaller, more frequent meals may spend more time excreting an alkaline urine than managed dolphins, which would reduce the risk of ammonium urate stone formation in free-ranging dolphins. Managed dolphins consuming larger, less frequent meals may excrete more alkaline urine after each meal but will excrete a more acidic urine for a longer period of time between meals than free-ranging dolphins consuming smaller meals. A more acidic urine would favor precipitation of uric acid, and, therefore, lower concentrations of ammonium ions would be needed to facilitate ammonium urate stone formation in the urine once a nidus has formed.[9] Unfortunately, it is unknown how post-prandial urinary ammonium ion and urate concentrations compare between managed and free-ranging dolphins with respect to their different meal sizes.

Energy intake

Like other carnivores, bottlenose dolphins consume energy dense foods, and the energy provided by their diet then limits the quantity of food dolphins need to maintain a lean body condition.[203, 204] The amount of fish, therefore, a dolphin consumes in a day to meet its energy needs dictates the total nutrient concentrations consumed, metabolized, and subsequently excreted.

As previously addressed, an average 160 kg non-lactating, non-pregnant adult dolphin may have an average daily energy requirement ranging from approximately 16 Mcal/day in the winter to 22 Mcal/day in the summer, according to preliminary data by Costa et al. (2013).[174] On the other hand, non-pregnant, non-lactating adult dolphins cared for at one resort facility consume on average about 10 Mcal/day when maintaining a stable body weight, and individual animals may consume between 25% less to 45% more than that amount.(Ardente, unpublished data) In another facility, a 160 kg non-pregnant, non-lactating adult dolphin would consume approximately 5.5 to 10.5 Mcal/day, or if that dolphin was still growing, approximately 8.5 to 13 Mcal/day.[177]

Although there is considerable variability among individual managed dolphins, overall it appears that free-ranging dolphins have greater maintenance energy requirements than managed dolphins. The difference in maintenance energy requirements between free-ranging and managed dolphin populations is likely a consequence of different activity levels, water temperatures, and reproductive status. Free-ranging dolphins may be consuming, metabolizing, and excreting up to twice the amount of nutrients as some managed dolphins. This might make ammonium urate stone formation in dolphins more likely to occur in free-ranging dolphins, which we know is not the case. It is important, therefore, to consider the energy requirements, food intake, and overall nutrient intake of the whole diet offered to managed dolphins when assessing the risk factors for stone development.

Water intake and electrolyte homeostasis

Hydration is an important consideration in the development of ammonium urate stones because the concentration of total solutes in urine depends on the amount of free water excreted by the kidney. Although surrounded by water, the ocean

environment is akin to that of a desert, with a lack of free water available for consumption.[183] Thus, marine mammals have adapted unique mechanisms for handling water and electrolytes, which may play an important role in the development of ammonium urate stones in managed dolphins. Common bottlenose dolphins can tolerate a wide range of salinities, from the ocean, to estuaries, to fresh water rivers.[138, 150, 151] Ocean water contains 450 mEq/L of Na^+ and 540 mEq/L of Cl^- and has an osmolarity of greater than 800 mOsm/kg.[205-207] Despite their hyperosmolar environment, the plasma osmolality of bottlenose dolphins is only slightly greater than that of human beings, 325 mOsm/kg and 280-295 mOsm/kg, respectively.[180, 208] Furthermore, bottlenose dolphins maintain circulating sodium and chloride concentrations similar to those of other mammals, 150-158 mEq/L Na^+ and 108-125 mEq/L Cl^-.[209, 210]

The kidney is the primary organ responsible for regulating urine volume and concentration. Bottlenose dolphins have reniculated kidneys that are comprised of hundreds of individual units called renules.[211] Each renule contains a separate cortex, thick medulla, papilla, calyx, and blood supply.[212] Contrary to the seemingly efficient renal anatomy, unfed dolphins concentrate their urine similarly to human beings, with a urine:plasma ratio of 3.8 compared to 4.6, respectively.[180, 213] After consumption of a fish meal, however, dolphins excrete Na and Cl concentrations equal to or greater than those of sea water, increasing their urine:plasma osmolality ratio to 5.0.[180, 183] These increases in urine osmolality and Na and Cl concentrations indicate that the dolphins' physiologic response to a meal is likely to conserve free water available from its food and excrete excess salt in the urine; therefore, the osmoregulatory capacity of the

dolphin kidney likely depends on the extent of salt intake, via the diet and sea water ingestion.[183]

Another organ by which dolphins can potentially regulate water and electrolyte balance is the skin. One study performed by Hui et al. (1981) suggested that free water flux is possible through the skin but that electrolytes cannot travel percutaneously.[209] Hui et al. used percutaneous transfer of tritiated water to measure the total rate of bi-directional flow of water through the skin, which was approximately 4 L/day for fasting dolphins. The experiment, however, was performed on only one dolphin, and several factors were not considered in the experimental design, including the salinity of the water surrounding the dolphin, possible variations in water flux depending on body region, the degree of subdermal vascularization, and other variable active processes on-going in the skin.[209] This type of experiment was also not repeated or published by any other authors to date, so the role of skin in total body water balance in dolphins has yet to be truly established.

Water loss is also regulated by excretion in the feces; therefore the gastrointestinal tract also plays a role in water balance. The fecal moisture content for dolphins ranges from 60 to 85%, but electrolytes excreted in the feces are isotonic with stable plasma concentrations. Thus, the gut can affect the amount of water lost but has little effect on the regulation of plasma osmolarity.[214] Other means of water loss include respiratory evaporation, tear production, and milk production in lactating females.[183, 214] Respiratory water loss has not been quantified for marine mammals, but it is estimated that a variable amount, approximately 30 to 75% less than that which is lost by terrestrial mammals of similar size, contributes to water flux. Lower evaporative loss is

likely another strategy to assist in water conservation.[215] Water lost to tear production is thought to be minimal because tears are viscous, and only a small amount is secreted for corneal lubrication. Lastly, the milk of *T. truncatus* is composed of approximately 70% water, which is higher than in other marine mammals. The high water and lower fat content of bottlenose dolphin milk may reflect a lesser need for fat deposition in their subcutaneous blubber layer considering their temperate to subtropical habitats, compared to other marine mammals.[214, 216, 217]

Dolphins may take in water by drinking seawater or consuming whole fish which provides moisture both by its water content and as generated as a byproduct of fat and protein metabolism. Dolphins reportedly consume 12-14 mL/kg, or up to 1.5 L per dolphin, of seawater per day, but it is not well understood whether the water is actively ingested or passively consumed during foraging for fish.[209, 214] To gain free water from ingestion of seawater, however, dolphins need to excrete concentrations of Na^+ and Cl^- greater than those in the ocean. Concentrations of electrolytes in dolphin urine are similar to those in seawater, which makes any physiologic advantage for seawater consumption unclear.[183, 209, 218]

The whole fish and invertebrate species consumed by free-ranging bottlenose dolphins vary in water, protein, and fat content, depending on the species, season, sex, age, and location.[110] Pinfish, pigfish, and striped mullet consumed by Sarasota Bay dolphins consist of 65 to 75% moisture ‘as fed’, so dolphins acquire most of their daily water needs directly from the fish they eat. Furthermore, these fish species contain 53-63% protein and 10-20% fat relative to dry matter, so in addition to providing a direct source of water, metabolic water is derived from the digestion of those energy-

containing nutrients.[113] More metabolic water is produced from the digestion of fat than from protein or carbohydrate, so a diet higher in fat, with a greater percentage of pinfish for example, would provide a greater indirect source of water.[113, 219]

Like dolphins, saltwater fish are hypotonic to their environment, whereas invertebrates are isotonic with seawater.[183] In pinfish, pigfish, and mullet, sodium content varies from 0.15 to 0.5% relative to dry matter. This can be compared to the much greater sodium content of West Coast Loligo squid (*Loligo opalescens*) which is 2% sodium relative to dry matter.[113] Generally, the species fed to managed dolphins, including squid, herring (*Clupea spp.*), and capelin (*Mallotus spp.*), have a considerably more sodium content compared to free-ranging fish species, ranging from 0.75 to 2% relative to dry matter. The discrepancy in sodium content between fish species consumed by free-ranging dolphins and fish species fed to managed dolphins is likely the result of the processing in which a brine solution is applied before freezing and storing the fish for human consumption.[101-103] Nevertheless, when related to water, 'as fed' water-to-sodium ratios were lower in pigfish, capelin, and squid, compared to pinfish, mullet, and herring, which indicates that dolphins would have to excrete more sodium in order to obtain free water after pinfish, mullet, and herring were consumed.[113] Because dolphins can excrete concentrations of sodium and chloride that are equal to or greater than the concentrations in ocean water, an increased sodium and chloride content in certain fish species may not pose a problem for dolphins maintaining water and electrolyte homeostasis.[180]

The way in which bottlenose dolphins regulate and process different quantities of consumed water, protein, and electrolytes has been studied by Ridgway et al. (1972,

2010) and Ortiz et al (2010). [180, 214, 220] Ridgway's trials, however, were performed on dolphins cared for by the MMP that were trained to accept placement of an indwelling urinary catheter; therefore, data could be collected over 16-24 hours to better determine the effects of fasting, fresh and sea water ingestion, and meal consumption on urine flow rate, urine volume, plasma and urine osmolality, and electrolyte and urea excretion.

Fresh water is not available to free-ranging dolphins, but fresh water may be administered by staff to dolphins under human care for hydration purposes or be accessible within the dolphins' environment. Oral administration of fresh water to managed dolphins results in excretion of a more dilute urine (820 mOsm/kg) at a greater flow rate, with lower concentrations of sodium, chloride, and potassium when compared to the urine of unfed control dolphins. Small decreases in plasma osmolality and plasma electrolyte concentrations also occur, indicating a likely total body diuresis. Thus, there may be a dose effect for oral administration of fresh water, where the dolphins' kidneys can conserve electrolytes and excrete free water to a point before causing a total body diuresis with potential health implications.[180]

Ingestion of salt water also increased the urine flow rate, but the urine osmolality was greater than that of ocean water (~1,700 mOsm/kg) with similar sodium and chloride concentrations. No change in plasma osmolality was observed, but the plasma sodium concentration increased just slightly beyond the upper limit of the reference range for bottlenose dolphins (>159 mEq/L). Thus, if dolphins are naturally consuming ocean water, it is likely consumed in smaller, more frequent amounts as they forage for fish, rather than in one large bolus, in order to avoid hypernatremia.[180]

Unlike the effect of saltwater alone, ingestion of a fish meal also resulted in sustained elevations in plasma osmolality and plasma sodium concentrations, indicating a mild hypernatremic effect.[180] Ortiz et al. (2010), however, did not report an increase in plasma sodium concentrations in fed dolphins, so the increase in urine sodium concentrations found by Ridgway was likely due the additional bolus of sea water accompanying the fish meal.[180, 220] The concentration of sodium and chloride in the urine also increased, however, to a concentration greater than that of ocean water (798 mEq/L Na and 823 mEq/L Cl) when dolphins ingested a fish meal and saltwater together. Additionally, post-prandial urine potassium and urea concentrations were elevated. In other mammals, greater excretions of urea produce osmotic medullary diuresis, which facilitates excretion of all other solutes, including electrolytes, and results in a greater urine osmolality.[220-224]

The increased solute concentrations in the urine may also be a consequence of free water conservation, considering the dolphins' need to hydrate in their hyperosmotic environment. Nevertheless, the effect of a meal on osmotic and electrolyte balance resolved 5-10 hours post-prandially, which is a more rapid return to homeostasis than is found in terrestrial mammals.[225, 226] Thus, dolphins ingesting a large bolus of a high salt fish meal or salt water, rather than smaller, more frequent meals, may impact the relative solubility of ammonium and urea from increased solute excretion, and therefore be an important risk factor in the development of ammonium urate stones.

Purine metabolism

Fish are rich in purines, but the total purine content of the fish and squid species commonly consumed by free-ranging dolphins and fed to managed dolphins are not known. Furthermore, as previously described in human beings, ingestion of certain

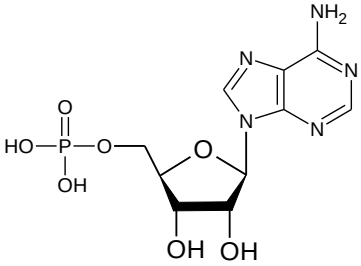
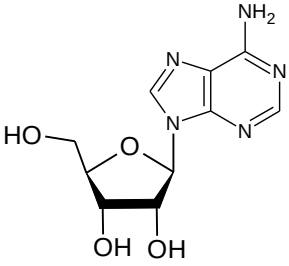
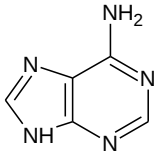
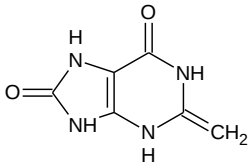
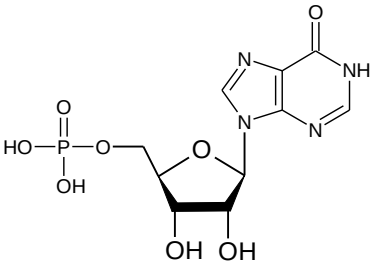
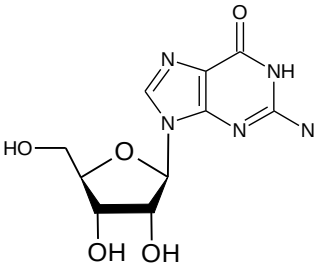
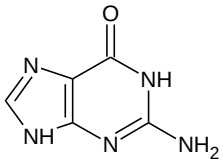
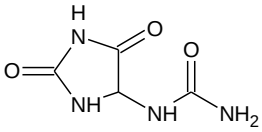
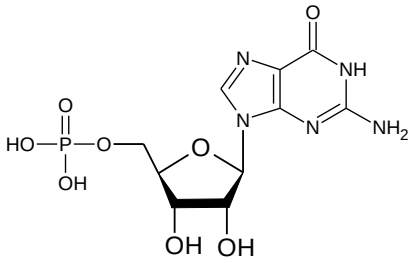
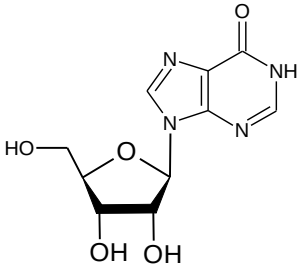
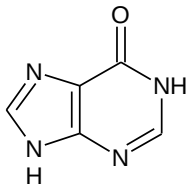
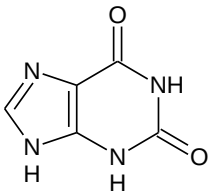
individual purine metabolites, like adenine and hypoxanthine, may cause more uric acid to be excreted in the urine of dolphins when compared to other purine metabolites. The total purine content and individual metabolite concentrations in fish can vary depending on species and post-mortem handling methods. Thus, it is reasonable to consider that the purine content of the frozen, stored, and thawed cold water species fed to managed dolphins may differ from the live temperate water fish species consumed by free-ranging dolphins, and therefore may be a risk factor for ammonium urate stone development in managed dolphins.

Considering the purine-rich diet consumed, it is important to understand how purines are metabolized by bottlenose dolphins. It is unknown whether the primary excretory end-product of purine metabolism in dolphins is uric acid or allantoin because allantoin has never been measured in the urine of dolphins. This is an important consideration because if dolphins excrete uric acid in greater concentrations than allantoin in their urine, ammonium urate stone development may be more likely given greater saturation of urine with the more insoluble end-product uric acid.

The extent to which purine metabolism has been studied in free-ranging and managed bottlenose dolphins is limited, but urinary uric acid concentrations have been quantified and compared among dolphins in both populations. One study reported lower uric acid concentrations relative to creatinine in the urine of fasted managed dolphins (mean of 142 mg/g creatinine) than in the urine of fed managed dolphins (392 mg/g creatinine) and free-ranging dolphins of unknown feeding status (349 ± 41 mg/g creatinine).[2] The effect of a purine-rich meal on post-prandial urinary uric acid concentrations in managed dolphins was also confirmed by Smith et al. (2014).[82] Thus,

the rise in urinary uric acid concentrations following a fish meal may indicate that hepatic uricase is overwhelmed in fed managed dolphins by the large quantity of purines consumed with bolus-style feedings.[82] Nevertheless, the similarity between uric acid concentrations in the urine of fed managed and free-ranging dolphins may further indicate that purines are metabolized via the same mechanisms in both populations, and either 1) hepatic uricase is not functional in dolphins and therefore allantoin is not produced; 2) bottlenose dolphins have a unique purine metabolic pathway and/or osmoregulatory mechanism that predisposes post-prandial hyperuricosuria; or 3) bottlenose dolphin renal tubules fail to reabsorb uric acid from acidic urine.[82]

Table 2-1. Chemical structures* of purine nucleotides, nucleosides, nucleobases, and two excreted by-products of purine metabolism.

Nucleotides	Nucleosides	Nucleobases	By-products
Adenine monophosphate	Adenosine	Adenine	Uric acid
			
Inosine monophosphate	Guanosine	Guanine	Allantoin
			
Guanosine monophosphate	Inosine	Hypoxanthine	Xanthine
			

*Chemical structure images created with ACDLABS Chemsketch Program (April 13, 2016)

Table 2-2. Comparison of reported* mean concentrations (mg/mL) of purine metabolites and end-products in the urine of mammals

Species/condition	Adenine	Guanine	Hypoxanthine	Xanthine	Uric acid	Allantoin
Humans						
Healthy adults [a]					0.250 – 0.749	0.0009 – 0.002
Healthy adults, low-protein diet [b]					0.336	
Healthy adults, high-protein diet [b]					0.562	
Dairy cow, healthy adults [c]			ND (< $1.36e^{-4}$)	ND (< $1.52e^{-4}$)	0.136 – 0.501	1.15 – 2.91
Sheep, healthy adults [c]			0.047 – 0.100	0.003 – 0.007	0.049 – 0.150	0.275 – 0.364
Rats, healthy adults, purine-free diet [d]	0.005	0.016	0.032	0.004		
Mice, healthy juveniles [e]					0.400	2.20
Dogs						
Healthy adult beagles, urinary care diet [f]					0.046	
Healthy adult beagles, growth diet [f]					0.160	
Cats						
Healthy males [g]					0.087	
Healthy females [g]					0.066	
Common bottlenose dolphins						
Managed healthy adults [h]					0.114	
Managed healthy adults, fasted [j]					0.269	
Managed adults, fasted [i†]					0.156	
Managed adults, 6-hours post-prandial [i†]					0.432	
Managed healthy adults, post-prandial [j]					0.314	
Managed adults, mild nephrolithiasis [h]					0.223	
Managed adults, adv. nephrolithiasis [h]					0.071	
Free-ranging healthy adults [j]					0.478	

* References: [a] Kim et al. 2009 J. Chrom. B.; [b] Fellstrom et al. 1983 Clin. Sci.; [c] Shingfield et al. 1999 J. Chrom. B.; [d] Clifford et al. 1976 J. Nutr.; [e] Wu 1994 Proc. Natl. Acad. Sci. USA; [f] Bartges et al. 1995 AJVR; [g] Cottam et al. 2002 J. Nutr.; [h] Venn-Watson et al. 2010 Dis. Aquat. Organ.; [i] Smith et al. 2014 J. Urol.; [j] Venn-Watson et al. 2010 Comp. Med.

† Fifty-percent of the dolphins included in this analysis were healthy and fifty-percent had nephrolithiasis (total n=8).

Table 2-3. Reported* gross energy density and protein, fat, and ash concentrations† relative to dry matter for species commonly fed to managed dolphins and species commonly consumed by free-ranging dolphins.

Species	Sample size	GE‡ kcal/100g DM‡	Dry matter % DM	Crude protein % DM	Crude fat % DM	Ash % DM
Managed diet species						
Capelin (*Mallotus villosus*)[1]	16	494 – 592	15-23	56 – 76	7 – 23	8 – 12
Capelin[2]	1	472	25	72 ± 1	17 ± 1	9 ± 0.6
Capelin, female[2]	2	503	23	69 ± 2	21 ± 2	8 ± 0.4
Capelin, male[2]	2	452	19	74 ± 4	14 ± 4	11 ± 0.4
Capelin[3]	45		21	67	19	10
Capelin[4]	ND‡	555	19	59	14	
Capelin[5]	ND		20	69	12	7
Herring (*Clupea spp.*)[1]	10	513 – 673	24 – 33	46 – 69	13 – 43	7 – 14
Herring[3]	71		28	59	31	10
Herring[4]	ND	633	28	45	34	
Herring[5]	ND		26	62	22	6
Atlantic herring (*Clupea harengus*)[1]	5	545 – 641	21 – 29	44 – 70	16 – 38	7 – 11
Mackerel (*Scomber spp.*)[3]	13		27	66	18	10
Pacific mackerel (*Scomber japonicus*)[1]	3	446 – 594	24 – 34	43 – 79	3 – 37	7 – 13
Squid (*Loligo spp*.)[1]	2	542 – 544	15 – 19	65 – 77	8 – 11	4 – 6
Squid[2]	ND	495	19	71 ± 3	20 ± 4	8 ± 0.7
Free-ranging diet species						
Striped mullet (*Mugil cephalus*)[3]	ND		29	58 ± 5	16 ± 7	23 ± 6
Striped mullet[5]	ND		36	56	14	7
Pinfish (*Lagodon rhomboides*)[5]	ND		33	53	20	8
Pigfish (*Orthopristis chrysoptera*)[5]	ND		25	62	11	10

*References: [1]Bernard et al. 2002 N.A.G. handbook; [2]Van Pelt et al. 1997 Comp. Bio. Phys. A; [3]Corse et al. 1999 N.A.G. proceedings; [4]Crissey et al. 1994 A.Z.A. penguin husbandry manual; [5]Slifka et al. 2013 Int. J. Vet. Med.

†Values represent reported means or ranges.

‡GE, gross energy; DM, dry matter; ND, not described

Table 2-4. Reported* macromineral concentrations† relative to dry matter for species commonly fed to managed dolphins and species commonly consumed by free-ranging dolphins.

		% DM‡					
Species	Sample size	Ca	P	Mg	Na	K	S
Managed diet species							
Capelin (*Mallotus villosus*)[1]	16	1.1 – 2.2	1.3 – 2.2	0.08 – 0.2	0.3 – 1.7	0.7 – 1.9	
Capelin[2]	14	1.5 ± 0.6	2.0 ± 0.6	0.1 ± 0.1	1.4 ± 0.4		
Capelin[3]	ND‡	1.6	0.3				
Capelin[4]	ND	1.5	1.8	0.09	0.7	1.7	1.1
Herring (*Clupea spp.*)[1]	6	1.6 – 6.4	1.2 – 2.3	0.09 – 0.2	0.3 – 1.0	1.1 – 1.7	
Herring[2]	18	1.8 ± 0.9	1.7 ± 0.6	0.2 ± 0.1	1.0 ± 0.4		
Herring[3]	ND	1.6	0.3				
Herring[4]	ND	1.5	1.5	0.12	1.2	1.7	1.0
Atlantic herring (*Clupea harengus*)[1]	5	1.5 – 1.8	1.0 – 2.1	0.1 – 0.2	0.3 – 0.8	0.9 – 1.9	
Mackerel (*Scomber spp.*)[2]	8	2.0 ± 0.9	2.0 ± 0.5	0.5 ± 0.7	1.0 ± 0.3		
Pacific mackerel (*Scomber japonicus*)[1]	3	1.5 – 3.0	1.2 – 2.6	0.09 – 0.2	0.3 – 1.1	0.7 – 1.6	
Squid (*Loligo spp.*)[1]	2	0.11 – 0.17	1.1 – 1.2	0.21 – 0.23	0.8 – 0.9	0.6 – 1.1	
Free-ranging diet species							
Striped mullet (*Mugil cephalus*) [3]	3	4.5 ± 0.5	2.7 ± 0.6	0.2 ± 0.08	0.8 ± 0.4	1.1 ± 0.06	
Striped mullet[4]	ND	3.1	2.2	0.07	0.19	0.70	0.82
Pinfish (*Lagodon rhomboides*)[4]	ND	3.9	2.7	0.06	0.16	0.50	0.85
Pigfish (*Orthopristis chrysoptera*)[4]	ND	4.8	3.3	0.10	0.40	0.90	1.1

*References: [1]Bernard 2002 N.A.G. husbandry manuals; [2]Corse 1999 N.A.G. proceedings; [3]Crissey 1994 A.Z.A. penguin husbandry manual; [4]Slifka 2013 Int.J.Vet.Med.

†Nutrient concentrations are reported as means ± one standard deviation or ranges.

‡DM, dry matter; Ca, calcium; P, phosphorous; Mg, magnesium; Na, sodium; K, potassium; ND, not described

Table 2-5. Reported equations used to determine the energy requirements for common bottlenose dolphins.

Equation	Reference	Average dolphin energy requirement (kcal/day)*
Resting metabolic rate†		
70 kcal·kg$^{-0.75}$·d^{-1}	Kleiber 1932	3,150
21 - 30 kcal·kg^{-1}·d^{-1}	Yeates 2008	3,360 – 4,800
40 - 70 kcal·kg^{-1}·d^{-1}	Williams 2001	6,400 – 11,200
Field metabolic rate		
Winter: 98 kcal·kg^{-1}·d^{-1}	Costa 2013	98 · 160 = 15,680
Summer: 140 kcal·kg^{-1}·d^{-1}	Costa 2013	140 · 160 = 22,240

*Sample calculation was performed assuming a common bottlenose dolphin weighing 160 kg.
† kg, body weight

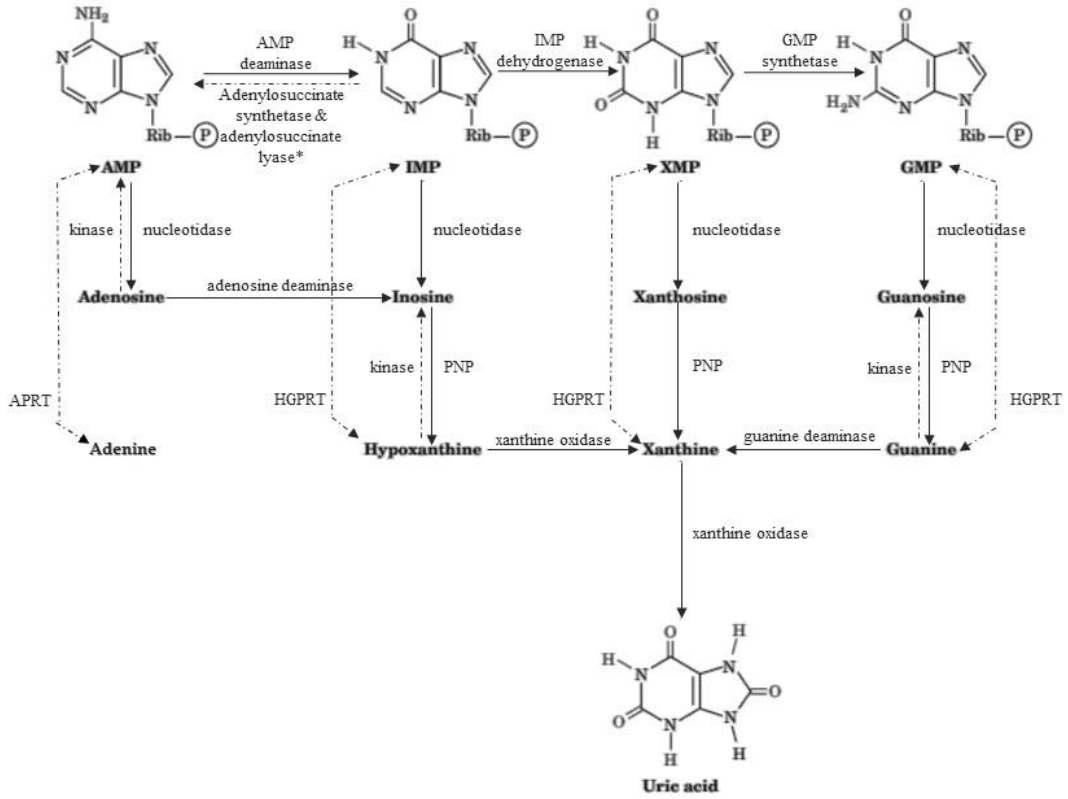


Figure 2-1. Degradation and salvage pathways of purines compiled from several resources (Green 1972; Datta 1994; Voet 2011; Nicholson 2013; Jurecka 2015). Black solid lines represent degradation pathway and black dotted lines represent salvage pathway. AMP, adenine monophosphate; IMP, inosine monophosphate; XMP, xanthine monophosphate; GMP, guanine monophosphate; APRT, adenosine phosphoribosyl transferase; HGPRT, hypoxanthine-guanine phosphoribosyl transferase; PNP, purine nucleoside phosphorylase.

CHAPTER 3
INVESTIGATING THE DIET OF BOTTLENOSE DOLPHINS

Introduction

Bottlenose dolphins, *Tursiops truncatus*, are carnivores that consume a diet of whole fish and invertebrates. The nutrient composition of the managed dolphin diet may differ from the free-ranging dolphin diet because fish nutrient content varies with species, sex, and age of the fish, and the season and location where fish are caught.[110, 118] In particular, the energy density can vary between fish species because the amount of fat and water can vary widely among fish. Water provides no energy, and fat provides more than twice the energy per gram as protein or carbohydrate. Additionally, fish fed to managed dolphins are frozen, stored and thawed, which may cause water and vitamin loss and fat oxidation.[117, 119, 227, 228] Water lost during processing may impact the dolphins' hydration status because sea water has a high salt content and the diet is an important alternative source of water.[113, 209, 214, 229] Changes in the energy density and nutrient content of fish consumed could affect body condition, the intake of vitamins and other essential nutrients, and the overall health of managed dolphins.[2, 96, 230, 231] Thus, it is important to accurately represent differences in nutrient composition among fish fed to managed dolphins.

The nutrient composition of diets can be reported in three ways: either "as fed", relative to dry matter (DM), or relative to metabolizable energy (ME).[232] "As fed" analyses report nutrient composition relative to the total weight of the food with water

---

This chapter has been previously published: Ardente AJ, Hill RC. The nutrient composition of the diet of bottlenose dolphins (*Tursiops truncatus*) is better assessed relative to metabolizable energy than dry matter. J of Zoo Wild Med. 2015; 46(2): 198-204.

included, but “as fed” nutrient comparisons can be misleading because water ‘dilutes’ nutrients to varying degrees among foods. Dry matter analyses take account of differences in water content and are useful for comparing forage-based herbivore diet that differ in water content but not ME density. Herbivore diets are also bulky and provide little energy so DM intake limits energy intake. In contrast, carnivores consume energy dense foods of variable ME density. Thus, ME intake, not DM, limits the amount of food that can be consumed if animals are to maintain a lean body condition.[203, 204] In carnivores, therefore, comparing nutrient concentrations relative to ME, not DM, provides the best measure of differences in nutrient composition of food.[203]

Reporting the nutrient composition relative to ME has become standard practice when evaluating the diets of dogs, cats, and human beings, but most published reports have compared the nutrient composition of whole fish fed to dolphins on a DM basis.[109, 110, 112, 113] These reports of fish composition do not account for ME density differences among fish and how those differences may alter nutrient intake depending on the amount of food a dolphin is consuming.

Thus, the first purpose of this paper is to illustrate that the ME content varies widely among fish consumed by dolphins and must be considered when determining how many fish by weight should be fed to dolphins to maintain a lean body condition. Secondly, this paper will demonstrate that that the nutrient composition of fish consumed by dolphins should be compared relative to ME, not DM, in order to more accurately determine the nutrient content of the diet. By adopting such a practice, managers will be better able to assess the diet and maintain the health of the dolphins under their care.

Materials and Methods

Two studies were selected which compared fish nutrient composition on a DM basis. The first study compared proximate analyses of two fish species commonly fed to managed dolphins, capelin (*Mallotus sp.*) and herring (*Clupea sp.*), and two fish species consumed by free-ranging dolphins, inland silverside (*Menidia beryllina*) and striped mullet (*Mugil cephalus*).[112] The second study compared the nutrient content of herring and capelin with three fish species commonly consumed by free-ranging dolphins in Sarasota Bay, Florida: pinfish (*Lagodon rhomboides)*, pigfish *(Orthopristis chrysoptera)*, and mullet.[113]

Ash content was not reported in the second study and carbohydrate was not measured in either study. To estimate the ME content of fish, therefore, two preliminary calculations had to be performed. Firstly, in the second study, total ash content was estimated as the sum of all reported mineral concentrations.[113] Secondly, in both studies, the carbohydrate content was estimated as nitrogen free extract (NFE), by subtracting the weight of crude protein, crude fat, ash and moisture from the total weight of the diet. The ME density of each fish species was then calculated using Atwater factors that are used for estimating the ME density of human food. These factors assume that protein provides 4 kcal ME/g crude protein, fat provides 9 kcal ME/g crude fat, and carbohydrate provides 4 kcal ME/g NFE.[203, 204] The ME density was calculated both relative to wet weight, i.e., “as fed”, and relative to DM, and then the nutrient content of each fish was calculated relative to ME.

In order to make nutrient content comparisons between fish species, the percent change in nutrient composition between fish species was calculated for both DM and ME analyses. Then, the percent change for each nutrient was compared between DM

and ME analyses to ascertain the magnitude of nutrient content discrepancies between the two methods of analysis. Statistical comparisons were not performed because only a single percent change was obtained for each nutrient compared.

To limit the number of comparisons while still illustrating the discrepancies between DM and ME analyses, the percent change in nutrient content for each fish species is compared with one baseline species, herring, in Tables 3-1 and 3-2. Herring was chosen as the baseline species because it was included in both reviewed studies and is a fish commonly fed to managed dolphins. Also, herring had the highest ME relative to DM and, therefore, best illustrates the difference between DM and ME analyses.

Finally, to better represent the water available to dolphins consuming fish, the total water provided by each fish species was calculated as the sum of the tissue moisture and the metabolic water generated during the oxidation of major nutrients.[203] Metabolic water was estimated as the sum of 0.41 mL/g crude protein, 1.07 mL/g crude fat, and 0.6 mL/g NFE.[219] The water provided by each fish species was then also reported relative to ME.

Results

The ME densities, total water content, and nutrient composition of fish from the two studies are reported in Tables 3-1 and 3-2. The percent difference in nutrient content for each fish species relative to herring is also shown.

The “as fed” ME density was greatest for herring (1.5 and 1.3 Mcal/kg, in the two studies respectively) and lowest for capelin (0.9 and 0.8 Mcal/kg, respectively). Herring contained more fat and less moisture than the other species, which contributed to its greater ME content. The ME density relative to DM was also greatest in herring (5.3 and

4.8 Mcal/kg DM, in the two studies respectively), lower in capelin (4.7 and 4.3 Mcal/kg DM, respectively) and lowest for inland silverside (3.8 Mcal/kg DM). Thus, capelin and other fish species contained up to 37% less ME/kg “as fed” and up to 28% less ME/kg DM than herring.

Total water available relative to ME was greatest for capelin (860 and 990 mL/Mcal ME, in the two studies respectively) and least for mullet (570 and 420 mL/Mcal ME, respectively). Herring also provided comparatively little total water (490 and 600 mL/Mcal ME, in the two studies respectively). Thus, capelin provides 64-74% more total water than herring on an ME basis but only appeared to provide 8-10% more moisture when these fish species were compared on an as fed basis.

For nutrient content comparisons between fish species, the disparity in percent difference between the DM and ME analyses was proportional to the difference in ME density relative to DM. When the ME density relative to DM was the same among fish species, the percent difference in nutrient content was the same for DM and ME analyses. However, the percent difference was often substantially different between DM and ME analyses when ME density relative to DM differed markedly between fish species. For example, in the second study, both pinfish and capelin provide about 4.3 Mcal/kg DM and therefore percent differences between DM and ME analyses were comparable. The calcium content on a DM basis was 15.9 g/kg DM in capelin and 39 g/kg DM in pinfish, a percent increase of 145%. A similar percent increase of 141% was found when the calcium content was compared on an ME basis (from 3.7 g/Mcal ME in capelin to 8.9 g/Mcal ME in pinfish). In the first study, however, the ME density relative to DM was 28% less in inland silverside than in herring because there was 69% less

crude fat and 162% more ash on a DM basis in inland silverside than in herring. As a consequence, the percent difference in nutrient content between herring and inland silverside differed substantially on an ME basis from that on a DM basis. For example, inland silverside contained only 8% more crude protein than herring on a DM basis but 50% more protein on an ME basis.

If a fish species contained more of a certain nutrient than herring on a DM basis, the percent difference was even greater when ME density was taken into account. For example, in the second study, the ME density relative to DM was up to 15% greater in herring than other fish species, with the greatest difference between herring and pigfish. As a consequence, pigfish contained 3.2 times more calcium than herring on a DM basis, but 3.8 times more calcium on an ME basis; pigfish also contained 14% more sulfur than herring on a DM basis but contained 34% more sulfur on an ME basis.

On the other hand, if a fish species contained less of a certain nutrient than herring, then the greater ME density of herring reduced the percent difference between the two fish species. For example, pigfish contained 63% less sodium, 25% less potassium, and 11% less magnesium than herring on a DM basis but contained only 56% less sodium, 12% less potassium, and 5% *more* magnesium than herring on an ME basis.

Discussion

This comparison revealed the marked differences in ME density of fish fed to managed and free-ranging dolphins because water, ash and fat content differed among different fish species. Capelin, for example, contained one third less energy per gram than herring because it contained one third less fat (DM basis) and 10% more water ("as fed" basis) than herring. Thus to meet a dolphin's energy requirement, managers

must feed more capelin by weight if capelin are substituted for herring in the diet. Energy density can also vary between fish of the same species because nutrient composition changes with handling practices and the season and location where fish are caught.[110, 118] The ME density of fish fed to dolphins at one facility over a 3 year period varied from 0.7-1.4 Mcal ME/kg “as fed” for capelin and 1.2-1.6 Mcal ME/kg “as fed” for herring. (Ardente, A.J. unpubl. data) It is important, therefore, to obtain a proximate analysis of each batch of fish and to calculate the average as fed ME density using Atwater factors before deciding how much to feed.

The ME density of fish was estimated using Atwater factors that are used for estimating the ME density of foods consumed by people and dogs.[203, 204, 233] These factors assume 91% digestibility for protein and 96% digestibility for fat and carbohydrate.[203, 204, 233] The digestibility of whole fish fed to dolphins and the amount of energy dolphins retain from their food has yet to be determined. Nevertheless, dolphins have a similar gastrointestinal anatomy to pinnipeds and pinnipeds digest protein and fat with similar efficiency to domestic dogs fed an unprocessed diet.[170, 234-236] It seems reasonable, therefore, to estimate the ME density of fish fed to dolphins using Atwater factors.

This paper also shows that changes in ME density can markedly affect nutrient intake and that a DM analysis does not necessarily give a good representation of nutrient intake. The ME requirement of a dolphin, not DM, determines how many Mcal must be taken in to maintain body condition, so changes in ME density relative to DM will greatly affect DM intake. Thus, comparing nutrient composition relative to ME gives a better representation of differences in nutrient intake than comparisons on a DM

basis. This paper shows that there is a marked discrepancy between nutrient percent differences on a DM versus ME basis when the ME density relative to DM differs between the two fish. To give a practical example, the difference in crude protein content between inland silverside and herring was only 8% on a DM basis, which substantially underestimated the 50% difference evident on an ME basis. Thus, an average healthy adult dolphin consuming 10 Mcal/day would consume 1.1 kg protein if fed exclusively herring and 1.7 kg protein (i.e. 50% more protein) if fed exclusively inland silverside. Such discrepancies need to be recognized because they may be metabolically important. More protein produces more urea and acid, which subsequently must be excreted in the urine.[176, 203, 237] Similarly, differences in the calcium, magnesium and sulfur content between pigfish and herring were substantially underestimated when assessed relative to DM. These minerals may be absorbed and then excreted in the urine, so changes in dietary amounts could impact urine pH and saturation and thus the potential for urolith formation. An average adult dolphin consuming 10 Mcal/day, for example, would ingest 31 g of calcium daily if fed exclusively herring but as much as 118 g of calcium if fed only pigfish.

This report also shows that sodium and water content relative to ME differs markedly among fish species. Bottlenose dolphins live primarily in a hyperosmotic environment. Unless dolphins frequent estuaries and rivers, they do not have access to freshwater. Water can only be obtained, therefore, from the moisture in food, from the metabolism of food, or by ingestion of sea water.[180] This water intake has to compensate for water excreted in the urine or lost through the skin by osmosis.[209] Any sodium and chloride taken in by mouth in the diet or as seawater also has to be

excreted in urine or feces.[180] Any seawater consumed is entirely excreted causing diuresis; therefore the amount of free water from food and its relative proportion to protein and minerals in the diet is critically important.[180, 183, 238] This study shows that the amount of dietary water varied widely relative to ME. For example, an average healthy adult dolphin consuming 10 Mcal of fish per day would obtain approximately 5 L of water from its food per day if it was fed exclusively herring or approximately 9 L of water per day if fed exclusively capelin. This difference may affect the concentration of solutes in the urine, but can only be appreciated using an ME based comparison.

This paper also confirmed that fish species fed to managed dolphins contain more sodium and potassium relative to ME than the fish consumed by free-ranging dolphins. Slifka et al. attributed these differences to several factors, including the fish processing methods, but the DM analysis did not accurately represent the degree of difference. Fish distributors generally apply a brine solution to the fish during processing prior to packaging and freezing.[113] Brining is a common practice because salt acts as a preservative and increases palatability and tenderness.[102] This difference between managed diet fish species and free-ranging diet fish species is potentially important because free-water has to be excreted with any sodium and chloride consumed.[180] A dolphin consuming 10 Mcal/day of a 50:50 capelin: herring diet (average 1 Mcal/kg) would consume 20 g sodium and approximately 8 L of water; however, the same dolphin consuming a diet of capelin and mullet (average 1.2 Mcal/kg) would consume only 12 g sodium and 7 L of water.

In summary, this comparative analysis shows that fish differ markedly in “as fed” ME density because water, ash and fat content varies greatly among fish. The ME

density determines how much a dolphin must consume to maintain body condition so managers must adjust the weight of fish fed to dolphins to accommodate changes in the “as fed” ME density of fish. In addition, nutrient comparisons made on a DM basis often do not accurately represent differences evident when nutrient concentrations are evaluated relative to ME. Differences in the nutrient content relative to ME of fish fed to managed dolphins may have important health implications.

Table 3-1. Metabolizable energy and nutrient comparisons among fish fed to managed dolphins and fish consumed by free-ranging dolphins, on as 'as fed', dry matter, and energy basis (Reference Corse et al. data)

Proximate analysis	Herring Nutrient content		Capelin Nutrient content		Capelin Percent change from herring		Inland Silverside Nutrient content		Inland Silverside Percent change from herring		Striped mullet Nutrient content		Striped mullet Percent change from herring	
	/kg as fed	/Mcal ME	/kg as fed	/Mcal ME	as fed	ME	/kg as fed	/Mcal ME	as fed	ME	/kg as fed	/Mcal ME	as fed	ME
Metabolizable energy (ME, Mcal)	1.49		0.94		-37%		1.01		-33%		1.28		-15%	
Dry matter (DM, g)	282	189	202	214	-28%	13%	262	261	-7%	38%	287	225	2%	19%
Moisture (g)	718	481	799	846	11%	76%	738	733	3%	53%	713	559	-1%	16%
Metabolic water (mL)	17	11	10	11	-38%	-1%	12	12	-28%	6%	15	11	-13%	2%
Total water (mL)	735	492	809	857	10%	74%	750	745	2%	51%	725	570	-1%	16%
	/kg DM	/Mcal ME	/kg DM	/Mcal ME	DM	ME	/kg DM	/Mcal ME	DM	ME	/kg DM	/Mcal ME	DM	ME
Metabolizable energy (ME, Mcal)	5.3		4.7		-12%		3.8		-28%		4.4		-16%	
Crude protein (g)	594	112	673	144	13%	28%	643	168	8%	50%	580	131	-2%	17%
Crude fat (g)	310	58	191	41	-38%	-30%	95	25	-69%	-58%	160	36	-48%	-38%
Ash (g)	95	12	100	15	6%	25%	248	27	162%	129%	235	20	148%	70%
Nitrogen free extract (g)	2	7	36	14	1685%	121%	14	42	610%	536%	25	39	1155%	489%

Table 3-2. Metabolizable energy density and nutrient comparisons among fish fed to managed dolphins and fish consumed by free-ranging dolphins, on as 'as fed', dry matter, and energy basis (Reference Slifka et al. data)

	Managed Diet Fish Species						Free-ranging Diet Fish Species											
Water and Mineral Content	Herring Nutrient content		Capelin Nutrient content		Capelin Percent change from herring		Pinfish Nutrient content		Pinfish Percent change from herring		Pigfish Nutrient content		Pigfish Percent change from herring		Mullet Nutrient content		Mullet Percent change from herring	
	/kg as fed	/Mcal ME	/kg as fed	/Mcal ME	as fed	ME	/kg as fed	/Mcal ME	as fed	ME	/kg as fed	/Mcal ME	as fed	ME	/kg as fed	/Mcal ME	as fed	ME
Metabolizable energy (Mcal)	1.25		0.83		-34%		1.42		13%		1.00		-20%		1.58		27%	
Dry matter (DM, g)	258		191		-26%		324		26%		244		-5%		35		38%	
Moisture (g)	742	594	809	981	9%	65%	676	478	-9%	-20%	756	752	2%	27%	645	407	-13%	-31%
Metabolic water (mL)	14	11	9	11	-34%	-0.4%	18	13	27%	12%	11	11	-19%	1%	18	12	32%	4%
Total water (mL)	756	605	818	992	8%	64%	694	490	-8%	-19%	767	764	2%	26%	663	419	-12%	-31%
	/kg DM	/Mcal ME	/kg DM	/Mcal ME	DM	ME	/kg DM	/Mcal ME	DM	ME	/kg DM	/Mcal ME	DM	ME	/kg DM	/Mcal ME	DM	ME
Metabolizable energy (Mcal)	4.8		4.3		-11%		4.4		-10%		4.1		-15%		4.5		-8%	
Calcium (g)	15.1	3.1	15.9	3.7	6%	18%	39	8.9	158%	186%	48.6	11.8	222%	279%	31.2	7.0	107%	124%
Phosphorous (g)	15.8	3.3	18.9	4.4	20%	34%	27.2	6.2	72%	90%	33.4	8.1	111%	148%	22.4	5.0	42%	54%
Sodium (g)	12.1	2.5	7.4	1.7	-39%	-31%	1.6	0.4	-87%	-86%	4.5	1.1	-63%	-56%	1.9	0.4	-84%	-83%
Potassium (g)	13.1	2.7	17.6	4.1	34%	51%	5.7	1.3	-56%	-51%	9.8	2.4	-25%	-12%	7.1	1.6	-46%	-41%
Magnesium (g)	1.2	0.2	0.9	0.2	-23%	-14%	0.6	0.1	-47%	-41%	1.0	0.3	-11%	5%	0.7	0.2	-35%	-30%
Sulfur (g)	10.1	2.1	11.1	2.6	9%	23%	8.6	2.0	-15%	-6%	11.5	2.8	14%	34%	7.6	1.7	-25%	-18%
Zinc (g)	0.08	0.02	0.08	0.02	1%	13%	0.05	0.01	-35%	-28%	0.05	0.01	-31%	-19%	0.06	0.01	-26%	-20%

CHAPTER 4
NUTRIENT DIFFERENCES BETWEEN DIETS CONSUMED BY FREE-RANGING COMMON BOTTLENOSE DOLPHINS (*TURSIOPS TRUNCATUS*) AND COMMON BOTTLENOSE DOLPHINS UNDER HUMAN CARE MAY EXPLAIN WHY SOME DOLPHINS ARE PRONE TO DEVELOP AMMONIUM URATE NEPHROLITHS

Introduction

Nephroliths composed of 100% ammonium urate have been reported to affect common bottlenose dolphins, *Tursiops truncatus*, managed under human care in facilities across the United States, but nephroliths are rare in free-ranging bottlenose dolphins.[1] These nephroliths can cause azotemia, ureteral and urethral obstruction, hydronephrosis, and renal failure.[1-3] Nephroliths have been identified using ultrasound and computed tomography (CT) imaging in 35% of dolphins in one managed population.[1] The reason for the difference in prevalence of nephroliths between managed and free-ranging dolphins is unknown, but the nutrient composition of the diet can influence ammonium urate urolith formation in other mammals, and may also affect urate stone formation in dolphins.[5, 8]

The tendency for ammonia and urate to complex and precipitate as ammonium urate crystals is determined by the relative concentrations of these solutes in urine, as well the presence of other solutes, and urine pH.[4, 10, 185] Given enough time and appropriate conditions, crystals may then aggregate to form stones.[10] Uric acid is a product of purine metabolism and whole fish, which comprise the bulk of the dolphin diet and are purine-rich.[239] Ammonium ions, produced by the action of glutaminase on glutamine, provide a mechanism by which protons are excreted by the kidney. In acidosis, the kidney excretes greater amounts of ammonia in urine to eliminate protons.[19, 196] The relative proportions of anions and cations from the diet that are excreted in the urine affects the number of protons and, thus, ammonium ions excreted

in urine. Absorption and excretion of positively charged cations, such as sodium, potassium, calcium, and magnesium, tend to make urine more alkaline, whereas excretion of negatively charged anions, such as chloride, phosphate, and sulfate, tend to make urine more acidic.[15, 32] Thus, consumption and metabolism of increased dietary protein, which generates sulfate and phosphate anions, makes urine more acidic.[197-199] It is possible, therefore, to obtain an indication of how a change in diet might affect the average pH of urine and excretion of ammonium ions by comparing the relative concentration of cations and anions in the diet and hence, the risk of forming uroliths. This dietary cation-anion difference (DCAD) has also been called the dietary anion gap (DAG) or potential renal acid load (PRAL).[36, 37] It has been used to design diets that alter urine pH to reduce the risk of urolith formation in cats, dogs, and people, and also to create diets that facilitate the release of calcium from bone in lactating cows.[37, 240, 241] It is possible, therefore, that an increase in anions relative to cations (a decrease in DCAD) in the diet of bottlenose dolphins under human care could increase proton and ammonium ion excretion, and increase the risk of ammonium urate nephrolith development.

All bottlenose dolphins consume a diet consisting primarily of whole fish, but fish nutrient content varies with species, sex, and age of the fish, and the season and location where fish are caught.[110, 118]Different fish are fed to managed dolphins than those consumed by free-ranging dolphins: managed dolphins are primarily fed a few cold-water species that are commercially caught during specific times of year, frozen, stored and then thawed before feeding, whereas free-ranging dolphins consume a wide variety of live, temperate water fish species across all seasons.[149, 161, 242] A brine

solution is also often applied to commercial fish fed to managed dolphins before the fish are sealed in packages and frozen. The fish are caught primarily for human consumption and brine is applied to protect the fish from freezer burn during frozen storage and to enhance the flavor, texture and palatability of the fish.[102, 104]The liquid applied depends on the commercial fishery and can consist of tap water, sodium chloride, or electrolyte-based solution and could affect the DCAD of the diet.[101, 243] All of these handling and storage processes are well-documented to effect water content, protein and fat oxidation, mineral content, and vitamin loss in food.[117, 228] Urine solute concentrations are also affected by the amount of water excreted in urine, which is dependent on the amount of water consumed either with food, by drinking, or from metabolism of dietary protein, fat, and carbohydrate.[24] Sodium in the diet and urea, from the metabolism of dietary protein, can also influence the amount of water excreted and may affect urine solute concentrations.[180, 244]

Differences in the nutrient composition of the diets consumed by the two dolphin populations may explain, therefore, why managed dolphins are prone to form ammonium urate nephroliths; however, the nutrient composition of whole fish consumed by free-ranging dolphins is unknown. Furthermore, the few reports of the nutrient composition of whole fish fed to dolphins under human care do not report enough information to assess DCAD.[110, 113] Thus, the purpose of this study was to measure and compare the nutrient composition of fish and one invertebrate typically consumed by the two populations of dolphins. Our hypothesis was that nutrient composition of a typical diet consumed by managed dolphins differs from that of a typical diet consumed by free-ranging dolphins, particularly with respect to metabolizable energy, protein, fat,

water, minerals, and DCAD. Further, we hypothesized that nutrient differences would follow trends that might promote ammonium urate nephrolith formation in dolphins under human care.

Methods

Sample Collection and Processing

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

The eight fish species most commonly consumed by free-ranging bottlenose dolphins residing in Sarasota Bay, FL ('free-ranging species'), including four abundant and four soniferous species (Table 4-1), were selected to represent the free-ranging dolphin diet.[148, 161, 245] Samples of these fish were caught between May and September 2013 from the waters off the west coast of Florida using a rod and reel, crab trap, cast net, or with a purse-seine net in Sarasota Bay. To mimic the rapid death of fish consumed by wild dolphins as closely as possible, fish were euthanized humanely by immersion in a bath containing 500ppm tricaine methanesulfonate (MS 222, Western Chemical, Ferndale, WA 98248). When death was confirmed by cessation of opercular movement for 10 minutes, fish were weighed and length was measured. Fish were then individually bagged and transported in dry ice to the UF laboratory where fish were stored at -80ºC for a maximum of 6 months before further processing.

Six fish species and one species of squid commonly fed to dolphins under human care ('managed species') were supplied by two bottlenose dolphin management facilities (Table 4-2). Fish and squid had been caught during one commercial fishing season, wrapped in plastic and frozen stored at -18ºC for 6 to 9 months. Then they were

shipped overnight on dry ice to the UF laboratory where they were stored at -20ºC, for a total frozen stored time of 6 to 9 months before further processing.

Five separate samples of each species were analyzed. To provide sufficient material to perform all the analyses on every sample, a minimum of 2 individual fish (or squid) were included in each sample; however, 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. The five samples of each species were individually ground using commercial meat grinders with 4.5 and 10 mm plates (Biro 6642, Marblehead, OH 43440, and 1.5 HP, LEM Products, West Chester, OH 45011).

Free-ranging fish species were thawed the minimum amount needed to allow grinding, whereas managed diet fish species were thawed more completely using the standard operating procedure of one dolphin management facility. Free-ranging fish species were air thawed in a temperature controlled cold room (11-12ºC) for approximately 1 hour, until fish thawed to a firm, slightly malleable texture. Managed diet species were air thawed in the cold room (11-12ºC), wrapped in plastic, for approximately 20 hours. Then individuals of each species were removed from their plastic bags and rinsed with cold water (approximately 16ºC). Both minimally and well-thawed fish or squid were then transported to a grinder in a cooler containing ice. Grinder equipment was thoroughly rinsed with water between each sample. Ground samples were homogenized by hand and stored in sample bags (Whirl-Pak® bags, Nasco, Fort Atkinson, WI 53538) at -80ºC until shipped overnight on dry ice to each laboratory for analysis.

Nutrient Analysis

Gross energy and nutrient analyses were performed by a commercial laboratory (Dairy One, Ithaca, NY 14850). Gross energy (GE) density (kcal/g) was measured using a bomb calorimeter (IKA C2000 basic Calorimeter System, IKA Works, Inc., Wilmington, NC). Crude protein (CP) was measured with a nitrogen/protein analyzer (AOAC 992.15, Leco FP-528, Leco Corporation, St. Joseph, MI 49085). Crude fat (CF) was measured by ether extraction (AOAC 2003.05), ash by combustion (AOAC 942.05), and moisture (dry matter) by oven drying (AOAC 930.15). When these macronutrients were added together, the total obtained was greater than 100% of the analyzed sample in all species. This suggested that the method used to measure CP over-estimated protein content and that all species contained negligible amounts of carbohydrate. Protein content was calculated, therefore, by difference from 100% and carbohydrate content was assumed to be zero. Calcium (Ca), phosphorus (P), magnesium (Mg), sodium (Na), potassium (K), and sulfur (S), were determined using an Inductively Coupled Plasma (ICP) Radial Spectrometer (Thermo iCAP 6300, Fisher Scientific, Waltham, MA 02454) after microwave digestion in a Microwave Accelerated Reaction System (CEM, Matthews, NC 28106). Chloride (Cl^-) was measured by potentiometric titration with silver nitrate and a silver electrode (Brinkmann Metrohm 716 Titrino Titration Unit, Metrohm USA, Riverview, FL 33578). The laboratory was blind as to the source of each sample duplicate.

The metabolizable energy (ME) density of each fish species was calculated using Atwater factors[246] and then the nutrient content of each fish was calculated relative to ME. Total water (TW) content was calculated by adding moisture in food to metabolic

water, calculated as the sum of 0.41 mL/g protein and 1.09 mL/g fat.[203] Ratios (v/w) of total water to protein and sodium content were also calculated.

The DCAD (mEq/Mcal) was calculated using four equations (Table 4-3). The first two equations, $DCAD_{short}$ and $DCAD_{long}$, have been found to have utility in several species but do not take account of differences in absorption of minerals.[33, 34, 36] $DCAD_{human,}$ which uses absorption coefficients derived from human studies, has been used to estimate the potential renal acid load (PRAL) in people. This equation was modified slightly because the sulfur in the fish and squid species analyzed was measured directly rather than being estimated from the sulfur-containing amino acid content of the diet.[37] Protein, from which most sulfur is probably derived, appears to be well digested by marine mammals, which is why Atwater factors were used to calculate ME.[246] The absorption of sulfur was assumed, therefore, to be the same (91%), as the digestibility of protein on which the Atwater factor for protein is based.[203] Human beings are not pure carnivores like dolphins, however, so a fourth equation was used to calculate $DCAD_{cat}$, using values for the apparent absorption of minerals obtained from studies of another pure carnivore, the domestic cat. Specifically, absorption of sodium, potassium, and chloride are reported to be greater than 90% in adult cats and 95% in human beings, so an absorption coefficient of 95% was used for these minerals.[37, 203] Absorption of sulfur was again assumed to be 91%. The absorption coefficients of 25% for Ca and 35% for P were obtained from linear regression equations correlating the concentration of dietary Ca and P to absorption in adult cats.[247] The Mg absorption coefficient was assumed to be 25% because the calcium content of whole bony fish is

high, and Mg absorption decreases in adult cats from 40% to 25% with increasing calcium in the diet.[203]

Model Dolphin Diets

Two model managed dolphin diets were formulated based on the relative proportions of fish species fed by two dolphin management facilities (Table 4-4). Facilities vary the total amount of fish or squid fed to dolphins to provide enough calories to maintain body weight or growth rate but wet weight of fish determines the relative proportion of each species that makes up the total diet.[246] To determine the proportion of the total ME of the diet provided by each species, therefore, the percent as fed weight (g/100g of diet) of each species was multiplied by the average 'as fed' ME density of that species. The ME contribution of each species was then calculated as a percent of the total Mcal provided by all the species in 100g of diet.

A model free-ranging bottlenose dolphin diet (Table 4-5) was derived from the proportions of fish species reported to be consumed by bottlenose dolphins in Sarasota Bay, FL.[132] Investigators have reported the number of each fish species or family group of fish as a percentage of the total number of fish consumed based on stomach content analyses, observation, and prey abundance studies conducted over more than 20 years.[132] Such reports have grouped some fish species into families. For example, Atlantic threadfin herring and menhaden have been grouped together as the Clupeid family, and pinfish and sheepshead have been grouped under the Sparid family. For the model diet, Atlantic threadfin herring and menhaden were assumed to contribute equally to the total number of Clupeid fish consumed, whereas 35% of pinfish and 3.1% of sheepshead were assumed to make up the total Sparid fish consumed because 11 times more pinfish are generally consumed than sheepshead.[149, 161, 162] In addition,

percentages of all of the fish species added together gave a total of only 85%. The additional 15% probably represents other unnamed species in the diet. For the model diet, the percent of each documented fish species was increased proportionately so that the total percent of all of the fish was 100. The ME provided by each fish species to the diet was then calculated by multiplying the percentage of each species in the diet by the average weight (g) of that fish species caught for this study and by the average ME density of that fish species (Mcal/kg as fed). The ME provided by each fish species was then calculated as a percentage of the total ME provided by all of the fish.

Statistical Analysis

Statistical comparisons were performed using statistical software (SAS® for Windows software version 9.4, SAS Institute Inc., Cary, NC, 27513) 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. Least square means were used to compare nutrient contents among model diets (SAS procedure lsmestimate).

Results

Species consumed by free-ranging dolphins contained more DM, protein and ash, less fat and TW, and the ME density was greater ($p \leq 0.05$) than in species fed to dolphins under human care (Tables 4-6 and 4-9). Nutrient concentrations also varied widely among individual fish and squid species ($p \leq 0.05$; Table 6). Spot and Pacific herring provided up to three-fold more ‘as fed’ ME, up to five-fold more CF and up to three-fold less protein relative to ME than other species. Gulf toadfish and Loligo squid

were the least energy dense and contained five-fold less CF and three-fold more protein relative to energy than other species.

All mineral concentrations, except S, differed significantly between the two groups of fish species ($p \leq 0.0001$) (Tables 4-7 and 4-9). The Ca and P concentrations were four and three-times greater, respectively, in free-ranging diet fish species than in managed diet fish species. Managed diet species contained 60% more Cl and 20% more Na than free-ranging species. In particularly, Canadian capelin contained up to three-fold more Na and up to eight-fold more Cl compared with other managed and free-ranging species.

The managed diet species provided more water relative to energy and crude protein compared to the free-ranging diet species, whereas the free-ranging diet species provided more water relative to sodium ($p \leq 0.05$; Tables 4-8 and 4-9). Total water relative to ME, protein, and Na also varied widely among individual species ($p \leq 0.05$; Table 4-8). Specifically, Loligo squid and Gulf toadfish provided up to 4 times more water per Mcal ME than other species, and spot, mullet, and Atlantic herring provided the least amount of water per Mcal ME. Mullet and spotted sea trout provided approximately twice the amount of water relative to sodium than was provided by Atlantic herring and Canadian capelin.

The DCAD calculated using the $DCAD_{short}$, $DCAD_{long}$, and $DCAD_{cat}$ equations was more positive in free-ranging diet species than for managed diet species, whereas $DCAD_{human}$ was more positive for the managed diet species than free-ranging diet species ($p \leq 0.05$; Table 8). The DCAD also varied widely among fish species within

groups depending on the equation used, but $DCAD_{long}$ was notable for being positive for all free-ranging diet fish species and negative for all managed diet species (Figure 4-1).

The managed and free-ranging model diets had comparable gross and metabolizable energy densities; however, the free-ranging model diet contained 8 to 25% more protein and 8 to 22% less fat ($p \leq 0.05$) than the managed model diets (Table 4-9). The free-ranging model diet also contained up to 400% more Ca and 150% more P than the model managed diets. On the other hand, 'Managed diet #2' contained approximately 60% more Na and Cl than the other managed diet and 40% more Na and 100% more Cl than the free-ranging model diet. 'Managed diet #2' also had 25-30% less TW relative to Na than the other two diets, and the model free-ranging diet had 13 to 20% less TW relative to protein than the model managed diets.

DCAD results varied depending on the equation used (Table 4-9). The $DCAD_{long}$ was strongly positive for the model free-ranging dolphin diet but strongly negative for both model managed diets. All other DCAD equations gave negative DCAD values for both managed and free-ranging diets, but $DCAD_{cat}$ was 14-30% less negative for the model free-ranging diet compared to the model managed diets and $DCAD_{short}$ was 25% less negative for the free-ranging diet than 'Managed diet #2' ($p \leq 0.05$). On the other hand, $DCAD_{human}$ was 10-20% more negative for the model free-ranging diet compared to the model managed diets. Furthermore, the nutrient content varied significantly for almost all nutrients between the managed model diets and the average managed diet and for the free-ranging model diet and the average free-ranging diet ($p \leq 0.05$). 'Managed diet #1' for example, was as much as 20% lower in protein and higher in fat than the average managed diet.

Discussion

To our knowledge, this study is the first to compare the nutrient content of the free-ranging dolphin diet to that of two diets commonly fed to dolphins under human care. Furthermore, previous studies have only compared the nutrient content of a few individual fish consumed by each group of dolphins and have not taken into account individual variation within a fish species or the relative proportions of each fish species in the total diet.[110, 113] We measured the nutrient composition of five samples of a wide range of species commonly consumed by dolphins in Sarasota Bay, FL. This enabled us to create a model diet based on the documented frequency that the fish species are found in the stomach of dolphins in that location. We also measured the nutrient composition of five samples of a range of fish and squid species fed to two large groups of dolphins under human care, which allowed us to establish the nutrient content of model diets fed at those two facilities and compare their composition to that of the model free-ranging diet. Comparison of the model diets allowed us to evaluate whether differences among the diets could explain why ammonium urate nephrolithiasis has been found in managed but not free-ranging bottlenose dolphins. The nutrient requirements of dolphins are currently unknown but the free-ranging model diet also provides an indication of what constitutes an adequate intake of essential nutrients. Thus, the nutrient content of the free-ranging model diet can be used as a guide to determine how the species or proportions of species fed within the managed diet should be changed in order to approximate the nutrient composition of the free-ranging model diet.

This study confirmed previous observations that the nutrient composition differs between the fish commonly consumed by free-ranging common bottlenose dolphins and

species fed to dolphins under human care. Nevertheless, individual differences among species were either magnified or minimized, depending on the percentage of each species in the model diets. Thus, the free-ranging model diet provided more ME, less total water, and greater protein than both managed dolphin model diets because it was comprised of more lean fish species, like pinfish, and fewer higher fat fish species, like spot and sheepshead. On the other hand, the managed model diets provided more fat than the free-ranging model diet because the managed diet species, like Pacific herring, generally contained more fat and contributed a larger percentage of calories to the whole diet than the fatty fish in the free-ranging diet. These changes in macronutrient composition would *not* be expected to decrease the risk of nephrolith formation, however, because the *greater* protein and *less* water in the free-ranging model diet would be expected to result in a *more* concentrated, *more* acidic urine containing more ammonia.

The tendency to form ammonium urate stones is also impacted, however, by mineral concentrations excreted in the urine. Mineral concentrations compared among fish species revealed comparatively greater concentrations of Ca and P for free-ranging species and higher concentrations of Na and Cl for managed diet species. These discrepancies in Ca, P, and Na content are similar to differences previously reported when small samples of pinfish, pigfish, and mullet were compared to capelin and herring, but Cl and consequently DCAD has not been compared previously.[113] Free-ranging fish species tend to be more bony and have teeth which would contribute to their greater Ca and P concentrations when compared to managed diet species. The greater Na and Cl content of managed diet species are likely caused by application of a

brine solution.[113] The composition of this brine solution, and the concentration in which it is applied, varies depending on the fishery, but generally contains sodium chloride at a concentration up to 25% (w/v).[248, 249] The greater Na and Cl concentrations of the managed model diets are accompanied by greater total water concentrations. For example, a large percentage of 'Managed diet #2' is comprised of Canadian capelin, which is the species with the greatest total water content and Na and Cl contents compared with all other species. Thus, the second managed model diet would generate an enhanced post-prandial diuresis as additional Na, Cl, and water are excreted in the urine, which may help to prevent ammonium urate nephrolith formation.[180]

The differences in Ca, P, Na, and Cl among free-ranging and managed diet species significantly impact the DCAD of the model diets but the value obtained depends on the equation used to calculate DCAD because each equation differs regarding the assumed relative absorption of minerals. The longest equation, $DCAD_{long}$, assumes 100% apparent absorption of *all* macrominerals, whereas the shortest equation assumes 100% absorption of Na, K, Cl, and S, but does not account for absorption of Ca, Mg, and P. It is unlikely, however, that minerals are either completely absorbed or not absorbed at all, so we also evaluated two additional equations: the $DCAD_{human}$ equation, which uses human mineral absorption coefficients; and, a $DCAD_{cat}$ equation based on macromineral absorption in adult domestic cats, which are also obligate carnivores like bottlenose dolphins.[37, 203, 247] Several authors have developed alternative equations for predicting the urine pH of cats by regressing dietary mineral and amino acid concentrations in the diet against urine pH.[250, 251] These alternative cat equations were not used because the coefficients imply more than 100% absorption of

some minerals, and the cat diets used in these studies include absorbable sources of sulfur and phosphorus that are added to the diet to lower urine pH.

The apparent gastrointestinal absorption of minerals in dolphins is unknown, so the DCAD values obtained must be interpreted very cautiously. Nevertheless, three of the equations predict that the managed diets would tend to produce on average a more acidic urine than the free-ranging diet. A more acidic urine would tend to result in more ammonium ion excretion and might explain why managed dolphins form ammonium urate nephroliths. The $DCAD_{long}$ provides the most striking difference because $DCAD_{long}$ was strongly positive for all but one of the free-ranging diet fish species, and the model diet was strongly negative for *all* of the managed diet species and model diets (Figure 1). This is partly because capelin, Pacific mackerel, and sardines fed to managed dolphins contain much more Cl than Na compared to any of the free-ranging species. More importantly, however, there was more Ca and P in the free-ranging fish, and the Ca:P ratio was about 1.6:1 for the free-ranging fish species, but only 1:1 in the managed species. The $DCAD_{long}$ reflects these differences because it assumes complete absorption of both Ca and P, whereas the other three equations reduce the effect of the increased Ca relative to P in the diet because they assume Ca and P are either not absorbed or only partly absorbed. Although $DCAD_{long}$ correlates well with urine pH in cats fed some feline diets, $DCAD_{long}$ suggests that 225 mEq/Mcal ME more cations than anions must be added to the managed model diets in order to match the free-ranging model diet $DCAD_{long}$.[34] In contrast, the $DCAD_{short}$ and $DCAD_{cat}$ equations suggest that a more reasonable addition of 10-30 mEq/Mcal ME of cations relative to anions would be sufficient achieve a similar DCAD among managed and free-ranging model diets. The

$DCAD_{human}$ suggests the opposite (DCAD is more negative in the free-ranging diet), because $DCAD_{human}$ assumes that intestinal absorption of P is three times greater than Ca absorption. When combined with the increased amount of both Ca and P in the fish consumed by free-ranging dolphins, the contribution of P anion to the diet is strongly favored over the Ca cation contribution. Unfortunately, only measuring mineral absorption in the intestine of dolphins under human care or measuring the total urinary excretion of minerals and urine pH over 24 hours during a controlled feeding trial will decide which of these DCAD equations best represents the effect of dietary acid-base balance on urine pH.

The study has several limitations. The nitrogen content of the fish measured as crude protein was likely overestimated, perhaps due to non-nitrogen sources including purines. Because the carbohydrate content of fish is negligible, we assumed the carbohydrate content of all fish species was zero and calculated the protein content based on the difference from 100, less the fat content.[252] It is likely that the nutrient content of fish depends on the location where fish are caught, as well as the species. Fish nutrient content can also vary considerably with season and frozen storage time. Within a given season, the protein and fat composition of fish changes based on water temperatures and spawning cycles.[98, 99] Due to financial constraints, nutrient analyses were only able to be performed on fish caught during one season, which was determined by practical considerations for free-ranging species collection and when commercial fisheries are active. The two managed dolphin model diets are relatively standard among management facilities, but nutrient analysis was limited to one lot, or one catch date, of each type of fish also because of financial constraints. Thus,

differences between fish lots caught at different times within a commercial catch season could not be determined, and this study did not account for seasonal variations in fish body composition. Furthermore, frozen storage time was set at 6-9 months for managed diet fish species. Frozen storage has been well-documented to affect the nutrient content of fish, particularly with respect to fatty acid oxidation and water loss; therefore, it is possible that storage times less than 6 months or greater than 9 months may have yielded different results for managed diet fish composition.[119, 253]

The free-ranging model diet also made assumptions regarding the species and relative proportions that are consumed by free-ranging dolphins. The free-ranging model diet was inferred from previous reported data because it is impractical to measure the actual intake of free-ranging dolphins but it is specific to inshore bottlenose dolphins residing in Sarasota Bay, FL.[132, 161] This population of dolphins was chosen as an example of a free-ranging population because they have been studied for more than 45 years, and there are more published reports of the fish consumed by these dolphins than any other free-ranging population. Nevertheless, this model diet does not account for individual variation based on age, sex, reproductive status, or prey preference, and other populations of free-ranging dolphins may consume diets with a different composition. It is also possible that the fish caught for this study were not representative of fish consumed by dolphins at different times of year. Nevertheless, all fish lengths fell within the reported range (50-300 mm, up to 1,027 mm) for fish consumed by free-ranging dolphins.[156, 161]

The model diets comparisons also assume an equal caloric intake among dolphin populations, whereas 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, FL, has an average daily energy requirement (measured using double labelled water method) ranging from approximately 16 Mcal/day in the winter to 22 Mcal/day in the summer.[174] Among dolphins under human care at one facility, however, non-pregnant, non-lactating adults have been reported to consume approximately 8.5 to 12 Mcal/day and growing male and female dolphins to consume approximately 8.5 to 16 Mcal/day.[177] These differences in energy requirements are likely a consequence of different activity levels, water temperatures, and reproductive status. Nutrient intake is affected by the amount of food consumed as well as the nutrient composition of the diet, so free-ranging dolphins may be consuming, metabolizing, and excreting more of some nutrients than some managed dolphins even when the managed diet contains less of those nutrients on an equal caloric basis.

In conclusion, this study was the first to compare model diets consumed by free-ranging common bottlenose dolphins and fed to dolphins under human care. By examining the total diet, it is possible to better understand the daily nutrient intake of dolphins relative to the energy they are consuming. Several nutrient differences between free-ranging and managed dolphin model diets may contribute to ammonium urate nephrolith development in dolphins under human care, including total protein, fat, water, and DCAD. Further studies are warranted in managed dolphins to determine whether altering those nutrients would affect solute excretion and saturation, urine pH, and ammonium urate stone development.

Table 4-1. Fish species commonly consumed by free-ranging dolphins, the size of fish caught and location where fish were caught

Fish species	Catch location	Wet weight (g)*	Length (mm)*
Abundant species			
Pinfish (*Lagodon rhomboides*)	Sarasota Bay, FL	70 (7-174)	143 (68-209)†
Striped mullet (*Mugil cephalus*)	Sarasota Bay, FL Roberts Bay, FL Gulf of Mexico, FL	615 (195-875)	333 (242-400)†
Sheepshead (*Archosargus probatocephalus*)	Sarasota Bay, FL	310 (165-560)	236 (188-294)†
Ladyfish (*Elops saurus*)	Sarasota Bay, FL Gulf of Mexico, FL	285 (134-919)	339 (253-600)†
Soniferous species			
Pigfish (*Orthopristis chrysoptera*)	Sarasota Bay, FL	65 (3-171)	143 (65-220)†
Spot croaker (*Leiostomus xanthurus*)	Sarasota Bay, FL Gulf of Mexico, FL	200 (132-310)	224 (202-260)†
Spotted sea trout (*Cynoscion nebulosus*)	Sarasota Bay, FL Gulf of Mexico, FL	293 (40-670)	313 (158-440)‡
Gulf toadfish (*Opsanus beta*)	Sarasota Bay, FL Gulf of Mexico, FL	119 (8-520)	157 (85-300)‡

*Values are medians with ranges in parentheses.
†Fork length measured from most anterior point of head to the deepest notch in tail fin.
‡Straight length measured from most anterior point of head to most caudal point of tail fin.

Table 4-2. Fish species commonly fed to managed dolphins, the location where fish were caught, and the date fish were caught

Fish and squid species	Catch location	Catch date
Pacific herring (*Clupea pallasii*)	Pacific coast, USA	December 2013
Atlantic herring (*Clupea harengus*)	East coast, USA	November 2013
Icelandic capelin (*Mallotus villosus*)	Iceland	March 2014
Canadian capelin (*Mallotus villosus*)	East coast, Canada	June 2014
Pacific mackerel (*Scomber japonicus*)	Pacific coast, USA	April 2014
Pacific sardine (*Sardinops sagax*)	Pacific coast, USA	October 2013
West coast Loligo squid (*Loligo opalescens*)	Pacific coast, USA	October 2013

Table 4-3. Mineral molecular weights, valences, and absorption coefficients used to calculate dietary cation-anion differences

Mineral	Molecular weight (g/mol)	Valence	Absorption coefficient	
			Human*	Cat†
Na	22.990	1+	0.95	0.95
K	39.098	1+	0.8	0.95
Ca	40.078	2+	0.25	0.20
Mg	24.305	2+	0.32	0.25
Cl	35.450	1-	0.95	0.95
S	32.060	2-	0.91‡	0.91‡
P	30.974	1.8-	0.63	0.35

*Absorption coefficients are those used by Remer et al., 1995 to estimate the potential renal acid load in human beings, with the exception of sulfur.

†Absorption coefficients were based on mineral absorption in domestic cats reported by NRC, 2006 for Na, K, Mg, and Cl, and by Mack et al., 2015 for Ca and P.

‡Absorption coefficient for sulfur was assumed to be 91%, which is the digestibility of protein predicted by Atwater factors.

Table 4-4. Proportions of fish and squid species in model managed common bottlenose dolphin diets

Fish and squid species	% 'as fed' weight	% Mcal ME
Managed diet #1		
Icelandic capelin	60	54.0
Pacific herring	20	31.9
Pacific mackerel	10	8.7
West coast Loligo squid	10	5.4
Managed diet #2		
Canadian capelin	60	47.4
Atlantic herring	10	15.2
Pacific herring	10	17.2
Pacific mackerel	10	9.4
Pacific sardine	10	10.8

Table 4-5. Proportions of fish species in a model free-ranging common bottlenose dolphin diet

Fish species	% total fish	% Mcal ME
Pinfish	41	27.1
Gulf toadfish	40	24.0
Sheepshead	4	9.2
Spot	3	11.4
Pigfish	3	1.6
Mullet	2	19.3
Ladyfish	2	2.9
Spotted sea trout	2	2.6
Menhaden	1	1.9
Atlantic threadfin herring	1	0.2

Table 4-6. Energy and macronutrient content of fish and squid commonly consumed by free-ranging and managed dolphins*

Fish and squid species	GE Mcal/kg 'as fed'	ME‡ Mcal/kg 'as fed'	DM‡ g/kg 'as fed'	Protein‡ g/Mcal ME	Fat§ g/Mcal ME	Ash‡ g/Mcal ME
Free-ranging diet†						
Pinfish	1.49 ± 0.07^{c}	1.24 ± 0.07^{d}	286 ± 5^{c,d}	136 ± 9^{e}	51 ± 4^{d}	46 ± 6^{b,c}
Gulf toadfish	0.87 ± 0.04^{f}	0.66 ± 0.03^{g}	201 ± 14^{f,g,h}	218 ± 6^{a}	14 ± 3^{h}	68 ± 12^{a,b}
Mullet	1.89 ± 0.08^{b}	1.67 ± 0.08^{b,c}	334 ± 8^{a}	101 ± 6^{f}	66 ± 3^{c}	33 ± 3^{d,e}
Spot	2.26 ± 0.12^{a}	2.06 ± 0.08^{a}	348 ± 11^{a}	73 ± 3.5^{h}	79 ± 2^{a}	18 ± 1^{g,h}
Sheepshead	1.17 ± 0.13^{d,e}	0.96 ± 0.10^{e,f}	272 ± 15^{c,d,e}	174 ± 16^{c,d}	34 ± 7^{e,f}	82 ± 11^{a}
Ladyfish	1.15 ± 0.12^{d,e}	0.89 ± 0.11^{e,f}	238 ± 15^{e,f}	208 ± 21^{a,b,c}	19 ± 9^{f,g,h}	38 ± 4^{c,d}
Spotted sea trout	1.01 ± 0.05^{e}	0.78 ± 0.05^{f}	216 ± 12^{f,g}	214 ± 7^{a,b}	16 ± 3^{g,h}	44 ± 6^{b,c,d}
Pigfish	1.42 ± 0.12^{c,d}	1.18 ± 0.12^{d,e}	259 ± 14^{d,e}	134 ± 11^{e}	52 ± 5^{d}	34 ± 4^{c,d,e}
Managed diet						
Canadian capelin	1.10 ± 0.021^{e}	0.83 ± 0.01^{f}	190 ± 3^{h}	156 ± 0.7^{d}	42 ± 0.3^{e}	28 ± 0.8^{e,f}
Icelandic capelin	1.27 ± 0.05^{d}	1.02 ± 0.05^{e}	201 ± 4^{g,h}	122 ± 3^{e}	57 ± 2^{d}	19 ± 1^{g}
Pacific herring	2.11 ± 0.02^{a}	1.82 ± 0.04^{b}	313 ± 4^{b}	85 ± 3^{g}	73 ± 1^{b}	12 ± 0.3^{j}
Atlantic herring	1.89 ± 0.02^{b}	1.61 ± 0.01^{c}	291 ± 2^{c}	96 ± 2^{f}	69 ± 1^{c}	16 ± 0.7^{h}
Pacific mackerel	1.34 ± 0.02^{d}	0.99 ± 0.01^{e}	246 ± 1^{e}	188 ± 5^{c}	28 ± 2^{f}	28 ± 1^{e,f}
Pacific sardine	1.49 ± 0.04^{c}	1.14 ± 0.06^{d,e}	265 ± 8^{d,e}	168 ± 7^{c,d}	37 ± 3^{e,f}	26 ± 2^{f}
Loligo squid	0.81 ± 0.03^{f}	0.61 ± 0.01^{g}	150 ± 1^{i}	206 ± 2^{b,c}	20 ± 0.9^{f,g}	14 ± 0.4^{i}

*Values are means ± one standard deviation. GE, gross energy; ME, metabolizable energy; DM, dry matter.

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

‡Nutrient concentrations were greater for free-ranging diet species than for managed diet species ($p \leq 0.05$).

§Energy content or nutrient concentrations were greater for managed diet species than free-ranging diet species ($p \leq 0.05$).

^{abcdefghij} Nutrient concentrations with different superscripts within a column are different among species ($p \leq 0.05$).

Table 4-7. Mineral concentrations of fish and squid consumed by free-ranging and managed dolphins*

Fish and squid species	Ca‡ g/Mcal ME	P‡ g/Mcal ME	Mg‡ g/Mcal ME	K‡ g/Mcal ME	Na§ g/Mcal ME	Cl§ g/Mcal ME	S g/Mcal ME
Free-ranging diet†							
Pinfish	11.2 ± 0.9^{b,c}	6.9 ± 0.5^{b,c}	0.43 ± 0.03^{c}	2.5 ± 0.2^{c,d}	1.4 ± 0.2^{c,d}	1.8 ± 0.2^{d}	2.1 ± 0.1^{c}
Gulf toadfish	17.1 ± 3.2^{a,b}	10.5 ± 2.0^{a,b}	0.69 ± 0.05^{a}	3.7 ± 0.3^{a,b}	2.6 ± 0.3^{a,b}	3.5 ± 0.7^{b,c}	3.0 ± 0.1^{b}
Mullet	6.6 ± 0.7^{d}	4.1 ± 0.5^{d,e}	0.24 ± 0.02^{e,f}	1.5 ± 0.1^{e}	0.7 ± 0.6 3^{e}	0.7 ± 0.2^{e,f}	1.3 ± 0.06^{e,f}
Spot	4.3 ± 0.6^{e,f}	2.8 ± 0.3^{f,g}	0.19 ± 0.02^{f,g}	1.2 ± 0.07^{f}	0.7 ± 0.05^{e}	0.3 ± 0.09^{f}	1.0 ± 0.05^{g}
Sheepshead	22.7 ± 3.0^{a}	12.3 ± 1.9^{a}	0.61 ± 0.08^{a,b}	2.9 ± 0.2^{b,c}	1.9 ± 0.3^{b,c}	2.4 ± 0.3^{c,d}	2.6 ± 0.2^{b,c}
Ladyfish	9.0 ± 1.3^{c,d}	6.8 ± 0.9^{b,c}	0.46 ± 0.05^{b,c}	3.8 ± 0.5^{a,b}	1.5 ± 0.3^{c,d}	2.2 ± 0.4^{c,d}	3.2 ± 0.6^{b}
Spotted sea trout	10.3 ± 1.9^{b,c,d}	7.7 ± 1.0^{b,c}	0.45 ± 0.02^{b,c}	4.1 ± 0.4^{a}	1.7 ± 0.07^{c}	2.5 ± 0.05^{c}	2.9 ± 0.2^{b}
Pigfish	9.4 ± 0.9^{c,d}	5.8 ± 0.6^{c}	0.36 ± 0.04^{c}	2.5 ± 0.4^{c,d}	1.5 ± 0.2^{c,d}	2.2 ± 0.2^{c,d}	2.0 ± 0.3^{c,d}
Managed diet							
Canadian capelin	3.6 ± 0.2^{f}	3.7 ± 0.07^{e}	0.50 ± 0.01^{b}	2.3 ± 0.06^{d}	3.1 ± 0.1^{a}	5.8 ± 0.1^{a}	2.1 ± 0.07^{c}
Icelandic capelin	2.6 ± 0.2^{g}	2.9 ± 0.1^{f}	0.26 ± 0.01^{e}	2.3 ± 0.08^{d}	1.5 ± 0.4^{c,d}	2.7 ± 0.06^{c}	1.6 ± 0.06^{d}
Pacific herring	2.0 ± 0.1^{h}	2.4 ± 0.01^{g}	0.16 ± 0.01^{g}	1.7 ± 0.05^{e}	0.7 ± 0.02^{e}	0.9 ± 0.04^{e}	1.2 ± 0.04^{f}
Atlantic herring	2.4 ± 0.2^{g}	2.4 ± 0.1^{g}	0.28 ± 0.01^{d}	1.7 ± 0.07^{e}	1.7 ± 0.06^{c}	2.6 ± 0.1^{c}	1.3 ± 0.03^{e}
Pacific mackerel	4.0 ± 0.1^{e,f}	4.4 ± 0.1^{d}	0.45 ± 0.01^{b,c}	3.3 ± 0.08^{b}	1.7 ± 0.03^{c}	3.4 ± 0.04^{b,c}	2.5 ± 0.06^{b,c}
Pacific sardine	4.5 ± 0.4^{e}	4.2 ± 0.2^{d}	0.37 ± 0.02^{c}	2.7 ± 0.06^{c}	1.4 ± 0.09^{d}	2.5 ± 0.2^{c}	2.2 ± 0.07^{b,c}
Loligo squid	0.3 ± 0.03^{i}	2.9 ± 0.2^{f}	0.44 ± 0.05^{b,c}	2.5 ± 0.15^{c,d}	2.4 ± 0.08^{b}	4.4 ± 0.7^{b}	4.5 ± 0.3^{a}

*Values are means ± one standard deviation. Ca, calcium; P, phosphorus; Mg, magnesium; K, potassium; Na, sodium; Cl, chloride; S, sulfur.
†Free-ranging diet species are listed in order of greatest to least contribution to the total energy content of the diet.
‡Nutrient concentrations are significantly greater in free-ranging diet species than in managed diet species ($p \leq 0.05$).
§Nutrient concentrations are significantly greater in managed diet species than in free-ranging diet species ($p \leq 0.05$).
abcdefg Nutrient concentrations with different superscripts within a column are significantly different ($p \leq 0.05$) among species.

Table 4-8. Total water relative to energy, protein, and sodium and dietary cation-anion differences among fish and squid species*

Fish and squid species	TW_{mL}:ME_{Mcal}†	TW_{mL}:CP_g†	TW_{mL}:Na_g‡	$DCAD_{short}$‡δ	$DCAD_{long}$‡δ	$DCAD_{human}$†δ	$DCAD_{cat}$‡δ
Free-ranging diet¥							
Pinfish	586 ± 39^{e}	4.3 ± 0.15^{d,e}	416 ± 35^{c}	-59 ± 1^{d,e}	136 ± 13^{b}	-161 ± 8^{e,f}	-70 ± 8^{c,d}
Gulf toadfish	1230 ± 77^{b}	5.6 ± 0.26^{c}	477 ± 73^{a,b,c}	-76 ± 24^{d,e,f}	223 ± 33^{a,b}	-232 ± 29^{g,h}	-94 ± 25^{d,e,f}
Mullet	413 ± 25^{f,g}	4.1 ± 0.05^{e,f}	620 ± 35^{a}	-31 ± 0.6^{b,c}	75 ± 5^{c}	-96 ± 2^{b,c}	-40 ± 1^{b}
Spot	329 ± 17^{h}	4.5 ± 0.25^{d,e}	468 ± 34^{b,c}	-13 ± 2^{a}	53 ± 9^{c}	-60 ± 6^{a}	-21 ± 5^{a}
Sheepshead	776 ± 95^{d,e}	4.5 ± 0.15^{d,e}	399 ± 20^{c}	-71 ± 13^{d,e,f}	400 ± 16^{a}	-221 ± 15^{f,g}	-71 ± 13^{c,d,e}
Ladyfish	877 ± 116^{c,d}	4.2 ± 0.16^{e,f}	592 ± 46^{a}	-101 ± 3^{e,f}	-12 ± 14^{d}	-228 ± 5^{f,g,h}	-128 ± 4^{e,f}
Spotted sea trout	1020 ± 83^{b,c}	4.8 ± 0.24^{d}	615 ± 59^{a}	-72 ± 18^{d,e,f}	32 ± 76^{c,d}	-218 ± 32^{f,g}	-106 ± 20^{e,f}
Pigfish	645 ± 85^{d,e}	4.8 ± 0.26^{d}	423 ± 20^{c}	-61 ± 3^{d,e}	102 ± 5^{b,c}	-147 ± 3^{d,e}	-69 ± 3^{c,d}
Managed diet							
Canadian capelin	983 ± 17^{c}	6.3 ± 0.13^{b}	317 ± 10^{d}	-96 ± 7^{e,f}	-92 ± 19^{f,g}	-173 ± 17^{e,f,g}	-116 ± 9^{e,f}
Icelandic capelin	792 ± 44^{d}	6.5 ± 0.33^{a,b}	521 ± 20^{a,b}	-48 ± 24^{d}	-62 ± 21^{e}	-116 ± 40^{c,d}	-68 ± 27^{c,d}
Pacific herring	392 ± 10^{g}	4.6 ± 0.08^{d}	586 ± 7^{a}	-27 ± 10^{b}	-54 ± 59^{e}	-88 ± 59^{b}	-49 ± 14^{b,c}
Atlantic herring	454 ± 4^{f}	4.7 ± 0.10^{d}	263 ± 8^{e}	-39 ± 5^{c}	-33 ± 14^{d}	-90 ± 7^{b}	-52 ± 5^{c}
Pacific mackerel	773 ± 11^{d,e}	4.1 ± 0.09^{e,f}	466 ± 7^{c}	-101 ± 3^{f}	-123 ± 15^{g}	-203 ± 11^{f,g}	-131 ± 5^{f}
Pacific sardine	660 ± 42^{d,e}	3.9 ± 0.14^{f}	490 ± 6^{a,b}	-79 ± 2^{e,f}	-69 ± 41^{e,f}	-169 ± 11^{e,f}	-103 ± 4^{e,f}
Loligo squid	1400 ± 40^{a}	6.8 ± 0.21^{a}	576 ± 8^{a}	-236 ± 4^{c,d}	-352 ± 11^{h}	-312 ± 10^{h}	-259 ± 5^{g}

*Values are means ± one standard deviation. TW, total water; ME, metabolizable energy; CP, crude protein; Na, sodium.

†Nutrient concentrations are significantly greater, or DCADs are more positive, in managed diet species than in free-ranging diet species ($p \leq 0.05$).

‡Nutrient concentrations are significantly greater, or DCADs are more positive, in free-ranging diet species than in managed diet species ($p \leq 0.05$).

δDCAD, dietary cation-anion difference calculated using four equations: $DCAD_{short}$ = (Na + K) – (Cl + S); $DCAD_{long}$ = (Na + K + Ca + Mg) – (Cl + S + P); $DCAD_{cat}$ = (0.95Na + 0.95K + 0.2Ca + 0.25Mg) - (0.95Cl + 0.35P + 0.91S); and $DCAD_{human}$ = (0.95Na + 0.8K + 0.25Ca + 0.32Mg) - (0.95Cl + 0.63P + 0.91S) where Na, K, Ca, Mg, Cl, S and P represent the mEq/Mcal metabolizable energy of sodium, potassium, calcium, magnesium, chloride, sulfur and phosphorus, respectively.

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

abcdefgh Nutrient concentrations with different superscripts within a column are significantly different ($p \leq 0.05$) among species.

Table 4-9. Energy and nutrient content, nutrient ratios, and dietary anion-cation differences for model managed and free-ranging diets*

Nutrient	Managed model diet #1	Managed model diet #2	Free-ranging model diet	Managed diet species	Free-ranging diet species
/kg 'as fed'					
GE (Mcal)	1.52 ± 0.01[a]	1.46 ± 0.01[b]	1.45 ± 0.01[b]	1.43 ± 0.005[†]	1.41 ± 0.02[†]
ME (Mcal)	1.25 ± 0.01[a]	1.17 ± 0.01[b]	1.23 ± 0.01[a]	1.15 ± 0.005[†]	1.18 ± 0.01[†]
DM (g)	238 ± 1[a]	240 ± 0.9[a]	276 ± 2b	237 ± 0.7[†]	269 ± 2[†]
/Mcal ME					
TW (L)	0.70 ± 0.01[a]	0.75 ± 0.04[b]	0.72 ± 0.01[ab]	0.78 ± 0.005[†]	0.73 ± 0.01
Protein (g)	120 ± 0.9[a]	139 ± 0.5[b]	150 ± 1.5[c]	146 ± 0.7[†]	157 ± 1.8[†]
Fat (g)	58 ± 0.4[a]	49 ± 0.2[b]	45 ± 0.7[c]	46 ± 0.3[†]	41 ± 0.8[†]
Ash (g)	17 ± 0.3[a]	23 ± 0.2[b]	48 ± 1.5[c]	20 ± 0.2[†]	45 ± 1.0[†]
Ca (g)	2.4 ± 0.04[a]	3.3 ± 0.04[b]	11.9 ± 0.4[c]	2.8 ± 0.03[†]	11.3 ± 0.3
P (g)	2.9 ± 0.03[a]	3.4 ± 0.02[b]	7.3 ± 0.2[c]	3.3 ± 0.02[†]	7.1 ± 0.2
Mg (g)	0.25 ± 0.003[a]	0.39 ± 0.003[b]	0.44 ± 0.008[c]	0.35 ± 0.004[†]	0.43 ± 0.007[†]
K (g)	2.2 ± 0.02[a]	2.3 ± 0.01[a]	2.6 ± 0.04[b]	2.4 ± 0.01[†]	2.8 ± 0.05[†]
Na (g)	1.3 ± 0.01[a]	2.1 ± 0.03[b]	1.5 ± 0.04[c]	1.8 ± 0.01[†]	1.5 ± 0.03
Cl (g)	2.3 ± 0.02[a]	3.8 ± 0.03[b]	1.9 ± 0.09[c]	3.2 ± 0.05[†]	2.0 ± 0.06
S (g)	1.7 ± 0.02[a]	1.9 ± 0.02[b]	2.1 ± 0.02[c]	2.2 ± 0.02[†]	2.3 ± 0.04[†]
TW (mL): Protein (g)	5.7 ± 0.08[a]	5.3 ± 0.03[b]	4.6 ± 0.04[c]	5.3 ± 0.03	4.6 ± 0.03
TW (mL) : Na (g)	540 ± 5[a]	388 ± 2[b]	487 ± 10[c]	460 ± 2[†]	501 ± 7
mEq/Mcal ME					
$DCAD_{short}$[‡]	-56 ± 1[a]	-74 ± 2[b]	-55 ± 2[a]	-90 ± 2[†]	-60 ± 2[†]
$DCAD_{long}$[‡]	-80 ± 3[a]	-77 ± 3[a]	152 ± 9[b]	-112 ± 3[†]	126 ± 6[†]
$DCAD_{human}$[‡]	-125 ± 2[a]	-149 ± 2[b]	-163 ± 4[c]	-165 ± 2[†]	-170 ± 4
$DCAD_{cat}$[‡]	-78 ± 1[a]	-95 ± 2[b]	-67 ± 2[c]	-111 ± 2[†]	-75 ± 2[†]

*Values are means ± standard error. Mean and standard error values for managed and free-ranging diet species are shown for comparison. GE, gross energy; ME, metabolizable energy; DM, dry matter; TW, total water; Ca, calcium;, P, phosphorous; Mg, magnesium; K, potassium; Na, sodium; Cl, chloride; S, sulfur.

[abc]Nutrient concentrations with different superscripts across rows are different among model diets ($p \leq 0.05$).

[†]Nutrient concentrations are different between managed diet species and free-ranging diet species ($p \leq 0.05$).

[‡]DCAD, dietary cation-anion difference calculated using four equations: $DCAD_{short} = (Na + K) – (Cl + S)$; $DCAD_{long} = (Na + K + Ca + Mg) – (Cl + S + P)$; $DCAD_{cat} = (0.95Na + 0.95K + 0.2Ca + 0.25Mg) - (0.95Cl + 0.35P + 0.91S)$; and $DCAD_{human} = (0.95Na + 0.8K + 0.25Ca + 0.32Mg) - (0.95Cl + 0.63P + 0.91S)$ where Na, K, Ca, Mg, Cl, S and P represent the mEq/Mcal metabolizable energy of sodium, potassium, calcium, magnesium, chloride, sulfur and phosphorus, respectively.

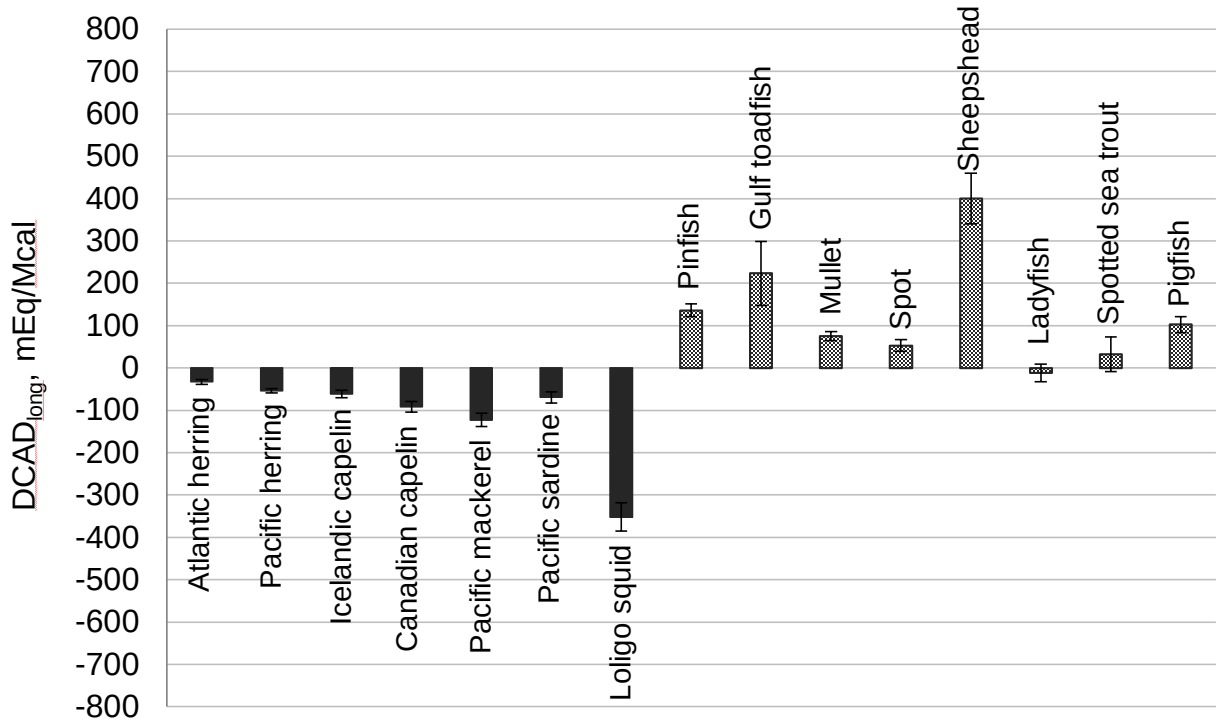


Figure 4-1. Individual $DCAD_{long}$ values for managed and free-ranging diet fish species. ($DCAD_{long}$ = (Na + K + Ca + Mg) – (Cl + S + P)). Solid bars represent managed diet species and textured bars represent free-ranging diet species.

CHAPTER 5
A TARGETED METABOLOMICS ASSAY TO MEASURE EIGHT PURINES IN THE DIET OF COMMON BOTTLENOSE DOLPHINS, *TURSIOPS TRUNCATUS*

Introduction

Purines contribute to the structure of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), adenine triphosphate (ATP) and guanine triphosphate (GTP). Purines are either made by the body, salvaged and recycled, or absorbed from food. They are metabolized to uric acid, which is excreted in the urine (Figure 5-1) [13, 55, 60, 239].

Dietary purines are found in high concentrations in organ meat and seafood.[60, 254] Consumption of a purine-rich diet increases the production and excretion of uric acid. When the concentration of uric acid in the urine reaches a threshold, uric acid precipitates out of solution and can aggregate to form urate-based stones in the urinary tract. These urate uroliths can be composed of uric acid, ammonium urate, monosodium urate, sodium calcium urate, or potassium urate. Human beings primarily develop uric acid uroliths, and the incidence of these uroliths has increased internationally as a consequence of increased consumption of meat [255, 256]. Individuals living in Taiwan, for example, experience a high rate of uric acid stones as a consequence of consuming purine-rich diets that include seafood and specifically grass shrimp [47]. Some Dalmatian dogs have a genetic predisposition to develop ammonium urate uroliths because of altered purine metabolism. For both species, consumption of a purine-restricted diet minimizes uric acid excretion and the risk of forming urate uroliths.

Foods are currently classified according to their total purine content, defined as the sum of the four nucleobases, adenine, guanine, hypoxanthine, and xanthine [47, 60]. However, individual metabolites have different propensities for causing hyperuricosuria and individual metabolite concentrations may vary widely among purine-rich foods [83, 89,

[90, 257]. Human beings, for example, fed a diet supplemented with adenine and hypoxanthine excreted greater urinary uric acid concentrations than when fed a diet supplemented with guanine and xanthine.[66] Thus, it may be more important to describe the individual purine metabolite composition of food rather than total purine content, when determining a food's uricogenic potential.

It also may not be sufficient to describe just the nucleobase content of foods. Additional metabolites, including nucleotides adenine 5'-monophosphate (AMP), inosine 5'-monophosphate (IMP) and the nucleoside inosine (Figure 1) are absorbed by the mammalian gastrointestinal tract and may also increase urinary uric acid concentrations [66, 258, 259]. Furthermore, the concentrations of purine metabolites are affected by food source and storage conditions [47, 126, 260]. For example, hypoxanthine concentrations progressively increase over time in pork stored at 2°C, but this effect is delayed at lower storage temperatures [258]. Also, IMP, hypoxanthine, xanthine, and inosine concentrations in fish fileted for human consumption have been demonstrated to vary with water temperature where fish were caught, cooking methods, storage methods and duration, and among species [47, 117, 127, 228]. Thus, it would seem important to measure more purine metabolites than the four nucleobases that are usually measured and also to report individual concentrations, when considering a food items' uricogenic potential.

This is particularly relevant when considering purines in whole fish and squid commonly consumed by dolphins. Common bottlenose dolphins (*Tursiops truncatus*) managed under human care develop ammonium urate kidney stones, but these stones rarely occur in free-ranging dolphins [2, 3]. Why ammonium urate nephroliths form in managed dolphins is unclear, but the purine-rich whole fish diet may contribute to

nephrolith development because the diets of free-ranging and managed bottlenose dolphins differ [110, 254].

The total purine content of the fish species consumed by dolphins has not been previously reported. The purine content of the filets of some fish species has been measured using high-performance liquid chromatography (HPLC) with ultraviolet detection but the authors are aware of only one study that has measured purines in a *whole* fish (gilted sea bream) [261]. Fish organs, including the liver and skin, are reported to be rich in purines; therefore, purines measured only in the muscle of fish may underestimate total purine content of whole fish.[254, 262]

We sought, therefore, to develop an expanded assay using HPLC with tandem mass spectrometry (MS/MS) that would accurately identify and quantify a broader range of purine metabolites in both fresh frozen fish commonly consumed by free-ranging dolphins and in stored frozen and thawed fish and squid commonly consumed by managed dolphins. We also hypothesized that it would not be possible to predict the total purine content obtained with the new assay using an assay that only measures four nucleobases. In particular, we suspected that the concentration of some of the additional metabolites would be lower and hypoxanthine concentrations would be higher for the frozen stored and thawed species fed to managed dolphins.

Materials and Methods

Chemicals

The stock solutions used for the mobile phase and for purine standards were prepared freshly at least monthly. The water used for mixing all solutions was HPLC-grade. These stock solutions included: 0.1% acetic acid (glacial) in water, 10% formic acid in water, 10 mM ammonium (NH_4) acetate in water, and 2 mM ammonium

hydroxide (NH_4OH) in water. All chemicals were purchased from Fisher Scientific Company, LLC (Suwanee, GA 30024).

Individual standard solutions were freshly prepared before each run. Purine metabolite solubility is highly pH dependent. Thus, AMP, IMP, adenine, and inosine were dissolved in 10mM NH_4 acetate (pH 6.5), whereas uric acid and xanthine were dissolved individually and hypoxanthine and guanine were dissolved together in 2 mM NH_4OH (pH 12, Sigma-Aldrich, St. Louis, MO). Each solution was then sonicated in a heated (35°C) water bath for 10-15 minutes to achieve complete dissolution. The individual standard purine solutions in NH_4 acetate were combined to generate one mixed standard solution with final concentrations of 0.01 mM for adenine, 0.02 mM for IMP, 0.05 mM for AMP, and 0.15 mM for inosine. Individual standard solutions dissolved in NH_4OH were also combined to generate another mixed standard solution with final concentrations of hypoxanthine and guanine at 0.23 mM, xanthine at 0.22 mM, and uric acid at 0.01 mM. An internal standard, $^{15}N_2$-xanthine (Cambridge Isotopes Laboratory, Inc., Tewksbury, MA 01876), was mixed with 2mM NH_4OH to achieve a concentration of 1.3 mM. The final concentrations of the individual metabolites in the combined solutions were half their original starting concentration.

Fish Sampling and Processing

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

Eight fish species commonly consumed by free-ranging bottlenose dolphins and six fish species and one squid species commonly fed to dolphins under human care were analyzed for purine metabolite content (Table 5-1).[161, 245] Free-ranging fish

samples were caught between May and September 2013 from the waters off the FL west coast by local fisherman using a rod and reel, crab trap, cast net, or with a purse-seine net during field surveys conducted by the SDRP team in Sarasota Bay. To mimic the rapid death of fish consumed by dolphins in the wild as closely as possible, fish were euthanized humanely by immersion in 500ppm tricaine methanesulfonate (MS 222, Western Chemical, Ferndale, WA 98248) in sea water.[263, 264] Death was confirmed by cessation of opercula movement for 10 minutes, and then fish were weighed, length was measured, and samples of fish were individually bagged, placed into a cooler of dry ice and transported to the UF nutrition laboratory where fish were stored at -80ºC until further processing.

Boxes containing six fish species and one species of squid commonly fed to dolphins managed under human care ('managed species') were supplied by two bottlenose dolphin management facilities (Table 5-2). Fish and squid were caught during one commercial fishing season, frozen stored at -18ºC for 6 to 9 months, and then shipped overnight on dry ice from the dolphin management facilities to UF. Upon arrival, fish were stored at -20ºC until further processing.

Five separate samples of each species were analyzed. To provide sufficient material to perform all the analyses on every sample, a minimum of 2 individual fish (or squid) were included in each sample; however, 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. The five samples of each species were individually ground using commercial meat

grinders with 4.5 and 10 mm plates (Biro 6642, Marblehead, OH 43440, and 1.5 HP, LEM Products, West Chester, OH 45011).

Free-ranging fish species were thawed the minimum amount needed to allow grinding, whereas managed diet fish species were thawed more completely using the standard operating procedure of one dolphin management facility. Free-ranging fish species were air thawed in a temperature controlled cold room (11-12ºC) for approximately 1 hour, until fish thawed to a firm, slightly malleable texture. Managed diet fish were removed from the cardboard boxes, maintained wrapped in plastic, and air thawed in the cold room for approximately 20 hours. Fish were then removed from the plastic and rinsed with cold water (approximately 16ºC). Both minimally and well-thawed fish were then transported to the grinder in a cooler containing ice. Grinder equipment was thoroughly rinsed between each sample to prevent contamination between samples. Ground samples were homogenized by hand and stored in sample bags (Whirl-Pak® bags, Nasco, Fort Atkinson, WI 53538) at -80ºC until shipped overnight on dry ice to each laboratory for analysis.

Fish Extraction

For each fish purine extraction, a 25g sample bag was removed from the -80ºC freezer where it had been stored and immersed in water at room temperature for 30-60 minutes until thawed. The aqueous extraction method previously published by Clariana, et al., was followed, but required several modifications.[260] Thawed fish tissue was mixed by hand by massaging the sample bag, and 2 g of mixed sample was transferred into a beaker with 20 mL of ultra-pure water and 500 µL of the internal standard solution. Following homogenization, sonication, heating, and cooling, as described by Clariana, et al., the extract supernatant was filtered through Whatman #1 filter paper

(Whatman Inc., Clifton, NJ 07014) with a glass funnel. In all subsequent steps, extract and solvent volumes were doubled to provide a greater final volume for standard additions to be performed. Thus, a 10 mL aliquot of the filtrate was combined with 10 mL of HPLC-grade hexanes (Fisher) and centrifuged at 6,500 rpm for 7 minutes at 20ºC. A 4 mL aliquot from the bottom layer of fluid was removed and combined with 4 mL of HPLC-grade methanol (Fisher), 4 mL of HPLC-grade acetone (Fisher), and 80 µL of 10% formic acid in water. Samples were centrifuged at 18,100 rpm for 17 minutes at 15ºC. Five aliquots of 1,500 µL each were pipetted into 5 mL conical snap-top tubes (Eppendorf, Fisher).

Standard additions were used to quantify individual purine concentrations in the fish extract. Thus, for each fish sample, the first 1,500 µL aliquot was considered the blank fish extract sample. To the remaining 4 aliquots, the NH_4 acetate mixed purine standard solution and the NH_4OH mixed purine standard solution were added in increasing quantities to provide standard additions of 2x, 4x, 6x, and 8x purine concentrations. Conical tubes were centrifuged at 3,000 rpm for 5 minutes at room temperature and then dried while heating to 35°C under a stream of nitrogen gas (MULTIVAP, Organomation, Berlin, MA 01503) for approximately 5-6 hours.

The dried samples were reconstituted with 500 µL of 10 mM NH_4 acetate. Conical tubes were centrifuged at 4,000 rpm for 15 minutes. Following centrifugation, if the extract had a distinct pellet at the bottom of the conical tube, supernatant was transferred to a 2 mL LC vial (Thermo Scientific, Waltham, MA 02451) for HPLC analysis. If the sample had floating particulate matter, the extract was re-mixed, transferred to a 2 mL microcentrifuge 0.22 µm nylon filter tube (Corning Incorporated,

Corning, NY 14831), and centrifuged at 11,884 rpm for 5 minutes. The supernatant was then transferred to a 2mL LC vial for HPLC-MS/MS analysis.

Chromatographic Conditions

Purine metabolite separation was carried out with an auto-sampler (Accela Open, Thermo) and HPLC system (Accela 1250, Thermo). Several HPLC columns were tested (Table 2) under various conditions, manipulating column and sample stack temperature and using several mobile phase solvent combinations, including 10 mM NH_4 acetate in water (pH adjusted with acetic acid from 4-6), 10mM NH_4 acetate in 90:10 acetonitrile in water, 100% acetonitrile, 90:10 acetonitrile in water, 0.1% acetonitrile in water, 0.1% formic acid in water, 0.1% acetic acid in water, 0.1% NH_4OH in water, and 100% methanol.

The column and compartment were maintained at ambient temperature. The injection volume was 10 µL with a flow rate of 500 µL/min. The elution conditions for separation of AMP, IMP, inosine, adenine, guanine, hypoxanthine, xanthine, and uric acid began with 97% A (0.1% acetic acid, pH 3.5) and 3% B (methanol) for 2 minutes, gradually decreased to 90% A and increased to 10% B from 2 to 4 minutes, 70% A and 30% B from 4 to 5.5 minutes, 50% A and 50% B from 6 to 8 minutes, with a final rapid return back to the original conditions at 8.5 minutes. The column was maintained under these conditions for re-equilibration for a further 6.5 minutes. In addition to testing different columns, several mobile phases were tested to determine which combination of solvents and timing of transitions achieved the best separation of the metabolites with similar masses. The maximum number of injections per day, including duplicate sample injections and blanks, was 84; therefore, the maximum daily run time was approximately 22.5 hours. To optimize the lifespan of the column, particularly considering the potential

for particulate build-up on the column, the column was flushed at the end of every long run with methanol at 500 µL/min for approximately 15-20 minutes. Additionally the guard column was changed after approximately 400 injections. For column storage between runs, the column was flushed at 500 µL/min with 65% acetonitrile and 35% water for at least 10 column volumes.

Mass Spectrometry Analysis

Purine metabolites were identified by mass and retention time with a triple quadrupole mass spectrometer (TSQ Quantum Access Max, Thermo) with heated electrospray ionization (HESI). The HESI source was operated in negative-ion mode with the following settings: spray voltage 4000V, vaporizer temperature 250°C, sheath gas pressure 50 arb, ion sweep gas pressure 0.0 arb, auxillary gas pressure 10 arb, and capillary temperature 300°C. Compounds were identified by comparing the retention time and selected reaction monitoring (SRM) pairs (Table 5-3) during one scan event with a 20 msec scan time, as well as through the addition of purine standards. Data was processed using computer software (Xcalibur Quan Browser, Thermo).

Purine Standard Stock Solution Stability

The stability of purine standard stock solutions was assessed by first preparing combined stock solutions as described above and spiking the solutions into 1000 µL of 10 mM NH_4 acetate to achieve equivalent 2x, 6x, and 8x standard addition concentrations. Samples were analyzed in triplicate immediately then stored at -80°C for 24 hours. Samples were then permitted to thaw for at least 1 hour until they approximated ambient temperature, vortexed, and re-analyzed in triplicate alongside freshly prepared standard solutions. This procedure was repeated once more for a total of 2 freeze/thaw cycles at 24 and 48 hours. In addition, stability over 24 hours while at

ambient temperature was assessed for standard stock solutions and fresh prepared Pacific herring and mullet samples, prepared using the method described above.

Method Validation

Repeatability was assessed on pooled samples of Pacific herring and striped mullet that were divided into separate bags and frozen at -80ºC. Purines were analyzed in these samples over 4 separate days to determine between-day variability. Additionally, 4 extracts were prepared from one sample bag for each fish species and analyzed on the same day to determine within-day variability.

Purine Concentration Quantification

For each sample, the ratio of peak area relative to internal standard was regressed against the concentration of purine metabolite after standard additions. The purine metabolite concentration (mM) in the fish extract was obtained from the absolute value of the x-intercept of the regression line. Total purine content (mM) was calculated either as the sum of adenine, guanine, hypoxanthine, and xanthine, representing the commercially available four-metabolite assay (TP4), or as the sum of those four metabolites plus uric acid, AMP, IMP, and inosine, representing the total obtained using this expanded eight-metabolite assay (TP8).

Statistical Analysis

Statistical comparisons were performed using statistical software (SAS® for Windows, version 9.4, Cary, NC, 27513). The coefficient of variation for within and between days (n=4) was calculated for individual samples of Pacific herring and striped mullet. The correlation between TP4 and TP8 was compared between groups of species using a general linear model procedure (SAS proc glm). A similar analysis was performed using logarithms of TP4 and TP8. The ratio of TP8:TP4 and the ratio of

inosine to hypoxanthine were compared among fish species' nested within species group using a general linear model (SAS glimmix) and post-hoc comparisons of least square means were performed with a Tukey-Kramer correction applied. Results are reported as means with ranges in parentheses.

The primary endpoint was to determine whether there is 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 [47, 265, 266].

Results

Purine metabolites were separated by mass and retention time using this HPLC/MS-MS method (Figure 5-2). Several of the metabolites were of similar mass: adenine and hypoxanthine, guanine and xanthine, and AMP and IMP. The reverse phase 100 Å, 150 mm x 3.0 mm column (Luna 5µm PFP(2), Phenomenex, Torrance, CA 90501) with a guard column (SecurityGuard for PFP HPLC, Phenomenex) provided the best metabolite separation and most stable retention times when used with the method conditions described above. This column also had sufficient lifespan (~800 injections) for fish tissue purine identification. All other columns did not achieve desired results (Table 5-2). Standard additions verified the location of each metabolite, particularly when concentrations in the fish extract were low. Each metabolite concentration in the fish extract was quantified as the x-intercept of the regression of concentration after standard additions with area ratio relative to that of the internal standard (Figure 5-3).

There was a 15-30% decrease in signal for all metabolites after the first 24 hour freeze and thaw cycle. There was no signal for adenine, AMP, and IMP following the second freeze/thaw cycle and the signal for the other metabolites decreased by an average of 25%. During a single 24 hour run, the signal for neat standards decreased by up to 90% for adenine, between 20-40% for AMP, IMP, and uric acid, and between 5-15% for all other metabolites. The decrease in signal for metabolites in the fish extract was less than 20% over a 24 hour run.

The coefficient of variation, measured both within and between days, for the Pacific herring and mullet samples were much greater for metabolites contained in minimal concentrations, like AMP, adenine, IMP, but was mostly below 20% for metabolites in greater concentration, like guanine and hypoxanthine (Table 5-4). The ratio of TP8:TP4 differed between fresh frozen free-ranging diet species and managed species [1.9 (1.1-2.6)] which had been frozen stored for several months and then thawed [1.5 (1.1-2.1)] and among individual fish species ($p \leq 0.0001$; Figure 4). The slope of linear correlations of TP8 to TP4 was steeper for fresh frozen fish commonly fed to free-ranging dolphins than in stored and thawed species commonly fed to managed dolphins ($p = 0.01$). Regression of the logarithm of TP8 with the logarithm of TP4 improved the fit, and the logTP8:logTP4 regression slopes were almost identical for both groups of fish (0.963 and 0.969, respectively, Figure 5-5). Values for TP8 were on average 24% higher relative to $TP4^{0.966}$ in free-ranging diet species than in managed diet species but variation was substantial. The difference between TP8 and TP4 was largely due to a change in the ratio of inosine to hypoxanthine, which was on average

greater for the free-ranging species [3.70 (0.1-9.2)] than for managed species [1.67 (0.2-4.9); $p \leq 0.0001$].

Discussion

This study describes a new method that quantified eight purine metabolites contained in whole fish and a squid commonly consumed by bottlenose dolphins. The four metabolites in greatest concentration were guanine, hypoxanthine, xanthine, and inosine, and the method provided satisfactory, repeatable results for those purines. Inosine is not measured by the four-nucleobase commercial assay, so the whole fish total purine content would be underestimated if only the usual four nucleobases, adenine, guanine, hypoxanthine, and xanthine, were measured.

The total purine content from eight metabolites cannot be predicted by measuring just four metabolites because the ratio of the total obtained from eight versus the total from four metabolites varied widely among species. The difference in ratio between the two groups of species may be due to inherent species differences in total purine content but differences in handling are more likely to be responsible. Inosine and IMP degrade to hypoxanthine during frozen storage and storage conditions can markedly affect purine degradation rates.[126, 227] In this study, fresh frozen fish had a greater inosine to hypoxanthine ratio than frozen, stored, and thawed fish. This likely represents the degradation of inosine to hypoxanthine over the six to nine month frozen storage time of managed diet species, whereas degradation was probably minimal for the free-ranging fish species that were quickly frozen at -80ºC after being euthanized.

These findings confirm our initial hypothesis that it is important to measure more than just four purine metabolites when comparing the purine intakes of managed and free-ranging bottlenose dolphins. All of the metabolites measured are absorbed through

the mammalian intestinal tract; however, the uricogenic importance of inosine is not well-described. In rats, it is suspected that intestinal absorption of inosine is saturable, but the threshold for saturation has not been established [61]. This may be important because even though fish concentrations of inosine are large, when compared to other purine metabolites, a limit to absorption may limit its impact on urine uric acid excretion. In dolphins, very little is known about purine metabolism. To our knowledge, this is the first attempt to quantify the purine content of the diet of dolphins, and the relationship between diet and uric acid excretion has not been established [2, 3]. At this time, therefore, all purine metabolites must be considered equally important in assessing the dolphin diet.

Measuring more purines with this assay may also be relevant for human medicine. Purine-rich foods are classified based on the total nucleobase content. Human beings susceptible to developing gout or uric acid stones are advised to avoid foods containing increased purines. This study shows that foods may be misclassified as to total purine content if the additional purines that are known to be absorbed by the mammalian intestinal tract are not measured. In particular, this study suggests that the total purine content may be underrepresented by the traditional assay especially in fresh foods that are not frozen and stored for long periods.

This method of analysis has some limitations. Fish were pooled and ground for analysis, and there was an inherent heterogeneity of the samples of some species because whole fish are composed of diverse tissues that respond differently to grinding. This was particularly true for some of the smaller, bonier species like pinfish, where it was challenging to ensure ground sample was well homogenized. The aqueous purine

extraction method was also very time consuming. Acid-hydrolysis is a more commonly used method for purine extraction partly because it takes less time; however, acid-hydrolysis extraction was found to reduce sensitivity of this HPLC-MS/MS method because it resulted in lower MS/MS signals for purine metabolites, including guanine and inosine, in a Pacific herring sample when compared to the aqueous extraction method.

The fish matrix seemed to have a protective factor and stabilized purine metabolites in the extracted sample. Nevertheless, lower MS signals for neat standards following the frozen storage and thawing experiments meant that purine standard stock solutions had to be prepared fresh for each experiment and added to experimental samples within 24 hours. Thus, each analytical run had to be completed within 24 hours. It was also necessary to use standard additions to quantify purine concentrations, which increased the number of runs necessary to analyze each sample and thus reduced the number of samples that could be analyzed in any one day. Standard additions were used for two important reasons. First, purines were soluble at different pH: AMP, IMP, inosine, and adenine dissolved only at a pH of approximately 7 (NH_4 acetate solvent), whereas guanine, hypoxanthine, xanthine, and uric acid dissolved at a pH of approximately 10 (NH_4OH solvent). Thus, the aqueous extraction conditions of this method did not provide soluble conditions for all purine standards. Additionally, the retention times of neat individual purine standards prepared in NH_4 acetate and NH_4OH solutions differed from retention times of purines found in the whole fish tissue; therefore, the matrix effect of this specific tissue type also did not permit accurate use of a standard calibration curve and isotopically labeled internal standards

for each compound were not available. Thus, standard additions of four increasing concentrations were used to verify and quantify metabolites present in the fish.

The purines included in this analyses were selected based on available information on their ability to be intestinally absorbed in mammals. While this is an expanded assay in comparison to the one that is commercially available, it is not inclusive of every metabolite in the purine degradation pathway that may lead to uric acid formation. The solubility of purine metabolites varies considerably depending on the pH of solution, making it especially difficult to include all purines in the analysis. Therefore, purine metabolites were selected for inclusion based on their reported abundance in fish tissue, their ability to be intestinally absorbed, and/or their uricogenic potential. On the adenine side of the degradation pathway, adenine was measured instead of adenosine because adenine is metabolized differently than all other purines and is known to be more uricogenic than its nucleoside or nucleotide forms [267]. Adenosine 5'-diphosphate (ADP) and adenosine triphosphate (ATP) were initially included in the analysis but were removed because concentrations in whole fish were below the assay's limit of detection (< 7.2 µmol/L). On the guanine side of the degradation pathway, guanine rather than guanosine was measured because of its abundance in the metallic scales of fish [262]. Guanine 5'-monophosphate (GMP) has been demonstrated to be intestinally absorbed. Nevertheless, IMP production in mammalian cells is under allosteric control preferring conversion from AMP rather than GMP. Any GMP is either trapped and either degraded to guanine or guanosine for intestinal absorption or readily converted to carbon dioxide [66, 75].

In conclusion, this method enables accurate and reproducible identification and quantification of the individual purine metabolites contained in whole fish. Furthermore, the inclusion of additional metabolites more accurately quantifies the total purine content of a food item and may more accurately represent a foods' uricogenic potential. In particular, accurately assessing differences in the purine content of diets consumed by managed and free-ranging bottlenose dolphins may help explain why managed dolphins are predisposed to forming ammonium urate nephroliths.

Table 5-1. Fish and squid species commonly consumed by free-ranging and managed bottlenose dolphins

Free-ranging diet species	Managed diet species
Pinfish (*Lagodon rhomboids*)	Pacific herring (*Clupea pallasii*)
Striped mullet (*Mugil cephalus*)	Atlantic herring (*Clupea harengus*)
Sheepshead (*Archosargus probatocephalus*)	Icelandic capelin (*Mallotus villosus*)
Ladyfish (*Elops saurus*)	Canadian capelin (*Mallotus villosus*)
Pigfish (*Orthopristis chrysoptera*)	Pacific mackerel (*Scomber japonicus*)
Spot croaker (*Leiostomus xanthurus*)	Pacific sardine (*Sardinops sagax*)
Spotted sea trout (*Cynoscion nebulosus*)	West coast Loligo squid (*Loligo opalescens*)
Gulf toadfish (*Opsanus beta*)	

Table 5-2. Columns tested for separation of purines and observed results

Manufacturer	Column	Chromatographic results
Waters Corporation (Milford, MA 01757)	Acquity UPLC HSS T3 1.8 µm,150x2.1 mm, with HSS T3 1.8 µm Vanguard	Failed after ~800 injections
	Symmetry C18 3.5 µm, 150x2.1 mm	Poor retention
ACE (Aberdeen, Scotland)	Excel C18-PFP 2 µm, 100x2.1 mm	Poor separation
	Excel C18-amide 2 µm 100x2.1 mm	Poor elution AMP*, IMP*
	Excel UltraCore Super PhenylHexyl 2.5 µm, 75x2.1 mm	Poor metabolite retention
	HALO-PFP 2.6µm, 150x2.1mm	Poor elution adenine
	HALO-HILIC 2.6µm, 150x2.1mm	Poor elution AMP*, IMP*
Phenomenex (Torrance, CA 90501)	Prodigy ODS 3 µm, 100x2 mm	Low sensitivity, except for AMP*, IMP*
	Luna PFP(2) 3µm, 150x3.0 mm†	Sufficient retention and separation, good sensitivity

*AMP, adenine 5'-monophosphate; IMP, inosine 5'-monophosphate
† Column selected for purine metabolite separation

Table 5-3. Purine metabolite mass spectrometer identification parameters

Metabolite	Precursor mass (g/mol)	Product mass (g/mol)	Collision energy (eV*)	Tube lens (V)
Adenine	134.00	107.0	24	74
Hypoxanthine	135.05	65.3	29	74
		95.2	18	74
Guanine	150.00	108.0	18	74
Xanthine	151.02	80.3	26	78
		108.2	18	78
$^{15}N_2$ - Xanthine	152.95	80.9	32	66
		109.2	18	66
Uric acid	167.00	124.2	16	65
Inosine	267.02	108.2	41	100
		135.2	25	100
AMP*	345.99	79.3	42	98
		134.2	39	98
IMP*	346.96	79.3	37	90
		135.1	34	90

*eV, electron volts; AMP, adenine 5'-monophosphate; IMP, inosine 5'-monophosphate

Table 5-4. Purine metabolite concentrations and assay variability for two whole ground fish samples

Metabolites	Within day Mean ± SD* (µmol/L)	Within day CV (%)†	Between day Mean ± SD* (µmol/L)	Between day CV (%)†
Pacific herring (*Clupea pallasii*)				
AMP*	5.46 ± 3.51	64	2.97 ± 3.21	108
Adenine	0.32 ± 0.09	27	0.14 ± 0.17	122
Guanine	201 ± 0.02	0	241 ± 40	17
IMP*	0.25 ± 0.36	147	0.10 ± 0.23	225
Inosine	228 ± 21	9	262 ± 63	24
Hypoxanthine	156 ± 24	15	166 ± 30	18
Xanthine	31.1 ± 4.8	15	34.4 ± 13.6	39
Uric acid	0.00 ± 0.00	N/A*	0.04 ± 0.13	316
Striped mullet (*Mugil cephalus*)				
AMP*	0.70 ± 0.09	13	0.77 ± 0.28	36
Adenine	1.34 ± 0.19	14	1.17 ± 0.50	43
Guanine	152 ± 17	11	165 ± 21	13
IMP*	0.95 ± 1.41	147	1.84 ± 2.16	117
Inosine	180 ± 15	8	191 ± 18	9
Hypoxanthine	35.9 ± 2.5	7	41.8 ± 6.7	16
Xanthine	12.2 ± 1.6	14	11.2 ± 2.5	22
Uric acid	0.00 ± 0.00	N/A	0.00 ± 0.00	N/A

*AMP, adenine 5'- monophosphate; IMP, inosine 5'-monophosphate; N/A, not applicable because concentration is zero

†Purine metabolite mean variability was calculated within day (n=4) and between days (n=4).

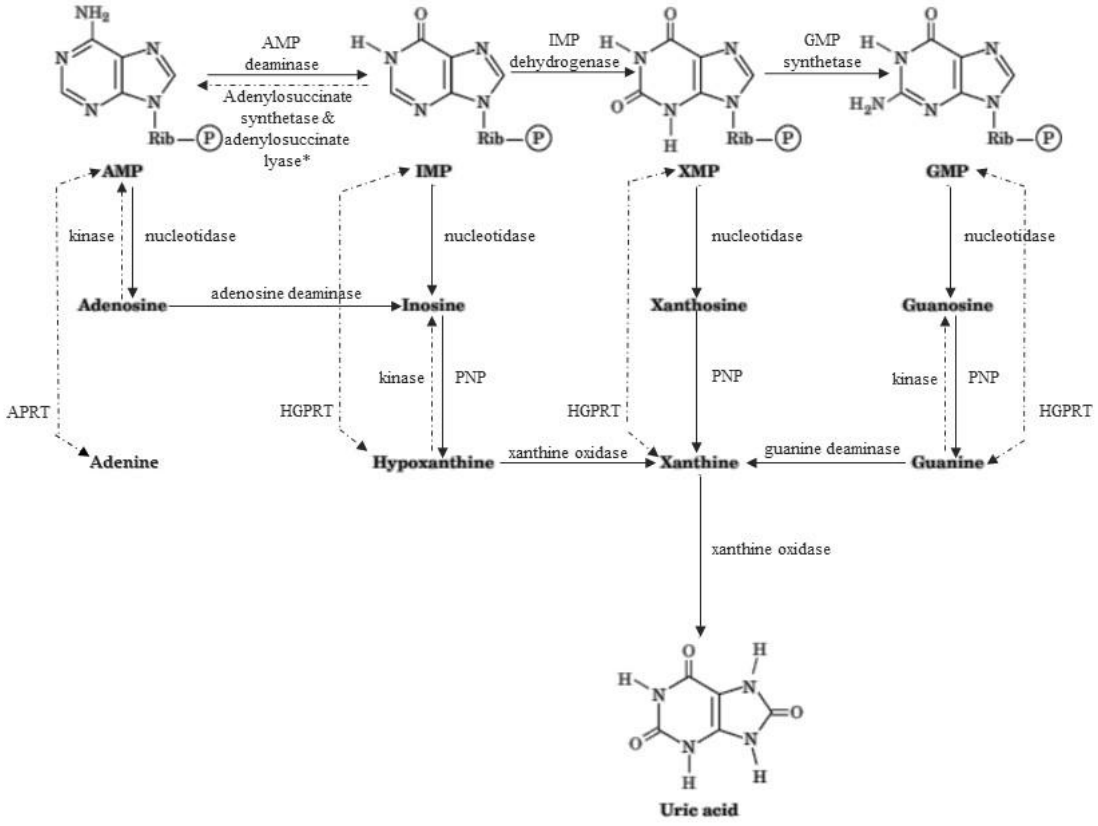


Figure 5-1. Degradation and salvage pathways of purines compiled from several sources (Green 1972; Datta 1994; Voet 2011; Nicholson 2013; Jurecka 2015). Black solid lines represent degradation pathways and black dotted lines represent salvage pathways. AMP, adenine monophosphate; IMP, inosine monophosphate; XMP, xanthine monophosphate; GMP, guanine monophosphate; APRT, adenosine phosphoribosyl transferase; HGPRT, hypoxanthine-guanine phosphoribosyl transferase; PNP, purine nucleoside phosphorylase.

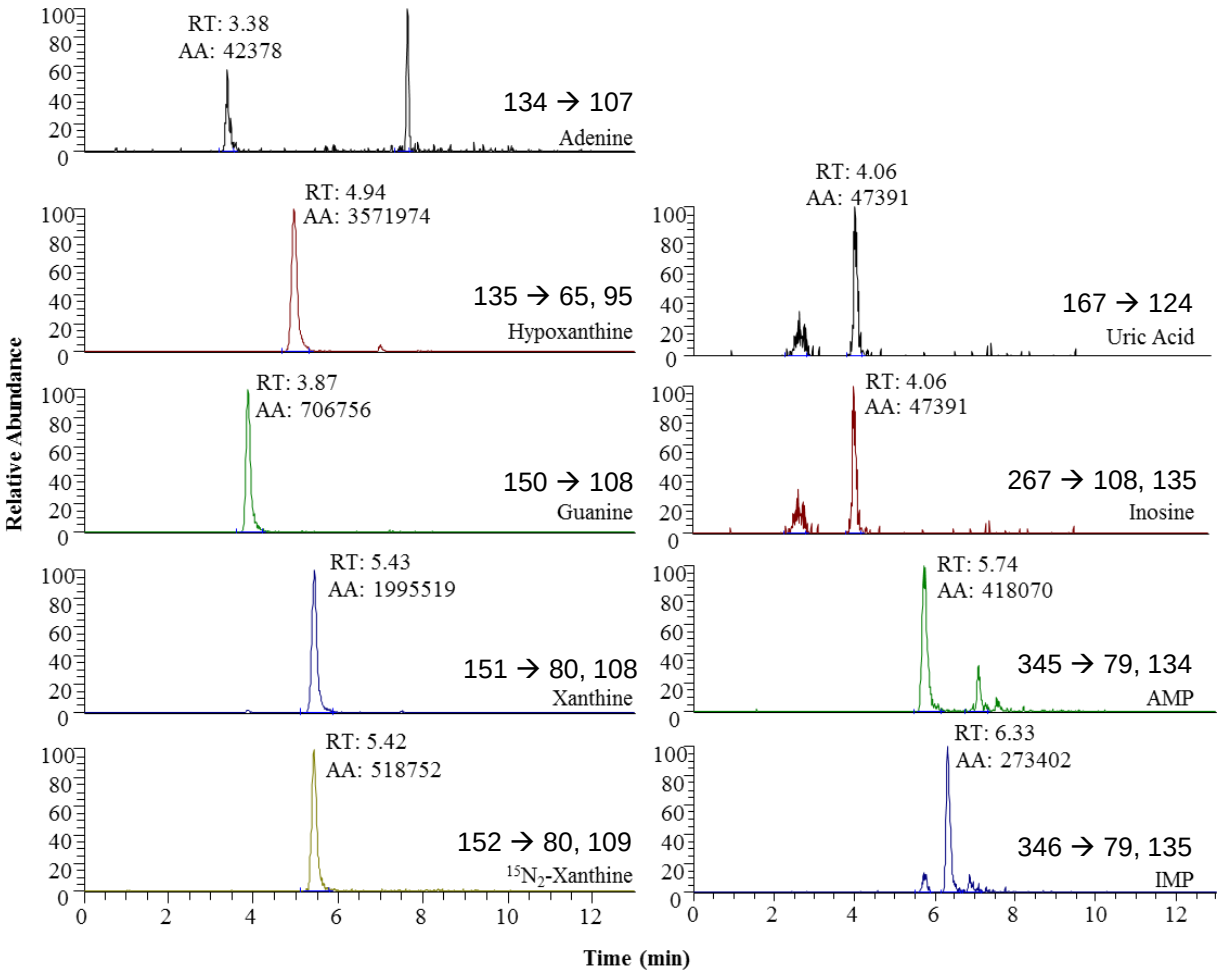


Figure 5-2. Chromatographic separation of eight purine metabolites in striped mullet with the addition of the 8x concentration standard. Time (minutes) and relative abundance are represented along the x-axis and y-axis, respectively. Each metabolite is labeled with purine name, precursor mass, and product mass(es). The retention time (RT) and the area under the curve (AA) are noted above each metabolite peak. AMP, adenine 5'-monophosphate; IMP, inosine 5'-monophosphate.

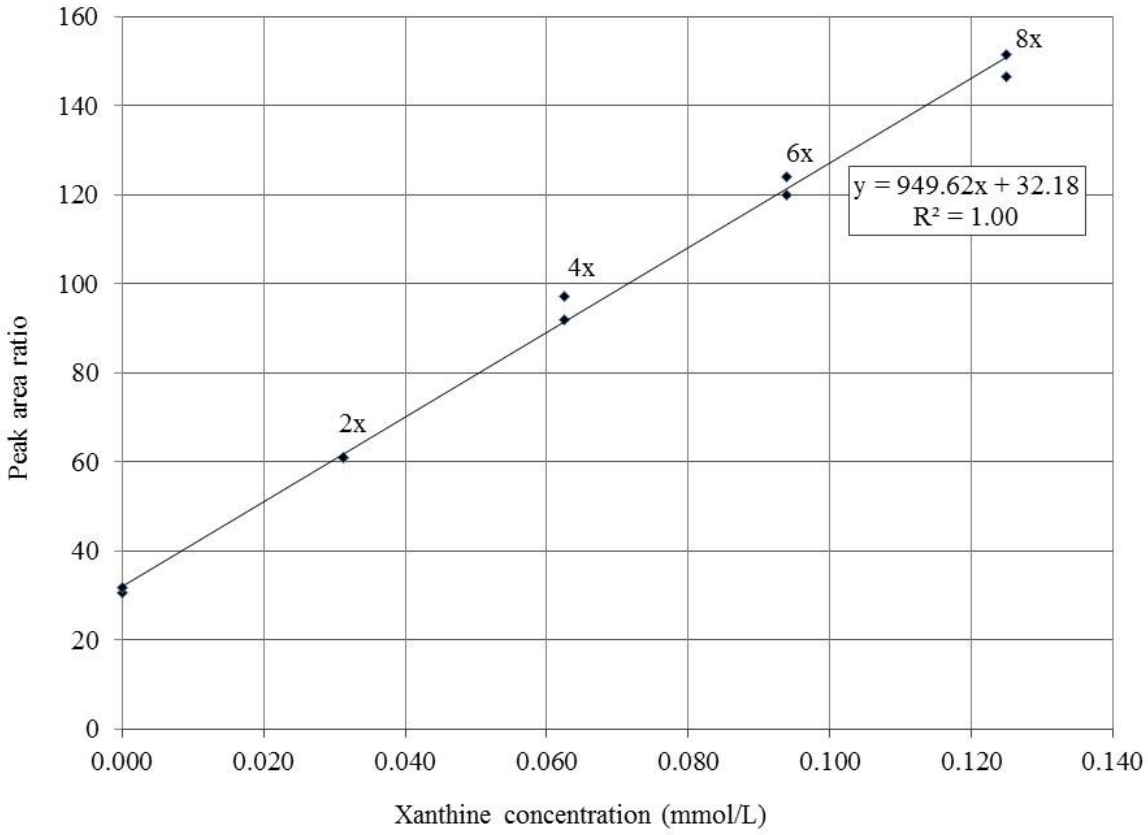


Figure 5-3. Example of a standard addition curve that shows the relationship between concentration and the ratio of peak area of xanthine to that of the internal standard that was used to establish the xanthine concentration in extract from a ground sample of striped mullet. Values are plotted either when no external standard was added or when increasing concentrations (2x, 4x, 6x, and 8x) of xanthine external standard were added to the fish extract. The xanthine concentration in the fish extract was calculated as the absolute value of the x-intercept of the linear regression line. Thus, for this curve x=(y-32.18)/949.6 (R^2=1.00) and xanthine concentration in the extract was determined to be |-32.18/949.6|=0.0339 mmol/L.

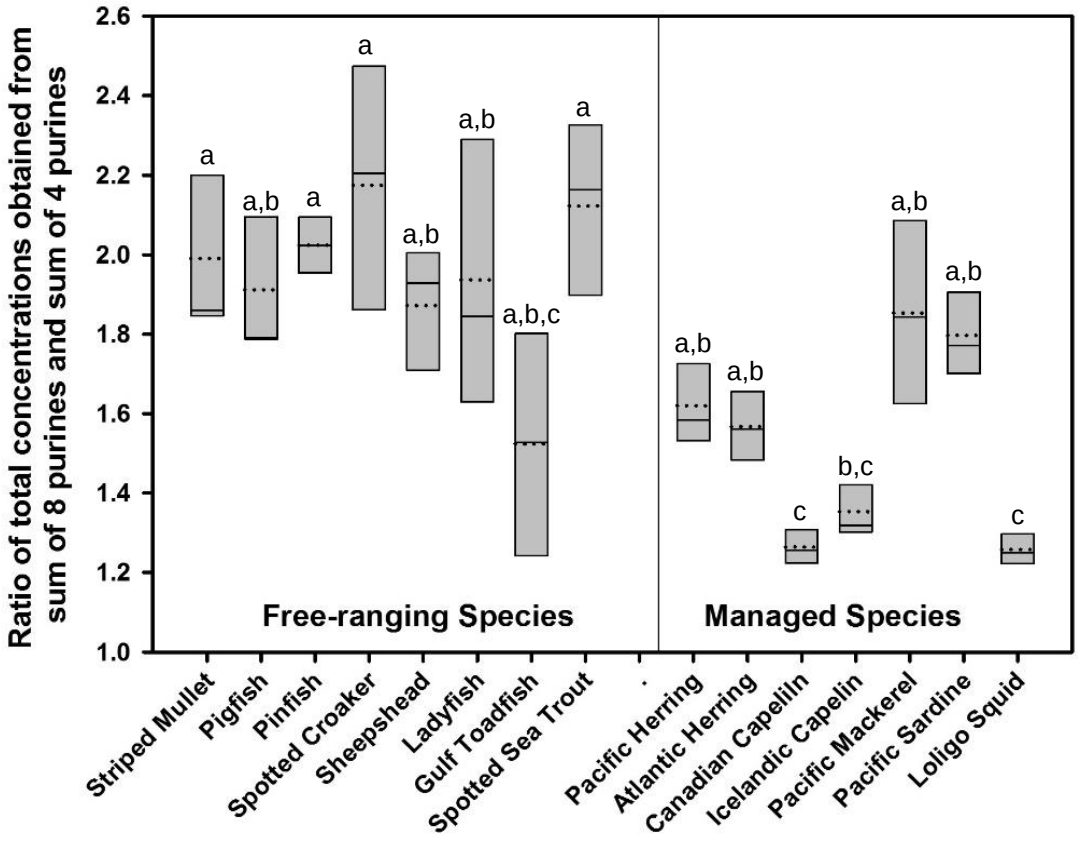


Figure 5-4. Ratios of the sum of concentrations of eight purine metabolites versus four purine metabolites in extracts of fish and squid samples of species commonly consumed by free-ranging dolphins and dolphins under human care. Boxes represent the range, the solid lie represents the median, and the dotted line is the mean. Free-ranging diet species overall have a greater ratio than managed diet species ($p < 0.05$), and ratios of individual species with different letter superscripts are significantly different ($p \leq 0.05$; n=5).

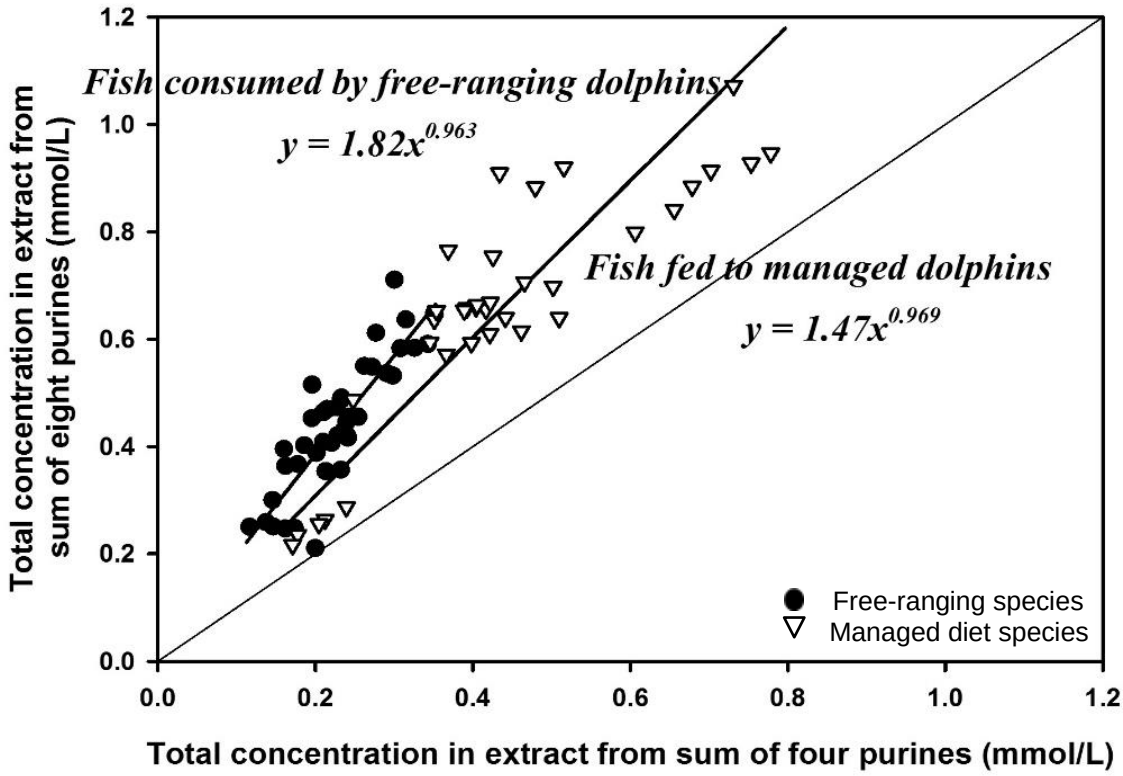


Figure 5-5. Comparison of the total concentration of eight purine metabolites (TP8) versus the total concentrations of four purine metabolites (TP4) among fish fed to free-ranging dolphins and species consumed by managed dolphins. Regression lines obtained by regressing the logarithm of TP8 with the logarithm of TP4 are shown. The exponents of the regression lines were approximately the same for both groups of fish but the ratio of TP8 to TP4 was on average 24% higher for free-ranging dolphins than managed dolphins.

CHAPTER 6
PURINE METABOLITES IN FISH, SQUID, AND MODEL DIETS COMMONLY CONSUMED BY MANAGED AND FREE-RANGING COMMON BOTTLENOSE DOLPHINS, *TURSIOPS TRUNCATUS*

Introduction

Common bottlenose dolphins (*Tursiops truncatus*) managed under human care develop ammonium urate kidney stones; however, these stones rarely form in free-ranging bottlenose dolphins.[1] The prevalence of nephroliths has not been determined in most facilities taking care of bottlenose dolphins, but one facility reports 35% prevalence among dolphins in their population.[1] The cause of nephrolith development in managed bottlenose dolphins is unknown, but it may be related to the fact that dietary purines absorbed through the gastrointestinal tract are metabolized to uric acid, which is excreted in urine.[55] In other mammals, a purine-rich diet can promote urate urolith formation.

Organs and seafood contain more purines than other foods, and the diet of bottlenose dolphins consists primarily of purine-rich whole fish.[60, 254] Nevertheless, the species of fish consumed by free-ranging bottlenose dolphins differ from the species fed to managed dolphins, and we hypothesized that purine metabolite composition may differ between the two groups of species. Free-ranging dolphins consume a great variety of whole live fish and invertebrates that are available within their temperate and subtropical environment.[132, 161] On the other hand, dolphins under human care within the United States are fed frozen, stored, and thawed cold-water fish and squid species of less variety.[95, 110] Purine content is reported to differ among fish species, and post-mortem changes in purine metabolite concentrations have been documented in fish filleted for human consumption.[127, 227] Specifically, concentrations of inosine 5'-

monophosphate (IMP), inosine, hypoxanthine, and xanthine in cold-stored fish, vary greatly with species, storage temperature, and storage time.[117, 125, 126] Thus, it is possible that the purine composition of the diet of managed dolphins may differ both in the relative proportions of purine metabolites and in total purine content when compared with the free-ranging dolphin diet. To date, there are no reports of either the total purine content or individual purine metabolite concentrations of the whole fish species consumed by dolphins.

Most commonly when the purine content of a food is reported, the concentrations of the four nucleobases, adenine, guanine, hypoxanthine, and xanthine, are measured and summed to provide the total purine content. This total is used to determine whether the food is rich, moderate, or low in purines, and it is commonly advised that foods rich in purines are avoided by individuals prone to urate stone development.[47, 60, 239] Nevertheless, other purine metabolites, in particular IMP, inosine, and adenine 5'-monophosphate (AMP), are absorbed from the diet by other mammals, and can be metabolized to uric acid and excreted in urine.[16, 55, 77] Thus, purine content reported as the sum of only four nucleobase concentrations may not accurately represent the total potential purine load ingested by dolphins.

Furthermore, total purine content may not accurately represent the uricogenic potential of a food source because metabolism of individual purines varies. For example, adenine and hypoxanthine affect the amount of uric acid excreted by human being more so than guanine and xanthine.[66] It is not known how dolphins metabolize purines, but measuring a range of individual purine metabolites as well as the total purine content of fish species consumed by the two groups of dolphins should give a

better appreciation of their uricogenic potential. Our first objective, therefore, was to quantify and compare individual and total concentrations of eight purine metabolites among fresh frozen fish species consumed by free-ranging dolphins and frozen stored species fed to managed dolphins.

The total daily purine intake of free-ranging and managed bottlenose dolphins depends on the amount of each species consumed as well as the purine content of each individual species. The quantity of fish dolphins consume is determined by the metabolizable energy (ME) dolphins need to maintain body condition. To determine how many purines dolphins are consuming, therefore, the purine content of each species was determined relative to ME and the relative proportions of ME provided by each species were estimated to generate model diets for managed and free-ranging dolphins.[246] Model managed dolphin diets were based on the relative proportions of species fed by two dolphin management facilities and a model free-ranging dolphin diet was based on proportions of fish species reported to be consumed by bottlenose dolphins in Sarasota Bay, FL (see Chapter 4). Our second objective, therefore was to compare individual metabolite concentrations and total purine content among model diets typically consumed by managed and free-ranging dolphins. We hypothesized that concentrations would vary among fish species 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. Furthermore, we hypothesized that the managed dolphin model diets would contain more purines than the free-ranging model diet, which may provide a reason why managed dolphins are prone to forming urate nephroliths.

Materials and Methods

Fish Sampling and Processing

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

All fish sample procurement and processing methods were performed as described by Ardente et al. (see Chapter 4). The eight fish species most commonly consumed by free-ranging bottlenose dolphins residing in Sarasota Bay, FL ('free-ranging species', Table 6-1) were selected to represent the free-ranging dolphin diet.[148, 161, 245] Fish were caught, humanely euthanized, weighed, length measured, individually bagged, and transported in dry ice to the UF laboratory where fish were stored at -80ºC until further processing. Six fish species and one species of squid commonly fed to dolphins under human care ('managed species') were supplied by two facilities that care for bottlenose dolphins (Table 6-2). Fish and squid had been caught during one commercial fishing season, wrapped in plastic and frozen stored at -18ºC for 6 to 9 months. Then they were shipped overnight on dry ice to the UF laboratory where they were stored at -20ºC until further processing.

Five separate samples of each species were analyzed. To provide sufficient material to perform all the analyses on every sample, a minimum of 2 individual fish (or squid) were included in each sample; however, 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. The five samples of each species were processed, thawed, and ground as described by Ardente et al. (see Chapter 4). Ground samples were homogenized by hand and stored

in sample bags (Whirl-Pak® bags, Nasco, Fort Atkinson, WI 53538) at -80ºC until purine analysis was performed. All samples were analyzed by a researcher who was blind as to their origin.

Purine Analysis

Purine metabolites were extracted, separated and quantified using high performance liquid chromatography mass spectrometry as previously described, using both an internal standard and standard additions of external standards (see Chapter 5). Purine concentrations (mmol/Mcal ME) were reported relative to the metabolizable energy (ME) content of the fish and squid species. The metabolizable energy (ME) density of each species was calculated using the protein and fat contents previously reported for each fish and squid species (see Chapter 4) and Atwater factors (4 kcal ME/g of protein and 9 kcal ME/g of fat).[246] The total purine content was calculated as the sum of adenine, guanine, hypoxanthine, xanthine, uric acid, AMP, IMP, and inosine.

Model Dolphin Diets

Two model managed bottlenose dolphin diets (Table 6-3) fed to dolphins at two dolphin management facilities were compared with a model free-ranging bottlenose dolphin diet (Table 6-4) derived from the proportions of fish species reported to be consumed by bottlenose dolphins in Sarasota Bay, FL.[132]

Statistical Analysis

Values are reported as means +/- one standard deviation. Comparisons among fish and diets were performed with statistical software (SAS® System for Windows 9.4, SAS Institute Inc., Cary, NC, USA). 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. 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 is 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.[47, 265, 266]

Results

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 in free-ranging diet species than managed diet species (Table 6-5 and Table 6-6). Purine metabolite concentrations also differed ($p \leq 0.05$) among individual species (Table 6-5). Adenine, uric acid, IMP and AMP concentrations were present in very small to negligible amounts in almost all species, with a few exceptions, like ladyfish which contained at least twice the IMP content of all other species. Guanine and inosine were present in high concentrations in all species except for Loligo squid and toadfish which contained almost no guanine. The concentration of hypoxanthine was greater than xanthine in all species, and up to four times greater in some species. Icelandic capelin, Canadian capelin, and Loligo squid among managed diet species and Gulf toadfish of free-ranging species contained the most hypoxanthine.

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 6-5 and Table 6-6). 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 three-fold higher than the total purine content of spot and mullet.

The median total purine content was two-fold greater for the model managed dolphin diets (6.96 and7.82 mmol/Mcal ME, respectively) than the model free-ranging dolphin diet (3.91 mmol/Mcal ME; $p \leq 0.0001$) (Figure 6-1 and Table 6-6). The two model managed diets had similar individual purine metabolite contents except that managed diet #1 contained more IMP, and less adenine and xanthine than managed diet #2. The free-ranging model diet contained more IMP but less guanine, inosine, hypoxanthine, and xanthine than the managed model diets.

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. The purine content of the model free-ranging dolphin diet also provides a guide as to the amount of purines that can be consumed by dolphins without inducing nephrolith formation. It also may be possible to reduce the uricogenic potential of managed dolphin diets by feeding species that have a lower total purine content. Not surprisingly, fish with the highest ME density (free-ranging species mullet and spot and managed species herring) contained the least

purines relative to ME and therefore could be a substitute for species in the managed dolphin diet that are less energy dense and contain more purines.

The uricogenic potential of dietary purines varies, however, among species consuming the food. In rats, for example, dietary hypoxanthine and xanthine result in greater allantoin concentrations in the urine but uric acid concentrations remain unchanged.[267] Human beings, however, excrete more urinary uric acid when fed a diet supplemented with adenine, hypoxanthine, AMP, and IMP, but do not have an increase in urinary uric acid concentrations when fed a diet supplemented with guanine.[66, 259] It may be important, therefore, to consider the contribution of each purine metabolite, to the production of urinary uric acid in dolphins, but it is not known how readily each metabolite is absorbed from the dolphin's intestine. For example, inosine content of all managed and free-ranging diet species was greater than the concentrations of all other metabolites. It has been speculated, however, that the gastrointestinal epithelial absorption of inosine is saturable in rats because luminal inosine concentrations increased with increasing oral doses of AMP. Thus, it may be possible that very high concentrations of dietary inosine are inconsequential to urinary uric acid production in dolphins if inosine transport is also saturable.[61] Nevertheless, gastrointestinal absorption of inosine in dolphins is not known, and inosine can be converted readily to absorbed hypoxanthine during frozen storage; therefore, the uricogenic potential of inosine must not be discounted. Guanine concentrations were also greater than most other metabolites in all fish species, likely due to the presence of metallic scales which are rich in guanine.[254, 268] Gulf toadfish and Loligo squid were the only two species with much lower guanine content than the other species, which is most likely due to their

lack of metallic scales covering their skin. Thus, if guanine is well absorbed through the dolphins' gastrointestinal tract and has a greater uricogenic potential in dolphins than in people, the guanine content of the diet may be of particular concern. Loligo squid or toadfish could then be fed to dolphins in greater proportions in order to decrease the total guanine content of the diet. Canadian and Icelandic capelin provide another example because they contained twice as much hypoxanthine as Atlantic and Pacific herring. Hypoxanthine is uricogenic in people, so replacing capelin, which often represents a large proportion of the managed diet, with herring may help decrease urinary uric acid excretion.[66] In this way, it may be possible to alter the species fed to managed bottlenose dolphins in order to both reduce the concentrations of certain targeted metabolites and the total purine content until the diet more closely approximates the free-ranging dolphin diet.

It is important to remember however, that individual purine metabolites may have different uricogenic potential in dolphins than in human beings because dolphins metabolize purines to a different end-point than people. Dolphins excrete urinary allantoin in concentrations that are a thousand-fold greater than allantoin concentrations in the urine of people but are comparable to allantoin concentrations in the urine of dogs.(Ardente, under review; see Chapter 7 and 8) This suggests that dolphins, like dogs but unlike human beings, have functional uricase, the enzyme that oxidizes uric acid to allantoin. Nevertheless, uric acid concentrations relative to allantoin concentrations increase after a meal in managed dolphins; therefore, although uricase may be functional, it is possible that its ability to convert uric acid to allantoin is saturable following ingestion of a purine rich meal (see Chapter 8). Further studies are

needed to determine how changes in purine intake affect uric acid concentrations in dolphin urine.

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.[227] Concentrations of IMP have been reported to be greater in fresh fish and to decrease over time, degrading to inosine and then hypoxanthine, as fish is maintained in chilled storage.[269] In our study, concentrations of IMP and the ratio of inosine to hypoxanthine were greater in free-ranging species than managed species even though concentrations of hypoxanthine and inosine were greater among managed species than free-ranging species. There were two species, however, where purine metabolite concentrations did not follow the same pattern as that observed in other species within their group. The first was ladyfish which had at least twice the IMP content of all other managed and free-ranging diet species. Ladyfish fought vigorously when they were caught and died more rapidly than other species – often these fish appeared dead before being placed in the MS-222 bath. During supramaximal anaerobic activity, an additional ATP and AMP can be generated from two adenosine diphosphate (ADP) molecules, whereupon AMP is broken down to IMP, inosine, and eventually hypoxanthine.[16] Vigorous muscle movements in the ladyfish may have utilized more ATP, therefore generating more IMP. Then rapid freezing may have prevented further metabolism. The other exceptional species was Gulf toadfish, which contained hypoxanthine at a concentration that was comparable to that in capelin, the managed diet species with the greatest hypoxanthine content, and much greater than that in any other free-ranging species. Toadfish were the only fish caught in crab pots by local

commercial crabbers. The fish were then transported alive back to shore in five gallon 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.[270]

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 fish are composed of diverse tissues that respond differently to grinding. 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 are sorted for uniform size and sex, which may have led to more variability among free-ranging species compared to managed species.[101] Seasonal variations in protein and fat are reported to occur in other species, but all species in this study, from both diets, were caught during one season; therefore, any seasonal variations that may affect protein content, and in turn possibly purine content, were not considered.[98, 99, 119] For the managed diet species, the duration of frozen storage was fixed at 6-9 months, 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 months or greater than 9 months may have yielded different results.[271]

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 of fish and squid varies within and among facilities, depending on the requirements of individual dolphins. The free-ranging dolphin model diet was based on published information from one population of free-ranging inshore dolphins residing in the Sarasota Bay region of FL. The species consumed by these and other dolphin populations probably changes with 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 almost certainly great variation in the energy needs of any individual or group of dolphins depending on activity levels, water temperature, and life stage. Preliminary data suggests that free-ranging dolphins may have higher energy requirements than managed dolphins. An average 160 kg free-ranging dolphin in Sarasota Bay, FL, has been reported to have an average daily energy requirement (measured using double labelled water method) ranging from approximately 16 Mcal/day in the winter to 22 Mcal/day in the summer.[174] Among dolphins under human care at one facility, however, non-pregnant, non-lactating adults have been reported to consume approximately 8.5 to 12 Mcal/day and growing male and female dolphins to consume approximately 8.5 to 16 Mcal/day.[177] Total purine intake is ultimately affected by the amount of food consumed, so free-ranging dolphins may be consuming, metabolizing, and excreting more purines than some managed dolphins, even when the free-ranging diet may contain less purines than the managed diets on an equal caloric basis.

In conclusion, the individual purine metabolites differ significantly between the fish species fed to managed bottlenose dolphins and those consumed by free-ranging bottlenose dolphins. Additionally, the managed dolphin model diets have a greater total purine content than the model free-ranging dolphin diet. These differences in individual and total purine concentrations may affect urate nephrolith formation, but further investigation is necessary to determine the uricogenic potential of purine metabolites in dolphins and whether consumption of a managed diet with greater total purine composition results in greater urinary uric acid concentrations than concentrations of uric acid that would be excreted if a diet with a lower total purine content similar to the free-ranging diet is fed to managed dolphins.

Table 6-1. Fish species commonly consumed by free-ranging dolphins, the size of fish caught and location where fish were caught between May and September 2013

Fish species	Catch location	Wet weight (g)*	Length (mm)*
Abundant species			
Pinfish (*Lagodon rhomboides*)	Sarasota Bay, FL	70 (7-174)	143 (68-209)†
Striped mullet (*Mugil cephalus*)	Sarasota Bay, FL Roberts Bay, FL Gulf of Mexico, FL	615 (195-875)	333 (242-400)†
Sheepshead (*Archosargus probatocephalus*)	Sarasota Bay, FL	310 (165-560)	236 (188-294)†
Ladyfish (*Elops saurus*)	Sarasota Bay, FL Gulf of Mexico, FL	285 (134-919)	339 (253-600)†
Soniferous species			
Pigfish (*Orthopristis chrysoptera*)	Sarasota Bay, FL	65 (3-171)	143 (65-220)†
Spot croaker (*Leiostomus xanthurus*)	Sarasota Bay, FL Gulf of Mexico, FL	200 (132-310)	224 (202-260)†
Spotted sea trout (*Cynoscion nebulosus*)	Sarasota Bay, FL Gulf of Mexico, FL	293 (40-670)	313 (158-440)‡
Gulf toadfish (*Opsanus beta*)	Sarasota Bay, FL Gulf of Mexico, FL	119 (8-520)	157 (85-300)‡

*Values are medians with ranges in parentheses.

†Fork length measured from most anterior point of head to the deepest notch in tail fin.

‡Straight length measured from most anterior point of head to most caudal point of tail fin. All fish lengths fell within the reported range (50-300mm, up to 1,027mm) for fish consumed by free-ranging dolphins (McCabe 2010, Allen 2001).

Table 6-2. Fish species commonly fed to managed dolphins, the date and location where fish were caught

Fish and squid species	Catch location	Catch date
Pacific herring (*Clupea pallasii*)	Pacific coast, USA	December 2013
Atlantic herring (*Clupea harengus*)	East coast, USA	November 2013
Icelandic capelin (*Mallotus villosus*)	Iceland	March 2014
Canadian capelin (*Mallotus villosus*)	East coast, Canada	June 2014
Pacific mackerel (*Scomber japonicus*)	Pacific coast, USA	April 2014
Pacific sardine (*Sardinops sagax*)	Pacific coast, USA	October 2013
West coast Loligo squid (*Loligo opalescens*)	Pacific coast, USA	October 2013

Table 6-3. Proportions of fish and squid species in model managed common bottlenose dolphin diets

Fish and squid species	% 'as fed' weight	% Mcal ME
Managed diet #1		
Icelandic capelin	60	54.0
Pacific herring	20	31.9
Pacific mackerel	10	8.7
West coast Loligo squid	10	5.4
Managed diet #2		
Canadian capelin	60	47.4
Atlantic herring	10	15.2
Pacific herring	10	17.2
Pacific mackerel	10	9.4
Pacific sardine	10	10.8

Table 6-4. Proportions of fish species in a model free-ranging common bottlenose dolphin diet

Fish species	% total fish	% Mcal ME
Pinfish	41	27.1
Gulf toadfish	40	24.0
Sheepshead	4	9.2
Spot	3	11.4
Pigfish	3	1.6
Mullet	2	19.3
Ladyfish	2	2.9
Spotted sea trout	2	2.6
Menhaden	1	1.9
Atlantic threadfin herring	1	0.2

Table 6-5. Metabolizable energy content and purine metabolite concentrations* in fish and squid species consumed by managed and free-ranging common bottlenose dolphins

Species	ME Mcal/kg*	AD†	GN	UA†	HXA†	XA†	AMP†	IMP‡	INO†	Total†
		mmol/Mcal ME								
Managed diet										
Icelandic capelin	1.0±0.02^{e}	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}
Canadian capelin	0.8±0.01^{f}	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}
Pacific herring	1.8±0.02^{b}	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}
Atlantic herring	1.6±0.01^{c}	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}
Pacific mackerel	1.0±0.01^{e}	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}
Pacific sardine	1.1±0.03^{d,e}	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}
Loligo squid	0.6±0.01^{g}	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}
Free-ranging diet										
Pinfish	1.2±0.03^{d}	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}
Gulf toadfish	0.7±0.01^{g}	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}
Sheepshead	1.0±0.04^{e,f}	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}
Spot	2.0±0.04^{a}	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}
Pigfish	1.2±0.05^{d,e}	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}
Mullet	1.7±0.03^{b,c}	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}
Ladyfish	0.9±0.05^{e,f}	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}
Spotted sea trout	0.8±0.02^{f}	0.02±0.001^{c}	1.9±0.08^{a}	0.002±0.001^{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}

*Values are means ± one standard deviation. ME Mcal/kg relative to 'as fed'. AD, adenine; GN, guanine; UA, uric acid; HXA, hypoxanthine; XA, xanthine; AMP, adenosine monophosphate; IMP, inosine monophosphate; INO, inosine; BLD = below limit of detection

^{abcdefg} Values with different superscripts within a column are significantly different ($p \leq 0.05$).

†Managed diet species have greater purine metabolite content than free-ranging diet species ($p \leq 0.05$).

‡Free-ranging diet species have greater IMP content than managed diet species ($p \leq 0.05$).

Table 6-6. Purine metabolite concentrations for model managed and free-ranging diets*

Purine metabolite (mmol/Mcal)	Managed model diet #1	Managed model diet #2	Free-ranging model diet	Managed diet species	Free-ranging diet species
AMP†	0.04 ± 0.00[a]	0.04 ± 0.00[a]	0.05 ± 0.00[a]	0.07 ± 0.00‡	0.05 ± 0.00
IMP†	0.07 ± 0.02[a]	0.01 ± 0.00[b]	0.15 ± 0.01[c]	0.03 ± 0.00‡	0.24 ± 0.02
Adenine	0.06 ± 0.01[a]	0.14 ± 0.02[b]	0.05 ± 0.00[a]	0.06 ± 0.01‡	0.04 ± 0.00
Guanine	2.01 ± 0.16[a]	1.63 ± 0.11[a]	1.09 ± 0.05[b]	1.66 ± 0.09	1.45 ± 0.05
Inosine	1.98 ± 0.06[ab]	2.19 ± 0.08[a]	1.61 ± 0.10[b]	2.12 ± 0.05‡	1.86 ± 0.09
Hypoxanthine	1.70 ± 0.13[a]	1.92 ± 0.15[a]	0.91 ± 0.05[b]	1.73 ± 0.08‡	0.75 ± 0.03
Xanthine	1.14 ± 0.08[a]	2.06 ± 0.22[b]	0.14 ± 0.01[c]	1.23 ± 0.01‡	0.16 ± 0.01
Uric acid	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.02 ± 0.00[b]	0.01 ± 0.00‡	0.02 ± 0.00
Total purines, 4 metabolites[δ]	4.92 ± 0.35[a]	5.74 ± 0.41[a]	2.20 ± 0.09[b]	4.69 ± 0.19‡	2.40 ± 0.08
Total purines, 8 metabolites[δ]	7.02 ± 0.38[a]	7.98 ± 0.46[a]	4.03 ± 0.13[b]	6.92 ± 0.21‡	4.56 ± 0.14

*Values are means ± standard error.

†AMP, adenine 5'-monophosphate; IMP, inosine 5'-monophosphate

‡Purine metabolite concentrations are different between managed diet species and free-ranging diet species ($p \leq 0.05$).

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

[δ]Total purines obtained using the traditional method of summing the content of four metabolites: adenine, guanine, hypoxanthine, and xanthine. Total purines obtained by summing the content of eight metabolites measured reported here.

CHAPTER 7
URINE PURINE METABOLITES EXCRETED BY FREE-RANGING COMMON BOTTLENOSE DOLPHINS

Purine nucleotides from DNA, RNA, and ATP are metabolized to uric acid, which is then oxidized in most mammals to allantoin by uricase. Human beings lack a functional gene for uricase, and excrete uric acid in urine in much higher concentrations compared to other mammals. Excretion of uric acid is only favored over allantoin in other mammals when there is a defect in purine catabolism. For example, in Dalmatian dogs, a hereditary defect results in reduced oxidation of uric acid by uricase and reduced reabsorption of uric acid by renal tubules.[87, 88]

As a result of increased urinary uric acid excretion, some human beings form uric acid uroliths and some Dalmatians form ammonium urate uroliths. Common bottlenose dolphins (*Tursiops truncatus*) under human care also develop ammonium urate nephroliths, but free-ranging common bottlenose dolphins do not, and the reason for this difference is unknown.[1] Both dolphins under human care and free-ranging dolphins consume a purine-rich diet of whole fish. Bottlenose dolphins excrete uric acid, but it is not known whether they excrete allantoin.[3, 272] We sought, therefore, to determine whether allantoin is present in urine of free-ranging bottlenose dolphins and to compare urinary uric acid and allantoin concentrations with those of non-Dalmatian dogs, which are known to convert uric acid to allantoin. The purine metabolites, hypoxanthine and xanthine, were also measured because they are excreted in urine by some species.[60]

Urine samples were collected by catheterization from 15 free-ranging bottlenose dolphins during health assessments performed during 2014 and 2015 by the Chicago Zoological Society's Sarasota Dolphin Research Program in Sarasota, FL.[245] All dolphins had fish matter identified in their forestomach by ultrasound examination. Urine

samples were collected from 5 unfed (for 12 hours) healthy dogs by free-catch. Samples were collected under the National Oceanic and Atmospheric Administration's National Marine Fisheries Service Scientific Research Permit No. 15543, and the auspices of Mote Marine Laboratory and University of Florida (UF) Institutional Animal Care and Use Committees.

Purine analysis was conducted in the UF Southeast Center for Integrated Metabolomics (SECIM) Core Laboratory (Gainesville, FL) using a high-performance liquid chromatography with tandem mass spectrometry (Thermo Accela 1250 with Thermo TSQ Quantum Access, Thermo Scientific, Waltham, MA, USA). Allantoin, uric acid, hypoxanthine, and xanthine were identified by mass and retention time, and metabolites were quantified using external standard concentration curves. Creatinine was measured using a chemistry analyzer (Dimension® Xpand® Plus, Siemens, Malvern, PA, USA) with a reagent cartridge (Dimension® Flex®, Siemens). Statistical analysis was performed using statistical software (SAS® 9.4 for Windows, SAS Institute Inc., Cary, NC, USA). Urine concentrations and relative ratios were compared between bottlenose dolphins and dogs using a Kruskal-Wallis test with a 5% probability of error when rejecting the null hypothesis. Concentrations were also compared to previously reported means in other species.[81, 82, 273-277]

Mean uric acid concentrations and uric acid to creatinine ratios were 2.5 and 5 times greater, respectively, in dolphin urine than in dog urine ($p \leq 0.01$). Dog values were similar to, or greater than, previously reported mean concentrations of uric acid in urine from dogs, cats, sheep, cows and human beings consuming a low protein diet, but similar to concentrations in urine from human beings fed a high protein diet (Table 7-1).

There was no evidence that creatinine or allantoin concentrations or the ratio of allantoin to creatinine differed between dogs and dolphins. The mean ratio of uric acid to allantoin was on average four-fold higher in dolphin urine than in dog urine ($p \leq 0.001$) (Figure 7-1). Hypoxanthine and xanthine concentrations in dolphin urine were small and below the limit of detection in dog urine.

Urine allantoin concentrations in common bottlenose dolphins were comparable to concentrations in species that make uricase and thousand-fold higher than have been previously reported in urine from human beings that do not make uricase. The greater uric acid concentrations in dolphin urine than dog urine may be because dolphins had eaten and dogs had not or because of differences in purine content of food consumed by dolphins and dogs. Bottlenose dolphins consume primarily whole fish which contain considerable amounts of protein and purines and almost no carbohydrate.[113] The dogs in this study were being fed commercial dry adult dog food containing substantial amounts of carbohydrate, which dilutes protein and purines in the diet. When compared with dog urine, the greater uric acid concentrations relative to allantoin concentrations in bottlenose dolphin urine suggest that either uricase is saturated or operating at a reduced rate in dolphins, or that the capacity of the kidney to reabsorb uric acid may be reduced in dolphins. In animals consuming a high purine diet, reduced retention may be a normal adaptive response in order to excrete excess uric acid. The mean fractional excretion of uric acid has been reported in healthy rats (17%), dogs consuming a meat-based diet (27%), and in healthy men (7%) and women (10%), and has been shown to increase in people with increased uric acid production. Bottlenose dolphins, however, appear to have a mean fractional excretion of uric acid

that is substantially higher (63% unfed and 89% post-prandial). (Bartges and others 1994;[82, 278-280]

In conclusion, free-ranging common bottlenose dolphins excrete allantoin, like most other mammals with functioning uricase. Nevertheless, the dolphins' capacity to metabolize and excrete uric acid as allantoin in the urine appears to be limiting, which may explain why dolphins consuming a high purine diet are at risk of developing urate nephroliths under certain circumstances.

Table 7-1. Urine creatinine and purine metabolite concentrations* of common bottlenose dolphins and dogs, with published values in other species

Species	Creatinine	Hypoxanthine	Xanthine	Uric Acid†	Allantoin	UA:CR†‡	AL:CR‡
	----------mmol/L----------					----------mmol:mmol----------	
Free-ranging bottlenose dolphins, post-prandial	12.9 (4.6-37.9)	1.5 (0.9-5.0)	0.3 (0.2-1.0)	3.3 (1.0-6.1)	6.6 (4.1-14.1)	0.3 (0.05-0.6)	0.5 (0.2-0.9)
Dogs, un-fed	24.0 (17.5-31.7)	ND‡	ND‡	1.3 (1.0-1.9)	8.4 (6.5-18.2)	0.06 (0.03-0.08)	0.4 (0.2-0.8)
Bottlenose dolphins under human care, fasted[a]	14.2			0.9		0.06	
Bottlenose dolphins under human care, post-prandial[a]	9.4			2.6		0.28	
Human beings, low-protein diet[b]	8.4			2.1		0.24	
Human beings, high-protein diet[b]	8.8			3.3		0.38	
Human beings[c]					0.008 ± 0.002		
Dog[d]	18.6 (2.6-28.4)	ND‡	ND‡	1.2 (0.3-2.4)		0.08 (0.05-0.1)	
Dog[e]						0.05 (0.01-0.38)	1.0 (0.16-7.9)
Cat, males[f]	16.2 (4.8-32.3)			0.52 (0.01-1.35)		0.04 (0.001-0.11)	
Cat, females[f]	12.0 (3.3-27.8)			0.39 (0.01-0.98)		0.04 (0.002-0.09)	
Cow[g]	5.7 ± 1.8	ND‡	ND‡	1.9 ± 1.1	12.8 ± 5.5		
Sheep[g]	3.2 ± 1.3	0.5 ± 0.2	0.03 ± 0.02	0.6 ± 0.3	1.7 ± 0.6		

[a]: Smith (2014) J. of Urology; [b]: Fellstrom (1983) Clin. Sci.; [c]: Kim (2009) J. Chrom. B; [d]: Gow (2011) Vet Record; [e]: Rivara (2013) Vet. Record; [f]: Cottam (2002) J. Nutr.; [g]: Shingfield (1999) J. Chrom. B.

*Values are medians with range in parentheses or means ± one standard deviation.

†Uric acid concentration and UA:CR are significantly different between free-ranging bottlenose dolphins and dogs ($p \leq 0.01$).

‡UA:CR, uric acid to creatinine ratio; AL:CR, allantoin to creatinine ratio; LOD, limit of detection (Hypoxanthine 0.012 mmol/L, Xanthine 0.005 mmol/L); ND, not detected

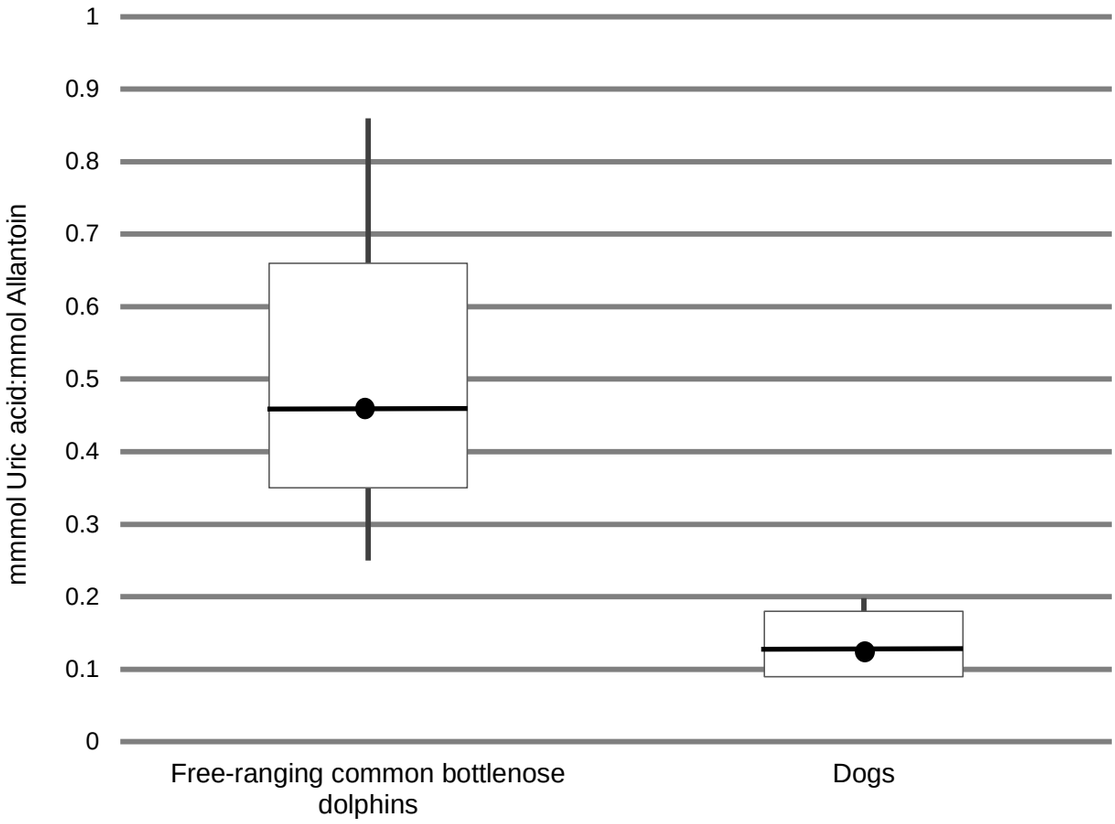


Figure 7-1 Uric acid to allantoin ratios in urine from bottlenose dolphins and dogs. The white line represents the median; boxes represent the 25th and 75th percentiles; whiskers represent the range. Bottlenose dolphins have greater urine uric acid concentrations relative to allantoin concentrations than dogs ($p \leq 0.05$).

CHAPTER 8
URINARY PURINE AND ALLANTOIN CONCENTRATIONS IN COMMON BOTTLENOSE DOLPHINS, *TURSIOPS TRUNCATUS*, UNDER HUMAN CARE

Introduction

Some common bottlenose dolphins (*Tursiops truncatus*) managed under human care develop ammonium urate nephroliths; however, these nephroliths rarely form in free-ranging bottlenose dolphins.[1] The prevalence of nephrolithiasis among managed bottlenose dolphins is poorly documented, but one facility reports 35% of their dolphins are affected.[1] The reason for nephrolith formation in managed dolphins is unknown, but the composition of the diet can influence ammonium urate urolith formation in other mammals, and may also affect urate stone formation in dolphins.[5, 8]

The tendency for ammonia and urate to complex and precipitate as ammonium urate crystals is determined by the relative concentrations of ammonium and urate ions in urine, as well the presence of other solutes, and urine pH.[4, 10, 185] Given enough time and appropriate conditions, crystals may then aggregate to form stones.[10] Uric acid is a product of purine metabolism. Purines contribute to the structure of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), adenine triphosphate (ATP) and guanine triphosphate (GTP). Purines are either made by the body, salvaged and recycled, or absorbed from food.[13, 55, 60, 239]. Foods that are purine-rich include organ meat and seafood.[60, 254] In most mammals, uric acid is excreted in urine but can be further oxidized to allantoin by uricase. Allantoin is more soluble in urine and excreted in much higher concentrations than uric acid.[17] Uric acid excretion is only favored over allantoin in mammals when there is a defect in purine catabolism. Human beings, for example, lack the functional gene for uricase, and excrete uric acid in much greater concentrations compared to other mammals. Some Dalmatian dogs also excrete

greater concentrations of uric acid than allantoin in their urine because they have a hereditary defect which causes inefficient oxidation of uric acid by uricase and reduced uric acid reabsorption by the renal tubules.[87, 88]

Consumption of a purine-rich diet is a risk factor for urolith formation in human beings and dogs.[8, 273, 276] Whole fish, which comprise the bulk of the managed and free-ranging dolphin diet, are purine-rich[60, 239] but the concentrations of purine metabolites excreted in the urine of dolphins has not been well documented.

We have recently reported that the urine of free-ranging bottlenose dolphins with food in their stomachs contains allantoin in concentrations that are comparable to concentrations in the urine of unfed (non-Dalmatian) dogs, a species which makes uricase. The concentrations of allantoin in dolphin urine were also much greater than those in the urine of human beings, a species which lacks functional uricase.[281] Furthermore, fed free-ranging dolphins had greater urinary uric acid concentrations relative to allantoin concentrations than were observed in the urine of unfed dogs.[281]A post-prandial rise in urinary uric acid concentrations has been reported to occur in managed dolphins,[82] and could explain why allantoin and uric acid concentrations were greater in free-ranging dolphins that had recently consumed a meal when compared with unfed dogs. Also, the whole fish consumed by bottlenose dolphins is much richer in purines than the commercial dry kibble diet of dogs so we hypothesized there may be saturation or decreased functional efficiency of uricase or a decreased capacity for uric acid reabsorption by the renal tubules after a meal in dolphins.[281]

Nevertheless, free-ranging dolphins rarely develop urate nephroliths, so it is possible that purine concentrations may be different in the urine of managed bottlenose

dolphins. Free-ranging and managed dolphins both consume whole fish, but free-ranging diet fish species contain less total purines relative to energy intake than managed dolphin diet species. Free-ranging dolphins also consume smaller, more frequent meals than managed dolphins. Thus, free-ranging dolphins likely consume less purines per meal than managed dolphins, which may be a reason why free-ranging dolphins do not develop nephrolithiasis (see Chapter 6).

It is also not known why some, but not all, dolphins under human care at a given facility develop ammonium urate nephrolithiasis. No risk factors have been identified that might explain the difference in disease prevalence among individuals in the same managed population, so there is likely a continuum of nephrolith risk among all managed dolphins.[2, 3, 82] Managed dolphins with nephrolithiasis do excrete greater concentrations of uric acid, however, than healthy managed dolphins.[3] The greater concentrations of uric acid may be due to the resulting kidney disease that occurs in dolphins with nephrolithiasis, reducing the ability of the renal tubules to reabsorb urate.

We also noted the presence of hypoxanthine and xanthine in addition to allantoin and uric acid in the urine of fed free-ranging dolphins. Hypoxanthine and xanthine are also products of purine metabolism but are usually metabolized to uric acid. Both hypoxanthine and xanthine have been reported in the urine of other species, including cows, sheep and rats, and in some instances have been associated with derangements in purine metabolism.[60, 277] For example, a genetic mutation causing hypoxanthine-guanine phosphoribosyl transferase deficiency in human beings results in greater concentrations of hypoxanthine and xanthine in the urine.[282] Another genetic mutation in people and Cavalier King Charles spaniels causes xanthine dehydrogenase deficiency

and therefore greater concentrations of xanthine to be excreted in the urine compared to uric acid.[283, 284]

Thus, our first objective was to determine whether managed dolphins with or without nephrolithiasis experience a post-prandial increase in allantoin and other purine concentrations in the urine. We hypothesized that post-prandial allantoin concentrations in the urine of fed managed dolphins may not increase to the same extent as urinary uric acid concentrations, and that concentrations might be different before and after feeding between managed dolphins with and without nephrolithiasis. We hypothesized, also, that fed managed dolphins might excrete *greater* concentrations of urinary uric acid relative to allantoin than fed free-ranging dolphins, because they may consume a more purine-rich diet.

Materials and Methods

Study Population

Urine samples from managed dolphins were a subset of samples collected for a larger research endeavor examining the pathophysiological basis for ammonium urate nephrolithiasis in bottlenose dolphins owned and cared for by the Navy Marine Mammal Program (MMP).[82] The Secretary of Navy Instruction 3900.41G directs that Navy marine mammals be provided the highest quality care. The MMP is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and adheres to the national standards of the United States Public Health Service Policy on the Humane Care and Use of Laboratory Animals and the Animal Welfare Act. Further, all samples were collected under a protocol approved by the MMP's Institutional Animal Care and Use Committee (IACUC) and the Navy's Bureau of Medicine. Urine sample handling and processing was approved by the University of

Florida's IACUC. The study population and study design have been described in detail previously, but are summarized here.[82]

The study population of managed dolphins included four, two male and two female, sexually mature healthy (non-stone forming) bottlenose dolphins and four, two male and two female, sexually mature stone forming bottlenose dolphins of similar body weight. Dolphins were considered to be healthy non-stone formers if they had no evidence of azotemia within the last 10 years and had no nephroliths detected with routine ultrasound examination; whereas stone forming individuals had previously developed azotemia and nephroliths had been detected with ultrasound, but were not currently azotemic. All dolphins were housed by the MMP in open-ocean enclosures within San Diego Bay, CA.

Urine was collected continuously for six hours from each dolphin, including two hours before and four hours after a meal. Prior to sample collection, dolphins were fasted for 12 hours overnight and then were lightly sedated with diazepam or midazolam. The bladder was catheterized, emptied and a closed collection system was attached to the catheter for continuous urine collection. While the urinary catheter was in place, dolphins were suspended in water within fleece-lined stretchers. Urine that was collected for the first two-hour period was analyzed as the unfed sample. The dolphins were then fed one-third (1.8-2.7 kg) of their total daily diet of frozen thawed whole fish, consisting of one-third Pacific herring (*Clupea pallasii*) and two-thirds Icelandic capelin (*Mallotus villosus*) (by as fed weight). Urine was then collected for four hours after the meal, and the aliquot taken two to four hours after feeding was analyzed as the post-prandial sample. All urine samples collected were frozen and stored at -80ºC. Samples

were shipped overnight on dry ice to the University of Florida (UF) College of Veterinary Medicine where they were maintained frozen at -80 ºC until the analysis was performed.

Purine metabolite concentrations and ratios were measured in each urine sample. Metabolite concentrations and ratios in the urine of the fed managed dolphins was also compared to previously reported concentrations and ratios of urine from 15 apparently healthy free-ranging bottlenose dolphins. Free-ranging dolphin urine samples were collected during health assessments performed during May 2014 and 2015 by the Chicago Zoological Society's (CZS) Sarasota Dolphin Research Program (SDRP) in Sarasota, FL.[245, 281] During health assessments led by the CZS SDRP, bottlenose dolphin body condition, morphometrics, genetics, life history and health data were obtained through capture-release research.[245] Selected dolphins were encircled with a 500m long x 4m deep seine net in the shallow, sheltered Sarasota Bay waters. One at a time, individual dolphins were placed in a sling, lifted aboard a specialized veterinary examination vessel, and placed on a shaded pad. The dolphin was kept wet with bay water while its behavior, respiratory pattern, and heart rate were continuously monitored. The dolphin was first weighed, measured for standardized lengths and girths, and ultrasonic measurements of blubber thickness were made at standardized sites. A full physical examination was then performed by experienced marine mammal veterinarians, which included ultrasound examination of internal organs and sex determination by examination of the genital region. Blood, urine, feces, milk, microbiological swabs, and biopsy samples were routinely collected. Upon completion of the physical examination and sample collection, the dolphin was marked with a freeze brand or tagged as appropriate, photographed, and released. For this study, 2

mL aliquots of urine were collected via sterile catheterization from 15 free-ranging dolphins. Urine samples were frozen in liquid nitrogen and stored at -80°C until purine analysis was performed. Fish matter was identified in the forestomach by ultrasound examination of all 15 free-ranging study dolphins. All samples, including the urine samples used in this study, were collected from dolphins under the National Oceanic and Atmospheric Administration's National Marine Fisheries Service Scientific Research Permit No. 15543, with approval by Mote Marine Laboratory's IACUC.

Purine Analysis

Chemical reagents

A 0.01mM sodium hydroxide (NaOH, pH 11.8, Fisher Scientific Company, Suwanee, GA 30024) solution and artificial urine were prepared freshly at least once every month. Artificial urine (pH ~5) was prepared by dissolving the following chemicals in water: potassium chloride, sodium chloride, urea, potassium phosphate, sodium hydroxide, sodium bicarbonate, and sulfuric acid (Fisher); and citric acid, ascorbic acid, and creatinine (Sigma-Aldrich, St. Louis, MO 63103).[285] A solution of 2% acetonitrile and 0.1% sodium azide (Fisher) in water was also prepared. The mobile phase solution 0.1% acetic acid (Fisher) in water was prepared at least every 48 hours. The water used for mixing all solutions and all solvents was HPLC-grade (Fisher Scientific Company, LLC, Suwanee, GA, USA).

All purine metabolites for external calibration standards were purchased from Sigma. Because purine metabolite solubility is highly pH dependent, separate stock solutions were prepared by dissolving allantoin in artificial urine, whereas uric acid, xanthine, and hypoxanthine were dissolved together in 0.01 mM NaOH. All solutions were heated (35°C) in a water bath and sonicated for 10-15 minutes for complete

dissolution. These two solutions were then combined to produce a stock solution containing final concentrations of allantoin at 0.425 mg/mL (2.7 mM), uric acid at 0.025 mg/mL (0.15 mM), xanthine at 0.025 mg/mL (0.16 mM), and hypoxanthine at 0.05 mg/mL (0.37 mM).

Internal standard stock solutions were prepared by dissolving 15N2-xanthine (Cambridge Isotopes Laboratory, Inc., Tewksbury, MA, USA) to a final concentration of 0.04 mg/mL (0.26 mM) in 0.01mM NaOH and DL-allantoin-5-^{13}C, 1-^{15}N (ISOTEC®, St. Louis, MO, USA) to a final concentration of 0.5 mg/mL (3 mM) in the 98% water/2% acetonitrile solution. Both external and internal standard solutions were prepared, then frozen in aliquots at -80ºC, and a fresh aliquot was thawed to room temperature each day an analysis was performed.

Calibration standards and sample preparation

A 150 µL aliquot of either undiluted or 1:2, 1:8, and 1:16 dilutions of the external standard stock solution in artificial urine was combined with 25 µL of allantoin internal standard, and 25 µL of xanthine internal standard, and then diluted to 1 mL in a 2 mL Eppendorf tube (Eppendorf, Fisher) to establish a calibration curve. Dolphin urine samples were thawed to room temperature in tap water. Creatinine concentrations in an 150 µL aliquot of urine was measured using a chemistry analyzer (Dimension® Xpand® Plus, Siemens, Malvern, PA, USA) with a reagent cartridge (Dimension® Flex®, Siemens). Urine samples were diluted either 1:4, 1:15, 1:30 or 1:60 with 98% water and 2% HPLC-grade acetonitrile (Fisher) solution and then 150 µL was mixed with 25 µL of each internal standard solution and diluted to 1 mL in a 2 mL Eppendorf tube. Samples and standards were vortexed for 30 seconds and then for each individual standard solution or sample, a 480 µL aliquot was transferred to a 500 µL centrifuge tube with a

0.2 µm nylon filter insert (Costar® Spin-X®, Corning, NY 14831) and centrifuged at 5,000 rcf for 5 minutes (Eppendorf centrifuge 5417R, Sigma).

Urine from one adult male-neutered dog (collected free-catch) was analyzed alongside every dolphin urine analysis to ensure the method was working consistently. Aliquots of dog urine were frozen at -80ºC and thawed similarly to dolphin urine samples. A 20 µL aliquot of urine was added to 930 µL of water/acetonitrile and 25 µL of each internal standard solution in a 2 mL conical tube. The dog urine sample was then processed the same way as dolphin urine samples.

Chromatographic analysis

Purine metabolite separation in urine was performed with high performance liquid chromatography (Accela 1250 HPLC system, Thermo Scientific, Waltham, MA 02451) using a reverse phase Luna 5µm PFP(2) column (100 Å, 150 mm x 3.0 mm, Phenomenex, Torrance, CA 90501) with guard column (SecurityGuard for PFP HPLC, Phenomenex). The column and compartment were maintained at ambient temperature. The injection volume was 10 µL with a flow rate of 500 µL/min. The elution conditions for separation of purine metabolites began with 97% A (0.1% acetic acid in water, pH 3.5) and 3% B (methanol) for 2 minutes, gradually decreased to 90% A and increased to 10% B from 2 to 4 minutes, 70% A and 30% B from 4 to 5.5 minutes, 50% A and 50% B from 6 to 6.5 minutes, with a final return to the original conditions at 9 minutes. The column was maintained under these conditions for re-equilibration for a further 6.5 minutes. For column storage between runs, the column was flushed at 500 µL/min with 65% acetonitrile and 35% water for at least 10 column volumes.

Mass spectrometry analysis

Purine metabolites were identified by mass and retention time (TSQ Quantum Access Max, Thermo) with a triple quadrupole mass spectrometer with heated electrospray ionization (HESI). The HESI source was operated in positive-ion mode for detection of allantoin, with the following settings: spray voltage 4000 V, vaporizer temperature 250°C, sheath gas pressure 50 arb, ion sweep gas pressure 10 arb, auxillary gas pressure 10 arb, and capillary temperature 300°C. The HESI source was operated in negative-ion mode for detection of uric acid, hypoxanthine, and xanthine, with the following settings: spray voltage 2500 V, vaporizer temperature 350°C, sheath gas pressure 50 arb, ion sweep gas pressure 0.0 arb, auxillary gas pressure 10 arb, and capillary temperature 350°C. Compounds were identified by comparing the retention time and selected reaction monitoring (SRM) pairs (Table 1) during one scan event with a 20 msec scan time, as well as through the addition of purine standards. Data was processed using computer software (Xcalibur Quan Browser, Thermo).

Statistical Analysis

SAS® software, Version 9.4 of the SAS System for Windows (Cary, NC, USA) was used to perform all statistical analyses. Differences in metabolite concentrations between unfed and fed managed dolphins, either healthy or with nephrolithiasis or for both groups of dolphins combined, were evaluated using a Wilcoxin Signed Rank test. Metabolite concentrations and ratios were also compared between unfed and fed healthy managed dolphins and unfed and fed dolphins with nephrolithiasis using a Wilcoxin Rank Sum exact test. Differences in metabolite concentrations and ratios were compared between fed managed dolphins (with or without nephrolithiasis) and fed free-ranging dolphins with a Wilcoxin Rank Sum exact test. A Bonferroni correction was

applied to each experiment to maintain type 1 experiment-wise error ≤0.05. Thus, for example, only values ≤0.01 were considered significant when comparing values before or after feeding in managed dolphins with or without nephrolithiasis.

Results

Separation of purine metabolites hypoxanthine and xanthine, breakdown products uric acid and allantoin, and xanthine and allantoin internal standards was achieved with this HPLC-MS method (Figure 8-1). There was no evidence of a change in concentrations or concentration ratios of purines to creatinine or uric acid to allantoin for fed or unfed healthy managed dolphins compared with fed or unfed managed dolphins with nephrolithiasis ($p \geq 0.1$; Table 8-2). When both healthy dolphins and dolphins with nephrolithiasis were considered together, however, there was a trend towards a decrease in concentration of creatinine ($p = 0.05$) after a meal. Purine concentrations did not change to the same degree ($p \geq 0.01$) after the meal, and as a result, the ratio of the concentrations of xanthine, hypoxanthine, and uric acid to creatinine increased ($p \leq 0.008$), and there was a trend towards an increase in the ratio of allantoin to creatinine concentrations ($p = 0.015$) after a meal. Excretion of other purines increased more than allantoin, however, so the ratio of the concentration of uric acid and of the combined concentrations of hypoxanthine and xanthine relative to allantoin were higher post-prandially than before a meal ($p \leq 0.008$; Table 8-3). The ratio of the combined concentrations of xanthine and hypoxanthine to uric acid decreased after a meal ($p \leq 0.008$). There was no evidence of a difference in urine concentrations or ratios between managed dolphins and free-ranging dolphins after a meal ($p \geq 0.1$), except that post-prandially, free-ranging dolphins had greater

concentrations of hypoxanthine in their urine than managed dolphins ($p \leq 0.04$; Table 8-2).

Discussion

This study demonstrated that there was a post-prandial increase in uric acid, hypoxanthine and xanthine concentration relative to creatinine and allantoin concentrations in the urine of managed dolphins. These findings provide support for our previous suggestion that the oxidation of uric acid to allantoin by uricase and/or the efficiency of the renal tubules to reabsorb uric acid may be overwhelmed or saturated in dolphins, in this instance, following the intake of a purine-rich meal.[281] Consumption of a purine-rich meal has the potential, therefore, to increase urinary uric acid concentrations making urolith formation more likely, even though dolphins make allantoin. Furthermore, this study determined that hypoxanthine and xanthine concentrations relative to creatinine increase following a meal. Hypoxanthine and xanthine concentrations combined increase more than allantoin concentrations in fed dolphin urine, which may indicate xanthine oxidase, the enzyme responsible for converting hypoxanthine to xanthine and then xanthine to uric acid, is also overwhelmed or saturated. On the other hand, the concentration of hypoxanthine and xanthine to uric acid decreased which suggests that uricase not xanthine oxidase is the primary bottle-neck.

Fed free-ranging dolphins had similar uric acid and allantoin concentrations in their urine when compared with fed managed dolphins which suggests that there may be no difference, genetic or metabolic, in how free-ranging and managed dolphins metabolize dietary purines. Nevertheless, the response to a meal has not been assessed in free-ranging dolphins, and the post-prandial concentration of uric acid excreted in the urine may be affected by the size and frequency of the meal and the

relative quantities of purines ingested. Free-ranging bottlenose dolphins consume fish in smaller, more frequent meals throughout both the day and night, whereas managed dolphins are fed 3-8 meals over the duration of a 8-9 hour business day.[82, 132, 193, 194] Thus, larger fish meals consumed as a bolus by managed dolphins may provide a larger dose of purines that must be digested and metabolized over a shorter time period. This bolus of purines may cause uric acid to be excreted in greater quantities in the urine at one time compared to concentrations of uric acid in the urine of free-ranging dolphins that are consuming small frequent meals.

This study was also the first to quantify and compare the urine purine metabolite concentrations among healthy bottlenose dolphins and dolphins with nephrolithiasis under human care.[281] Though urine concentrations of creatinine, hypoxanthine, xanthine, and uric acid appeared to be lower in dolphins with nephrolithiasis than in healthy managed dolphins, sample size was very small, which limited the capacity to detect a difference even if one had been present. Nevertheless, in other mammals, nephrolithiasis can cause kidney damage and limit the capacity to concentrate urine.[286] It is plausible, therefore, that dolphins with nephrolithiasis had chronic kidney disease and were unable to concentrate their urine.

It is also interesting to note that hypoxanthine and xanthine are consistently present in the urine of both unfed and fed managed dolphins and free-ranging dolphins. Hypoxanthine and xanthine are not found in the urine of healthy people, dogs, and cats, but are present in cow, sheep and rat urine.[60, 277, 281] Dolphins, however, have urinary concentrations of hypoxanthine that are 20 to 50 fold greater and xanthine that are 55 to 70 fold greater than concentrations in the urine of cows, sheep, and rats.[281] Thus,

dolphins may have adapted to their purine-rich diet and excrete hypoxanthine and xanthine in their urine as a way of minimizing the amount of uric acid excretion.

The greatest limitation of this study design was the small sample size of managed dolphins. Many of the differences observed in urine creatinine and purine metabolite concentrations and relative ratios among dolphin populations were not statistically significant, but differences may have been detected if the population sample sizes were larger. Only a few dolphins were studied because each dolphin had to be acclimated to a smaller pool and trained to accept catheterization for continuous collection of urine over six hours.

In conclusion, managed bottlenose dolphins experience a greater post-prandial rise in urinary uric acid concentrations than allantoin concentrations, which suggests that the metabolic pathway for conversion to allantoin may be overwhelmed by a the large intake of purines consumed with a fish meal. Nevertheless, fed managed bottlenose dolphins excrete similar concentrations of uric acid and allantoin in their urine as free-ranging dolphins.

Table 8-1 Purine metabolite mass spectrometer identification parameters

Metabolite	Precursor mass (g/mol)	Product mass (g/mol)	Collision energy (eV*)	Tube lens (V)
Positive ion mode				
Allantoin	159.067	73.3	18	79
		116.1	5	79
DL-allantoin-5-^{13}C, 1-^{15}N	161.10	61.5	10	79
		74.3	18	79
		118.1	5	79
Negative ion mode				
Hypoxanthine	135.05	65.34	29	74
		95.22	18	74
Xanthine	151.02	80.28	26	78
		108.16	18	78
$^{15}N_2$-xanthine	152.95	80.95	32	66
		109.20	18	66
Uric acid	167.00	124.24	16	65

*eV, electron volts

Table 8-2. Urine creatinine and purine metabolite concentrations* of common bottlenose dolphins.

Dolphin population	Sample size (n)	Unfed/Fed	Nephroliths	Creatinine	Hypoxanthine	Xanthine	Uric Acid	Allantoin
Managed	4	Unfed	No	29 (16.5-31.9)	1.4 (0.96-1.9)	0.42 (0.35-0.67)	2.4 (1.5-3.5)	8.3 (5.8-11.5)
	4	Fed	No	14 (2.9-17.9)	1.3 (0.33-2.1)†	0.35 (0.09-0.61)	4.0 (1.1-4.5)	6.6 (1.8-9.1)
	4	Unfed	Yes	12 (8.7-16.1)	0.6 (0.45-0.85)	0.22 (0.15-0.27)	1.4 (1.0-2.2)	7.3 (3.6-8.5)
	4	Fed	Yes	9 (5.6-15.2)	0.9 (0.82-1.1)†	0.30 (0.22-0.42)	3.5 (2.8-3.9)	6.0 (4.7-8.3)
Free-ranging[δ]	15	Fed	No	13 (4.6-37.9)	1.5 (0.9-5.0)†	0.31 (0.18-0.95)	3.3 (1.0-6.1)	6.6 (4.1-14.1)

*Values for concentrations (mmol/L) are medians with ranges in parentheses.
†Fed free-ranging dolphins have greater concentrations of hypoxanthine in the urine than fed managed dolphins, including both healthy dolphins and dolphins with nephrolithiasis ($p \leq 0.04$).
[δ]Reference Ardente 2016 Aquatic Mammals (under review)

Table 8-3. Relative ratios of urine creatinine and purine metabolites*of managed common bottlenose dolphins.

Status	N	AL:CR	UA:CR	HXA:CR	XA:CR	UA:AL	HXA+XA:AL	HXA+XA:UA
Unfed	8	0.42 (0.19-0.87)	0.11 (0.08-0.14)[a]	0.06 (0.04-0.07)[a]	0.02 (0.01-0.02)[a]	0.25 (0.13-0.60)[a]	0.15 (0.1-0.37)[a]	0.68 (0.47-0.92)[a]
Fed	8	0.56 (0.32-0.86)	0.31 (0.23-0.61)[b]	0.11 (0.06-0.15)[b]	0.03 (0.02-0.04)[b]	0.55 (0.39-0.91)[b]	0.22 (0.16-0.40)[b]	0.38 (0.30-0.64)[b]

*Values for molar ratios are medians with ranges in parentheses. Managed dolphins included both healthy dolphins (n=4) and dolphins with nephrolithiasis (n=4). CR, creatinine; AL, allantoin; UA, uric acid; HXA, hypoxanthine; XA, xanthine.
[ab] Medians with different superscripts within a column are significantly different ($p \leq 0.008$).

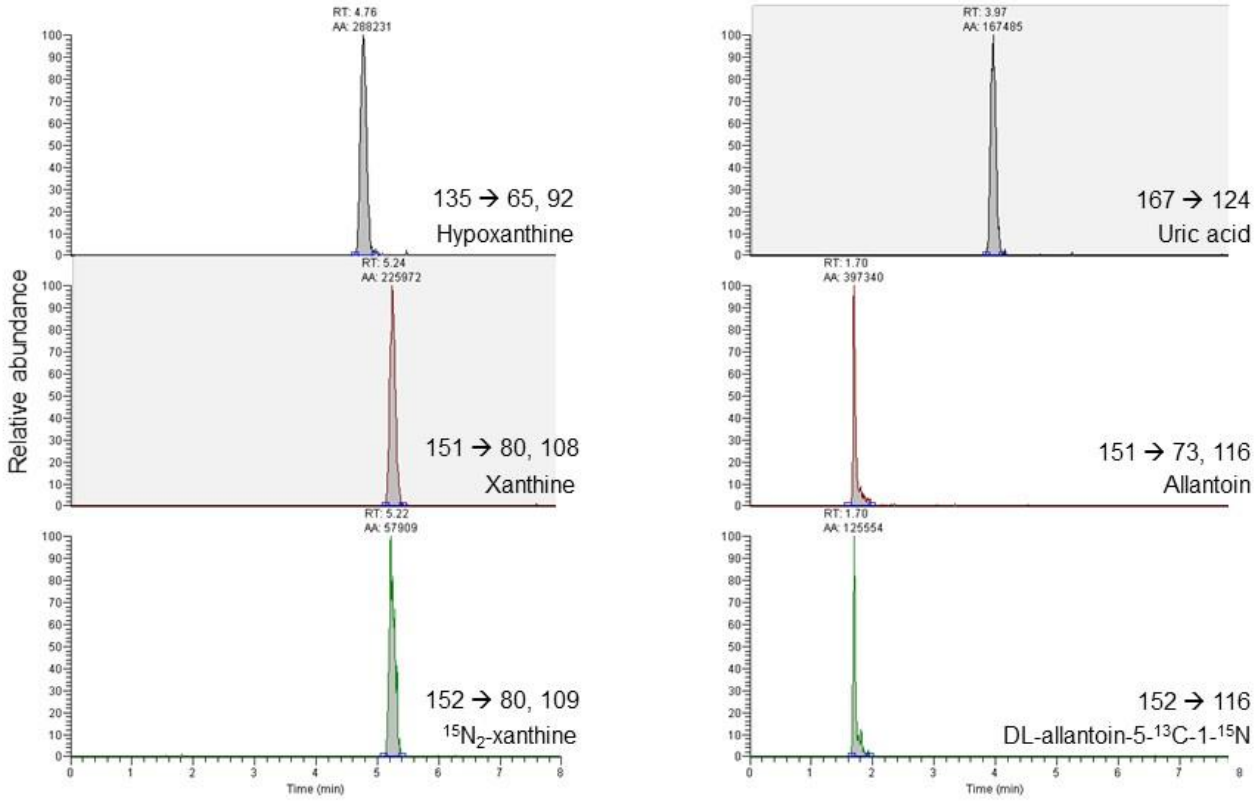


Figure 8-1. Chromatographic separation of hypoxanthine, xanthine, uric acid, allantoin, and internal standards in urine from a bottlenose dolphin under human care. Time (minutes) and relative abundance are represented along the x-axis and y-axis, respectively. Each metabolite is labeled with purine name, precursor mass, and product mass(es). The retention time (RT) and the area under the curve (AA) are noted above each metabolite peak.

CHAPTER 9
CONCLUSIONS, LIMITATIONS, AND FUTURE DIRECTIONS

This dissertation research explored the role nutrition may play in the development of ammonium urate kidney stone disease in common bottlenose dolphins under human care. The nutrient content relative to ME of fish and squid species most commonly fed to managed dolphins was compared with that of fish species commonly consumed by free-ranging bottlenose dolphins in Sarasota Bay, FL. This study, therefore, was the first to provide a comprehensive review of the nutrient intake of free-ranging bottlenose dolphins. This study was also the first to develop and compare model diets for managed and free-ranging dolphins, taking into consideration the relative proportions of fish in the whole diet, in order to better assess total nutrient intake. The free-ranging model diet provided an indication of what constitutes an adequate intake of essential nutrients and therefore can be used as a guide to determine how the species or proportions of species fed within the managed diet could be changed in order to approximate the total nutrient composition of the free-ranging dolphin model diet. Furthermore, little is known about how dolphins metabolize their purine-rich whole fish diet, so this research quantified the purine metabolites excreted in the urine of free-ranging dolphins, healthy managed dolphins, and managed dolphins with nephrolithiasis because greater concentrations of urinary uric acid may promote ammonium urate stone development.

Nutrient analyses focused on the key nutrients of concern in the development of ammonium urate stones, including water, protein, macromineral, and purine content. Nutrient concentrations were compared relative to metabolizable energy, which is more appropriate than comparing nutrients relative to dry matter for carnivorous species, like

dolphins, for which food intake is limited by its energy needs to maintain body condition. The fish and squid species fed to managed dolphins contained more total water and fat relative to energy compared with the free-ranging diet fish species, whereas the free-ranging fish species were more energy dense, containing more dry matter, protein, and ash than the managed diet species. In light of ammonium urate kidney stone disease, these nutrient findings did not provide a potential reason for development of stones in managed dolphins over free-ranging dolphins. The greater water and lesser protein contents of the managed dolphin diet would, in theory, provide for a more dilute, less saturated urine compared to the urine of free-ranging dolphins consuming a more energy dense diet with a greater protein content.

The macromineral content differences between the species consumed by managed and free-ranging dolphins and the model diets may provide a reason for stone development in dolphins under human care. Species fed to managed dolphins provided more sodium and chloride relative to energy than free-ranging diet species, which was likely a consequence of brining prior to frozen storage. On the other hand, free-ranging diet species provided more calcium and phosphorous relative to energy than managed diet species, which was likely due to their leaner and more bony composition.

The mineral contents of the fish and squid species were then put into context for how they may contribute to ammonium urate stone development by calculating the dietary cation-anion gap for both individual species and for the model dolphin diets. DCAD may contribute to ammonium urate stone formation if more anions are provided by the diet relative to cations, resulting in excretion of a more acidic urine. To buffer the acidic urine, it is possible that dolphins will excrete more ammonium ions in the urine,

promoting supersaturation and crystallization of ammonium with urinary uric acid. Four equations were used to calculate and describe the DCAD for individual fish species and the model diets. Managed diet species and the model managed dolphin diets had a more negative DCAD than free-ranging diet fish species (providing more anions relative to cations), except when DCAD was calculated using the human mineral absorption coefficients. The human equation for DCAD assumes that two-thirds more phosphorous is absorbed than calcium. Because the extent to which minerals are absorbed through the gastrointestinal tract of dolphins is unknown, it is difficult to state with certainty which equation would be most appropriate to predict the effect diet may have on urine acid-base balance. The $DCAD_{long}$ equation provided the starkest contrast between free-ranging and managed diet species and model diets, with a very positive DCAD for the free-ranging diet (152 ± 9 mEq/Mcal) and very negative DCAD for the managed diets (-77 to -80 ± 3 mEq/Mcal). $DCAD_{long}$, however, assumes equal absorption of all anions and cations, which is unlikely to be true based on reports in human beings, dogs, and cats of mineral absorption efficiencies being less than 100%.[31, 203, 247] Thus, it seems the better equation to predict DCAD for the dolphin diet may be $DCAD_{cat}$, which takes into account mineral absorption in a cat, a carnivorous species like the dolphin. $DCAD_{cat}$ predicted a negative DCAD for both the managed (-78 to -95 ± 2 mEq/Mcal) and free-ranging (-67 ± 2 mEq/Mcal) diets, but the DCAD of the managed diets was still 15-30% more negative compared to the free-ranging diet DCAD, indicating that the managed diets may indeed promote excretion of a more acidic urine.

Thus, it may be possible to make the $DCAD_{cat}$ more positive for the managed dolphin diets by substituting managed diet species with free-ranging species that have a

more positive DCAD. For example, mullet and spot might be good alternatives to Canadian capelin, Pacific mackerel, or Pacific sardines. If mullet replaces two-thirds of the capelin in 'Managed Diet #2', the $DCAD_{cat}$ is -70 ± 1 mEq/Mcal, which is much closer to the free-ranging diet $DCAD_{cat}$. It is important, therefore, to account for the relative proportions of fish when assessing the $DCAD^{cat}$ for the whole diet, and also to keep in mind that altering fish species and proportions may affect the concentrations of other important nutrients in the whole diet. Urinary alkalinizers, like potassium or sodium citrate and sodium bicarbonate, could also be used to bring the $DCAD_{cat}$ of the managed diets closer to that of the free-ranging diet. Managed dolphins can consume about 8 Mcal/day, depending on the individual and the facility, so alkanizing agents need to be dosed based on that average daily caloric intake. Potassium citrate and sodium bicarbonate are available in tablet form which could easily be hidden inside of food fish and fed to dolphins. Nevertheless, potassium citrate provides 10mEq of alkali per 1080mg tablet, so approximately 22 tablets per day would be required to approximate the $DCAD_{cat}$ of the free-ranging diet for the managed diet for a dolphin fed 8 Mcal/day. One 650mg tablet of sodium bicarbonate provides 7.7 mEq of alkali, so approximately 29 tablets of sodium bicarbonate would be needed to alter the managed diet $DCAD_{cat}$ if the dolphin is consuming 8 Mcal/day. Thus, it may not be practically feasible or cost effective to administer the number of tablets required of either alkalinizing agent to alter the $DCAD_{cat}$ of the managed dolphin diet to approximate that of the free-ranging diet. Sodium citrate is another alkalinizing agent that is available in a powder form, so a paste could be made for oral administration to dolphins; however, administration could be challenging depending on the consistency and quantity of the

paste and the individual animal's willingness to accept the medication. Sodium citrate provides 98 mg/mEq of sodium, so approximately 220 g of powder would be required to adjust the $DCAD_{cat}$ of the managed diet to that of the free-ranging diet. Nevertheless, the efficacy of these urinary alkalinizers is still dependent on the efficiency of mineral absorption through the intestinal tract. Further research would be needed to investigate the dose effect of each urinary alkalinizer on actual urinary pH.

The purine content of the fish and squid commonly fed to managed dolphins and fish commonly consumed by free-ranging dolphins was measured with a new assay developed by this research team. The four metabolites in greatest concentration were guanine, hypoxanthine, xanthine, and inosine, and the method provided satisfactory, repeatable results for those purines. We confirmed our hypothesis that it is important to measure more than just four purine metabolites, as measured by the commercially available assay, when comparing the purine intakes of managed and free-ranging bottlenose dolphins. The commercial assay measures only four nucleobases, so would underestimate the whole fish total purine content. Furthermore, we justified use of this expanded analysis because it would not be possible for one to predict the total purine content from the measurement of just four metabolites because the ratio of the total purine content obtained from eight versus four metabolites varied among species and was greater for free-ranging species than for managed species. This difference in ratio between the two groups of species may be explained by species differences in the total purine content and by handling and processing method differences among managed and free-ranging diet species.

In this study, the individual and total purine contents varied among species and between free-ranging and managed diet groups. 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. Concentrations of IMP were greater in free-ranging species than managed species, whereas concentrations of hypoxanthine and inosine were greater among managed species than free-ranging species. Furthermore, fresh frozen free-ranging diet fish species had a greater inosine to hypoxanthine ratio than frozen, stored, and thawed managed diet species, likely representing the more rapid degradation of inosine to hypoxanthine over the six to nine month frozen storage time of managed diet species. This may be important if hypoxanthine is more readily absorbed than inosine.

We also compared the total purine content among the model managed and free-ranging dolphin diets. The total purine content of the free-ranging dolphin diet was approximately half the total purine content of the model managed dolphin diets. Thus, the model free-ranging diet could provide a guide for the amount of purines that can be consumed by dolphins without inducing nephrolith formation. In order to lower the total purine content of the managed dolphin diet, species commonly fed to managed dolphins could be replaced, or their proportion in the diet decreased, by substituting a free-ranging or managed diet species with a less uricogenic profile or a lower total purine content. Dietary hypoxanthine, for example, produces excretion of more urinary uric acid in human beings and rats. Thus, managed diet species with high concentrations of hypoxanthine relative to ME, like capelin, could be replaced with species containing lower quantities of hypoxanthine relative to ME, like managed diet species Pacific

sardine or free-ranging diet species like pinfish. Nevertheless, it is unknown how dolphins absorb dietary purines through their gastrointestinal tracts and the extent to which those metabolites may affect excreted concentrations of urinary uric acid; therefore, it is likely that the uricogenic potential of all metabolites must be equally considered until further research is performed to determine otherwise. The total purine content of the managed dolphin diet, however, could be reduced to better approximate the total purine content of the model free-ranging diet. Fish with the highest ME density, like free-ranging diet mullet and spot and managed diet herring, contained the least total purine content relative to ME and therefore could be a substitute for species in the managed dolphin diet that are less energy dense and contain more purines, like capelin. For example, if the species and proportions of the managed diet were adjusted to provide 18% of Icelandic capelin, 16% of Pacific herring, 6% Loligo squid, and 60% mullet relative to Mcal of ME, the total purine content of the managed diet would be 4.07 mmol/Mcal ME, which would be equivalent to the total purine content of the model free-ranging diet (4.03 mmol/Mcal ME). Nevertheless, further research would be needed to determine how this addition of mullet to the managed diet may affect all other nutrients of concern and whether obtaining mullet in that quantity would be logistically feasible.

Considering the purine-rich whole fish diet of bottlenose dolphins, the last aim of this research was to determine whether dolphins excreted greater concentrations of allantoin than uric acid in their urine, like other carnivorous mammals and unlike people, and also to quantify the other purine metabolites excreted in the urine of dolphins. We therefore began by measuring and comparing uric acid, allantoin, hypoxanthine, and xanthine concentrations and relative ratios in the urine of free-ranging common

bottlenose dolphins and dogs. Free-ranging common bottlenose dolphins excrete allantoin in concentrations that are similar to dogs that have functioning uricase and much greater than human beings who do not have functional uricase. Nevertheless, when compared with dog urine, dolphins had greater uric acid concentrations in their urine relative to allantoin concentrations, which is suggestive that either uricase is saturated or operating at a reduced rate in dolphins, or that the capacity of the kidney to reabsorb uric acid may be reduced in dolphins. Furthermore, the greater ratio of uric acid to allantoin in dolphin urine compared with dog urine may explain why dolphins consuming a high purine diet are at risk of developing urate nephroliths under certain circumstances.

We then compared the purine metabolite concentrations found in the urine of free-ranging dolphins with concentrations in the urine of managed dolphins, and further compared the effect nephrolithiasis and feeding on urine purine concentrations among managed dolphins. Urine concentrations of creatinine, hypoxanthine, xanthine, and uric acid appeared to be lower in dolphins with nephrolithiasis than in healthy managed dolphins, but the sample size was very small, limiting our capacity to detect a difference even if one had been present. Nevertheless, post-prandial increases in uric acid, hypoxanthine, and xanthine, but not allantoin, were evident in the urine of managed dolphins and were comparable to concentrations in the urine of fed free-ranging dolphins. This finding provided further support that the oxidation of uric acid to allantoin by uricase is likely overwhelmed by a large dietary purine load and/or the efficiency of the renal tubules to reabsorb uric acid after dolphins consume a purine-rich meal is limited. Similar urine purine contents of fed free-ranging and fed managed dolphins

indicates that there may be no difference, genetic or metabolic, as to how the two populations of dolphins metabolize dietary purines. Furthermore, consumption of a purine-rich meal has the potential to increase urinary uric acid concentrations making urolith formation more likely, even though dolphins make allantoin.

There were several limitations that affected various components of this research. All nutrient analyses in this study were performed on fish caught during one season and for the managed diet species, was further limited to one lot of fish. Thus, seasonal differences within species could not be determined. Furthermore, free-ranging species varied in size and sex based on availability of what was caught; whereas commercially caught fish species are sorted for uniform size and sex, which may have led to more variability among free-ranging species compared to managed species. Frozen storage and thawing practices also impact nutrient content of fish, and for this study, the frozen storage time for the managed diet species was set at 6-9 months. With respect to processing fish samples prior to nutrient analysis, all fish and squid species were pooled and ground for analysis, so there was an inherent heterogeneity of the samples of some species because whole fish are composed of diverse tissues that respond differently to grinding. This was particularly true for some of the smaller, more bony species like pinfish, where it was challenging to ensure ground sample was well homogenized.

The model free-ranging diet also had limitations based on assumptions that had to be made regarding the species and relative proportions that are consumed by free-ranging dolphins. Furthermore, the model diet was based on the fish species and proportions consumed by free-ranging dolphins in Sarasota Bay, FL, which may or may not be representative of what all other inshore bottlenose dolphins or pelagic bottlenose

dolphins consume. The model diet does also not account for individual variation based on age, sex, reproductive status, or prey preference (species and size) and other populations of free-ranging dolphins may consume diets with a different composition.

The model diet comparisons also assume an equal caloric intake among dolphin populations. Preliminary data, however, suggest that free-ranging dolphins may have higher energy requirements than managed dolphins, likely a consequence of different activity levels, water temperatures, and reproductive status of the managed dolphins selected for this research. Nutrient intake is affected by the amount of food consumed as well as the nutrient composition of the diet so free-ranging dolphins may be consuming, metabolizing, and excreting more of some nutrients than some managed dolphins even when the managed diet contains less of those nutrients on an equal caloric basis.

Fish meal size and feeding frequency differ between managed and free-ranging dolphin populations, which may also impact total nutrient intake at a given time and affect the ability of dolphins to metabolize their purine-rich diet. Free-ranging bottlenose dolphins consume fish in smaller, more frequent meals throughout both the day and night, whereas managed dolphins are fed larger, less frequent meals over the course of daylight hours. Thus, larger fish meals consumed as a bolus by managed dolphins may provide a larger dose of nutrients all at once. For example, if a bolus feeding of Canadian capelin is fed to a managed dolphin, the high sodium and purine content of that fish species would likely result in an increased excretion of sodium, chloride, urea, and uric acid in the urine and greater overall urine osmolality, as was evident in the

Ridgway et al. studies.[180] Thus, it is probable that feeding method is a risk factor for development of ammonium urate stones in dolphins under human care.

Lastly, the greatest limitation of the urine purine analysis study design was the small sample size of managed dolphins. Urine concentrations of creatinine, hypoxanthine, xanthine, and uric acid appeared to be lower in dolphins with nephrolithiasis than in healthy managed dolphins, but due to the small sample size, the ability to detect a significant difference was limited. If the sample size had been larger, the trends toward a more dilute urine excreted by dolphins with nephrolithiasis may have been confirmed, indicating that dolphins may be experiencing chronic renal failure secondary to kidney stone disease that limits their urine concentrating ability, similar to what is seen in other mammals. On the other hand, it also may have been possible to prove that urine purine concentrations did not differ among managed healthy dolphins and dolphins with nephrolithiasis, indicating that there is a continuum of risk present for all managed dolphins to develop nephrolithiasis.

Future directions of this research, therefore, could begin by analyzing the urine purine content in a greater number of managed dolphins with nephrolithiasis and compare concentrations to purine concentrations excreted in the urine of healthy managed dolphins in order to determine whether nephrolithiasis alters the ability for the dolphin kidney to concentrate the urine and/or reabsorb uric acid. Furthermore, in order to better understand how the meal size, feeding frequency, energy intake, and nutrient content of the managed dolphin diet affects ammonium urate stone development, feeding trials could be performed in at least one population of managed dolphins. One feeding trial could test the effect of meal size and feeding frequency using the current

fed diet, in order to determine the effect of feeding smaller, more frequent meals over a 24-hour time frame on urine pH and uric acid excretion. A follow-up trial then could be performed to test a trial diet formulated to provide a greater moisture content, lesser protein and purine content, and a more positive $DCAD_{cat}$ relative to ME. The urine of dolphins receiving the trial diet could then be collected pre- and post-feeding to determine whether there is a decrease in ammonium urate supersaturation. Although the studies may be difficult to conduct, logistically, more information is also needed on the nutrient digestibility of dolphins, particularly with respect to mineral and purine metabolite absorptions, and on the energy requirements of dolphins, both free-ranging and managed populations. The only way to really compare the diets of the two populations is to understand the quantity of nutrients they are actually consuming relative to their total energy intake.

In summary, we now have a better understanding of the nutrients provided by the free-ranging and managed dolphin diets, particularly with respect to nutrients that may promote development of ammonium urate nephroliths. We also know that dolphins excrete allantoin, as other mammals, but excrete greater concentrations of uric acid relative to allantoin, which suggests a potential difference in their purine metabolic pathway compared to other mammals. Thus, it is will be possible in future studies to manipulate the managed dolphin diet to approximate the model free-ranging diet, which may help in the prevention or treatment of ammonium urate nephrolithiasis in dolphins under human care.

LIST OF REFERENCES

1. Smith C, Venn-Watson S, Wells R, Johnson S, Maffeo N, Balmer B, Jensen E, Townsend F, Sakhaee K: **Comparison of Nephrolithiasis Prevalence in Two Bottlenose Dolphin (Tursiops truncatus) Populations.** *Frontiers in Endocrinology* 2013, **4:**145.

2. Venn-Watson S, Townsend F, Daniels R, Sweeney J, McBain J, Klatsky L, Hicks C, Staggs L, Rowles T, Schwacke L, et al: **Hypocitraturia in common bottlenose dolphins (Tursiops truncatus): Assessing a potential risk factor for urate nephrolithiasis.** *Comp Med* 2010, **60:**149-153.

3. Venn-Watson S, Smith C, Johnson S, Daniels R, Townsend F: **Clinical relevance of urate nephrolithiasis in bottlenose dolphins, *Tursiops truncatus*.** *Dis Aquat Organ* 2010, **89:**167-177.

4. Moran M: **Uric acid stone disease.** *Front Biosci* 2003, **8:**s1339-1355.
5. Soble J, Hamilton B, SB S: **Ammonium acid urate calculi: a reevaluation of risk factors.** *J Urol* 1999, **161:**869-873.

6. Dear JD, Shiraki R, Ruby AL, Westropp JL: **Feline urate urolithiasis: a retrospective study of 159 cases.** *J Feline Med Surg* 2011, **13:**725-732.

7. Kruger J, Osborne C: **Etiopathogenesis of uric acid and ammonium urate uroliths in non-Dalmatian dogs.** *Veterinary Clinics of North America Small Animal Practice* 1986, **16:**87-126.

8. Bartges J, Osborne C, Lulich J: **Canine urate urolithiasis: etiopathogenesis, diagnosis, and management.** *Veterinary Clinics of North America Small Animal Practice* 1999, **29:**161-191.

9. Bowyer RC, McCulloch RK, Brockis JG, Ryan GD: **Factors affecting the solubility of ammonium acid urate.** *Clin Chim Acta* 1979, **95:**17-22.

10. Werness P, Brown C, Smith L, Finlayson B: **EQUIL 2: a basic computer program for the calculation of urinary saturation.** *J Urol* 1985, **134:**1242-1244.

11. Teotia M, Sutor DJ: **Crystallisation of ammonium acid urate and other uric acid derivatives from urine** *Br J Urol* 1971, **43:**381-386.

12. Hsu TC: **Ammonium acid urate lithiasis - Experimental observations.** *J Urol* 1966, **96:**88.

13. Richette P, Bardin T: **Gout.** *The Lancet* 2010, **375:**318-328.

14. Asper R: **Epidemiology and socioeconomic aspects of urolithiasis.** *Urol Res* 1984, **12:**1-5.

15. Klohn M, Bolle JF, Reverdin NP, Susini A, Baud CA, Graber P: **Ammonium urate urinary stones.** *Urol Res* 1986, **14:**315-318.

16. Voet D, Voet J: *Biochemistry.* 4th edn. Hoboken: John Wiley and Sons, Inc.; 2011.

17. Cameron J, Simmonds H: **Uric acid, gout, and the kidney.** *J Clin Pathol* 1981, **34:**1245-1254.

18. Curthoys NP, Watford M: **Regulation of glutaminase activity and glutamine metabolism.** *Annu Rev Nutr* 1995, **15:**133-159.

19. Halperin M, Ethier J, Kamel K: **The excretion of ammonium ions and acid base balance.** *Clin Biochem* 1990, **23:**185-188.

20. Konieczna I, Żarnowiec P, Kwinkowski M, Kolesińska B, Frączyk J, Kamiński Z, Kaca W: **Bacterial Urease and its Role in Long-Lasting Human Diseases.** *Curr Protein Pept Sci* 2012, **13:**789-806.

21. Adeva MM, Souto G: **Diet-induced metabolic acidosis.** *Clin Nutr* 2011, **30:**416-421.

22. Buffington C: **The role of diet in feline struvite urolithiasis syndrome.** In *Nutrition of the Dog and Cat.* Edited by Burger I, Rivers J. Cambridge: Cambridge University Press; 1989: 357-380.

23. Kok DJ, Poindexter J, Pak CYC: **Calculation of titratable acidity from urinary stone risk factors.** *Kidney Int* 1993, **44:**120-126.

24. Babcock S: *Metabolic water: its production and role in vital phenomena.* The University of Wisconsin Agricultural Experiment Station; 1912.

25. Kleeman CR, Epstein FH, White C: **The effect of variations in solute excretion and glomerular filtration on water diuresis.** *J Clin Invest* 1956, **35:**749-756.

26. Joliffe N, Smith H: **The Excretion of Urine In The Dog: I. The Urea and Creatinine Clearances on a Mixed Diet.** *Am J Physiol* 1931, **98:**572-577.

27. Van Slyke D, Rhoads C, Hiller A, Alving A: **The relationship of the urea clearance to the renal blood flow.** *The American Journal of Physiology* 1934, **10:**387-391.

28. Danowski T, Elkinton J, Winkler A: **The deleterious effect in dogs of a dry protein ration.** *J Clin Invest* 1944, **23:**816-823.

29. Hawthorne AJ, Markwell PJ: **Dietary Sodium Promotes Increased Water Intake and Urine Volume in Cats.** *The Journal of Nutrition* 2004, **134:**2128S-2129S.

30. Lennon EJ, Lemann J, Jr., Relman AS: **The effects of phosphoproteins on acid balance in normal subjects.** *J Clin Invest* 1962, **41:**637-645.

31. Remer T, Manz F: **Estimation of the renal net acid excretion by adults consuming diets containing variable amounts of protein.** *Am J Clin Nutr* 1994, **59:**1356-1361.

32. Asplin J, Parks J, Lingeman J, Kahnoski R, Mardis H, Lacey S, Goldfarb D, Grasso M, Coe F: **Supersaturation and stone composition in a network of dispersed treatment sites.** *J Urol* 1998, **159:**1821-1825.

33. Frassetto LA, Todd KM, Morris RC, Sebastian A: **Estimation of net endogenous noncarbonic acid production in humans from diet potassium and protein contents.** *The American Journal of Clinical Nutrition* 1998, **68:**576-583.

34. Kienzle E, Schuknecht A, Meyer H: **Influence of Food Composition on the Urine pH in Cats.** *The Journal of Nutrition* 1991, **121:**S87-S88.

35. Stipanuk MH, Ueki I: **Dealing with methionine/homocysteine sulfur: cysteine metabolism to taurine and inorganic sulfur.** *J Inherit Metab Dis* 2011, **34:**17-32.

36. Block E: **Manipulation of dietary cation-anion difference on nutritionally related production diseases, productivity, and metabolic responses of dairy cows.** *J Dairy Sci* 1994, **77:**1437-1450.

37. Remer T, Manz F: **Potential renal acid load of foods and its influence on urine pH.** *J Am Diet Assoc* 1995, **95:**791-797.

38. Chou Y-H, Huang C-N, Li W-M, Huang S-P, Wu W-J, Tsai C-C, Chang A-W, Chen S-M, Lin Y-L, Lin Y-P: **Clinical study of ammonium acid urate urolithiasis.** *The Kaohsiung Journal of Medical Sciences* 2012, **28:**259-264.

39. Hossain RZ, Ogawa Y, Hokama S, Morozumi M, Hatano T: **Urolithiasis in Okinawa, Japan: A relatively high prevalence of uric acid stones.** *Int J Urol* 2003, **10:**411-415.

40. Mandel NS, Mandel GS: **Urinary-tract stone disease in the United States veteran population. 1. Geographical frequency of occurrence** *J Urol* 1989, **142:**1513-1515.

41. Herring LC: **Observations on analysis of 10 thousand urinary calculi** *J Urol* 1962, **88:**545.

42. Cifuentes JM, Pourmand G: **Mineral-composition of 103-stones from Iran.** *Br J Urol* 1983, **55:**465-468.

43. Shekarriz B, Stoller ML: **Uric Acid Nephrolithiasis: Current Concepts and Controversies.** *The Journal of Urology* 2002, **168:**1307-1314.

44. Vanwaeyenbergh J, Vergauwe D, Verbeeck RMH: **Infrared spectrometric analysis of endemic bladder stones in Niger.** *Eur Urol* 1995, **27:**154-159.

45. Mandel NS, Mandel GS: **Urinary-tract stone disease in the United States veteran population. 1. Geographical analysis of variations in composition** *J Urol* 1989, **142:**1516-1521.

46. Robertson WG: **Renal stones in the tropics.** *Semin Nephrol* 2003, **23:**77-87.

47. Lou S-N: **Purine Content in Grass Shrimp during Storage as Related to Freshness.** *J Food Sci* 1998, **63:**442-444.

48. Saxena A, Sharma RK: **Nutritional aspect of nephrolithiasis.** *Indian Journal of Urology : IJU : Journal of the Urological Society of India* 2010, **26:**523-530.

49. Dennison S, Gulland F, Haulena M, De Morais H, Colegrove K: **Urate nephrolithiasis in a northern elephant seal (Mirounga angustirostris) and a California sea lion (Zalophus californianus).** *Journal of Zoo and Wildlife Medicine* 2007, **38:**114-120.

50. Simpson V, Tomlinson A, Molenaar F, Lawson B, Rodgers K: **Renal calculi in wild Eurasian otters (*Lutra lutra*) in England.** *Vet Rec* 2011, **169:**49.

51. Calle P: **Asian small-clawed otter (Aonyx cinerea) urolithiasis prevalence in North America.** *Zoo Biol* 1988, **7:**233-242.

52. Boehm J, Greenwell M, Coe F: **Dietary management in the treatment of uric acid urolithiasis in a Pacific white-sided dolphin (*Lagenorhynchus obliquidens*).** In *International Association for Aquatic Animal Medicine 28th annual conference; Hardervijk,* Netherlands; 1997.

53. Berg J, Tymoczko J, Stryer L: **Nucleotide biosynthesis.** In *Biochemistry (Mosc).* 5th edition. New York: W.H. Freeman and Company; 2002.

54. Murray AW: **The Biological Significance of Purine Salvage.** *Annual review of biochemistry* 1971, **40:**811-826.

55. Ho CY, Miller KV, Savaiano DA, Crane RT, Ericson KA, Clifford AJ: **Absorption and metabolism of orally administered purines in fed and fasted rats.** *The Journal of Nutrition* 1979, **109:**1377-1382.

56. Yamamoto S, Inoue K, Murata T, Kamigaso S, Yasujima T, Maeda J, Yoshida Y, Ohta K, Yuasa H: **Identification and functional characterization of the first nucleobase transporter in mammals: Implication in the species difference in the intestinal absorption mechanism of nucleobases and their analogs between higher primates and other mammals.** *The Journal of Biological Chemistry* 2010, **285:**6522-6531.

57. Hellsten-Westing Y, Balsom P, Norman B, Sjodin B: **The effect of high-intensity training on purine metabolism in man.** *Acta Physiol Scand* 1993, **149:**405-412.

58. Zieliński J, Rychlewski T, Kusy K, Domaszewska K, Laurentowska M: **The effect of endurance training on changes in purine metabolism: a longitudinal study of competitive long-distance runners.** *Eur J Appl Physiol* 2009, **106:**867-876.

59. Carver J: **Dietary nucleotides: effects on immune and gastrointestinal systems.** *Acta Pediatrica Supplement* 1999, **430:**83-88.

60. Clifford AJ, Story DL: **Levels of purines in foods and their metabolic effects in rats.** *J Nutr* 1976, **106:**435-442.

61. Salati LM, Gross CJ, Henderson LM, Savaiano DA: **Absorption and metabolism of adenine, adenosine-5'-monophosphate, adenosine, and hypoxanthine by the isolated vascularly perfused rat small intestine.** *The Journal of Nutrition* 1984, **114:**753-760.

62. Carson DA, Kaye J, Seegmiller JE: **Lymphospecific toxicity in adenosine deaminase deficiency and purine nucleoside phosphorylase deficiency: Possible role of nucleoside kinase(s).** *Proceedings of the National Academy of Sciences* 1977, **74:**5677-5681.

63. Gray J, Owen R, Giacomini K: **The concentrative nucleoside transporter family, SLC28.** *Pflügers Archiv* 2004, **447:**728-734.

64. Baldwin S, Beal P, Yao SM, King A, Cass C, Young J: **The equilibrative nucleoside transporter family, SLC29.** *Pflügers Archiv* 2004, **447:**735-743.

65. Van Den Berghe G, Bontemps F, Vincent MF: **Cytosolic purine 5'-nucleotidases of rat liver and human red blood cells: Regulatory properties and role in AMP dephoshorylation.** *Adv Enzyme Regul* 1988, **27:**297-311.

66. Clifford AJ, Riumallo JA, Young VR, Scrimshaw NS: **Effect of oral purines on serum and urinary uric acid of normal, hyperuricemic and gouty humans.** *The Journal of Nutrition* 1976, **106:**428-450.

67. Enomoto A, Kimura H, Chairoungdua A, Shigeta Y, Jutabha P, Cha SH, Hosoyamada M, Takeda M, Sekine T, Igarashi T, et al: **Molecular identification of a renal urate-anion exchanger that regulates blood urate levels.** *Nature* 2002, **417:**447-452.

68. Unger S, Tausche AK, Kopprasch S, Bornstein SR, Aringer M, Grassler J: **Molecular basis of primary renal hyperuricemia. Role of the human urate transporter hURAT1.** *Z Rheumatol* 2007, **66:**556.

69. Caulfield MJ, Munroe PB, O'Neill D, Witkowska K, Charchar FJ, Doblado M, Evans S, Eyheramendy S, Onipinla A, Howard P, et al: **SLC2A9 Is a High-Capacity Urate Transporter in Humans.** *PLoS Med* 2008, **5:**1509-1523.

70. Vitart V, Rudan I, Hayward C, Gray NK, Floyd J, Palmer CN, Knott SA, Kolcic I, Polasek O, Graessler J, et al: **SLC2A9 is a newly identified urate transporter influencing serum urate concentration, urate excretion and gout.** *Nat Genet* 2008, **40:**437-442.

71. Griffith DA, Jarvis SM: **Characterization of a sodium-dependent concentrative nucleobase-transport system in a guinea-pig kidney cortex brush-border membrane vesicles.** *Biochemistry Journal* 1994, **303:**901-905.

72. Griffith DA, Jarvis SM: **High affinity sodium-dependent nucleobase transport in cultured renal epithelial cells (LLC-PK1).** *J Biol Chem* 1993, **268:**20085-20090.

73. Theisinger A, Grenacher B, Scharrer E: **Na+ gradient-dependent transport of hypoxanthine by calf intestinal brush border membrane vesicles.** *Journal of Comparative Physiology B* 2003, **173:**165-170.

74. Goldwasser E: **The enzymic conversion of adenine to adenosine phosphates.** *Biochim Biophys Acta* 1954, **13:**341-346.

75. Green H, Ishii K: **On the Existence of a Guanine Nucleotide Trap, the role of Adenosine Kinase and a Possible Cause of Excessive Purine Production in Mammalian Cells.** *J Cell Sci* 1972, **11:**173-177.

76. Al-Khalidi U, Chaglassian T: **The species distribution of xanthine oxidase.** *J Biochem (Tokyo)* 1965, **97:**318-320.

77. Savaiano DA, Ho CY, Chu V, Clifford AJ: **Metabolism of orally and intravenously administered purines in rats.** *The Journal of Nutrition* 1980, **110:**1793-1804.

78. Usuda N, Reddy MK, Hashimoto T, Rao MS, Reddy JK: **Tissue specificity and species differences in the distribution of urate oxidase in peroxisomes.** *Lab Invest* 1988, **58:**100-111.

79. Keebaugh AC, Thomas JW: **The genomes of the South American opossum (Monodelphis domestica) and platypus (Ornithorhynchus anatinus) encode a more complete purine catabolic pathway than placental mammals.** *Comparative Biochemistry and Physiology D-Genomics & Proteomics* 2009, **4:**174-178.

80. Bartges JW, Osborne CA, Felice LJ, Brown C, Allen TA, Koehler L, Unger L, Bird K, Chen ML: **Influence of 2 amounts of dietary casein on uric-acid, sodium urate, and ammonium urate urinary activity product ratios of healthy beagles** *Am J Vet Res* 1995, **56:**893-897.

81. Cottam YH, Caley P, Wamberg S, Hendriks WH: **Feline Reference Values for Urine Composition.** *The Journal of Nutrition* 2002, **132:**1754S-1756S.

82. Smith CR, Poindexter JR, Meegan JM, Bobulescu IA, Jensen ED, Venn-Watson S, Sakhaee K: **Pathophysiological and Physicochemical Basis of Ammonium Urate Stone Formation in Dolphins.** *The Journal of Urology* 2014, **192:**260-266.

83. Bartges JW, Osborne CA, Felice LJ, Allen TA, Brown C, Unger LK, Koehler LA, Bird KA, Chen MG: **Diet effect on activity product ratios of uric-acid, sodium urate, and ammonium urate in urine formed by healthy beagles.** *Am J Vet Res* 1995, **56:**329-333.

84. Bartges JW, Osborne CA, Felice LJ, Unger LK, Chen MG: **Influence of allopurinol and 2 diets on 24-hour urinary excretions of uric-acid, xanthine, and ammonia by healthy dogs.** *Am J Vet Res* 1995, **56:**595-599.

85. Kuster G, Shorter RG, Hallenbe.Ga, Dawson B: **Uric-acid metabolism in Dalmatians and other dogs - role of liver** *Arch Intern Med* 1972, **129**.

86. Benedetti E, Kirby JP, Asolati M, Blanchard J, Ward MG, Williams R, Hewett TA, Fontaine M, Pollak R: **Intrasplenic hepatocyte allotransplantation in dalmatian dogs with and without cyclosporine immunosuppression.** *Transplantation* 1997, **63:**1206-1209.

87. Kessler RH, Hierholzer K, Gurd RS: **Localization of urate transport in the nephron of mongrel and Dalmatian dog kidney** *Am J Physiol* 1959, **197:**601-603.

88. Bannasch D, Safra N, Young A, Karmi N, Schaible R, Ling G: **Mutations in the SLC2A9 gene cause hyperuricosuria and hyperuricemia in the dog.** *PLoS Genet* 2008, **4**.

89. Hardy R, Klausner J: **Urate calculi associated with poral vascular anomalies.** In *Current Veterinary Therapy VIII.* Edited by Kirk R. Philadelphia: WB Saunders; 1983: 1073-1076.

90. Brockis JG, Levitt AJ, Cruthers SM: **The effects of vegetable and animal protein diets on calcium, urate and oxalate excretion** *Br J Urol* 1982, **54:**590-593.

91. Giesecke D, Stangassinger M: **Effects of purine-rich nutrition in the renal and extrarenal excretion of purine catabolites in Dalmatian dogs.** *Z Ernahrungswiss* 1990, **29:**208-218.

92. Appel SL, Houston DM, Moore AEP, Weese JS: **Feline urate urolithiasis.** *The Canadian Veterinary Journal* 2010, **51:**493-496.

93. Ridgway S: **History of Veterinary Medicine and Marine Mammals: A Personal Perspective.** *Aquatic Mammals* 2008, **34:**471-513.

94. Ceta-Base: **Captive Cetaceans (List): United States and Canada.** http://www.ceta-base.org/captive/cetacean/list/us-canada.php2016, April 20.

95. Venn-Watson S, Smith C, Stevenson S, Parry C, Daniels R, Jensen E, Cendejas B, Balmer B, Janech M, Neely B, Wells R: **Blood-based indicators of insulin resistance and metabolic syndrome in bottlenose dolphins (*Tursiops truncatus*).** *Frontiers in Endocrinology* 2013, **4:**1-8.

96. Venn-Watson SK, Ridgway SH: **Big Brains and Blood Glucose: Common Ground for Diabetes Mellitus in Humans and Healthy Dolphins.** *Comp Med* 2007, **57:**390-395.

97. Schmitt T: **Feeding frequency and activities of managed dolphins at Sea World, San Diego, CA.** (Ardente A ed.); 2015.

98. Vollenweider J, Heintz R, Schaufler L, Bradshaw R: **Seasonal cycles in whole-body proximate composition and energy content of forage fish vary with water depth.** *Marine Biology* 2011, **158:**413-427.

99. Henderson RJ, Sargent JR, Hopkins CCE: **Changes in the content and fatty acid composition of lipid in an isolated population of the capelin Mallotus villosus during sexual maturation and spawning.** *Marine Biology* 1984, **78:**255-263.

100. Mårtensson PE, Gotaas ARL, Norddy ES, Blix AS: **Seasonal changes in energy density of prey of northeast Atlantic seals and whales.** *Marine Mammal Science* 1996, **12:**635-640.

101. Davis K: **McRoberts Sales Co.** (Ardente A ed.) Ruskin, FL; 2012.

102. Asli M, Mørkøre T: **Brines added sodium bicarbonate improve liquid retention and sensory attributes of lightly salted Atlantic cod.** *LWT - Food Science and Technology* 2012, **45:**196-202.

103. Weinberg Z, Regenstein J, Baker R: **Effects of different salts on water-retention and heat initiated binding-properties of comminuted cod muscle.** *Journal of Food Biochemistry* 1984, **8:**215-227.

104. Strasburg G, Xiong Y, Chiang W: **Physiology and Chemistry of Edible Muscle Tissues.** In *Fennema's Food Chemistry.* 4th edition. Edited by Damodaran S, Parkin K, Fennema O. Boca Raton, FL: CRC Press, Taylor & Francis Group; 2008: 923-974.

105. Fennema O, Damodaran S, Parkin K: **Introduction to Food Chemistry.** In *Fennema's Food Chemistry.* 4th edition. Edited by Damodaran S, Parkin K, Fennema O. Boca Raton: CRC Press Taylor & Francis Group; 2008.

106. Prester L, Macan J, Varnai VM, Orct T, Vukusic J, Kipcic D: **Endotoxin and biogenic amine levels in Atlantic mackerel (Scomber scombrus), sardine (Sardina pilchardus) and Mediterranean hake (Merluccius merluccius) stored at 22 degrees C.** *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* 2009, **26:**355-362.

107. Bando M: **Comparing the nutritional quality of Stellar Sea Lion (*Eumetopias jubatus*) diets.** University of Alaska Fairbanks; 2002.

108. Van Pelt TI, Piatt JF, Lance BK, Roby DD: **Proximate composition and energy density of some north pacific forage fishes.** *Comparative Biochemistry and Physiology Part A: Physiology* 1997, **118:**1393-1398.

109. Crissey S, McGill P: **Association of Zoos and Aquariums Penguin Husbandry Manual; Chapter 5: Diet and Nutrition.** (Ellis S, Branch S eds.), 1st edition. Bethesda, MD; 1994.

110. Bernard J, Allen M: **Feeding captive piscivorous animals: nutritional aspects of fish as food.** In *Nutrition Advisory Group Handbook* (Baer D, Crissey S, Ullrey D eds.). pp. 12: Nutrition Advisory Group; 2002:12.

111. Montevecchi W, Piatt J: **Inshore spawning capelin (*Mallotus villosus*): Implications for seabird predators.** *Comp Biochem Physiol* 1984, **78:**15-20.

112. Corse M, Glick-Bauer M, Saul B, Dierenfeld E: **Nutrient composition of locally-obtained native fishes (St. Catherine's Island Wildlife Survival Center, GA, USA) compared with fish commonly purchased for North American zoo feeding programs.** In *Third Conference of the American Zoo and Aquarium Association (AZA) and Nutrition Advisory Group (NAG) on Zoo and Wildlife Nutrition; Columbus, OH*1999: 127-135.

113. Slifka K, Wells R, Ardente A, Crissey S: **Comparative diet analysis of fish species commonly consumed by managed and free-ranging bottlenose dolphins (*Tursiops truncatus*).** *The Internet Journal of Veterinary Medicine* 2013, **10**.

114. Parkin K: **Enzymes.** In *Fennema's Food Chemistry.* 4th edition. Edited by Damodaran S, Parkin K, Fennema O. Boca Raton, FL: CRC Press, Taylor & Francis Group; 2008: 331-436.

115. Ngapo TM, Babare IH, Reynolds J, Mawson RF: **Freezing and thawing rate effects on drip loss from samples of pork.** *Meat Sci* 1999, **53:**149-158.

116. Reid D, Fennema O: **Water and Ice.** In *Fennema's Food Chemistry.* 4th edition. Edited by Damodaran S, Parkin K, Fennema O. Boca Raton: CRC Press Taylor & Francis Group; 2008.

117. Aubourg SP, Piñeiro C, Gallardo JM, Barros-Velazquez J: **Biochemical changes and quality loss during chilled storage of farmed turbot (Psetta maxima).** *Food Chemistry* 2005, **90:**445-452.

118. Leu S, Jhaveri S, Karakoltsidis P, Constantinides S: **Atlantic mackerel (*Scomber scombrus, L.*): Seasonal variation in proximate composition and distribtuion of chemical nutrients.** *J Food Sci* 1981, **46:**1635-1638.

119. Nunes M, Batista I, Morao de Campos R: **Physical, chemical, and sensory analysis of sardine (*Sardina pilchardus*) stored in ice.** *J Sci Food Agric* 1992, **59:**37-43.

120. Dam H: **Interrelations between Vitamin E and Polyunsaturated Fatty Acids in Animals.** In *Vitam Horm. Volume* Volume 20. Edited by Robert S. Harris IGWGFM, Kenneth VT: Academic Press; 1962: 527-540.

121. Valk E, Hornstra G: **Relationship between vitamin E requirement and polyunsaturated fatty acid intake in man: a review.** *Int J Vitam Nutr Res* 2000, **70:**31-42.

122. Hayes KC, Nielsen SW, Rousseau JE: **Vitamin E Deficiency and Fat Stress in the Dog.** *The Journal of nutrition* 1969, **99:**196-209.

123. Lehmann J, Martin HL, Lashley EL, Marshall MW, Judd JT: **Vitamin E in foods from high and low linoleic acid diets.** *J Am Diet Assoc* 1986, **86:**1208-1216.

124. Bieri J, Thorp S, Tolliver T: **Effect of dietary polyunsaturated fatty acids on tissue vitamin E status.** *The Journal of Nutrition* 1978, **108:**392-398.

125. Digre H, Erikson U, Aursand IG, Gallart-Jornet L, Misimi E, Rustad T: **Rested and Stressed Farmed Atlantic Cod (Gadus morhua) Chilled in Ice or Slurry and Effects on Quality.** *J Food Sci* 2011, **76:**S89-S100.

126. Fraser D, Dingle J, Hines J, Nowlan S, Dyer W: **Nucleotide degredation, monitored by thin-layer chromatography, and associated postmortem changes in relaxed cod muscle.** *Journal of the Fisheries Research Board of Canada* 1967, **24:**1837-1841.

127. Lou S-N, Lin C-D, Benkmann R: **Changes in Purine Content of *Tilapia mossambica* during Storage, Heating, and Drying.** *Food Science and Agricultural Chemistry* 2001, **3:**23-29.

128. Jhaveri S, Leu S, Constantinides S: **Atlantic mackerel (Scomber scombrus, L.): Shelf life in ice.** *J Food Sci* 1982, **47:**1808-1810.

129. Savage A, Warris P, Jolley P: **The amount and composition of the proteins in drip from stored pig meat.** *Meat Sci* 1990, **27:**289-303.

130. Pigott G, Tucker B: **Effects on Processing of Nutrients.** In *Seafood - Effects of technology on nutrition.* New York: Marcel Dekker, Inc.; 1990: 68-69.

131. Wells RS: **Learning From Nature: Bottlenose Dolphin Care and Husbandry.** *Zoo Biol* 2009, **28:**635-651.

132. Wells R, McHugh KA, Douglas D, Shippee S, McCabe E, Barros N, Phillips G: **Evaluation of potential protective factors against metabolic syndrome in bottlenose dolphins: feeding and activity patterns of dolphins in Sarasota Bay, FL.** *Frontiers in Endocrinology* 2013, **4:**1-16.

133. Wells R, Scott M, Irvine A: **The social structure of free-ranging bottlenose dolphins.** In *Current mammalogy.* Edited by Genoways H. New York: Plenum Press; 1987: 247-305.

134. Mitchell E: *Porpoise, dolphin, and small whale fisheries of the world.* England: International Union for Conservation of Nature and Natural Resources; 1975.

135. Read A, Wells R, Hohn A, Scott M: **Patterns of growth in wild bottlenose dolphins, *Tursiops truncatus*.** *Journal of Zoology London* 1993, **231:**107-123.

136. Ridgway S, Fenner C: **Weight-length relationships of wild-caught and captive Atlantic bottlenose dolphins.** *J Am Vet Med Assoc* 1982, **181:**1310-1315.

137. Mead J, Potter C: **Natural history of bottlenose dolphins along the central Altantic coast of the United States.** In *The bottlenose dolphin.* Edited by Leatherwood S, Reeves R. San Diego: Academic Press, Inc.; 1990: 165-195.

138. Wells R, Irvine A, Scott M: **The Social Ecology of Inshore Odontocetes.** In *Cetacean Behavior: Mechanisms and Processes.* Edited by Herman L: John Wiley & Sons, Inc.; 1980: 263-317.

139. Sergeant D, Caldwell D, Carldwell M: **Age, growth, and maturity of bottlenosed dolphin (*Tursiops truncatus*) from Northeast Florida.** *Journal of the Fisheries Research Board of Canada* 1973, **30:**1009-1011.

140. Perrin W, Reilly S: **Reproductive parameters of dolphins and small whales of the family Delphinidae.** *Report of the International Whale Commission* 1984.

141. Tolley K, Read A, Wells R, Urian K, Scott M, Irvine A, Hohn A: **Sexual dimorphism in wild bottlenose dolphins (*Tursiops truncatus*) from Sarasota, Florida.** *J Mammal* 1995, **76:**1190-1198.

142. Bryden MM: **Growth and development of marine mammals.** In *Functional Anatomy of Marine Mammals. Volume* 1. Edited by Harrison RJ. New York: Academic Press, Inc.; 1972: 1-24.

143. Cockcroft V, Ross A: **Age, growth, and reproduction of bottlenose dolphins, Tursiops truncatus, from the east coast of southern Africa.** *Fishery Bulletin* 1990, **88:**289-302.

144. Cheal A, Gales N: **Body mass and food intake in captive, breeding bottlenose dolphins, *Tursiops truncatus*.** *Zoo Biol* 1991, **10:**451-456.

145. McBride A, Kritzler H: **Observations on pregnancy, parturition, and post-natal behaviour in the bottlenose dolphin.** *J Mammal* 1951, **32:**251-266.

146. Sweeney J, Stone R, Campbell M, McBain J, St. Leger J, Xitco M, Jensen E, Ridgway S: **Comparative survivability of *Tursiops* neonates from three U.S.**

institutions for the decades 1990-1999 and 2000-2009. *Aquatic Mammals* 2010, **36:**248-261.

147. Wells R, Scott M: **Chapter 7: Bottlenose Dolphin *Tursiops truncatus* (Montagu, 1821).** *Handbook of Marine Mammals* 1999, **6:**137-182.

148. Barros N, Odell D: **Food habits of bottlenose dolphins in the southeastern United States.** In *The Bottlenose Dolphin.* Edited by Leatherwood S, Reeves R. San Diego: Academic Press; 1990: 309-328.

149. Barros N, Wells R: **Prey and feeding patterns of resident bottlenose dolphins (*Tursiops truncatus*) in Sarasota Bay, Florida.** *J Mammal* 1998, **79:**1045-1059.

150. Irvine A, Scott M, Wells R, Kaufmann J: **Movements and activities of the Atlantic bottlenose dolphin, *Tursiops truncatus*, near Sarasota, Florida.** *Fishery Bulletin* 1981, **79:**671-688.

151. Caldwell M, Caldwell D: **Behavior of marine mammals.** In *Mammals of the Sea Biology and Medicine.* Edited by Ridgway S. Springfield: Charles C. Thomas; 1972: 419-465.

152. Leatherwood S: **Some observations of feeding behavior of bottle-nosed dolphins (*Tursiops truncatus*) in the Northern Gult of Mexico and (*Tursiops* cf *T. gilli*) off Southern California, Baja California, and Nayarit, Mexico.** *Marine Fisheries Review* 1975, **37:**10-16.

153. Connor R, Wells R, Mann J, Read A: **The Bottlenose Dolphin: Social relationships in a fission-fusion society.** In *Cetacean Societies Field of Studies of Dolphins and Whales.* Edited by Mann J, Connor R, Tyack P, Whitehead H. Chicago: The University of Chicago Press; 2000: 91-126.

154. Barros N: **Feeding ecology and foraging strategies of bottlenose dolphins on the central east coast of Florida.** University of Miami; 1993.

155. Nowacek D: **Sequential foraging behaviour of bottlenose dolphins, *Tursiops truncatus*, in Sarasota Bay, FL.** *Behaviour* 2002, **139:**1125-1145.

156. Allen MC, Read AJ, Gaudet J, Sayigh LS: **Fine-scale habitat selection of foraging bottlenose dolphins *Tursiops truncatus* near Clearwater, Florida.** *Marine Ecology Progress Series* 2001, **222:**253-264.

157. Wells R, Boness D, Rathbun G: **Behavior.** In *Biology of Marine Mammals.* Edited by Reynolds III J, Rommel S. Washington: Smithsonian Institution Press; 1999: 324-422.

158. Nowacek DP: **Acoustic ecology of foraging bottlenose dolphins (Tursiops truncatus), habitat-specific use of three sound types.** *Marine Mammal Science* 2005, **21:**587-602.

159. Burros NB, Myrberg AA: **Prey detection by means of passive listening in bottlenose dolphins (Tursiops truncatus).** *The Journal of the Acoustical Society of America* 1987, **82:**S65-S65.

160. Gannon DP, Barros NB, Nowacek DP, Read AJ, Waples DM, Wells RS: **Prey detection by bottlenose dolphins, Tursiops truncatus: an experimental test of the passive listening hypothesis.** *Anim Behav* 2005, **69:**709-720.

161. McCabe E, Gannon D, Barros N, Wells R: **Prey selection by resident common bottlenose dolphins (*Tursiops truncatus*) in Sarasota Bay, FL.** *Marine Biology* 2010, **157:**931-942.

162. Dunshea G, Barros NB, Berens McCabe EJ, Gales NJ, Hindell MA, Jarman SN, Wells RS: **Stranded dolphin stomach contents represent the free-ranging population's diet.** *Biol Lett* 2013, **9**.

163. Shane S: **Behavior and ecology of the bottlenose dolphin at Sanibel Island, Florida.** In *The bottlenose dolphin.* Edited by Leatherwood S, Reeves R. San Diego: Academic Press, Inc.; 1990: 245-266.

164. Sogard S, Powell G, Holmquist J: **Utilization of fishes of shallow, seagrass-covered banks in Florida Bay: 1. Species composition and spatial heterogeneity.** *Environmental Biology of Fishes* 1989, **24:**53-65.

165. McCabe E, Westgate AJ, Wells R: **Prey energy density and bottlenose dolphin foraging implications.** In *19th Biennial Conference on the Biology of Marine Mammals; Tampa, FL*. The Society for Marine Mammalogy; 2011.

166. Paul A, Paul J: **Spring and summer whole-body energy content of Alaskan juvenile Pacific Herring.** *Alaska Fishery Research Bulletin* 1998, **5:**131-136.

167. Dierenfeld E: **Multi-feed and requirement nutrient comparison.** Saint Louis, MO; 2005.

168. Kleiber M: *The fire of life: an introduction to animal energetics.* 2nd edn. New York: John Wiley and Sons, Inc.; 1975.

169. Worthy G: **Nutrition and Energetics.** In *CRC Handbook of Marine Mammal Medicine.* 2nd edition. Edited by Dierauf, Gulland. New York: CRC Press; 2001: 791-827.

170. Williams TM, Haun J, Davis RW, Fuiman LA, Kohin S: **A killer appetite: metabolic consequences of carnivory in marine mammals.** *Comparative Biochemistry and Physiology a-Molecular and Integrative Physiology* 2001, **129:**785-796.

171. Yeates LC, Houser DS: **Thermal tolerance in bottlenose dolphins (Tursiops truncatus).** *The Journal of Experimental Biology* 2008, **211:**3249-3257.

172. Hampton I, Whittow G, Szekerczes J, Rutherford S: **Heat transfer and body temperature in the Atlantic bottlenose dolphin, *Tursiops truncatus*.** *Int J Biometeorol* 1971, **15:**247-253.

173. McGinnis S, Whittow G, Ohata C, Huber H: **Body heat dissipation and conservation in two species of dolphins.** *Comparative Biochemistry and Physiology A* 1972, **43:**417-423.

174. Costa D, Worthy G, Wells R, Read A, Waples D, Scott M, Irvine A: **Patterns of seasonal metabolic rate variation for bottlenose dolphins in Sarasota Bay, Florida.** In *Society for Marine Mammalogy 20th Biennial Conference; Dunedin, New* Zealand; 2013.

175. Cates MB, Schroeder JP: **The nutrition of acclimated vs. newly captured *Tursiops truncatus*.** *Aquatic Mammals* 1986, **12:**17-20.

176. Hand M, Thatcher C, Remillard R, Roudebush P, Novotny B (Eds.): **Small Animal Clinical Nutrition**, 5th edition. Topeka, KS: Mark Morris Institute; 2010.

177. Reddy M, Kamolnick T, Curry C, Skaar D: **Energy requirements for the bottlenose dolphin (*Tursiops truncatus*) in relation to sex, age, and reproductive status.** *Marine Mammals: Public Display and Research* 1994, **1:**26-31.

178. Kastelein RA, Vaughan N, Walton S, Wiepkema PR: **Food intake and body measurements of Atlantic bottlenose dolphins (Tursiops truncates) in captivity.** *Marine Environmental Research* 2002, **53:**199-218.

179. Schmitt TL, Sur RL: **Treatment of Ureteral Calculus Obstruction with Laser Lithotripsy in an Atlantic Bottlenose Dolphin (Tursiops truncatus).** *Journal of Zoo and Wildlife Medicine* 2012, **43:**101-109.

180. Ridgway S, Venn-Watson S: **Effects of fresh and seawater ingestion on osmoregulation in Atlantic bottlenose dolphins (Tursiops truncatus).** *Journal of Comparative Physiology B* 2010, **180:**563-576.

181. Venn-Watson S, Smith C, Dold C, Ridgway S: **Use of a serum-based glomerular filtration rate prediction equation to assess renal function by age, sex, fasting, and health status in bottlenose dolphins (*Tursiops truncatus*).** *Marine Mammal Science* 2008, **24:**71-80.

182. Harms C, Lo Piccolo R, Rotstein D, Hohn A: **Struvite penile urethrolithiasis in a pygmy sperm whale (*Kogia breviceps*).** *J Wildl Dis* 2004, **40:**588-593.

183. Ortiz RM: **Review: Osmoregulation in Marine Mammals.** *The Journal of Experimental Biology* 2001, **204:**1831-1844.

184. Jackson M: **Renal System.** In *Veterinary Clinical Pathology: An Introduction.* Ames, IA: Blackwell Publishing; 2007.

185. Osborne CA, Lulich JP, Bartges JW, Unger LK, Thumchai R, Koehler LA, Bird KA, Felice LJ: **Canine and feline urolithiasis: relationship of etiopathogenesis to treatment and prevention.** In *Canine and Feline Nephrology and Urology.* Edited by Osborne CA, Finco DR. Philadelphia, PA: Lea & Febiger; 1995: 798-888.

186. Argade S, Smith CR, Shaw T, Zupkas P, Schmitt TL, Venn-Watson S, Sur RL: **Solubility of ammonium urate nephroliths from bottlenose dolphins (*Tursiops truncatus*)** *Journal of Zoo and Wildlife Medicine* 2013, **44:**853-858.

187. Pak CY, Sakhaee K, Fuller C: **Successful management of uric acid nephrolithiasis with potassium citrate.** *Kidney Int* 1986, **30:**422-428.

188. Sakhaee K, Nicar M, Hill K, Pak CYC: **Contrasting effects of potassium citrate and sodium citrate therapies on urinary chemistries and crystallization of stone-forming salts** *Kidney Int* 1983, **24:**348-352.

189. Pinheiro VB, Baxmann AC, Tiselius HG, Heilberg IP: **The effect of sodium bicarbonate upon urinary citrate excretion in calcium stone formers.** *Urology* 2013, **82:**33-37.

190. Ngo T, Assimos D: **Uric acid nephrolithiasis: Recent progress and future directions.** *Rev Urol* 2007, **9:**17-27.

191. **Canine Urate Uroliths** [http://www.cvm.umn.edu/depts/minnesotaurolithcenter/prod/groups/cvm/@pub/@cvm/@urolith/documents/asset/cvm_asset_107727.pdf]

192. Smith C, Meegan J, Bailey J, Scott G, Sur R, L'Esperance J, Ivancic M, Cotte L, Cendejas V, Sakhaee K, et al: **Case report: Surgical management of a partial ureteral obstruction under general anesthesia in a geriatric bottlenose dolphin.** In *International Association for Aquatic Animal Medicine; Chicago,* IL; 2015.

193. Jensen E: **United States Navy - managed diet.** (Ardente A ed. San Diego, December 2011.

194. Sweeney J: **Dolphin Quest - managed diet.** (Ardente A ed. San Diego, December 2011.

195. Massey LK: **Dietary Animal and Plant Protein and Human Bone Health: A Whole Foods Approach.** *The Journal of nutrition* 2003, **133:**862S-865S.

196. Spallholz J, Boylan L, Driskell J: **Proteins.** In *Nutrition Chemistry and Biology.* 2nd edition. Edited by Wolinsky I. New York: CRC Press; 1999: 41-52.

197. Reddy ST, Wang CY, Sakhaee K, Brinkley L, Pak CYC: **Effect of low-carbohydrate high-protein diets on acid-base balance, stone-forming propensity, and calcium metabolism.** *Am J Kidney Dis* 2002, **40:**265-274.

198. Breslau N, Brinkley L, Hill K, Pak C: **Relationship of animal protein-rich diet to kidney stone formation and calcium metabolism.** *J Clin Endocrinol Metab* 1988, **66:**140-146.

199. Sakhaee K, Maalouf N, Sinnott B: **Special feature, Clinical Review. Kidney stones 2012: Pathogenesis, diagnosis, and management.** *J Clin Endocrinol Metab* 2012, **97:**1847-1860.

200. Liangos O, Jaber B: **Kidney stones.** In *Nutrition in Kidney Disease.* Edited by Byham-Gray L, Burrowes J, Chertow G. Totowa, NJ: Humana Press; 2008: 513-530.

201. Ekeruo W, Tan Y, Young M, Dahm P, Maloney M, Mathias B, Albala D, Preminger G: **Metabolic risk factors and the impact of medical therapy on the management of nephrolithiasis in obese patients.** *The Journal of Urology* 2004, **172:**159-163.

202. Pak CYC, Holt K, Britton F, Peterson R, Crowther C, Ward D: **Assessment of pathogenic roles of uric-acid, monopotassium urate, monoammonium urate and monosodium urate in hyperuricosuric calcium-oxalate nephrolithiasis** *Miner Electrolyte Metab* 1980, **4:**130-136.

203. NRC: *National Research Council: Nutrient Requirements of Dogs and Cats.* Washington, DC: The National Academies Press; 2006.

204. Merrill A, Watt B: **Energy value of foods... basis and derivation.** (USDA ed., vol. Agriculture Handbook No. 74. pp. 109. Washington, D.C.: Agricultural Research Service; 1973:109.

205. Castro P, Huber M: *Marine Biology.* New York City: McGraw-Hill; 2009.

206. Barnes H: **Some Tables for the Ionic Composition of Sea Water.** *J Exp Biol* 1954, **31:**582-588.

207. Lovett DL, Tanner CA, Glomski K, Ricart TM, Borst DW: **The effect of seawater composition and osmolality on hemolymph levels of methyl farnesoate in the green crab Carcinus maenas.** *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 2006, **143:**67-77.

208. Robertson G: **Posterior pituitary.** In *Endocrinology and metabolism.* 2 edition. Edited by Feldman E, Nelson R, Reusch C, Scott-Moncrieff J. New York: McGraw Hill; 1987: 338.

209. Hui CA: **Seawater consumption and water flux in the common dolphin Delphinus delphis** *Physiol Zool* 1981, **54:**430-440.

210. Bossart G, Reidarson T, Dierauf L, Duffield D: **Clinical Pathology.** In *CRC Handbook of Marine Mammal Medicine.* 2nd edition. Edited by Dierauf L, Gulland F. New York: CRC Press; 2001: 383-436.

211. Green R: **Observations on the anatomy of some cetaceans and pinnipeds.** In *Mammals of the Sea: Biology and Medicine.* Edited by Ridgway S. Springfield: Charles C. Thomas; 1972: 247-297.

212. Cave AJE, Aumonier FJ: **Morphoogy of cetacean reniculus** *Nature* 1962, **193:**799.

213. Vander A: *Renal Physiology.* New York: McGraw Hill, Inc.; 1995.

214. Ridgway S: **Homeostasis in the Aquatic Environment.** In *Mammals of the Sea: Biology and Medicine.* Edited by Ridgway S. Springfield: Charles C. Thomas; 1972: 590-747.

215. Coulombe H, Ridgway S, Evans W: **Respiratory water exchange in two species of porpoise.** *Science* 1965, **149:**86.

216. Peddemors V, De Muelenaere H, Devchand K: **Comparative milk composition of the bottlenosed dolphin (*Tursiops truncatus*), humpback dolphin (*Sousa plumbea*) and common dolphin (*Delphinus delphis*) from southern African waters.** *Comparative Biochemistry and Physiology Part A: Physiology* 1989, **94:**639-641.

217. Oftedal O: **Milk composition, species comparisons.** In *Encyclopedia of Animal Science.* Edited by Pond W, Bell A. New York: Marcel Dekker; 2005: 625-628.

218. Albrecht C: **Toxicity of sea water in mammals.** *Am J Physiol* 1950, **163:**370-385.

219. Rowntree L: **The water balance of the body.** *Physiology Review* 1922, **2:**116.

220. Ortiz RM, Long B, Casper D, Ortiz CL, Williams TM: **Biochemical and hormonal changes during acute fasting and re-feeding in bottlenose dolphins (Tursiops truncatus).** *Marine Mammal Science* 2010, **26:**409-419.

221. Hiatt EP, Hiatt RB: **The effect of food on the glomerular filtration rate and renal blood flow in the harbor seal (Phoca Vitulina L.).** *Journal of Cellular and Comparative Physiology* 1942, **19:**221-227.

222. Kerr W: **Maximum clearances (GFR and ERPF) in dogs.** *J Urol* 1958, **80:**205-207.

223. Manitius A, Pigeon G, Epstein FH: **Mechanism by which dietary protein enhances renal concentrating ability.** *American Journal of Physiology -- Legacy Content* 1963, **205:**101-106.

224. Hostetter TH: **Human renal response to meat meal.** *American Journal of Physiology - Renal Physiology* 1986, **250:**F613-F618.

225. Bie P: **Sustained Water Diuresis in Anesthetized Dogs: Antidiuresis in Response to Intravenous and Bilateral Intracarotid Infusion of Hyper-Osmolar Solutions of Sodium Chloride.** *Acta Physiol Scand* 1977, **101:**446-457.

226. Irving RA, Noakes TD, Buck R, van Zyl Smit R, Raine E, Godlonton J, Norman RJ: **Evaluation of renal function and fluid homeostasis during recovery from exercise-induced hyponatremia.** *J Appl Physiol (1985)* 1991, **70:**342-348.

227. Aubourg SP, Quitral V, Angélica Larraín M, Rodríguez A, Gómez J, Maier L, Vinagre J: **Autolytic degradation and microbiological activity in farmed Coho salmon (Oncorhynchus kisutch) during chilled storage.** *Food Chemistry* 2007, **104:**369-375.

228. Bennour M, El Marrakchi A, Bouchriti N, Hamama A, El Ouadaa M: **Chemical and microbiological assessments of mackerel (*Scomber scombrus*) stored in ice.** *J Food Prot* 1991, **54:**789-792.

229. Telfer N, Cornell LH, Prescott JH: **Do dolphins drink water?** *J Am Vet Med Assoc* 1970, **157:**555-558.

230. Geraci J, St. Aubin D: **Nutritional disorders of captive fish-eating animals.** In *The Comparative Pathology of Zoo Animals.* Edited by Montali R, Migaki G. Washington, D.C.: Smithsonian National Press; 1980: 41-49.

231. Geraci J: **Marine mammals (Cetacea, Pinnipedia, and Sirenia); nutrition and nutritional disorders.** In *Zoo and Wild Animal Medicine.* 2nd edition. Edited by Fowler M. Philadelphia: WB Saunders Co.; 1986: 760-764.

232. Thatcher C, Hand M, Remillard R: **An Iterative Process.** In *Small Animal Clinical Nutrition.* 5th edition. Edited by Hand, Thatcher, Remillard, Roudebush, Novotny. Topeka: Mark Morris Institute; 2010: 3-21.

233. Case L, Carey D, Hirakawa D, Daristotle L: *Canine and Feline Nutrition: A Resource for Companion Animal Professionals.* 2nd edn. St. Louis, MO: Mosby; 2000.

234. Williams T: **Physiological and Ecological Consequences of Extreme Body Size in Whales.** In *Whales, Whaling, and Ocean Ecosystems.* Edited by Estes J, DeMaster D, Dok D, Williams T, Brownell J. Berkley, CA: University of California Press; 2006: 191-201.

235. Stevens CE, Hume ID (Eds.): **Comparative Physiology of the Vertebrate Digestive System**, 2nd edition. New York: Cambridge University Press; 1995.

236. Clauss M, Kleffner H, Kienzle E: **Carnivorous Mammals: Nutrient Digestibility and Energy Evaluation.** *Zoo Biol* 2010, **29:**687-704.

237. Atinmo T, Beaton G, Calloway D, Xue-cun C, Debry G, Durnin J, Ferro-Luzzi AF, GB, Garby L, Inoue G, Munro H, et al: **Energy and Protein Requirements.** In *Joint FAO/WHO/UNU expert consultation report* (Protection AaC ed. Geneva, Switzerland: World Health Organization; 1985.

238. Ridgway S (Ed.). **Mammals of the Sea**. Springfield, IL: Charles C. Thomas Publisher; 1972.

239. Choi HK, Liu SM, Curhan G: **Intake of purine-rich foods, protein, and dairy products and relationship to serum levels of uric acid - The Third National Health and Nutrition Examination Survey.** *Arthritis Rheum* 2005, **52:**283-289.

240. Ender F, Dishington I: **Etiology and prevention of paresis pueperalis in dairy cows.** In *Parturient Hypocalcemia.* Edited by Anderson J. New York: Academic Press; 1970: 71-79.

241. Kealy R, Lawler D, Monti D, Biery D, Helms R, Lust G, Olsson S, Smith G: **Effects of dietary electrolyte balance on subluxation of the femoral head in growing dogs.** *Am J Vet Res* 1993, **54:**555-562.

242. Venn-Watson S, Smith CR, Gomez F, Jensen ED: **Physiology of aging among healthy, older bottlenose dolphins (Tursiops truncatus): comparisons with aging humans.** *J Comp Physiol B* 2011, **181:**667-680.

243. Jittinandana S, Kenney P, Slider S, Kiser R: **Effect of brine concentration and brining time on quality of smoked rainbow trout fillets.** *Food Chemistry and Toxicology* 2002, **67:**2095-2099.

244. Hinchcliff KW, Reinhart GA, Burr JR, Swenson RA: **Exercise-associated hyponatremia in Alaskan sled dogs: urinary and hormonal responses.** *J Appl Physiol (1985)* 1997, **83:**824-829.

245. Wells RS, Rhinehart HL, Hansen LJ, Sweeney JC, Townsend FI, Stone R, Casper DR, Scott MD, Hohn AA, Rowles TK: **Bottlenose dolphins as marine ecosystem sentinels: Developing a health monitoring system.** *EcoHealth* 2004, **1:**246-254.

246. Ardente A, Hill R: **The nutrient composition of the diet of bottlenose dolphins (Tursiops truncatus) is better assessed relative to metabolizable energy than dry matter.** *Journal of Zoo and Wildlife Medicine* 2015, **46:**198-204.

247. Mack J, Alexander L, Morris P, Dobenecker B, Kienzle E: **Demonstration of uniformity of calcium absorption in adult dogs and cats.** *J Anim Physiol Anim Nutr* 2015, **99:**801-809.

248. Duerr J, Dyer W: **Proteins in fish muscle, IV. Denaturation by salt.** *Journal of the Fisheries Research Board of Canada* 1952, **8c:**325-331.

249. Gallart-Jornet L, Barat J, Rustad T, Erikson U, Escriche I, Fito P: **A comparative study of brine salting of Altantic cod (*Gadus morhua*) and Atlantic salmon (*Salmo salar*).** *Journal of Food Engineering* 2007, **79:**261-270.

250. Yamka R, Friesen K, Schakenraad H: **The prediction of urine pH using dietary cations and anions in cats fed dry and wet foods.** *The International Journal of Applied Research in Veterinary Medicine* 2006, **4:**58-66.

251. Pires C, Saad F, Carciofi A, Santos J: **Inter-relacao entre balanco cation-anionico do alimento e o pH urinario de gatos.** *Archives of Veterinary Science* 2011, **16**.

252. U.S. Department of Agriculture ARS: **USDA National Nutrient Database for Standard Reference.** Washington, D.C.: USDA; 2012.

253. MacCallum W, Adams D: **Newfoundland capelin lipids: fatty acid composition and alterations during frozen storage.** *Journal of the Fisheries Research Board of Canada* 1969, **26:**2037-2060.

254. Choi H, Atkinson K, Karlson E, Willett W, Curhan G: **Purine-rich foods, dairy and protein intake, and the risk of gout in men.** *The New England Journal of Medicine* 2004, **350:**1093-1103.

255. Serio A, Fraioli A: **Epidemiology of nephrolithiasis.** *Nephron* 1999, **81:**26-30.

256. Sakhaee K, Maalouf NM: **Metabolic syndrome and uric acid nephrolithiasis.** *Semin Nephrol* 2008, **28:**174-180.

257. Sarwar G, Brule D: **Assessment of the uricogenic potential of processed foods based on the nature and quantity of dietary purines.** *Prog Food Nutr Sci* 1991, **15:**159-181.

258. Spann WK, Gröbner W, Zöllner N: **Effect of Hypoxanthine in Meat on Serum Uric Acid and Urinary Uric Acid Excretion.** In *Purine Metabolism in Man-III. Volume* 122A. Edited by Rapado A, Watts RWE, Bruyn CMM: Springer US; 1980: 215-219: *Advances in Experimental Medicine and Biology*].

259. Brulé D, Sarwar G, Savoie L: **Changes in serum and urinary uric acid levels in normal human subjects fed purine-rich foods containing different amounts of adenine and hypoxanthine.** *J Am Coll Nutr* 1992, **11:**353-358.

260. Clariana M, Gratacós-Cubarsí M, Hortós M, García-Regueiro JA, Castellari M: **Analysis of seven purines and pyrimidines in pork meat products by ultra high performance liquid chromatography–tandem mass spectrometry.** *J Chromatogr A* 2010, **1217:**4294-4299.

261. Huidobro, A., Pastor A, Tejada M: **Adenosine Triphosphate and Derivatives as Freshness Indicators of Gilthead Sea Bream (Sparus aurata).** *Food Sci Technol Int* 2001, **7:**23-30.

262. Levy-Lior A, Pokroy B, Levavi-Sivan B, Leiserowitz L, Weiner S, Addadi L: **Biogenic guanine crystals from the skin of fish may be designed to enhance light reflectance.** *Crystal growth and design* 2008, **8:**507-511.

263. Jacobs J: **NOAA/NCCOS/Oxford Lab - tricaine methanesulfonate (MS-222) effect on tissue mineral concentrations.** (Ardente A ed. Oxford, MDDecember 2012.

264. Posner L: **North Carolina State University College of Veterinary Medicine, Anesthesiologist - tricaine methanesulfonate (MS-222) effect on tissue mineral concentrations.** (Ardente A ed. RaleighDecember 2012.

265. Piñeiro-Sotelo M, Rodríguez-Bernaldo de Quirós A, López-Hernández J, Simal-Lozano J: **Determination of purine bases in sea urchin (Paracentortus lividus) gonads by high-performance liquid chromatography.** *Food Chemistry* 2002, **79:**113-117.

266. **Power Analysis** [http://www.statmethods.net/stats/power.html]

267. Brule D, Sarwar G, Savoie L, Campbell J, Vanzeggelaar M: **Differences in uricogenic effects of dietary purine bases, nucleosies and nucleotides in rats.** *J Nutr* 1988, **118:**780-786.

268. Sumner FB: **Vision and Guanine Production in Fishes.** *Proc Natl Acad Sci U S A* 1944, **30:**285-294.

269. Ryder JM: **Determination of adenosine triphosphate and its breakdown products in fish muscle by high-performance liquid chromatography.** *J Agric Food Chem* 1985, **33:**678-680.

270. Marklund N, Ostman B, Nalmo L, Persson L, Hillered L: **Hypoxanthine, uric acid, and allantoin as indicators of in vivo free radical reactions. Description of a HPLC method and human brain microdialysis data.** *Acta Neurochir (Wien)* 2000, **142:**1135-1142.

271. Ackman RG, Ke PJ, MacCallum WA, Adams DR: **Newfoundland Capelin Lipids: Fatty Acid Composition and Alterations During Frozen Storage.** *Journal of the Fisheries Research Board of Canada* 1969, **26:**2037-2060.

272. Smith C, Poindexter J, Meegan J, Bobulescu I, Jensen E: **Pathophysiological and physicochemical basis of ammonium urate stone formation in dolphins.** In *Unpublished*2013.

273. Fellstrom B, Danielson BG, Karlstrom B, Lithell H, Ljunghall S: **The influence of a high dietary intake of purine-rich animal protein on urinary urate excretion and supersaturation in renal stone disease** *Clin Sci* 1983, **64:**399-405.

274. Kim K, Henderson G, Frye R, Galloway C, Brown N, Segal M, Imaram W, Angerhofer A, Johnson R: **Simultaneous determination of uric acid metabolites allantoin, 6-aminouracil, and triuret in human urine using liquid-chromatography-mass spectrometry.** *Journal of Chromatography B: Analytical Technologies in Biomedical Life Science* 2009, **877:**65-70.

275. Gow A, Fairbanks L, Simpson J, Jacinto A, Ridyard A: **Xanthine urolithiasis in a Cavalier King Charles spaniel.** *Vet Rec* 2011, **169**.

276. Rivara C, Johnson C, Lulich J, Osborne C, Murtaugh M: **The effect of disease on the urinary purine metabolite concentrations in dogs.** *Vet Rec* 2013, **173**.

277. Shingfield KJ, Offer NW: **Simultaneous determination of purine metabolites, creatinine and pseudouridine in ruminant urine by reversed-phase high-performance liquid chromatography.** *Journal of Chromatography B: Biomedical Sciences and Applications* 1999, **723:**81-94.

278. Bartges J, Osborne C, Felice L, Unger L, Bird K, Koehler L, Chen M: **Reliability of single urine and serum samples for estimation of 24-hour urinary uric acid excretion in six healthy Beagles.** *Am J Vet Res* 1994, **55:**472-476.

279. Steinhauslin F, Burnier M, Magnin J, Munafo A, Buclin T: **Fractional excretion of trace lithium and uric acid in acute renal failure.** *J Am Soc Nephrol* 1994, **4:**1429-1437.

280. Yang H, Gao L, Niu Y, Zhou Y, Lin H, Jiang J, Kong X, Liu X, Ling L: **Mangiferin inhibits renal urate reabsorption by modulating urate transporters in experimental hyperuricemia.** *Biol Pharm Bull* 2015, **38:**1591-1598.

281. Ardente A, Garrett T, Wells R, Colee J, Hill R: **Urine purine metabolites excreted by free-ranging common bottlenose dolphins.** pp. 5. Under review: Veterinary Record: University of Florida; 2016:5.

282. Sorensen LB: **Mechanism of excessive purine biosynthesis in hypoxanthine-guanine phosphoribosyltransferase deficiency.** *J Clin Invest* 1970, **49:**968-978.

283. van Zuilen CD, Nickel RF, van Dijk TH, Reijngoud DJ: **Xanthinuria in a family of Cavalier King Charles spaniels.** *Vet Q* 1997, **19:**172-174.

284. Levartovsky D, Lagziel A, Sperling O, Liberman U, Yaron M, Hosoya T, Ichida K, Peretz H: **XDH gene mutation is the underlying cause of classical xanthinuria: a second report.** *Kidney Int* 2000, **57:**2215-2220.

285. Calafat A, Sampson E: **Laboratory Procedure Manual: Benzophenone-3, bisphenol A, 2,4-dichlorophenol, 2,5-dichlorophenol, ortho-phenylphenol, mehtyl-, ethyl-, propyl-, and butyl parabens, 4-tert-octylphenol, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol, triclosan.** In *Bisphenol A and other environmental phenols in urine*. pp. 35: Centers for Disease Control, Environmental Health; 2009:35.

286. Coe F, Evan A, Worcester E: **Kidney stone disease.** *The Journal of Clinical Investigation* 2005, **115:**2598-2608.

BIOGRAPHICAL SKETCH

Dr. Amanda Ardente was born in Hatfield, Pennsylvania in 1980. She completed her Bachelor of Science in Veterinary Medical Technology at Wilson College in 2002. She went on to complete her Doctor of Veterinary Medicine at North Carolina State University (NCSU) College of Veterinary Medicine (CVM) in 2009. Dr. Ardente then stayed on at NCSU CVM for a one-year nutrition-focused small animal rotating internship. Following completion of her internship, she worked as an emergency veterinarian for one year in a companion animal private practice in Valley Cottage, NY, before returning to academia in fall 2011 to begin pursuit of her Doctor of Philosophy with the University of Florida's College of Veterinary Medicine Aquatic Animal Health program.