# ABSTRACT

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| Title of Thesis: | DIETARY CONTROL OF CYSTINURIA IN MANED WOLVES (CHRYSOCYON BRACHYURUS) |
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| Degree and year: | Master of Science, 1998 |
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The maned wolf (*Chrysocyon brachyurus*) is an endangered South American species of canid, whose numbers in both the wild and captivity are threatened in part by a condition called cystinuria. In humans and rats, cystinuria is caused by mutations of the rBAT gene, expressed in intestinal mucosal cells and renal tubules. In dogs, only renal tubules are affected. Cystinuric individuals exhibit impaired capacity for reabsorption of the sulfur amino acid cystine. Consequently, excess cystine accumulates in the urine. In acidic conditions, cystine may precipitate into uroliths, potentially obstructing the urethra and ultimately causing bladder rupture or kidney damage. Treatments include medical management and dietary modification.

Test diets differing only in levels of cystine were fed to 15 maned wolves in a cross-over design. Apparent digestibility calculations suggest the diets were well received, well utilized, and improved fecal quality. The low cystine diet significantly reduced urinary excretion of cystine ($P<0.05$). Mean urinary concentrations of cystine were $341.8 \pm 45.87$ mg/L or $2.78 \pm 0.326$ % total urinary nitrogen for the low cystine

diet; and 475.8 ± 47.80 mg/L or 4.01 ± 0.342 % for the high cystine diet.

Water balance studies was determined three maned wolves, using orally administered deuterium oxide. Urine samples were collected daily for seven days, purified by sublimation, and analyzed by infrared spectrometry. It was determined that total body water, water turnover rate and body composition were 17.2 L, 2.3 L/d, and 20.5% fat; 79.5% lean, respectively. These results are comparable to published values for dogs.

DIETARY CONTROL OF CYSTINURIA

IN MANED WOLVES

*(CHRYSOCYON BRACHYURUS)*

by

Jennifer Boniface

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland at College Park in partial fulfillment
of the requirements for the degree of
Master of Science
1998

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MD

DEPT OF ANIMAL SCIENCES

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# DEDICATION

To maned wolves everywhere, with the strong hope that they will not leave our planet too soon . . .

To my two best girls, KC and Tynee, the doberman companions who light up my life and inspire me to search for a better way.

To my true friends, both old and new, and family. Your unwavering support and belief in me have carried me through a very tough three years. I literally could not have done it without each one of you cheering me on. Guess what, you were right: I made it!

Last but not least, to me! For being brave enough to embark on this journey, and determined enough to see it through to completion. The end of this project is the beginning of my new career.

# ACKNOWLEDGMENTS

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# CHAPTER 1.   Literature Review

## The Maned Wolf

The maned wolf (*Chrysocyon brachyurus*) is the largest of the South American canids and can be found in Brazil, Peru, Argentina, Paraguay and Bolivia (Bueler 1974). Maned wolf ecology and social organization have been studied mainly in Brazil, where the species is considered endangered (Dietz 1984). One report estimated that between 1964 and 1967 there were only 1,500 to 2,200 maned wolves living in the wild in Brazil (DaSilveira 1968). Thirty years later, the International Union for the Conservation of Nature and Natural Resources upholds their classification as vulnerable, defining "Vulnerable" as "taxa believed likely to become Endangered in the near future if the causal factors of its decline continue operating" (IUCN 1990).

Over the years, the maned wolf has been assigned to three different genera and five different species (Osgood 1934). Science has long regarded the taxonomy of the maned wolf as a "mystery" but today most experts agree it is the oldest of the modern South American canid lines and unique enough to be assigned to its own genus (Bueler 1974). The maned wolf shares chromosomal similarities with true wolf-like canids (gray wolf, coyote, African hunting dog) and anatomical similarities such as feet, nose pad, and cecum, with the bush dog (Dietz 1984).

In the wild, the maned wolf's diet consists of approximately 49% animal matter, made up of small prey such as mice, birds, armadillos, and various insects, and 51% plant matter, comprised of native fruits and grasses (Dietz 1987). The main staple of the maned wolf diet in the wild is *Solanum lycocarpum*, or "lobiera," which means "fruit of

the maned wolf." This fruit resembles a tomato and is available year round (Dietz 1984). Other fruits are more seasonally available. The way in which the wild maned wolf adapts its diet to wet/dry seasonal variation and availability of food items suggests it is more of an omnivore than its standard treatment in captivity presumes (Dietz 1984).

Twenty years ago, there were approximately 100 maned wolves in captivity in 25 facilities world wide (Brady and Ditton 1978). Although successful reproduction is rare, with high mortality rates among the offspring produced, today there are approximately 500 maned wolves in 125 institutions reporting to the Studbook Keeper; 90 of these wolves are kept at 28 zoos in the US (Rodden 1998). The diets fed to these captive wolves vary greatly among facilities. Historically in zoos, captive maned wolves have been fed a frozen, meat-based diet. Compared to their omnivorous feeding pattern observed in the wild, feeding captive maned wolves as carnivores may be a misguided effort (Barboza et al 1994).

One of the largest threats to the health of maned wolves in captivity is a condition called cystinuria. While most wild maned wolves apparently have and can live with cystinuria, captive maned wolves tend to develop potentially fatal complications from the condition. Eighty percent of wolves tested in zoos both in the U.S. and abroad, as well as wild-caught animals who were tested, were found to be affected (Montali and Bush 1982, Segal and Thier 1983). European and Australian zoos have a much lower incidence of problems related to cystinuria, probably due to the fact that their maned wolf diets contain much higher levels of plant fiber and lower levels of meat protein than diets fed to maned wolves in the U.S. (Allen 1995).

# Cystinuria

In dogs and humans, cystinuria is an inherited, metabolic disorder. The dibasic, non-essential, sulfur-containing amino acid cystine is composed of two molecules of cysteine, the precursor of which is the sulfur-containing amino acid methionine. Normally present in low concentrations, cystine is filtered through the kidney and then reabsorbed in the tubules. Individuals affected with cystinuria exhibit impaired capacity for reabsorption of cystine in the proximal tubules. The exact mechanism of reabsorption of cystine in the tubules is not well understood. Calonge and co-workers provided convincing evidence establishing the human rBAT gene as a cystinuria gene, by characterizing several cystinuric-specific mutations. The rBAT gene is expressed in the brush border membranes of the proximal tubules, and "the most common mutation nearly abolishes the amino acid transport activity of rBAT" (Calonge et al 1994).

In some cases, excess cystine not reabsorbed by the kidneys is excreted in the urine. In other cases, excess cystine precipitates to form uroliths, also called calculi, or stones. These uroliths can lead to urethral blockage, infections, and ultimately to kidney insufficiency (Bovee and Kronfeld 1981). Any of these conditions can be fatal.

Cystinuria has been studied in humans, dogs, and maned wolves. The incidence of the condition in humans is one in 7,000; in dogs it is less than one in 1,000; in maned wolves it is remarkably higher (Calonge et al 1994, Segal 1983). In one study, maned wolves, some wild, and some captive, were tested. Researchers found that 34 out of 42 had excessive cystine in their urine; four of these had calculi composed of cystine, and

three of those four died as a result of urinary obstruction and subsequent bladder rupture (Bovee and Bush 1978).

Although somewhat rare, cystinuria does affect and has been studied in humans. These studies have revealed both similarities and differences between the mechanisms of the condition in humans and dogs. In humans, cystinuria is associated with an inherited disorder of intestinal transport of cystine, as well as a renal tubular transport defect (Calonge et al 1994, Sakhaee 1989). This is not the case in dogs, where cystinuria has no known association with any intestinal transport defects (Bovee 1986). Human cystinuria has been classified into three types, based on intestinal mucosal transport patterns for dibasic amino acids, as described by Segal and Thier (1983). The majority of human cystinuria patients are Type I, where no cystine or other dibasic amino acids are absorbed by the intestine. Type II patients absorb some cystine, but no other dibasic amino acids. In both Type I and Type II patients, serum levels of cystine cannot be raised by oral cystine loading, while in Type III, oral cystine loading does result in normal elevation of serum cystine levels. Type III is also characterized by some, but still less than normal absorption of both cystine and other dibasic amino acids. Very few human patients fall into the Type III category (Segal and Their 1983).

In addition to the formation of cystine uroliths, some human cystinuria patients have been shown to develop a cysteine deficiency. Martensson and co-workers measured urinary excretion of the degradation products of cysteine --inorganic sulfate, and taurine-- as well as concentrations of glutathione, which functions as an intracellular reservoir of cysteine. The results indicated that the patients studied did have a cysteine

deficiency, which was partially characterized by deficiencies of taurine and glutathione. This is an important finding, as deficiencies of taurine and glutathione in humans may result in development of abnormal retinal functions (Martensson et al 1990).

Cystinuria has been studied extensively in dogs and seems to be most prevalent in certain breeds such as Dachshunds, Irish Terriers, and Bulldogs (Hoppe et al 1993. It has also been found in Basset Hounds, Chihuahuas, and Yorkshire Terriers (Lewis and Morris 1984). Genetic studies have been done mostly with Newfoundlands (Casal et al 1995). While none of these breeds is closely related to the maned wolf, we use the domestic dog as the closest model for the maned wolf.

Since it is an inherited condition, cystinuria in dogs is of importance to breeders. Identification of carriers is vital in order to prevent the condition from being passed on to future generations. Phenotypically normal parents can produce affected offspring, with both males and females being affected, but males display clinical signs more frequently and severely (Casal et al 1995, Tsan et al 1972). It has recently been determined that cystinuria is an autosomal recessive trait in dogs, and may be detected via urine testing as early as two days of age (Casal et al 1995). There is currently no carrier detection test available, but research efforts are attempting to develop molecular screening tests (Casal et al 1995). It is recommended that affected dogs, their parents, and their offspring be excluded from breeding programs (Casal et al 1995). Presumably the same recommendation extends to the captive maned wolf breeding program. The development of screening tests may be of benefit to this species as well.

Although cystinuria can be detected in both male and female dogs and maned wolves, problems associated with cystine uroliths have been reported mainly in males. Cystinuria sometimes goes undetected in female canids, presumably due to their ability to pass some smaller uroliths through their comparatively short, wide urethras (Tsan et al 1972). The smaller, narrower male urethra is less able to pass uroliths. Bush and Bovee necropsied a male maned wolf who had died of complications following surgical removal of cystine calculi. They found that the male maned wolf has a narrower urethral lumen than a dog of comparable size, indicating a greater susceptibility of maned wolves to obstruction by uroliths (Bush and Bovee 1978).

Surgically removed uroliths have been found mostly in the bladder and urethra, and their composition analyzed as approximately 93 - 99% pure cystine (Bovee 1978, Casal et al 1995, Tsan et al 1972). Cystine crystals are described as hexagonal, yellow-brown in color, round to oval in shape, with a typical "maple sugar crystal" surface, and ranging in size from 2 mm to 1.6 cm (Bovee 1978, Casal et al 1995).

Because not all cystinuric individuals form cystine crystals, cystinuria is a predisposing factor rather than the primary cause of urolith formation (Hoppe et al 1993). It should be noted that excessive cystine in the urine is not necessarily a health risk as long as it is in a soluble form, which can be excreted in urine. Loss of solubility of excess cystine may lead to the presence of cystine uroliths. The fact that maned wolves in the wild live with, rather than die from, the condition of cystinuria supports the assertion that the condition itself is not life-threatening. Rather, it is the formation of cystine uroliths that is the source of potential problems in the maned wolf.

One possible explanation for naturally occurring high levels of cystine in the urine of maned wolves in the wild is that the resultant strong odor associated with sulphocysteine compounds could play a role in scent marking. The presence of high levels of sulfur-containing amino acids in the urine of mammals is not necessarily abnormal or even unhealthy in several species.

Among the mammals known to excrete sulfur-containing compounds in their urine are the genet (*Genetta genetta*), the bobcat (*Lynx rufus*), the red fox *(Vulpes vulpes)*, and the grey, or timber wolf *(Canis lupus)* (Crawhall and Segal 1965, Henry 1978, Mattina et al 1991, Raymer et al 1984). None of these species is known to have problems with cystinuria or any other amino aciduria. Each of them exhibits scent marking behavior, and studies have been done on this phenomenon, specifically regarding the role of the sulfur-containing compounds in scent marking.

Scent marking behavior is thought to serve two purposes. Strongly scented urine acts to warn and thereby repel prey animals. It also serves to mark the territory, letting possible intruders know that this property is already taken. There is no published literature available on scent-marking, sulphocysteine compounds in urine, or urine marking in the maned wolf. It is improbable that urine marking by wild maned wolves is related to repelling prey, since prey animals are a relatively small part of the diet. However, marking boundaries of territories may well be an important factor. Maned wolves in the wild are mainly solitary dwellers rather than pack animals, and their territories are fairly large. Also, since maned wolves pair bond, with both male and female participating in raising young, urine marking may serve a very important function

in communicating the pair's existence and establishing its territory. Possibly, the existence of excess cystine in the urine of maned wolves is not as abnormal as has been believed.

## Treatment for Cystinuria

Studies of cystinuria and cystine uroliths in dogs have suggested controlling the condition by using dietary modification, medical management, or some combination of the two.

Drug therapy for cystinuria is aimed at dissolving cystine uroliths that already exist, as well as preventing formation of new uroliths. This may be accomplished by altering the pH of the urine, and/or increasing the volume of urine, both of which will serve to increase solubility of the uroliths (Osborne et al 1989, Segal and Their 1983).

Thiol-containing drugs are the most commonly used group for treatment of cystinuria. The one most referred to in the literature is d-penicillamine, or dimethyl-cysteine, a nonmetabolizable degradation product of penicillin. This drug combines with cystine to form cysteine-d-penicillamine disulfide, a compound that is reported to be up to fifty times more soluble than free cystine (Osborne et al 1989).

Adverse reactions to the drug in dogs include fever, nausea and vomiting, while in humans it is associated with fever, skin rash, muscular aches, headache, weakness, anorexia, vomiting, and impaired liver function (McDonald and Henneman 1965, Segal and Thier 1983). D-penicillamine causes reduced activity of the transsulfuration pathway, resulting in decreased conversion of methionine to cysteine and leading to problems with the inhibition of pyridoxine, or vitamin $B_6$ (Martensson et al 1990). This

deficiency may be treated by supplementing with pyridoxine phosphate (Segal and Thier 1983). In addition, due to the chelating properties of penicillamine, there may be an increase in the urinary excretion of copper and zinc, as well as effects on calcium, mercury, and iron excretion (Segal and Thier 1983). Chelates are a complex of an organic ligand (electron donor) and a metal (electron acceptor), in a ring structure formed by the positive-negative attraction of the electrons (Kratzner 1986, Soares 1997). The affinity of penicillamine for molybdene may also lead to a molybdene deficiency, characterized by an increased output of thiosulfate in the urine (Martensson et al 1990).

A second, thiol-containing drug used to treat cystinuria is N-2-mercaptopropionyl-glycine, or MPG. This drug serves to decrease the concentration of cystine similarly to the actions of d-penicillamine, via a thiol-disulfide exchange reaction (Osborne et al 1989). It is thought that MPG may be more effective than penicillamine in the disulfide exchange reaction, due to its higher oxidation-reduction potential (Segal and Thier 1983). MPG is reportedly highly effective and less toxic than d-penicillamine (Osborne et al 1989), although it is not without side effects such as skin rash, fever, nausea, and diarrhea (Segal and Thier 1983). It is thought that MPG may be tolerated by some patients who are sensitive to penicillamine, and that it may therefore be used as an alternative treatment for some cystinuric individuals (Segal and Thier 1983).

A third drug has been suggested for treatment of cystinuria in humans. N-acetylcysteine (NAC) has been reported as a strong solvent for renal cystine calculi, as it too forms a mixed disulfide with cysteine (Martensson et al 1990). However, a recent

study showed that NAC is rapidly metabolized in humans and cannot be recommended for treatment to reduce cystine output (Martensson et al 1990).

Surgical removal of cystine calculi is possible. Surgery may be indicated in cases where drug therapy has been unsuccessful in dissolving uroliths, or where there is a physical abnormality of the urinary tract that surgery can correct (Caywood and Osborne 1986). The goals of surgery are to remove the uroliths, eliminating blockage of urine flow, while preserving organ function. However, surgical removal is not usually a satisfactory resolution of the problem. Uroliths tend to recur in those individuals who form them, within one year following surgery (Caywood and Osborne 1986). The Newfoundland breed studied by Casal and co-workers exhibited an exceptionally rapid recurrence of formation not seen in other breeds (Casal et al 1995). For this reason, it is important to evaluate the situation prior to surgery, and select a course of preventive treatment to begin postoperatively (Caywood and Osborne 1986).

As one of several inherited aminoacidurias, cystinuria is among those with the most significant nutritional consequences. Dietary modification shares the same goals as drug therapy, to dissolve crystals that are present while preventing the formation of new ones. This is accomplished through alkalinization of the urine and increasing its volume. Cystine is the least soluble, naturally occurring amino acid (Osborne et al 1989). Osborne and co-workers reported that cystine crystals are insoluble in acidic urine, but their solubility increases as the urine pH increases to become alkaline. Normal canine urine pH is in the range of 5.5 to 7.0, and cystine in this pH range is marginally soluble, but is readily soluble in urine with a pH of 7.5 or higher (Osborne et al 1989). The pH of

urine is affected by the diet consumed (Hu et al 1993). Given that urinary pH in excess of 7.5 may predispose maned wolves to cystitis, it is also important not to attempt to raise urinary pH higher than 7.5 (Allen 1997).

In both humans and dogs, cystinuria complications have been reduced by making dietary modification. The main dietary adjustment found to be effective is to lower the overall protein content. Bartges and co-workers (1995) studied healthy beagles to determine the influence of diet on the activity product ratios of urates. Both the source of dietary protein and the level of protein in feed were examined.

In the first experiment, when the level of protein was held constant at approximately 11% for each of four diets containing different sources of protein, it was found that 24-hour urine pH was significantly higher when dogs consumed an egg-based diet. The other three diets were casein-based, chicken based, and chicken-based, liver-flavored. Also, 24-hour creatinine clearance was significantly lower when dogs consumed the casein-based diet. The diets differed primarily in protein source, and had similar contents of protein, carbohydrate, fat, minerals, and electrolytes. All diets also contained 1% potassium citrate to promote alkalinization of urine, which probably accounts for the association of all four diets with the production of alkaline urine.

The effects of the level of dietary protein were examined in the second experiment, by feeding each of two different diets to the same group of dogs used in the first study. As compared to the high-protein, meat-based diet, the low-protein, casein-based diet was found to minimize changes in urine that predispose dogs to urate urolithiasis. Activity product ratios of urates and creatinine clearance were lower, while

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24-hour urine pH was higher when dogs consumed the low-protein, casein-based diet. It is uncertain whether the effect on pH was attributable to supplementation with 1% potassium citrate or to a reduction of acid metabolites associated with reduced dietary protein.

The results of these two studies on protein source and level suggest that incorporation of a diet containing low levels of a non-meat based protein source may be beneficial in the dissolution and prevention of urate uroliths in dogs (Bartges et al 1995).

Both human and dog patients switched from high animal-protein diets to vegetarian diets showed significant reduction in urinary cystine excretion (Chesney 1989). It is believed that a animal protein based diet may play a role in fostering the formation of cystine uroliths, due to the acidic urine produced by this type of diet. Historically, such a diet has been fed to captive maned wolves in the US. In contrast, most plant-based foods are known to be alkaline, and to reduce urine acidity (Hu et al 1993). It follows then, that a plant-based diet, with a plant source of protein, should promote a more alkaline urine, where cystine is more soluble and does not precipitate into uroliths (Chesney 1989).

For the captive maned wolf, dietary modification is also centered around adjusting the protein content of the diet. Studies on cystinuria in captive maned wolves have indicated that elevated protein levels in the diet are responsible for complications (Barboza et al 1994). One male maned wolf sustained a ruptured bladder following an increase in dietary meat protein consumption in conjunction with pup rearing (Rasmussen and Tilson 1984). The wolf was treated surgically to repair the rupture, and

his diet was modified; he recovered and suffered no further problems (Rasmussen and Tilson 1984).

In formulating a diet for captive maned wolves, there were several issues to consider. The overall intent was to provide a palatable diet, containing a complete amino acid profile, that would not exacerbate the apparent predisposition of maned wolves to cystinuric complications. An important concept was that the diet contain minimal levels of cystine and its precursor, methionine. It was thought that this would minimize crystal formation associated with excess levels of cystine. Studies of human patients with cystinuria have centered around decreasing urinary excretion of cystine, thereby reducing the frequency of urolith formation (Martensson et al 1990). Manipulation of dietary levels of methionine is one possible way to accomplish this, and has yielded varied results. Restriction of dietary methionine, in conjunction with a low-protein diet, results in decreased cystine in the urine (McDonald and Henneman 1965). One human patient on a low methionine diet had a complete disappearance of cystinuria (Segal and Thier 1983). In a study performed on cystinuric dogs, Hess and Sullivan (1942) reported that urinary cystine excretion increased sharply with increased protein intake. The protein source was casein-based and the levels studied were 5, 10, 25, and 50%. The same experiment also found that ingestion of methionine and cysteine hydrochloride led to the excretion of excessive cystine. This effect was much more pronounced at the 5% and 10% levels of protein than at the 25% or 50% levels.

One other area of concern in formulating a diet for captive maned wolves is their tendency to be lean and have difficulty maintaining body mass. This is probably

attributable to the low fat content of some of the commercially available "low protein" dog foods commonly fed. Maned wolves appear to require more energy dense diets in order to maintain their weight.

Low sodium intake has also been reported to reduce the excretion of cystine and other dibasic amino acids (Chesney 1989). Jaeger and co-workers (1986) showed that there is a significant positive correlation between sodium intake and cystine excretion in the urine. The mechanism underlying this observation is not clear, but one explanation may be that long term sodium depletion resulting from a low sodium diet might stimulate thirst (Nutrition Reviews 1987). Greater water intake would likely result in a more voluminous and diluted urine, which in turn reduces the overall concentration of cystine, rendering urolith formation less likely to occur (Jaeger et al 1986).

## Water Balance

The question has been raised of whether the maned wolf's tendency to produce loose, watery and poorly formed stools could be a contributing factor to the formation of uroliths. Excessive water loss in the stool rather than via the kidney might conceivably lead to a more concentrated urine which would foster precipitation of cystine into stones. This then becomes another consideration in formulating a maned wolf diet, with emphasis on including ingredients that will promote good stool formation.

Zentek and Meyer (1995) compared fecal water loss in a large dog breed (Great Dane), to that in a medium breed (Beagle). Their report is relevant since the Great Dane has a body size and composition similar to the maned wolf. Differences between the two breeds evaluated were attributed to the relationship between body weight and

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gastrointestinal tract, and to differences in transit time, especially in the large intestine. Other factors possibly affecting fecal water loss are: physiological conditions such as disease, stress, pregnancy; type of gut flora; and breed of dog.

Fecal quality is also influenced by dietary factors such as the source of carbohydrate and protein, and both the quantity and quality of fiber provided. These diet components may affect water binding capacity of the feces; if this capacity is high, less free (unbound) water is excreted in the feces, and feces are more well-formed. Vegetable sources of protein, especially gluten, lead to well-formed feces, while protein derived from diets rich in meat, with large amounts of connective tissue and slaughter offal, tends to lead to formation of softer feces (Zentek and Meyer 1995).

This study concluded that larger breeds tend to produce softer, more watery feces. This holds true even when there is no evidence of gastrointestinal upset, and the diet consumed is identical to that of medium sized breeds, which do not exhibit soft feces. Furthermore, nutrient absorption rates were lower in the larger breed, although utilization was apparently the same. This was probably due to the relatively smaller size of the digestive tract compared to smaller breeds. A smaller digestive tract results in a faster rate of passage, with increased water loss. High fiber diets might also play a role in accelerating the transit time of ingesta through the gastrointestinal tract.

The measurement and calculation of total body water, daily water turnover rate, and the half life of water in the body are permitted by the use of heavy water, containing deuterium oxide ($D_2O$). $D_2O$ is said to be an ideal tracer for body water because it essentially behaves like water, becoming uniformly distributed throughout the body

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within a relatively short period of time (Faller et al 1955). Heavy water is administered either via injection or orally, in a measured dose based on the body weight of the subject. Subsequent collections of either blood, urine, or both are made. These samples may then be analyzed for their concentration of $D_2O$.

A variety of methods may be used to analyze for $D_2O$. These techniques include the falling-drop method, elevation of the freezing point, gas chromatography, mass spectrometry, and infrared spectrometry. Analysis by infrared spectrometry is usually preferred since sample preparation is relatively easy, and the method offers superior sensitivity and accuracy when $D_2O$ is present in low concentrations (Zweens et al 1980).

As $D_2O$ equilibrates in the body fluids, the deuterium oxide is present in two molecules: $H_2O$ and HOD, according to the following equation: $H_2O + D_2O \rightleftharpoons 2$ HOD (Zweens et al 1980). The absorbance of the OD vibrational band is what the infrared spectrometry measures; from this measurement, the concentration of deuterium may be determined. Since there may sometimes be interfering substances in the sample which absorb near the OD band, use of infrared spectrometry requires the samples to be purified, in order to remove such substances (Zweens et al 1980). Purification of biological fluid samples may be accomplished either by distillation or sublimation (Faller et al 1955). Once purified, samples are read on the infrared spectrophotometer, which measures their transmittance and absorbance as compared to a reference cell containing pure, distilled water, with no deuterium.

## Objectives

Cystinuria-related complications are a threat to the health of maned wolves, an endangered species with a small captive population. Because it is probably an inherited disorder, affected individuals will perpetuate this problem, especially since the gene pool is relatively small. The long term and widespread use of various drugs to discourage cystine precipitation and induce urolith dissolution may have far reaching impact on the species. There might be associated effects, unknown at this time, which could irreparably harm this endangered species and jeopardize its future in captivity. For these reasons, it is important that we obtain a better understanding of cystinuria, and devise safer methods to control the occurrence of potentially lethal urolith formation associated with the condition.

This research project was designed to develop a palatable diet which would meet nutritional requirements without exacerbating an possible predisposition to a potentially fatal condition. The overall goal was to determine whether manipulation of dietary content is a feasable method of decreasing urinary cystine content, thereby reducing the likelihood of cystine urolith formation. Test diets containing differing levels of sulfur amino acids were developed and tested for digestibility as well as ability to lower cystine excretion in captive maned wolves. Effect of the test diets on stool quality was also evaluated, using a canine fecal grading sheet based on subjective assignation of numerical scores. Finally, water balance studies were conducted, in an attempt to obtain quantitative data on water turnover rates and body compositions of maned wolves.

# CHAPTER 2. Determination of Digestibility and Overall Suitability of Diets

## Abstract

Many individuals in both wild and captive populations of maned wolves appear to demonstrate high levels of urinary cystine, possibly indicative of cystinuria. Cystinuria-related complications arise when excess cystine precipitates in the urine, forming uroliths. These uroliths have the potential to cause blockages of the urethra. Uroliths form in acidic conditions, so one way to discourage their formation is to alkalinize urinary pH sufficiently to maintain cystine in solution. Urinary pH is largely responsive to the diet fed. It is possible that by managing the diet of captive maned wolves, the incidence of cystinuria-related problems may be reduced. Other issues that are of importance for maned wolves in captivity are their observed tendency to excrete loose, watery stools, and their difficulty in maintaining body weight. These problems may also respond to dietary management.

Two experimental diets were formulated to be isocaloric and isonitrogenous, differing only in their levels of the sulfur-containing amino acids cystine and methionine. The diets were developed to contain fairly high levels of fat and fiber, and a moderate level of protein, compared to maintenance formulas for adult dogs. Chromic oxide was used as a marker. Digestion trials were performed using 15 wolves at two locations in a Latin square, cross-over design. Digestion trials lasted 10 days each. Intake data for each subject were recorded daily. Fecal samples were collected daily and pooled in two groups, representing Days 1-5 and Days 6-10, for analysis.

Daily intakes for each diet ranged between 15.0-17.0 g/kg body weight, with means of $15.9 \pm 0.83$ for the high cystine diet and $15.1 \pm 0.83$ for the low cystine diet. Mean apparent digestibilities for the high cystine and low cystine diet respectively were: dry matter 66.8, 67.5% $\pm$ (0.75); crude protein 74.0, 74.5% $\pm$ (0.87); fat 89.6, 86.3% $\pm$ (2.10); neutral-detergent fiber (NDF) 37.9, 40.1% $\pm$ (1.49); acid-detergent fiber (ADF) 27.4, 28.5% $\pm$ (1.39); and gross energy 71.8, 74.5% $\pm$ (1.04). These values indicate that the test diets were equally digestible, with no significant differences between them ($P>0.05$). It appears that captive maned wolves accept this diet and may be maintained sufficiently on it. In addition, fecal quality scores were greatly improved over the term of the study.

## Introduction

Captive maned wolves are prone to complications resulting from the presence of excess cystine in their urine (Allen 1995, Barboza et al 1994, Bovee et al 1981). This has been one reason for their reputation as unthrifty in captivity. Problems arise when excess cystine forms into uroliths, potentially obstructive to the process of urination. Uroliths may lead to infections, kidney damage, or bladder rupture, and death (Bovee et al 1978). Uroliths form most often when the pH of urine becomes too acidic, such that the cystine precipitates cannot dissolve. Normal urinary pH in the dog is usually 5.5 - 7.0, but cystine uroliths are generally insoluble in urine with a pH below the range of 7.0 - 7.5 (Osborne et al 1986). The normal urinary pH of the maned wolf is undetermined; however it is suspected that raising the pH above 7.5 might predispose the animal to cystitis (Allen, 1997). Diet consumed has a direct effect on the pH of urine (Hu et al

1993). By controlling the diet, and thereby the urinary pH, it may be possible to reduce the likelihood of urolith formation.

An additional concern when feeding captive maned wolves is their tendency to be quite lean. Historically, it has been difficult to maintain the body weight of these animals in captivity (Allen 1995, Pojeta 1997). This difficulty can reduce successful reproduction efforts and rearing of puppies. Captive maned wolves also tend to produce loose, watery stools, which are universally problematic for animal caretakers.

The Species Survival Program (SSP) has a limited gene pool of approximately 90 captive maned wolves in this country, and is challenged to maintain the existence of as healthy a captive population as is possible. This research project was designed to develop a palatable diet that would meet nutritional requirements without exacerbating a predisposition to a potentially fatal condition. The intention was to test whether dietary control of cystinuria can be effective, and therefore preclude the use of pharmacologic agents. In addition, the outcome of this research contributes to the effort to define amino acid requirements of maned wolves. The main objective in formulating this diet was to reduce urinary cystine output by restricting dietary sources of the sulfur amino acids, cystine and methionine. A secondary objective of this study was to test the effects of this diet on stool consistency.

## Materials and Methods

Number and location of subjects. Sixteen maned wolves were initially used in this experiment; one developed health problems and was subsequently dropped from the study as he could not be fed the test diet exclusively while recuperating. Of the fifteen

participating maned wolves, eleven were located at the Conservation Research Center, National Zoological Park, Smithsonian Institution in Front Royal, VA (CRC); the remaining four were located at the Wild Canid Survival Center in Eureka, MO. Basic descriptive information about the wolves in this study is detailed in Table 2.1.

Table 2.1. Description of population of maned wolves participating in digestion trials.

| Sex | n | Body Weight (BW) Range | Average BW (kg) | Age Range (Years) | Average Age (Years) |
|---|---|---|---|---|---|
| Males | 7 | 24.0-32.8 kg | 29.6 | 2-10 | 5.1 |
| Females | 8 | 21.5-30.4 kg | 26.1 | 2-9 | 4.6 |

Every effort was made to ensure that all experimental conditions were kept the same for each subject in the trial, to the extent possible. This study was approved by the Institutional Animal Care and Use Committee of the CRC, and by the Animal Care and Use Committee of the University of Maryland. The daily routines of the maned wolves were carefully considered and disturbed as little as possible, to minimize undue stress.

Formulation of Diets. Experimental diets were balanced to meet or exceed nutrient requirements of adult dogs at maintenance, according to the National Research Council recommendations (NRC 1974, 1985), with the following exception. The low cystine diet contained a lower level of total sulfur amino acids, methionine and cystine, than the high cystine diet. The two diets were isocaloric, isonitrogenous, and identical in every other respect. Both diets contained ~ .05% chromic oxide ($Cr_2O_3$) as a non-absorbable, non-digestible marker. Diets were mixed and extruded by Purina Mills, Inc. in St. Louis, MO, and labeled with the brand name Mazuri. Ingredient composition of the diets is listed in Table 2.2. Chemical composition of the diets is listed in Table 2.4.

Experimental Design. Where possible, wolves were confined indoors, on cement floors, for the duration of the digestion trials. This eliminated the possibility of any food items being ingested other than the test diet. Confinement was not always practical. Four of the animals at the CRC as well as the four in MO are housed in areas that do not provide the option of complete indoor confinement for any length of time. In these cases, no food items were offered other than the experimental diet; however, the ingestion of grass, plants, insects or other small prey was a possibility that could not be controlled. Statistical analysis addressed the issue of whether intakes and digestibilities were affected by this variable.

The digestion trials were conducted as a Latin square, cross-over design. Since wolves were housed in pairs, it was decided that each pair would receive the same diet during the same trial, to simplify the feeding schedule and minimize the potential for errors. Outside of this stipulation, the assignment of the diets to the pairs of wolves was random. The digestion trials were conducted in accordance with the published recommendations of the Association of American Feed Control Officials standard protocols for measuring apparent digestibility of diets for dogs (AAFCO 1993). A transition period of two weeks was allowed for the wolves to acclimate to the test diets. The first trial continued for ten consecutive days. Following a minimum five day adaptation period to the opposite diet, the second trial was begun, with each pair receiving the opposite diet of that fed during the first trial. The second trial also lasted for ten consecutive days. The only difference between the two trials was the diet fed. All animals had free access to water at all times.

Table 2.2. Ingredient composition of diets fed to maned wolves.

| Ingredient | % As Fed[1] |
|---|---|
| Ground Corn | 12.3 |
| Poultry Bi-product Meal | 3.5 |
| Corn Gluten Meal | 8.6 |
| Rice Polishings | 9.1 |
| Meat Meal | 10.7 |
| Poultry Fat[2] | 6.0 |
| Dried Beet Pulp | 12.9 |
| Animal Fat[3] | 7.8 |
| Animal Digest | 1.0 |
| Brewers Dried Yeast | 2.6 |
| Dried Whey | .5 |
| Dried Whole Egg | .5 |
| Soybean Oil | .5 |
| Soybean Hulls | 9.3 |
| Apple Pomace | 8.0 |
| Tomato Pomace | 4.0 |
| Acid Casein | 1.0 |
| Mineral Mix[4] | .6 |
| Vitamin Mix[5] | .8 |
| Ethoxyquin | .02 |
| Chromic Oxide[6] | .3 |
| Total | 100.0 |

[1] Estimated percentages.

[2] Preserved with ethoxyquin.

[3] Preserved with BHA.

[4] Contains (as grams per kilogram of mix): calcium 14.0; phosphorus 14.8; potassium 6.0; magnesium 1.3; sulfur 1.7; sodium 5.5; chlorine 9.0; trace minerals (as micrograms per kilogram of mix): iron 340; zinc 190; manganese 60; copper 14; cobalt .65; iodine 1.6; chromium 1.9; selenium .24

[5] Contains (as ppm): Vitamin K (as menadione) 3.0; thiamin 15; riboflavin 15; niacin .08; pantothenic acid 26; choline chloride 2,000; folic acid 3.0; pyridoxine 16; biotin .56; $B_{12}$ 69; Vitamin A 7,568; Vitamin $D_3$ 100; Vitamin E 230.

[6] As an indigestible marker.

Daily Intake Calculation.  Individuals of pairs were physically separated during the trials, allowing each animal to access only its own food dish.  Food offered each day was weighed to the nearest tenth of a gram prior to feeding, with weights recorded on a daily log, by the animal caretaker responsible for feeding.  Food offered was available for 24 hours, after which time any uneaten diet (orts) was collected and stored in labeled bags in the freezer at -20°C.  Orts were later oven dried at 55°C and weighed to the nearest tenth of a gram.  The difference in weights between the food offered, and orts collected the following day, was presumed to be the amount of food actually eaten by the individual wolf.  All weights were corrected to reflect a dry matter basis.  From these data, daily intake was computed, according to the individual body weights of each wolf, and expressed as grams of food consumed per kilogram of body weight.

Collection of Samples.  Fecal collections of approximately 200 - 300 grams per animal were made daily.  The use of chromic oxide as a marker in the diet allowed partial fecal collections.  Daily fecal samples were collected to obtain the cleanest possible samples from the center of each deposit of feces.  The sampling utensil was cleaned between animals, to avoid any cross contamination.  Samples were placed into clean tupperware containers which were sealed and labeled with the animal's identification and date, and then frozen at -20°C until analyzed.  Fecal consistencies were assessed and recorded on a daily log,  at the time of sample collection.  A fecal grading sheet developed by nutritionists at a leading canine research facility was used to grade the feces of each subject (Waltham).

Preparation of Samples. Fecal samples were lyophilized at a temperature of -80°C for two weeks. Samples were placed in aVirtis 100 freeze drier in the same containers in which they were collected, with the covers removed. Once completely dried, the samples were weighed to the nearest tenth of a gram, then pooled in five day groups by animal, representing Days 1-5 and Days 6-10 for each digestion trial. The four pooled samples for each subject were then homogenized into a fine powder using a Wiley mill with a 1 mm screen and stored at room temperature in labeled plastic bags for later analysis.

Chemical Analysis of Samples. Analysis was performed on all diet and feces samples for determination of dry matter, crude protein, fat, acid-detergent fiber (ADF), and neutral-detergent fiber (NDF). Dry matter was determined using oven drying at 55°C for 5 days for diets, and freeze drying at -80°C for 14 days for feces. Crude protein was calculated from Kjeldahl N (N x 6.25) for all samples (AOAC, 1990). Fat content was determined by ether extract, using the Soxhlet method (AOAC 1990). ADF and NDF were determined using the method of Goering and Van Soest (1975). Gross energy content of diets and feces was determined by bomb calorimetry using the Adiabatic Calorimeter and Parr 1710 Calorimeter Controller, both by the Parr Instrument Co., Moline, IL. Samples were also analyzed for content of chromic oxide, to allow computation of the apparent digestibility of each nutrient. Following digestion with a mixture of nitric ($HNO_3$) and perchloric ($HclO_4$) acids, chromium analysis was done using ultraviolet spectrometry with a Perkin-Elmer Lambda 2 UV/VIS

Spectrometer. Amino acid analysis of diets was accomplished by ion-exchange chromatography, using the method of Schram, et al (1954).

Statistical Analysis of Samples. Data were analyzed as a Latin square cross-over design using the SAS statistical analysis package (SAS Version 6.12, SAS Institute, Cary, NC). The SAS proc mixed procedure provided ANOVA least squares means, SEM, and P values. The SAS univariate procedure was used to check assumptions of normality.

<p style="text-align:center">Results</p>

Figure 2.1 presents the daily intake in g/kg of body weight (BW) for each wolf, for each of the diets tested. Amounts are presented on a dry matter basis. The mean daily intake in g/kg BW for the low cystine diet was $15.1 \pm 0.83$, and for the high cystine diet was $15.9 \pm 0.83$. There was no significant difference between the two diets tested with regard to the amount of food consumed ($P>0.05$). A comparison of intakes between sexes and diets consumed is presented in Table 2.3.

Table 2.3. Comparison between sexes of mean intakes (in g/kg body weight), ages (in years), and body weights (in kg) of maned wolves fed experimental diets differing in amounts of cystine.[1]

| Sex | n | Mean Age (Years) | Mean BW (Kg) | Mean Intake Low Cys Diet | Mean Intake High Cys Diet |
|---|---|---|---|---|---|
| ♂ | 7 | 5.1 | 29.6 | 14.7 g/kg | 16.1 g/kg |
| ♀ | 8 | 4.6 | 26.1 | 15.9 g/kg | 16.3 g/kg |

[1] n = number of subjects, BW = body weight, cys = cystine

Figure 2.1. Daily intakes of maned wolves fed two experimental diets differing in levels of cystine. Intakes presented on a dry matter basis, as grams of food eaten per day per kilogram of body weight of the individual. Data shown above each column represent means over a ten day collection period.

**Average Daily Intake of Maned Wolves on Experimental Diets**

☒ Low Cystine Diet  ■ High Cystine Diet
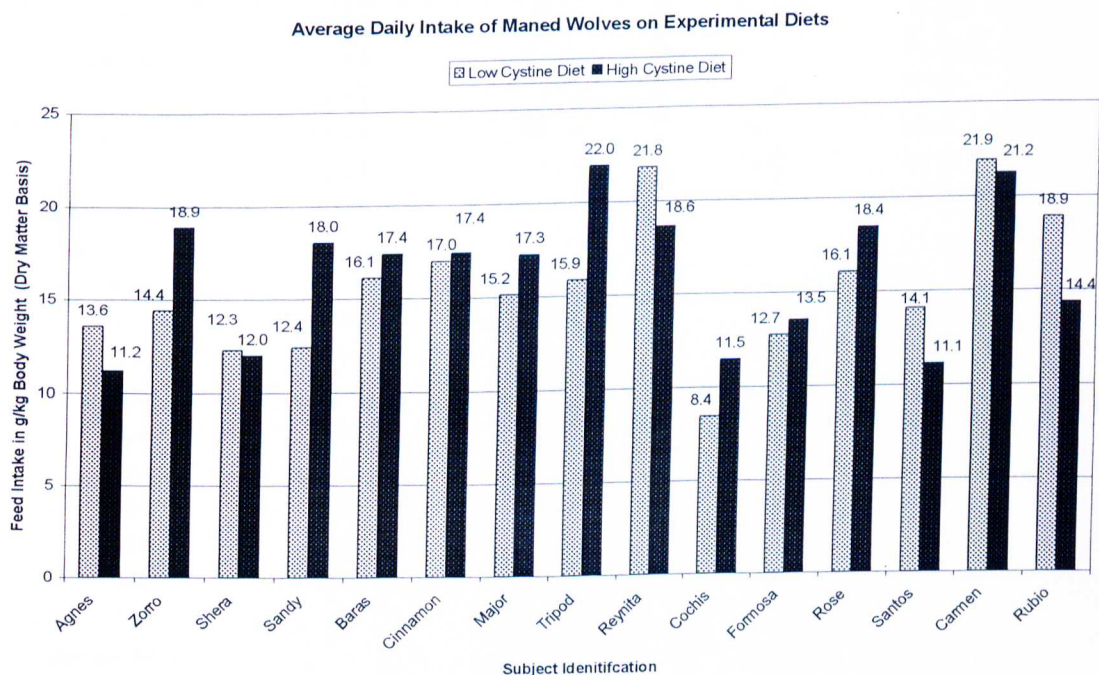


The chemical composition of diets fed to maned wolves is presented in Table 2.4. Pre-experimental diets are shown here to serve as a reference to which the test diets may be compared. Pre-experimental diets included a combination of canned dog food and two types of dry dog food, one low protein (16%), the other high protein (25%). Test diets were isonitrogenous at 21% protein, and isocaloric at 5.14 kcal/g. The difference between the two test diets was the level of total sulfur-containing amino acids. Each test diet included ~ 0.05% chromic oxide as a part of the feed formulation, as an indigestible marker to facilitate the digestion trial.

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Table 2.4. Chemical composition of diets fed to maned wolves, on a dry matter basis.

| Ingredient | ----------Pre-Experimental Diets---------- | | | --Experimental Diets--- | |
|---|---|---|---|---|---|
| | Canned | Low Protein | High Protein | Low Cys | High Cys |
| Dry Matter | 21.22 | 93.74 | 95.00 | 96.59 | 95.49 |
| Crude Protein | 41.82 | 17.96 | 24.30 | 21.04 | 21.05 |
| Fat | 34.76 | 1.99 | 9.41 | 13.61 | 13.77 |
| NDF | 34.65 | 21.94 | 20.61 | 20.66 | 20.61 |
| ADF | 4.35 | 3.51 | 2.92 | 13.92 | 13.71 |
| GE, kcal/g | 6.15 | 4.61 | 5.03 | 5.14 | 5.14 |
| Amino Acids | | | | | |
| Essential | | | | | |
| Arg | 1.78 | 1.23 | 1.54 | .63 | .59 |
| His | 2.15 | .67 | 1.19 | 1.45 | 1.50 |
| Ile | .48 | .27 | .41 | .35 | .38 |
| Leu | 3.46 | 1.34 | 2.10 | 1.56 | 1.57 |
| Lys | 2.46 | .71 | 1.31 | 1.39 | 1.46 |
| **Met** | **.61** | **.25** | **.36** | **.17** | **.19** |
| Phe | 1.43 | .60 | .92 | .80 | .80 |
| Thr | 1.66 | .59 | .98 | .78 | .74 |
| Val | .85 | .37 | .52 | .51 | .57 |
| Amino Acids | | | | | |
| Nonessential | | | | | |
| Ala | 2.83 | 1.11 | 1.55 | 1.24 | 1.27 |
| Asp | 2.99 | 1.25 | 2.13 | 1.87 | 1.88 |
| **Cys** | **.38** | **.28** | **.36** | **.32** | **.44** |
| Glu | 5.19 | 2.84 | 4.13 | 3.41 | 3.37 |
| Gly | 4.49 | 1.42 | 1.98 | 1.89 | 1.94 |
| Pro | 1.97 | 1.05 | 1.39 | 1.18 | 1.19 |
| Ser | 1.77 | .74 | 1.16 | .86 | .81 |
| Tyr | .84 | .29 | .47 | .39 | .39 |
| TSAA[1] | .99 | .53 | .72 | .49 | .63 |

[1] TSAA=Total sulfur-containing amino acids.

Average apparent digestibilities of the test diets are presented in Table 2.5.

Table 2.5. Mean nutrient intakes and apparent digestibilities ± SEM of maned wolves fed two experimental diets.

| Item | Low Cystine | High Cystine |
|------|-------------|--------------|
| Intake, g/d | | |
| Dry Matter | 418.9 ± 9.25 | 438.5 ± 8.66 |
| Crude Protein | 88.2 | 92.3 |
| Fat | 57.0 | 60.4 |
| NDF | 86.6 | 90.4 |
| ADF | 58.3 | 60.1 |
| Gross Energy, kcal/d | 2153.2 | 2253.9 |
| Digestibility, % | | |
| Dry Matter | 67.5 ± 0.75 | 66.8 ± 0.75 |
| Crude Protein | 74.5 ± 0.87 | 74.0 ± 0.87 |
| Fat | 86.3 ± 2.10 | 89.6 ± 2.10 |
| NDF | 40.1 ± 1.49 | 37.9 ± 1.49 |
| ADF | 28.5 ± 1.39 | 27.4 ± 1.39 |
| Gross Energy | 74.5 ± 1.04 | 71.8 ± 1.04 |

The feces of each test subject were assigned quality scores which were recorded daily by animal keepers at the time of collection of that day's samples. Scoring was done according to the Waltham Centre's Dog Fecal Grading Sheet. Scores range from 1 to 5, where 1 represents hard, crumbly feces and 5 represents extremely watery diarrhea. A score of 2 to 3 is considered desirable. Daily scores for animals consuming the low cystine diet were added together and averaged, as were those for the high cystine diet. These scores are represented in Figure 2.2. Overall fecal quality was improved over the course of the digestion trials, with the scores generally following a decreasing pattern over time. Fecal quality scores were not influenced differently between the two test

diets.  Both of the test diets had beginning average scores of 3.6 and ending average

scores of 2.5

Figure 2.2. A comparison of fecal quality scores, from 1 (best) to 5 (worst), for maned wolves fed two experimental diets differing in cystine levels.  Data represent daily means for n=11 test subjects.



**Comparison of Fecal Quality Scores for Maned Wolves Fed Experimental Diets**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| low cys | 3.6 | 3.3 | 3.2 | 3.1 | 3.1 | 2.9 | 3.0 | 2.7 | 2.7 | 2.5 |
| high cys | 3.6 | 3.4 | 3.0 | 3.2 | 3.1 | 3.1 | 3.1 | 3.2 | 2.8 | 2.5 |

Days in Digestion Trial

Daily Fecal Quality Scores, Averaged for all Wolves
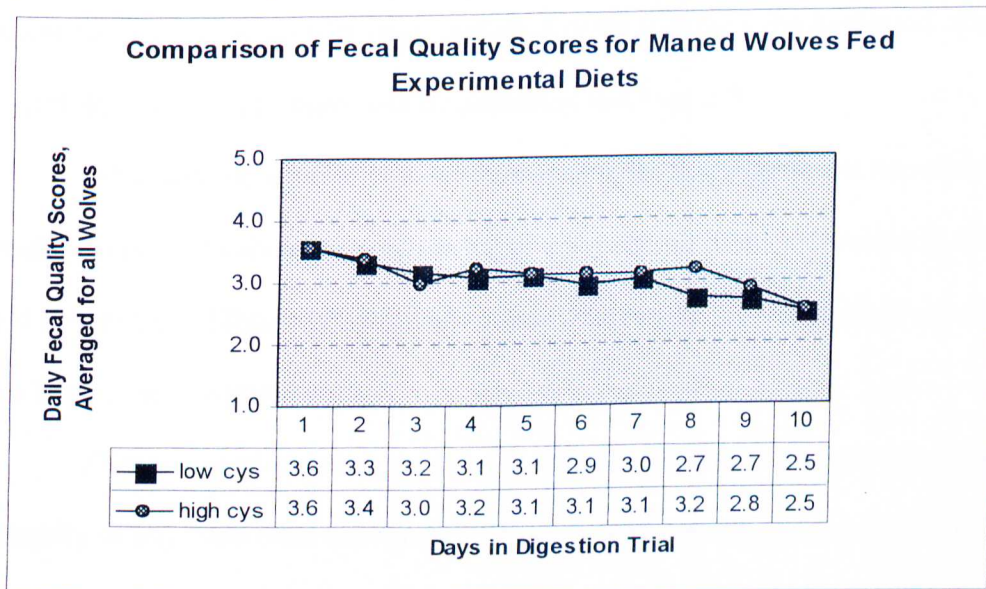
## Discussion

Development of a diet that is digestible and palatable for maned wolves, while providing essential nutrients and maintaining good health is an important challenge. The goal of this project was to accomplish these objectives in addition to attempting to lower the concentration of urinary cystine in order to reduce the likelihood of cystine urolith formation. The latter will be addressed in Chapter 3.

Although the number of subjects available for this experiment was relatively small, it represents about 17% of the captive population of maned wolves in this country and covers two different geographical areas. The results are therefore of significance to the larger, captive population.

Elimination of as many outside variables as possible is necessary to preserve the integrity of any controlled experiment. Working with an endangered species can be a challenge in this respect, as limits are placed on certain procedures. Confining the wolves indoors for a ten day period was extremely stressful for them, but necessary in order to assure that the only food ingested was the test diet. However, it would be highly unusual for any facility keeping captive maned wolves to confine them to an indoor area permanently. It therefore was relevant to test the performance of these diets on wolves who had access to the outdoors. An earlier experiment using the same CRC facility concluded that digestibility was "probably not affected by small intakes of grass by wolves" (Barboza et al 1994). The 15 wolves used in this experiment were evenly divided between those who were confined (n=7) and those who were not confined (n=8), such that one could serve as a control group for the other. In addition, the cross over

design of the experiment allows each subject to act as its own control. Statistical analysis showed that there were no significant effects (P>0.05) of the housing variable, either between wolves inside and outside, or between wolves in Virginia and Missouri. Digestibility results found in this experiment may then be expected to apply to maned wolves in other locations than the ones studied.

Daily intakes fluctuated for each individual over the ten day trials, but averaged to consistent amounts, with no significant difference between the two test diets. Apparent digestibilities of the test diets were not significantly different (P>0.05). From this, it may be concluded that these test diets were equally well accepted and digested by maned wolves. Table 2.6 compares some results from this experiment to an earlier maned wolf experiment by Barboza and co-workers, who conducted digestion trials on maned wolves at CRC using two different extruded dog foods. Mean intakes of the Mazuri maned wolf diet approximate mean intakes calculated for typical dog diets.

Table 2.6. Comparison of dry matter intakes and digestibility by maned wolves fed different experimental diets.

| | Dry Matter Intake grams/day | Dry Matter Digestibility (%) |
|---|---|---|
| Mazuri Maned Wolf Diet[1] | 429 ± 8.9 | 67 ± 0.8 |
| Laboratory Canine Diet[1,2] | 584 ± 127 | 73 ± 1.0 |
| Purina Pro Plan, Adult [2,3] | 445 | 73 ± 1.0 |

[1] Purina Mills, St. Louis, MO
[2] Barboza et al 1994
[3] Ralston Purina, St. Louis, MO

One of the issues addressed in formulating a maned wolf diet was that of poor fecal quality. Captive maned wolves have an observed tendency to pass loose, watery

stools (Allen 1997, personal observation 1997, Pojeta 1997). Aside from being a nuisance for animal caretakers, there is a concern that this characteristic may be a contributing factor to the expression of cystinuria-related complications. Excess water excreted in the feces could be partially responsible for production of a concentrated, low volume of urine which would probably provide an environment conducive to the formation of cystine uroliths. The test diets were formulated to contain high levels of fiber in order to resolve this problem. Increasing the fiber content of the diet, especially with vegetable protein such as gluten, seems to result in increased water binding capacity and a better formed feces (Zentek & Meyer 1995). As shown in Table 2.4, the level of ADF is approximately 4 times greater in the test diets compared to the pre-experimental diets. The two test diets had almost identical effects on fecal grading scores, with a general improvement of stool quality during the duration of the digestion trials. All of the maned wolves from this experiment have been maintained on the low cystine diet since the conclusion of the digestion trials, and fecal quality improvement has been sustained with approximately the same scores as those achieved at the conclusion of the digestion trials (Bishop 1998, Pojeta 1998). It may not be possible or even advisable to attempt to improve the fecal quality of maned wolves beyond what was achieved with these test diets. A certain degree of softness is apparently normal for this species, and is in keeping with the conclusion that larger breed dogs tend to produce a looser feces than smaller breeds (Zentek and Meyers 1995).

Captive maned wolves tend to be lean, which could contribute to health problems. To address this concern, the diet formulation included high levels of fat for

energy, compared to most dog foods. The nutrient concentrations presented in Table 2.4 illustrate the significantly higher amount in the test diets compared to the pre-experimental diets. Captive maned wolves are not often weighed, as this process involves the stress of capture, confinement, and sedation. Unless there is a need to sedate the animal for some other reason, body weights are generally obtained on an approximately annual basis (Pojeta 1997). It was therefore necessary to use subjective visual examination to determine whether these test diets were successful in maintaining body weight. The duration of the digestion trial periods was not long enough to provide a fair evaluation of this aspect of the test diets, but none of the wolves appeared to be either more or less thin at the conclusion of the experiment.

The general conclusion of this part of the experiment is that both of the test diets were well accepted and digested by maned wolves, and could be considered suitable for long term maintenance of captive wolves.

# CHAPTER 3.  Effect of Diet on Cystine Content of Urine

## Abstract

Captive maned wolves often exhibit characteristics associated with cystinuria, a condition causing improper reabsorption of the amino acid cystine in the kidney tubules. This results in a urine that contains excessive levels of cystine, which may then precipitate into uroliths. Uroliths pose the threat of urethral blockage, causing the animal to strain in an attempt to urinate. In some cases, uroliths may have more serious implications such as kidney damage or bladder rupture.

Research suggests that control of the cystinuric condition is possible via dietary management designed to maintain urinary pH at a level that will discourage precipitation of excess cystine as well as resulting in lower levels of urinary cystine. Cystine is insoluble in acidic urine, characteristic of an animal protein based diet. Uroliths are less likely to form in alkaline urine, and pH of urine is influenced by diet consumed. Two diets were formulated and fed to maned wolves. The diets were isocaloric and isonitrogenous, differing only in their levels of total sulfur amino acids. Urine samples were obtained from each of 11 maned wolves to represent baseline values resulting from pre-experimental diets, and after exposure to each of the two test diets. Urine samples were analyzed for cystine, creatinine, and nitrogen.

Results indicate that urinary cystine excretion was significantly lower for the low cystine diet than the high cystine diet. ($P < 0.05$). Mean concentrations of cystine in mg/L were $459.8 \pm 45.87$, $341.8 \pm 45.87$, and $475.8 \pm 47.80$ for pre-experimental, low and high cystine diets, respectively. Similarly, mean concentrations of cystine expressed as a

percentage of total nitrogen were 3.76 ± .326, 2.78 ± .326, 4.01 ± .342. Cystine in nmol/mg creatinine was 0.53 ± .180 for the low cystine diet, and .81 ± .189 for the high cystine diet. These results were not significantly different (P>0.05). Comparisons to pre-experimental diets were not expressed in nmol/mg creatinine.

## Introduction

Cystinuria is a condition whereby the sulfur amino acid cystine is not properly reabsorbed in the kidney tubules, leading to the presence of an excessive level of cystine in the urine of affected individuals. Excess urinary cystine may, under certain conditions, precipitate in the urine, giving rise to uroliths. A deciding factor in whether excess cystine will precipitate is urinary pH; cystine is insoluble in acidic urine (pH < 5.5), marginally soluble at the normal canine urine pH range of 5.5 - 7.0, and soluble at pH of 7.5 or higher (Osborne et al 1989). Osborne also reported that the solubility of cystine at a urine pH of 7.8 is double that at a urine pH of 5.0 (Osborne et al 1989). The pH of urine is largely influenced by the diet consumed (Hu et al 1993). Thus there is reason to believe that a diet selected to maintain urine pH in the alkaline range may play a role in preventing the formation of cystine uroliths.

The condition of cystinuria presents a problem in captive maned wolves, an endangered species with limited numbers in captive breeding programs. It is thought that complications arising from cystinuria may be partially controlled via dietary manipulation. Cystine is a nonessential amino acid, derived via the transsulfuration pathway as a conversion of methionine and homocysteine (Brody 1994). Levels of the essential amino acid methionine must therefore be considered when developing diets

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designed to decrease cystine levels in urine. Segal and Their studied the response of urinary excretion of cystine to dietary amino acid manipulation. They found that while feeding less methionine did not always result in a decreased concentration of urinary cystine (1983), feeding more methionine or cysteine resulted in greater urinary excretion of cystine (1989). Perhaps then, by lowering dietary levels of cystine, the concentrations of urinary cystine may be decreased, thereby reducing the risk of formation of cystine uroliths.

Cystine uroliths, like other uroliths, may range in size from small crystals to large stones. While small crystals may be passed in the urine, larger stones may cause blockages of the kidney or urethra, which could result in kidney damage or bladder rupture (Bovee et al 1981, Bovee 1986). Males are more often affected than females, probably due to their relatively longer and narrower urethras (Segal and Thier 1989, Tsan et al 1972). Casal and co-workers studied a group of Newfoundland dogs, and found that both males and females are affected, although males more often exhibit symptoms of dangerous blockages caused by cystine uroliths (Casal et al 1995). Bush and Bovee documented the case of a male maned wolf, who had died of bladder rupture as a result of urethral obstruction from cystine uroliths. Necropsy revealed a urethra that is less than one-half the diameter of that in a dog of comparable size (Bush and Bovee 1978).

Symptoms associated with cystinuria are more prevalent in males, and for this reason it was once thought to be inherited via an X-linked recessive gene (Bush and Bovee 1978, Cornelius et al 1967, Tsan et al 1972). It has recently been established that

the condition is inherited by an autosomal recessive mode, in both dogs and humans (Calonge et al 1994, Casal et al 1995).

Two test diets were formulated and fed to maned wolves in an attempt to test whether a lower level of total sulfur amino acids, cystine and methionine, in the diet fed would result in lower levels of cystine excreted in the urine. The diets were developed to provide no less than the minimum daily nutrient requirements for maintenance of adult dogs, according to National Research Council (NRC) recommendations (NRC 1974, 1985). The only difference between the two test diets was the level of total sulfur amino acids. Table 3.1 shows the sulfur amino acid analysis of both test diets, as well as pre-experimental diets. Digestibility results (Chapter 2) previously determined that each of the test diets was well accepted and utilized by maned wolves.

Table 3.1. Sulfur amino acid content of diets fed to maned wolves. % total sulfur amino acids is sum of % cystine and % methionine.

| Diet | % Cystine | % Methionine | % Total Sulfur Amino Acids |
|------|-----------|--------------|----------------------------|
| Pre-Experimental[1] | .34 | .52 | .86 |
| Low Cystine | .32 | .17 | .49 |
| High Cystine | .44 | .19 | .63 |

[1] Pre-experimental diets analyzed were two different extruded dog foods and one canned dog food. Results for the three diets were combined and their mean % of total sulfur amino acids shown here.

## Materials and Methods

Number and location of subjects. Twelve maned wolves from the Conservation Research Center, National Zoological Park, Smithsonian Institution (CRC) in Front

Royal, VA, were initially used in this experiment. One wolf was eliminated from the study due to illness. Ages and sexes of the subjects are shown in Table 3.2.

Table 3.2. Age and sex distribution of 11 maned wolf subjects fed two diets containing different levels of cystine and methionine.

| Sex | n | Body Weight (BW) Range | Average BW (Kg) | Age Range (Years) | Average Age (Years) |
|-----|---|------------------------|-----------------|-------------------|---------------------|
| ♂ | 5 | 25.7-32.6 kg | 30.2 | 2-10 | 5.6 |
| ♀ | 6 | 22.0-30.4 kg | 26.6 | 2-9 | 4.8 |

Experimental Design. Diet trials were conducted as a Latin square, cross-over design. Animals were given two weeks to adapt to the test diets before beginning a ten-day digestion trial. They were then switched to the opposite diet, given two weeks to adapt, and a second ten-day digestion trail was conducted.

Collection of Samples. One urine sample was collected from each animal prior to beginning the digestion trials, to establish baseline values for levels of cystine. One urine sample was collected from each animal again at or near the conclusion of each of the digestion trials. It is a normal reaction for maned wolves to urinate upon sighting their caretakers, or sometimes upon sighting a stranger. They also tend to urinate in response to stress, as when temporarily confined in a small space. The collection technique used for this experiment was to confine each wolf individually until it had urinated. Urine samples were then collected off the hard floor surface (either painted wood, or concrete) using a 6 ml plastic syringe and a 19 gauge hypodermic needle.

Preparation of Samples. For the initial urine collection period, the following procedure was followed: immediately after collection, urine samples were transferred

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from the syringe into clean plastic test tubes, labeled with the animal's name and date of collection. A 5% solution of Sulfosalycilic Acid (SSA) from Sigma Chemicals was added, in equal measure to the sample volume, effectively diluting it by half. Tubes were then centrifuged at -4°C for 4 minutes to precipitate any extraneous materials in the sample. Supernatant was pipetted off and stored in 2 ml Eppendorf tubes. Tubes were labeled with the animal's name and date of collection, then frozen at -20°C until analyzed.

After the initial urine collections in June, 1997, but before the urine collections made in July and August, 1997, it was discovered that the addition of the SSA solution to the urine samples was likely to interfere with the analysis for creatinine content (Brown 1997). According to a study that established reference values for amino acid concentrations in plasma and urine of dogs: "After determination of creatinine content, urine samples were diluted and deproteinized by addition of an equal volume of 3% sulfosalicylate, followed by centrifugation" (Blazer-Yost and Jezyk 1979). Therefore, the sample preparation procedure for the two subsequent urine collection periods was modified as described below.

Prior to treatment with the SSA solution, the urine sample was divided into two equal portions. One of these portions was treated with SSA as described above; the second portion was untreated. All tubes were centrifuged and supernatant pipetted as described above. Both sets of samples were then analyzed.

Analysis of Samples. Urine samples were analyzed for cystine, creatinine, and total nitrogen. It is common to report urinary cystine values in relation to urinary

creatinine values. Creatinine is a product of muscle metabolism, which enters the bloodstream and is excreted by the kidney (Bovee and Joyce 1979). The production of creatinine is constant for an individual, and is unaffected by diet consumed or physical activity of the individual (Block and Schoenheimer 1939, Bovee and Joyce 1979). Total nitrogen output in urine is also constant for an individual, and some studies that report amino acid content of dog urine have done so based on total nitrogen content of urine (Brand et al 1939, Clark and Cuddeford 1971).

Cystine was determined as cysteic acid, using ion-exchange chromatography, following the method of Schram et al (1954). According to Schram and co-workers, 90% of the amino acids cystine and cysteine are converted to cysteic acid as a result of oxidation of the sample. Incomplete recovery is due to side reactions that occur during the oxidation process. Cysteic acid remains a stable compound during the acid hydrolysis step of the sample preparation procedure, and may then be determined by chromatography. The final result does not distinguish between cystine and cysteine, and is reported as cystine (Schram et al 1954).

Creatinine determination was accomplished using the alkaline picrate method (Bonsnes and Taussky 1945). Standard solutions were made from stock solutions containing known concentrations of creatinine, to which were added equal amounts of distilled water (dH2O), .75N sodium hydroxide (NaOH) and picric acid. The standard solutions were read on a Dinatech Microplate Reader in duplicate, a regression analysis performed, and a standard curve plotted. Samples were prepared in the same manner as

standard solutions, following the same additions of dH2O, .75N NaOH and picric acid. Samples were read in duplicate.

Total nitrogen was determined using microkjeldahl analysis (AOAC 1990) and a Labconco Rapid Distillation Unit.

Statistical Analysis. Data were analyzed using the SAS statistical analysis package (SAS Version 6.12, SAS Institute, Cary, NC). The proc mixed procedure was used to perform an ANOVA and obtain least squares means, standard errors, and differences between means with related P values. Proc univariate was used to check normality of assumptions.

### Results

Subjects exhibited lower urinary cystine output in response to the low cystine diet compared to the high cystine diet. Differences in urinary cystine concentration between the low and high cystine diets were significant ($P<0.05$) in all cases except when expressed in relation to creatinine. Table 3.3 presents the concentration of urinary cystine in mg/L, as a percentage of total nitrogen, and as nmol/mg creatinine. Cystine concentration of one urine sample could not be determined, and was entered as a missing value for statistical analysis purposes, lowering n to 10 rather than 11 for this group of samples.

Table 3.3. Urinary cystine concentration in maned wolves. Values given are mean ± SEM.[1]

| Maned Wolves [2] | n | mg/L | % of Total Nitrogen | nmol/mg Creatinine |
|---|---|---|---|---|
| | | | ------------Concentration of Cystine in Urine------------ | |
| Pre-exper. | 11 | $459.8 \pm 45.87^a$ | $3.76 \pm 0.326^a$ | not determined |
| Low Cys Diet | 11 | $341.8 \pm 45.87^b$ | $2.78 \pm 0.326^b$ | $0.53 \pm 0.180^a$ |
| High Cys Diet | 10 | $475.8 \pm 47.80^a$ | $4.01 \pm 0.342^a$ | $0.71 \pm 0.189^a$ |

[1] Values not having a common letter are significantly different ($P<0.05$).
[2] Age and sex distribution detailed in Table 3.2

### Discussion

Excessive levels of urinary cystine are of concern in captive maned wolves, an endangered species. When cystine excretion is high, the possibility of formation of cystine uroliths is increased. Uroliths can vary in size, and may cause blockages of the urethra that could lead to bladder rupture, and death. The likelihood of cystine precipitating into uroliths is partly dependent on urinary pH; cystine is soluble in alkaline urine, with a pH of 7.5 or higher. Urinary pH is largely influenced by diet consumed, especially levels and amino acid composition of dietary protein sources.

The dog is often used as the closest model for the maned wolf. The scope of research performed on dogs far exceeds that performed on maned wolves; it is difficult to know what is normal or ordinary for this species. It may not necessarily be the case that numerical results between the two species will be comparable. Values obtained for maned wolf urine samples were much higher than those reported in the literature for both normal and cystinuric dogs.

Comparisons between normal and cystinuric dogs and maned wolves are also complicated by the fact that urinary cystine values vary over such a wide range (Bovee et al 1974, Clark and Cuddeford 1971, Tsan et al 1972). Dogs classified as normal may exhibit very high concentrations of urinary cystine without forming uroliths (Clark and Cuddeford 1971). Conversely, cystinuric dogs, with confirmed histories of cystine uroliths, may demonstrate very low concentrations of urinary cystine (Hoppe et al 1993, Tsan et al 1972). In most cases, cystinuric dogs had significantly elevated cystine excretion when compared to a control group (Clark and Cuddeford 1971, Hoppe et al 1993). Tsan and co-workers arbitrarily chose the urinary concentration of 75 mg cystine/g of creatinine as the dividing line between normal and cystinuric dogs, and considered dogs demonstrating concentrations greater than this to be cystinuric. "This value is approximately one standard deviation above the mean value for dogs not having cystine stones, and one standard deviation below the mean value of dogs having cystine stones" (Tsan et al 1972). Other researchers cited defined normal dogs as those with no history of cystine uroliths, and cystinuric dogs as those with past histories of cystine uroliths (Blazer-Yost and Jezyk 1979, Clark and Cuddeford 1971, Hoppe et al 1993). Table 3.4 presents literature values for cystine analysis of dog urine by various research teams.

Data on cystine levels in the urine of maned wolves is scarce. One reference on a single maned wolf reported the concentration of cystine in the urine as 5,089 nmol/ml (Bush and Bovee 1978). This value is approximately three times as high as the mean value of 1,759 nmol/ml calculated for the 11 maned wolves used in this study. One

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explanation for the large variance might be that the wolf in Bush's report was known to be suffering from blockages of cystine stones, while the wolves in this experiment all appeared to be in good health and not suffering from any blockages due to uroliths. Another explanation is that interpretation based on single urinary samples may be misleading due to the wide variation in cystine concentrations demonstrated in dogs. When working with maned wolves, it is very difficult to obtain more than a single, random urine sample, and 24 hour collections are not feasible. The mean value of 1,759 nmol/ml reported here is based on three urine samples per subject, and was determined without respect to which diet the animal was consuming at the time of collection. The calculation was made in order to compare results to another, published source on maned wolves.

Results for cystine analysis of urine samples collected while the wolves were being fed pre-experimental diets were presented for comparative purposes. An important goal of the experiment was to reduce overall urinary cystine output produced by either of the test diets in comparison to the pre-experimental diets, and ultimately to show that the low cystine diet had the greatest effect on urinary cystine concentration. Pre-experimental diets were varied in composition. Each animal was fed a different combination of food items, subject to change daily. The base diet consisted of one of two dry dog foods, differing in level of protein and manufactured by different companies. To the dry food was added any combination of the following: canned dog food (various varieties), cooked rice, boiled chicken, freshly killed mice, fresh fruits (various varieties) and a powdered dog supplement. Because these pre-experimental diets were so varied

among individuals, it was not possible to obtain exact amounts of cystine and methionine being fed. However, the base diets of dry and canned food were analyzed for amino acid content as a point of reference. These results were presented in Table 3.1.

Unfortunately, the addition of SSA to the preliminary urine samples collected caused unreliable results for creatinine analysis. There was an interference of the SSA with the creatinine content of the samples, causing creatinine results for the SSA-treated samples to be disregarded. Although these preliminary samples could not be analyzed for creatinine, they were analyzed for total nitrogen as an alternate reporting method.

The samples collected during this study were stored for several months prior to analysis. It is possible that analyzed values for cystine and total nitrogen may have been even higher. One research team observed degradation of some amino acids, notably 1/2-cystine, in urine samples left longer than one week after collection before being analyzed (Blazer-Yost and Jezyk 1979).

The overall results of this experiment demonstrate that the low cystine diet significantly lowered urinary concentrations of cystine from pre-experimental diets conditions. Two out of three reporting methods indicate that feeding the low cystine diet to captive maned wolves resulted in significantly lower urinary cystine concentrations compared to the high cystine diet in the same group of animals.

Table 3.4. Urinary cystine concentrations in normal and cystinuric dogs.

| Cystine Concentration | n[1] | Normal Dogs | n | Cystinuric Dogs | Reference |
|---|---|---|---|---|---|
| % (mg/L) | 13 | 40 ± 16.0 | 13 | 180 ± 45.0 | Clark and Cuddeford 1971[2] |
| | | | 1 | 170 | Brand et al 1939[3] |
| % total Nitrogen | 13 | 0.148 ± 0.0543 | 13 | 0.998 ± 0.3395 | Clark and Cuddeford 1972[2] |
| | | | 1 | 3.778 | Brand et al 1939[3] |
| nmol/mg Creatinine | 15 | 0.039 ± 0.0255 | 24 | 0.368 ± 0.2908 | Hoppe et al 1993[4] |
| | 28 | 0.125 ± 0.125 | 11 | 0.791 ± 0.5160 | Tsan et al 1972[5] |
| ½ cystine, nmol/mg Creatinine | 6 | 139 ± 89 | | | Blazer-Yost and Jezyk 1979[6] |

[1] n=number of dogs

[2] Various breeds and ages of dogs, all males. Normal dogs had no previous history of urinary tract disease. All cystinuric dogs had had surgical treatment for cystine urolithiasis.

[3] A single, cystinuric, male Irish Terrier. Standard error not reported.

[4] Various breeds of dogs, mean age 5 years, all male. Cystinuric dogs had verified cystine uroliths consisting of pure cystine.

[5] Various breeds and ages of dogs, sexes not given. Normal dogs had no cystine stones; cystinuric dogs had cystine stones.

[6] Various breeds of dogs, age > 5 years, sexes not given.

# CHAPTER 4. Water Balance Studies Using $D_2O$

## Abstract

Determination of the total body water content and calculation of the lean body mass or percent body fat of an individual are possible using heavy water, or deuterium oxide, either orally or intravenously, as a tracer for body fluids. Deuterium, or labelled hydrogen, in the heavy water becomes evenly distributed throughout all body fluids fairly quickly, and may then be measured in samples of blood or urine using infrared spectrometry. Concentrations of deuterium decrease over time, causing a change in its absorbance. Regression analysis of log transformed data allows for calculation of total body water content, turnover rates, half lives, lean body mass, and percent body fat.

Three maned wolves were given oral doses of deuterium oxide at ~ 3.0 ml/kg and urine samples were collected daily over the next seven day period. Samples were purified using vacuum sublimation, and infrared spectrometry was used to determine concentrations of deuterium. Two males had similar turnover rates and half lives of 2.4 and 2.9 L/d, and 4.2 and 4.6 days, respectively. The female had a lower turnover rate of 1.5 L/d, and a higher half life of 8.3 days. Body compositions of subjects were determined to be 74.1-84.9% lean; 15.1-25.9% fat.

## Introduction

Water balance studies are generally easy to perform, non-invasive, and provide information that enables the calculation of the individual's total body water content (TBW) and water turnover rate, or rate at which water is utilized by the body. The individual's lean body mass (LBM), percent body fat, and half life of water in the body

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may also be computed based on the assumption that water is associated with lean body mass, and not with fat..

Commonly used techniques of conducting water balance studies include the use of urea, antipyrine, and water labelled with tracers such as $^{18}O$, deuterium ($^2H$), or tritium ($^3H$) (DiBartola et al 1992). Of these, deuterium oxide ($D_2O$) has been termed "ideal," as it is quickly assimilated and uniformly distributed in body fluids (Faller et al 1955) and because it behaves like water for all practical purposes (Zweens et al 1980). In addition, deuterium oxide is a stable and non-toxic isotope of water (Oftedal and Iverson 1987); in fact, some $D_2O$ is inherently present in all individuals, since it is a naturally occurring compound (Baer 1996, Oftedal 1998). The method is not absolute, since the isotopes involved may exchange with hydrogen in the body tissues, producing overestimation of the water turnover rate (Oftedal and Iverson 1987, Prentice et al 1952, Streit 1982).

Deuterated water, commonly called "heavy water," may be administered either orally or via injection; the dose is calculated based on body weight of the subject. Following an equilibration period, samples, generally either blood or urine, are collected regularly over a period of time. $D_2O$ becomes uniformly distributed in all body fluids, and concentrations of deuterium oxide analyzed in urine samples are not significantly different from those analyzed in plasma samples from the same subject (Faller et al 1955). Therefore, researchers may rely on urine samples rather than blood samples for the analysis of $D_2O$ concentrations and water balance calculations. This finding is useful

in situations where it is impractical or impossible to obtain blood samples, as when studying wild animals like the maned wolf.

The decrease in the concentration of labelled hydrogen in the samples is measured over time. Studies on dogs and cats determined that $D_2O$ equilibrates throughout plasma in less than one minute, with distribution equilibration reached in 2-4 hours (Edelman 1952). Through analysis of the samples and application of mathematical formulas, calculation of TBW may be made. From this, LBM may be calculated as well.

Methods for analyzing the $D_2O$ content of samples include the falling-drop method, elevation of the freezing point, gas chromatography, mass spectrometry, and infrared spectrometry. Infrared spectrometry is preferred, since it has greater sensitivity and accuracy at low $D_2O$ concentrations. Also, preparation of samples for this method is easier than for other methods. Thornton and Condon (1950) described the use of infrared spectrometry to determine deuterium oxide concentration in water. Turner and co-workers (1960) developed a rapid determination method for deuterium in biological fluids, utilizing a double-beam infrared absorption spectrophotometer.

Infrared spectrometry measures the absorbance of the OD vibrational band, which occurs when $D_2O$ and $H_2O$ combine to yield 2HOD (Zweens et al 1980). There can be other substances in the sample that also absorb near this OD band, preventing the determination of $D_2O$ concentration. Such interfering substances can be removed either by distillation or sublimation (Zweens et al 1980).

The determination of TBW and turnover rate in maned wolves is of interest because a better understanding of how they utilize water may help to explain the occurrence of cystinuria-related problems in this species. One of the aims of both medical and dietary management of cystinuria is to increase the volume of urine, since cystine is more likely to precipitate in low volume, highly concentrated urine (Osborne et al 1989, Segal and Thier 1983). If maned wolves do not drink much water, which appears to be the case according to observations of captive wolves, it follows that their urine must be low volume. Another possible explanation for the low urine volume in maned wolves may be their tendency to produce loose, watery stools. A watery feces could be preventing a higher volume urine. Studies of water balance can help determine daily water intake and may shed light on a possible contributing factor to the occurrence of uroliths in some individuals.

## Materials and Methods

Number and Location of Subjects. Three maned wolves from the Conservation Research Center, National Zoological Park, Smithsonian Institution, (CRC) in Front Royal, VA were used in this experiment. Subjects were selected without regard to age, sex, or diet; the study was conducted opportunistically, since the administration of $D_2O$ necessitated the use of anesthetic. Animals who had to be anesthetized for annual physical examinations, or minor medical attention, were given deuterium oxide at the same time.

Method of $D_2O$ Administration. The same protocol was followed for each subject. While under anesthesia, the wolf was weighed, and body weight recorded to the

nearest tenth of a kilogram. A stomach tube was inserted. Heavy water containing 99.9% $D_2O$, lot # CAS [7789-20-0] from Cambridge Isotope Laboratories, Andover, MA was used. Syringes of $D_2O$, pre-weighed to contain a dose of approximately 3.0 ml/kg of body weight, were attached to the stomach tube via a three-way stopcock. With the stopcock open, the $D_2O$ was delivered in a steady stream into the wolf's stomach. The syringe was then rinsed with plain distilled water, to ensure that all of the $D_2O$ was delivered into the stomach. The exact time of administration was recorded.

Complications arose during the procedure for the female subject. A pre-weighed syringe containing $D_2O$ was dropped on the floor prior to being given to the subject. A substitute syringe was filled and administered in place of the pre-weighed one. The weight of the substitute syringe could not be determined prior to administering it to the subject. Its weight was later estimated as closely as possible, based on the volume of $D_2O$ in the syringe.

Collection of Samples.  Urine samples were collected as often as possible, but no less than once daily for a period of seven days following the administration of heavy water. The method of collection was as described in Chapter 3. Once collected, urine was transferred into clean 5 ml plastic tubes, labeled with animal's name, date, and time of day when voided. Samples were frozen at $-20°C$ until the time of analysis.

Purification of Samples. Samples were sublimated, using the method of Byers (1979). Frozen urine samples were placed under vacuum, where they boil at a low temperature ($-20°C$) Water boiled off the sample was collected in tubes and subsequently analyzed by infrared spectrometry.

<u>Preparation of Standards</u>. Standard calibration solutions were prepared by weighing known amounts of $D_2O$ into clean nalgene bottles, then diluting the $D_2O$ with distilled $H_2O$ ($dH_2O$) to a pre-determined volume, verified by weighing. Mass fractions of $D_2O$ were converted into volume fractions by utilizing the densities of $D_2O$ and $dH_2O$. These standard solutions were measured on the infrared spectrometer, and a regression analysis performed to generate a standard curve.

<u>Measurement of Samples</u>. A Perkin-Elmer 1420 ratio recording infrared spectrophotometer was used. The cell in the reference beam contained pure distilled water. With distilled water in the sample cell, the spectrophotometer was set at 90% transmittance at the wavelength $\lambda^{-1} = 2510$. Prior to introducing each new sample, the sample cell was rinsed with distilled water, then vacuum dried. Samples were read in duplicate.

<u>Calculation of Total Body Water</u>. $D_2O$ concentrations in each sample were converted to natural logarithms and their regression analysis plotted. The scale for time was expressed as fractions of days, calculated as total hours elapsed since the administration of $D_2O$ (time zero). Concentration of $D_2O$ at time zero was estimated via extrapolation. The total amount of body water for each animal was then calculated using the following formula:

$$k = Q_{D2O} / P_{H2O}$$

where k is the equilibrium concentration, or estimated concentration of $D_2O$ already present in the body at the time of dosing, Q = the total grams of $D_2O$ administered, and

P = the pool of total body water (Baer 1996 and 1998, Kleiber 1961, Oftedal and Iverson 1987).

Calculation of Lean Body Mass and Percent Body Fat. TBW represents the entire volume of water in the body, in liters, and may be expressed as a percentage of the body weight. Estimation of the percentage of water in the body is known to include an overestimation error of approximately a 3-5%, due to the exchange of deuterium with hydrogen in other compounds besides water (Baer 1996, Kleiber 1961, Oftedal and Iverson 1987), and has therefore been corrected using 4% (Oftedal 1998). By convention, and based on previous work done in this lab, LBM is calculated as 73.2% of body water (Baer 1996, DiBartola 1992, Oftedal 1998). Since LBM + body fat = TBW, it follows that percentage of body fat may be calculated as the difference between LBM and 100 (Kleiber 1961).

Calculation of Turnover Rate and Half Life. The daily turnover rate of water as a percentage was calculated from the regression output generated for each subject. Turnover rates were expressed as litres per day and ml per kg of BW for each subject, using mathematical formulas. The half life of water in the body was calculated as the natural logarithm of 2 divided by the turnover rate (Oftedal 1998).

Results

Calculated TBW, LBM, % fat, turnover rates and half lives for the three subjects are presented in Table 4.1. Plots of the regression lines of $D_2O$ concentrations against time for each subject are shown in Figure 4.1.

Table 4.1. Results of water turnover study in three maned wolves given deuterium oxide ($D_2O$) orally at a dose of ~3.0 ml/kg. BW=Body weight; TBW=Total Body Water; LBM=Lean Body Mass.

| Name | Sex | BW (kg) | TBW (L) | LBM (%) | Fat (%) | Turnover Rate (L/d) | Turnover Rate (ml/kg) | Half Life (Days) |
|------|-----|---------|---------|---------|---------|---------------------|------------------------|-------------------|
| Zorro | ♂ | 30.5 | 17.8 | 74.1 | 25.9 | 2.9 | 95.6 | 4.2 |
| Shera | ♀ | 28.6 | 19.7 | 89.0 | 11.0 | 1.6 | 57.6 | 8.3 |
| Hayden | ♂ | 24.1 | 15.9 | 84.9 | 15.1 | 2.4 | 99.7 | 4.6 |

Figure 4.1. Regression analysis of $D_2O$ concentrations over time in urine of three maned wolves.



Zorro: y= -.16x - .05

Shera: y= -.08x - .78

Hayden: y= -.15x - .47

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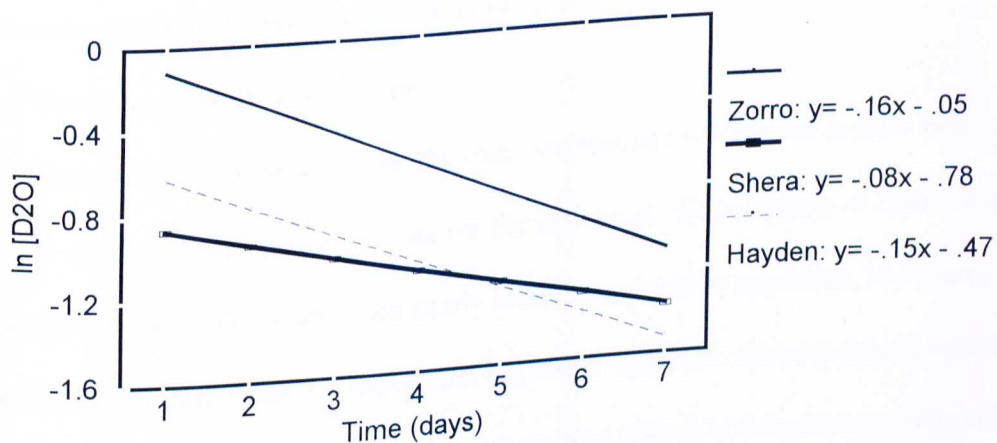
## Discussion

Captive maned wolves are thought to drink very little water, yet they have also been observed to produce loose, watery stools on a normal, daily basis (personal observation 1997, Pojeta 1997). These observations may explain why maned wolf urine is more highly concentrated, which may contribute to the formation of cystine crystals.

During a previous, unpublished water balance study also using maned wolves at the CRC, researchers encountered the presence of an unknown compound in the urine, which interfered with the $D_2O$ absorbance reading and determination by infrared spectrometry (Allen 1997). Their samples had been prepared via heat distillation. To avoid these difficulties, an alternative method of sample purification was used. Vacuum-sublimation satisfactorily removed the interfering compound, allowing complete recovery of $D_2O$ from maned wolf urine samples.

Estimations must be made for the concentration of $D_2O$ present at time zero, and are based on the calcuated turnover rate for the individual. Examination of Figure 4.1 shows that the two male subjects had nearly identical regression equations, indicating similar turnover rates. Table 4.1 shows that the males' turnover rate was approximately double that of the female. There is not enough data to draw a conclusion regarding this difference as a sex-related one; however, in many mammalian species, including the maned wolf, the larger sex is often the male, and it is the male who often has a higher food intake. Comparing the two males in this experiment is difficult since Hayden was not a part of the digestion trials due to illness, so there is no intake data available for him.

While turnover rate is not synonymous with water intake, it is largely dependent on diet; the amount of water consumed increases as the amount of water in the diet decreases (DiBartola 1992). Intake data for diets show that Zorro was eating approximately twice as much as Shera during the time of the $D_2O$ trials (Chapter 2). It is therefore logical to expect that he would be drinking more water, especially since the diet being fed contained < 10% water (Chapter 2).

A comparison of maned wolf data with research conducted on dogs is presented in Table 4.2. Where turnover rates are provided as L/d, literature values for dogs are comparable to maned wolf values determined in this study. However, where expressed as ml/kg, turnover rates appear to be very different, with dogs having rates 5-6 times greater than wolves.

Whether water intakes in captive wolves approximate those of their wild counterparts is questionable, since many factors are involved. Wild wolves are likely more active, if only in their search for food. Additionally, their diet has a higher water content since it consists of fresh food items rather than dried pre-formulated chows. Despite the small sample size (n=3) and the procedural complications encountered, the knowledge gained from this experiment may provide baseline data for maned wolf water balance.

Table 4.2. Comparison of water turnover rates and half lives in maned wolves and dogs.[1]

| | Body Weight (kg) | Turnover rate (L/d) | Turnover rate (ml/kg) | Half life (d) | Researcher |
|---|---|---|---|---|---|
| Dogs | 10.6 | 0.9 ± .12 | | 5.6 | Richmond et al 1962 |
| Dogs, corrected [2] | 26.5 | 2.3 | | | Richmond et al 1962 |
| Canine, adult | | | 589 | | Edelman 1952 |
| Male mongrels | | | 626 | | Zweens et al 1980 |
| Beagles | | 2.1 | 631 | | Sheng and Huggins 1986 |
| Maned Wolves: | | | | | |
| Zorro (♂) | 30.5 | 2.9 | 96 | 4.2 | |
| Shera (♀) | 28.6 | 1.5 | 58 | 8.3 | |
| Hayden (♂) | 24.1 | 2.4 | 100 | 4.6 | |
| Means | 27.7 | 2.3 | 84 | 5.7 | |

[1] Missing values were not reported.

[2] Data from Richmond et al x 2.5, to facilitate comparison.

# CHAPTER 5. Conclusion

Given that both captive and wild populations of maned wolves have been tested and found to excrete high levels of cystine in their urine, it is common to diagnose the condition of cystinuria. From this follows the belief that virtually all the individuals of this endangered species suffer from this inherited condition, and are predisposed to the formation of cystine uroliths. It seems prudent then to search for methods to help control the incidence of complications that may arise in the captive population. The least invasive, and probably the least harmful method over the long term, is dietary modification. It seems logical that if we were to provide captive maned wolves a diet similar to that of their wild counterparts, there would be fewer incidences of cystinuria-related health problems.

Observation of wild maned wolves in Brazil has revealed that their native diet is an omnivorous one, with a main staple being the fruit called *Solanum lycocarpum*, nicknamed "fruit of the maned wolf" (Dietz 1984). In 1989, specimens of this fruit were imported from Brazil and analyzed by the Animal Science Comparative Nutrition Laboratory of Michigan State University, East Lansing, MI. The analysis shows that this item is a low protein, low fat, high fiber food. Since this fruit is so important to wild maned wolves, it may be useful to consider its composition when formulating a diet for captive maned wolves.

Accordingly, the test diets used in this experiment were formulated to contain high levels of fiber, which are in parallel to the levels analyzed in the sample fruits. Mineral analysis of the test diets was not independently confirmed, but the formula

accompanying the diet was available. Acknowledging that the fruit is only one component, albeit a very important one, of the wild maned wolf's diet, while the Mazuri diet is a complete and balanced diet specially formulated for captive maned wolves, a comparison of the two is revealing. Table 5.1 lists comparative compositions of the two foods, for those components or ingredients for which information is available.

Table 5.1. Comparison of analyzed compositions of an important fresh diet staple of the wild maned wolf, *Solanum lycocarpum*, and a customized dry diet for the captive maned wolf, Mazuri brand. Amounts given on a dry matter basis, in percentages except where noted as ppm.

| Diet Component | *Solanum lycocarpum* | Mazuri Maned Wolf Diet |
|---|---|---|
| Dry Matter | 20.6 | 96.5 |
| Protein | 6.2 | 21.0 |
| Fat | 1.6 | 13.7 |
| NDF | 14.2 | 20.6 |
| ADF | 14.2 | 13.8 |
| GE, kcal/g | 4.2 | 5.1 |
| Potassium | 2.1 | 0.60 |
| Phosphorous | .13 | 0.80 |
| Selenium, ppm | .02 | 0.24 |
| Copper, ppm | 6.3 | 14.0 |
| Iron, ppm | 20.0 | 340.0 |
| Zinc, ppm | 8.9 | 190.0 |
| Manganese, ppm | 3.1 | 60.0 |
| Calcium | 0.05 | 1.40 |
| Magnesium | 0.03 | 0.13 |
| Sodium | 0.002 | 0.55 |

*Solanum* appears to have quite a low sodium content. Research on human cystinuria patients has showed that lower sodium diets have an anticystinuric effect (Jaeger et al 1986). It is speculated that perhaps long-term sodium depletion may stimulate thirst, due in part to a resultant loss of urinary concentration (Jaeger et al 1986). Whether the wild maned wolf's diet is sodium-deplete is unknown, as is its normal water intake. Water balance studies (Chapter 4) indicate the captive wolf's water intake may be higher than was originally thought. Nevertheless, lowering the sodium content of the Mazuri diet might be a consideration for future formula revisions.

Considering how important the role of the domestic dog has been in studying the condition of cystinuria in maned wolves, it seems relevant to point out that the Mazuri maned wolf diet is also being tested for use in cystinuric dogs. Full details and results of this study are not yet available. However, results for a single, confirmed cystinuric Newfoundland are as follows: baseline urinary cystine analysis 1,283.0 nmol/ml; 14 days post diet 310.0 nmol/ml; 28 days post diet 302.5 nmol/ml (Giger 1998). Composition of the pre-experimental diet of this dog is unknown, as is what supplementation was used, if any, in conjunction with the test diet, which was the low cystine diet. The maned wolf diet would probably not be suitable for long term maintenance of dogs, given its high fat and fiber content, although the fiber may be of benefit to larger breed dogs prone to softer feces. Even so, it is interesting to note the decrease in cystine concentration obtained in this dog, which appears to be significant. This suggests the maned wolf diet may have farther reaching impact than just for the maned wolf.

Published studies on the maned wolf are relatively few, especially those concerned with nutrition of the captive population. It is therefore interesting to compare the results obtained in this digestibility study to those obtained by Barboza and co-workers in 1994, especially since that team conducted their research at the same facility, probably using some of the same individual animals. Their study was "initiated to assess methods of dietary management for improving oral health and reducing excess dietary protein" with the assumption that "diets high in prey and meat items may provide sulfur amino acids in excess of their requirements" (Barboza et al 1994). Their study compared pre-experimental diets to two different extruded dog chows in terms of intakes and digestibility. Pre-experimental diets consisted of a mix of fresh items including meat, grains, and fruits. The protein source of one dog chow was soy and cereal based, while the other chow was poultry by-products and cereal based. Chows were not fed exclusive of other diet items, but replaced some of the meat and grains in the pre-experimental diets. Table 5.2 compares diet analyses for the chows of the Barboza experiment, and the Mazuri maned wolf diets presented in Chapter 2.

Table 5.2. Comparison of diet compositions from two digestibility studies with captive maned wolves.[1]

|  | Soy Dog Chow (n=2) | Poultry Dog Chow (n=1) | Mazuri Maned Wolf Diet (n=15) |
|---|---|---|---|
| Daily Intake (g) | 584 ± 127 | 445 | 429 ± 9 |
| Protein (%) | 28.9 | 29.8 | 21.0 |
| Fat (%) | 10.1 | 21.2 | 13.7 |
| Fiber (%) | 3.7 | 3.3 | 8.0 |

[1] On a dry matter basis

The Barboza study stated that the diets used were calculated to contain 0.9% total sulfur amino acids, and acknowledged that the level may be excessive for adult maned wolves. They concluded that "further research should assess the levels of sulfur amino acids appropriate for long-term feeding of maned wolves predisposed to cystinuria" (Barboza et al 1994).

Are all maned wolves predisposed to cystinuria? An interesting occurrence happened during one of the digestion trials. One male maned wolf passed multiple stones with no discernable urine, on a single occasion. The stones were approximately 1 mm in diameter and their smooth, round shape and tan color appeared to fit the standard description of cystine stones (Bush and Bovee 1978). The stones were not analyzed. The maned wolf in question had a prior history of cystine stone formation, but not blockages (Pojeta 1997). He did not appear to suffer any ill effects following the discovery of the stones. He was not dropped from the study, and continued to eat, drink, and urinate normally without further occurrences or any signs of discomfort. This incident would have gone unnoticed except for the fact that this individual had been temporarily confined in an attempt to collect a urine sample. The stones were discovered when they appeared instead of urine. They evidently did not cause blockages, and have not recurred since (Pojeta 1998). It seems possible that such incidents may occur but go unnoticed, suggesting that some maned wolves may regularly pass cystine stones as a normal occurrence.

It is possible that the mere presence of high levels of cystine in the urine of maned wolves is not indicative of a cystinuria-related health risk, but is normal for this

species. Indications are that the normal status of this species includes levels of cystine that would be considered very high for dogs of similar size. When Bovee and co-workers used paper chromatography as a detection method, they found that 34 out of 42 maned wolves had high levels of cystine in their urine, and concluded that these individuals were cystinuric. Exact levels of cystine were not reported. 34 of the captive maned wolves tested were captive, from zoos in six countries; 8 were live trapped in Brazil. Equal numbers of males and females were affected (Bovee et al 1981).

It is unknown why such a large percentage of the maned wolf population, both wild and captive, is apparently affected with cystinuria. How is it that a wild species has survived and prospered without human interference for so many years if virtually every individual is born with an inherited condition that would likely limit its chances to live out a normal life span, including reproduction? It is possible that by using established urinary cystine concentrations measured in domestic dogs as a yardstick for maned wolves, we are applying unnatural standards to this wild species. There must therefore be some other explanation for what we quantify as "high" levels of urinary cystine excretion found in most maned wolves tested.

One such explanation is that a high level of cystine in the urine results in a strong odor that may play a role in scent-marking. The phenomenon of scent-marking, specifically urine marking, is widespread and has been studied in many mammalian species, including beaver, puma, reindeer, and elk as well as canids (Gosling 1982).

Although it has no disorder in amino acid excretion, the genet's (*Genetta genetta*) urine contains high concentrations of cystine. Similar concentrations in

cystinuric humans cause calculi formation, yet there is no evidence of calculi formation in the genet (Crawhall and Segal 1965). A link between urinary sulphocysteine excretion and diet was observed when the genet was fed raw horse meat and urinary concentration of cystine increased. During this study, the concentration of cystine in the genet's urine was high but did not exceed its solubility; therefore no calculi were formed (Crawhall and Segal 1965).

Another species, the bobcat (*Lynx rufus*) exhibits sulfur-containing compounds in its urine and yet has no history of cystinuria. The bobcat has been studied as a mammalian predator, whose strong smelling urine serves to repel its prey, namely hare, deer, and woodchucks (Mattina et al 1991).

A third species that excretes sulfur-containing compounds in its urine is the red fox (*Vulpes vulpes*). Although the role of these compounds as pheremones is unknown, it has been observed that the odor of red fox urine in both males and females becomes intensified with the onset of breeding season, stays strong for the duration of the season (approximately three weeks) and then gradually diminishes during the following two months (Henry 1978). One hypothesis is that these strong, persistent olfactory markers in the urine may signal an increase of steroid hormone synthesis in preparation for the mating season (Jorgenson et al 1978). It has also been shown that these compounds, when synthetically reproduced and used to mimic the studied marking patterns of wild foxes, served to induced scent-marking behavior (Whitten et al 1980). The same and similar compounds have been found in wolves (*Canis lupus*) where they are mostly associated with intact males and peak during breeding season (Raymer et al 1984). These

sulfur-containing compounds have a close chemical relationship to the scent-marking

substance secreted by the anal gland of the mink (*Mustela vison*), the polecat (*Mustela*

*putorius*) and the striped skunk (*Mephitis mephitis*)  (Jorgenson et al 1978).

The primary functions of scent-marking in canids are to express dominance and

indicate possession of territory.  Communicating territorial boundaries in wild canids

reduces competition for natural resources and ensures lone males remain isolated from

areas of established pairs (Gosling 1982). Urine marking has also been connected to

establishing pair-bonding in both wolves and jackals, and to communicating reproductive

status in dogs  (Anisko 1976).

Studies of both wild and captive wolves (*Canis lupus*) have revealed that urine

marking behavior is conducted by both males and females, and is predominantly

performed by dominant or alpha individuals urinating with a raised leg  (Peters and Mech

1975). Female maned wolves have been reported to urinate in the raised leg pattern

typically associated with males (Anisko 1976). Wild wolves tend to increase the number

of urine marks used during the breeding season, (Peters and Mech 1975) supporting the

theory that urine marking plays a role in reproduction.

There is no published literature available on scent-marking  or urine marking in

the maned wolf.  However, this seems like an area worthy of further investigation, as it

may help to explain the presence of excess cystine in the urine of maned wolves

unaffected by cystine urolith formation and other health problems associated with

cystinuria.  Territorial marking seems the most likely use for urine marking by maned

wolves.   The fact that wild maned wolves inherently excrete high levels of cystine in

their urine, yet remain largely unaffected by health concerns related to cystinuria, suggests we should look further for possible explanations such as scent-marking implications.

Water balance studies have not been published for the maned wolf, and results obtained in this experiment are probably not reliable for extrapolation to the species as a whole, since only three animals were used and two of the three included a number of errors that likely affected the final outcome (Chapter 4).

Water turnover rates are known to vary with activity level. In a water turnover study using alaskan sled dogs, Hinchcliff and co-workers demonstrated a five-fold difference between sedentary and exercising dogs. Both groups were described as physically fit, but when sedentary the mean water turnover rate was $0.91 \pm 0.1$ L/d, compared to mean rate of $5.03 \pm 0.59$ L/d for the exertion group (Hinchcliff et al 1996). The maned wolves studied had varied levels of activity; those confined indoors were obviously more sedentary while those who lived outdoors were more active. It seems reasonable to expect the differences in activity levels to average out, since approximately half the animals were indoors while half were outdoors.

Water intakes probably also vary with composition of the diet; a drier diet results in higher intake of water, while a diet containing more fresh foods decreases the need for water consumption. Pre-experimental diets of the maned wolves in this study were based on dry diets, but also contained several different fresh ingredients (Chapter 3). This may partially explain the observation of relatively little water consumption prior to changing the wolves' diet to the experimental, dry diets with no fresh items included.

Throughout the discussion of cystinuria, its causes and treatments, the pH of the urine has been a central focus. Urine alkalinization is an objective of both drug and dietary treatments of the cystinuric condition, since cystine uroliths are less likely to present a problem in an environment conducive to their dissolution. Yet the impact of the test diets on urinary pH has not been directly addressed thus far. Accurate measurement of urinary pH is crucial, yet difficult to obtain. Attempts to record pH values were made during the first round of urine collections, using pH paper strips in the range of 5-7. It was discovered that obtaining an accurate reading was impossible. The method of collection that must be used for these animals mandates that voided urine is exposed to the influence of the temperature differential of the floor as well as the lime and calcium contents of the cement composition of the floor. There is also a lapse of time involved, even if the caretaker is present while the animal is urinating, because the animal must be isolated elsewhere before the caretaker can enter the enclosure to collect urine. Additionally, reading a value off paper pH strips is highly subjective, and may give inconsistent results. Any or all of these things could impact the determination of pH, making it nearly impossible to evaluate any effect of diet on this variable. This is an area worthy of future investigation, for a study with an experimental design focused specifically on the pH effect of diet, and equipped to determine this measurement precisely.

Finally, the importance of this project to maned wolves lies in furthering collective knowledge of this species, its nutritional requirements, and how they impact the incidence of cystinuria-related complications. "Knowledge of this condition in

maned wolves now demands breeding strategies to perpetuate the species" (Montali and Bush 1982). Individuals found to be affected with cystinuria may be carriers of the mutation for cystinuria, and should not be used in breeding programs. The question has been raised here of whether the presence of high levels of cystine in the urine is adequate to diagnose an individual of this species as cystinuric. Current research is developing practical methods to test dogs for genetic proof of the condition (Casal et al 1995). If successful, this non-invasive, simple testing method could prove useful for captive maned wolf breeding programs. In the meantime, it is encouraging to know that dietary management may be successful as a prophylactic measure to prevent the formation of cystine uroliths in maned wolves prone to high levels of urinary cystine.

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