The effect of inorganic dietary phosphorus on the digestibility of the diet and renal health of the domestic feline (*Felis catus*)

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Abstract

With the increasing prevalence of feline chronic kidney disease (CKD), there is a growing focus on dietary intervention to reduce the incidence and slow the progression of this disease in domestic cats. Studies investigating the effects of feeding high phosphate levels have been of particular interest, as this has been closely associated with reduced renal function in healthy cats and dogs. Phosphate additive supplementation is common practice in pet foods to improve texture or palatability, and the nutritional guidelines for cats only regulate a minimum P requirement. Given the adverse effects of excessively high dietary P on the renal function of various species (e.g., cats, dogs, and humans), there is clear need for a determined maximum or "safe upper limit" of P intake to be implemented for feline health. There is also a need to determine how different P sources (inorganic vs organic) affect animal health, as high concentrations of the more bioavailable inorganic P (Pi) have been shown to be particularly problematic for renal health.

This study aimed to investigate the effects of feeding diets with differing P sources on indicators of feline renal health. Eight healthy young and desexed domestic shorthair cats (four males and four females) from the Centre of Feline Nutrition (Massey University, Palmerston North, New Zealand) were used for this study. The cats were aged 1-4 years (mean ± SEM: 3.05 ± 0.10 years) and weighed 2.4-5.6 kg (mean ± SEM: 4.26 ± 0.24 kg). All cats were sequentially fed three dietary treatments: control diet (CON; diet containing 3.75 g organic P/kg), test diet 1 (T1; diet containing 3.75 g organic P/kg and 2.39 g Pi/kg), and test diet 2 (T2; diet containing 3.75 g organic P/kg and 3.25 g Pi/kg), all in the form of canned moist food. Each diet was considered complete and balanced according to the AAFCO guidelines and was formulated to have different Ca:P ratios (CON: 1.6:1; T1: 0.9:1; T2: 0.6:1) in order to determine whether the Ca:P ratio affected P digestibility. Each diet was fed for 26 days, beginning with a 7-day adaption phase, and ending with a 6-day apparent nutrient digestibility assessment. The cats had *ad libitum* access to the food and water, which were both replaced daily, throughout the study.

The total P and Pi/Mcal differed in each test diet (Total P: T1: 6.19 g/kg DM, T2: 7.65 g/kg DM; Pi/Mcal: T1: 0.53 g/mcal, T2: 0.68 g/mcal). Blood sampling took place on day 26 of each feeding block, and included a preprandial/fasted sample (12 h minimum) and two postprandial samples (3 h and 5 h after food intake). Quantitative collection of faeces and urine took place during the six-day apparent nutrient digestibility period at the end of each diet block.

The effects of dietary Pi supply on calcium (Ca) and P balance were assessed through blood, faecal and urine analyses, and changes in renal markers (i.e., creatinine, symmetric dimethylarginine, fibroblast growth factor-23 (FGF23), parathyroid hormone (PTH) and specific gravity) were investigated to determine the implications of feeding Pi on the feline kidney.

Based on blood analyses and frequent weighing and monitoring, all eight cats remained clinically healthy for the duration of the study. The average body weights of the cats decreased throughout both the CON and T1 blocks, but increased gradually during the feeding of T2. These body weight changes were likely at least partly due to normal seasonal body weight changes. There was a significant increase in P intake in the T1 and T2 trial diets compared to the CON diet. The higher level of NaH_2PO_4 and Pi/Mcal in diet T2 led to the greatest P intake (mg/kg BW) and apparently digested P (mg/kg BW). The CON trial provided the baseline assessment of renal function. The addition of Pi in both trial diets (T1 and T2) caused an unexpected drop in serum concentrations of P and corresponding levels of FGF23. Despite the low serum P concentrations observed, there was a significant increase in both PTH concentrations and renal P excretion. In addition to this, urine analyses indicated that specific gravity and pH levels were lowest during the T2 trial. This study indicated that when Pi was added to the feline diet in moderate amounts, there were significant changes in phosphate metabolism. Additionally, the diet containing a Ca:P ratio lower than the reference range and a higher level of NaH_2PO_4 and Pi/Mcal (T2) induced a greater renal response, especially the renal excretion of P. This suggests that the potential for renal damage may be increased if the dietary Pi had been added in greater quantities.

The evident effects of including moderate levels of highly soluble Pi and a low Ca:P ratio in the feline diet emphasises the need for further investigations into feeding these Pi sources for longer periods and potentially assessing further parameters of renal health.

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List of abbreviations

AAFCO – Association of American Feed Control Officials

ACVM - Agricultural Compounds and Veterinary Medicines

Ca – Calcium

FEDIAF – European Pet Food Industry Federation

FGF23 – fibroblast growth factor-23

GI – Gastrointestinal

GFR – Glomerular filtration rate

H_3PO_4 – phosphoric acid

Mcal - megacalories

NaH_2PO_4 – sodium dihydrogen phosphate

NRC – National Research Council

P – phosphorus

Pi – Inorganic phosphate

PTH – Parathyroid hormone

SDMA – Symmetric dimethylarginine

SG- Specific Gravity

T1 – test diet 1

T2 – test diet 2

Chapter 1

General introduction

Chapter 1 - General Introduction

The domestic cat (*Felis catus*) belongs to the order carnivora and currently represents one of seven families making up the feliformia suborder (Johnson et al., 2006). Domestic cats are obligate carnivores and have evolved consuming a diet of prey that was high in protein, had a low to moderate fat content, and contained a minimal amount of carbohydrate that was consumed in small quantities many times per day (Zoran, 2002). A diet consisting of animal tissue provides an organic source of minerals such as calcium (Ca) and phosphorus (P). The cat was domesticated approximately 8,000 years ago, making them one of the more recently domesticated species of mammals (Hart, 1985). They are currently the most common companion animals in New Zealand, with 41% of households having at least one cat (Companion Animals NZ, 2020). With a sizeable domestic population of cats, it is not surprising the rate at which the international pet food industry is growing and becoming increasingly competitive. The dietary requirements of the domestic cat are highly specific and the types of food made available to them has changed considerably throughout their domestication (Bradshaw, 2006). It is important that diets manufactured by the pet food industry meet the requirements of domestic cats.

There are many nutrient requirements specific to the domestic cat including protein and amino acids, essential fatty acids and micromineral (e.g., vitamin A, D and E) requirements. These requirements are dependent on life stage and the associated energy needs of the cat. Nutritional recommendations have been set by the National Research Council (NRC) (2006) to ensure pets obtain sufficient nutrient intake to meet their requirements. Over the past few decades, investigations into the mineral requirements of both cats and dogs have become of great interest for researchers, particularly that of Ca and P dietary intake. These are the most abundant macrominerals in the body and are two of the most important minerals when it comes to bone and metabolic health of pets (Case et al., 2010). Dietary P is either present as naturally occurring ‘organic’ P or derived from phosphoric acid and its salts, i.e., inorganic P (Pi), which are commonly used as feed and food additives. Inorganic sources of P tend to differ in solubility and are much more soluble compared to organic sources, making them more available for absorption in the small intestines. Inorganic P salts (e.g., calcium phosphate or sodium phosphate) are routinely applied to improve texture, bind water, enhance flavour and act as acidifiers in commercial pet foods and foods produced for human consumption (Kalantar-Zadeh et al., 2010, Noori et al., 2010). These Pi sources have to be legally approved and their

use in pet foods are subject to regulation under the Agricultural Compounds and Veterinary Medicines (ACVM) Act 1997. These additives have been found in levels exceeding the daily allowance of P recommended for cats by the NRC (2006) (Gagné et al., 2013; Summers et al., 2020). Over the past few decades, many studies have shown that inorganic forms of P are more bioavailable than organic forms, and tend to be associated with adverse health effects when fed in high levels (Pastoor et al.., 1995; Dobenecker et al. 2018a, b; Coltherd et al., 2019; Dobenecker et al., 2021b).

Currently, nutritional guidelines provide a minimum requirement for inclusion of P in domestic canine and feline diets to ensure maintenance requirements are met. There are currently no maximum levels or 'safe upper limits' for dietary Ca and P in guidelines for domestic cats provided by either the AAFCO (2023), NRC (2006), or the European Pet Food Industry Federation (FEDIAF, 2021). The AAFCO (2023) guidelines for dogs indicate that levels exceeding 2.5% dry matter (DM) and 1.6% DM of Ca and P, respectively, have been found to have detrimental effects on canine health. Laflamme and Jowsey (1972), Jowsey et al., (1974), Schneider et al., (1980) and Dobenecker et al., (2021a, b) were some of many studies that reported high levels of dietary P intake to be detrimental to canine renal health. Unfortunately, the current evidence in domestic cats (Böswald et al., 2018; Alexander et al., 2019; Steffan and Dobenecker, 2023) is insufficient to prove that this is also applicable for feline health. With no regulations around the maximum inclusion of dietary P, commercial cat food is being formulated currently without considering this potential renal health risk.

It is increasingly clear that feline renal health continues to deteriorate (Dobenecker et al., 2021b), with chronic kidney disease (CKD) now being the leading cause of mortality in cats (Chen et al., 2020). Over the past few decades, research has shown that there are nutritional factors contributing to this decline in renal health, with recent studies indicating that high levels of dietary P intake may be a leading contributor towards the progression of feline CKD (Kalantar-zadeh et al., 2010; Geddes et al., 2013; Dobenecker et al., 2018a). More specifically, there is a clear association between increased intake of inorganic P and the progression of CKD in humans, cats and dogs (Nouri et al., 2010; Böswald et al., 2018; Chen et al., 2020), demonstrating the potential effect Pi may have on renal function. This potential risk for feline renal health associated with the feeding of high levels of Pi additives in commercial diets may indicate potential causation, especially with the prevalence of feline CKD increasing. Studies trialling dietary restriction of Pi in cats at differing stages of CKD suggest that dietary

intervention may prove to be a promising method to slow the progression of the disease (Kidder and Chew, 2009; Geddes et al., 2013b). This only reinforces the need to investigate the restriction of dietary inclusion of Pi in the effort to reduce the incidence of CKD in domestic cats. The effects of Pi in the feline diet requires further investigation before dietary limits can be implemented in nutritional guidelines and elicit change in the formulation of commercial cat food.

Chapter 2

Review of Literature and Overview of Thesis

Chapter 2 – Literature Review

2.1 Introduction

2.1.2 Evolution of the felid (domestication)

Felis catus, commonly referred to as the domestic cat, is believed to be derived from the north African wildcat *F. silvestrus lybica* (Bradshaw, 2006). This carnivorous species is known to be a more specialised predator than the wolf (*Canis lupus*) and has been domesticated for a much shorter period of time (Bradshaw, 2006). Domestication of the dog is said to have begun approximately 14,000 years ago according to archaeological evidence. It was during this time that the dog first became distinguishable in appearance from wolves (Bradshaw, 2006). In contrast, according to Macdonald et al.., (1984), the domestication of the cat began approximately 8,000 years ago. With their primal hunting instincts, the wild cat fed solely on prey (e.g., rodents, birds, fish, insects etc.) prior to domestication, whereas the ancestors of dogs had a more diverse diet of mammals, fish, birds, amphibians and vegetable matter (Legrand-defretin, 1994). The dog is more of a scavenging or facultative carnivore and can sustain a diet consisting of both animal and plant matter to meet their nutrient requirements, whereas the cat is an obligate carnivore, and will preferentially consume a high-protein diet, consisting primarily of animal tissue and low levels of carbohydrates (Salaun et al., 2017).

Today, most domestic cats obtain a balanced diet from human provisioning as well as the occasional hunted meal if they have outdoor access. The evolution of the cat has contributed significantly to the types and amounts of nutrients they now require in their diets. The feline dietary requirements are further constrained by an absence of certain metabolic enzymes, which were lost in the common ancestor of all extant species in the Felidae family (Bradshaw, 2006). As cats have evolved to consume prey, many mutations that have occurred to reduce the levels of these metabolic enzymes such as cysteine dioxygenase (required for synthesis of taurine) do not affect them. Instead, they have direct access to nutrients from the prey. As a result, they have highly regulated dietary requirements which means diets need to be carefully formulated in an effort to improve their health and longevity as pets. First and foremost, cats have a high dietary protein requirement.

2.2 Protein requirements of domestic cats

A requirement for dietary protein represents the need for essential amino acids and adequate non-essential amino acids to supply nitrogen and maintain body protein. The natural diet of the cat consists of animal tissue which contains little carbohydrate; hence cats have adapted

metabolically to favour the use of protein and moderate amounts of fat for energy (Zoran, 2002). Protein in the diet of adult dogs, adult cats, puppies, and kittens is necessary for the replacement of protein losses in the skin, hair, digestive enzymes, and mucosal cells, as well as losses from normal cellular protein catabolism (Case et al., 2010). The amount of dietary protein that must be consumed each day to replace the obligatory protein loss (through urea, ammonia, urine, faeces, and the sloughing of epithelial cells) is termed the maintenance protein requirement (Roudebush et al., 2000). The NRC (2006) recommends a minimum protein requirement of 2.5 g of protein per kg BW for maintenance in the adult cat. It is important to note that a higher-quality protein and a higher digestibility will reduce the protein requirements, and vice versa (Case et al., 2010). Cats have a much greater requirement for dietary protein when compared to the dog which suggests that cats have an increased requirement for essential amino acids or a higher basal requirement for nitrogen (Zoran, 2002). This idea is reinforced by Case et al., (2010) who stated that the elevated protein requirement results from the inability of certain catabolic enzymes in the cat's liver to down regulate in response to changes in dietary protein intake.

Dietary protein provides essential amino acids, which cannot be synthesised in the body and must be ingested, and are necessary for the synthesis of other metabolic proteins. There are 10 amino acids that are considered essential in cats: arginine (Arg), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr), tryptophan (Tr), and valine (Val). According to Case et al., (2010), with the exception of Leu, Thr, Met and Arg, and a unique requirement for taurine, the cat's requirement for specific essential amino acids is not significantly higher than those of other species such as the rat, dog or pig. When it comes to diet formulation it is important to note that for a carnivorous species like the domestic cat several offals will meet their minimum protein requirements. However, these do not account for other important dietary needs such as their mineral requirements (Case et al., 2010). In addition to a high protein requirement, cats are highly dependent on a balanced dietary mineral uptake. It is important to consider these other essential nutrients and their metabolism when it comes to optimising feline diets.

2.3 Companion animal mineral nutrition

There is a general classification scheme which divides minerals into two groups; macrominerals and microminerals (Case et al., 2010). Macrominerals are abundant in the body and account for most of the body's mineral content. These include calcium (Ca), phosphorus

(P), magnesium (Mg), sulphur (S) and iron (Fe), and the electrolytes sodium (Na), potassium (K) and chloride (Cl). Microminerals (e.g., selenium and iron), also referred to as trace minerals, tend to be present in much smaller quantities in the body. When it comes to nutrient requirements of the cat, it is important to consider their requirements for essential minerals such as Ca and P – as these are the most abundant macrominerals in the body and account for most of the body's mineral content (Adedokun and Adeola, 2013). Both Ca and P are present in a range of foods and each play important roles in animal nutrition and physiology.

2.3.1 Biological functions of calcium and phosphorus

Minerals have a variety of functions in the body ranging from activating enzymatically catalysed reactions to providing skeletal support and maintaining water and electrolyte balance (Case et al., 2010). Calcium and P are both essential for bone health and energy metabolism. Calcium is an essential inorganic component of bone with as much as 99% of the body's Ca found in the skeleton in the form of hydroxyapatite crystals (Finch, 2016). The Ca in bone is constantly mobilised and deposited to allow for the necessary bone growth and maintenance to occur (Case et al., 2010). Calcium in bone provides structural integrity to the skeleton and also contributes to the maintenance of blood Ca levels through ongoing resorption and deposition. In addition, the body's need for Ca fluctuates and circulating concentrations are strictly controlled through homeostatic mechanisms that are independent of the animal's dietary Ca intake (Case et al., 2010). Phosphorus, like Ca, provides structural support to the skeleton and is released into the bloodstream under homeostatic regulation. The largest pool of P is also located in the skeletal tissue of vertebrates, making up approximately 85% of total P stores which acts as a storage depot with P being absorbed and released as required (Stockman and Villaverde, 2020). When it comes to skeletal support, vitamin D is also of utmost importance.

The active form of vitamin D (1,25-$(OH)_2D_3$), commonly referred to as calcitriol targets the intestine, bone tissue, parathyroid gland and kidneys (Zafalon et al., 2020). In the kidneys, calcitriol promotes renal phosphate and Ca reabsorption. In the intestine, calcitriol acts on passive paracellular and transcellular Ca transport. Lastly, calcitriol regulates the secretion of parathyroid hormone (PTH). Each of these functions allow calcitriol to indirectly regulate the mineralisation and growth of bone (Zafalon et al., 2020). Proszkowiec-Weglarz and Angel (2013) found that Ca and P deficiencies and an overall imbalance of these minerals resulted in reduced growth rates and bone mineralisation in broiler chickens. This led to an increased

incidence of skeletal abnormalities such as rickets, which resulted in lameness and increased morality rates (Proszkoweic-Weglarz and Angel, 2013).

Extracellular P concentrations are regulated primarily by urinary excretion, intestinal absorption and the deposition and release of P from bound sources in bone and various other body tissues (Laflamme et al., 2020). Phosphorus is distributed throughout all cell components, including the nucleus, mitochondria, and the outer membrane. Additionally, phosphorylation is vital for regulating enzyme activity, and in the activation and inactivation of many key enzymes through the addition or removal of phosphate groups (Stockman and Villaverde, 2020). In fact, over 44% of all metabolic molecules are phosphorylated (Srinivasa and Morowitz, 2009). Many metabolic processes are highly reliant on P, and there are many specific properties of P which make it so critical to biochemical functions including: solubility; ionisation; energetics and site solubility just to name a few.

When it comes to evaluating feeds as sources of minerals, we must not only consider the dietary content, but also the absorbability and bioavailability of the mineral (Roudebush et al., 2000). This is especially important for Ca and P because many factors influence the bioavailability of these minerals (Case et al., 2010). These include: 1) chemical form (which affects solubility), 2) the amount of other dietary components with which it interacts with metabolically, 3) mineral intake and body stores, 4) whether the mineral source is organic or inorganic, 5) physiological status of the animal and 6) absorption rates in the lumen of the gut (Roudebush et al., 2000; Cline, 2012). The more bioavailable a nutrient is, the greater the quantity that can be digested and absorbed from the diet in the gastrointestinal tract (GIT).

2.3.2 Sources of Phosphorus

Dietary P is either present as naturally occurring organic P or derived from the added inorganic P (Pi). Organic P sources can be found in the animal products (e.g., meat and bone meals) and plant-derived ingredients (e.g., grain) that are commonly used in commercial pet foods (Bump, 2016). The source of P influences its apparent digestibility, which can range from 0% to 80% (Laflamme et al., 2020). In cereals, a significant fraction of P may be present in the form of phytate ($C_6H_{18}O_{24}P_6$), which is less bioavailable than other forms of P (Roudebush et al., 2000) and only has a digestibility of up to 40% (Laflamme et al., 2020). This is because the P bound to phytate is insoluble in water and is therefore less available for monogastric animals (Lineva et al., 2019). Humer et al., (2015) found that phytate-bound P was either poorly utilised or

entirely unavailable to monogastric animals due to the very low level of phytase activity found in the small intestine. Phosphorus from animal sources is primarily present as calcium phosphates such as hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) and phosphate esters (i.e., organophosphates). Inorganic P is commonly used as a dietary P-based additive and is significantly more available for absorption from the small intestine than organic forms in humans (Coltherd 2019; Dobenecker et al., 2021b). The difference in bioavailability may be due to the P contained in organic sources being bound to intracellular signalling molecules and *in vivo* proteins, whereas the Pi tends to be added to diets in a soluble form which can readily disassociate and be absorbed (Coltherd, 2019). Examples of Pi sources include mined phosphate rock, phosphoric acid, or various phosphate salts (see Table 1). The P contained in these inorganic food additives tend to have an intestinal uptake of 80% or greater (Laflamme et al., 2020). Many studies in cats have indicated that the higher the P content of the diet, the higher the amount of P excreted in the urine and faeces (Kienzle et al., 1998; Pastoor et al., 1995; Fettman et al., 1992). However, this relationship is not straightforward, as the total P content of the diet and the Ca: P ratio both appear to influence bioavailability, and the source of dietary P may also play a role in this process (Mack et al., 2017; Coltherd et al., 2019).

2.3.3 Mineral requirements of companion animals

A minimum requirement has been established for the inclusion of Ca and P in the diet to ensure the body can maintain the levels of both minerals necessary for normal metabolic functions in the cat. The adult cat has a maintenance requirement of 0.6% on a dry matter basis (DM) and 0.5% DM for Ca and P respectively (AAFCO, 2023). These numbers are similar to those provided by other nutritional guidelines. For P requirements, the National Research Council (NRC, 2006) recommends an allowance of 0.035 g P/kg BW, and the European Pet Food Industry Federation (FEDIAF, 2021) recommends 0.5-0.6% DM. For minimum Ca requirements FEDIAF (2021) recommends 0.5-0.6% DM and the NRC (2006) provide a recommended allowance of 0.040 g Ca/kg BW. The current nutrient profiles provided by AAFCO (2023) are more practical than other guidelines as their numbers are based on the use commercial ingredients and hence, suggest a higher minimum requirement for the inclusion of both Ca and P in commercially prepared foods. There are currently no maximum levels for dietary Ca and P cited in these nutritional guidelines for cats, unlike that of the dog (Summers et al., 2020). The AAFCO (2023) guidelines for dogs indicate that levels exceeding 2.5% DM and 1.6% DM of Ca and P, respectively, have been found to have detrimental effects on canine health. Unfortunately, there is insufficient evidence to determine whether this is also applicable

for feline health. Summers et al (2020) compared analysed P and Ca concentrations to the minimum amounts provided on the product labels of 80 non-prescription commercial cat foods. The investigation revealed that there was a high number of products with high levels of P and low levels of Ca, reinforcing the need for pet food regulatory reform (Summers et al., 2020).

2.3.4 Importance of Ca to P ratio

Calcium and P are generally discussed together as their metabolism and homeostatic mechanisms regulating the circulating concentrations of both minerals in the body are closely interrelated, emphasising the need for suitable Ca:P ratios in the diet. Complete and balanced commercial diets manufactured for domestic cats have a Ca:P ratio requirement as a preventative measure for deficiencies and toxicities of both minerals. A dietary Ca:P ratio between 1.2:1 and 1.4:1 is considered optimal by most nutritionists (Case et al., 2010). Many commercial diets tend to supplement Ca to ensure the ratio to P is suitable for the targeted life stage and lifestyle (Cline, 2012). The ratio can differ markedly with the form and availability of either the Ca or P supplied in the diet. For example, animals eating foods high in phytate require greater P intake to meet their needs (Roudebush et al., 2000) as this form of P is less bioavailable than other forms (Graf, 1986).

The ratio of these minerals has a significant effect on the bioavailability of P as it influences the amount that can be absorbed in the intestinal tract. Kienzle et al. (1998) investigated the P requirement of cats and found that Ca:P ratios of 2:1 significantly reduced the apparent absorption of P regardless of concentration of each of the minerals in the diet. With respect to dietary interactions, there is ample evidence to suggest that increasing the dietary concentration of Ca decreases the absorption of P (Kienzle et al., 1998; NRC, 2006; Dobenecker et al., 2018a). It is important to note that this is most evident with Ca:P ratios greater than 2:1 in both cats and dogs (Case et al., 2010). When this ratio is not appropriately maintained, it can lead to issues such as hypocalcaemia (decreased ionised Ca) and hyperphosphatemia (increased ionised P) which can both have detrimental effects on feline health (Potts and Juppner, 1998).

2.3.5 Phosphorus in commercial pet foods

With cats being such finicky eaters, it can be a challenge to provide a complete and balanced diet that is also highly palatable. Feed additives are widely used in both human and pet foods due to their various properties. Phosphate additives are commonly used in pet foods not only for its contribution to nutritional adequacy, but also for palatability. Phosphate additives are not protein bound; they exist as salts that readily dissociate and are absorbed in the small

intestines (Kalantar et al., 2010). There is a wide range of inorganic phosphate salts used as dietary additives (see Table 1).

Table 1. Chemical formulae and names of phosphate salts (retrieved from: Laflamme et al., 2020)

Chemical formula	Common name	Alternate names
NaH_2PO_2	Sodium hypophosphite	
Multiple	Sodium phosphate	Used as generic term for mono-, di- and tri-sodium phosphates
NaH_2PO_4	Monosodium phosphate	Sodium Dihydrogen phosphate, SDHP; Sodium phosphate monobasic, Sodium dihydrogen orthophosphate
Na_2HPO_4	Disodium phosphate	Disodium hydrogen phosphate, Sodium phosphate dibasic
Na_3PO_4	Trisodium phosphate	Sodium orthophosphate, Tribasic sodium phosphate; Trisodium orthophosphate, TSP
$Na_5P_3O_{10}$	Sodium tripolyphosphate	Pentasodium triphosphate, STPP
$Na_4P_2O_7$	Tetrasodium pyrophosphate	TSPP, Sodium pyrophosphate
$(NaPO_3)_6$	Sodium hexametaphosphate	Sodium polymetaphosphate
H_3Po_4	Phosphoric acid	Orthophosphoric acid
$CaH_4(PO_4)_2$; OR $Ca(H_2PO_4)_2$	Monocalcium phosphate	Calcium biphosphate, Calcium monobasic phosphate, Calcium dihydrogen phosphate
$Ca_3(PO_4)_2$	Calcium phosphate	Tricalcium orthophosphate, penta-Calcium hydroxide triphosphate, Calcium phosphate tribasic
$CaHPO_4$	Dicalcium phosphate	Calcium hydrogen phosphate, Calcium phosphate dibasic
$Ca_3(PO_4)_2$	Tricalcium phosphate	Calcium phosphate
$Ca_{10}(PO_4)_6(OH)_2$	Calcium apatite	Hydroxyapatite
$Ca_4Na(PO_4)_3$	Defluorinated phosphate	Defluorinated feed phosphate
KH_2PO_4	Monopotassium phosphate	Potassium dihydrogen phosphate
K_2HPO_4	Dipotassium phosphate	Potassium phosphate dibasic
K_3PO_4	Tripotassium phosphate	Potassium phosphate, Potassium phosphate tribasic
$K_5P_3O_{10}$	Potassium tripolyphosphate	Pentapotassium triphosphate, Potassium triphosphate
$K_4P_2O_7$	Potassium pyrophosphate	Potassium diphosphate, Tetrapotassium diphosphate; Tetrapotassium pyrophosphate

In the United States, the most common P sources added to dry foods are phosphoric acid and dicalcium phosphate, while tricalcium phosphate, dicalcium phosphate and sodium tripolyphosphate are the most common forms added to wet foods (Laflamme et al., 2020). Phosphoric acid is one example of an additive applied to cat food to enhance palatability especially in dry food (Laflamme et al., 2000). Additionally, it also functions as a preservative, texturiser and urine acidifier. Urine acidifiers are essential in feline diets to maintain pH balance in the blood and urine (Case et al., 2010). Many commercial pet foods are formulated to produce acidic urine even if the diet is not promoted as acidifying. It is important to note that certain properties of the diet will have a naturally acidifying effect. For example, methionine is a major component of animal protein, being naturally present in food sources such as meat, fish and legumes. L-methionine is metabolised to L-cysteine producing sulphate and hydrogen ions, changing the cation/anion balance, causing the pH of the urine to decrease (Jacobs et al., 2001). Acidifiers tend to be included to prevent struvite urolithiasis in cats (Spears et al., 2003). Other phosphate additives such as sodium hexametaphosphate aid in reducing the accumulation of dental tartar (Hennet et al., 2007). Past studies have shown that the vast majority of pet food products on the market tend to contain the major mineral P in amounts far exceeding the recommended daily allowance (Davies et al., 2017; Dobenecker 2021c).

The level of risk associated with dietary P depends on the source and its bioavailability. In particular, there tends to be more risk associated with the use of Pi which are highly bioavailable. Indeed, the P burden from pet food additives derived from Pi is disproportionally high relative to its dietary content which predominantly consists of organically (animal and plant) derived P sources (Kalantar-Zadeh et al., 2010). Excessive levels of P in the diet can be detrimental to the renal health of cats and dogs. The intake of excessive amounts of highly bioavailable dietary Pi may lead to a high absorption from the small intestine and results in a correspondingly high renal excretion of P (Dobenecker et al., 2018b; Steffan and Dobenecker, 2023). Increased renal excretion of P occurs as a result of increased parathyroid hormone (PTH) stimulation which decreases the density of renal P transporters (Murer et al., 1998; Böswald et al., 2018). The degree of renal P excretion is the major regulating pathway, with 80-90% of P reabsorbed by the kidneys under normal conditions. In addition to PTH, fibroblast growth factor-23 (FGF23) is likely to be constantly stimulated when dietary P is high and this has been known to decrease tubular reabsorption of P leading to an increase in tubuli P concentrations

and potentially tubular damage (Kalantar-Zadeh et al., 2010). This emphasises the importance of maintaining healthy concentrations of circulating P to avoid adverse health effects.

2.4 Mineral metabolism

2.4.1 Absorption and digestion

Under normal conditions, when an animal consumes food, dietary P is absorbed in the small intestine. Approximately 70% of P absorption occurs in the duodenum and jejunum in response to the release of a hormone called calcitriol. The process of intestinal absorption of Ca is species dependent. Transcellular absorption only occurs in the duodenum and is activated when there is reduced Ca intake in both the cat and dog (Cline, 2012). This process involves Ca entering intestinal epithelial cells through voltage-insensitive transient receptor potential channels. This transport across the brush boarder membrane is enhanced by the carrier protein Calbindin (see Figure 2.1). Calcium-Adenosine triphosphate (ATP)ase then pumps Ca out of the basolateral membrane. Inducing the Ca-ATPase and synthesising Calbindin are both dependent on vitamin D (Cline, 2012). Kalantar et al. (2010) found that more than 90% of Pi is absorbed in the small intestine, as opposed to 40-60% of naturally present organic P. Once P is absorbed through the small intestine it enters the bloodstream and travels to the renal glomerulus for filtering. Phosphorus is filtered freely across the glomerulus, and of this approximately 80-90% of the P that reaches the renal nephron tubules is reabsorbed with excess P eliminated predominantly via renal excretion (Stockman and Villaverde, 2020). In the healthy animal, the total renal P excretion is balanced to dietary P intake under hormonal regulation to maintain fairly stable circulating P concentrations.

Figure. 2.1 Active transport of Ca across an epithelial cell in the duodenum (retrieved from: Cline, 2012).

According to Mack et al., 2015, the adult cat and dog may only adapt to low Ca supply by altering gastrointestinal (GI) Ca absorption if challenged for an extended period. Over shorter periods, Ca stores in bone may be released to maintain Ca homeostasis in the blood in the case of deficiency (Mack et al., 2015). It seems that, at maintenance, adult cats and dogs may have little need to increase true Ca digestibility when dietary Ca supply is low. Mack et al., (2015) explored the potential evolutionary reason for this. The ancestors of both cats and dogs were carnivorous, ingesting a diet consisting of prey which means bones were regularly consumed. If intake of Ca was low, this would likely be combined with low food intake and potentially starvation (Mack et al., 2015). This would suggest that the animal would not metabolically favour expending energy to increase efficiency of GI Ca absorption.

2.4.2 Calcium and phosphorus homeostasis

As mentioned previously, the homeostatic control of P and Ca blood concentrations are influenced by many of the same regulatory mechanisms that are dependent on the secretion of PTH, calcitriol (active vitamin D) and calcitonin (inactive vitamin D) (Stockman and Villaverde, 2021). The primary controllers of PTH secretion are the abundant calcium-sensing receptors (CaSR) in the chief cells of parathyroid glands and renal tubules (Centeno et al., 2019). When circulating Ca concentrations decrease, CaSR in the parathyroid glands release PTH into the bloodstream to stimulate the synthesis of calcitriol in the kidneys and increase the resorption of Ca and P from bone (see Figure 2.2) (Case et al., 2010).

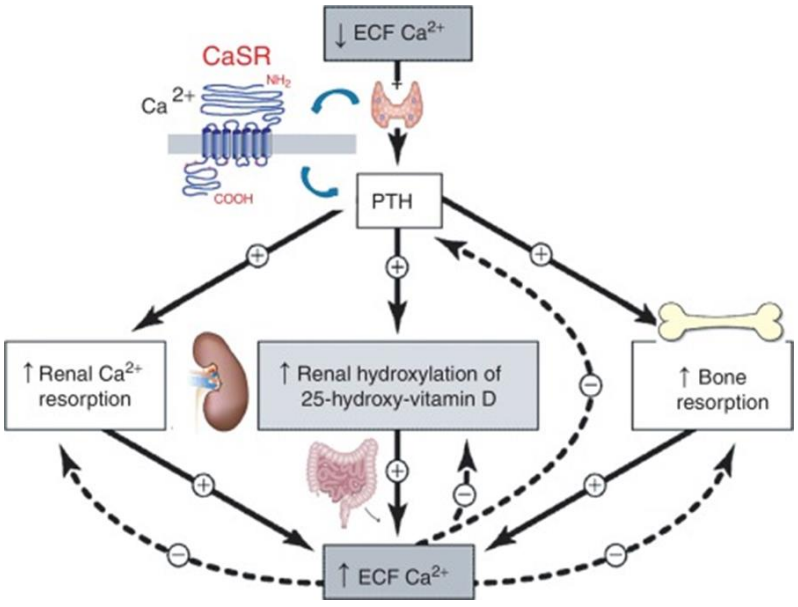

Figure. 2.2 Calcium metabolism (retrieved from: Diaz Soto et al., 2016)
CaSR: Calcium sensing receptor; ECF: extracellular fluid; PTH: parathyroid hormone

Calcium and P can be mobilised from calcium phosphate stores located in the extracellular fluid of bone, but these stores deplete rapidly (Schenck et al., 2006). Osteoclastic bone resorption must then be activated to allow for more prolonged release of Ca or P, a process that occurs through the signalling of PTH in conjunction with calcitriol. Osteoclasts secrete proteases and hydrochloric acid that cause the dissolution of the mineralised matrix of bone and allows Ca and P to be mobilised (Schenck et al., 2006). Geddes et al. (2013a) demonstrated that PTH not only regulated plasma Ca concentrations, but also increased Ca and P ion efflux from bone to blood, which in turn stimulated calcitriol production in the kidney. Within the kidney, calcitriol acts in an exocrine manner to prevent urinary Ca loss by further promoting renal transport mechanisms that are activated by PTH to increase tubular reabsorption of Ca from glomerular filtrate (Schenck et al., 2006). When blood Ca levels are within a normal range, PTH secretion is inhibited through a negative feedback mechanism (see Figure 2.2), and calcitonin acts to reduce Ca levels by increasing osteoblastic activity and reducing osteoclastic activity in bone tissue (Case et al., 2010). When PTH levels are low, calcitriol increases bone uptake of minerals to maintain Ca concentrations within the bone (Finch, 2016).

The regulation of P in the body is interconnected with the regulatory mechanisms mentioned above. The metabolism of Ca and P are closely interrelated, and there is currently little evidence of a specific phosphate sensing mechanism like that of CaSR and Ca (Centeno et al., 2019). Currently, the method by which mammals detect changes in circulatory phosphate concentrations is largely unclear (Michigami et al., 2018). The ability of a cell to sense changes in phosphate levels in its environment is a vital step in the regulation of P homeostasis (Kumar, 2009). Martin et al. (2005) fed uremic rats a pre-experimental diet that was high in P for one month, then gave a low P diet on the day of experiment. It was found that serum PTH and P concentrations dropped significantly within two hours of the rats being switched to the low P diet, but serum Ca concentrations were unchanged. Given that there was no corresponding change in Ca, the authors concluded that this response was likely due to the phosphate-sensing mechanism in the parathyroid glands (Martin et al., 2005). This hypothesis is further supported by the fact that an intravenous infusion of P in normal rats fed low P diets caused a slight increase in serum P and PTH concentrations, but no changes in serum Ca (Martin et al., 2005). Additionally, an infusion of phosphonoformic acid, a phosphate uptake inhibitor, into the duodenum caused a rapid increase in PTH and no significant change in serum P (Martin et al., 2005). Collectively, these data suggest a P-sensing mechanism that is independent of serum Ca may exist in the parathyroid glands (Martin et al., 2005). However, recent studies tend to

suggest that this mechanism may exist in multiple organs as it can be influenced by both FGF23 and PTH (Kro-o, 2008; Kumar, 2009; Michigami et al., 2018). Michigami et al. (2018) suggested that signals triggered by low or excessive levels of P in animals may need to be integrated from multiple organs (e.g., parathyroid glands, kidneys, intestines, and bone) to maintain systemic phosphate homeostasis.

Like that of Ca, the regulation of P is influenced by PTH and low serum P concentrations stimulate intestinal absorption through activation of calcitonin to calcitriol via the 1α-hydroxylase enzyme (Gaasbeek and Meinders, 2005; Stockman and Villaverde, 2021). Calcitriol enhances the P transport from the intestinal lumen to intestinal capillaries (Schenck et al., 2006) and increased plasma P concentrations stimulate PTH secretion and inhibits the formation of calcitriol in the kidneys, forming homeostatic feedback loops (Geddes et al., 2013a). However, the homeostatic mechanisms regulating both blood Ca and P, which are vital for feline health, can be disrupted by the intake of excess Pi via both PTH and FGF23.

2.4.3 Importance of FGF23

Fibroblast growth factor 23 (FGF23) are a group of peptides which are primarily expressed and secreted from osteocytes in the bone (Kuro-o, 2008). Fibroblast growth factor-23 is considered a phosphaturic hormone which induces negative phosphate balance in regulating phosphate homeostasis (Kuro-o, 2008). Phosphate balance is considered positive when P intake exceeds P loss and negative when P intake is less than P loss. The secretion is activated with increased serum P concentrations, and FGF23 activity is dependent on it signalling through FGF receptor (FGFR) tyrosine kinases with the aid of klotho (Kuro-o, 2008; Stockman and Villaverde, 2021). Klotho is a transmembrane co-transporter protein which is prominent in both the kidneys and parathyroid glands. Several FGFR isoforms bind with klotho to form complexes with enhanced affinity for FGF23 which act as FGF23-specific receptors (Kurosu et al., 2006). Fibroblast growth factor-23 requires Klotho as a co-receptor to induce renal P excretion and suppress calcitriol biosynthesis in the kidneys through the inhibition of 1α-hydroxylase activity, which reduces intestinal P absorption (Erben, 2016; Kuro-o, 2008). Additionally, PTH acts in conjunction with FGF23 to reduce the expression of the sodium-phosphate transporters in the renal tubules, and thus reducing renal P reabsorption (see Figure 2.3).

Figure. 2.3 Fibroblast growth factor-23 involvement in phosphorus homeostasis (retrieved from: Hardcastle and Dittmer, 2015). *FGF-23: fibroblast growth factor-23; Pi: inorganic phosphate; FGF23-klotho-FGFR1: fibroblast growth factor-23 klotho-fibroblast growth factor receptor 1; PTG: parathyroid gland; Pi: inorganic phosphate; GIT: gastrointestinal tract; Na: sodium; MAPK: mitogen-activated protein kinase; ERK1/2: extracellular signal-related kinase-1/2; EGR: early growth response 1,25(OH)$_2$D$_3$: calcitriol*

2.5 Phosphorus in pet health and disease

In animals, the internal pool of P is kept in a state of equilibrium via the kidneys, intestines, bone, and soft tissue, where P intake and storage are equal to P loss (Michigami et al., 2018). Phosphorus toxicity is commonly referred to as hyperphosphatemia (Case et al., 2010). As the regulation of Ca and P are connected, the effect of a Ca deficiency, also known as hypocalcaemia, is the likely development of nutritional secondary hyperthyroidism due to the depletion of Ca stores in the cat as it attempts to restore Ca homeostasis (Cline, 2012). Past studies have found that excessively high levels of dietary P can lead to nutritional secondary hyperthyroidism on account of the low Ca:P ratio and the reduced levels of Ca absorbed in the small intestine (Böswald et al., 2018). Additionally, this condition has been found to increase the amount of bone resorption taking place in an attempt to return the high circulating Ca levels to normal (Böswald et al., 2018; Potts and Juppner, 1998). In this state, PTH is stimulated resulting in an increase in renal P and an indirect increase in blood calcitriol levels which in turn causes an increase in bone resorption. Chronic Ca deficiency can cause major bone mineral content reduction, and can result in significant skeletal abnormalities (NRC, 2006). In addition to affecting skeletal health, excessive dietary P intake can also affect cardiovascular and renal health. Osuka and Razzaque (2012) found that reducing the P burden and maintaining P balance through adequate dietary intake is crucial for normal health in cats, as P toxicity can cause irreversible organ damage like that in the renal tubules.

On another note, Ca levels in the body exceeding physiological requirements leads to hypercalcaemia. Over supplementation of Ca occurs occasionally in cats and dogs (Case et al., 2010). The development of clinical signs of hypercalcemia in companion animals depends on the magnitude of the Ca elevation, how quickly it occurs, and its duration (Cline, 2012). The physiological response involves decreased production of PTH, increased blood calcitonin levels, and decreased calcitriol production in the kidneys due to direct inhibition and the reduction in PTH stimulation. In a state of hypercalcaemia there is a reduced release of Ca and P from bone due to low PTH levels, increased renal excretion of Ca, and decreased intestinal absorption of Ca (Finch, 2016).

2.5.1 Chronic kidney disease

In the past couple of decades, the prevalence of CKD has increased significantly in both humans and cats (Dobenecker et al., 2021b), leading to a peak in interest surrounding the cause and onset of CKD within the research sector. Researchers have been delving into the connection that seems to exist between diet and the onset of CKD in pets (Bartges, 2012; Dobenecker et al., 2021b; Elliott et al., 2000; Kalantar et al., 2010).

Feline CKD is a progressive renal disease that affects up to 80% of geriatric cats, and is now the leading cause of feline mortality (Chen et al., 2020). Medically, it is defined as "the structural and/or functional impairment of one or both kidneys for approximately three months or longer" (Bartges, 2012). The loss of renal function may stabilise for a period of time after diagnosis; however, the loss is irreversible, and CKD is ultimately a progressive condition (Bartges, 2012). The International Renal Interest Society (IRIS) has provided a staging system for CKD in cats and dogs. The system was created on the basis that fasted plasma creatinine levels in a stable patient diagnosed with CKD can be used as a marker for glomerular filtration rate (GFR) (Geddes et al., 2013a).

As mentioned above, P homeostasis is dependent on a balance between dietary intake, renal excretion, and exchange of P between extracellular and bone storage pools (Geddes et al., 2013a). In the kidney of a healthy patient, phosphate ions are filtered freely in the glomerulus and P excretion is controlled by an overflow mechanism. When plasma P concentrations increase, this stimulates PTH secretion causing the level of P reabsorption from the glomerular filtrate to decrease in the renal tubules. Parathyroid hormone does this by reducing the capacity of the proximal tubules to reabsorb phosphate ions, increasing P concentrations in the tubuli (Kalantar et al., 2010) and thus increasing phosphate loss via urine (Geddes et al., 2013a).

In CKD, the GFR decreases due to a decline in the number of functioning nephrons, reducing the level of urinary phosphate ion excretion that can take place. Hence, the tubular damage tends to continue to increase with high plasma P and the associated constant secretion of PTH and FGF23. To a certain degree, the remaining intact glomeruli tend to mask the deteriorating state of the kidneys (Dobenecker et al., 2021a). In some cases, the proportion of damaged renal tissue can be as high as 66-75% by the time of initial diagnosis (Elliott and Barber, 1998).

It has been established that the nutrient content of the diet, particularly P levels, can impact the renal health of pets. There is a growing body of evidence that suggests that crude amounts of dietary P do not reflect accurate P exposure because of the variability in bioavailability (St-Jules et al., 2017). As discussed earlier, foods differ in P bioavailability based on whether the P source is plants, animals or food additives. There are important implications for research linking dietary P to the progression and dietary guidelines for managing the effects of diseases such as CKD (St-Jules et al., 2017). With CKD being the leading cause of feline mortality, further investigation of this dietary factor may allow better understanding of the cause and progression of this disease in both pet and human medicine.

2.6 Thesis aims and objectives

This thesis intended to investigate the effects of feeding highly available inorganic P on indicators of feline renal health and assess the digestibility of three diets fed consecutively with increasing levels of highly bioavailable Pi. The control (CON) diet consisted of organic P and contained a Ca:P ratio within the recommended reference range (1.6:1). The first test diet (T1) consisted of Pi and contained a Ca:P which sat at the lower end of the reference range (0.9:1), and the second test diet (T2) also consisted of Pi and contained a lower Ca:P below the reference range (0.6:1). The main objectives of this thesis were: (1) to assess the digestibility of two test diets containing phosphoric acid (H_3PO_4) in combination with sodium dihydrogen phosphate (NaH_2PO_4): (2) investigate the effects of dietary Pi supply on Ca and P balance through blood, faecal and urine analyses and; (3) to analyse markers of renal health (e.g., Creatinine, SDMA, FGF23, PTH, and urine specific gravity) to determine the effect of feeding increasing levels of dietary Pi on the feline kidney. The overall goal of this thesis was to further knowledge of the impact of feeding highly available Pi on feline renal function. I aim to support the growing pool of evidence revealing a very prominent connection between dietary Pi supply and the onset of feline CKD. At large, I intend to contribute to the collective effort being made to communicate the need for maximum P restrictions in current pet food nutritional guidelines.

Chapter 3

Materials and Methods

Chapter 3: Materials and Methods

3.1 Study species and site

This study was conducted using domestic cats (*Felis catus*) housed at the Centre for Feline Nutrition (Massey University, Palmerston North, NZ). The colony consists of 19 sheltered outdoor pens (see Figure 3.1) with each pen holding up to eight cats, and a total colony population of 135 cats. The colony consists of adult short-haired domestic cats, specifically 38 entire females, 26 spayed females, six entire males (housed separately), and 65 neutered males. All husbandry complied with the Massey University Animal Ethics Committee (MUAEC) protocol number 21/25 and the Animal Welfare (Cats) Code of Welfare (2018).

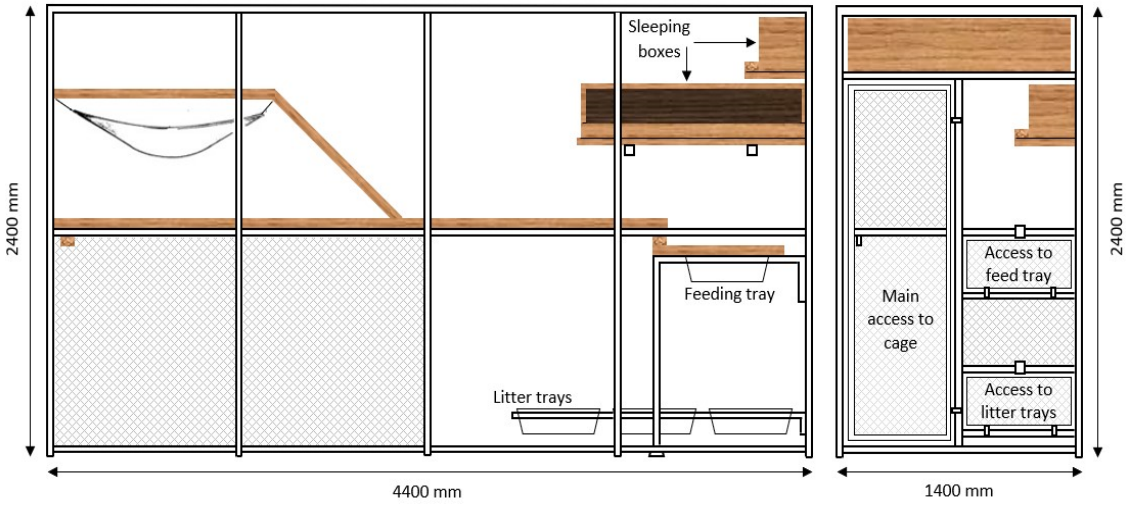

Figure. 3.1 Diagram of the colony housing at the Massey University Centre for Feline Nutrition used to house the cats.

The centre was staffed from 07:30 h to 15.00 h on weekdays and 08:00 h to 12:00 h on weekends. All pens were washed out and the sawdust litter in multiple litter trays (see Figure 3.1) were replaced daily between 07:30 h and 09:30h. Fresh food was provided daily between 10:00am and 11:00am. Cats at the colony are generally fed *ad libitum* with both wet (canned) and dry Chef (Heinz Wattie's Ltd, Hastings, NZ) cat foods. However, during this study, the cats were fed a complete and balanced (AAFCO, 2023) moist (canned) diet manufactured at the Massey University Pilot Plant (Palmerston North, New Zealand) further diet details are provided in Section 3.4. The cats were provided *ad libitum* access to food, which was approximately 350 g per cat per day to ensure their adult maintenance energy requirements were exceeded.

Each pen of cats had one hour of 'playtime' each week which occurred only during weekdays. During this time, the cats were moved into a 'playroom' containing many enrichment items including cat trees, a running wheel and a number of toys. While in the playroom, staff interacted with the cats, weighed them and observed them for any general health concerns (e.g., dental problems or injury).

During the digestibility trial collection periods, cats were housed in single pens which were maintained at the same standard for cleaning and feeding and contained single litter boxes (see Figure 3.3). The cats were also provided with fresh food and water daily.

3.2 Research animals

Initially, 14 cats were blood sampled to provide a full biochemistry analysis prior to being fed the control diet to assess markers of renal function (e.g., creatinine, urea, SDMA) and rule out any potential pre-existing renal issues and to ensure they were in good health. From these 14 cats, eight healthy young domestic shorthair cats (four desexed males and four desexed females) were selected for this trial. The selected cats were aged 1 - 4 years (mean + SEM: 3.05 ± 0.10 years), and weighed 2.4 - 5.6 kg (mean + SEM: 4.26 ± 0.24 kg). Throughout the trial (78 days) the body condition of the cats was visually assessed weekly, and weighing was conducted twice per week. The trials began in early March 2023 (early autumn) and were completed at the end of May 2023 (early winter), a cooler period when the cats tend to naturally lose weight. This study was approved by the Massey University Animal Ethics Committee (protocol number 22/78).

3.3 Experimental design

The cats were sequentially fed three dietary treatments: control (CON), treatment diet 1 (T1), and treatment diet 2 (T2). The diets are described in Section 3.4. Each diet was fed for 26 days, beginning with a 7-day adaptation phase and ending with a 6-day digestibility assessment. The digestibility trial was conducted to allow quantitative collection of urine and faeces and to record individual food consumption. Blood sampling occurred on day 26 of each diet block, commencing with a preprandial/fasted sample (minimum 12 h after last meal) sample and two postprandial samples (3 h and 5 h after food intake).

3.4 Diet manufacturing

The three diets were produced in the Massey University Food Pilot Plant between December 2022 and April 2023, and formulated to be complete and balanced. The control trial was intended to determine the basal values of all parameters for each individual cat. The dietary P

in CON was derived entirely from organic sources and met the recommended daily allowance of 2.6g/kgDM for P (National Research Council, 2006). Inorganic phosphates were added to CON to produce each of the two treatment diets (T1 and T2). I had to consider the effect of using phosphoric acid (H_3PO_4) as the Pi source when it came to diet formulation. Phosphoric acid was unable to be used exclusively to meet the intended level of Pi, as this may have compromised the palatability of the diets. Instead, sodium dihydrogen phosphate (NaH_2PO_4) was used to replace some of the phosphoric acid. To prevent the diet from becoming excessively acidic from the use of phosphoric acid, potassium hydroxide (KOH) was added to neutralise the acid and obtain a dietary pH between 5.85 - 6.40 across all three diets. A pH spear (Eutech, Singapore) was used to measure pH of the diet during production. The probe was inserted into six different sections of the diet as it was being minced to ensure an accurate representation of the diet pH. The three diets were formulated with a Ca:P of 1.6:1, 0.9:1 and 0.6:1, two of which fell within the recommended range of 1:1 to 2:1 for adult maintenance (AAFCO, 2023). The T2 diet fell below the recommended range for Ca:P to demonstrate the effect of high levels of dietary Pi on feline renal parameters. All three diets produced for the trials were fed *ad libitum*, and fed fresh every morning.

The Massey University Food Pilot Plant is fully equipped for the production of a wet feline diet derived from offal. The offals used included lamb heart, kidney, and liver, all of which were provided by Alliance Group (Dannevirke, New Zealand) in frozen or semi-frozen form for diet production. The offals used were delivered directly to the pilot plant where I utilised a Guardian 400R Bandsaw (Guardian, Wellington, New Zealand) and commercial Biro mincer/mixer (Model AFMG-24; Biro manufacturing company, Ohio, United States of America.) for producing the diet. All dry ingredients (e.g., source of carbohydrates from ground wheat and cellulose), oils (sunflower and linseed) and Pi supplements were pre-weighed/prepared and added to the minced offal as it was blended to a homogenised state in the Biro mincer/mixer which had a capacity for preparing the diet in 25 kg batches. The diet was canned in this same facility and a retort machine (Steriflow S.A.S Heat Exchanger CODAP 2000; Steriflow, Roanne, France) was used to cook it (see Figure 3.2). The cooking process used normal commercial retort conditions for thermal processing. The retort machine used was a horizontal water cascade which requires less water and energy to heat than other retort machines. In this type of autoclave, the pressure was controlled at 0.05 bar. An F0 value of 32 was targeted for each batch, with F0 being defined as the lethality time required to eliminate all microorganisms present in foods by exposing them to a temperature of 121.1℃. The cans

of diet were cooked in seven batches, each containing a mixture of the three diets, with cooking conditions replicated to further avoid any variation in thermal processing between the three diets. In each batch of cans that were cooked, three cans contained temperature loggers to ensure the appropriate temperatures were achieved during cooking across different areas within the retort machine.

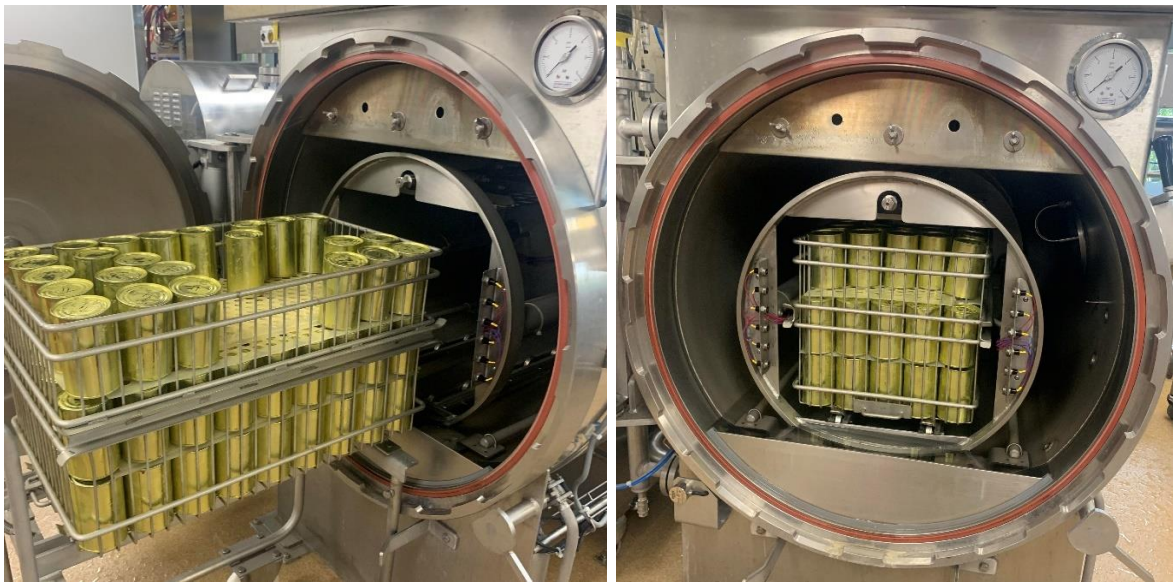

Figure 3.2 Steriflow Heat Exchanging Retort Machine (CODAP 2000) used to cook the trial diets at Massey University Food Pilot Plant.

3.4.1 The inorganic phosphorus sources

Test diet one (T1) contained phosphoric acid (H_3PO_4) and was supplemented with sodium dihydrogen phosphate (NaH_2PO_4) and potassium hydroxide (KOH). The H_3PO_4 was diluted with MilliQ water in a fumigation hood. The diluted solution was then added to the diet gradually in the Biro mincer/mixer to ensure adequate homogenisation. Potassium hydroxide was prepared in a laboratory where chips of the ingredient were ground down into a fine powder for addition with the dry ingredients. The addition of KOH ensured a balanced pH of the diets. The mineral/vitamin premixes used contained NaH_2PO_4. Test diet one (T1) contained 1.21 g of NaH_2PO_4 per kg of diet (mineral premix), 0.87 g of H_3PO_4 per kg of diet and 1.68 g KOH per kg of diet. Test diet 2 (T2) contained 2.07 g of NaH_2PO_4 per kg of diet (mineral premix), 0.96 g of H_3PO_4 per kg of diet and 3.27 g KOH per kg of diet (see Table 3.1).

Table 3.1 Analysed nutrient content of each diet (CON, T1 and T2) with a focus on phosphate content between diets.

Nutrient content	Unit	Control	T1	T2
Basal ingredients	%	Lamb (heart, liver, kidney) 65.1 Wheat flour 30.7 Cellulose 0.8 Sunflower oil 0.8 Linseed oil 1.10 Mineral/vitamin premix 1.5		
NaH_2PO_4	g/kg diet	0	1.21	2.07
H_3PO_4	g/kg diet	0	0.87	0.96
KOH	g/kg diet	0	1.68	3.27
GE	MJ/kg DM	22.92	22.92	23.14
ME calculated	MJ/kg DM	18.8	18.8	19.2
ME calculated	Mcal/kg DM	4.50	4.50	4.59
Pi (calculated)	g/kg DM	0	2.08	3.18
Ca:P		1.6	0.8	0.6

DM = dry matter, GE = gross energy, Ca = calcium, P = phosphorus, Ca:P = Ca-P ratio, Mcal = megacalorie, Pi = inorganic phosphorus, ME = metabolisable energy, NaH_2PO_4 = sodium dihydrogen phosphate, H_3PO_4 = phosphoric acid, KOH = potassium hydroxide

3.5 Sample collection and storage Section

3.5.1 Blood samples

Three blood samples were taken from each cat on day 26 of each treatment block. In total, each cat provided nine blood samples over the 78-day period. A total of 1 ml of blood was collected per sample via jugular venepuncture using a 25-gauge needle. The area was shaved and cleaned with methylated spirits on a cotton swab before application of xylocaine (2%) gel (topical anaesthetic) to the skin 30 min prior to sampling. Preprandial blood samples were taken from the cats in a fasted state (12h after last meal). The cats were then fed 50% of their daily ration and given approximately 60 min to eat. Post-prandial blood samples were then taken 3h and 5h after feeding. The blood samples were placed in red top vacutainers and left to sit at room temperature for two to four hours prior to centrifuging. The samples were spun at 448 x g (4000 rpm) in the centrifuge, before the serum was aliquoted into tubes and frozen at -80 °C before analysis.

3.5.2 Faecal and urine samples

During the first 17 days of the trial all eight cats were housed in a single group. The cats ate as a group during this period, so overall intake was averaged to estimate intake per cat. From day 18 to 26, the cats were housed in single cages of which the cats were very familiar with (see

Figure 3.3). The single cages were located outdoors in the same environment (i.e., the same air temperature, humidity and lighting) as their group pen. *Ad libitum* treatment diet was provided fresh each day and fresh water was provided at all times. The single cages were equipped with a single sleeping box and blanket, toys and a metabolic tray (see Figure 3.1). The cats were allowed two 45 min slots of activity in the playroom per week while being housed in the single cages.

Faecal samples were collected quantitatively at 08.30 h daily for the duration of each 6-day digestibility trial. Samples were weighed and stored frozen at -17℃ until freeze-drying. After freeze-drying, faecal samples were ground, pooled for each animal and sent for analysis (Nutrition laboratory, School of Food and Advanced Technology, Massey University, Palmerston North, NZ). For urine collection, metabolic trays were used which had an inner tray with a mesh lining for faecal collection which allowed urine to pass into the outer tray (see Figure 3.2). The trays contained 30 ml of paraffin oil (Vetpak Ltd, Te Awamutu, NZ) to preserve urine pH as samples remained in trays for up to 24 h. Samples were also collected quantitatively, and weighed taking into account the addition of paraffin oil. Individual samples were separated into two vials, the first refrigerated at 7℃ and six samples per animal were sent for analysis after a maximum of five days (IDEXX Vet Med laboratories, Massey University, Palmerston North, NZ). The second vials were stored frozen in the -80℃ freezer prior to thawing and being sent for analysis for Ca, P and creatinine content (Nutrition laboratory, School of Food and Advance Technology, Massey University, Palmerston North, New Zealand).

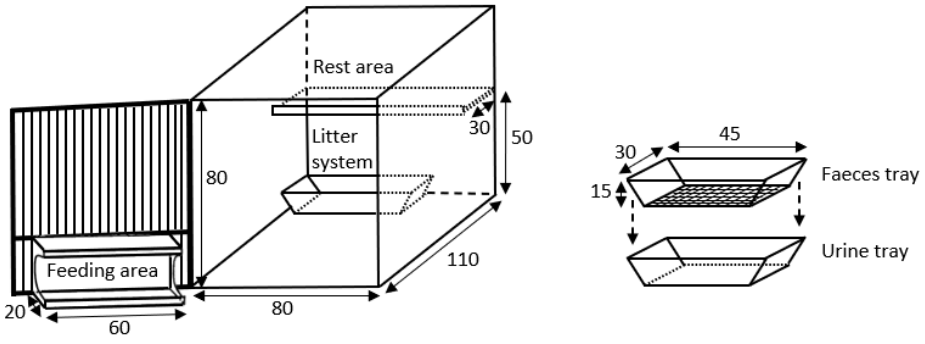

Figure. 3.3 Diagram of the metabolic cages at the Massey University Centre for Feline Nutrition used to house the cats for digestibility collections.

3.5.3 Dier, faecal and urine analyses

The crude nutrient content in the faeces and diet samples (including Ca and P) were determined at the Nutrition laboratory, School of Food and Advanced Technology, Massey University, Palmerston North, New Zealand. Samples of each diet were also transported to Ludwig-Maximilians-University, Munich, Germany for analysis. The diet and faeces were analysed for moisture, dry matter, crude ash, nitrogen, crude fat and crude fibre content according to AOAC International approved analytical methods (Horwitz and Latimer, 2005). Gross energy content was assessed using bomb calorimetry. The dry matter and moisture content were determined using a convection oven at 135℃ (AOAC 930.15). Crude ash content was determined using a furnace at 600℃ (AOAC 942.05). The nitrogen content was determined according to the combustion method (AOAC 92.15) using the Elementar rapid MAX N exceed (Elementar Analyesensysteme GmbH, Langenselbold, Germany). Compressed helium gas (99.9%) was used as a carrier gas and ethylenediaminetetraacetic acid (EDTA) was used as a nitrogen standard for calibration. Nitrogen content was converted to crude protein by multiplying by 6.25. The crude fat content of the diet was determined using the gravimetric (Mojonnier) method (AOAC 954.02), while the crude fat content of the faeces was determined using the Randal modification of the standard Soxhlet extraction (AOAC 200.06). The crude fibre content was determined using bomb calorimetry. In bomb calorimetry a premeasured amount of food or faeces was completely combusted, which resulted in a rise in temperature of a known amount of water in the bomb calorimeter. The metabolizable energy (ME) of the diet was calculated using the gross energy (GE) of the diet and faeces (AAFCO< 2023). Calcium content of the urine was determined using Arzenzo III, and Pi content of the urine was determined using the UV method.

3.5.4 Biochemistry Health Screen

A feline biochemistry health panel was conducted commercially on the serum samples by IDEXX Vet Med Laboratories, Palmerston North, New Zealand. The panel provided symmetric dimethylarginine (SDMA), creatine kinase (CK), aspartate aminotransferase (AST), alkaline phosphatase (ALP), bilirubin, total protein, albumin, globulin, urea, creatinine, phosphate, calcium, cholesterol, sodium, potassium, and chloride as an initial health screen and between each diet block.

3.5.5 Parathyroid hormone

Serum parathyroid hormone (PTH) concentrations were assessed using a feline-specific, commercially available enzyme linked immunosorbent assay (ELISA) (Cat No.

MBS16033584; MyBiosource, San Diego, California, USA). The assay had a sensitivity of 0.05 pmol/L and standard curve range of 0.1-6.4 pm/L (standards were 0.0, 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 pmol/L). The samples and standards were analysed according to the manufacturer's instructions and optical density was measured at 450nm using the BioTek 800TSUV plate reader (Millenium Science NZ Ltd., Auckland, New Zealand). All standards were analysed in duplicate, with an intraassay CV of 8.03%. The standard curve modelled using a four-parameter logistic curve. Most samples were analysed as a single sample. However, a total of nine serum samples were analysed in duplicate; the intraassay CV for feline serum samples was 10.1%.

3.5.6 Fibroblast growth factor (FGF) -23

Serum fibroblast growth factor-23 (FGF23) concentrations were assessed using a commercially available two-site ELISA (CY-4000; KAINOS Laboratories Inc., Tokyo, Japan). Two specific murine monoclonal antibodies bind to full-length FGF-23. One antibody is immobilised onto the microtiter plate well for capture. The other antibody is conjugated to horseradish peroxidase (HRP) for detection. The assay had a high sensitivity with a minimum detection limit of 3 pg/ml as well as a wide quantification range of 3-800 pg/ml. The assay standards ranged from 0-800 pg/ml (0, 10, 50, 100, 250, 500, 800 pg/ml). The samples and standards were analysed according to the manufacturer's instructions and optical density was measured at 450 nm using the BioTek 800TSUV plate reader (Millenium Science NZ Ltd., Auckland, New Zealand). All standards were analysed in duplicate, with an intraassay CV of 4.37%. The standard curve modelled using a linear regression. Most samples were analysed as a single sample. However, a total of nine serum samples were analysed in duplicate; the intraassay CV for feline serum samples was 6.01%.

3.6 Data evaluation and statistical analysis

All statistical analyses were conducted using RStudio (RStudio Team. RStudio: Integrated Development for R, 4.1.1; RStudio: Boston, MA, USA, 2021) and a significance level of $P<0.05$. The Shapiro-Wilk normality test was used to check the normality of all data. Paired t-tests were performed to compare values between diets and for pre- and post-prandial serum and urine values. In some cases, this caused the data to be non-parametric. In an attempt to make the data normally distributed we looked at the proportional change from the mean baseline values. This removed any variability between the cats, uncovering any significant differences in serum values between trial diets without having to transform the data.

The proportion of change from the mean baseline was assessed for serum parameters (Ca, P, creatinine, FGF23, PTH and Ca/P). These parameters were analysed using a repeat measures ANOVA procedure to determine whether trial diet had a significant effect on preprandial (fasted) and postprandial (3h and 5h) serum concentrations. Apparent digestibility was calculated using the equation: aD [%] = (nutrient $intake_{feed}$ – nutrient $excretion_{faeces}$)/nutrient $intake_{feed}$.

Chapter 4

Results

Chapter 4: Results

4.1 Body weight, intake, and digestibility

All eight cats remained clinically healthy for the duration of the study. The addition of Pi in T1 and T2 caused a significant difference in the average apparent digestibility (aD) of Ca between the trial diets (Table 4.1: p = 0.017). The average aD of Ca was significantly lower in T2 compared to CON (p = <0.001). This corresponded with the significant difference in aD of P in diet blocks caused by the addition of Pi in T1 and T2 (Table 4.1: p <0.001), and a significant difference in apparently digested P between the CON and both test diets (T1 and T2) (Table 4.1: p = <0.001). There was a difference in P intake between the trial diets, with P intake being lower in the CON compared to both the T1 and T2 diet blocks (Table 4.1: p = <0.001). The added dietary Pi resulted in higher NaH_2PO_4 intake in T2 (0.141 g/day) than T1 (0.083 g/day). As such, the H_3PO_4 intake was also higher for T2 (1.55 ml/day) than T1 (1.45 ml/day). The higher P intake in the T1 and T2 diet revealed no effect of the lower Ca:P ratios on the amount of apparently digested P when comparing these two diets (see Figure 4.3). There was no significant difference in Ca intake between all three diets (p = 0.835). The diet analysis determined the levels of phosphate sources in the trial diets, measuring the amount of organic phosphate, NaH_2PO_4 and H_3PO_4, which allowed for comparison between the fed Ca:P ratios (see Table 4.1).

Table 4.1 Measured levels of phosphate sources in each trial diet (CON, T1 and T2). Diet analysis was completed at Ludwig-Maximilians-University, Munich, Germany.

Nutrient content	Unit	Control	T1	T2
P sources				
Organic P	g/kg (P/DM)	3.75	4.11	4.62
NaP	g/kg (P/DM)		1.21	2.07
H_3PO_4	g/kg (P/DM)		0.87	0.96

P =phosphorus, org = organic, NaP = sodium phosphate, H_3PO_4 = phosphoric acid, g = gram, kg = kilogram, DM = dry matter

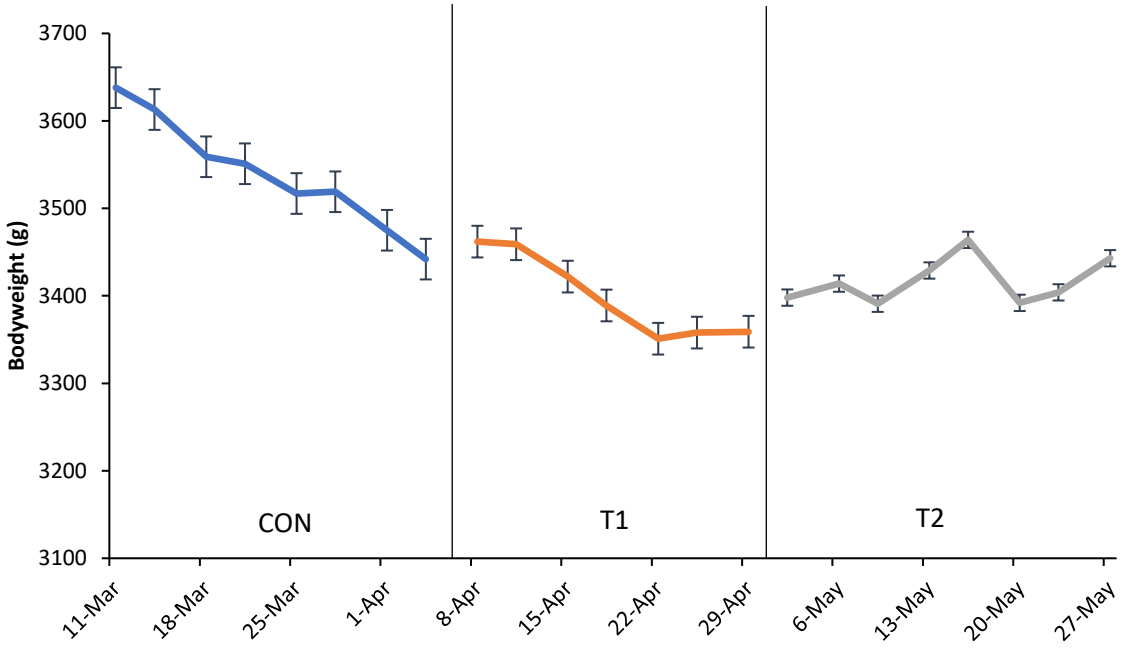

Figure 4.1. Average bodyweight (g) for each diet block (CON: blue line; T1: orange line; and T2: grey line) from 11-March to 27-May.

The average body weights of the cats decreased throughout both the CON and T1 trial periods, but increased gradually in the trial of T2 (see Figure 4.1). Average dry matter intake did not differ significantly between diet blocks (CON = 107.07 ± 2.81, T1 = 121.82 ± 4.81, T2 = 125.25 ± 3.24 DM per kg BW; see Figure 4.2).

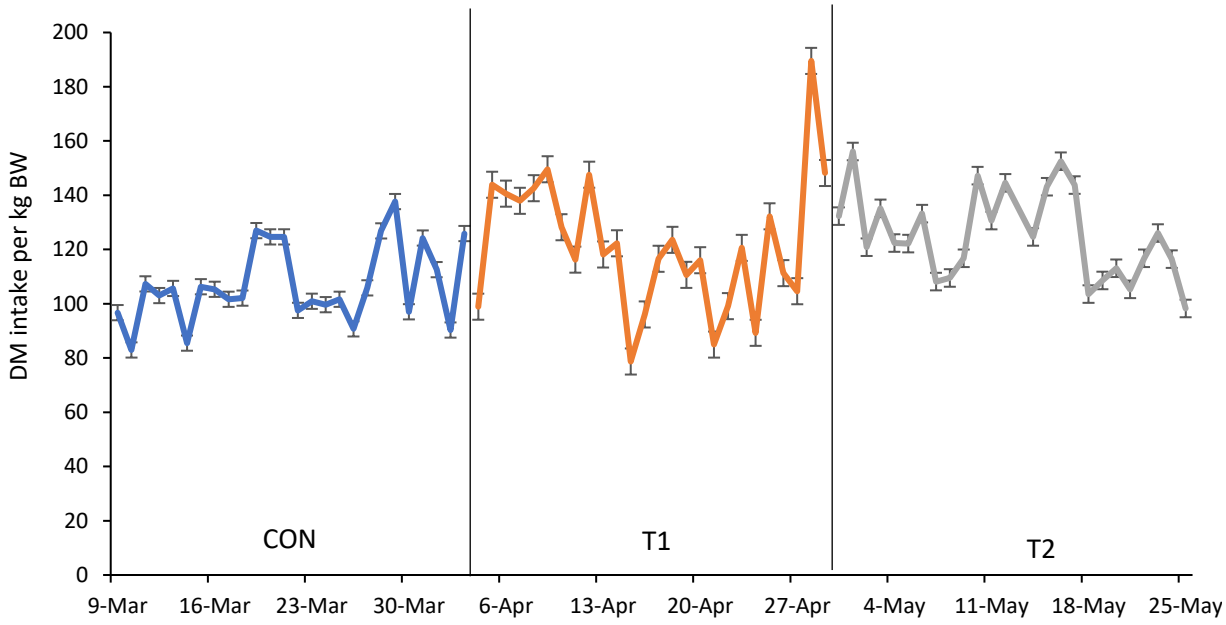

Figure. 4.2 Mean ± SEM group food intake (DM/kg BW/day) for each diet block (CON – blue line, T1 – orange line and T2 – grey line) from 09 March to 25 May.

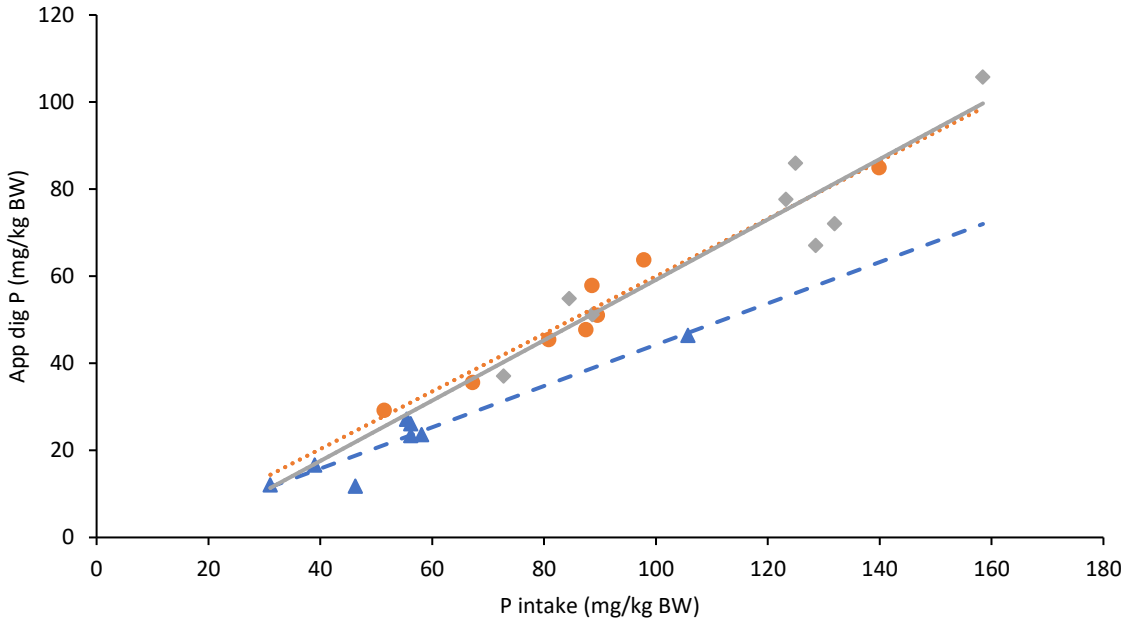

Figure 4.3 The effect of phosphorus intake on apparently digested phosphorus between CON (blue triangles with dashed blue regression line, y = 0.4742x – 3.1528, R^2 = 0.9118), T1 (orange dots with orange regression, y = 0.6613x – 6.1416, R^2 = 0.9566), and T2 (grey diamonds with solid grey regression line, y = 0.6933x – 10.167, R^2 = 0.8741) containing different Ca:P ratios.

The apparent digestibility (aD) of dry matter was relatively consistent throughout all three trials (p = 0.1284). Of the three diets, T1 had the greatest dry matter digestibility of 81.57%. The energy digestibility of the trial diets increased in the diets with lower Ca:P ratios (CON: 77.6%, T1: 81.25%, T2: 80.57%). Protein digestibility varied between the three trial diets (CON: 71.94, T1: 74.60%, T2: 70.12%), with the highest protein digestibility found in the T1 diet (0.9:1). The fat digestibility of the trial diets increased with a reduction in the Ca:P ratios (CON (1.6:1): 87.6%, T1 (0.9:1): 91.62%, T2 (0.6:1): 94.27%).

4.2 Mineral excretion

Faecal P excretion differed between the trial diets (p = 0.358), with concentrations being significantly higher for the T2 diet than the CON diet (p = 0.012: see Table 4.2). However, faecal P excretion for T1 did not differ from either CON or T2 (see Table 4.2). The urine concentrations of P increased with the addition of Pi, with mean urinary P concentrations being significantly different between all three trial diets (p = <0.001: see Table 4.4), despite an increase in urine volume produced by the cats in the T2 trial compared to CON (p = 0.034). Aligned with this, renal excretion of P was significantly different between each trial diet (p = <0.001: see Table 4.2). Faecal Ca excretion showed no significant difference between trial diets with the addition of Pi in T1 and T2 (see Table 4.2). While urine Ca and creatinine

concentrations appeared to be lower for the T1 and T2 diets, levels were not significantly different between trial diets (see Table 4.4). There was no significant difference in renal excretion of Ca between trial diets (p = 0.295). Mineral retention (Ca and P) decreased significantly (p = 0.002, and p = 0.02, respectively) during the T2 trial diet compared to the CON diet, but did not differ between the CON and T1 diets (see Table 4.2). On average, the group experienced negative retention of both Ca and P during the T2 trial (P: -11.96 ± 2.71, and Ca: -9.78 ± 4.90 mg/kg BW).

Table. 4.2 Intake, apparent digestibility, renal and faecal excretion, and retention of calcium and phosphorus (control, test diet 1, and test diet 2) (mean ± SEM)

	Diet	Intake mg/kg BW	Faecal exc. mg/kg BW	aD %	App.digest mg/kg BW	Renal exc. mg/kg BW	Retention mg/kg BW
P	CON	48.89 ± 3.92^{a}	28.77 ± 1.98^{a}	40.70 ± 2.87^{a}	20.12 ± 2.13^{a}	16.72 ± 1.01^{a}	4.34 ± 2.10^{a}
	T1	89.62 ± 9.53^{b}	36.51 ± 3.34^{ab}	58.60 ± 1.65^{b}	53.11 ± 6.56^{b}	45.46 ± 4.47^{b}	7.65 ± 4.44^{a}
	T2	114.16 ± 10.31^{b}	45.18 ± 4.15^{b}	59.88 ± 2.44^{b}	68.98 ± 7.65^{b}	80.95 ± 6.71^{c}	-11.96 ± 2.71^{b}
Ca	CON	77.95 ± 5.45^{a}	71.67 ± 12.79^{a}	7.04 ± 4.19^{a}	6.28 ± 3.01^{a}	0.34 ± 0.09^{a}	5.31 ± 3.05^{a}
	T1	76.39 ± 8.13^{a}	71.49 ± 6.90^{a}	4.85 ± 4.41^{a}	4.90 ± 3.43^{a}	0.22 ± 0.02^{a}	4.69 ± 3.03^{a}
	T2	72.10 ± 6.51^{a}	81.70 ± 7.22^{a}	-15.20 ± 6.82^{b}	-9.59 ± 4.89^{b}	0.24 ± 0.04^{a}	-9.83 ± 4.90^{b}

Different superscripts above indicate statistical significance (P < 0.05).

4.3 Blood parameters

4.3.1 Serum phosphorus

Preprandial serum values allowed initial comparisons to be made between the three trial dietary treatments. The preprandial serum P concentrations were significantly lower during T2 compared to CON and T1 (p = <0.001, see Table 4.3). The effects of the dietary treatments (T1 and T2) were then compared by assessing the postprandial serum values. Blood samples taken 3 h postprandially indicated that serum P concentrations remained the same (see Table 4.3) after feeding a Ca:P ratio of 0.9:1 (T1). The results also indicated that serum P concentrations remained significantly lower during T2 compared to the other diet treatments both 3 h and 5 h postprandially (3 h: p = 0.012; 5 h: <0.001, see table 4.3). The proportional change from the mean baseline of serum P concentrations (see Figure 4.4), indicated a significant effect of dietary treatment on both preprandial serum P (p = <0.001, see Table 4.3) and 5 h postprandial serum P (p = 0.022, see Table 4.3).

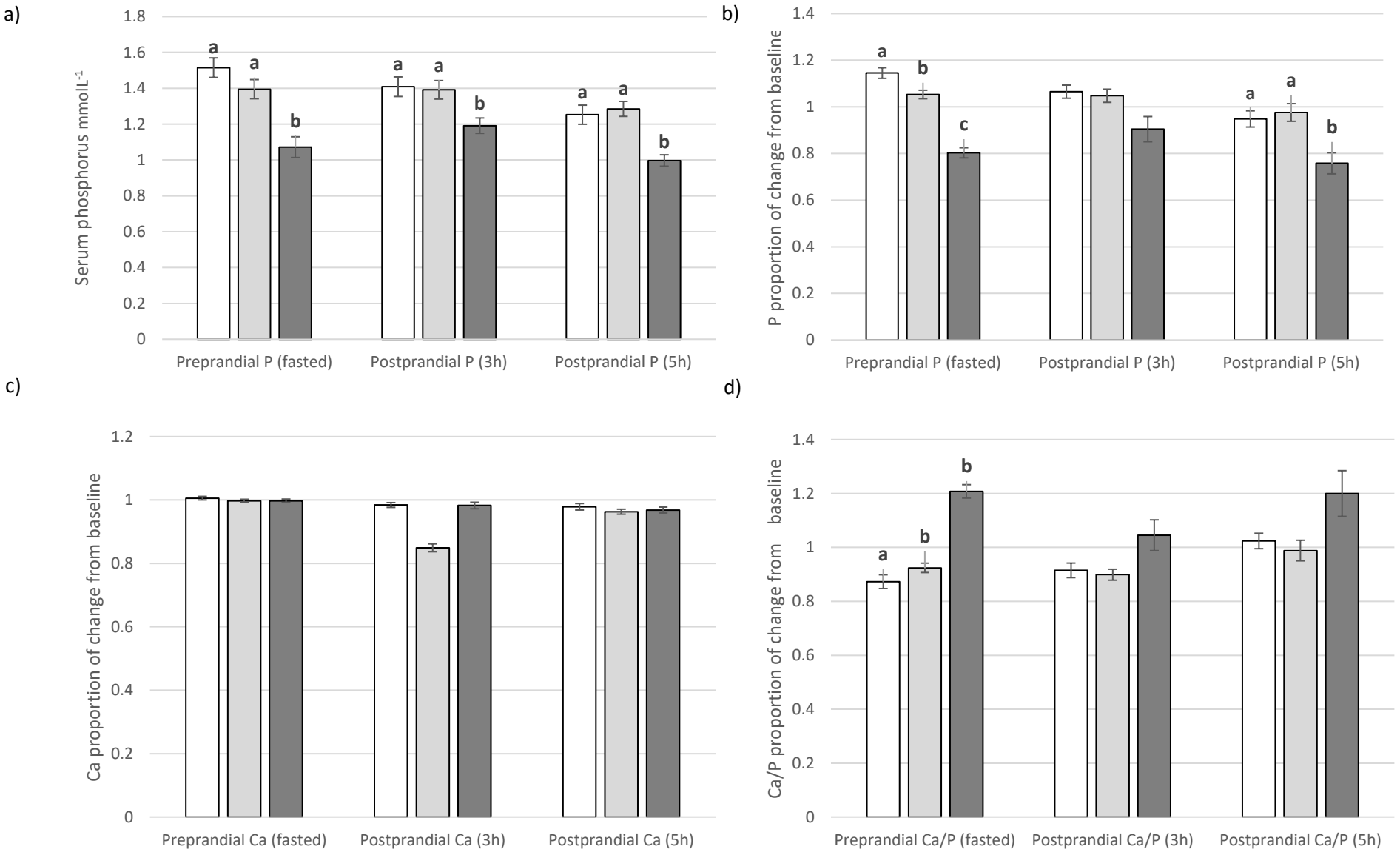

Figure. 4.4. Mean ± SEM (CON: white bars, T1: light grey bars; T2: dark grey bars), (a) serum phosphorus concentrations (b) proportional change from mean baseline serum phosphorus concentrations (c) proportional change from mean baseline serum calcium concentrations and (d) proportional change from mean baseline Ca/P ratios in samples taken after 12h of fasting (preprandial), 3h after feeding (postprandial 3h), and 5h after feeding (postprandial 5h) following 25 days of each diet. Differences between diets are indicated by different superscripts ($p < 0.05$)

4.3.2 Serum Ca/P ratio

Preprandial serum Ca:P ratio concentrations were significantly higher during T2 compared to CON and T1 ($p = <0.001$, see Table 4.3). Blood samples taken 3 h preprandially indicated that the serum Ca:P ratio after feeding T1 dropped lower than levels measured after feeding CON. The serum analysis also indicated that serum Ca:P concentrations during T2 continued to be significantly higher than CON and T1 at both 3 h and 5 h postprandially (3 h: $p = 0.02$; 5 h: $p = 0.008$, see Table 4.3).

4.3.3 Serum calcium

Preprandial serum Ca concentrations did not differ significantly between dietary treatments (see Table 4.3). There was also no significant difference in serum Ca concentrations between dietary treatments to note when looking at the proportional change from mean baseline serum Ca concentrations (see Figure 4.4).

4.3.4 Serum creatinine

Preprandial serum creatinine concentrations were significantly lower for T1 and T2 when looking at the proportional change from the baseline mean of these values compared to CON ($p = <0.001$, see Table 4.3). Serum creatinine concentrations measured after feeding T1 and T2 postprandially (3 h and 5 h) increased slightly compared to levels present in the preprandial serum (see Figure 4.5). The proportional changes in serum creatinine concentrations from the mean baseline continued to be significantly lower for both treatment groups (T1 and T2) compared to CON at both 3 h and 5 h postprandially (3 h: $p =<0.001$; 5 h: $p = <0.001$, see Table 4.3).

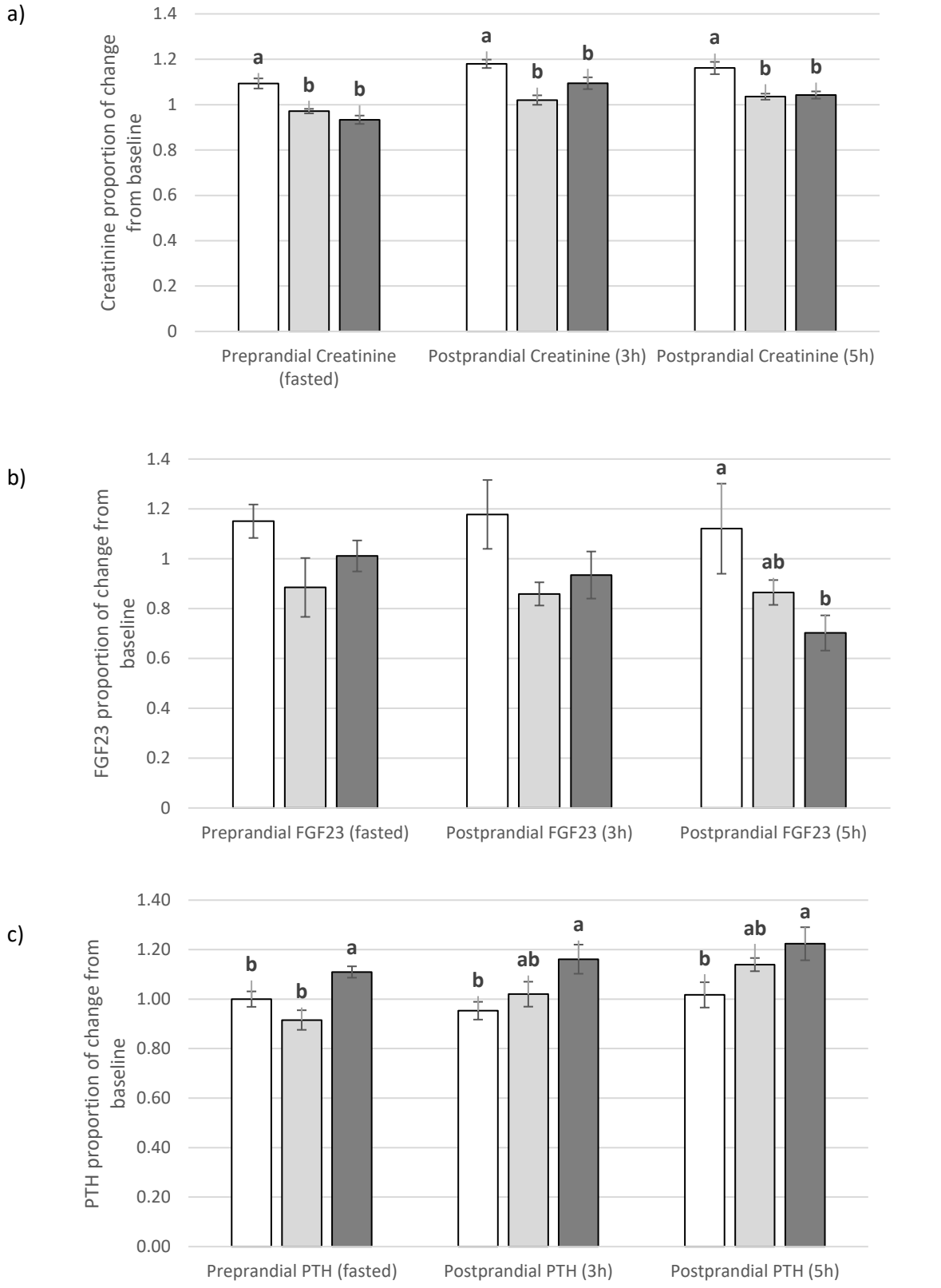

Figure. 4.5. Mean ± SEM (CON: white bars; T1: light grey bars; T2: dark grey bars), (a) proportional change from mean baseline serum creatinine concentrations (b) proportional change from mean baseline serum fibroblast growth factor-23 (FGF23) concentrations (c) proportional change from mean baseline serum parathyroid hormone (PTH) concentrations in samples taken after 12h of fasting (preprandial), 3h after feeding (postprandial 3h), and 5h after feeding (postprandial 5h) following 25 days of each diet. Differences between diets are indicated by different superscripts ($p < 0.05$)

4.3.5 Serum parathyroid hormone

The results of the ELISA provided a standard curve with a four-parameter logistic regression (R^2 = 0.974) from which I calculated PTH concentrations. Preprandial serum PTH concentrations were significantly higher during T2 compared to the other dietary treatments when looking at the proportional change from the mean baseline of these values (p = 0.01, see Table 4.3). The proportional changes in serum PTH concentrations from the mean baseline continued to be significantly higher during T2 compared to CON at both 3 h and 5 h postprandially (3 h: p = 0.019; 5 h: p = 0.03, see Table 4.3).

4.3.6 Serum fibroblast growth factor-23

The results of the ELISA provided standard curve with a strong linear regression (R^2 = 0.994) from which serum FGF23 concentrations were calculated. It is important to note that seven (all control samples) of the values obtained from the assay were above the level of detection capability of the plate reader and thus, these values were excluded from all analyses. Preprandial blood samples and 3 h postprandial samples indicated that serum FGF23 concentrations were not significantly different between dietary treatments. The proportional changes in serum FGF23 concentrations from the baseline mean values were significantly lower for T2 compared to CON at 5 h postprandially (p = 0.006, see Table 4.3). Serum FGF23 concentrations were consistently lower for both dietary treatments (T1 and T2) compared to CON for both postprandial samples (3 h and 5 h) (see Figure 4.5).

4.4 Urine parameters

The average urine volumes differed for each trial period (CON: 11.65 ± 5.71, T1: 11.29 ± 4.58, T2: 14.69 ± 5.97 ml/day per kg BW). There was a significant difference in average urine volumes during CON compared to T2 (p = 0.034). Urine pH decreased significantly during T2 compared to T1 and CON (p = 0.014). The average specific gravity of urine decreased gradually across all three trial diets with the lowest measured in the T2 trial (CON: 1.052, T1: 1.045, T2: 1.045).

Table. 4.3: Preprandial (12h fasted) and post-prandial (3h and 5h) serum parameters for three diet blocks (CON, T1 and T2). Values acquired from repeated measures ANOVA and presented as mean ± SEM.

	Ref range	CON	T1	T2	P-value
Preprandial					
P (mmol/l)	1.3-2.8	1.52 ± 0.055^{a}	1.39 ± 0.053^{a}	1.07 ± 0.058^{b}	<0.001
P prop		1.14 ± 0.023^{a}	1.05 ± 0.018^{b}	0.80 ± 0.022^{c}	<0.001
Ca (mmol/l)	1.81-2.7	2.29 ± 0.046	2.29 ± 0.035	2.29 ± 0.026	NS
Ca prop		1.01 ± 0.006	0.99 ± 0.005	0.99 ± 0.006	NS
Ca/P ratio		1.57 ± 0.058^{a}	1.68 ± 0.064^{b}	2.19 ± 0.097^{b}	<0.001
Ca/P prop		0.87 ± 0.025^{a}	0.92 ± 0.017^{b}	1.21 ± 0.025^{b}	<0.001
FGF23 (pg/ml)		188.98 ± 17.501	138.83 ± 42.312	195.94 ± 21.48	NS
FGF23 prop		1.15 ± 0.067	0.89 ± 0.118	1.01 ± 0.062	NS
PTH (pg/ml)		4.17 ± 0.322	3.89 ± 0.285	4.73 ± 0.308	NS
PTH prop		0.98 ± 0.031^{b}	0.92 ± 0.039^{b}	1.11 ± 0.023^{a}	0.01
Creatinine (umol/l)	70-159	107.14 ± 7.334	92.88 ± 5.851	89.13 ± 5.636	NS
Creatinine prop		1.09 ± 0.022^{a}	0.97 ± 0.009^{b}	0.93 ± 0.018^{b}	<0.001
Post-prandial (3hr)					
P (mmol/l)	1.3-2.8	1.41 ± 0.055^{a}	1.39 ± 0.052^{a}	1.19 ± 0.043^{b}	0.012
P prop		1.07 ± 0.028	0.92 ± 0.133	0.90 ± 0.054	NS
Ca (mmol/l)	1.81-2.7	2.25 ± 0.038	2.25 ± 0.044	2.26 ± 0.016	NS
Ca prop		0.98 ± 0.007	0.85 ± 0.122	0.98 ± 0.010	NS
Ca/P ratio		1.65 ± 0.058^{a}	1.63 ± 0.045^{a}	1.87 ± 0.077^{b}	0.02
Ca/P prop		0.92 ± 0.07	0.89 ± 0.020	1.05 ± 0.057	NS
FGF23 (pg/ml)		234.88 ± 48.842	177.46 ± 23.979	178.48 ± 19.334	NS
FGF23 prop		1.18 ± 0.138	0.86 ± 0.047	0.94 ± 0.094	NS
PTH (pg/ml)		4.04 ± 0.242	4.55 ± 0.479	4.94 ± 0.359	NS
PTH prop		0.95 ± 0.036^{b}	1.02 ± 0.050^{ab}	1.16 ± 0.059^{a}	0.019
Creatinine (umol/l)	70-159	115.43 ± 7.438	96.29 ± 7.174	104.25 ± 5.999	NS
Creatinine prop		1.18 ± 0.019^{a}	1.02 ± 0.021^{b}	1.09 ± 0.026^{b}	<0.001
Post-prandial (5hr)					
P (mmol/l)	1.3-2.8	1.25 ± 0.053^{a}	1.29 ± 0.042^{a}	0.99 ± 0.032^{b}	<0.001
P prop		0.95 ± 0.035^{a}	0.98 ± 0.037^{a}	0.76 ± 0.045^{b}	0.002
Ca (mmol/l)	1.81-2.7	2.23 ± 0.041	2.22 ± 0.394	2.23 ± 0.019	NS
Ca prop		0.98 ± 0.010	0.96 ± 0.008	0.97 ± 0.009	NS
Ca/P ratio		1.84 ± 0.035^{a}	1.78 ± 0.034^{a}	2.15 ± 0.126^{b}	0.008
Ca/P prop		1.02 ± 0.029	0.99 ± 0.038	1.19 ± 0.085	NS
FGF23 (pg/ml)		246.02 ± 56.884	172.03 ± 24.863	140.63 ± 22.411	NS
FGF23 prop		1.12 ± 0.181^{a}	0.87 ± 0.049^{ab}	0.70 ± 0.071^{b}	0.0056
PTH (pg/ml)		4.30 ± 0.260	4.87 ± 0.368	5.17 ± 0.348	NS
PTH prop		1.02 ± 0.051^{b}	1.14 ± 0.027^{ab}	1.22 ± 0.067^{a}	0.03
Creatinine (umol/l)	70-159	113.43 ± 7.138	98.5 ± 5.085	99.63 ± 6.248	NS
Creatinine prop		1.16 ± 0.027^{a}	1.035 ± 0.013^{b}	1.04 ± 0.016^{b}	<0.001

P: phosphorus; Ca: calcium; FGF23: fibroblast growth factor-23; PTH: parathyroid hormone. Differences between diets are indicated by different superscripts ($p < 0.05$)

Table. 4.4 Urine parameters for three diet blocks (CON, T1 and T2) (Mean ± SEM)

Urine	CON	T1	T2	P-value
P (mmol/L)	53.14 ± 5.52[a]	127.94 ± 9.35[b]	170.4 ± 4.89[c]	<0.001
Ca (mmol/L)	0.7 ± 0.14[a]	0.49 ± 0.061[a]	0.41 ± 0.07[a]	NS
Creatinine (mmol/L)	32876.25 ± 2203.03[a]	30135.88 ± 2141.34[a]	29149.13 ± 2116.43[a]	NS

P = phosphorus; Ca = calcium

Chapter 5

Discussion

Chapter 5: Discussion

Commercial pet foods often contain levels of phosphates (P) that exceed the recommended daily allowance according to the AAFCO (2023) nutritional guidelines (Davies et al., 2017; Gagne et al., 2013; Dobenecker et al., 2021a). The prevalence of feline chronic kidney disease (CKD) continues to be the leading cause of mortality in geriatric cats. Although CKD is most common in older cats, it can occur in cats of all ages (Coltherd et al., 2021). Excessive levels of dietary P have been linked to the progression of this ultimately fatal disease, which suggests that the levels of P in commercial pet foods may need to be evaluated further in an attempt to reduce the rate of feline CKD (Geddes et al., 2013a). Highly processed feeds (e.g., dry kibbles) tend to be supplemented with large quantities of highly soluble P such as phosphoric acid (Laflamme et al., 2020). Phosphoric acid is one of the most common forms of inorganic P (Pi) used for preservation and palatability of commercial pet foods. As a highly soluble form of P, there is concern regarding the safety and regulation of phosphoric acid in commercial pet foods, as negative implications of its usage are relatively unknown (Steffan and Dobenecker, 2023). With the wide use of highly available P in commercial cat food, concerns have been raised as these substances have been proven to have adverse health effects in both dogs and humans (Bird and Eskin, 2021; Dobenecker et al., 2021a). The aim of this study was to investigate the effects of feeding Pi on indicators of feline renal health and assess the digestibility of three diets fed consecutively with increasing levels of highly bioavailable Pi. In common with Coltherd et al (2019) and Steffan and Dobenecker (2023), I found that the two Pi containing trial diets (T1 and T2) caused marked effects on Ca and P homeostasis. I also delved into the associated effects on indicators of renal function such as creatinine, FGF23 and PTH.

5.1. Nutrient intake and digestibility

Firstly, the palatability of the diet, assessed by average dry matter (DM) intake did not change significantly with the lower Ca:P ratios (T1 and T2). There was a slight increase in DM intake during the T2 trial compared to the T1 trial, however this was consistent with the observed gradual increase in average body weights of the cats. It is important to consider that this project was conducted during autumn which is typically a period where domestic cats tend to start experiencing increased voluntary feed intake and corresponding increased weight gain (Bermingham et al., 2013; Serisier et al., 2014). During the T1 and T2 diet blocks, we observed a significant increase in P uptake compared to CON. This supports Kalantar-Zadeh et al (2010) who found that feeding high levels of inorganic P-additives led to a significant rise in P intake compared to the diet with no P-additives. In this study, the greatest intake of DM occurred

during CON, however, there was a significantly higher level of P intake for the diets with lower Ca:P ratios (i.e., T1 and T2) compared to CON. This is likely to be a result of T1 and T2 having significantly greater levels of apparently digestible P when compared to CON. This supports the idea that inorganic sources of P are more readily available, have greater absorption efficiency, and hence, have greater digestibility compared to organic sources. Laflamme et al (2020), suggests the intake of highly digestible sources of P leads to increased renal P excretion, which was also observed in the present study. It is likely that the highly bioavailable P sources in T1 and T2 contributed to the lower dietary Ca:P ratios than observed for CON. It is evident that there was no difference between the 0.9:1 and 0.6:1 Ca:P dietary treatments when looking at the effect on apparently digested P (see Figure 4.1), suggesting that there is no correlation between P intake and apparently digested P.

Surprisingly, the addition of $NaH_2PO_4$and H_3PO_4 in T1 and T2 did not result in a positive correlation between intake of P, the amount of apparently digested P, and serum P concentrations in postprandial samples. Steffan and Dobenecker (2023) observed a positive correlation between these measures with additional dietary Pi. This may be due to Steffan and Dobenecker (2023) feeding higher levels of Pi, providing greater correlation between P intake and serum P concentrations when compared to this study. However, I still expected to see a relationship between P intake and serum P concentrations in results of the T1 and T2 dietary treatments. It is important to note that using NaH_2PO_4 allowed us to rule out the potential effect of using a phosphate salt with a higher sodium (Na) content. Phosphate salts such as disodium phosphate with a higher Na content have been found to increase P absorption by stimulating Na^+ -dependent uptake of P (Marks et al., 2015). This was observed by Finco et al (1989) who compared a diet with a P component derived from 100% organic P against one with a P component comprised of 63.5 % Pi (disodium phosphate) and 36.5 % organic P. Finco et al (1989) showed a marked increase in urinary P excretion with the addition of Pi. Sodium dihydrogen phosphate was also selected by Dobenecker et al (2021b) as an inorganic source for one of their high P diets. As a highly water-soluble P source, it was noted to be more readily available for absorption. Dobenecker et al., (2021b) observed reduced apparent digestibility (aD) of P in the organic P diet compared to the high P diets. This supports the significant increase in the aD of P observed in the high P trial diets (T1 and T2) compared to the CON.

5.2 Calcium and phosphorus

It has been well established that Pi is more readily available than organic forms as these dietary additives are not protein bound and exist as salts which dissociate more readily allowing for

greater absorption in the intestinal tract (Bell et al., 1977). However, my results contradicted this as there were no increases in postprandial (3 h and 5 h) serum P concentrations in both diets containing Pi (T1 and T2) despite an increase in Pi intake. A recent study that fed healthy dogs diets supplemented with Pi (monosodium and monopotassium phosphate) found that postprandial serum P increased significantly and there were significantly lower preprandial serum P levels (Dobenecker et al., 2021b). In the current study, the higher Pi intake provided by T1 and T2 diets also resulted in lower preprandial serum P concentrations (see Figure 4.4). Dobenecker et al (2021b) also found that the Pi sources disrupted serum PTH and FGF23 concentrations, demonstrating a disruption to Ca and P homeostasis and potential adverse effects on renal health when compared to organic dietary P sources. This also aligns with Coltherd et al (2019), who demonstrated that adding Pi sources in the feline diet resulted in a greater peak in plasma P than natural (organic) sources. Organic P is bound to carbon and requires enzymatic cleavage to free the P for absorption, thus reducing the rate and efficiency of P absorption (Calvo and Tucker, 2013). The addition of highly water-soluble Pi (e.g., NaH_2PO_4) to the diet at high levels may introduce a P burden for the body to alleviate. Phosphoric acid may also be a common cause of P burden as it so widely used in commercial diets and approaches 100% absorption efficiency (Calvo and Tucker, 2013). A P burden is usually caused by increased P resorption through either calcification or renal excretion. The process of calcification involves the removal of soluble P through the formation of Ca-P complexes (e.g., soft tissue calcification) (Jono et al., 2000). Additionally, elevated serum P concentrations have been identified as a risk factor for cardiovascular diseases due to the association with PTH and calcitriol in mineral homeostasis (Anderson et al, 2011); and increased mortality in individuals diagnosed with CKD (Kalantar et al.,2010). There have been numerous studies that have observed some of the extreme adverse effects of Pi loading (e.g., renal failure and death) in both humans and animals (Jowsey et al., 1974; Marraffa et al., 1987; Ohnishi et al., 2010). Some of these studies involved the administration of Pi in amounts that were much greater than what is considered suitable for practical application in commercial diets (Jowsey et al., 1974; Marraffa et al., 1987). In contrast to these past studies, the serum P analyses of this study found that although P intakes increased with the added Pi (diets T1 and T2), there was a surprisingly significant drop in serum P concentrations.

In the present study, there was a notable difference observed in serum P concentrations irrespective to sample time between T1 and T2 trial diets (see Table 4.3). This suggests the addition of Pi to the T1 and T2 diets (H_3PO_4, and NaH_2PO_4) had a significant effect on serum

P concentrations when compared to the CON diet (organic P only). As the T1 and T2 diets were composed of different amounts of Pi sources (T1: 1.118% H_3PO_4 and 0.645g NaH_2PO_4 per kg of diet, T2: 1.102% H_3PO_4 and 1.005g NaH_2PO_4 per kg of diet), this suggests that the solubility of the dietary P source largely determines the physiological effect that P intake has on an animal.

The three diets were formulated with different Ca:P ratios. The CON diet contained a Ca:P of 1.6:1 which lies within the recommended refence range of 1:1 to 2:1 for safe levels feline diets to achieve adult maintenance (FEDIAF, 2019). T1 contained a Ca:P ratio of 0.9:1 which sits around the lower end of the reference range, and T2 contained a Ca:P ratio of 0.6:1 which is lower than the reference range, to provide insight to the effects of feeding high levels of Pi on indicators of feline renal health. Coltherd et al (2022), found that Ca:P ratio acutely affected plasma P concentrations, which reflected changes observed in apparent digestibility and subsequent urinary P excretion in past studies (e.g. Hoek et al., 1988). As previously mentioned, prolonged increases in blood P or PTH may have detrimental effects on Ca and P homeostasis (Finch et al., 2012). These findings have led to the discovery of ways to modulate the availability of P through the limitation of highly bioavailable forms of P or manipulation of Ca inclusion in the diet (e.g., through dietary Ca:P ratio: Dobenecker et al., 2018a; Laflamme et al., 2020). The previous study investigating the effect of feeding inorganic P fed diets containing different Ca:P ratios (Coltherd et al., 2019) and found that the Ca:P ratio may have had an effect on plasma P and PTH concentrations in addition to the inclusion of inorganic P (Coltherd et al., 2022). By accounting for the effect of different Ca:P on P digestibility and subsequent serum P values, I was able to focus solely on the effects of inorganic vs. organic P sources and the Ca:P ratio included in the diet.

It has been suggested that feeding diets containing low Ca:P ratios may increase the risk of renal dysfunction in cats (Alexander et al., 2019). Laflamme et al (2020) stated that intestinal absorption of P in cats increases up to 49% with low dietary Ca:P ratios. The effects of low Ca:P ratio in cats have been demonstrated through feeding a diet supplemented with NaH_2PO_4 * 2 H_2O and a Ca:P ratio of 0.3:1 with the cats exhibiting reduced creatinine clearance, indicative of an adverse effect on their renal function (Pastoor et al., 1995). Coltherd et al (2022) suggested that feeding diets with higher Ca:P ratios when highly-soluble forms of P are present may aid in the prevention of hypocalcaemia, which has many proven associated health concerns, including soft tissue calcification and an increased risk of cardiovascular events (Anderson et al., 2011). In the early stages of CKD, the decrease in renal Ca excretion is often

overlooked and one of the main roles of PTH is to prevent hypocalcaemia. According to Felsenfeld et al (2015), the lower amount of Ca filtered at the glomerulus contributes to decreased renal Ca excretion in CKD. Interestingly, the reduced amount of filtered Ca increases the risk of hypercalcaemia as a result of Ca loading which can lead to vascular and soft tissue calcification (Felsenfeld et al., 2015).

5.3 Mineral excretion

Past studies have indicated that the consumption of diets containing highly available P may compromise parameters of renal function in healthy cats (Coltherd et al., 2021; Dobenecker et al., 2018a; Pastoor et al., 1995). These studies also found that percentages of renal P excretion increase greatly. Firstly, the amount of P excreted by the kidneys is dependent on the bioavailability of the dietary P being ingested, as this determines how much can be absorbed in the small intestines (Laflamme et al., 2020). The amount of P excreted per nephron may be a major determinant of renal P toxicity in rats, with this effect being evident irrespective of whether rats were in a state of hyperphosphatemia or not (Haut et al., 1980). This finding is further supported by the fact that dietary Pi have been found to cause an increase in formation of calciprotein particles in the renal tubules (Kuro-o, 2013). Calciprotein particles are associated with changes in the endocrine axes involved with mediating the regulation of mineral metabolism, and are a blood parameter commonly assessed in the diagnosis of CKD (Kuro-o, 2013). Therefore, it is suggested that renal P excretion may be a leading contributor to P nephrotoxicity.

In this study, the intake of Pi did not lead to increased serum P concentrations. However, the urinalysis results did indicate a rise in renal P excretion, suggesting that the level of P absorption in the small intestine increased during the feeding of the T1 and T2 diets. There was a significant effect of these lower Ca:P ratios on renal excretion of P when compared to the 1.6:1 Ca:P ratio in the CON diet. The significant increase in renal P excretion during T2 was likely to be due to the higher Pi/total P (CON: 0, T1: 2.08, T2: 3.18 g/kg DM) which would indicate there was more P available from the added Pi as reflected in greater P intakes in T2. The significant increase in both apparently digested P and renal excretion in P caused when T1 and T2 diets were fed may suggest that the current 1 g Pi/mcal threshold (Ca:P ratio safe upper limit) suggested in nutritional guidelines (FEDIAF, 2019) may not be appropriate. Haut et al., (1980) suggested that an increased P excretion per nephron may be responsible for renal damage. The effects of T1 on renal excretion of P occurred over a short 28-day period and the diet contained a Ca:P ratio close to the lower end of the reference range. It is important to

consider the potential prolonged/lifelong effects of feeding a diet containing 0.9:1 or 0.6:1 (Ca:P). Studies done prior to the implementation of a safe upper limit of Ca:P ratio tended to use young, healthy cats, and hence, were not representative of the entire population where cats are at different stages of kidney function (Dobenecker et al., 2018a; Coltherd et al., 2019).

The rise in renal P excretion during T1 and T2 should have been associated with an increase in serum phosphatonins (PTH and FGF23), as this is said to contribute to reduced absorption of P in the proximal tubules and hence, may explain an observed rise in urinary P excretion (Alexander et al., 2021; Kalantar et al., 2010). A rise in serum FGF23 contributes to a reduced absorption of P in the proximal tubules and hence, would explain a rise in urinary P excretion (Blaine et al., 2015). Under normal circumstances, renal sodium phosphate transporters mediate the reabsorption of P in the proximal tubules of the kidneys (De Brito Galvao et al., 2013). Reductions in the expression of these renal cotransporter within the renal tubules have been linked to phosphaturia in the domestic cat (Coltherd et al., 2019). It is interesting to note, in an attempt to maintain adequate sodium reabsorption in the cat, the minimum level of P reabsorption is 30% regardless of FGF23 and PTH activity (Stockman and Villaverde, 2021). Surprisingly, the present study found that levels of serum FGF23 did not increase at any sample times (preprandially, 3 h and 5 h postprandially) with the addition of Pi (T1 and T2). As expected, there was a significant rise in serum PTH concentrations with the addition of Pi when looking at the proportional change from the mean baseline (see Table 4.3). However, this increase in serum PTH alone is not sufficient to explain the high levels of renal P excretion observed. With the high level of renal P excretion in T2, an increase in serum P and more significant rise in PTH was expected. There is speculation that this may be due to the selected postprandial blood sampling times (i.e., 3 h and 5 h) (refer to Section 5.5).

An increase in Pi intake has been found to cause constant stimulation of FGF23 and a subsequent decrease in tubular reabsorption of P (Kalantar-Zadeh et al., 2010). It has been well established that a main role of FGF23 is to increase renal excretion of P, inducing phosphaturia in individuals fed a high P diet (Isakova et al., 2011). The serum FGF23 results of this study may be explained by a potential error in the ELISA assay that was used (refer to Section 5.6).

One of the most common clinical signs of CKD is the retention of P and thus elevated circulating P (Schauf et al., 2021). Mineral retention occurs when dietary intake of that mineral is high relative to its renal excretion (Cupisti and Kalantar-Zadeh, 2010). In the present study, there was a significant difference in the retention of both minerals in T2 compared to CON and

T1, with a higher intake of P (T2 diet) resulting in decreased Ca and P retention. This finding is contradictory when compared to past literature. According to Pastoor et al (1995), high P retention is a consequence of feeding a high P diet and is indicative of potential renal damage. It was expected that P retention to be higher if the same Pi sources (H_3PO_4 and NaH_2PO_4) were fed in greater quantities as demonstrated by Steffan and Dobenecker (2023). The level of faecal and renal excretion of P was much higher than expected considering the amount of Pi included in T1 and T2, and low levels of serum P measured, irrespective of the higher aD of P and P intake. The level of renal Ca excretion was considerably lower during the T2 trial compared to the CON trial, and with a significant reduction in Ca retention this may be explained by the negative aD of Ca observed in results obtained during the T2 diet block. Overall, the results indicate the increased level of Pi had little effect on renal and faecal excretion of Ca. These results are supported by Mack et al (2015), who found no indication of a decrease in the amount of faecal Ca excretion in relation to intake in feed trials with low dietary Ca intake. Mack et al (2015) observed a linear relationship between Ca intake and Ca digestibility, and between P intake and P digestibility in adult cats. This suggests that cats do not efficiently alter their GI Ca absorption when challenged with a low or high Ca intake.

Phosphorus intake in cats has been found to play a role in urolithiasis or struvite stone formation (Pastoor et al., 1995). With P being one of the main components of struvite stones, diets containing high levels of P have been linked to increased formation of struvite (Alexander et al., 2019). The amount of struvite increased gradually with the samples taken after feeding the higher P diets (T1 and T2). However, the sediment results from urinalysis of the present study may not have been reliable due to the urine samples being frozen prior to analysis which can cause varying degrees of cellular disruption (Schultz et al., 2000). Mean urinary pH was between 6.3 and 6.5 for CON and T1 samples, both of which lie within the recommended range to reduce the risk of struvite crystal formation (Lulich et al., 2016). According to Alexander et al (2019), urinary pH needs to be neutral or alkaline for struvite formation to take place, however formation can also occur when mineral concentrations are significantly high. With an average pH of 6.07, samples from the T2 trial were found to have increased levels of struvite, a likely consequence of the addition of dietary Pi and higher levels of renal P excretion.

5.4 Creatinine

Creatinine concentrations are a common parameter routinely used for assessment of renal function. In cats, creatinine in the blood is filtered by the glomeruli of the kidneys, and rather than being re-absorbed and re-excreted by the kidneys, is excreted via the urine (Deguchi and

Akuzawa, 1997). Glomerular filtration rate (GFR) is considered one of the best markers of renal function (Dobenecker et al., 2018a). If GFR persistently declines it is usually indicative of direct damage to the renal tubules and potential loss of functioning nephrons (Braun and Lefebvre, 2008). In this study, there was an evident drop in urine creatinine concentrations with the addition of highly soluble P in the diet (T1 and T2). When looking at the proportional change from the mean baseline, serum creatinine concentrations decreased significantly, in both T1 and T2 diet blocks compared to CON, irrespective to sample time. Past studies found blood creatinine concentrations did not change significantly, but urine concentrations decreased significantly when cats were fed a higher P diet. Serum concentrations tend to be a more accurate representation of the GFR compared to urine creatinine concentrations (Hosten, 1990). Hart (2005), stated that serum creatinine values are commonly utilised for assessment and is widely accepted as an index of GFR in both animals and humans. It is important to note that unlike in cats, inulin is regarded as the analyte used as an index of value GFR in dogs (Hart, 2005).

According to Hall et al (2014), early detection of GFR decline in cats with CKD may be essential to providing dietary or medical intervention when serum creatinine concentrations are still within a healthy range. Observed serum creatinine concentrations were significantly different in samples obtained during the lower Ca:P ratio diet blocks (T1 and T2); however, they remained within the normal range. Hart (2005) discussed the possibility that decreased creatinine levels in the urine in conjunction with an increased renal excretion of P may indicate the presence of renal damage. Indeed, Chen et al (2020), found GFR is correlated with serum P concentrations as P is freely filtered by the glomeruli. In fact, even an acute decrease in GFR has been shown to cause a proportional increase in serum P concentrations (Chen et al., 2020).

Individuals with CKD have reduced renal mass which worsens with progression of the disease. Tubular damage in the kidneys tend to cause the level of renal dysfunction to increase over an extended period. In the early stages of CKD, the impaired function of the kidneys is compensated for by an increased GFR and glomerular pressure through the activation of the renin-angiotensin aldosterone system (Ames et al., 2019). Additionally, Ca and P balance is maintained through regulatory mechanisms influenced by FGF23 and PTH. According to Schauf et al (2021), these compensatory mechanisms may become mal-adaptive, and could contribute to the progression of renal damage and incidence of cardiovascular disease in humans.

5.5 Fibroblast growth factor-23 and parathyroid hormone

After feeding the T2 diet, a significant increase in serum PTH levels was observed, independent of sampling time. Coltherd et al (2019), also found that adding Pi to a feline diet caused a temporary increase in postprandial plasma P concentrations, and a subsequent rise in PTH and urinary P excretion. Parathyroid hormone is an integral component of P homeostasis. Secretion of PTH is stimulated when plasma Ca concentrations drop, triggering an increase in renal tubular Ca absorption and renal excretion of P through the action of PTH in conjunction with FGF23 (Anderson et al., 2011; Blaine et al., 2015; Stockman and Villaverde, 2021). The release of PTH causes the downregulation of sodium-phosphate cotransporters (Npt2a, Npt2c, and PiT-2) in the renal proximal tubule, resulting in decreased renal reabsorption of and phosphaturia (Blaine et al., 2015). Chronic PTH elevation may increase the risk of hyperparathyroidism caused by renal failure, and is associated with hypertension and cardiovascular events (Anderson et al., 2011). Additionally, increased PTH could contribute to the development of hypocalcaemia in CKD patients, resulting in an increased demand for PTH with progressive parathyroid gland hyperplasia (Felsenfeld et al., 2015). Hyperplasia of parathyroid glands tend to be characterised by the loss of vitamin D receptors (VDR) and calcium-sensing receptors (CaSR), which reduce the inhibitory effect of Ca and 1,25-$(OH)_2$-D_3 (calcitriol) on parathyroid function (Felsenfeld et al., 2015).

However, the serum PTH concentrations measured during T1 and T2 were surprisingly high considering the drop in serum P concentrations that were observed. With the high level of renal P excretion observed in T2, an increase in serum P and consequential increase in PTH secretion was expected. There is speculation that the observed serum P and PTH concentrations may have been due to the selected postprandial blood sampling times of 3 and 5 h. Coltherd et al., 2022, fed diets containing Pi in the form of NaH_2PO_4 to healthy cats and found that serum P and PTH generally peaked 120 min postprandially. In this study, T1 and T2 contained both NaH_2PO_4 and H_3PO_4 as sources of Pi. As H_3PO_4 is a highly soluble form of Pi, it is possible that serum P concentrations peaked earlier than 3 h postprandially and caused subsequent PTH secretion to occur earlier which would not have been captured due to the selected sampling times. This also suggests that the cause for high levels of renal P excretion were likely due to the Pi source rather than the Ca:P ratio (0.6:1) of T2.

In recent studies involving high P intake in dogs, cats, and humans, the rise in PTH concentrations tended to occur in conjunction with an increase in FGF23 secretion (Alexander et al., 2019; Coltherd et al., 2021; Steffen and Dobenecker, 2023). In healthy individuals,

FGF23 is secreted in response to dietary P loading or elevated levels of calcitriol, leading to phosphaturia and reduced PTH and calcitriol levels in the blood (Isakova et al., 2011). However, these effects are confounded as the decline in the circulating levels of calcitriol leads to relative hypocalcaemia, which in turn stimulates the synthesis and secretion of PTH in the attempt to restore Ca homeostasis (Blaine et al., 2015; Cupisti and Kalantar-Zadeh, 2013). Fibroblast growth factor-23 only binds to its receptor (FGFR) when α-klotho is present. When FGF23 levels increase in a healthy individual, FGF/ α-klotho complex expression rises, increasing the affinity for FGF23, eventually leading to a drop in PTH secretion (Stokman and Villaverde, 2021). In late stages of CKD, the level of α-klotho synthesis drops which in turn increases circulating FGF23, having little effect on the hyperphosphatemia in the individual (De Borst et al., 2011). As CKD progresses, glomerular filtration rate (GFR) steadily drops, FGF23 levels rise, and renal P excretion continues to be stimulated (see Figure 5.1).

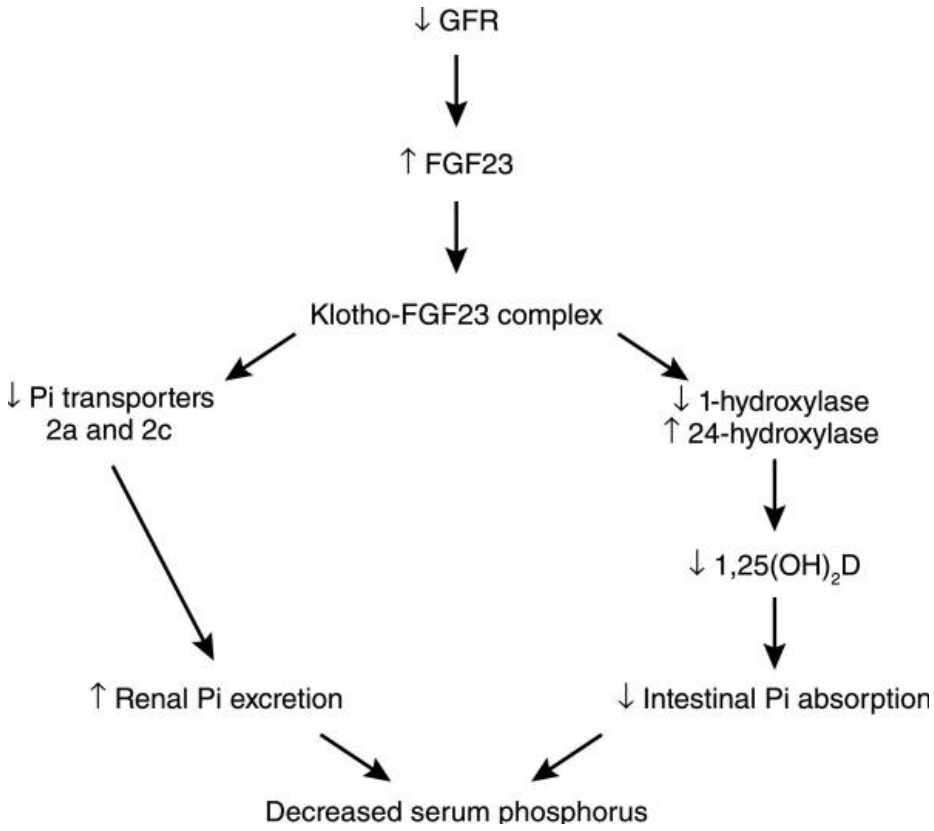

Figure 5.1. Role of fibroblast growth factor-23 in the regulation of blood phosphorus concentrations (retrieved from Blaine et al., 2015).

Under normal conditions, FGF23 suppresses PTH secretion, allowing the counterbalance of phosphaturia. However, klotho and FGFR1 (receptor) expression decreases as GFR deteriorates, which may induce resistance of FGF-receptors in the parathyroid gland (Felsenfeld et al., 2015; Steffan and Dobenecker, 2023, see Figure 5.1). At this stage, the extent

to which high levels of FGF23 contribute to the progression of CKD have not been established, and instead have just been reported as an indication of the disease and its progression.

Unfortunately, serum analyses in this study contradicted the well-established trend of FGF23 evident in past literature as there was no detected rise in FGF23 concentrations after increasing dietary Pi in T1 and T2. Instead, like that of serum P concentrations, serum FGF23 concentrations seemed to drop. The results indicated that a diet composed of a Ca:P ratio lower than the recommended reference range (T2) led to a significant drop in serum FGF23 levels. This is consistent with the drop in serum P concentrations, although surprising when considering serum FGF23 levels were higher in postprandial samples during the CON diet block. Figure 5.1 demonstrates how elevated serum P levels normally cause increased FGF23 secretion which in turn increases renal excretion of P. Given the highly unexpected findings with the FGF23 results of the present study, it was likely that there may have been a problem with the ELISA rendering the results unreliable. Whether this was due to poor assay sensitivity for feline FGF23 or an unexpected antibody cross-reactivity remains unclear. However, given the uncertainty around the FGF23 results, I advise these data be disregarded for this study.

5.6 Limitations of the study

Upon assessment of serum during each trial diet period, there were many significant differences between diet blocks (CON, T1, and T2) in many parameters. When exploring the effects of feeding diets containing low Ca:P ratios on Ca and P homeostasis, serum analyses provided some interesting results. The mean difference in serum parameters (Ca, P, Creatinine, Ca/P, PTH, and FGF23) were unexpected in some cases potentially due to the high amount of intra-cat variation for some parameters.

One of the main blood parameters currently used to assess renal function is FGF23. This study aimed to assess the serum concentrations of FGF23 to demonstrate the effect of feeding Pi on kidney function, and more specifically on renal excretion of P. Unfortunately, our FGF23 results were unreliable, and the problem seemed to be assay-specific. Seven of the FGF23 values obtained from the assay were at levels beyond the detection capability of the plate reader which questions the validity of the assay.

The selected blood sampling times may have limited our ability to observe changes in serum concentrations of P and PTH. As T1 and T2 contained highly soluble phosphoric acid, when using this dietary Pi source in future research it may be suggested that postprandial blood

samples are drawn earlier than three hours postprandially to account for potential early changes in serum concentrations.

The initial dietary transition proved to be more difficult than anticipated. The cats were unfamiliar with the texture of the trial diets which were slightly drier than expected. This may have been down to the thermal processing of the diet during manufacture. Water was added during feeding preparations to improve moisture levels and palatability of the diets for the cats. This may have restricted the level of food intake in the initial stages of the CON diet trial.

Chapter 6

Conclusions and future research

Chapter 6: Conclusions and future research

6.1 Key findings of this thesis

This thesis aimed to investigate the effects of feeding highly available Pi on indicators of feline renal health and assess the digestibility of three diets fed consecutively with increasing levels of highly bioavailable Pi in the form of H_3PO_4 and NaH_2PO_4. The CON diet consisted of organic P and contained a Ca:P ratio of 1.6:1. The T1 diet contained a Ca:P ratio that sat near the lower end of the recommended reference range (0.9:1), and the T2 diet contained a Ca:P ratio below the reference range (0.6:1). One of the main objectives was to investigate the effects of dietary Pi supply on Ca and P balance through blood, faecal and urine analyses.

The ratio of Pi sources in the T1 diet proved to be best for overall DM digestibility, although DM intake was highest with the T2 diet. The lowest Ca:P in the T2 diet proved to have the greatest impact when increasing apparent digestibility of P and led to the highest intake of both Pi sources despite the lower level of H_3PO_4 included in the T2 diet compared to T1. The CON diet did not contain Pi, which provided the baseline concentrations of parameters being measured to assess renal function. It is possible that the serum P concentrations increased earlier than 3 h postprandially as a result of the use of highly soluble phosphoric acid. This would suggest an increase in serum P may have taken place prior to the first postprandial blood sample taken three hours after food consumption. This would explain the rise in PTH and significant increase in renal excretion of P during the T2 diet block. With serum FGF23 being a major indicator of renal damage and now commonly used to assess the progression of CKD, it is unfortunate that the FGF23 results obtained during this study were unreliable. It was interesting to note that although levels of Pi added to the T2 were not considered to be relatively high in the sense of practical application, the rise in stimulation and secretion of PTH was great enough to cause a significant increase in renal excretion of P. High levels of renal P excretion for prolonged periods commonly lead to potential renal damage or dysfunction (Coltherd et al., 2019). The observed effect of feeding a Ca:P ratio of both 0.9:1 and 0.6:1 for a duration of 26 days was cause for concern, especially for T1 which contained a Ca:P ratio sitting near the lower end of the recommended reference range, suggesting that feeding a Ca:P ratio of 1:1 may not be as safe as we think. There is need for further investigations into feeding these Ca:P ratios for prolonged periods to improve our understanding of the potential detrimental effects of lifelong feeding of these diets. In addition to this, the addition of Pi in T2 caused a significant drop in serum creatinine concentrations when looking at the proportional change from the mean

baseline values. Together, serum creatinine concentrations and the renal P excretion observed may suggest there was potential renal dysfunction with the feeding of a Ca:P ratio of 0.6:1 in T2. The specific gravity and pH of urine decreased with the addition of Pi, with lowest levels observed during T2. Both of these measures are also indicative of a potential drop in renal function (Hart, 2005).

6.2 Future research:

In an extension of this current study, it may be worthwhile to investigate the effects of the same Pi sources (NaH2PO4 and H3PO4) fed in higher quantities. This study has allowed me to observe the effects of feeding a diet consisting solely of organic P versus diets with similar amounts of added Pi additives in slightly different ratios. The results of this study provided confounding results, with the added Pi causing serum P to drop, and renal P excretion increasing despite low levels of serum FGF23. In addition to this serum PTH concentrations were relatively high and serum creatinine concentrations were relatively low which does not align with the lower serum P concentrations observed in past studies. Replicating this study and feeding the same Pi additives at the same ratio but in larger quantities may allow us to observe the effects of Pi on a greater scale, still within a range suitable for practical application in commercial cat food. This is especially important in regards to this study, as renal excretion of P was high despite only moderate amounts of Pi being added. It would be interesting to see how this level of P excretion would change if serum P and corresponding FGF23 secretion were to also increase.

In an attempt to assess the effects of feeding high levels of Pi, there are adjustments that could be made to improve the trial diets and potentially increase diet intakes, in turn this may lead to more consistent and interpretable changes to indicators of renal function. Feeding these Pi additives in higher concentrations is likely to paint a clearer picture of the effect Pi has on the renal function of the cats, allowing us to further understand the implications regarding the lack of commercial diet restrictions around inclusion of P. Firstly, the aim would be to improve the moisture content and texture of the diet to encourage intake, as the initial transition onto the control diet was more difficult than anticipated. In order to do this, more water could be added to the diet prior to cooking to improve moisture levels. It may also be possible to make changes to thermal processing methods to improve the overall texture of the diets, in turn improving palatability and potentially increasing food intake. However, saying this the cats consumed sufficient amounts of all three diets that were manufactured for the present study.

Ultimately, there is a clear need for more research into high P diets, specifically high Pi diets, as I showed that when Pi is only feed at moderate levels, renal P excretion increased significantly and indicated the potential for renal dysfunction. This suggests that fed in greater quantities, this may lead to increased potential for renal damage in the cat. There is need for further investigations into feeding the Ca:P ratios present in T1 and T2 for prolonged periods due to the significant their significant effect on renal P excretion when only fed for 26 days. Feeding these Ca:P ratios, especially 0.9:1 which sits near the lower end of the recommended reference range for the safe upper limit of Ca:P ratios in adult feline diets, for an extended period may allow to improve our understanding of the potential detrimental renal effects of lifelong feeding of these diets. The treatment diets (T1 and T2) fed during this study did cause changes to renal markers over a very short period in young cats, which is cause for concern when considering the potential impact of feeding these same diets for longer to older cats.

It is important that further studies assessing the effects of feeding high levels of Pi be undertaken, especially that of the role of FGF23 as an indicator of deteriorating renal function and progression of CKD. Without further evidence of the negative health implications of feeding high levels of Pi, regulation of dietary P inclusion cannot be implemented in feline nutritional guidelines.

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