Investigating the domestic cat's apparent predisposition to chronic kidney disease and hyperthyroidism

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Abstract

Chronic kidney disease (CKD) and hyperthyroidism are two age-related diseases that are especially prevalent in domestic cats, compared to other species. This thesis explored potential reasons for the relatively high prevalence of these diseases in domestic cats. In particular, consumption of high levels of fish by domestic cats, relative to other companion animal species, such as dogs, and their wildcat ancestors, was considered. Study 1 investigated whether pro-oxidant minerals, such as arsenic and mercury, that are abundant in fish and which may instigate or progress CKD via oxidative stress mechanisms, may accumulate in domestic cat kidneys and associate with the presence of chronic interstitial nephritis (CIN). Although domestic cats, compared to dogs and Scottish wildcats, have greater arsenic concentrations in their kidneys and urine, suggesting increased arsenic consumption, the predominant arsenic species in fish-containing cat foods with high total arsenic content is arsenobetaine and therefore, presents low risk of renal toxicity. Arsenic concentration did not associate with the presence of CIN and mercury and iron concentrations were lower in cat kidneys, than in dogs. Domestic cat kidneys appeared to have depleted protective and/or antioxidant trace elements, including copper, zinc, rubidium and strontium, relative to dog kidneys, which may contribute to the increased prevalence of CIN in aged domestic cats' kidneys.

In other species, CKD may result from renal accumulation of intracellular lipid, which has a lipotoxic effect on the cells. However, the renal proximal tubule epithelial cells of healthy cats naturally contain an abundance of cytoplasmic lipid droplets, of which the composition is unknown. Study 2 aimed to quantify and characterise the intracellular lipids within the domestic cat's kidney, compared to kidneys of dogs and Scottish wildcats. Domestic cat and Scottish wildcat kidneys contained a similar amount of lipid and significantly more than dogs at all ages studied. However, domestic cat renal intracellular lipid droplets were found to contain an additional lipid molecular class, monoalkyldiacylglycerol (MADAG), which is comparatively reduced in Scottish wildcat kidneys and absent from dog kidneys. The branched-chain fatty acid

composition of the identified MADAGs, which are unusual in mammalian tissues, implies that they may originate from plant oils or fish-based ingredients that are incorporated into commercial pet foods. The cat, as an obligate carnivore whose ancestors evolved in an arid environment, may not be physiologically well-adapted to metabolise such ingredients. These findings indicate a fundamental tendency for felines to accumulate certain renal lipids, which may be exacerbated by chronic consumption of a commercial diet and domesticity.

The pathogenesis of feline hyperthyroidism is unknown, however, cats that eat canned, or fish-flavoured, foods may have increased risk of developing the condition. Such foods may have high iodine content. Due to the similarities between feline hyperthyroidism and human toxic nodular goitre (TNG), a similar aetiology of fluctuating dietary iodine intake was considered for feline hyperthyroidism. Study 3 explored whether domestic cats, consuming commercial pet foods and with relatively high intake of fish, are more likely than dogs to experience large fluctuations in their dietary iodine intake, which may underpin the high prevalence of feline hyperthyroidism. Although most complete diets that were analysed in this study complied with FEDIAF nutritional guidelines for iodine, a few had levels of iodine that exceeded the legal maximum, or were below the recommended minimum concentration. We established that even feeding identical foods over time would introduce significant flux in iodine intake, as the iodine content of subsequent batches varies considerably. Nonetheless, from a survey of cat owners, we found that most pet cats experience dietary changes, most commonly alternating between wet and dry foods and a variety of flavours on a daily basis. Iodine concentration did not associate with any particular flavour of food, including fish, or with wet foods packaged in cans versus other packaging types. This study highlights that cats may occasionally consume diets that are deficient or excessive in iodine content. However, urinary iodine analysis suggested that, on average, the daily iodine intakes of cats and dogs are not different. Nonetheless, hair iodine concentration, as a biomarker of long-term iodine intake, was different in euthyroid, versus hyperthyroid, cats. A causative

implication of dietary iodine in feline hyperthyroidism remains to be established, however.

These studies, which employed a series of histological, chromatographic and mass spectrometry methods, showed that domestic cats differ from dogs and Scottish wildcats with regard to the mineral and lipid composition of their kidneys, suggesting that a species and environmental interaction may be responsible for the high prevalence of CKD in domestic cats. Further studies are required to establish the origin and role of the unusual renal lipid in the domestic cat. Some ‘complete’ pet foods contain levels of certain minerals, which may be involved in CKD or hyperthyroidism development, that fall outside of the recommended range to ensure adequate nutrition. Thus, stricter regulation of pet food manufacture is required, to ensure that domestic cats receive an appropriate diet, which might include avoiding certain ingredients, which, in high levels that the cat is not evolutionarily adapted to consume, may promote disease.

Publications and Presentations

Publications

Davies, M., Jones, L., Alborough, R., Davis, C., Williams, C. & Gardner, D. S. 2017. Mineral analysis of complete dog and cat foods in the UK and compliance with European guidelines. *Scientific Reports*. DOI: 10.1038/s41598-017-17159-7.

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Abbreviations

AAFCO	American Association of Food Control Officers
AKI	Acute kidney insult/injury
ANOVA	Analysis of variance
AsB	Arsenobetaine
As(V)	Arsenate
As(III)	Arsenite
ATP	Adenosine triphosphate
BPA	Bisphenol A
BUN	Blood urea nitrogen
cAMP	Cyclic adenosine monophosphate
CIN	Chronic interstitial nephritis
CKD	Chronic kidney disease
CRM	Certified reference material
CV	Coefficient of variation
DAG	Diacylglyceride
d.f.	Degrees of freedom
DHA	Docosahexaenoic acid
DM	Dry matter
DMA	Dimethylarsinic acid
DNA	Deoxyribonucleic acid
DSH	Domestic shorthair
DPX	Dibutylphthalate polystyrene xylene
ECM	Extracellular matrix
eGFR	Estimated GFR
EMT	Epithelial-to-mesenchymal transition
EPA	Eicosapentaenoic acid
ESRD	End stage renal disease
FA	Free fatty acids
FAME	Fatty acid methyl ester
FAO	Fatty acid oxidation
FAOH	Hydroxyl-fatty acids
FEDIAF	Fédération Européene de l'Industrie des aliments pour Animaux Familiers / The European Pet Food Industry Federation
FFPE	Formalin-fixed paraffin-embedded
FS	Free sterol
fT3	Free triiodothyronine
fT4	Free thyroxine
GC	Gas chromatography
GC-FID	Gas chromatography-flame ionisation detector
GC-MS	Gas chromatography-mass spectrometry
GFR	Glomerular filtration rate
H_2O_2	Hydrogen peroxide
HE	Haematoxylin and eosin
HNO_3	Nitric acid
HPLC	High performance liquid chromatography
HPT	Hypothalamic-pituitary-thyroid (axis)
ICP-MS	Inductively-coupled plasma-mass spectrometry
IMS	Industrial methylated spirit

IQR	Interquartile range
IRIS	International Renal Interest Society
LoD	Limits of detection
LoQ	Limits of quantification
MADAG	Monoalkyldiacylglycerol
MMA	Monomethylarsinic acid
MT	Masson's trichrome
m/z	Mass-to-charge ratio
NRC	National Research Council
ORO	Oil Red O
PBB	Polybrominated biphenyl
PBDE	Polybrominated diphenyl ether
PCB	Polychlorinated biphenyl
PUFA	Polyunsaturated fatty acid
QC	Quality control
RAAS	Renin angiotensin aldosterone system
RBC	Red blood cell
REML	Restricted maximum likelihood
ROS	Reactive oxygen species
RPTEC	Renal proximal tubule epithelial cell
SDMA	Symmetric dimethylarginine
SE	Sterol ester
sn	Stereospecific number
SNGFR	Single nephron glomerular filtration rate
SOD	Superoxide dismutase
TAG	Triacylglyceride
TAs	Total arsenic
T3	triiodothyronine
T4	thyroxine
TBBPA	Tetrabromobisphenol A
TIBC	Total iron binding capacity
TIC	Total ion chromatogram
TLC	Thin layer chromatography
TLE	Total lipid extract
TMAH	Tetramethylammonium hydroxide
TNG	Toxic nodular goitre
TPO	Thyroid peroxidase
TRH	Thyrotropin-releasing hormone
TSH	Thyroid stimulating hormone
UK	United Kingdom
UoN-VPS	University of Nottingham Veterinary Pathology Service
UP	Unknown peak
UPLC-MS/MS	Ultra-performance liquid chromatography-tandem mass spectrometry
USA	United States of America
VLFA	Very long chain fatty acid
χ^2	Chi-square

Chapter 1. Introduction

1.1. Pet food regulation

All companion animals should be fed a complete and balanced diet, meaning one that provides all the required nutrients for a daily ration in the correct amounts and proportions (Regulation EU No. 767/2009, adapted to pet food)(FEDIAF, 2019). Nutritional needs vary with species, animal size, level of activity and life-stage, such as growth, pregnancy, lactation and adult maintenance (Zicker, 2008). Disease can also affect an animal's ability to absorb and use nutrients and therefore, can alter nutritional requirements. Indeed, treatment of many diseases, such as chronic kidney disease (CKD) (Ross et al., 2006, Elliott et al., 2000) and hyperthyroidism (van der Kooij et al., 2014, Hui et al., 2015), includes prescription diets with higher or lower levels of certain nutrients. A nutritional requirement is defined as the lowest concentration of a particular nutrient that will support the physiological functions of a particular species at a specific life-stage, for which the food is designed (Zicker, 2008). In contrast, nutritional recommendations cover a range of concentrations of specific nutrients that a complete pet food should contain, in order to meet the animal's nutrient requirement, while avoiding complications associated with excess intake of the nutrient (Zicker, 2008).

The European Pet Food Industry Federation (FEDIAF, Fédération Européene de l'Industrie des aliments pour Animaux Familiers) sets guidelines for the maximum and minimum levels of nutrients that complete and complementary pet foods should contain. FEDIAF has 15 member associations of the pet food industry from 18 countries; Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Hungary, Ireland, Italy, Netherlands, Norway, Poland, Romania, Spain, Sweden, Switzerland and the United Kingdom (UK), as well as five company members (Affinity Petcare, Hill's Pet Nutrition, Mars Petcare, Nestlé Purina PetCare and Wellpet). FEDIAF aims to ensure that pet foods are well-balanced and nutritious. To achieve this, FEDIAF collaborates with a Scientific Advisory Board, including the American Association of Food Control Officers (AAFCO) and the National Research Council (NRC), and regularly updates its recommendations for nutrient levels in pet foods based

on the latest, peer-reviewed scientific research (Zicker, 2008). In turn, pet food manufacturers that are FEDIAF members should adjust their products in compliance with the latest FEDIAF nutritional guidelines (FEDIAF, 2019).

FEDIAF provides pet food manufacturers with guidelines for nutritional minimum and maximum recommended levels of macronutrients (e.g. protein and fat), vitamins and 12 minerals (calcium, phosphorus, potassium, sodium, chloride, magnesium, copper, iodine, iron, manganese, selenium and zinc) as well as calcium-to-phosphorus ratio (Ca:P), in complete pet foods (FEDIAF, 2019). The minimum recommended level is defined as the least amount of a particular nutrient that a complete pet food should contain, to ensure adequate intake of the nutrient. This means that by consuming a food that fulfils the minimum recommendation of a nutrient, the nutrient requirement of nearly all healthy individuals of the animal species for which the food is intended, should be met (Zicker, 2008, FEDIAF, 2019). The nutritional maximum recommendation is the highest concentration of a nutrient in complete pet food that is not associated with adverse effects in nearly all healthy animals of the species for which the food is intended (Zicker, 2008). Nutrient levels greater than the nutritional maximum may be safe, however, there is no scientific data to evidence this (FEDIAF, 2019). Furthermore, FEDIAF reports a legal maximum for some nutrients which are added to pet foods as nutritional additives (e.g. trace elements and vitamin D). This is a compulsory upper limit of a nutrient that pet foods must not exceed, in accordance with EU legislation (Regulation 1831/2003/EC) but only applies when the nutrient is added as a feed additive; if the total amount in the pet food comes entirely from feed materials, the nutritional maximum applies instead (FEDIAF, 2019). For the heavy metals, arsenic, cadmium, chromium, lead and mercury, which are toxic to the environment and biological systems even at low concentrations (Tchounwou et al., 2012), the recommended maximum levels are governed by the European Commission (EU directive 2002/32/EC).

1.2. Some pet foods do not meet recommendations

To be labelled as ‘complete’, foods have to be tested during and after manufacture and even after periods of storage in different conditions, to monitor for nutrient losses. This should ensure that nutritional recommendations and thus nutrient requirements of the intended animal at a particular life-stage are fulfilled (Zicker, 2008). In spite of the regulations surrounding pet food manufacture, previous research, conducted by us, identified some (94% [91/97] wet and 61% [46/80] dry) complete cat and dog foods that are commercially available in the UK with levels of certain minerals above the maximum and below the minimum FEDIAF guidelines (Davies et al., 2017a). Some diets also had Ca:P imbalance, which could be detrimental to the animal’s health if fed consistently for long periods of time (Davies et al., 2017a). In a Brazilian study of wet foods, phosphorus levels in some dog foods and sodium levels in cat and dog foods exceeded the FEDIAF-defined safe upper limits (Brunetto et al., 2019). Our previous analysis of mineral concentrations in pet foods revealed that wet foods have greater sodium and phosphorus content than dry foods (Davies et al., 2017a). Some of these UK-sold, wet foods for cats had sodium levels that exceeded the safe upper limit, and some wet foods for dogs exceeded the FEDIAF nutritional maximum recommendation, too (Davies et al., 2017a). There is neither a nutritional, nor a legal, maximum phosphorus concentration in the FEDIAF guidelines for cat foods, however, it is noted that high phosphorus intake may impact kidney function (FEDIAF, 2019). Adverse effects on renal function have been observed in healthy cats with dietary phosphorus intake as low as >3.0g/1000kcal metabolisable energy (ME) (Dobenecker et al., 2018). Excessive phosphorus intake may be linked with development of feline CKD (Boswald et al., 2018, Alexander et al., 2018). It is believed that high levels of sodium and phosphorus in wet cat and dog foods are due to their high meat content (Brunetto et al., 2019) but pet foods may also contain more bioavailable inorganic phosphorus salts due to inclusion of additives (Finco et al., 1989). Higher bioavailability, coupled with increased intake, of phosphorus may further increase the risk of renal toxicity (Coltherd et al., 2019). Additionally, high sodium may increase phosphorus absorption (Marks et al.,

2015), thus diets with levels of sodium exceeding the safe upper limit, may contribute to renal disease. Indeed, higher levels of phosphorus and sodium, as in maintenance diets for cats, may cause progression of existing renal disease, while renal therapeutic diets aim to slow the decline in renal function via reduced phosphorus and sodium content (Ross et al., 2006).

Furthermore, in line with other studies that have measured high levels of arsenic in fish (Filippini et al., 2018), fish-containing pet foods analysed by Davies et al. (2017a) had higher arsenic levels than foods without fish; arsenic concentrations in three foods were above the nutritional maximum and two of these were above the legal maximum. There was a positive correlation between the percentage of fish and the amount of arsenic in the foods (Davies et al., 2017a). High levels of mercury (Cheng et al., 2009) and iodine (Dahl et al., 2004) are also found in fish, however, these minerals were not measured in pet foods previously by us. Others have, however, found that some wet cat foods, particularly those with fish as the main ingredient (Luippold and Gustin, 2016), have levels of total mercury greater than the European legal maximum of 0.34 mg/kg dry matter (DM; EU directive 2002/32/EC, Commission Regulation (EU) 2015/186 of 6 February 2015). Mercury levels exceeding the legal maximum have been reported in foods for both cats and dogs, but particularly in wet foods, as opposed to dry foods (Paulelli et al., 2018). For clarification purposes, dry foods are those that contain less than 11% water, while wet foods have higher water content. Wet foods can be further divided into semi-moist, with 25-35% water content, and moist, with 60-87% water content (Zicker, 2008). Wet foods may be packaged in cans, pouches or trays. Cat foods, especially wet foods and predominantly those packaged in cans, have been found to have highly variable iodine content (Edinboro et al., 2013, Ranz et al., 2002, Johnson et al., 1992). The iodine content of some of these foods exceeds the recommended upper limit set for adult cat diets and others fail to meet the minimum recommended iodine concentration (Johnson et al., 1992, Edinboro et al., 2013). Thus, domestic cats may have variable iodine intakes from their diet and indeed more variable iodine intake than cats in the wild that do not eat canned foods or fish. To our knowledge, iodine has not been measured in pet foods that are commercially available in the UK.

Therefore, one element of this programme of study, investigating potential reasons for the high prevalence of CKD and hyperthyroidism in cats relative to other species, is to measure mercury (Chapter 2, Study 1) and iodine (Chapter 4, Study 3) concentrations in pet foods that are commercially available in the UK, to investigate an association with fish content and compliance with minimum and maximum nutritional recommendations and legal regulations. Additionally, arsenic speciation of pet foods with known high total arsenic content will be conducted (Chapter 2, Study 1), to determine the risk of toxicity from high arsenic intake due to high consumption of fish.

1.3. Origin of the domestic cat: evolution and domestication

Primates and carnivores are believed to have diverged from a common ancestor about 95 million years ago (Murphy et al., 2007). The cat and dog are both members of carnivora, however, these two species diverged from a common carnivore ancestor around 55 million years ago (Murphy et al., 2007, Springer et al., 2003). Whole genome mapping has shown that the domestic cat's genome is relatively well conserved since its divergence from humans, compared to other species (Murphy et al., 2000) including dogs (Murphy et al., 2007, Springer et al., 2003). Genes within the cat's genome have primarily been reordered, via intrachromasomal inversions. In comparison, the dog genome has experienced greater change by interchromasomal modifications. The reduced amount of interchromasomal change in the cat's genome is likely to be responsible for the greater conservation of the common mammalian ancestor's genetics in the cat, compared to the dog (Murphy et al., 2007, Lindblad-Toh et al., 2005).

Earlier domestication and thus earlier exposure to selection pressures in dogs, compared to cats, may be partly responsible for the greater rate of change in the canine, than the feline, genome, compared to their shared carnivore ancestor (Nash et al., 2001, Axelsson et al., 2013). Domestic dogs (*Canis familiaris*) are believed to have originated from the grey wolf, which gradually became domesticated around 15,000 years ago in East Asia (Savolainen et al., 2002) and 18,800–32,100 years ago in Europe (Thalmann et al., 2013). In comparison, domestication of the cat occurred relatively recently. Based on

DNA analyses, the domestic cat (*Felis silvestris catus*) is thought to have evolved from the Near Eastern wildcat (*Felis silvestris lybica*) between 107,000 and 155,000 years ago (Driscoll et al., 2007). However, based on archaeological findings, domestication of the wildcat, which is the increasing co-habitation with humans, began much later; approximately 9500 years ago (Vigne et al., 2004). Cats started living amongst humans to exploit grain stores, which attracted rodent prey. In doing so, farmers appreciated the wildcats for vermin control and the cats became more tolerant of humans. As cats became popular with humans, they were traded around the world (Hu et al., 2014). The 30–40 breeds of domestic cat that exist today developed over a much shorter time-frame, approximately the last 150 years, as humans interfered with cat breeding, selecting for aesthetic, rather than functional, traits (Montague et al., 2014).

While domestic cats today share a common ancestor with the Near Eastern wildcat, other wildcat subspecies do exist. These include the European wildcat (*F. s. silvestris*), South African wildcat (*F. s. cafra*), Central Asian wildcat (*F. s. ornata*), Chinese desert cat (*F. s. bieti*) and Scottish wildcat (*F. s. grampia*). The wildcat subspecies are thought to have shared a single common ancestor 230,000 years ago (Driscoll et al., 2007). Domestic cats can be distinguished from wildcats by their behaviour, tameness and coat colour (Driscoll et al., 2007). However, wildcats typically have a tabby pattern, making them difficult to differentiate from tabby domestic cats, particularly those that are feral (Driscoll et al., 2007). Moreover, introgression, which is the transfer of genetic information between species due to hybridisation between them and repeated back-crossing, has been evidenced between (likely feral) domestic cats and the European (Steyer et al., 2018), African, Central Asian (Driscoll et al., 2007) and Scottish (Beaumont et al., 2001) wildcats. This further increases the difficulty in distinguishing wildcats from domestic cats and threatens the conservation of the wildcat subspecies (Steyer et al., 2018). Nonetheless, in the UK, wildcats have not inhabited England, Wales or southern Scotland since the mid-19th century; in fact, the Scottish wildcat is endemic to northern Scotland (Taylor, 1946). Therefore, introgression with domestic cats in other parts of the UK is unlikely. Indeed, organisations such as Wildcat Haven are

working to conserve the Scottish wildcat, mainly by preventing hybridisation due to breeding between Scottish wildcats and feral domestic cats. The Scottish wildcat can be distinguished from domestic cats, hybrid cats and other wildcats based on coat colour, tail shape and markings, skull measurements and gut length (Corbett, 1979). Yet, there are few chromosomal differences between the domestic cat and other members of the Felidae family (Wurster-Hill and Centerwall, 1982, O'Brien et al., 2002). Therefore, throughout this programme of work, I use the Scottish wildcat as a within-species comparator for domestic cats, but without the environment of domesticity. I have used dogs as a comparator for the domestic cat within the domestic environment but with reduced incidence of renal and thyroidal disease.

1.4. Cats are obligate carnivores

While domestication of the dog was perhaps aided by adaptation to starch-rich diets and moving away from the carnivorous diet of their wolf ancestors, allowing dogs to benefit from the agricultural environment in human settlements (Axelsson et al., 2013), domestic cats have retained their hyper-carnivorous ancestors' dietary needs. Being from wildcat lineage, cats are adapted to hunt and consume prey and have thus evolved to survive on protein-replete, carbohydrate-deplete diets (Morris, 2002). Cats are, in fact, termed obligate carnivores (Wester et al., 2015), meaning that they have specific nutrient requirements that can only be met by consuming tissues of other animals (Morris, 2002). This is due to different activity levels, or an altogether lack, of specific enzymes involved in the metabolism or endogenous synthesis of certain nutrients in cats, compared to other species (Morris, 1985, Rogers and Phang, 1985, Edgar et al., 1998, Morris, 2002, How et al., 1994, Morris, 1999, Suhadolnik et al., 1957, Nishizuka and Hayaishi, 1963).

Nutrients are considered essential when they optimise or maximise a physiological function or maintain a metabolic variable, such as the circulating concentration of the particular nutrient (Morris and Rogers, 1994). Essential nutrients for cats, whether domestic or wild, which are non-essential in the diet of most other animals, include the amino acids, arginine and taurine and the vitamins A, D and B3 (Morris, 2002, Morris, 1999). Cats also require the fatty

acid, arachidonic acid, or higher levels of its precursor, linoleic acid, in their diets, due to low activity of delta-6 desaturase enzymes involved in the conversion (Bauer, 2006). Furthermore, domestic cats have been found to have upregulated systems for lipid metabolism compared to other species, which may be an adaptation to the strictly carnivorous diet of the cat (Montague et al., 2014). Indeed, cats are able to tolerate high dietary levels of saturated and polyunsaturated fatty acids, associated with high meat consumption (Thompson, 2008, Verbrugghe and Hesta, 2017), without exhibiting changes in plasma lipid concentrations (Butterwick et al., 2012) as would occur in humans, often alongside cardiovascular complications (Lind et al., 2018). Moreover, the feline kidney is laden with intracellular lipid, without apparent evidence of renal damage (Bargmann et al., 1977) as occurs in other organs and in the kidneys of other species (Bobulescu, 2010, Escasany et al., 2019). Notwithstanding, the origin and role of intracellular lipid in the feline kidney are unestablished.

Additionally, adult cats require two- and three-times more protein for maintenance than omnivorous species, such as dogs and rats, respectively (National Research Council, 2006, National Research Council, 1995). This is because cats, compared to other mammalian species, rely primarily on amino acids, rather than glucose, for energy and have a lower rate of endogenous protein synthesis, to conserve amino acids for energy production (Wester et al., 2015, Russell et al., 2003, Eisert, 2011). Furthermore, cats are unable to downregulate protein metabolism as effectively as other species, so endogenous nitrogen excretion remains high in cats, even if they consume a low protein diet (Hendriks et al., 1997, Morris, 2002). These are potential obligate carnivore adaptations to a high protein, low carbohydrate diet.

1.4.1. Greater consumption of certain nutrients in the obligate carnivore's diet

Due to almost exclusive consumption of meat, obligate carnivores, such as the cat, have evolved with essential requirements for certain nutrients that are found solely in animal tissues. Therefore, cats must consume tissues of other

animals in order to meet these essential nutrient requirements. Consequently, a greater proportion of the diet of cats, compared to omnivorous species such as dogs, is meat. Thus, alongside essential nutrients, cats may also consume high levels of other nutrients that are more abundant in animal tissues than in plant-based ingredients. For example, meat, particularly red meat such as beef, contains greater amounts of high quality protein, relative to vegetable matter (Wu et al., 2014, Mafra et al., 2018, Williams, 2007). Hence, red meat may be incorporated into feline diets more readily than canine diets, due to the higher protein requirement in cats. Yet, greater consumption of red meat, although almost entirely pork, and higher protein intake, independently, have been associated with renal disease progression in humans (Lew et al., 2017). This may be linked with the high renal acid load provided by animal- versus plant-proteins (Remer and Manz, 1995, Remer, 2001). Metabolic acidosis, resulting from increased dietary acid load, promotes renal disease progression (Rebholz et al., 2015, Banerjee et al., 2015). Interestingly, replacing one serving of red meat with any other protein source has been shown to significantly reduce the risk of end stage renal disease (ESRD) developing; the risk of ESRD was reduced most by substituting with poultry, then plant proteins, and least with fish (Lew et al., 2017).

Furthermore, meat is a good source of bioavailable haem-iron, zinc, potassium, phosphorus (Karp et al., 2012a) and magnesium (Wyness, 2016, Mafra et al., 2018). Plant material not only contains lower concentrations of these minerals; the iron (Wyness, 2016) and phosphorus (Karp et al., 2012b) are less bioavailable too. However, while these minerals are essential in the diet, they may also exert negative effects if consumed in excess. For example, iron has pro-oxidant activity and therefore, in excess, promotes ROS formation and oxidative damage to tissues (Gutteridge, 1986, Halliwell and Gutteridge, 1984, Nankivell et al., 1994, Nakatani et al., 2018). Iron-deficient diets or diets with iron chelators can prevent, or reduce, tubulointerstitial damage in the kidneys (Alfrey et al., 1989, Nankivell et al., 1994, Alfrey, 1992), which corroborates the role of iron in the pathogenesis of renal disease.

Additionally, fish is incorporated into pet foods as a source of taurine (Roe and Weston, 1965), vitamin D (Schmid and Walther, 2013) and essential minerals,

such as iodine (Dahl et al., 2004) and selenium (Marriott et al., 2019). Due to the cat's higher requirement for some of the nutrients found in high levels in fish and a believed taste-preference for fish (Houpt and Smith, 1981), domestic cats may be fed foods containing fish more commonly than dogs. Domestic cats certainly eat more fish than their wildcat ancestors who were adapted to an arid environment where fish consumption would be a rarity. Thus, domestic cats may also have greater dietary intake of other minerals that are found in high levels in fish, including arsenic (Marriott et al., 2019), mercury (Cheng et al., 2009) and iodine (Nerhus et al., 2018). Arsenic and mercury have no known biological role and can be toxic to tissues, such as the kidney, even at low levels of exposure (Tchounwou et al., 2012). Indeed, arsenic and mercury have both been associated with renal disease in humans (Chang and Singh, 2018, Park and Zheng, 2012). The only role for iodine in the body is in the production of thyroid hormone; departures from recommended levels of dietary iodine intake are known to cause thyroid disorders in humans (Laurberg et al., 2010). Cats may be maladapted to tolerate high levels of these nutrients, and hence fish, in their diet due to their evolution from the desert-dwelling wildcat and consequent loss of certain metabolic pathways (Montague et al., 2014).

1.5. Chronic kidney disease and hyperthyroidism are particularly common in domestic cats

Not only does the domestic cat differ in its dietary requirements, additionally, the cat appears to have a predisposition to certain diseases, particularly CKD and hyperthyroidism. The prevalence of these diseases is far greater in cats than in other species (Brown et al., 2016, Mooney and Peterson, 2012). These diseases are also more common in older animals (Peterson et al., 1994, Bartges, 2012), suggesting they may develop due to chronic exposure to risk factors over the animal's lifetime. Dietary risk factors for both conditions have been proposed. As discussed, high dietary intake of certain minerals may be involved in the development of these diseases in cats, as in other species such as humans (Laurberg et al., 2010, Chang and Singh, 2018, Park and Zheng, 2012). However, this requires investigation. Therefore, here, we investigate

associations of pro-oxidant minerals (Study 1) with feline CKD, and dietary iodine with feline hyperthyroidism (Study 3).

1.5.1. Chronic Kidney Disease

Chronic kidney disease is a long-term and progressive condition, where one or both kidneys are structurally and/or functionally impaired, usually due to damage occurring over a period of three months or more (Polzin, 2017). When working normally, the kidneys filter the blood and excrete foreign substances, such as drugs; waste products of metabolism, such as urea and creatinine; and excess water and electrolytes, which may have been obtained through the diet, from the body via the urine (O'Callaghan, 2017). Simultaneously, the kidneys reabsorb necessary components of the blood in order to maintain homeostasis. In this way, the kidney has important roles in regulating body fluid volume and blood pressure, body fluid osmolality to maintain normal cell volume, and acid-base balance to enable normal metabolic processes (O'Callaghan, 2017). The kidney also has endocrine functions; it produces and secretes erythropoietin to control the production of red blood cells (RBCs) (Erslev, 1974), renin for the control of blood pressure (Siragy and Carey, 2010) and active vitamin D (1,25-dihydroxyvitamin D_3), which is important in calcium and phosphate metabolism (Norman et al., 1971, Gray et al., 1971, O'Callaghan, 2017).

The kidney broadly contains two regions, the cortex on the outside and the medulla internally (O'Callaghan, 2017). Nephrons, the functional units of the kidney, span both the cortex and the medulla, being surrounded by the cortical and medullary renal interstitium (O'Callaghan, 2017). The renal interstitium contains cells, including fibroblast-like cells in the cortex and outer medulla, lipid-laden interstitial cells in the inner medulla, macrophages, interstitial dendritic cells and pericytes (Lemley and Kriz, 1991). These cells produce the extracellular components of the interstitium, which include collagen fibres, proteoglycans, glycoproteins and interstitial fluid (Lemley and Kriz, 1991). The renal interstitium provides structural support for the kidney, contributes to hormone synthesis and regulates nearly all of the exchanges that occur between the tubules and the vasculature along the nephron (Lemley and Kriz,

1991). Each nephron comprises a glomerulus and a series of renal tubules which lead into the collecting duct and ultimately drains urine through the renal papilla into the renal pelvis of the ureter (Figure 1) (O'Callaghan, 2017). The glomerulus is an extensive capillary system, surrounded by Bowman's capsule, a part of the glomerular filtration barrier through which blood contents are selectively filtered into Bowman's space and then flow into the renal tubules (O'Callaghan, 2017, Arif and Nihalani, 2013). The renal tubules, a continuum of proximal tubule (convoluted then straight), loop of Henle (thin descending limb, then thin ascending limb, then thick ascending limb) and distal convoluted tubule, are where substances, including nutrients such as mineral elements, are secreted into, and reabsorbed out of, the tubular fluid within the tubular lumen (O'Callaghan, 2017, Rhodin, 1958).

Figure 1. Structure of the kidney and nephron.

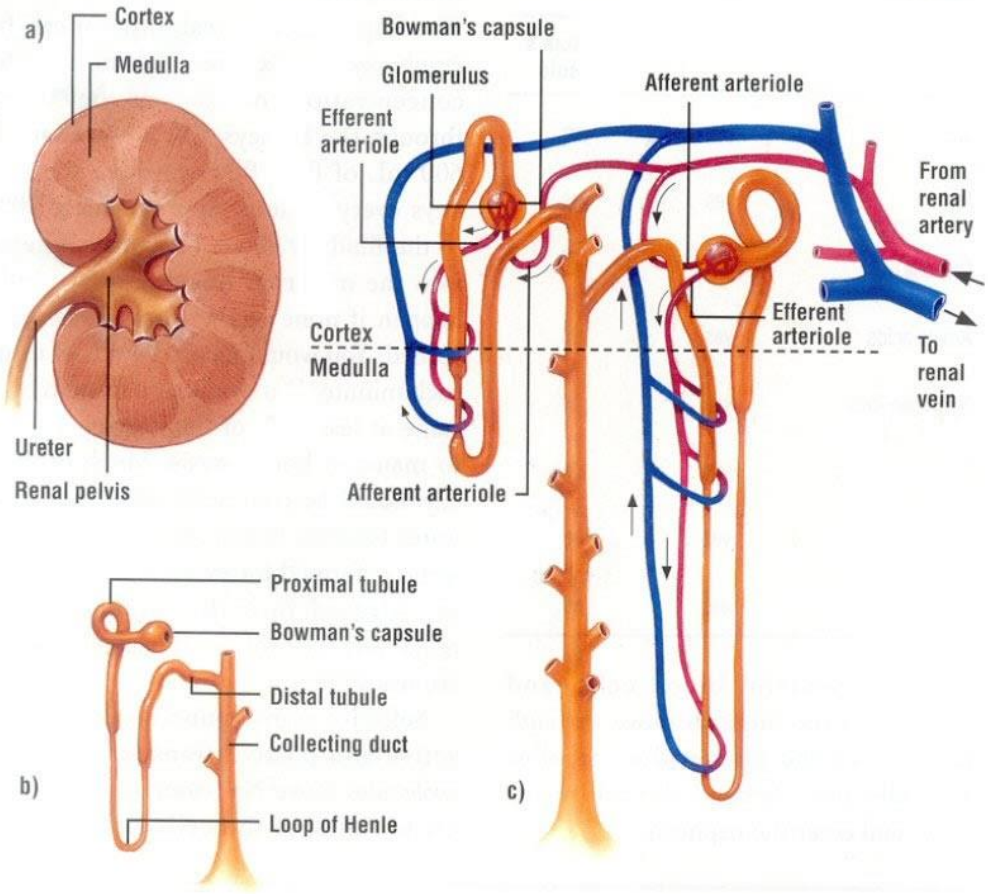


Figure 1. (a) A sagittal section through the kidney, showing the renal cortex and medulla, renal pelvis and ureter. **(b)** A schematic diagram of the renal tubule system of the nephron. **(c)** A more detailed depiction of the nephron, indicating the location of nephron segments in the renal cortex and medulla and showing the flow of blood through the renal vascular network. Image taken from Google images labelled for non-commercial reuse (http://drkupe.blogspot.com/2011/02/acute-kidney-injury.html, accessed 18th May 2020).

Each kidney has a large number of nephrons, which, along with the size of the glomeruli and thus the filtration surface area, correlates with the body surface area of the animal (Kunkel, 1930, Nyengaard and Bendtsen, 1992). The number of functioning nephrons declines with age, a phenomenon termed senescence (Denic et al., 2016, Tan et al., 2009), and when the kidney is damaged, as in CKD (Wang and Garrett, 2017). The functional nephrons that remain have to work harder in order to maintain the kidneys' regulatory functions. This has been shown experimentally in cats (Brown and Brown, 1995), dogs (Brown et al., 1990, Brown et al., 1991) and Sprague-Dawley rats

(Finn, 1982), and in senescent renal allografts from aged human donors (Tan et al., 2009) to involve increases in single nephron glomerular filtration rate (SN-GFR) and intra-glomerular hydrostatic pressure, as well as glomerular hypertrophy, of the remnant nephrons. At first, renal function remains stable but eventually, the remnant nephrons' ability to adapt to the increased workload is overwhelmed. As a result, the remnant nephrons too become damaged, total glomerular filtration rate (GFR) decreases and renal function declines (Denic et al., 2016, Bartges, 2012). The pathology underlying the non-functionality of the nephrons includes glomerulosclerosis, tubular atrophy and interstitial inflammation and fibrosis (Denic et al., 2016). Consequently, the kidney's filtration, reabsorption and secretion functions are impaired, which can result in fluid volume and electrolyte disturbances and acid-base imbalances (Bartges, 2012). Hence, patients with CKD commonly present with polyuria and polydipsia, dehydration, anorexia, loss of body weight, body condition and muscle mass, lethargy, halitosis and vomiting (Piyarungsri and Pusoonthornthum, 2017, Bartges, 2012). Systemic hypertension is also common, with a reported 61% incidence in cats with CKD (Kobayashi et al., 1990). Furthermore, hormone production is affected during renal disease, which can lead to conditions such as anaemia and secondary hyperparathyroidism (Bartges, 2012).

However, the degree of functional impairment does not always reflect the extent of structural disruption as, in some cases, CKD is worsened by concurrent pre- and/or post-renal complications. Unlike CKD, these extra-renal complications may be reversible, so managing these can improve the condition of the patient with renal disease (Bartges, 2012). However, often, the adaptive mechanisms that have established to maintain homeostasis, are actually maladaptive in the long-run and result in a vicious cycle of ongoing damage to the kidneys, further loss of nephrons and continued progressive decline in renal function (Polzin, 2017). Indeed, CKD is irreversible and treatment can only slow the progression to increase survival time with CKD, and ameliorate the clinical signs to improve quality of life, rather than cure the disease. Therefore, the earlier that CKD is diagnosed and treatment is initiated, the

greater the chance of slowing the disease progression (Greene et al., 2014, Brown et al., 1997).

Once diagnosed, CKD in cats and dogs can be staged according to a system devised by The International Renal Interest Society (IRIS) (International Renal Interest Society, 2019). The IRIS staging system is based on repeated measurements of serum creatinine and symmetric dimethylarginine (SDMA) concentrations in the fasted, stable CKD patient (Relford et al., 2016, International Renal Interest Society, 2019). Both serum creatinine and SDMA increase as CKD progresses through the IRIS stages (Table 1), reflecting decreasing GFR (Sparkes et al., 2016). CKD can be further sub-staged based on the degree of proteinuria, as indicated by the urine protein to creatinine ratio, and systolic blood pressure (Table 2) (International Renal Interest Society, 2019). Regularly discerning the stage of CKD is useful for monitoring the disease progression and can help to inform the most appropriate treatment options for managing the condition as it progresses in the particular patient (Polzin, 2013). For example, initiation of feeding a renal diet can be effective for cats in IRIS stages 2 and 3, but perhaps not in later stages of CKD (Ross et al., 2006). Moreover, proteinuria is not effectively reduced by feeding a renal diet so other management options, such as inhibiting the renin-angiotensin-aldosterone system (RAAS) using angiotensin-converting enzyme inhibitors, angiotensin receptor blockers or aldosterone receptor antagonists, may be required in proteinuric patients (Maniaki and Finch, 2018). Staging and sub-staging can also be used to determine the prognosis for the patient: survival time decreases with increasing severity of the disease and therefore, with progression through the IRIS stages and sub-stages (Sparkes et al., 2016).

IRIS stage	Serum creatinine					Serum SDMA		
	µmol/l		mg/dl		Description	µg/dl		Description
	Dogs	Cats	Dogs	Cats		Dogs	Cats	
1	<125	<140	<1.4	<1.6	Normal	<18	<18	Normal or mild increase
2	125–250	140–250	1.4–2.8	1.6–2.8	Mild azotaemia	18–35	18–25	Mild increase
3	251–440	251–440	2.9–5.0	2.9–5.0	Moderate azotaemia	36–54	26–38	Moderate increase
4	>440	>440	>5.0	>5.0	Severe azotaemia	>54	>38	Severe increase

Table 1. The International Renal Interest Society staging system for CKD in dogs and cats, based on serum creatinine and symmetric dimethylarginine (SDMA) concentrations. Adapted from www.iris-kidney.com.

Urine protein:creatinine		Proteinuria sub-stage	Systolic blood pressure (mmHg)	Blood pressure sub-stage	Risk of future target organ damage
Dogs	Cats		Dogs & Cats		
			<140	Normotensive	Minimal
<0.2	<0.2	Non-proteinuric	140–159	Pre-hypertensive	Low
0.2–0.5	0.2–0.4	Borderline proteinuric	160–179	Hypertensive	Moderate
>0.5	>0.4	Proteinuric	≥180	Severely hypertensive	High

Table 2. The International Renal Interest Society sub-staging systems for CKD in dogs and cats, based on the degree of proteinuria, as assessed by urine protein:creatinine measurement, and systolic blood pressure. Adapted from www.iris-kidney.com.

1.5.1.1. Prevalence of CKD in cats and dogs

Feline CKD is commonly diagnosed in small animal veterinary practice (Brown et al., 2016). In the domestic cat population as a whole, the prevalence of CKD has been reported as between 1.6% and 20% (Polzin, 2017) and as high as 50% (Marino et al., 2014), while in dogs it ranges between 0.5% and 7% (Polzin, 2017). The variation in prevalence estimates reflects the differences between patient populations studied, however, the consensus is that CKD is more common in cats than in dogs (Brown et al., 2016). CKD can occur at all ages, however, due to the progressive nature of the disease and the difficulty in diagnosing CKD before 75% of renal function has been lost, owing to the substantial renal functional reserve (Brown et al., 1997), it is more commonly seen in older animals (Bartges, 2012). It has been reported that the prevalence

of CKD in domestic cats positively correlates with increasing age from 5-6 years onwards (Brown et al., 2016). Lulich et al. (1992) studied four different age groups of cats and found the same pattern of increasing prevalence with increasing age: 13% of cats <4 years, 24% of cats aged 4-10 years, 31% of cats aged 10-15 years and 32% of cats ≥15 years old had CKD. In contrast, Marino et al. (2014) conducted a similar investigation and found that CKD prevalence did not increase with age in cats up to 15 years old, but increased thereafter, being as high as 80% in cats older than 15 years. Although CKD is more common in older animals and renal senescence occurs with age, CKD is not an inevitable consequence of aging; GFR decreases naturally with aging but this does not associate exactly with age-related renal lesions (Denic et al., 2016). Aging alone does not instigate CKD but it is possible that the renal changes that occur with age increase the susceptibility to the development of the disease (Brown et al., 2016). Nevertheless, it has been suggested that the age-related renal changes are an adaptation to increase longevity in cats, since cats with tubulointerstitial disease are older at death than cats without renal tubulointerstitial lesions (Lawler et al., 2006).

1.5.1.2. Renal pathology in feline CKD

Compared to the normal feline kidney, those with CKD are smaller and, macroscopically, appear granular with a “pitted” surface (Brown et al., 2016, Lucke, 1968). Microscopically, the most common renal lesions in cats with CKD are interstitial inflammation and fibrosis (McLeland et al., 2015). Acute inflammation involves polymorphonuclear leukocytes, such as neutrophils, and macrophages (Ysebaert et al., 2000). However, as the inflammatory reaction continues, mononuclear cells, primarily lymphocytes but also plasma cells and macrophages, take over (Yabuki et al., 2010, McLeland et al., 2015). This inflammatory infiltrate in the renal interstitium, together with fibrosis and tubular atrophy, is termed chronic interstitial nephritis (CIN) and occurs in all cats with CKD. However, the severity of these lesions is greater in the later stages of the disease process (Brown et al., 2016).

In a study that scored the renal lesions in cats at various stages of CKD, interstitial inflammation and fibrosis scores were found to correlate

(Chakrabarti et al., 2013). In that study, interstitial fibrosis score correlated with the severities of various markers of renal dysfunction, including azotaemia, hyperphosphatemia, anaemia and proteinuria. Interstitial inflammation scores also correlated with azotaemia, hyperphosphatemia and anaemia, although these correlations were weaker than those with fibrosis scores (Chakrabarti et al., 2013). In contrast, another study found increasing amounts of interstitial inflammation with IRIS stage progression in cats, reflecting a successive reduction in renal function with increasing inflammation (McLeland et al., 2015). There was significantly more fibrosis in stage 4 than in all other stages of CKD, however, the amount of fibrosis did not increase between stages 1 and 3 (McLeland et al., 2015). Furthermore, more fibrosis was observed in kidneys from geriatric control cats than from cats with IRIS stage 1 CKD, whereas inflammation was consistently greater in CKD cats than in controls (McLeland et al., 2015). This may suggest greater sensitivity of detecting CKD by evaluating inflammation, rather than fibrosis. Inflammation scores were generally higher than fibrosis scores in this latter study, too (McLeland et al., 2015), which may be because inflammation occurs prior to fibrosis in the damaged kidney (Hewitson, 2009). Indeed, inflammation can lead to the development of fibrosis; interstitial fibrosis is implicated in the progression of CKD to ESRD, with poor prognosis in most cases, regardless of the cause of the renal disease (Chakrabarti et al., 2013, Prunotto et al., 2011).

Other histological findings associated with CKD include thickening or mineralisation of Bowman's capsule and tubular basement membranes, tubular atrophy, interstitial lipid, glomerulosclerosis and arteriosclerosis (Chakrabarti et al., 2013, Lucke, 1968). However, while cases of canine (Schneider et al., 2013, Vaden, 2011, Macdougall et al., 1986, Muller-Peddinghaus and Trautwein, 1977, Minkus et al., 1994) and human (Sumnu et al., 2015, Kopp, 2013a) CKD frequently involve diseases of the glomeruli, in the majority of cases of feline CKD, the primary lesions are in the tubulointerstitium; glomerular lesions, if present, tend to be secondary (Chakrabarti et al., 2013, McLeland et al., 2015, Yabuki et al., 2010).

1.5.1.3. Development of renal fibrosis

The kidney has an intrinsic ability to repair itself after sustaining acute injury (Lawson et al., 2015). This involves proliferation and migration of the damaged cell type to replace cells that are damaged and can achieve complete restoration of normal tissue structure and function (Wynn, 2007). Furthermore, resident renal cells, activated by the initial injury, release pro-inflammatory cytokines which recruit innate inflammatory cells (Liu, 2006). Initially, these are polymorphonuclear cells such as neutrophils and, predominantly, macrophages (Ysebaert et al., 2000), which phagocytose any foreign material, bacteria and damaged cells (Hutchison et al., 2013). However, as already mentioned, in the progression of CKD, fibrosis can follow on from inflammation. If not brought under control, the innate immune cells that were initially activated by the damage secrete chemokines which attract, and cytokines which activate, adaptive immune cells, such as lymphocytes (Hutchison et al., 2013). Lymphocytes, too, produce pro-inflammatory, as well as, pro-fibrotic cytokines. Consequently, resident renal cells are activated to become mesenchymal and differentiate into myofibroblasts, which produce connective tissue, or extracellular matrix (ECM), to replace the injured renal cells (Hutchison et al., 2013). The ECM composition will vary according to the focus of the injury and the particular resident renal cells that are activated. For example, interstitial fibroblasts, tubular epithelial cells, glomerular mesangial cells, vascular pericytes and endothelial cells may be activated (LeBleu et al., 2013, Hutchison et al., 2013) to produce proteins, proteoglycans and glycoproteins, such as collagen (types I, III and V), fibronectin and tenascin-C, which form the ECM (Tomasek et al., 2002, Rodemann and Muller, 1991, Fu et al., 2016). Furthermore, a cascade of interactions between these molecules and cells increases ECM synthesis. For example, fibronectin, which is the first matrix glycoprotein to be synthesised, facilitates ECM deposition by attracting fibroblasts and creating an adhesive framework over which other ECM components can be laid down (Qi et al., 2006, Gharaee-Kermani et al., 1996). Tenascin-C promotes the proliferation of fibroblasts (Fu et al., 2016). The process of excess ECM deposition is termed fibrosis. However, in acute injury, this fibrosis is transient and largely resolves due to degradation by proteases,

which are also secreted by fibroblasts (Hutchison et al., 2013, Strutz and Zeisberg, 2006), and remodelling of the ECM, to restore tissue structure and function (Liu, 2006).

If the injurious agent persists (i.e. the damaging stimulus is chronic), the production of growth factors, proteolytic enzymes, angiogenic factors and fibrogenic cytokines is sustained. As a result, fibrosis continues past the point of normal wound-healing, becoming irreversible and pathologic, disrupting the normal tissue architecture and subsequently, function (Hutchison et al., 2013, Wynn, 2007). Furthermore, tubular epithelial cells transition to mesenchymal-type fibroblasts and myofibroblasts (epithelial-to-mesenchymal transition, EMT) to a greater extent in the chronically fibrotic kidney than after acute injury (Liu, 2006). In addition to greater deposition of normal matrix components (Tomasek et al., 2002), chronic fibrosis results in an accumulation of other matrix components, such as collagen IV and laminin, which are usually exclusive to tubular basement membranes (Eddy, 1996). Furthermore, these aberrant ECM constituents usually become cross-linked, gaining resistance to degradation by protease enzymes (Liu, 2006). Moreover, there are increased levels of protease inhibitors in fibrotic kidneys, compared to normal. Thus, defective ECM degradation adds to the progressive fibrosis of CKD (Eddy, 1996). Disruption of the normal tissue structure in this way hinders kidney function (Liu, 2006).

Reactive oxygen species (ROS) are also generated by inflammatory cells in response to renal damage and as a result of intracellular signalling induced by some matrix-degrading proteases (Liu, 2006, Radisky et al., 2005). The effect that ROS have on a particular cell depends on both the cell type and the concentration of ROS. For example, renal interstitial cells are more resistant to the detrimental effects of ROS than tubular epithelial cells (Kim et al., 2010). Furthermore, at high concentrations, ROS cause oxidative damage to DNA, creating further damage and inducing cell death, but at lower concentrations, they may promote cell survival and proliferation (Droge, 2002), with a role in EMT (Cheng and Lovett, 2003). Moreover, ROS can trigger chemokine and cytokine secretion by renal cells, which enhances the inflammatory response and increases fibrosis (Liu, 2010). Since the pathophysiologic mechanisms

occurring in the fibrotic kidney are simultaneous and interdependent, it is clear why CKD is a progressive disease process of gradual, ongoing and increasing disruption to the kidney's structure and function.

1.5.1.4. Aetiology and risk factors

The renal lesions associated with feline CKD are relatively non-specific (Chakrabarti et al., 2013) and may result from one or, more commonly, a combination of genetic, individual or environmental factors causing damage to the kidneys (Jepson, 2016, Finch et al., 2016). The numerous aetiologies of feline CKD can be assigned to one of two categories: the initiation or the progression of the disease (Brown et al., 2016). However, initiating causes often persist so can also be progression factors in the pathogenesis of CKD. In fact, the initial cause of the renal damage is usually the main contributor to renal disease progression (Brown et al., 1997). Once the kidney has sustained significant damage, the kidney adaptations that have enabled maintenance of renal function thus far, stimulate a vicious cycle of self-perpetuating renal disease. As a result, progression of CKD also occurs autonomously from the instigating cause (Brown et al., 1997). Consequently, while it is beneficial to resolve any underlying inciting cause, often, CKD progresses to ESRD nonetheless (Brenner et al., 1982). Moreover, in the individual patient, the specific, initiating cause of CKD often remains unidentified (Reynolds and Lefebvre, 2013, Minkus et al., 1994).

Known initiation factors for CKD include chronic primary renal diseases, such as lymphoma, polycystic kidney disease, immune complex glomerulonephritis, feline leukaemia virus, feline infectious peritonitis and bacterial pyelonephritis, among others (Brown et al., 2016); aging-associated diseases, or renal senescence (Brown et al., 2016), and acute kidney insults (AKI), such as ischaemia (Schmiedt et al., 2016). Environmental factors, including frequent vaccination (Finch et al., 2016, Lappin et al., 2005, Whittemore et al., 2010) and stresses of the domestic lifestyle, such as confinement and sharing resources and space with other cats, animals or humans (Brown et al., 2016) have also been associated with increased risk of CKD development and may

initiate the disease in cats. Initiation factors commence the renal damage, leading to a reduced number of functioning nephrons (Brown et al., 2016).

Once CKD has developed, progression factors may add to the structural and functional deterioration in the kidney (Finch et al., 2016). Inherent progression factors, i.e. the spontaneous repair processes occurring in the kidney, are activated if the initial renal injury is unresolved. Furthermore, consequences of the renal disease, such as proteinuria (Chakrabarti et al., 2012, Syme et al., 2006, Jacob et al., 2005), anaemia (Chakrabarti et al., 2012), systemic (Jepson, 2016, Jacob et al., 2003) and intraglomerular hypertension (Brown et al., 1997), RAAS activation (Jepson, 2016, Macconi et al., 2014, Nangaku and Fujita, 2008) and renal tubulointerstitial hypoxia (Nangaku and Fujita, 2008, Liu et al., 2018, Nangaku, 2006), may also accelerate the damage to the kidneys.

Additionally, there are external influences on the progression of renal disease. As well as the environmental factors already mentioned, commercial cat foods (DiBartola et al., 1993) and specific dietary components, including lipid (Brown et al., 1996), protein, phosphorus (Chakrabarti et al., 2012, Geddes et al., 2013, Brown et al., 1997), sodium (Buranakarl et al., 2004) and potassium (DuBose, 2017), may contribute to the progression of CKD. Similarly, disordered homeostasis of these nutrients, which may result from defective reabsorption and secretion functions in the damaged kidney, may further advance the renal disease (DuBose, 2017, Adrogue and Madias, 2014, Geddes et al., 2013). Moreover, some minerals, namely the toxic heavy metal elements, arsenic and mercury, are non-essential and serve no obvious function in the body and, instead, may induce oxidative damage in tissues, including the kidney (Valko et al., 2005). Indeed, arsenic and mercury have been associated with renal disease in humans (Chang and Singh, 2018, Park and Zheng, 2012). Such minerals may be consumed due to environmental contamination of food stuffs, including fish (Filippini et al., 2018) and rice (Rasheed et al., 2018), which are common ingredients in pet foods. It is important to note that many of the risk factors listed here have been concluded from in-vitro studies or investigations in other species such as humans.

Furthermore, most of the studies investigating risk factors for CKD show associations, rather than cause and effect relationships. It is unclear whether all of these risk factors are involved in the pathogenesis of feline CKD. In Study 1 (Chapter 2) of this programme of work, the mineral composition of kidney tissue and urine from cats and dogs with and without renal disease will be assessed, to determine whether cats have greater renal accumulation of pro-oxidant minerals, including arsenic and mercury. It is hypothesised that such findings may relate to greater fish consumption and associate with CKD. Despite heterogeneous aetiology, and irrespective of the inciting cause, the majority of cases of feline CKD progress to cause renal dysfunction via inflammation and fibrosis in the tubulointerstitium (Jepson, 2016, Yabuki et al., 2010). Hence, in Studies 1 and 2 of this thesis, renal interstitial inflammation and fibrosis were assessed to identify cases of CKD.

1.5.1.5. Lipotoxicity and renal disease

In species other than cats, evidence suggests that accumulation of intracellular lipid droplets within the kidney can lead to CKD. Indeed, ectopic accumulation of fat within organs, including the kidney, has been shown to induce inflammation, oxidative stress and fibrosis (Escasany et al., 2019, Kang et al., 2015, Izquierdo-Lahuerta et al., 2016). Furthermore, the products of lipid peroxidation, including hydroperoxides, aldehydes and polymeric materials, are cytotoxic and can cause apoptosis and cell death, which may contribute to renal proximal tubular atrophy (Khan et al., 2014, Li et al., 2009). The processes through which damage occurs following excessive lipid accumulation in non-adipose tissues are collectively termed lipotoxicity (Escasany et al., 2019). Intracellular lipotoxicity in the kidney has been associated with reduced renal function (Muller et al., 2019); in humans (Herman-Edelstein et al., 2014) and mice (Li et al., 2009) with renal disease, abnormally large quantities of lipid have been identified in the renal tubular epithelial cells. Conversely, inhibition of lipid uptake into the proximal tubule epithelial cells has been shown to attenuate renal damage (Yokoi and Yanagita, 2016, Souza et al., 2016). Thus, it is clear that intracellular

accumulation of lipid within the kidney may be involved in the pathogenesis of renal disease.

Free fatty acids are used by mitochondria to produce adenosine triphosphate (ATP) for cellular energy, in a process called beta (β) oxidation (Lazarow, 1978). Beta-oxidation of fatty acids also occurs within peroxisomes, however, ATP is not generated during this process (Lodish et al., 2000). Very long chain fatty acids (VLFAs) with more than 20 carbons are oxidised in peroxisomes (Lodish et al., 2000) whereas shorter chain fatty acids (including short-, medium- and some long-chain fatty acids) are metabolised by the mitochondria (Lazarow, 1978). Peroxisomes can metabolise VLFAs to shorter chain fatty acids, which can then be oxidised to completion by mitochondria (Reddy and Hashimoto, 2001). Fatty acid oxidation (FAO) occurs in cells along the entire length of the nephron, but to the greatest extent in the proximal tubule epithelial cells (Le Hir and Dubach, 1982). This is because renal proximal tubule epithelial cells (RPTECs) have the highest density of mitochondria and peroxisomes and the highest activity of the enzymes that are involved in the β-oxidation pathways in these organelles, compared to cells in other segments of the nephron (Kang et al., 2015, Le Hir and Dubach, 1982). RPTECs lack the enzymes involved in glycolysis, so energy production via glucose metabolism is limited in the RPTECs (Wirthensohn and Guder, 1986). Therefore, RPTECs preferentially use fatty acids for the production of energy (Kang et al., 2015). Moreover, FAO produces six times more ATP than occurs with glucose oxidation (Lodish et al., 2000). Thus, through β-oxidation of fatty acids, the high energy demand of RPTECs can be met (Kang et al., 2015, Le Hir and Dubach, 1982).

Normally, uptake, oxidation and synthesis of fatty acids are well-balanced so renal intracellular accumulation of lipid is prevented (Kang et al., 2015). The kidney is not a normal fat depot and such ectopic deposition of lipid is usually caused by obesity or metabolic syndrome (Bobulescu et al., 2014). In these cases, an excess of fat overwhelms the storage capacity of adipose tissue, spills over into the circulation and may enter other tissues, including muscle, the liver, the pancreas and the kidney (Escasany et al., 2019, Izquierdo-

Lahuerta et al., 2016). If the amount of fatty acids entering these tissues overwhelms the capacity of the mitochondria and peroxisomes to conduct β-oxidation, the fatty acids may instead be esterified and stored as triacylglycerides in intracellular lipid droplets (Escasany et al., 2019). Additionally, defects in FAO within organs, for example, due to decreased expression of the enzymes or key regulators involved, may exacerbate cytoplasmic lipid accumulation (Jao et al., 2019). This has been evidenced in human and mice kidneys, where it was found to eventually lead to tubulointerstitial fibrosis (Jao et al., 2019, Kang et al., 2015). Moreover, agonists of peroxisome proliferator-activated receptor-alpha (PPAR-α), a key regulator of β-oxidation, have been shown to reduce lipid accumulation and tubulointerstitial fibrosis and improve renal function after renal injury (Jao et al., 2019, Li et al., 2009). Thus, clearly, normal levels of FAO are important in order to avoid renal lipid accumulation and the associated consequences.

Furthermore, during renal disease, uptake of fatty acids into renal cells may increase due to damage to the glomerular filtration barrier. In circulation, fatty acids are either esterified as triacylglycerides or are bound to plasma proteins such as albumin in order to be solubilised and transported (Khan et al., 2018). Normally, lipid uptake into RPTECs, for use in ATP generation, occurs at the basolateral membrane (Trimble, 1993). Albumin-bound fatty acids must first dissociate from the albumin (Khan et al., 2018). However, increased permeability of the glomerular filtration barrier in the diseased kidney enables albumin-bound fatty acids to enter the glomerular filtrate. Then, fatty acids can be reabsorbed by the RPTECs at the apical membrane as well as the basolateral membrane (Khan et al., 2018). Consequently, more lipid may accumulate within the RPTECs during renal disease. In turn, this may increase the risk of lipotoxic-induced renal damage, worsening the renal disease and progressively reducing renal function (Khan et al., 2018, Kamijo et al., 2002). Albumin-bound fatty acids have been shown to promote greater production of inflammatory cytokines and ROS and a greater reduction in antioxidant defences, compared to albumin alone, indicating that the lipid, rather than the protein, in RPTECs is primarily responsible for the damage (Ishola et al., 2006, Kamijo et al., 2002). Clearly, even if renal intracellular lipid accumulation

doesn't initiate the CKD in a particular individual, it may occur as a consequence of the renal damage and may contribute to the progression of the disease through lipotoxic events.

In contrast to other mammals, in cats, the renal proximal tubules contain an abundance of intracellular lipid droplets, without showing evidence of lipotoxic damage (Lucke, 1968, Bargmann et al., 1977). Furthermore, unlike other species, the amount of lipid within the feline kidney is not associated with body weight (Modell, 1933). In fact, even young cats have lipid droplets within their RPTECs (Modell, 1933), which might suggest that the lipid does not accumulate, but is instead innately present. It is unclear why cats would require increased amounts of substrate for ATP production in the kidney, compared to other species. Indeed, cats have upregulated systems for lipid metabolism compared to other species (Montague et al., 2014) and a lack of certain enzymes involved in lipid metabolism, which causes certain lipids to be essential nutrients in cats, but not in other species (Morris, 2002). However, it is unknown whether lipid metabolism is upregulated in the feline kidney and is associated with high renal intracellular lipid content. Certain types of lipids, such as triacylglycerides, phospholipids and cholesterol, have been detected in the cat's kidney (Bargmann et al., 1977). It has been suggested that the type and/or amount of lipid in the cat's kidney may be related to the diet (Mottram, 1916) and under hormonal influence (Lobban, 1955) but this is uncertain. Despite the known lipotoxic effect of lipid accumulation in the kidney and a high prevalence of renal disease in domestic cats, an association between renal intracellular lipid and CKD in cats has not yet been investigated. Instead, excess intracellular lipid content appears to be regarded as a normal, non-pathological phenomenon in the feline kidney (Bargmann et al., 1977).

On the other hand, deposits of lipid within the renal interstitium are associated with renal disease in cats (Lucke, 1968). Lipid may accumulate in the renal interstitium due to tubular atrophy or degeneration, which causes the release of intracellular lipid (Lucke, 1968). In cats, considering that intracellular lipid is mainly within the proximal tubules, it is not surprising that interstitial lipid accumulation is seen primarily within the kidney cortex (Martino-Costa et al.,

2017). Additionally, these pathological interstitial lipid deposits are associated with chronic inflammatory cells (Lucke, 1968). In fact, it has been suggested that interstitial lipid may trigger the inflammatory reaction that occurs in the renal interstitium and may therefore contribute towards the chronic interstitial nephritis of CKD (Martino-Costa et al., 2017). Taking these factors into account, it is hard to believe that the cat is unique in having RPTECs that are resistant to lipotoxic episodes; especially considering the contrastingly high prevalence of renal disease in cats, compared to other species that have lower levels of renal intracellular lipid. Certainly, there is no evidence that excess lipid accumulation is protective against renal damage. Thus, perhaps the cat, due to its naturally high renal lipid content, is innately predisposed to CKD. There is a paucity of recent studies on the intracellular lipids in the feline kidney, highlighting that this is an area for further research. In particular, although it is known that cats have more intracellular lipid droplets in their kidneys compared to other mammals, it is unknown whether the composition of the lipids within these droplets is different in cats, compared to other species. Moreover, to our knowledge, an association between cytoplasmic lipid and pathology in cat kidneys has not been studied. Therefore, in Study 2 (Chapter 3) of this programme of work, we quantity and characterise the lipid within the kidneys of domestic cats, a species with high prevalence of CKD, versus dogs with lower CKD prevalence and Scottish wildcats as a within-species comparator; aiming to establish whether the domestic cat may be particularly susceptible to CKD due to the lipid environment within the kidney.

1.5.2. Hyperthyroidism

1.5.2.1. Normal thyroid gland structure and function

The thyroid gland is a bi-lobed endocrine organ, located in the neck. Its ultimate function is to produce thyroid hormones, triiodothyronine (T3) and thyroxine (T4), which in turn affect metabolic processes occurring in most tissues throughout the body (Mooney, 2017). Within each lobe of the thyroid gland are numerous follicles. Follicles are spaces lined by thyroid epithelial cells (also known as follicular cells or thyrocytes) and filled with colloid, a fluid that contains stores of the prohormone thyroglobulin (Ortiga-Carvalho et al.,

2016). Thyroglobulin is produced by follicular cells and secreted into the colloid using the enzyme thyroid peroxidase (TPO) (Mooney, 2017). In fact, TPO catalyses most of the stages in the production of thyroid hormone (Mooney, 2017).

Thyroid hormone production is controlled by the hypothalamic-pituitary-thyroid (HPT) axis (Figure 2) (Ortiga-Carvalho et al., 2016). The hypothalamus secretes thyrotropin-releasing hormone (TRH), which stimulates thyrotropin (thyroid-stimulating hormone; TSH) production and secretion by cells of the anterior pituitary gland. TSH then binds to the thyrotropin receptor on the thyroid gland (Mooney, 2017). A sodium iodide symporter is stimulated via a cAMP-dependent, G-protein-mediated signal transduction pathway and iodide in circulation is taken up into the follicular cell (Kopp, 2013b). Iodide is transported across the follicular cell membrane into the colloid where it is oxidised to iodine in the of presence hydrogen peroxide (H_2O_2) and TPO (Kopp, 2013b). In the colloid, iodine combines with tyrosine residues of thyroglobulin, in a process termed iodination, to form mono- and di-iodotyrosines (Ortiga-Carvalho et al., 2016). Oxidative coupling of one mono-iodotyrosine and a di-iodotyrosine, or two di-iodotyrosines, forms the thyroid hormones T3 and T4, respectively (Ortiga-Carvalho et al., 2016). These steps are also dependent on TPO and H_2O_2 (Dumont et al., 2016). Thyroid hormone-bound thyroglobulin then passes into the follicular cell by endocytosis of colloid (Kopp, 2013b). Intracellular vesicles fuse with lysosomes in which enzymes cleave the thyroid hormones from thyroglobulin (Kopp, 2013b). Thyroglobulin molecules are recycled and thyroid hormones are free to be secreted from the thyroid follicular cells into the bloodstream upon stimulation by TSH (Figure 3) (Mooney, 2017).

Nearly all thyroid hormone in circulation is bound to proteins such as thyroxine-binding globulins, transthyretin, albumin and lipoproteins, thus constituting a reservoir of thyroid hormone for delivery to tissues. Only free, unbound thyroid hormone is metabolically active (Mooney, 2017). T3 is more potent than T4, however, most of the thyroid hormone produced by the thyroid gland is T4 and a lesser proportion is T3 (Ortiga-Carvalho et al., 2016). Most of the T3 in the body is produced by monodeiodination of T4 in peripheral tissues, not by the

thyroid gland (Mooney, 2017). A system of negative feedback on the HPT axis operates to regulate the level of thyroid hormone production. When circulating levels of free T4 (fT4) and T3 are excessive, negative feedback to the hypothalamus and pituitary to reduce the production and secretion of TRH and TSH, respectively, in turn decreases thyroid hormone synthesis (Mooney, 2017, Ortiga-Carvalho et al., 2016).

Figure 2. Hypothalamic-pituitary-thyroid axis.

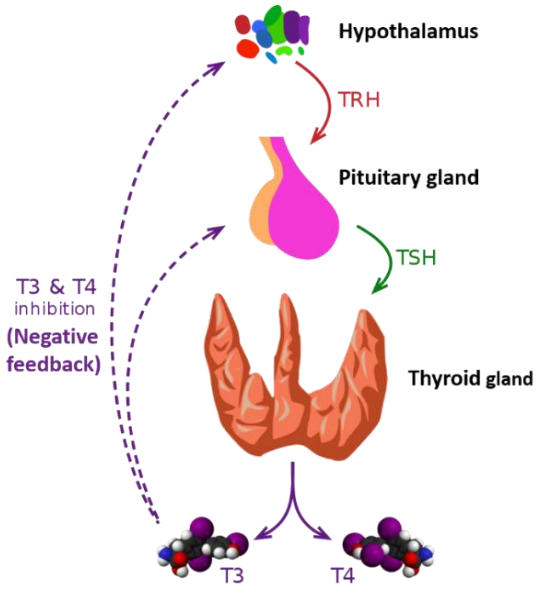


Figure 2. Thyrotropin-releasing hormone (TRH), secreted from the hypothalamus, stimulates the anterior pituitary gland to secrete thyroid-stimulating hormone (TSH), which acts on the thyroid gland to induce the production and secretion of thyroid hormone, T3 and T4. When circulating levels of T3 and T4 are high, negative feedback on the hypothalamus and pituitary gland acts to inhibit TRH and TSH secretion, to reduce thyroid hormone synthesis. Image modified from Wikimedia Commons, labelled for non-commercial reuse (https://commons.wikimedia.org/wiki/File:Thyroid_vector.svg, accessed 29th June 2020).

Figure 3. Thyroid hormone synthesis.

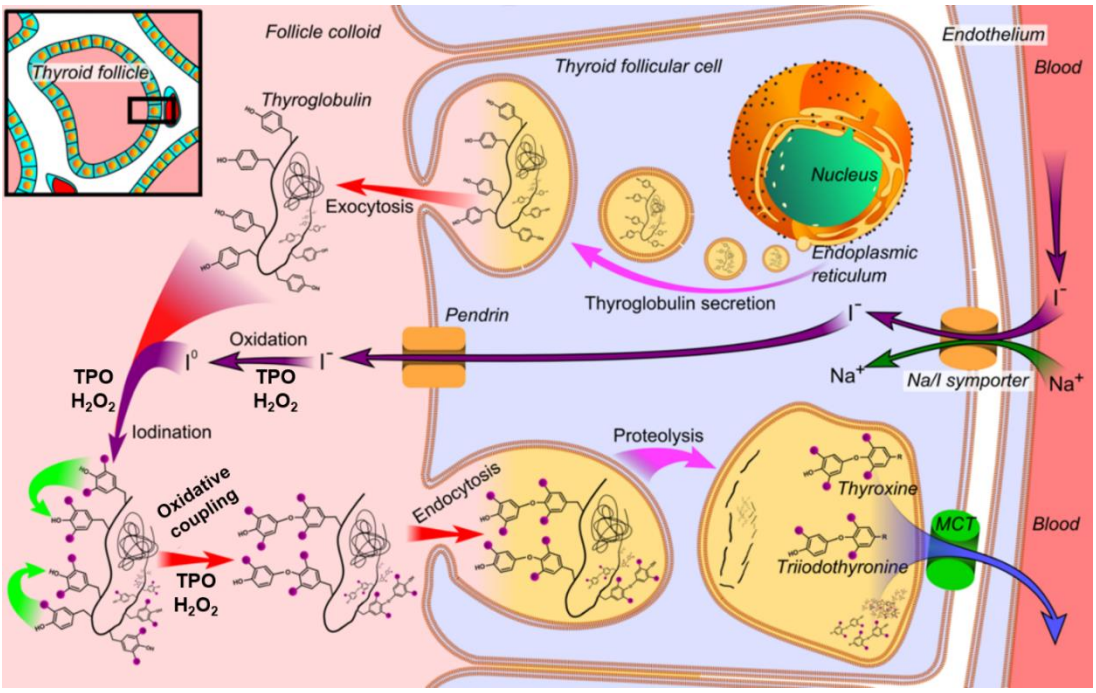


Figure 3. Thyroglobulin is synthesised in the rough endoplasmic reticulum and secreted from the follicular cell into the colloid in the follicular cell lumen. Sodium and iodide are transported from the blood into the thyroid follicular cell by a sodium/iodide (Na/I) symporter. Iodide (I^-) is transported across the follicular cell membrane into the colloid, where it is oxidised to iodine (I^0), then iodinated to tyrosine residues of thyroglobulin, in the presence of thyroid peroxidase (TPO) and hydrogen peroxide (H_2O_2), to form mono-iodotyrosine and di-iodotyrosine. Thyroid hormones (triiodothyronine [T3] and thyroxine [T4]) are formed by oxidative coupling of mono-iodotyrosines and di-iodotyrosines, endocytosis of these complexes into the follicular cell and proteolysis to free the T3 and T4 molecules, which can then leave the thyroid gland and enter the circulation. Image modified from Wikimedia Commons, labelled for non-commercial reuse (https://commons.wikimedia.org/wiki/File:Thyroid_hormone_synthesis.png, accessed 29th June 2020).

1.5.2.2. Feline Hyperthyroidism

Hyperthyroidism, a condition in which the thyroid gland is overactive and unresponsive to negative feedback, resulting in incessantly elevated thyroid hormone production, is common in cats (Graves, 2017). Thyroid carcinomas account for only 1–3% of cases of feline hyperthyroidism, while the majority (97–99%) of cases result from benign adenomatous hyperplastic nodules in the thyroid gland (Gunn-Moore, 2005). These nodules are autonomous in growth and function, meaning that the thyroid gland continues to secrete thyroid hormone, despite TSH suppression due to feedback on the

hypothalamus and pituitary by elevated circulating levels of thyroid hormone (Peter et al., 1987). In addition, some follicles have upregulated systems for iodide uptake (Peter et al., 1987) and mutations in the thyrotropin receptor or its associated G-proteins, facilitating increased thyroid hormone production (Peeters et al., 2002, Watson et al., 2005). In most (71%) cases of feline hyperthyroidism, the pathology is bilateral; adenoma or adenomatous hyperplasia affects only one lobe of the thyroid gland in 29% of cases (Peterson et al., 1983). In contrast, in dogs, thyroid disease most commonly presents as malignant thyroid carcinoma (Barber, 2007). However, this causes clinical symptoms associated with being a space-occupying lesion and rarely clinical hyperthyroidism (Leav et al., 1976, Barber, 2007). Thyroid adenomas do occur in dogs but are usually too small to cause clinical signs of hyperthyroidism (Barber, 2007). In addition, feeding raw meat containing thyroid gland tissue has been reported to cause hyperthyroidism in a small number of dogs (Zeugswetter et al., 2013, Kohler et al., 2012). Overall, hyperthyroidism is uncommon in dogs (Barber, 2007).

As thyroid hormones affect the metabolic processes in most organs in the body, the clinical signs associated with hyperthyroidism are multi-systemic and highly variable (Graves, 2017). They can include weight loss despite increased appetite, increased activity or restlessness, aggressive behaviour, poor hair coat, tachycardia, polyuria and polydipsia, periodic vomiting, increased defaecation and difficulty breathing (Peterson et al., 1983, Gunn-Moore, 2005). Clinical signs are slowly progressive in most cases of hyperthyroidism (Gunn-Moore, 2005). Additionally, hyperthyroid cats often develop concurrent conditions such as CKD and cardiac hypertrophy, due to the effect of thyroid hormones on the associated organs (Stephens et al., 2014, Vaske et al., 2016). The clinical presentation and histopathological features of hyperthyroidism in cats (Hoenig et al., 1982) resemble those of toxic nodular goitre (TNG), a form of hyperthyroidism that occurs in humans in iodine-deficient areas and especially affects the elderly population (Stanbury et al., 1998, Laurberg et al., 2010).

1.5.2.3. Prevalence of Feline Hyperthyroidism

Hyperthyroidism was first reported in cats in the USA in 1979 (Peterson et al., 1979). Shortly after, there were further reports of feline hyperthyroidism being diagnosed in the USA (Holzworth et al., 1980) and in other countries, including New Zealand (Jones and Johnstone, 1981). Feline hyperthyroidism probably occurred before this time, as suggested by post-mortem histopathological findings in cats in the UK (Lucke, 1964), however, the clinical significance of these findings was unknown at the time. Additionally, due to low awareness of hyperthyroidism in cats, even in the early 1980s, it is likely that cases were misdiagnosed (McLean et al., 2014). Nonetheless, since its first definitive diagnosis in 1979, the worldwide prevalence of feline hyperthyroidism has steadily increased (Peterson, 2012) and at a faster rate than that of other common feline diseases, such as diabetes mellitus and renal disease (Edinboro et al., 2004). For example, the prevalence apparently increased from 0.3 to 4.5% between 1979 and 1985 (Scarlett et al., 1988), but from 0.1 to 2% between 1978-1980 and 1993-1997 in the USA (Edinboro et al., 2004). Hyperthyroidism is now the most common endocrinopathy of domestic cats (Mooney and Peterson, 2012).

Particularly, hyperthyroidism is a disease of middle-aged to old cats and it is not commonly diagnosed in cats younger than 10 years old (Peterson et al., 1994). In the US, the prevalence of hyperthyroidism in cats seen at a veterinary hospital was found to increase with increasing age (Edinboro et al., 2004). Stephens et al. (2014) also exemplified this by reporting a hyperthyroidism prevalence of 2.4% in all cats (aged 6 to 25 years), but an 8.7% prevalence in cats ≥10 years old, in primary care veterinary practices in England. However, the prevalence of feline hyperthyroidism appears to vary with geographical location. To contrast the findings of Stephens et al. (2014), in primary care veterinary practices in Hong Kong in 2007 (De Wet et al., 2009) and Dublin, Ireland, in 2012 (Bree et al., 2018), respectively, 3.9% and more than 20% of cats aged over 10 years were diagnosed with hyperthyroidism. With similar, or greater, prevalence to that recorded in the UK (Stephens et al., 2014) and Ireland (Bree et al., 2018), feline hyperthyroidism is said to be common in Australia, New Zealand, Japan (McLean et al., 2014) and other European

countries such as Germany (Kohler et al., 2016) and Poland (Gójska-Zygner et al., 2014). It is less common in Hong Kong (De Wet et al., 2009) and Spain (Wakeling et al., 2005, McLean et al., 2014). However, it is not possible to make direct comparisons between the prevalences reported by different studies, due to different study populations (e.g. referral vs. first opinion practices), different ages of cats, different parameters for diagnosing the condition and the recency of the studies. Indeed, the difference in these results could also be an effect of time, since the prevalence of feline hyperthyroidism is believed to have increased since its first definitive diagnosis in 1979 (Peterson, 2012). Of course, the increase in reported prevalence that has occurred over time may in part be due to increased awareness of the disease, better availability of thyroid hormone assays used to diagnose the condition, longer life-span of cats and more aged cats visiting veterinary practices for geriatric health screening due to the importance of this highlighted by the media. However, the prevalence has seen such a large and rapid increase that it is improbable that these factors alone could be responsible (Edinboro et al., 2004, McLean et al., 2014). Nonetheless, it is known that many cats will develop hyperthyroidism as they age. Therefore, research into potential ways of preventing the condition seems important, in order to maintain good quality of life for more cats as they age.

1.5.2.4. Aetiology of, and risk factors for, feline hyperthyroidism

The aetiology of adenoma or adenomatous hyperplasia that results in feline hyperthyroidism is unknown (McLean et al., 2014). However, numerous researchers have investigated potential risk factors for development of the disease, and many risk factors have been identified. Thus, the pathogenesis of feline hyperthyroidism is almost certainly multifactorial and not simply caused by a single genetic, environmental or dietary factor. However, individual studies fail to consistently determine the same risk factors, so, for many, the significance of their contribution to the pathogenesis remains uncertain (McLean et al., 2014).

There is a consensus that the risk of developing hyperthyroidism is greater for older cats (Bree et al., 2018, Wakeling et al., 2009b). There is also much

agreement that non-purebred cats, such as the domestic shorthair (DSH), are at higher risk of developing hyperthyroidism than purebred cats, such as the Siamese, Himalayan, Burmese and Persian (Scarlett et al., 1988, Kass et al., 1999, Stephens et al., 2014, Olczak et al., 2005, Wakeling et al., 2009b). This suggests that there might be a genetic basis for the development of the disease. Alternatively, purebred cats, in general, may be older, or don't survive for as long, when they do develop the disease, compared to non-purebred cats, meaning hyperthyroid purebred cats are less readily available for such epidemiological studies (Stephens et al., 2014). A genetic aetiology is further supported by Kass et al. (1999) finding that hyperthyroid cats, compared to control cats in their case-control study, were more likely to have a relative with hyperthyroidism. However, they did not obtain information on all cats' relatives in their study. Furthermore, other factors may come into play here, since related cats possibly share a common household and are therefore exposed to the same environmental risk factors.

Environmental factors that have been associated with the development of feline hyperthyroidism, albeit with conflicting reports from different researchers, include the use of cat litter (Wakeling et al., 2009b, Kass et al., 1999), use of flea-control products on cats (Scarlett et al., 1988, Kass et al., 1999) or on cats' bedding (Olczak et al., 2005) and application of pesticides and fertilisers in the cat's immediate outdoor environment (Scarlett et al., 1988). The increased risk of hyperthyroidism in cats that use cat litter may be related to increased time spent indoors, since indoor cats (odds ratio [95% confidence interval]: 4.0 [1.3, 12.1]) and cats that spend most of their time indoors (11.2 [2.6, 48.0]) appear to have an increased risk of developing hyperthyroidism compared to those that are able to venture outside (Scarlett et al., 1988). Interestingly, the odds of hyperthyroidism was lower in cats that spent all of their time indoors, versus those that had a 'predominantly' indoor lifestyle, however, the 95% confidence interval was larger in the latter group, reflecting a lower degree of precision in the odds ratio (Szumilas, 2010). Moreover, Kass et al. (1999) who found that the use of cat litter, irrespective of brand, increased the odds of developing hyperthyroidism 3-fold, found no

link between the amount of time cats spent indoors and their susceptibility to the disease.

1.5.2.4.1. Environmental contaminants in the home and feline hyperthyroidism

Increased risk of hyperthyroidism for indoor cats may alternatively be caused by endocrine disruptors in their environment. For example, polybrominated biphenyls (PBBs), tetrabromobisphenol A (TBBPA) and polybrominated diphenyl ethers (PBDEs) have been used as flame retardants in furniture, fabrics and electric appliances since the 1970s (Alaee et al., 2003), just before the first reports of diagnosed cases of feline hyperthyroidism. Polychlorinated biphenyls (PCBs) were introduced as early as 1929 for use in paints, floor finishes and window sealants in the household setting (Serpe et al., 2018). PCBs and other similar compounds, including PBBs, are now banned due to their risk of environmental contamination and toxicity. However, they are still present in the environment due to their stability (Alaee et al., 2003). Other compounds, such as some PBDEs, are still in use today. PBDEs are classed as additive flame retardants because they are blended with, rather than chemically bound to, the polymers in the particular product, to confer fire resistance. Thus, they can more easily leave the products that contain them and enter the environment (Alaee et al., 2003).

High levels of PBDEs (Guo et al., 2012) and PCBs (Serpe et al., 2018) are also found in house dust. It has been reported that indoor cats could be exposed to twice the amount of PCBs than cats with outdoor access, just through inhalation (Serpe et al., 2018). Interestingly, Mensching et al. (2012) reported that dust PBDE levels were significantly greater in the houses of hyperthyroid cats, compared to those of euthyroid cats. Furthermore, a correlation between concentrations of PBDEs in dust and total serum T4 was found and client-owned cats with indoor access had higher serum PBDE concentrations than feral cats (Mensching et al., 2012), perhaps due to greater exposure to house dust containing PBDEs. PBDEs interfere with thyroid hormone production, however, the mechanism for this is not fully understood (Makey et al., 2016). Normally, T4 is bound to proteins, such as transthyretin,

in the blood, to be transported throughout the body to target tissues. However, PBDEs and other halogenated fire retardants have similar structures to thyroid hormones and, when hydroxylated, are able to bind to transthyretin instead (Meerts et al., 2000). In doing so, serum T4 levels decrease, which may trigger negative feedback on the hypothalamus and pituitary, and hyperactivity of the thyroid cells to increase thyroid hormone levels (Hallgren and Darnerud, 2002). Studies in rats (Wang et al., 2019, Zhou et al., 2001, Hallgren and Darnerud, 2002) and humans (Makey et al., 2016, Meeker et al., 2009, Turyk et al., 2008) have shown that PBDE exposure affects serum concentrations of thyroid hormones and sometimes TSH. The results are inconsistent however, and some studies show the effect in favour of hypothyroidism, rather than hyperthyroidism, development (Wang et al., 2019). Reports from similar investigations in cats are also conflicting: Mensching et al. (2012) and Guo et al. (2012) found that serum PBDE levels in hyperthyroid and euthyroid cats do not seem to differ or correlate with serum total T4 or TSH concentrations, while Walter et al. (2017) and Guo et al. (2016) later established a possible link between greater serum PBDE levels and hyperthyroidism in cats. However, the pharmacokinetics of PBDEs may be altered in hyperthyroid, compared to euthyroid, cats, which may mean that serum PBDE levels do not truly reflect exposure to PBDEs (Guo et al., 2012). Nonetheless, cats have been shown to have 20- to 100-fold greater serum PBDE levels than humans, despite the two species sharing the same household environment (Dye et al., 2007, Guo et al., 2016). This likely reflects greater exposure and even ingestion of PBDEs in cats compared to humans; probably a consequence of their sleeping, walking and grooming behaviours (Guo et al., 2016). Indeed, in one study, cats that slept on the floor were 6.6 times more likely to have a diagnosis of hyperthyroidism (Olczak et al., 2005), which may be due to closer contact with PBDEs in the carpets and soft furnishings and ingestion of house dust when grooming. However, cats and dogs share the same household environment, therefore should have similar exposure to PBDEs through their interactions with the environment. Yet, the prevalence of hyperthyroidism is much lower in dogs than it is in cats. Therefore, environmental contamination with endocrine disruptors is unlikely to be the primary risk factor for hyperthyroidism.

Exposure to endocrine disrupting contaminants including PBDEs, PCBs and bisphenol A (BPA) may also occur through the diet. Several epidemiological studies have found that cats whose diet comprises a greater proportion of canned, wet foods may have higher risk of developing hyperthyroidism (Scarlett et al., 1988, Kass et al., 1999, Wakeling et al., 2009b). More specifically, feeding food from cans with easy-open, pop-top lids has been associated with higher risk of feline hyperthyroidism (Edinboro et al., 2004). Wet food packaged in pouches, rather than cans, appears less likely to induce hyperthyroidism (Wakeling et al., 2009b). Higher levels of contaminants in canned, than in pouched, foods may account for this difference. For example, BPA is commonly added into can linings to prevent corrosion of the can by the food that it is in contact with (Edinboro et al., 2004). Furthermore, BPA leaches from the can into the food, particularly those with high fat or oil content (Hammarling et al., 2000), during heat processing, which is more intensive for canned versus pouched foods, and storage at higher temperatures (Munguia-Lopez and Soto-Valdez, 2001, Kang and Kondo, 2002). Wet food that is packaged in cans, particularly of the pop-top variety, does tend to have higher fat content than dry food (Edinboro et al., 2004). Additionally, Dye et al. (2007) concluded that cats consuming canned foods would have greater dietary intake of PBDEs than cats consuming dried foods.

Furthermore, PCB concentrations measured in wet and dry foods for cats were higher than in those for dogs. Cats also had greater serum PCB concentrations than dogs (Mizukawa et al., 2016). However, conversely, Kang and Kondo (2002) measured greater BPA concentrations in canned foods for dogs, than those for cats, despite hyperthyroidism being rare in dogs. Nonetheless, the concentrations of BPA measured in both cat and dog foods in that study were much lower than the maximum level of BPA contamination that is accepted in the European Union (Kang and Kondo, 2002). Yet, species-differences in the metabolism of these compounds may account for greater susceptibility to the toxic effects in cats compared to dogs (Mizukawa et al., 2016, Kunisue et al., 2005). For example, BPA and similar contaminants are mainly eliminated from the body via glucuronidation (Volkel et al., 2002, Yokota et al., 1999), a process

that is less efficient, and almost non-existent, in cats compared to other species (van Beusekom et al., 2014, Court and Greenblatt, 1997).

Furthermore, canned cat foods of fish and other seafood flavours have been found to have higher PBDE content than those of non-seafood flavours (Dye et al., 2007). Similarly, a study investigating the PBDE content of different human food groups found that fish contained the highest levels of these contaminants, followed by meat (Schecter et al., 2006). This is because PBDEs and other contaminants, such as PCBs, accumulate in the oceans and consequently in marine organisms, with biomagnification along the aquatic food chain. In contrast, levels of these contaminants in terrestrial flora and fauna are generally low (Shaw and Kannan, 2009). Indeed, serum levels of hydroxylated PBDEs determined in cats are similar to those in marine mammals, which implies that consumption of fish, a common ingredient in commercial cat foods, increases exposure to these contaminants in cats (Mizukawa et al., 2013). However, another study in which the PDBE content of some fish-containing canned cat foods was measured, the levels were much lower than those found in house dust, suggesting that the diet is a comparatively minor source of PBDE exposure for domestic cats (Mensching et al., 2012). Nonetheless, greater risk of developing hyperthyroidism has been associated with cats that have a preference for fish, as well as liver and giblets, flavoured canned cat food (Martin et al., 2000).

1.5.2.4.2. Dietary iodine and feline hyperthyroidism

The greater risk of hyperthyroidism in cats fed canned and fish-flavoured pet foods may be due to the iodine content of these foods. Dietary iodine is implicated in the pathogenesis of TNG, the form of human hyperthyroidism that most resembles feline hyperthyroidism. Particularly, an excessive intake of iodine after a period of iodine deficiency triggers human TNG (Stanbury et al., 1998). The iodine content of canned, wet cat food shows huge variation. A study conducted in New Zealand (NZ) measured a >100-fold difference in the mean iodine concentration in wet and dry cat foods (reported range <1.48 – 167 µmol/kg dry weight, equivalent to 187.96 – 21,209 µg/kg DM [molecular mass of iodine = 127.0]). Three foods (all wet) had a mean iodine

concentration above the recommended upper limit set for adult cat diets at the time of the study (23.6 µmol/kg DM, equivalent to 2997.2 µg/kg DM) and others had iodine concentrations below the limit of detection for the analysis (Johnson et al., 1992). A more recent American study also found widest (almost 200-fold; 840–154,550 µg/kg DM) variation in the iodine content of canned foods, compared to 11-fold variation among pouched wet foods (880–10,110 µg/kg DM) and 31-fold variation among dry foods (200–6260 µg/kg DM) (Edinboro et al., 2013). A similar study was conducted in Germany, with the same main findings of a 30-fold difference in iodine concentration overall and highest variability among canned foods (29-fold; 218–6356 µg/kg DM vs. 6.7-fold; 473–3181 µg/kg DM for dry foods) (Ranz et al., 2002). Evidently, there is variability in the ranges of iodine measured in cat foods in different countries (Ranz et al., 2002, Edinboro et al., 2013, Johnson et al., 1992) and even in different regions within a single country (Edinboro et al., 2013). This may reflect the geographical differences in feline hyperthyroidism prevalence. Due to the other similarities between feline hyperthyroidism and human TNG, it has been proposed that hyperthyroidism could be triggered in cats fed a high iodine diet after consuming an iodine deficient diet for a period of time. Thus, the wide variation in iodine concentrations in canned cat foods may be implicated in the development of feline hyperthyroidism. However, to our knowledge, no independent study has assessed iodine concentrations of commercially available pet foods in the UK.

Fish are known to have high iodine content (Nerhus et al., 2018). Moreover, there can be wide inter- and intra-species variation in the concentration of iodine in the tissues of fish, which perhaps reflects their own different dietary iodine intakes and environments (Julshamn et al., 2001, Nerhus et al., 2018). This may account for some of the variability in the iodine content of pet foods. Indeed, in the aforementioned American pet food investigation, foods containing fish had higher iodine concentrations than foods without fish. Although, when calculated as iodine intake on a dry matter or daily basis, median iodine concentration was greater for foods without fish, than for those with fish listed as lower than the fifth ingredient by weight (Edinboro et al., 2013). Furthermore, while some epidemiological studies have reported an

increased risk of feline hyperthyroidism with consumption of fish-containing foods (Martin et al., 2000, Wakeling et al., 2009b), others have not found this association (Kass et al., 1999, Edinboro et al., 2004). Instead of iodine, the association between fish consumption and hyperthyroidism may be due to high levels of other minerals, such as arsenic and mercury (Filippini et al., 2018, Molin et al., 2017), or organic pollutants like PBDEs and PCBs, in fish, which could act as goitrogens. Nonetheless, considering the similarities between feline hyperthyroidism and human TNG, the dietary provision of iodine to domestic cats should be investigated. If fish-containing pet foods have excessive iodine content with respect to the legal maximum iodine level recommended for complete foods, or if cat foods have wide variation in iodine content overall, greater intake of such foods may account for the increased occurrence of hyperthyroidism in cats compared to dogs.

1.6. Summary

Cats have specific dietary requirements. As obligate carnivores, cats eat a lot of meat. Cats are believed to enjoy fish, more so than dogs, but may not be very well adapted to consume and process a diet high in fish, as fish would not form part of the natural diet in the wild. Domestic cats eat more fish than wildcats. Greater fish consumption might provide domestic cats with damaging amounts of certain toxic minerals, such as arsenic and mercury. These minerals can cause oxidative damage to the kidney. Renal oxidative damage can be implicated in the pathogenesis of CKD. Thus, perhaps the high prevalence of CKD in domestic cats is partly due to high fish consumption and, therefore, high exposure of the kidney to pro-oxidant minerals.

Cats are also adapted to consume moderate amounts of fat, due to the abundance of animal tissues in their diet. As animal tissue supplies abundant arachidonic acid and linoleic acid, cats have evolved lacking the metabolic pathways needed for endogenous synthesis of these fatty acids, which are thus essential nutrients in the cat's diet. Cats appear to tolerate higher dietary intake of fat compared to other animals. Intracellular lipid accumulation can cause tissue damage via lipotoxic mechanisms. In other species, intracellular

lipid has been reported to cause CKD. Cat kidneys are fundamentally similar to the kidneys of other mammalian species but, curiously, they appear to deposit large amounts of intracellular lipid from an early age. Although the intracellular lipid in the cat's kidney has not been associated with renal disease, the role of the intracellular lipid in the feline kidney has not been established. Yet, cats have a higher prevalence of CKD and a higher renal intracellular lipid content than other mammals, so the association between these two features should be investigated.

Hyperthyroidism is also common in cats, relative to other species. Feline hyperthyroidism resembles human TNG, a form of hyperthyroidism in humans that is caused by excess dietary iodine intake after a period of iodine deficiency. Iodine has not been implicated in the pathogenesis of feline hyperthyroidism, although dietary risk factors, including canned foods and fish consumption, have been proposed. The risk of feline hyperthyroidism associated with the consumption of these foods may be due to the variable iodine content. The iodine content of pet foods that are commercially available in the UK has not been analysed. It seems necessary to evaluate the compliance of UK cat foods with recommended guidelines for iodine content, to establish whether cats receive variable dietary iodine intake and to determine the involvement of dietary iodine in the development of feline hyperthyroidism.

1.7. Aims and hypotheses of this project

The work detailed within this thesis aims to address the cat's apparent predisposition to CKD and hyperthyroidism. Three separate studies are presented. Two studies within this thesis focus on CKD. The first study follows on from previous work conducted by us, which showed that pet foods containing fish have higher, and sometimes excessive, arsenic content than foods that do not contain fish. We aim to determine the arsenic species responsible for the high total arsenic concentration in these foods, to determine the risk of arsenic-induced toxicity in cats with high fish intake. We also propose that the concentration of mercury, which is high in fish for human

consumption, may also be high in fish-containing pet foods, relative to foods that do not contain fish. We aim to assess the compliance of mercury levels in pet foods with EU guidelines. We suggest that the increased prevalence of CKD in domestic cats, relative to other species, may be due to increased accumulation of pro-oxidant minerals in the kidneys, due to increased consumption of fish. We will investigate the associations between levels of pro- and anti-oxidant minerals with renal disease in domestic cats, dogs and Scottish wildcats. The Scottish wildcat will be included to provide an insight into the effect that commercial diet, compared to the more natural wild-type obligate carnivore diet, might have on renal accumulation of minerals.

Our second study will focus on the difference between the domestic cat, dog and Scottish wildcat kidneys with respect to the content of intracellular lipid. We aim to quantify and characterise the lipid within the kidneys of these animals. High levels of intracellular lipid are known to cause tissue damage and, in the kidney, may facilitate the development of CKD. Therefore, we propose that the amount and nature of the lipid in the domestic cat's kidney creates an environment that predisposes the feline kidney to damage and progression of CKD.

The third study detailed in this thesis will focus on feline hyperthyroidism. As feline hyperthyroidism resembles human TNG, which is caused by excessive dietary iodine intake after a period of iodine deficiency, we suggest that high variability in iodine content of commercial cat foods may contribute to the high prevalence of hyperthyroidism in cats. We intend to analyse cat and dog foods that are commercially available in the UK, with respect to their iodine content and compliance with FEDIAF nutritional recommendations. We suggest that domestic cats are more likely than dogs to experience excessive dietary intake of iodine due to consumption of fish.

Chapter 2. Study 1: Renal accumulation of minerals and the relationship with CKD in domestic cats versus dogs and Scottish wildcats

2.1. Introduction

2.1.1. Renal handling of minerals

The kidney filters blood that is in circulation. It has an important role in reabsorbing components of the blood, such as the essential major and trace elements, so that they are not lost from the body and remain available to carry out their functions as required (Aitio et al., 2007). The kidney and the gastrointestinal tract are the major routes of excretion of mineral elements, especially toxic metal elements, which serve no biological purpose (Mertz, 1981). Certain minerals, such as iodine, selenium and chromium are almost exclusively excreted in urine by the kidneys, while manganese homeostasis is primarily controlled by intestinal excretion (Mertz, 1981). For certain metal elements, the levels excreted in the urine reflect circulating levels and tissue concentrations. Hence, urinary concentrations of minerals are often used to indicate exposure to, and/or body burden of, the minerals (Aitio et al., 2007, Diaz et al., 2015).

Arsenic and mercury are two elements that are primarily excreted by the kidney (Hazell, 1985, Bridges and Zalups, 2010). Some arsenic and mercury is also excreted in the faeces or bile, via the intestines (Hazell, 1985, Park and Zheng, 2012). Mercury in the blood is mostly absorbed across the basolateral membrane of the renal tubular cells and then secreted across the apical membrane into the tubular lumen to be excreted in the urine (Bridges and Zalups, 2010). To our knowledge, the mechanism of renal arsenic excretion has not been proposed. Nonetheless, due to renal excretion of arsenic and mercury, high-level or chronic exposure to these toxic minerals can lead them to accumulate in the kidney (Park and Zheng, 2012, Cui and Okayasu, 2008).

In contrast to arsenic and mercury, iron, copper and zinc carry out essential functions. Therefore, the kidney has an important role in the reabsorption of these minerals, so that their excretion in urine is minimal (van Raaij et al., 2019,

Smith and Thevenod, 2009, Mertz, 1981, Linder and Hazegh-Azam, 1996). Iron is a component of many proteins and enzymes in the body, thus it has many functions, including oxygen transport, mitochondrial respiration, metabolism of lipids and xenobiotics, cell signalling and antioxidant defence (Dev and Babitt, 2017). Most iron in the body is found within haemoglobin in RBCs (Jomova and Valko, 2011) and is recycled, not lost, at the end of the RBCs' lifespan (Dev and Babitt, 2017). About a quarter of iron in the body is stored, bound to the proteins ferritin and hemosiderin, mostly in the liver (Jomova and Valko, 2011, Thevenod and Wolff, 2016). Iron in circulation, which constitutes only about 0.1% of total body iron, is mostly bound to transport proteins, such as transferrin, albumin and lactoferrin (Jomova and Valko, 2011, Thevenod and Wolff, 2016). However, protein-bound iron is able to cross the intact glomerular filtration barrier and enter the nephron (Burne et al., 1999, Thevenod and Wolff, 2016). In fact, the kidney plays a key role in iron homeostasis (Thevenod and Wolff, 2016). Using iron transport proteins, iron that passes into the renal tubules is mostly reabsorbed, to be recycled for its functions in the body (Martines et al., 2013). The kidney excretes less than 1% of the iron that it filters daily (Thevenod and Wolff, 2016). The primary loss of iron from the body occurs through the shedding of duodenal enterocytes which contain iron (Green et al., 1968). However, this iron loss is also minimal and is normally replaced by intestinal absorption of the mineral from the diet, so body iron concentrations normally remain constant (Ganz and Nemeth, 2016). Nevertheless, excess dietary supply of iron may increase the amount of iron entering the kidney, which is able to respond by reducing reabsorption and increasing excretion of iron (Wareing et al., 2003, van Raaij et al., 2019).

In contrast to iron, most of the copper in circulation exists within complexes that are too large to filter through the glomerulus (Linder and Hazegh-Azam, 1996). Only a small proportion of the total body copper is free and able to be filtered (Linder and Hazegh-Azam, 1996). In fact, homeostasis of both copper and zinc is primarily controlled by the liver, rather than the kidney. Indeed, zinc and copper both appear to accumulate in cat livers more than kidneys (Andreani et al., 2010). In the kidneys and liver, zinc and copper are both mostly associated with metallothionein, which provides storage pools of these

essential minerals for release when they are required, and to protect against the cytotoxic effects of excess unbound zinc and copper (Andreani et al., 2010). Additionally, in the kidney, copper levels particularly are kept within narrow limits (Andreani et al., 2010). Thus, zinc and copper toxicities are rarely seen in companion animals (Wills et al., 1994).

2.1.2. Minerals associated with renal disease

Certain metal elements, which may be obtained through the diet, are important components of metalloenzymes, which are needed for physiological buffering of cellular ROS. For example, copper and zinc or manganese form part of the hydrolytic core of superoxide dismutase; iron is present in catalase and peroxidase enzymes (Ratliff et al., 2016) and selenium is required for glutathione peroxidase (Locatelli et al., 2003). As a result, the kidney is rich in antioxidant trace metal elements (Shimamura et al., 2013). This is important in order to counteract ROS generation, which occurs as a result of the high metabolic activity of the kidneys normally, and which is increased by other factors involved in CKD progression (Brown, 2008, Kim et al., 2009). Increased oxidative stress has been induced in animals fed diets that are deficient in antioxidant nutrients, such as vitamin E and selenium, and, in these animals, there is increased ROS synthesis (Gao et al., 2019) as well as glomerular and tubulointerstitial damage (Nath and Salahudeen, 1990). Thus, it is clear that adequate dietary supply of antioxidant minerals is important.

The RPTECs have high density and oxidative activity of mitochondria, in order to generate sufficient ATP for the cells' transport and reabsorptive functions. During this mitochondrial energy production, ROS are generated (Ratliff et al., 2016). Additionally, studies in rats have shown that RPTECs exhibit a limited capacity for endogenous synthesis of glutathione, a key component of the antioxidant enzyme, glutathione peroxidase (Visarius et al., 1996). Instead, RPTECs rely primarily on the glutathione in circulation, to buffer the abundance of ROS produced by the mitochondria (Visarius et al., 1996). Consequently, the RPTECs may be particularly susceptible to oxidative damage (Ratliff et al., 2016). If ROS production exceeds the reducing capacity

of the resident antioxidant defences, oxidative stress ensues (Locatelli et al., 2003). Oxidative stress involves damage to DNA, altered gene expression and intracellular protein function and lipid peroxidation (Brown, 2008, Ratliff et al., 2016) and may result in apoptosis and necrosis of cells (Brown, 2008). In the kidney, renal structural and functional impairment can thereby be initiated or progressed (Locatelli et al., 2003). Importantly, ROS may induce the inflammatory processes that underpin the development of tubulointerstitial nephritis and renal fibrosis (Agarwal, 2003, Kim et al., 2009). Furthermore, during renal disease, the inflammatory cascade and the presence of interstitial fibrosis enhance ROS generation (Locatelli et al., 2003, Brown, 2008). Ultimately, if not brought under control, or if the vicious cycle of renal injury is initiated, CKD may follow (Ratliff et al., 2016).

In contrast to the minerals with antioxidant roles, several trace elements are pro-oxidant and increase the production of ROS. Some minerals, such as iron, copper, chromium, cobalt, vanadium and nickel, are termed “redox-active” and induce ROS formation directly, in what are known as Fenton reactions (Ratliff et al., 2016, Ercal et al., 2001). Others, including the heavy metal elements, arsenic, mercury, lead and cadmium, are “redox-inactive” and increase ROS by actively depleting cellular antioxidants or by binding to antioxidants or their cofactors to inhibit their actions (Ercal et al., 2001, Ratliff et al., 2016). The liver and kidney are the two organs where these elements, particularly cadmium and arsenic, are most concentrated (Thevenod and Lee, 2013, Chang and Singh, 2018, Lewchalermvong et al., 2018). Through oxidative stress pathways or otherwise, these minerals, especially inorganic arsenic, mercury and cadmium, can be nephrotoxic in excess (Thevenod and Lee, 2013, Thevenod and Wolff, 2016, Ercal et al., 2001).

Most studies on the oxidative status of the kidney in CKD have focused on humans (Brown, 2008). The extremely short half-lives of ROS makes them difficult to measure (Locatelli et al., 2003). However, in humans, there is a strong correlation between the severity of renal disease and the levels of products of oxidative damage (Ratliff et al., 2016). It is uncertain whether imbalance of pro- and anti-oxidant compounds, resulting in increased ROS

formation and renal oxidative stress, is involved in CKD progression in cats and dogs (Brown, 2008).

2.1.2.1. Pro-oxidant minerals

2.1.2.1.1. Arsenic

Chronic kidney disease is particularly prevalent in countries such as Bangladesh and India (Abraham et al., 2016, Hasan et al., 2018) where people have high arsenic intake due to arsenic-contamination of the water that is used for drinking and cooking (Ohno et al., 2007, Mandal et al., 1998, Mazumder et al., 2013). However, in the studies that have been conducted so far, evidence for the causality of arsenic in CKD is limited (Zheng et al., 2014). Nonetheless, there appears to be a strong association between arsenic exposure and the development of CKD experimentally (Zheng et al., 2014). Indeed, increased oxidative stress in the kidney has been observed in mice exposed to arsenic (Li et al., 2010). Arsenic has been shown to increase inflammation (Escudero-Lourdes et al., 2010), oxidative stress and vascular dysfunction (Barchowsky et al., 1996), thus, renal arsenic exposure may lead to CKD via these mechanisms (Zheng et al., 2014). Indeed, recently, it was shown that chronic arsenic exposure can induce fibrosis in human kidney epithelial cells (Chang and Singh, 2018).

The kidney is the primary organ involved in arsenic excretion from the body (Chang and Singh, 2018). In humans, high urinary total arsenic, reflecting dietary intake of the mineral (Mazumder et al., 2013, Bae et al., 2017), has been associated with increased risk of renal dysfunction. In fact, a positive correlation between arsenic exposure and renal dysfunction has been noted (Zheng et al., 2014, Chen et al., 2011). Thus, it seems important that arsenic intake from the diet is kept within reasonable limits. Arsenic concentrations are highest in fish (Bae et al., 2017), rice and wheat (Rasheed et al., 2018), which are common ingredients used in pet foods. Indeed, arsenic concentration is higher in fish-containing pet foods that are commercially available and commonly fed to pet cats (Davies et al., 2017a, Squadrone et al., 2017). Moreover, the total arsenic content of some of these foods is above the legal

maximum arsenic concentration that pet foods should contain (Davies et al., 2017a). Thus, while cats are perceived to have a taste-preference for fish (Houpt and Smith, 1981), consuming large quantities of this ingredient may expose them to high levels of arsenic. This may have damaging consequences for the kidneys.

Different forms of arsenic exist however, with organic arsenic species being less toxic than the inorganic forms (Jomova et al., 2011). Many studies have identified the arsenic in marine and freshwater fish as predominantly arsenobetaine, an organic arsenic species of relatively low toxicological concern (Komorowicz et al., 2019, Sele et al., 2015, Kalantzi et al., 2017, Miyashita et al., 2009). However, the evidence for arsenic species composition within freshwater fish is conflicting: some studies have found a preponderance of other organic arsenic species, dimethylarsinic acid (DMA) and monomethylarsinic acid (MMA), as well as higher concentrations of toxic, inorganic arsenate (As[V]) and arsenite (As[III]), compared to marine fish (Yang et al., 2017b, Jankong et al., 2007). The forms of arsenic that exist within a particular fish appears to depend on the environment in which the fish lives; the amounts of these arsenic species may also be affected by the age, weight, lipid content, species and trophic level of the fish (Komorowicz et al., 2019, Kalantzi et al., 2017). Arsenic bioaccumulates through the food chain (Komorowicz et al., 2019). However, while inorganic forms of arsenic are apparently most common in the environment (Jomova et al., 2011) and are present in the phytoplankton and microalgae that fish consume (Caumette et al., 2012), they are metabolised within the fish to relatively innocuous methylated organic arsenic species (Yang et al., 2017b, Kalantzi et al., 2017). Often, the end-product of arsenic metabolism is arsenobetaine, which explains why it is the most abundant arsenic species in most fish (Komorowicz et al., 2019). Consequently, the arsenic content of these fish, despite being high, may pose little risk to the humans, or animals, that consume them (Komorowicz et al., 2019). Nonetheless, the composition of arsenic species in cat foods is unknown and requires investigation to determine the risk of high dietary arsenic intake via consumption of fish-containing foods for cats.

2.1.2.1.2. Mercury

Mercury is a non-essential mineral in the body (Park and Zheng, 2012). Like arsenic, there are different forms of mercury with different levels of toxicity. These are elemental mercury, inorganic mercury and organic mercury (Park and Zheng, 2012). Elemental mercury exposure occurs primarily through inhalation (Park and Zheng, 2012). Inorganic and organic forms of mercury are consumed with some foods. Total mercury concentrations are particularly high in fish, compared to other foods, and this is predominantly organic methylmercury (Maulvault et al., 2015, Cheng et al., 2009). Meat and poultry products may contain inorganic mercury, as methylmercury in fish products used as animal feed can be metabolised to inorganic mercury in the mammalian body (Clarkson and Magos, 2006, Suda and Hirayama, 1992). Thus, cats and dogs may be exposed to both organic and inorganic mercury through their diet.

It is possible that cats even consume more mercury than dogs, due to greater consumption of fish (Sakai et al., 1995). An American study found wide variation in the mercury content of a range of wet and dry, cat and dog foods; the foods with the highest mercury content all contained fish (Luippold and Gustin, 2016). In an analysis of dry dog foods, Kim et al. (2018) also detected significantly higher mercury concentrations in fish-, compared to poultry- or red meat-, based diets. However, the American study found that mercury content of cat foods was generally greater than that of dog foods; in particular, they found that some fish-containing, wet cat foods had excessive levels of mercury in relation to the recommendations (Luippold and Gustin, 2016). This is in contrast to findings from a study conducted in Brazil where the mercury content of cat and dog foods was similar (Paulelli et al., 2018). However, in that study, wet (canned) foods were found to have significantly greater mercury concentrations than dry foods and some contained more mercury than the maximum tolerable level for pets (Paulelli et al., 2018). Sakai et al. (1995) also detected higher levels of mercury in wet, versus dry, cat foods and greater mercury concentrations still in fresh and canned tuna and dried sardines produced for human consumption, which are often fed to pet cats but

infrequently to dogs (Sakai et al., 1995). High mercury concentrations were not associated with any particular flavour in the Brazilian study (Paulelli et al., 2018). However, a greater proportion of the canned cat foods, compared to the canned dog foods, that were analysed, contained fish as the main protein source. Moreover, the canned cat foods had higher average and maximum mercury concentrations, than the canned dog foods (Paulelli et al., 2018), which may be related to the fish content. In one study, although mercury concentrations measured in the kidneys were low, they were significantly higher in the kidneys from household dogs compared to stray dogs (Serpe et al., 2012). Consumption of commercial diets and ingredients such as fish could underpin this difference (Serpe et al., 2012). Domestic versus wild cats could exhibit the same distinction but, to our knowledge, this has not been investigated. Hence, this was an aim of the current study.

The species of mercury within the pet foods was not determined in any of the aforementioned studies, but was assumed to be mostly methylmercury in the American study (Luippold and Gustin, 2016). Another study, analysing dog foods exclusively, but measuring both total, and methyl-, mercury concentrations, had quite contrasting results: only three of the 24 diets evaluated contained mercury and, of these, only the one with the lowest total mercury concentration contained fish. Indeed, in that fish-based food, methylmercury was detected. On the contrary, methylmercury was not present in either of the other two foods that contained mercury in that study (Sires et al., 2019). Despite the contrasts, however, the results of these studies suggest that the risk of excessive mercury intake, likely through consumption of wet and/or seafood-flavoured diets, may be greater for cats than it is for dogs (Beynen, 2018, Kim et al., 2018). Indeed, cats have been shown to have higher total mercury concentrations in their hair, suggesting greater dietary intake of mercury, compared to dogs (Sakai et al., 1995). Furthermore, mercury concentrations in hair were also higher in older, compared to younger, cats, suggesting a cumulative effect in the body of chronic mercury exposure (Sakai et al., 1995). Thus, the risk of mercury toxicity may be increased by consistent long-term feeding of the same complete diet, which is believed to be standard practice in companion animals (Luippold and Gustin, 2016).

Inorganic mercury is water-soluble and, after ingestion, it primarily targets the kidney; particularly the proximal tubules, where it may cause damage (Park and Zheng, 2012, Bridges and Zalups, 2010). In contrast, organic methylmercury is lipid soluble and mainly targets the brain (World Health Organisation, 1990), causing neurological damage (Carocci et al., 2014, Albanus et al., 1972). Methylmercury is able to enter body tissues by forming hydrophilic complexes with amino acids, proteins and certain peptides (Clarkson and Magos, 2006, Simmons-Willis et al., 2002). Thus, it may enter renal tubule epithelial cells in this way (Bridges and Zalups, 2010). Furthermore, lipid-soluble organic mercury may readily accumulate in renal tubule epithelial cells due to the presence of lipid droplets. This may pose a greater problem for the domestic cat since renal intracellular lipid droplets are particularly abundant in this species (Modell, 1933). To our knowledge, direct methylmercury toxicity within the kidney has not been shown. Instead, organic methylmercury entering the kidney may have a toxic effect once it is converted to inorganic mercury compounds via oxidation (Bridges and Zalups, 2010). Methylmercury, obtained through the diet, can also be converted to inorganic mercury elsewhere in the body, such as in the gastrointestinal tract (Clarkson and Magos, 2006) and liver (Suda and Hirayama, 1992) and also by phagocytic cells in blood (Suda et al., 1993), prior to reaching the kidneys (Bridges and Zalups, 2010). Indeed, in cats fed mercury as almost exclusively methylmercury and less than 1% inorganic mercury, only 60% of the total mercury that ended up in the kidney was methylmercury, suggesting that the remainder was converted to other mercury compounds; possibly inorganic (Albanus et al., 1972). Even so, it is evident that the typically neurotropic, lipid-soluble, organic methylmercury can accumulate in the kidney. Additionally, it is clear that methylmercury exposure through consumption of fish, which is likely to be the greatest source of mercury in cats, may increase the inorganic mercury burden in the kidneys. Indeed, cats fed tuna had significantly higher mercury levels in their kidneys than cats fed beef (Houpt et al., 1988). Greater renal accumulation of mercury may consequently increase the risk of mercury-induced oxidative damage in the kidney. It is thought that mercury causes depletion of intracellular antioxidants, which leads to mitochondrial dysfunction, and binds to thiol groups present in certain amino acids, proteins,

enzymes and lipoic acids, which increases ROS production (Carocci et al., 2014, Antunes Dos Santos et al., 2018). In summary, it seems that increased mercury consumption could increase the mercury concentration in the kidney, which, itself, may lead to renal disease. Nonetheless, to our knowledge, no prior study has compared the kidney tissue mercury content of cats, a species with likely high mercury intake due to fish consumption and a high prevalence of CKD, to that of dogs, a species with lower fish consumption and lower prevalence of CKD. Hence, this comparison was made in the current study.

2.1.2.1.3. Iron

Different forms of iron exist: haem and non-haem iron. Haem iron is a component of haemoglobin and myoglobin, thus it is found within animal tissues and obtained through the diet by consuming meat (World Health Organization, 1988). This form of iron is more bioavailable than non-haem iron, which is found in cereals, grains and vegetables (Wyness, 2016, World Health Organization, 1988). Cats, as obligate carnivores, are likely to have greater dietary intake of the more bioavailable, haem iron than dogs, which are omnivores. Thus more iron may reach the cat's kidney than reaches the dog's kidney. Andreani et al. (2010) found that cat liver iron content correlates with the age of the cat and suggested that some diets for cats may supply excessive levels of iron, which may accumulate in the tissues over time. An age-associated increase in iron concentration has also been observed in rat kidneys (Nankivell et al., 1994, Shimamura et al., 2013). Increased dietary intake of iron in cats relative to dogs might cause increasingly higher iron content of the feline kidney over time.

Free iron, as opposed to protein-bound iron, is toxic to cells, as it is a catalyst of Fenton reactions and reacts with H_2O_2 to form highly reactive hydroxyl (OH^-) radicals, one type of ROS (Locatelli et al., 2003, Jomova and Valko, 2011). Interestingly, in the kidneys, mitochondria contain a form of the iron-storage protein, ferritin, which may help to reduce oxidative damage by free iron in these highly active cells (Thevenod and Wolff, 2016, Santambrogio et al., 2007). Nonetheless, increased iron levels have been found in kidney tissue,

associated with oxidative stress (van Raaij et al., 2018), tubular damage and proteinuria (Nankivell et al., 1992, Wang et al., 2001, Nankivell et al., 1994). Urine iron concentrations have also been found to correlate with markers of renal oxidative stress in human CKD patients (Nakatani et al., 2018). Furthermore, the inflammation and oxidative stress that are common features of chronically diseased kidneys, may cause upregulation of iron transport proteins, which may increase iron uptake into the renal tubule cells and, in turn, promote further oxidative damage (Martines et al., 2013). Few studies have measured iron in cats, although it has been reported that serum iron concentrations in cats with CKD and healthy cats are not different (Gest et al., 2015, Javard et al., 2017). Although serum iron concentrations do not reflect the total body stores or tissue concentrations of iron, these results may suggest that the cat's kidney does not receive an increased iron load during CKD (Gest et al., 2015). However, total iron binding capacity (TIBC) has been shown to be significantly lower in cats with CKD than in healthy cats (Gest et al., 2015). This may imply that there is more highly-reactive free iron in circulation and thus reaching the kidneys in cats with CKD, which may increase renal oxidative damage. Nonetheless, direct measurement of renal tissue iron concentrations in healthy cats, compared to cats with CKD, would be preferable to analysis of serum samples.

2.1.2.2. Antioxidant minerals

2.1.2.2.1. Zinc and copper

Zinc (Vallee and Falchuk, 1993) and copper are both essential structural and functional components of many proteins (Andreani et al., 2010). In fact, together, zinc and copper form part of the SOD1 and SOD3 isoforms of the antioxidant enzyme, superoxide dismutase (SOD) (Ratliff et al., 2016). SOD1 is active in the cytosol while SOD3 activity occurs extracellularly (Mates, 2000). The former constitutes 80% of the total SOD activity in the mammalian kidney (Marklund, 1984). Furthermore, zinc and copper are required for growth and development, tissue repair and immune responses (Mertz, 1981). Copper also has roles in iron metabolism, erythropoiesis, angiogenesis, cardiovascular

health, bone health and neurological function (Linder and Hazegh-Azam, 1996).

Zinc and copper are obtained through the diet; particularly with consumption of meat (Hazell, 1985). Deficiencies of copper and zinc may be more common than toxicities in companion animals. This may be through inadequate dietary intake of these nutrients or interactions with other mineral elements. For example, despite both minerals having similar antioxidant functions, zinc and copper compete for metallothionein binding in the gastrointestinal tract, so high levels of one can impair gastrointestinal absorption of the other (Yanagisawa et al., 2014). Indeed, excess zinc intake has been shown to induce copper deficiency and consequently diminish SOD, which can lead to increased ROS formation, oxidative stress and subsequent reduced renal function, systemic hypertension and reduced renal blood flow in rats (Yanagisawa et al., 2014). Iron also competes with copper and zinc for absorption, so high dietary iron intake may lead to copper and/or zinc deficiency. Mercury can also interact with copper and zinc, reducing their absorption in the gastrointestinal tract (Hazell, 1985). Therefore, if cats have greater intake of bioavailable iron and mercury, compared to dogs, due to the obligate carnivore diet and increased consumption of meat and fish as we suggest, they may too have lower levels of copper and zinc in their kidneys. Copper absorption may also be reduced by calcium-induced pH changes in the gastrointestinal tract (Hazell, 1985) as well as by interactions with cadmium, silver, and lead (Davies, 1979). Zinc absorption may also be hindered by calcium, cadmium, selenium and molybdenum (Hazell, 1985, Davies, 1979), but enhanced by a higher intake of protein and consumption of animal, as opposed to plant, proteins (Shah and Belonje, 1984). Copper absorption is facilitated by the presence of specific amino acids provided by the breakdown of dietary proteins (Hazell, 1985).

Cats up to one year old have been found to have greater concentrations of zinc in the renal medulla and copper in the renal cortex, compared to older cats (Passlack et al., 2014). However, in a separate study, feline kidney zinc and copper contents were not associated with age (Andreani et al., 2010). Similar methods were used in these two studies and both involved the use of

post-mortem tissue from cats with various pathologies. Although no conclusions about the effect of any particular medical conditions, other than CKD in the study by Passlack et al. (2014), on tissue mineral contents were drawn, the different array of pathologies within each study population of cats may account for the contrasting findings of an age effect. As well as reduced zinc concentrations with age, Passlack et al. (2014) also measured lower zinc concentrations in the kidney cortex and medulla from cats with CKD compared to cats without CKD. Since CKD is more common in older cats, these two findings, of age- and CKD-associated reduction in renal zinc content, may be confounding. However, they may suggest that cats have reduced zinc-dependent antioxidant activity in their kidneys with age and CKD. Supporting the notion that antioxidant capacity within organs may be influenced by mineral nutrition, lower concentrations of two copper-dependent enzymes, SOD and cytochrome oxidase, were detected in copper deficient tissues, including the kidney, in mice with a dietary copper deficiency (Prohaska, 1983). However, while another study found that kittens fed a copper deficient diet had reduced levels of copper and SOD in the liver, compared to kittens with dietary copper supplementation, kidney tissue copper content was not different between kittens fed different levels of dietary copper (Doong et al., 1983). Therefore, it is unclear whether kidney copper content reflects dietary copper intake. Nonetheless, Passlack et al. (2014) suggest that dietary intake of copper varies substantially between cats, since they measured a wide range of copper concentrations in the livers. Although Passlack et al. (2014) did not analyse the copper content of the diets directly, their conclusion is supported by a separate dietary analysis which revealed the copper content of some commercially-available complete cat and dog foods to be above the European legal limit and below the FEDIAF minimum recommended level in others (Davies et al., 2017a). In contrast to copper, concentrations of zinc in the kidney have been found to correlate with dietary zinc intake in cats, dogs (Drinker et al., 1927a) and rats (Drinker et al., 1927b) and commercially-available, complete foods for cats and dogs appear to comply with maximum and minimum recommended levels of zinc (Davies et al., 2017a).

2.1.3. Aims and hypotheses of Study 1

For companion animals, particularly cats that have rates of CKD that far exceed other mammals, the elemental profile of kidneys in relation to kidney disease has not been extensively studied (Andreani et al., 2010, Passlack et al., 2014). Chronic interstitial nephritis is the main renal lesion observed in cats with CKD and can be induced by oxidative stress, which itself may be promoted by an imbalance of minerals involved in ROS formation and antioxidant defences in the kidney. Arsenic (Marriott et al., 2019, Squadrone et al., 2017, Davies et al., 2017a) and mercury (Cheng et al., 2009, Luippold and Gustin, 2016) are two such pro-oxidant, metallic elements often found in high concentrations in fish and fish-flavoured pet foods, which are more often fed to cats than dogs (Houpt and Smith, 1981). However, cats may be maladapted to this diet, since their ancestors originated from the savannah, where fish as part of a normal diet would be a rarity (Driscoll et al., 2007, Montague et al., 2014). Iron is another pro-oxidant mineral that may be high in the diet of obligate carnivores, such as the cat (World Health Organization, 1988). Moreover, increased dietary intake of minerals such as iron and mercury, as we suggest is the case in cats relative to dogs, may reduce the levels of antioxidant elements, for example copper and zinc, that are available to the kidney (Hazell, 1985). We propose that renal accumulation of pro-oxidant elements, including arsenic, mercury and iron, and depletion of antioxidant elements, such as copper and zinc, underpin the high incidence of CIN in domestic cats.

Aims

1) In this chapter, I will assess kidney tissue from domestic cats, dogs and Scottish wildcats for histological evidence of chronic interstitial nephritis. I will semi-quantify the observed chronic tubulointerstitial renal lesions (inflammation and fibrosis), to determine the prevalence and extent of these lesions in our study populations.
2) The primary aim of this study is to measure the concentrations of pro-oxidant minerals that exist at high levels in fish-based foods and/or may promote renal oxidative stress through increased ROS formation, such

as arsenic, mercury and iron, as well as concentrations of minerals with antioxidant functions, such as copper and zinc, in the kidneys and urine of domestic cats and dogs and kidneys of Scottish wildcats.

3) We also aim to determine the mercury content of a range of commercially-available, complete, wet and dry, cat and dog foods that we have analysed previously for their content of other minerals. We will assess the compliance of these foods with the European legal limit of mercury that pet foods can contain.
4) Another aim of this study is to identify the species of arsenic within fish-based pet foods that have known high total arsenic content.

Hypotheses

1) Chronic interstitial nephritis is more prevalent and often of greater severity in domestic cats, compared to dogs and Scottish wildcats (*to address Aim 1*).
2) Renal accumulation of pro-oxidant elements, such as arsenic, mercury and iron, and depletion of antioxidant elements, including copper and zinc, underpin the high incidence of CIN in domestic cats (*Aim 2*).
3) Commercially-available, complete cat and dog foods that contain fish have higher concentration of mercury than complete pet foods that do not contain fish (*Aim 3*).
4) The known high total arsenic concentration in fish-based commercially-available complete wet cat foods is predominantly relatively non-toxic arsenobetaine (*Aim 4*).

2.2. Materials and Methods

2.2.1. Ethics

This study was approved by the University of Nottingham School of Veterinary Medicine and Science Research Ethics Committee (REC 2074 170721). Consent from pet owners to use tissue from their euthanised or deceased animals for research was taken at source by a veterinarian not associated with this project. All methods described were performed in compliance with the relevant guidelines and regulations for good laboratory practice.

2.2.2. Source of animal material

Kidneys were obtained from recently deceased or euthanised domestic cats (n=72) and dogs (n=70) submitted to The University of Nottingham Veterinary Pathology Service (UoN-VPS) for necropsy by board-certified veterinary pathologists. If present, urine was collected from the bladders of these animals (domestic cats, n=27; dogs, n=36) at the same time. Kidneys from recently deceased Scottish wildcats (n=17) were collected at necropsy at National Museums Scotland, frozen at -80°C and sent, on ice, in accordance with UN3373, to The University of Nottingham, where they were received next-day.

2.2.3. Renal histology

2.2.3.1. Sample preparation

At necropsy, a representative block of kidney tissue, containing both cortex and medulla, was fixed in 10% neutral-buffered formalin. Fixed tissue was rinsed twice with paraffin wax, then embedded in cassettes containing paraffin wax. Formalin-fixed paraffin-embedded (FFPE) tissue was cut to a thickness of 5 µm using a microtome and sections were mounted onto glass polylysine slides labelled with the corresponding animal identification number.

2.2.3.2. Haematoxylin and eosin staining

Prepared sections of kidney tissue were stained with haematoxylin and eosin (HE), according to a standard procedure in the pathology department at The

University of Nottingham. In short, sections were dewaxed in xylene for 10 minutes, then rehydrated through four baths containing 100% ethanol, for 2 minutes in each. Slides were then rinsed in tap water before being stained in Harris' haematoxylin solution for 2 minutes. Tap water was used again for 30 seconds to rinse the slides before differentiation in acid alcohol for a few seconds until the background was clear. Further 30 second rinses with tap water were completed before and after bluing the sections in Borax for 30 seconds. Then the sections were counterstained in 1% acidified eosin solution for 10 seconds, washed in tap water, dehydrated back through the four 100% ethanol baths and cleared in xylene for 10 minutes. Finally, the sections were mounted with glass coverslips using the resinous mounting medium, dibutylphthalate polystyrene xylene (DPX).

2.2.3.3. Masson's trichrome staining

A subset of domestic cats (with CIN, n=7; without CIN, n=3), dogs (with CIN, n=6; without CIN, n=9) and Scottish wildcats (n=7) were selected at random for semi-quantitative assessment of renal fibrosis. A second section of renal tissue from these animals was prepared as described (Section 2.2.3.1) and stained with Masson's trichrome (MT) following a routine protocol in the pathology department at The University of Nottingham. Differential staining of ECM components, such as collagen, and smooth muscle fibres as blue and red, respectively (Hernandez-Morera et al., 2016, Bargi et al., 2017), allowed assessment of fibrosis. In brief, sections were dewaxed in xylene for 10 minutes, rehydrated by soaking consecutively for 2 minutes in each of four separate 100% ethanol baths and then washed in running tap water. Next, slides were stained in freshly prepared Harris' haematoxylin for 5 minutes, then washed in tap water, then in acid alcohol and finally in ammoniated water. After this, the sections were stained with ponceau fuchsin trichrome stain for 5 minutes and rinsed with distilled water before being treated for a further 5 minutes with a freshly prepared solution of 1:1:2 (v/v/v) phosphotungstic acid: phosphomolybdic acid: distilled water. Slides were rinsed once again in distilled water, then counterstained in Light Green stain for 5 minutes. Following this, slides were immersed for 5 minutes in 1% acetic acid solution,

which was discarded after each single use. Finally, slides were rinsed again in water, dehydrated back through a series of four baths containing 100% ethanol and cleared in xylene over a total of 10 minutes. The sections were then mounted with glass coverslips using DPX mountant.

2.2.3.4. Semi-quantitative scoring of chronic interstitial nephritis

The HE-stained kidney tissue sections were viewed under a light microscope and scored for their content of chronic interstitial nephritis (CIN). A semi-quantitative scoring system was developed based on systems reported in the literature (Chakrabarti et al., 2013, McLeland et al., 2015). While others have assessed glomerular damage alongside tubulointerstitial lesions (Lawler et al., 2006), in the present study, only tubulointerstitial lesions were scored as these are the primary histopathological feature in the kidneys of cats with CKD (Yabuki et al., 2010, Lawler et al., 2006). Only lymphoplasmacytic inflammatory infiltrates were evaluated in the current study. The presence of polymorphonuclear cells, such as eosinophils or neutrophils, would indicate a more acute process, such as a drug toxicity reaction or an ascending bacterial pyelonephritis (John and Herzenberg, 2009).

Whole kidney tissue sections were evaluated systematically, beginning at the bottom left of the section, moving the field of view up to the top and then back down to the bottom of the tissue and working from left to right of the section. Two researchers, blinded to the species from which each sample came, independently evaluated the sections and recorded their estimate of the percentage coverage of each section with lymphoplasmacytic inflammation (CIN). Their estimates were then compared. Where there was a discrepancy of <10% recorded by the two researchers individually, an average of their two scores was taken to give the final score for a particular case. If the individual percentage scores for a particular case differed by ≥10%, the section was re-evaluated by both researchers together and a unanimous decision reached. The defining limits of the CIN score categories were then decided based on the distribution of the cases across the observed range of tissue coverage with CIN. In brief, scores of 0 (absent), 1 (mild), 2 (moderate) and 3 (severe) were given where 0%, <5%, 5-25% and >25% of the tissue section, respectively,

was affected by CIN. Score 1 corresponded to animals with very few mononuclear cell infiltrates within the renal interstitium (<5%) therefore, it is likely that they have little pathological significance. Thus, animals with CIN scores of 0 and 1 were grouped together as 'no CIN' while scores 2 and 3 were grouped together as 'CIN' in later analyses.

The animals' necropsy reports were obtained and descriptive data, such as breed, age and neutered status, as well as any diagnoses, were recorded. Cases with a known renal infectious and/or nephrotoxic aetiology of CIN, such as canine herpes virus (Robertson, 1986), feline infectious peritonitis (FIP), bacterial pyelonephritis (Brown et al., 2016) or ethylene glycol toxicity (Amoroso et al., 2017), as stated in the necropsy report or determined through microscopic assessment of the tissue, were excluded. In contrast, cases with histological evidence of renal infarcts were retained for analysis, since chronic infarction can be a consequence (Oh et al., 2016), as well as a cause (Yang et al., 2016), of CKD. Accordingly, CIN scores from n=62 domestic cats and n=70 dogs were retained for further analysis. Necropsy reports were not available for the Scottish wildcats. The only information available for these animals was their species identification as a Scottish wildcat, determined by National Museums Scotland, the location in which they were found and the macroscopic appearance of the kidneys. These animals were, most likely, roadkill and so the tissue from them was mostly of a poorer quality than the quality of kidney tissue from the domestic cats and dogs in this study. Nevertheless, if the histopathological appearance of the tissue did not allow assessment of CIN, the Scottish wildcat, to which the tissue belonged, was excluded. A total of n=17 Scottish wildcat kidneys were retained for further analysis.

2.2.3.5. Semi-quantitative scoring of fibrosis

Masson's trichrome (MT) stained tissue sections were scored semi-quantitatively for fibrosis when viewed under a light microscope. The same scoring system used to grade CIN in the current study, was applied to tubulointerstitial fibrosis. To clarify, this involved the same systematic

approach for examining the tissue section and the same limits for the score categories; scores of 0 (absent), 1 (mild), 2 (moderate) and 3 (severe) were given where 0%, <5%, 5-25% and >25% of the tissue section, respectively, was covered with fibrosis, determined by blue staining with MT.

2.2.4. Mineral analysis of kidneys and urine

2.2.4.1. Sample preparation

Gloves were worn at all times during sample handling, to avoid contamination. Kidneys, collected at necropsy, were stored frozen at -80°C. Approximately 1 gram (g) of frozen tissue, comprising predominantly renal cortex with some outer medulla, was then cut and freeze-dried at -55°C over a period of 48-72 hours, using the ScanVac CoolSafe 55-9 freeze-dryer according to the instruction manual.

Urine samples were also stored frozen at -80°C. Each sample was thawed at room temperature, centrifuged for 5 minutes at 800 g and then 500 µl was pipetted into an appropriately labelled 14 ml (105mm x 16.8mm) polypropylene tube (Sarstedt, Leicester UK) ready for elemental analysis by inductively-coupled plasma-mass spectrometry (ICP-MS). A certified reference material (CRM) of SERO210705 Seronorm Trace Elements Urine L-2 (LGC Standards) was used and added in duplicate per ≤60 urine samples submitted for ICP-MS.

2.2.4.2. Acid digestion of kidney tissue

Freeze-dried kidney tissue samples, weighing between 100 and 250 mg, were added into 50 ml inert PTFE-TFM-lined digestion vessels and then acid digested using a modification of the procedure described in detail in a publication from the United States Environmental Protection Agency (USEPA., 1995). This converted the solid tissue samples into a liquid state suitable for analysis by ICP-MS. In brief, each sample was left to incubate for 1 hour in 3.0 ml of 68% trace analysis grade HNO_3, 2.0 ml of 30% H_2O_2 (both Fisher Scientific UK Ltd, Loughborough, UK) and 3.0 ml deionised milli-Q water (18.2 MΩ.cm). Two tubes containing 100-200 mg of a CRM (NIST, National Institute

of Standards and Technology, Gaithersburg, MD, USA; 1577c bovine liver) and two ‘blank’ tubes containing only the digestion chemicals, were included alongside every 20 digestion vessels containing samples. ‘Blank’ tubes allowed for control of unexpected sources of contamination, for example, from the acids or water added during the acid-digestion step of kidney sample processing. Use of a CRM enabled control of variability between batches of samples on separate ICP-MS runs.

Next, the digestion vessels were loaded into the 24HVT50 rotor of a microwave reaction system (Multiwave PRO, Anton Parr, Austria), the directions of use for which are outlined in the manufacturer’s guide (Anton Paar Limited, 2016). Microwave-assisted digestion, which involved simultaneous rapid heating and pressure increase, accelerated digestion of the samples by the acid matrix applied, over a total of 45 minutes. This included heating the samples to 140°C over 10 minutes, holding this temperature for 20 minutes and then cooling to 55°C over the remaining 15 minutes. The samples were then allowed to cool to room temperature. After digestion, the resulting liquid samples were diluted to 15.0 ml by addition of 7.0 ml deionised milli-Q water. For each prepared kidney tissue and urine sample and the prepared CRMs and ‘blanks’, 500 µl was pipetted into a correspondingly-labelled 14 ml (105mm x 16.8mm) polypropylene tube (Sarstedt, Leicester UK) and capped ready for multi-element analysis by ICP-MS.

2.2.4.3. Inductively-coupled plasma-mass spectrometry

Acid-digested kidney samples and neat urine samples (500 µl) were diluted 1 in 20 by addition of 9.5 ml of double line 0.5% HNO_3 for multi-element analysis (31 elements; Table 3) by ICP-MS (Thermo-Fisher iCAP-Q, Thermo-Fisher Scientific, Loughborough, UK). ICP-MS has been used in previous studies for multi-elemental profiling of various biological fluids and tissues (Zhang et al., 2016, Goulle et al., 2005) and food materials (Joy et al., 2015). It is considered a method with sensitivity and specificity greater than with techniques traditionally used for mineral analyses, such as gas chromatography mass spectrometry and liquid chromatography coupled to a diode array detector (Goulle et al., 2005). Diluted samples were introduced via a covered

autosampler (Cetac ASX-520) through a 1317090 pfa-st nebulizer (ESI; Thermo-Fisher Scientific, Loughborough, UK). ICP-MS used a 'Flatopole collision cell', which was charged with helium gas for all elements except selenium, where hydrogen gas was used instead; in-sample switching allowed for simultaneous measurement. The collision cell was upstream of the analytical quadrupole to reduce polyatomic interference. For all elements, the internal standards used included scandium (Sc; 50 µg/L), germanium (Ge; 20 µg/L), rhodium (Rh; 10 µg/L) and iridium (Ir; 5 µg/L) in the preferred matrix of 2% HNO_3. Internal standards were introduced to the sample stream via a T-piece. External calibration standards were all in the range of 0-100 µg/L (ppb) for trace elements and 0-100 mg/L (ppm) for major elements. Sample processing was undertaken using 'Qtegra software' (Thermo-Fisher Scientific, Loughborough, UK).

For each batch (i.e. for every 20 kidney and every ≤60 urine samples analysed), the ICP-MS results of individual elements were blank-corrected and then corrected to the percentage recovery of each mineral element in the CRM. Blank correction involved calculating the average concentration of each element across the two blanks and subtracting it from the levels measured in each sample. CRM-correction, which followed, involved determining how much of each element was measured by ICP-MS in the CRM, as a percentage of the amount of each particular element the CRM was known to contain. This percentage of each element's concentration, for every sample in the batch was then calculated and these corrected element concentrations are presented. Limits of detection (LoD) of each element were determined from the standard deviation of element concentrations measured in n=3 blanks for kidney tissue and n=10 blanks for urine. For each of the major and trace elements concentrations in the CRM, recovery was >95% with <10% coefficient of variation (n=24 separate analyses). Intra-assay variability for all elements presented in this study was <2%.

Major element		Trace element					
Name	**Symbol**	**Name**	**Symbol**	**Name**	**Symbol**	**Name**	**Symbol**
Boron	B	Titanium	Ti	Cobalt	Co	Molybdenum	Mo
Sodium	Na	Lithium	Li	Nickel	Ni	Silver	Ag
Magnesium	Mg	Aluminium	Al	Copper	Cu	Cadmium	Cd
Phosphorus	P	Beryllium	Be	Zinc	Zn	Caesium	Cs
Sulphur	S	Vanadium	V	Arsenic	As	Barium	Ba
Potassium	K	Chromium	Cr	Selenium	Se	Thallium	Tl
Calcium	Ca	Manganese	Mn	Rubidium	Rb	Lead	Pb
		Iron	Fe	Strontium	Sr	Uranium	U

Table 3. Major and trace elements measurable by inductively-coupled plasma-mass spectrometry after sample digestion in an acid matrix.

2.2.4.4. Measurement of urine creatinine concentration to correct for different volumes and dilutions among the spot samples

Creatinine concentration was measured in each neat, urine sample (200-500 µl) using an automated assay, based on the enzymatic reaction of creatinine with picric acid, producing a colorimetric product (RX-Imola; Randox). The assay and standards were used according to the manufacturer's instructions (Randox Laboratories Limited, 2018). Human-based serum (Clinical Chemistry Calibration Serum Level 3, CAL2351) and 100% human urine (Assayed Urine Control Level 3, AU2353) with known creatinine contents were used as quality controls (QCs) to correct for intra- and inter-assay variability. The former QC was used to take into account the very dilute samples with creatinine concentrations at the low end of the assay. The urine QC was used neat (with creatinine concentration ~ 16,000 µmol/L) and as 1-in-2 and 1-in-10 dilutions to cover the full expected range of creatinine concentrations in the samples. Concentrations of creatinine measured in urine samples were converted from µmol/L to g/L, using the molecular weight of creatinine (88.42 g/mol). Then, the concentration (in mg/L or µg/L) of each element measured by ICP-MS in each urine sample was divided by the creatinine concentration in the particular urine sample (in g/L) to give the element concentration in the urine sample as mg/g creatinine or µg/g creatinine.

2.2.5. Further analyses of toxic minerals associated with fish

2.2.5.1. Mercury analysis of pet foods and kidneys

Mercury concentrations were measured in wet (n=87) and dry (n=74) complete foods for cats (n=106) and dogs (n=55) that had been analysed previously for their content of other minerals (Davies et al., 2017a). Mercury concentration was also measured in renal medulla from dogs (n=10), domestic cats (n=16) and Scottish wildcats (n=7) and in renal cortex from dogs (n=10) and domestic cats (n=10). Cortical tissue was not available from Scottish wildcat kidneys due to its use for other experiments in this study.

Sealed packages of pet food were opened and then a representative sample (100-200 g) was collected into a 250 ml solvent-resistant container (Sarstedt, UK) labelled with the appropriate sample number allocated to the particular food. Hence, for most wet foods, which were packaged in small (≤200 g) pouches, cans/tins and trays, the entire contents was taken. Pet food samples and kidney tissue samples (~1 g) were frozen, freeze-dried at -55°C for 48-72 hours (ScanVac CoolSafe 55-9 freeze-dryer), then ground down to homogenous powders using a pestle and mortar. The freeze-dried, homogenised foods were stored in sealed containers, in the dark at 4°C, for approximately 1 year between our initial research and the current project.

Homogenised pet foods and kidneys were sampled (100-200 mg) and transferred to metal boats, which were loaded into the sample tray of the Direct Mercury Analyser for determination of mercury content. The DMA-80 program, which involved heating the samples to 650°C, was run to completion, which took six minutes. Mercury concentrations measured in pet food and kidney tissue samples were corrected to the average recovery of mercury in duplicate 100-200 mg samples of fish muscle ERM-BB422 CRM.

Duplicate samples of 25 (15.5%) of the pet foods were analysed to check the accuracy of the machine and reproducibility of measurements**.** Five replicates of four foods were also analysed. For these repeated foods, the mean of the mercury measurements was calculated, along with the standard deviation and coefficient of variation (CV). The mercury content of nine (17.0%) kidney samples was measured in duplicate and the CV was calculated to evaluate the

reproducibility of the results. The mercury concentration measured in each pet food was plotted against the European legal limit for pet food mercury content (340 µg/kg DM) (Beynen, 2018).

2.2.5.2. Determining proportions of arsenic species in cat foods with high total arsenic

2.2.5.2.1. Cat food preparation for total arsenic concentration measurement

Four pet foods with high arsenic content, as determined previously by myself and others (Davies et al., 2017a), were selected. These were all commercially available, fish-containing, complete wet foods for adult cats. The samples were freeze-dried, homogenised and stored as described in section 2.2.5.1, then acid digested as described in section 2.2.4.2, ready for total arsenic concentration measurement by ICP-MS as described in section 2.2.4.3. The only methodological differences were that, for pet foods, triplicate samples were prepared and run alongside three 'blanks' and 100-200 mg of a CRM of fish muscle ERM-BB422, also in triplicate. Total arsenic was also measured, using ICP-MS, in these four pet foods, the fish muscle CRM and 'blanks', all in triplicate, after water-extraction as described below.

2.2.5.2.2. Water-extraction of arsenic species in cat foods

The same four cat foods were prepared for determination of their content of the arsenic species, dimethylarsinic acid (DMA), monomethylarsinic acid (MMA), arsenobetaine (AsB), arsenite (As[III]) and arsenate (As[V]). Purite water was used to extract the arsenic species, following a procedure outlined by Komorowicz et al. (2019). In that study, microwave-assisted extraction of arsenic species in freshwater fish with water, as opposed to a water-methanol solution, gave the best recoveries, which ranged from 92% to 99% (Komorowicz et al., 2019). In brief, in the current study, 8 ml of purite water was added to each 200-300 mg sample of freeze-dried, homogenised pet foods and a fish muscle CRM (ERM-BB422). Each pet food and the CRM was extracted in triplicate. The microwave (Multiwave PRO, Anton Parr, Austria),

at 800W power, was ramped to 80°C over 10 minutes, held at this temperature for 15 minutes and then cooled over 10 minutes. After this, the contents of the microwave vessels were decanted into appropriately labelled centrifuge tubes and rinsed with 1 ml deionised water, which was then added to the appropriate sample. The samples were centrifuged at 2000 g for 10 minutes and the supernatant was transferred to universal tubes using a Pasteur pipette. Care was taken to avoid collecting any of the undissolved sediment.

2.2.5.2.3. High performance liquid chromatography-coupled inductively-coupled plasma-mass spectrometry for arsenic speciation in cat foods

Following preparation by water-extraction, 600 µl of each sample was transferred into a separate plastic HPLC PolyVial with filter cap (both Thermo Scientific™, Waltham, MA, USA). Arsenic speciation was then undertaken using high performance liquid chromatography coupled to inductively coupled plasma mass spectrometry (HPLC-ICP-MS; HPLC 5000 series, Thermo Scientific™). The HPLC-ICP-MS system used a PRP-X100 anion exchange column (PS-DVB/Trimethyl ammonium exchanger; 5 µm particle size; 4.6 mm internal diameter, ID; 250 mm length) and the eluent was 20 mM $NH_4H_2PO_4$ and $(NH_4)_2HPO_4$ at pH = 5.6, pumped at 1.5 ml/minute in isocratic mode. The temperature was controlled at 40°C. Standards included 5.0 µg/L As(III) and As(V) (Spex Certiprep, Stanmore, U.K.), and 5.0 µg/L DMA, MMA and AsB (Sigma/Merck, Darmstadt, Germany). Each pet food sample and the fish muscle ERM-BB422 CRM was also spiked with 5.0 µg/L of the AsB standard in an attempt to separate the inevitably overlapping peaks of AsB and As(III) in the samples. The retention times of these two arsenic species are slightly different but the overlap is still too great to see small concentrations of As(III) against a large AsB peak. Chromatography run-time was around 13 minutes per sample. The output of the ICP detector, an electron multiplier detector, was the number of pulse counts (ions arriving) per second (counts per second, cps). Interpolation between calibration standards, which were run periodically, gave individual sensitivity values, in cps *per* parts per billion (cps/ppb), for each sample.

2.2.6. Statistical analysis

All data were analysed using GenStat v19 (VSNi Ltd., Rothamsted, UK). Pearson chi-square test was used to assess whether the proportion of domestic cats, versus dogs, with CIN (scores 1, 2 and 3) was significantly different, rather than being due to chance. Similarly, Pearson chi-square test was used to establish the significance of the number of cats, versus the number of dogs, with more extensive CIN (scores 2 and 3). Lin's concordance coefficient was used to evaluate the concordance between CIN and fibrosis scores in kidneys that were scored for both lesions (<0.25 = no agreement; >0.75 = strong agreement).

Due to unknown information, for example sex or neutered status, for a small number of cases, the experimental data (mineral concentrations [excluding mercury] in kidney tissue and urine) were analysed by linear mixed model, restricted maximum likelihood (REML). This assumed that missing values are at random and therefore do not bias the estimates. In turn, this meant that the particular case did not need to be excluded from all analyses. Restricted maximum likelihood models are able to take into account both fixed and random effects (Bolker et al., 2009). For this reason, the REML model, linear mixed model, was used for kidney tissue and urine data. The fixed effects were CIN (scores 0 and 1 grouped as 'No CIN', versus scores 2 and 3 grouped as 'CIN'), species (dog vs. domestic cat) and the interaction between the two. The random model was the patient ID as, in some cases, multiple samples from one animal were analysed.

Additionally, for some analyses, the design was unbalanced, as none of the Scottish wildcats had CIN (when CIN scores 0 and 1 were grouped together as 'No CIN'). Therefore, to compare domestic cat (without CIN) and Scottish wildcat kidney minerals data, the two-sample non-parametric Mann-Whitney U test was used. Residuals were checked and if the data were found to have a non-normal distribution, they were first log-transformed (log_{10}) to normalise the distribution before statistical analysis. Statistical significance was accepted at $P<0.002$ (Bonferroni correction for 18 elements of interest and with concentrations above the LoD). Data are presented as box plots showing the

median, interquartile range and 10th to 90th percentiles as well as individual values, using GraphPad Prism version 8 for Windows. Where data had been log-transformed, they were subsequently anti-logged and the raw values were plotted on a logarithmic scale using GraphPad Prism.

For mercury concentrations measured in pet foods and kidney tissue, data were log_{10} transformed to normalise the distribution prior to statistical analysis. General ANOVA for unbalanced experiment design was used to statistically evaluate the difference in mercury content between cat and dog foods (Species), between all wet and all dry foods (Wet/Dry) and between wet versus dry foods for cats and dogs separately (Sp*Wet/Dry). General ANOVA for unbalanced experiment design was also used to compare the mercury content of foods that contained fish muscle, fish oil, both fish muscle and fish oil, or neither fish muscle nor fish oil. The Bonferroni test was used to identify which groups of foods, based on incorporation of fish-based ingredients, had significantly different mercury contents to each other. General ANOVA was used to assess the effect of species (dog vs. domestic cat) on renal cortex mercury content and (dog vs. domestic cat vs. Scottish wildcat) on renal medulla mercury content. For renal medulla mercury content, as there were three species groups, the Bonferroni test was additionally used to identify which groups were significantly different from each other. General ANOVA, with animal ID as a blocking factor, was also used to evaluate the difference between cortical and medullary mercury concentrations in dogs and, separately, in cats. Statistical significance was accepted at $P<0.05$. The log-transformed data were presented on an anti-log scale as violin plots showing the full range of data, the median, interquartile range and individual data points, using GraphPad Prism version 8 for Windows.

2.2.7. Power and sample size, veterinary study

Sample size for the study was estimated from a pilot study of arsenic concentration in 35 kidney tissue samples (n=15 dogs and n=20 domestic cats). In these samples, the kidney tissue arsenic concentration (µg/g DM) in domestic cats without CKD (No-CIN) was 0.164 ± 0.037 versus cats with CIN (0.612 ± 0.350). Using a replication ratio of 1.0 and treatment mean effect size,

standard deviation and variance of 0.550, 1.10 and 1.21 µg/g DM, respectively, it was estimated that at least 51 individuals would be required per treatment group to be able to reject the null hypothesis that the population means are equal with probability (power) 0.80, if the true difference in the sample means of control vs. CIN kidney was >0.550 µg/g DM. The Type I error probability associated with this test of the null hypothesis is 0.05.

2.3. Results

2.3.1. Chronic interstitial nephritis and fibrosis in cats and dogs

From the sample of domestic cat, dog and Scottish wildcat kidneys that were evaluated, based on the interstitial inflammation, the incidence of CIN *per se* was greater in domestic cats (77% [48/62]) than dogs (51% [36/70]; χ^2 [1 d.f., n=132] =9.60, P=0.002). Moreover, domestic cats had a greater incidence of moderate-to-severe CIN (52% [32/62]) than dogs (16% [11/70]; χ^2 [1 d.f., n=132] =19.29, P<0.001; Figure 4). The majority of Scottish wildcats had no evidence of inflammatory cells within the renal interstitium (score 0), but 3 of 17 (18%) showed few multifocal, small aggregates of mononuclear cells within the interstitium (score 1).

An increased amount of interstitial collagenous tissue (fibrosis), which was made apparent by staining with MT, was seen in all (100% [10/10]) domestic cats and 80% (12/15) dogs. Two of seven Scottish wildcats showed a mild increase in the amount of interstitial collagenous tissue but neither of these had any inflammatory infiltrate within the renal interstitium. Similar to the amount of interstitial inflammation, moderate-to-severe fibrosis was identified in a greater proportion of domestic cat (90% [9/10]) than dog (40% [6/15]) kidneys (Figure 5). There was good concordance in kidneys scored for both interstitial mononuclear inflammation and fibrosis (0.65; where <0.25 = no agreement and >0.75 = strong agreement; Lin's concordance coefficient). Representative images of mild (score 1), moderate (score 2) and severe (score 3) CIN and fibrosis identified in kidney tissue sections are shown in Figure 6.

Retrospective analysis of our database of animals, from which we had obtained tissue, indicated that the average age (in months) of domestic cats and dogs with CIN was much older, as expected, than those without CIN (Cats: no-CIN, 58 [17-96] vs. CIN, 163.5 [107-206] months; Dogs: no-CIN, 42 [11.75-84] vs. CIN, 72 [27.25-159] months; median [IQR], P=<0.001, ANOVA). The proportion of males within each category was not different in cats (no-CIN, 16 of 30 [53%] vs. CIN, 17 of 32 [53%]) but was higher in dogs with CIN (no-CIN, 31 of 59 [53%] vs. CIN, 10 of 11 [91%]; χ^2 [1 d.f., n= 69] =5.38, P=0.020). The

majority of cats (50 of 62 [81%]) but less than half of the dogs (32 of 70 [46%]) had been neutered.

Figure 4. Dog, domestic cat and Scottish wildcat CIN scores.

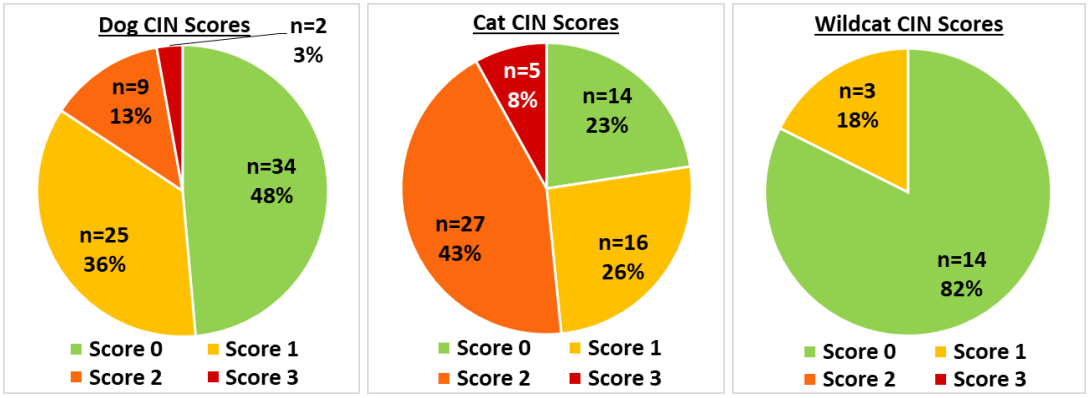


Figure 4. Haematoxylin and eosin (H&E) stained kidney tissue sections from dogs (n=70), domestic cats (n=62) and Scottish wildcats (n=17) were scored semi-quantitatively for chronic interstitial nephritis (CIN). Scores 0, 1, 2 and 3 were given where 0%, <5%, 5-25% and >26% of the section, respectively, was affected by mononuclear leukocyte (lymphocytes and plasma cells) infiltration in the renal interstitium.

Figure 5. Dog, domestic cat and Scottish wildcat renal interstitial fibrosis scores.

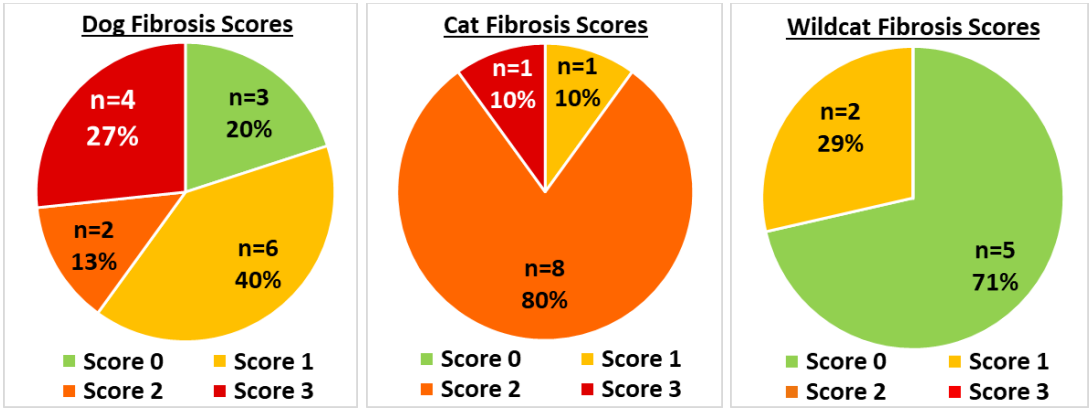


Figure 5. Masson's Trichrome stained kidney tissue sections from dogs (n=15), domestic cats (n=10) and Scottish wildcats (n=7) were scored semi-quantitatively for tubulointerstitial fibrosis, as identified by positivity for collagenous tissue (blue stain). Scores 0, 1, 2 and 3 were given where 0%, <5%, 5-25% and >26% of the section, respectively, was affected by increased deposition of fibrous connective tissue.

Figure 6. Representative images of severe, moderate and mild chronic interstitial nephritis and interstitial fibrosis in domestic cat and dog kidneys.

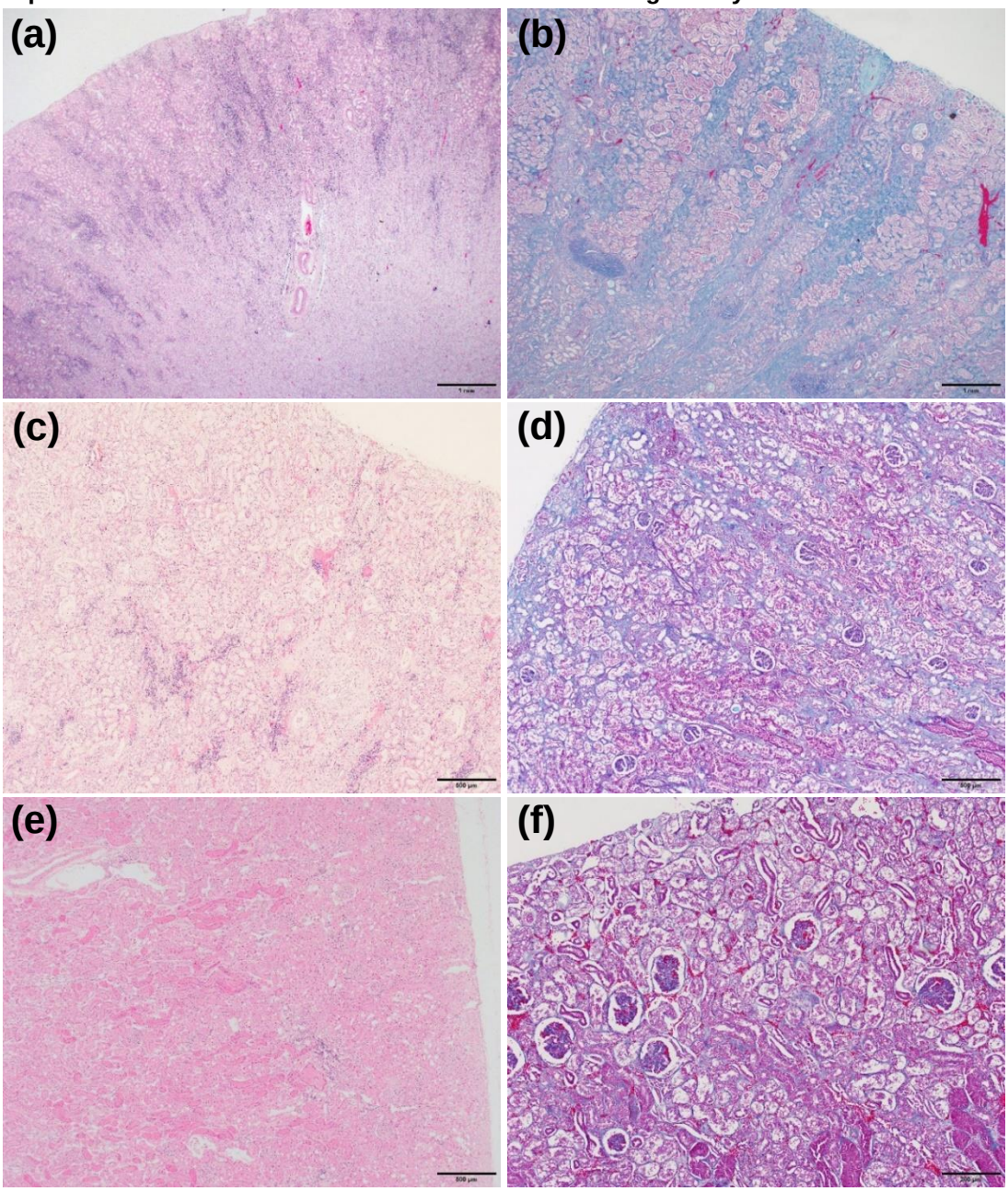


Figure 6. Representative images of severe (score 3), moderate (score 2) and mild (score 1) chronic interstitial nephritis (CIN; left, **(a)**, **(c)** and **(e)**; haematoxylin and eosin [H&E] stain) and interstitial fibrosis (right, **(b)**, **(d)** and **(f)**; Masson's trichrome [MT] stain), as determined by positivity for mononuclear leukocyte infiltration or collagenous fibrous tissue (blue stain), respectively. **(a)** Severe CIN (score 3), domestic cat. Abundant, multifocal to coalescing infiltration of lymphocytes and plasma cells within the interstitium of mostly the renal cortex, affecting more than 25% of the tissue section. H&E. **(b)** Severe fibrosis (score 3), dog. Abundant and diffuse deposition of collagen (blue stain) within the renal interstitium, associated with marked loss and atrophy of renal tubules, affecting more than 25% of the tissue section. MT. **(c)** Moderate CIN (score 2), dog. Low to moderate numbers of lymphocytes and plasma cells multifocally distributed within the renal interstitium, affecting between 5 and 25% of the tissue section. H&E. **(d)** Moderate fibrosis (score 2), domestic cat. Multifocal to coalescing deposition of a moderate amount of collagen within the renal interstitium, associated with renal tubular atrophy, affecting between 5 and 25% of the tissue section. MT. **(e)** Mild CIN (score 1), domestic cat. Low numbers of lymphocytes and plasma cells are multifocally present within the renal interstitium, affecting less than 5% of the tissue section. H&E. **(f)** Mild fibrosis (score 1), domestic cat. Multifocal deposition of a low amount of collagen within the renal interstitium, affecting less than 5% of the tissue section. MT.

2.3.2. Mineral content of domestic cat, dog and Scottish wildcat kidneys

2.3.2.1. Included and excluded elements

Elements with concentrations near to, or below, the LoD in all, or many, samples are not reported, due to lack of confidence in these results and/or the great variability that this caused in the dataset. This includes aluminium, barium, beryllium, boron, lead, lithium, nickel, silver, titanium and uranium. The concentrations of 21 of the 31 elements measured in kidney tissue by ICP-MS were detectable with levels above the LoD. However, a few of the detectable mineral elements (e.g. Cs, Tl, V) are not reported because they have no obvious known biological role or because we had no a priori information in regard to these minerals in the context of renal disease. Alongside the minerals of interest in the current study, some essential major (e.g. S) and trace elements (e.g. Cr, Co, Mn, Mo) that have known biological roles, but not necessarily in relation to the kidney and/or renal disease, are reported for completeness in Table 4, but will not be discussed further.

2.3.2.2. Pro-oxidant trace elements of concern

Levels of total arsenic (As) in renal tissue from dogs and Scottish wildcats were close to the limits of quantification (LoQ). Kidney As content was greater in domestic cats than dogs, regardless of a histopathological diagnosis of CIN ($P=<0.001$), and Scottish wildcats ($P=0.009$; Figure 7a). Iron concentration (Figure 7b) was significantly ($P=<0.001$) lower in domestic cat versus dog kidneys. Scottish wildcat kidney tissue iron levels were similar to those of domestic cats ($P=0.055$). In both domestic cats and dogs, iron concentration was lower in kidneys with CIN, compared to those without CIN ($P=0.010$; Figure 7b). Cadmium concentration was higher in kidneys from cats with CIN, but lower in dogs with CIN, compared to controls ($P=0.005$; Table 4).

2.3.2.3. Anti-oxidant trace elements of importance

Copper (Figure 7c) and zinc (Figure 7d) concentrations were significantly (P=<0.001) lower in domestic cat versus dog kidneys. There was no difference in kidney copper or zinc contents between domestic cats and Scottish wildcats. Copper was reduced in CIN kidneys, compared to kidneys without CIN, but particularly in dogs, as opposed to domestic cats (P=<0.001; Figure 7c).

2.3.2.4. Differences in kidney contents of major elements

The concentrations of all major elements (Ca, Mg, P, K, Na and S) measured in kidney tissue showed a significant species effect (P=<0.001), being higher in dogs than domestic cats. The presence of CIN significantly increased the calcium concentration in both dog and domestic cat kidneys, with a significantly greater increase observed in dogs (8.8-fold [dog] vs. 1.6-fold [domestic cat] P=<0.001). Scottish wildcats had renal Mg, P and K concentrations similar to domestic cats, but Ca, Na and S concentrations were significantly lower in Scottish wildcats than in domestic cats (Table 4).

2.3.2.5. Other trace elemental differences observed in the current study

Molybdenum and rubidium concentrations were significantly lower in domestic cat versus dog kidneys (P=<0.001; Table 4). Rubidium, chromium and cobalt concentrations were also significantly lower in domestic cat than in Scottish wildcat kidneys (Table 4). In contrast, kidney strontium concentration was significantly (P=<0.001) higher in domestic cats than in Scottish wildcats, where the median strontium concentration was below the LoQ. Strontium concentration was significantly increased in kidneys of domestic cats and dogs with CIN, compared to those without CIN, but to a greater extent in dogs (P=<0.001; Table 4). Kidney tissue manganese concentration increased with the presence of CIN in domestic cats but was reduced in dogs with CIN compared to dogs without CIN (P=<0.001; Table 4).

Major element (mg/kg)	Dogs (n=54)		Cats (n=56)		Wildcats (n=17)	P-value			
	No CIN (n=44)	CIN (n=10)	No CIN (n=30)	CIN (n=26)	No CIN (n=17)	Species	CIN	Species * CIN	C vs. WC
Calcium	685.7 (516.9, 1029)	6056 (2437, 15274)	418.8 (303.7, 547.8)	627.7 (455.0, 1054)	258.1 (238.2, 306.9)	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Magnesium	681.6 (640.9, 740.2)	788.0 (705.1, 1199)	558.8 (463.6, 685.0)	537.1 (462.0, 632.6)	506.8 (467.6, 595.2)	**<0.001**	0.159	0.004	0.195
Phosphorus	10492 (9665, 11268)	11776 (10944, 17246)	8865 (7372, 10590)	8799 (7481, 9931)	9634 (9181, 11127)	**<0.001**	0.185	0.009	0.121
Potassium	9470 (8645, 10928)	9596 (8551, 11125)	8343 (7035, 9917)	8149 (6639, 9274)	8043 (7487, 8522)	**<0.001**	0.378	0.981	0.696
Sodium	11609 (10184, 13663)	12637 (10389, 14579)	10133 (7702, 11181)	9766 (8221, 11949)	6143 (5236, 7611)	**<0.001**	0.267	0.662	**<0.001**
Sulphur	9763 (8802, 11216)	9947 (8741, 10897)	8488 (7073, 9575)	8730 (7658, 9725)	7227 (6857, 7487)	**<0.001**	0.791	0.693	**0.001**
Trace element (µg/kg)									
Arsenic	0.025 (0.013, 0.058)	0.021 (0.019, 0.070)	0.100 (0.067, 0.177)	0.088 (0.050, 0.210)	0.034 (0.016, 0.125)	**<0.001**	0.678	0.829	0.009
Cadmium	1.296 (0.629, 1.697)	0.582 (0.291, 1.572)	0.304 (0.169, 0.677)	1.030 (0.787, 1.703)	0.253 (0.154, 0.408)	0.146	**<0.001**	0.005	0.251
Chromium	0.189 (0.0877, 0.346)	0.359 (0.185, 0.632)	0.186 (0.111, 0.379)	0.194 (0.105, 0.405)	0.086 (0.050, 0.166)	0.612	0.624	0.081	**0.002**
Cobalt	0.064 (0.047, 0.086)	0.044 (0.033, 0.056)	0.061 (0.043, 0.081)	0.048 (0.043, 0.060)	0.045 (0.034, 0.049)	0.228	0.025	0.560	**0.001**
Copper	44.63 (29.12, 64.47)	12.58 (10.30, 27.44)	10.26 (8.246, 14.11)	9.654 (8.387, 11.24)	11.33 (9.598, 11.80)	**<0.001**	**<0.001**	0.010	0.547
Iron	657.3 (503.2, 974.1)	437.3 (387.8, 534.2)	260.8 (164.6, 408.0)	210.5 (158.4, 279.9)	210.5 (167.8, 248.7)	**<0.001**	0.010	0.590	0.055
Manganese	4.745 (3.985, 6.363)	3.069 (2.119, 3.805)	3.888 (3.153, 4.795)	4.042 (3.443, 4.442)	3.691 (2.897, 4.190)	0.160	0.004	**<0.001**	0.268
Molybdenum	1.011 (0.775, 1.333)	0.736 (0.517, 1.102)	0.468 (0.359, 0.711)	0.463 (0.330, 0.577)	0.645 (0.567, 0.742)	**<0.001**	0.224	0.469	0.023
Rubidium	19.62 (15.05, 23.65)	17.03 (15.30, 19.85)	11.65 (8.464, 15.73)	11.10 (8.368, 12.72)	23.60 (17.44, 48.18)	**<0.001**	0.222	0.836	**<0.001**
Selenium	4.555 (3.874, 5.751)	3.871 (3.176, 5.613)	4.585 (3.522, 5.019)	4.090 (3.149, 5.112)	4.313 (3.743, 5.798)	0.118	0.622	0.599	0.638
Strontium	0.419 (0.313, 0.719)	3.975 (1.274, 9.122)	0.275 (0.208, 0.396)	0.503 (0.265, 1.098)	0.129 (0.094, 0.170)	0.012	**<0.001**	**<0.001**	**<0.001**
Zinc	113.1 (92.62, 155.6)	128.4 (99.55, 145.7)	71.11 (54.66, 93.73)	73.59 (60.65, 83.87)	73.33 (58.94, 77.99)	**<0.001**	0.481	0.829	0.890

Table 4. Elemental composition of kidney tissue from dogs and domestic cats with or without CIN and from Scottish wildcats, all without CIN. Data of 18 major and trace elements of interest and with concentrations above the limit of detection (LoD) are presented as median (IQR: 1st to 3rd quartile) for each element. Freeze-dried samples (100-200mg) of renal tissue were acid-digested and subsequently analysed by ICP-MS to measure mineral element concentrations. Data were log_{10}-transformed prior to statistical analysis, in order to normalise skewed distributions and residual plots. Linear mixed models were used to compare data between dogs and domestic cats (Species) and between 'No CIN' and 'CIN' in dogs and domestic cats combined (CIN), as well as between 'No CIN' and 'CIN' in dogs, versus 'No CIN' and 'CIN' in cats (Species*CIN). The Mann-Whitney U test was used to compare data from domestic cats with 'No CIN' and Scottish wildcats (C vs. WC). Statistical significance was accepted at P<0.002 (Bonferroni correction for 18 elements of interest and with concentrations above the LoD).

Figure 7. Renal tissue arsenic, iron, copper and zinc concentrations in dogs, domestic cats and Scottish wildcats.

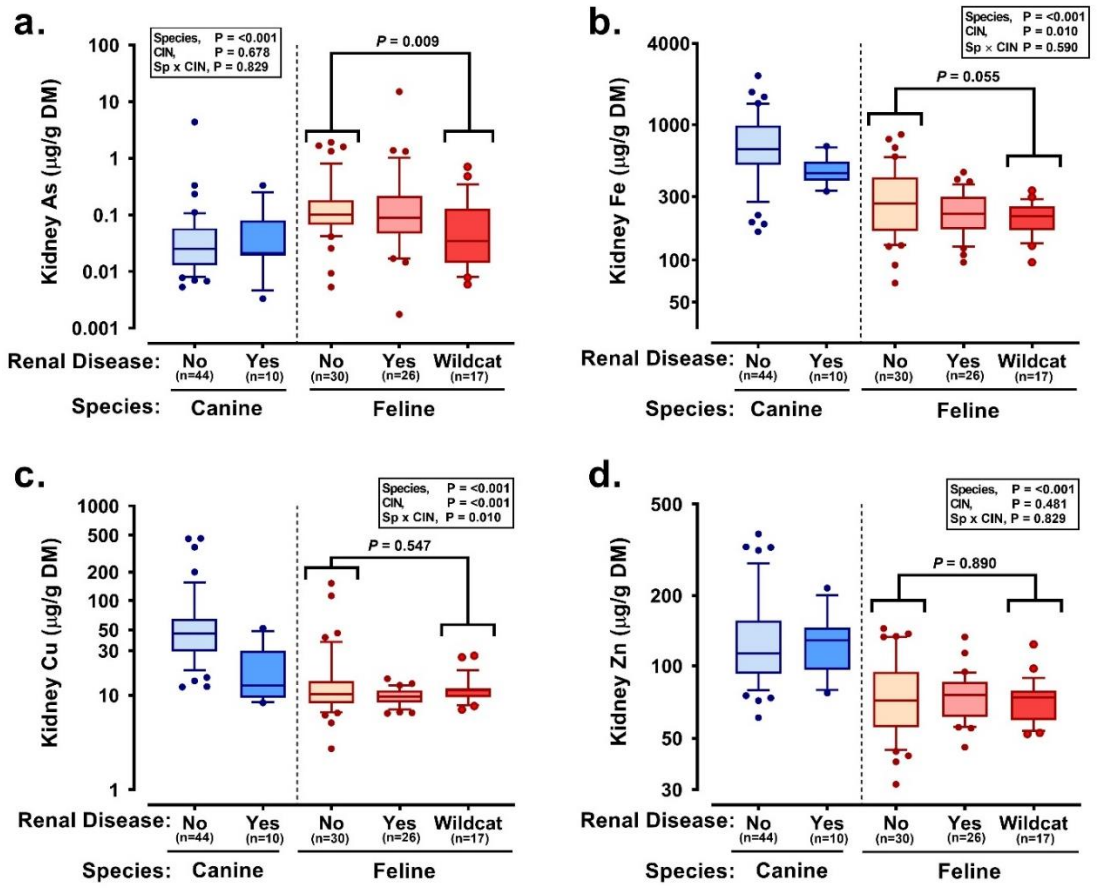


Figure 7. Renal tissue **(a.)** arsenic, **(b.)** iron, **(c.)** copper and **(d.)** zinc concentrations in dogs (Canine) and domestic cats (Feline), with (Renal Disease: Yes) and without (Renal Disease: No) CIN, and Scottish wildcats (Wildcat) all without CIN. Freeze-dried samples (100-200mg) of kidney tissue were acid-digested and subsequently analysed by ICP-MS. Log_{10}-transformed data of element concentrations are presented as box and whisker plots on a logarithmic scale, where boxes indicate the median, lower and upper quartiles and whiskers represent the 10^{th} to 90^{th} percentiles. Data outside of this range are presented as individual dots. Linear mixed models were used to compare data between dogs and domestic cats (Species), between no CIN and CIN in dogs and domestic cats combined (CIN) and between no CIN and CIN in dogs, versus no CIN and CIN in cats (Species x CIN); the resultant P values are indicated in the boxes at the top of each graph. The statistical significance of the difference between domestic cat (No CIN) and Scottish wildcat kidney mineral concentrations was analysed by the Mann-Whitney U test and is indicated above the brackets connecting the two groups. Statistical significance was accepted at P=<0.002 (Bonferroni correction for 18 elements of interest and with concentrations above the LoD).

2.3.3. Elemental composition of domestic cat and dog urine (corrected to creatinine)

2.3.3.1. Included and excluded elements

The concentrations of 27 of 31 elements measured in urine were above the LoD. Concentrations of aluminium, beryllium, cadmium and silver measured in some urine samples were below the LoD so are not reported. Along with the minerals of interest in the current study (e.g. As, Fe, Cu, Zn), some essential major (e.g. B, S) and trace elements (e.g. Cr, Co, Mn, Mo) that have known biological roles, but not necessarily in relation to the kidney and/or renal disease, are reported for completeness in Table 5, but will not be discussed further. Other trace elements that were measured but have little known biological importance (e.g. Ba, Cs, Pb, Li, Ni, Ti, Tl, U, V) are not reported. Statistical significance, accepted at $P<0.002$ due to multiple comparisons, was only found for As and Cr data (Table 5).

2.3.3.2. Pro-oxidant trace elements of concern

Urinary As concentration was significantly ($P=<0.001$) higher in cats compared to dogs overall, with particularly low urinary As concentrations measured in dogs without CIN. Domestic cats without CIN had urinary As levels more similar to their CIN counterparts (Figure 8). Urine iron concentration was higher in cats compared to dogs ($P=0.021$) and seemed to increase in dogs with CIN but decrease in cats with CIN, compared to the respective controls ($P=0.447$; Table 5).

2.3.3.3. Anti-oxidant trace elements of importance

As in kidneys, zinc and copper concentrations appeared to be lower in the urine of domestic cats relative to dogs (Table 5). Furthermore, urinary zinc concentration was 3-fold higher in dogs with CIN compared to dogs without CIN, but 3-fold lower in domestic cats with CIN compared to domestic cats without CIN ($P=0.093$; Table 5). Numerically, the urinary copper concentration was also reduced in domestic cats with CIN versus those without CIN, but was unchanged in dogs with CIN versus dogs without CIN ($P=0.260$; Table 5).

2.3.3.4. Major elements in the urine

There was no statistically significant effect of species or CIN on any of the major element concentrations in urine (Table 5).

2.3.3.5. Effect of CIN on urine mineral content

The presence of CIN in the kidney did not have a statistically significant effect on the concentration of any element in the urine of domestic cats or dogs, with the exception of chromium, which was increased in dogs with CIN compared to dogs without CIN, but decreased in domestic cats with, versus without, CIN (P=<0.001; Table 5).

Major element (mg/g creatinine)	Dogs (n=36)		Cats (n=27)		P-value		
	No CIN (n=30)	CIN (n=6)	No CIN (n=14)	CIN (n=13)	Species	CIN	Species*CIN
Boron	0.104 (0.035, 1.046)	0.495 (0.479, 1.797)	0.346 (0.229, 1.110)	0.322 (0.226, 0.729)	0.228	0.472	0.125
Calcium	38.36 (20.82, 69.93)	21.32 (14.63, 277.2)	29.29 (15.57, 127.6)	34.95 (12.99, 44.86)	0.453	0.534	0.573
Magnesium	38.77 (16.13, 93.38)	196.8 (89.65, 343.9)	22.97 (13.22, 76.06)	21.0 (15.14, 62.65)	0.095	0.094	0.349
Phosphorus	1104 (346.6, 1654)	1678 (1392, 2982)	1885 (1219, 2946)	952.2 (739.9, 1961)	0.080	0.963	0.074
Potassium	2453 (1405, 4352)	6208 (3715, 16046)	4257 (2327, 7663)	2610 (1539, 4666)	0.453	0.856	0.025
Sodium	1835 (575.1, 4164)	1761 (814.3, 15464)	2143 (1660, 4459)	3475 (1117, 5221)	0.322	0.670	0.747
Sulphur	1035 (708.4, 1605)	2045 (1049, 4522)	1336 (913.7, 2026)	1135 (753.5, 1586)	0.965	0.890	0.187
Trace element (µg/g creatinine)							
Arsenic	16.87 (8.776, 38.59)	103.3 (30.02, 166.0)	77.45 (18.52, 115.4)	95.87 (61.11, 169.9)	**<0.001**	0.105	0.877
Chromium	0.322 (0.080, 1.016)	2.023 (1.824, 39.63)	2.268 (1.245, 3.085)	0.534 (0.0158, 1.122)	0.130	0.857	**<0.001**
Cobalt	0.907 (0.431, 1.716)	3.286 (1.556, 13.00)	1.326 (0.750, 2.295)	0.509 (0.358, 0.706)	0.700	0.370	0.003
Copper	134.0 (52.11, 436.0)	125.1 (84.60, 2773)	122.6 (57.67, 550)	68.97 (33.78, 125.5)	0.419	0.628	0.260
Iron	111.0 (64.97, 377.3)	171.6 (49.45, 2403)	514.7 (372.0, 3133)	377.3 (83.62, 1150)	0.021	0.295	0.447
Manganese	2.465 (0.890, 4.998)	4.194 (2.928, 28.03)	6.183 (2.839, 9.370)	3.128 (0.427, 6.338)	0.677	0.818	0.150
Molybdenum	18.45 (6.744, 58.54)	132.1 (44.96, 161.7)	27.21 (14.29, 56.13)	14.01 (5.609, 30.57)	0.275	0.504	0.023
Rubidium	2299 (1101, 4746)	4090 (3396, 19156)	3182 (1862, 4852)	2196 (973.5, 2980)	0.606	0.904	0.028
Selenium	73.43 (44.92, 111.1)	99.26 (56.97, 369.9)	95.00 (38.65, 225.9)	65.62 (53.89, 96.75)	0.554	0.870	0.415
Strontium	129.3 (50.80, 168.2)	132.3 (83.05, 317.7)	41.35 (28.64, 170.6)	101.5 (29.20, 436.6)	0.362	0.420	0.947
Zinc	897.8 (521.0, 3897)	2685 (1411, 4420)	967.7 (667.8, 4278)	323 (227.9, 925.9)	0.305	0.360	0.093

Table 5. Elemental composition of urine from dogs and domestic cats with or without CIN. Data for 18 major and trace elements of interest and with concentrations above the limit of detection (LoD) are presented as median (IQR: 1st to 3rd quartile) for each element. Mineral element concentrations were measured in neat urine samples (0.50 ml) by ICP-MS and adjusted to urinary creatinine concentrations measured using RX-Imola. Data were $\log_{10}$-transformed prior to statistical analysis by linear mixed models, in order to normalise skewed distributions and residual plots. Statistical comparisons were made between dogs and domestic cats (Species), between 'No CIN' and 'CIN' in dogs and domestic cats combined (CIN), as well as between 'No CIN' and 'CIN' in dogs, versus 'No CIN' and 'CIN' in cats (Species*CIN). Statistical significance was accepted at P <0.002 (Bonferroni correction for 18 elements of interest and with concentrations above the LoD).

Figure 8. Urinary arsenic concentrations in dogs and domestic cats.

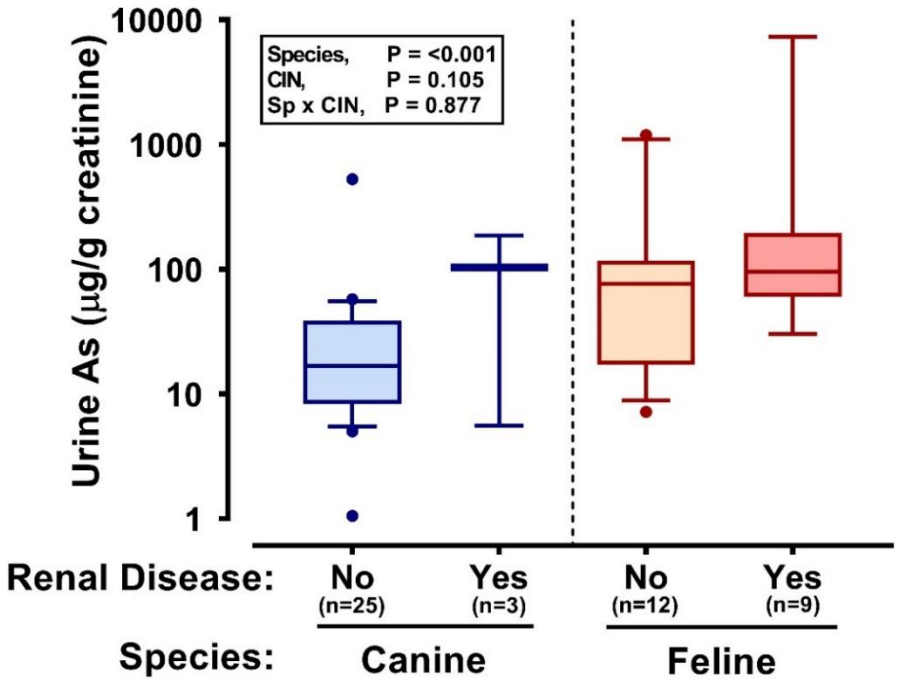


Figure 8. Urine arsenic concentration in dogs and domestic cats, with (Renal Disease: Yes) and without (Renal Disease: No) CIN. Mineral element concentrations were measured in neat urine samples (0.50 ml) by ICP-MS and adjusted to urinary creatinine concentrations measured in the particular urine sample using RX-Imola. Data were log_{10}-transformed to normalise skewed distributions and residual plots, then analysed by linear mixed models. Statistical comparisons were made between dogs and domestic cats (Species), between 'No CIN' and 'CIN' in dogs and domestic cats combined (CIN), as well as between 'No CIN' and 'CIN' in dogs, versus 'No CIN' and 'CIN' in cats (Species*CIN). Statistical significance was accepted at P<0.002 (Bonferroni correction for 18 elements of interest and with concentrations above the LoD). Log-transformed data of element concentrations are presented as box and whisker plots on a logarithmic scale, where boxes indicate the median, lower and upper quartiles and whiskers represent the 10^{th} to 90^{th} percentiles. Data outside of this range are presented as individual dots.

2.3.4. Mercury content of pet foods

Mercury recovery in the fish muscle CRM was 94%. There was good correlation between repeat mercury measurements of individual samples of pet foods (Figure 9). The mean standard deviation across the duplicated samples was 4.225 (range: 0.023–39.295) µg/kg DM and across the quintupled samples was 0.7224 (range: 0.3579–1.1503) µg/kg DM. The mean CV across the duplicated samples was 20% (range: 1–65%) and across the quintupled samples was 14% (range: 8–24%). There was no significant

difference in mercury concentration between foods for cats and foods for dogs, however, the mercury content of wet foods was significantly greater than that of dry foods (P=<0.001; Figure 10a). Foods containing fish muscle had significantly greater mercury content than those that did not contain fish muscle (P=<0.001; Figure 10b). All foods, apart from one wet food, had mercury concentrations below the European legal limit of 340 µg/kg DM (Beynen, 2018) (Figure 11).

Figure 9. Correlation between mercury concentrations in pet food sample repeats.

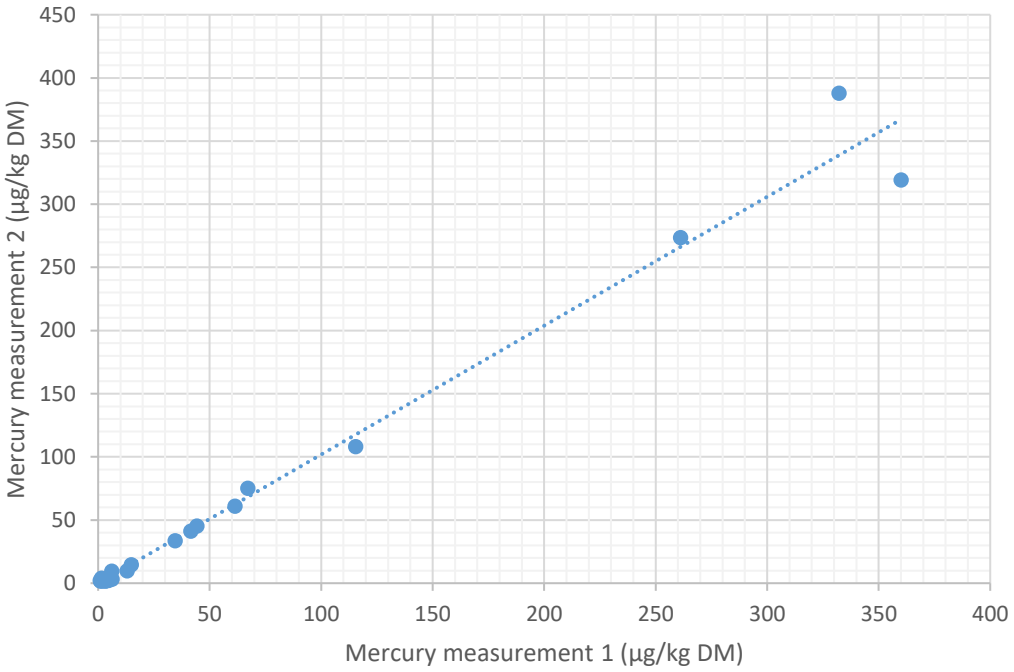


Figure 9. Wet and dry foods were freeze-dried and then ground down to homogenous powders, sampled (100-200 mg) and mercury content was measured using the Direct Mercury Analyser DMA-80 program. Mercury measurements in samples were corrected to the recovery of mercury in fish muscle ERM-BB422 certified reference material. The mercury concentrations measured in duplicate samples of 25 foods are plotted against each other to show the correlation between repeat analyses of mercury concentration in homogenised samples and the repeatability of the method used here for mercury determination.

Figure 10. Mercury content of dry and wet foods for dogs and cats and its association with fish-based ingredients.

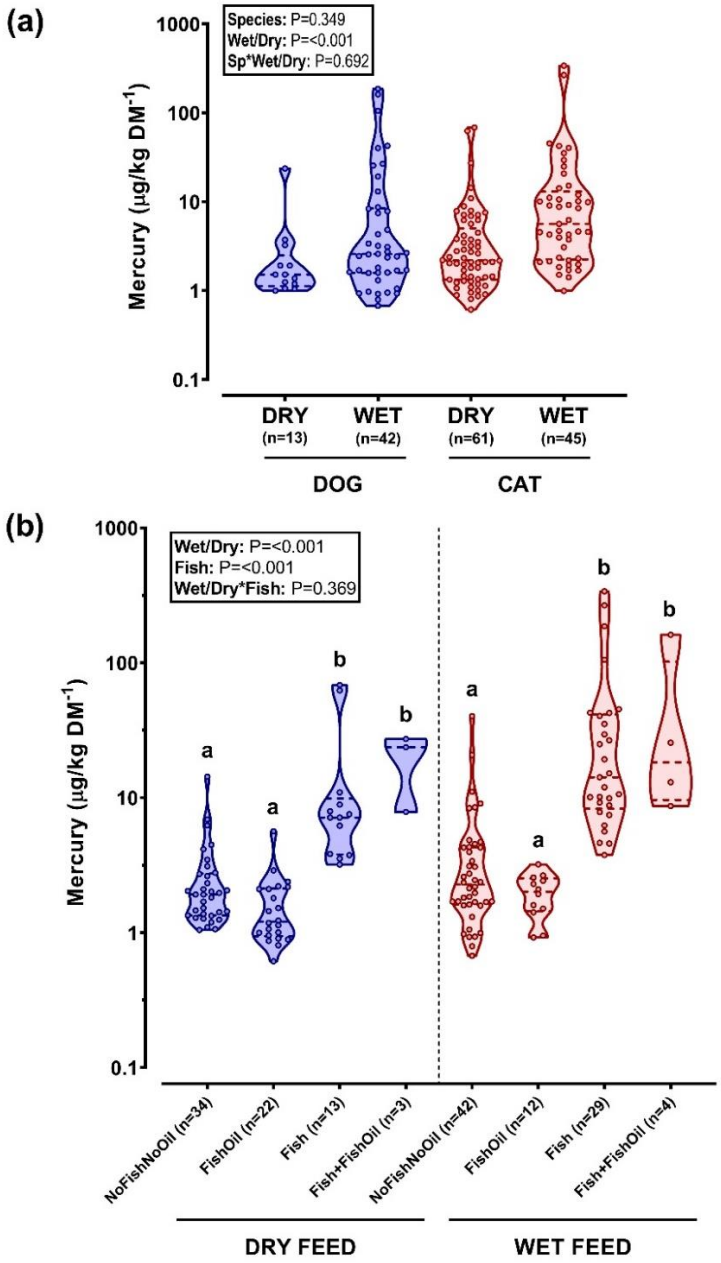


Figure 10. Mercury content of dry (n=74) and wet (n=87) complete foods for dogs (n=55) and cats (n=106) and the association of pet food mercury content with fish-based ingredients. Wet and dry foods were freeze-dried, then ground down to homogenous powders, sampled (100-200 mg) and mercury content was measured using the Direct Mercury Analyser DMA-80 program. Mercury measurements in samples were corrected to the recovery of mercury in fish muscle ERM-BB422 certified reference material. Data were $\log_{10}$-transformed to normalise the distribution, then general ANOVA for unbalanced experiment design was used to statistically evaluate the difference in mercury content **(a)** between cat and dog foods (Species), between all wet and all dry foods (Wet/Dry) and between wet versus dry foods for cats and dogs separately (Sp*Wet/Dry) and **(b)** between all wet and all dry foods (Wet/Dry), between foods grouped according to content of fish-based ingredients (fish muscle [Fish], fish oil [FishOil], both Fish muscle and fish oil [Fish+FishOil] or no fish-based ingredients [NoFishNoOil] when all wet and dry foods were considered together (Fish) and separately (Wet/Dry*Fish). The Bonferroni test was used to identify which groups of foods, based on incorporation of fish-based ingredients, had significantly different mercury contents (groups labelled 'a' are significantly different from groups labelled 'b'). Statistical significance was accepted at P<0.05. Log-transformed data are presented as violin plots on a logarithmic scale, showing the distribution of the full range of the data, with median and lower and upper quartiles represented by dashed lines. All individual mercury measurements are represented as dots.

Figure 11. Compliance of dry and wet complete pet foods with the European legal limit for mercury inclusion.

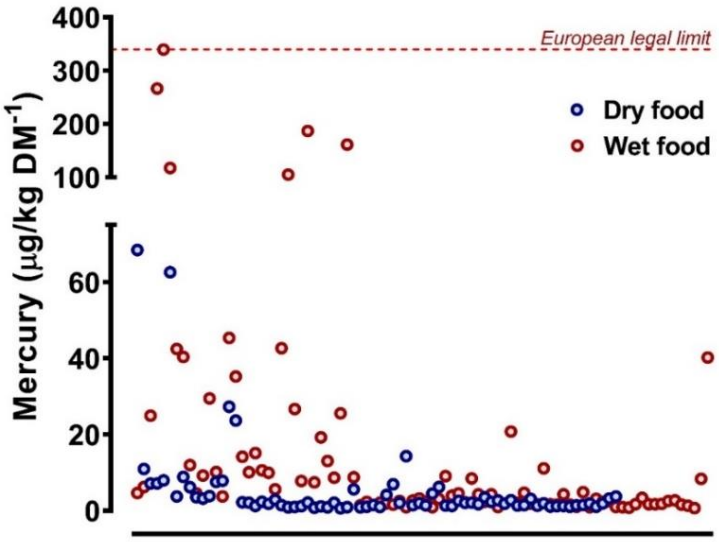


Figure 11. Mercury content of dry and wet foods for dogs and cats in comparison to the European legal limit for mercury inclusion. Wet and dry foods were freeze-dried and then ground down to homogenous powders, sampled (100-200 mg) and mercury content was measured using the Direct Mercury Analyser DMA-80 program. Mercury measurements in samples were corrected to the recovery of mercury in fish muscle ERM-BB422 certified reference material, then compared to the European legal limit for pet food mercury content (340 µg/kg DM; dashed line). Each dot represents a different dry (blue dot) or wet (red dot) food.

2.3.5. Mercury content of cat and dog kidneys

Mercury content in renal medulla was significantly greater for dogs than for domestic cats (P=0.009). Renal medulla mercury concentrations of domestic cats and Scottish wildcats were not significantly different (Figure 12). Mercury concentration in renal cortex was also greater in dogs than in cats (P=0.010; Figure 13). In all dogs (Figure 14) and domestic cats (Figure 15), mercury content was significantly greater in renal cortex than in renal medulla (P=<0.001). The mean coefficient of variation across the nine samples in which mercury was measured in duplicate was 2.90% (range: 0.091 to 9.30 %), indicating good reproducibility in the results.

Figure 12. Mercury content of dog, domestic cat and Scottish wildcat renal medulla.

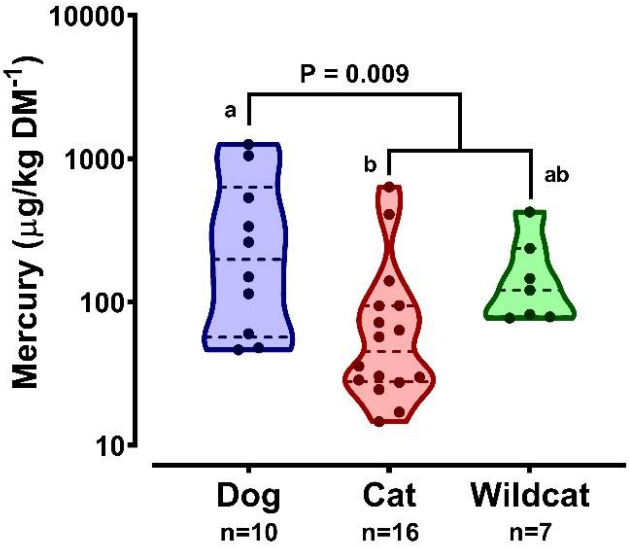


Figure 12. Mercury content of dog, domestic cat and Scottish wildcat renal medulla. Frozen tissue (~1g) was freeze-dried, ground, sampled (100-200mg) and mercury content was measured using the Direct Mercury Analyser DMA-80 program. Mercury measurements in samples were corrected to the recovery of mercury in fish muscle ERM-BB422 certified reference material. Data were log_{10}-transformed to normalise the distribution, then analysed using general ANOVA. Bonferroni test was used to identify which species had significantly different renal medulla mercury contents from each other ('a' is significantly different from 'b', while 'ab' is not significantly different from either 'a' or 'b'). Statistical significance was accepted at $P<0.05$. Log-transformed data are presented as violin plots on a logarithmic scale, showing the distribution of the full range of the data, with median and lower and upper quartiles represented by dashed lines. All individual mercury measurements are represented as dots.

Figure 13. Mercury content of dog and domestic cat renal cortex.

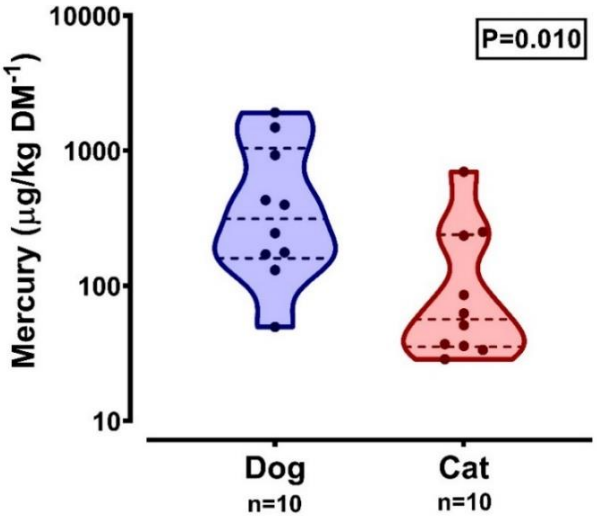


Figure 13. Mercury content of dog and domestic cat renal cortex. Frozen tissue (~1g) was freeze-dried, ground, sampled (100-200mg) and mercury content was measured using the Direct Mercury Analyser DMA-80 program. Mercury measurements in samples were corrected to the recovery of mercury in fish muscle ERM-BB422 certified reference material. Data were analysed using general ANOVA after log_{10}-transformation to normalise the distribution. Statistical significance was accepted at $P<0.05$. Log-transformed data are presented as violin plots on a logarithmic scale, showing the distribution of the full range of the data, with median and lower and upper quartiles represented by dashed lines.

Figure 14. Mercury content in renal cortex and medulla from ten dogs.

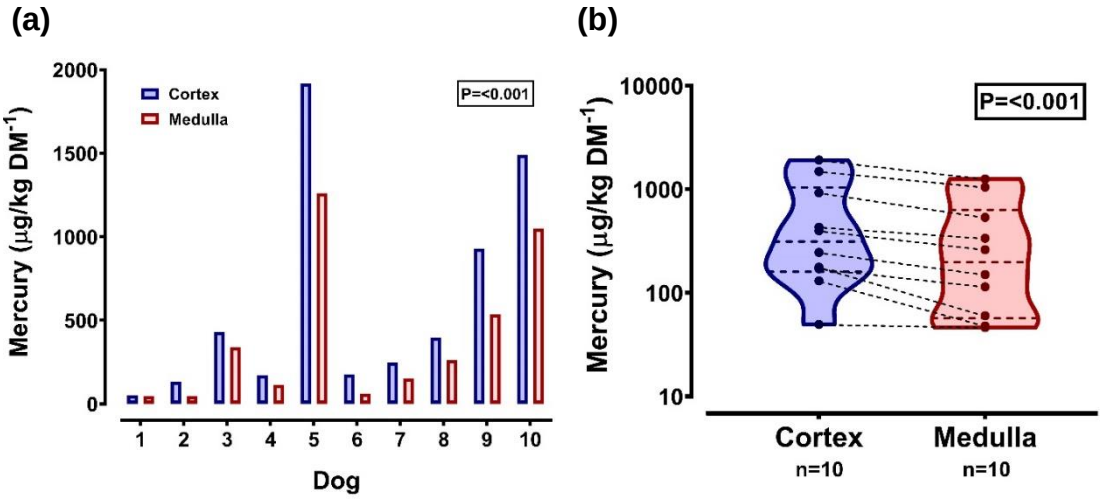


Figure 14. Mercury content in renal cortex and medulla from ten dogs. Frozen tissue (~1g) was freeze-dried, ground, sampled (100-200mg) and mercury content was measured using the Direct Mercury Analyser DMA-80 program. Mercury measurements in samples were corrected to the recovery of mercury in fish muscle ERM-BB422 certified reference material. Data were analysed using general ANOVA, with animal ID as a blocking factor, after $\log_{10}$-transformation to normalise the distribution. Statistical significance was accepted at $P<0.05$. Data are presented as **(a)** a bar chart showing cortex and medulla mercury contents from each dog side-by-side and **(b)** a violin plot showing the distribution of the full range of the log-transformed data, presented on a logarithmic scale, with the median and lower and upper quartiles represented by dashed lines within the plots. Mercury measurements (dots) from cortex and medulla from each individual dog are linked by black dotted lines.

Figure 15. Mercury content in renal cortex and medulla from ten cats.

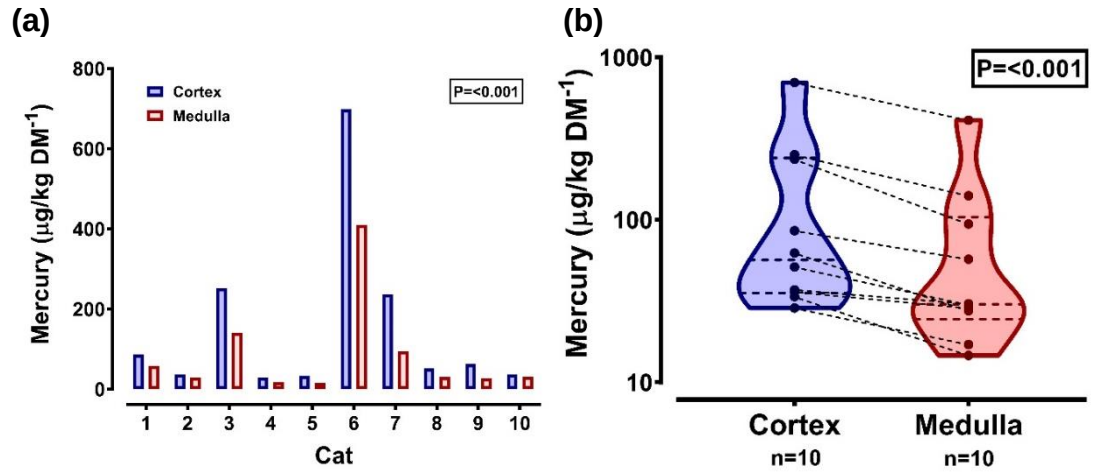


Figure 15. Mercury content in renal cortex and medulla from ten cats. Frozen tissue (~1g) was freeze-dried, ground, sampled (100-200mg) and mercury content was measured using the Direct Mercury Analyser DMA-80 program. Mercury measurements in samples were corrected to the recovery of mercury in fish muscle ERM-BB422 certified reference material. Data were analysed using general ANOVA, with animal ID as a blocking factor, after $\log_{10}$-transformation to normalise the distribution. Statistical significance was accepted at $P<0.05$. Data are presented as **(a)** a bar chart showing cortex and medulla mercury contents from each dog side-by-side and **(b)** a violin plot showing the distribution of the full range of the log-transformed data, presented on a logarithmic scale, with the median and lower and upper quartiles represented by dashed lines within the plots. Mercury measurements (dots) from cortex and medulla from each individual cat are linked by black dotted lines.

2.3.6. Total arsenic and arsenic species in fish-containing pet foods

Total arsenic (TAs) concentrations measured in acid-digested and water-extracted samples were similar, but were largely greater in the water-extracted samples (Table 6). The mean TAs concentrations measured in acid-digested and water-extracted fish muscle ERM-BB422 CRM were compared to the certified value of TAs for the CRM (12.8 ± 0.7 µg/g DM) and were 1.50- and 1.53-times higher, respectively. However, the main aim of this set of experiments was to determine the proportions of the arsenic species that made up the TAs concentration in the samples; the comparison between TAs concentrations measurable after acid-digestion and water-extraction of samples was to compare the extraction efficiencies of these two methods. Therefore the TAs concentrations measured in the pet food samples were not adjusted by the 1.5 correction factor and are presented, as measured, in Table 6.

The sum of all the arsenic species measured in each sample was calculated but was found not to equal the TAs concentrations measured in these foods. Percentage recoveries ranged from 72.44 to 84.72% and from 65.59 to 74.62% in the different pet foods when the sum of arsenic species measured was calculated as a percentage of the TAs concentration measured in acid digested and water-extracted samples, respectively.

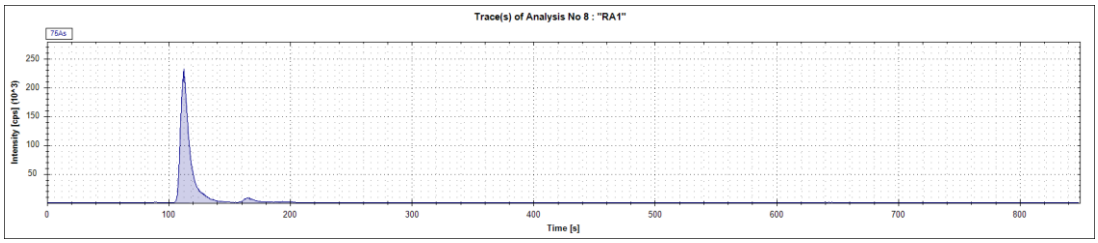
As predicted, AsB was the predominant arsenic species detected in all pet food samples, with DMA, MMA and As(V) constituting much smaller proportions of the sum of all arsenic species measured (Table 6). Spiking samples with the AsB standard failed to separate the extensively overlapping peaks of AsB and As(III) and a single, large peak was seen on the sample chromatograms at retention time ~110 s (Figure 16). However, the chromatograms of mixed AsB and As(III) standards (Figure 17) helped somewhat with peak identifications; by comparing retention times in standards and samples, the apparent single peak in the AsB/As(III) region on the chromatograms for each pet food sample was categorised as AsB. Although quantification of As(III) was not possible, due to masking by the large AsB peak, it was concluded that only very small amounts of this species could be present in the samples, if any.

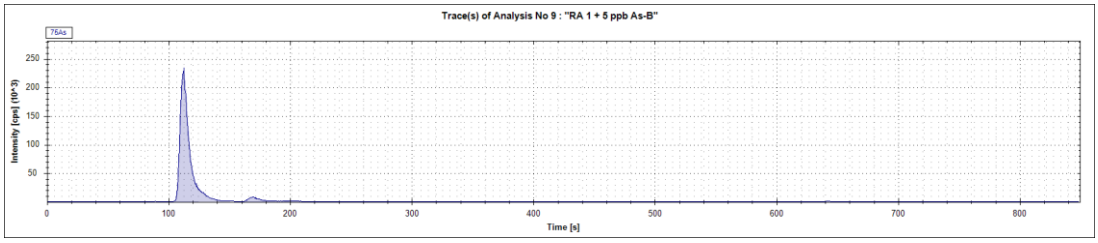
Sample	Mean Concentration ± Standard Deviation (mg/100g DM)							As Recovery (%)	
	D TAs	E TAs	E AsB	E DMA	E MMA	E As(V)	ΣAs Species	D AR	E AR
Pet food 1	0.9926 ± 0.040	1.1269 ± 0.103	0.7870 ± 0.040	0.0399 ± 0.002	0.0048 ± 0.0007	0.0093 ± 0.001	0.8410 ± 0.040	84.72	74.62
Pet food 2	0.7315 ± 0.026	0.9032 ± 0.048	0.5773 ± 0.019	0.0061 ± 0.0002	0.0036 ± 0.0006	0.0054 ± 0.0006	0.5924 ± 0.020	80.98	65.59
Pet food 3	0.5524 ± 0.033	0.5510 ± 0.082	0.3215 ± 0.012	0.0696 ± 0.004	0.0069 ± 0.0007	0.0021 ± 0.0008	0.4002 ± 0.015	72.44	72.63
Pet food 4	0.3242 ± 0.004	0.3936 ± 0.014	0.2579 ± 0.024	0.0036 ± 0.0002	0 ± 0	0 ± 0	0.2615 ± 0.024	80.67	66.44
Fish muscle CRM	1.9059 ± 0.029	1.9493 ± 0.047	1.2524 ± 0.017	0.0015 ± 0.0002	0 ± 0	0 ± 0	1.2538 ± 0.017	65.79	64.32

Table 6. Concentration of total arsenic and arsenic species in selected wet cat food samples and Fish muscle CRM. Total arsenic (TAs) and arsenic species (AsB – arsenobetaine; DMA – dimethylarsinic acid; MMA – monomethylarsinic acid; As(V) – arsenate) concentrations (mg/100g dry matter [DM]) in selected samples (n=4) of wet cat food with high TAs content and fish muscle ERM-BB422 certified reference material (CRM) are presented as mean (n=3) ± standard deviation. TAs concentration was measured by ICP-MS after microwave-assisted acid digestion (D) or water extraction (E) of samples. Concentrations of arsenic species were determined by HPLC-ICP-MS after sample preparation by water-extraction. Arsenic recovery (AR) was calculated as the percentage of the TAs concentration that the sum of arsenic species (ΣAs Species) amounted to.

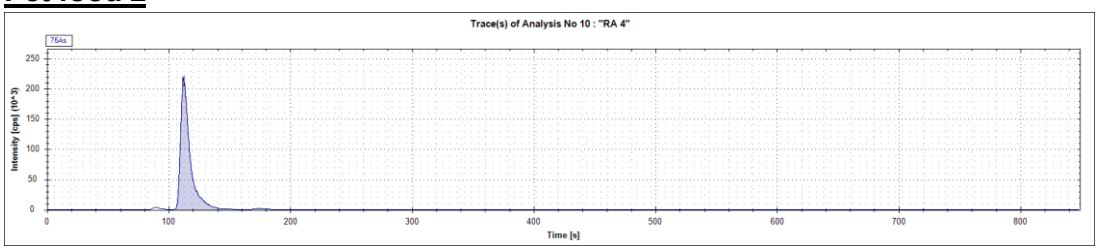
Figure 16. Arsenic species in pet food samples.

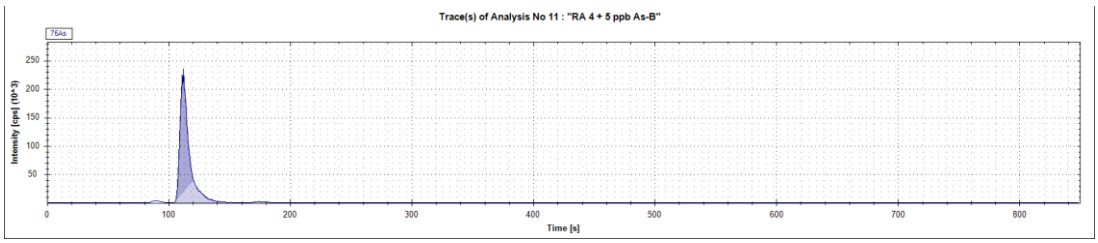
Pet food 1



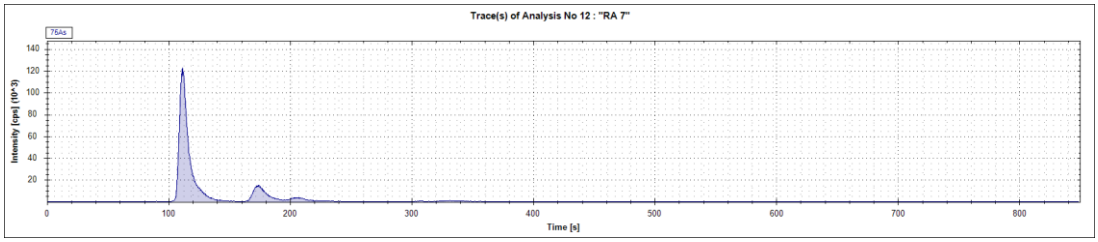


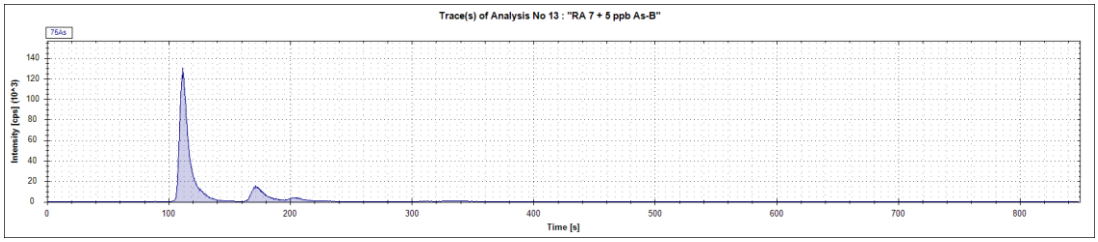
Pet food 2



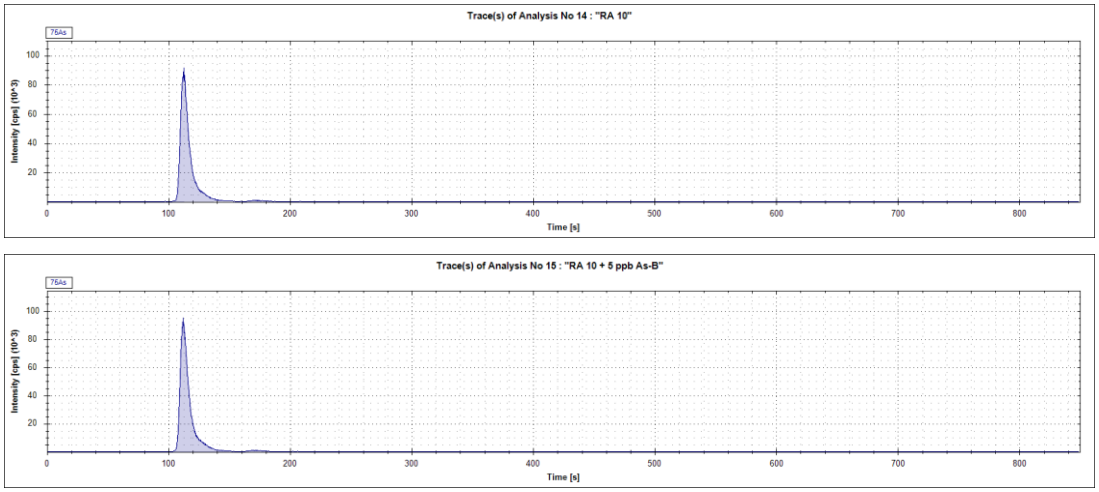


Pet food 3





Pet food 4



Fish muscle ERM-BB422

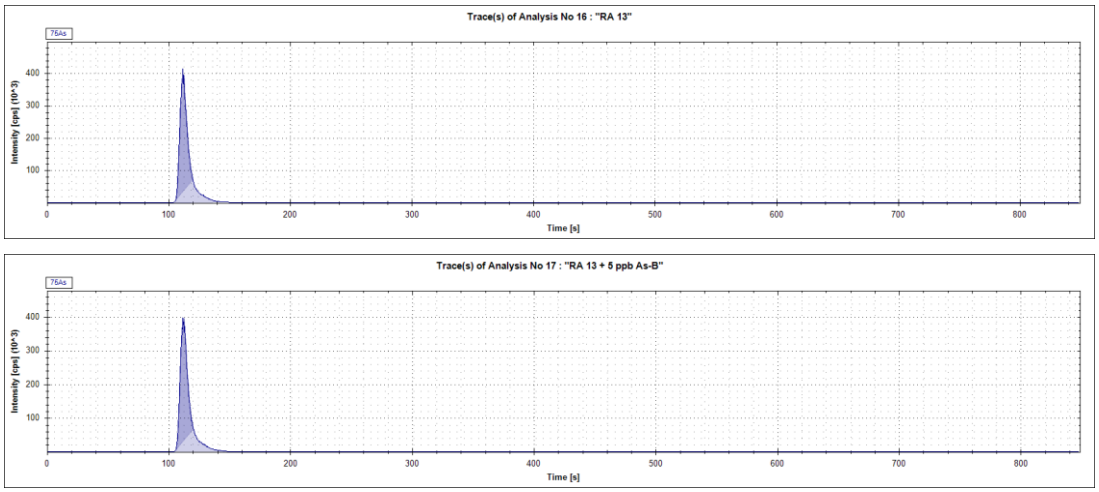
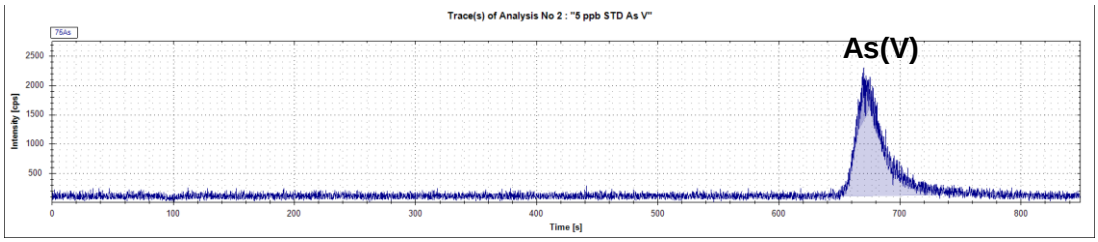


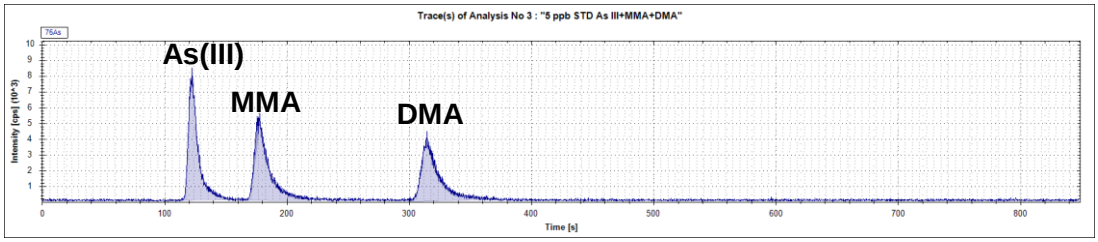
Figure 16. Representative chromatograms of arsenic species profiles in each wet cat food sample (n=4) and fish muscle ERM-BB422 certified reference material, with (above) and without (below) spiking with 5.0 µg/L arsenobetaine (+ 5 ppb As-B). Samples were prepared by microwave-assisted water-extraction, then arsenic species were detected by high performance liquid chromatography coupled to inductively coupled plasma mass spectrometry (HPLC-ICP-MS; HPLC 5000 series, Thermo Scientific™) using a PRP-X100 anion exchange column (PS-DVB/Trimethyl ammonium exchanger; 5µm particle size; 4.6mm internal diameter, ID; 250mm length) in isocratic mode. Arsenic species were identified in the samples by comparing the retention times of the peaks in the samples with those in the standard solutions (Figure 17). Peaks indicating the presence of arsenobetaine, arsenite, dimethylarsinic acid, monomethylarsinic acid and arsenate appeared at retention times ~110s, ~125s, ~175s, ~310s and ~660s, respectively. Intensity (counts per second [cps], $\times 10^3$) of some of these species was too low for peaks to be seen on the profiles. Arsenite was not detected in any sample.

Figure 17. Chromatograms of arsenic species standards.

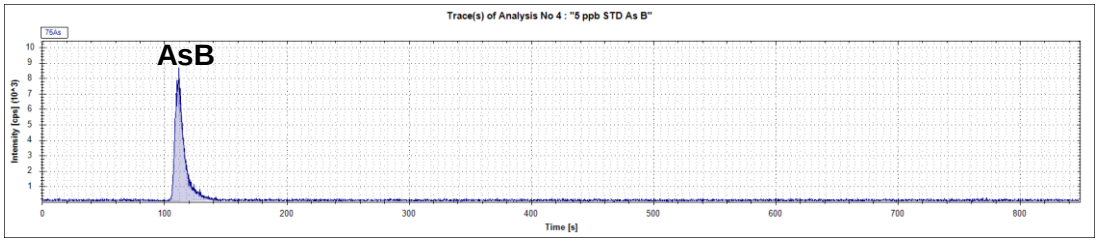
(a) Standard 1



(b) Standard 2



(c) Standard 3



(d) Standard 4

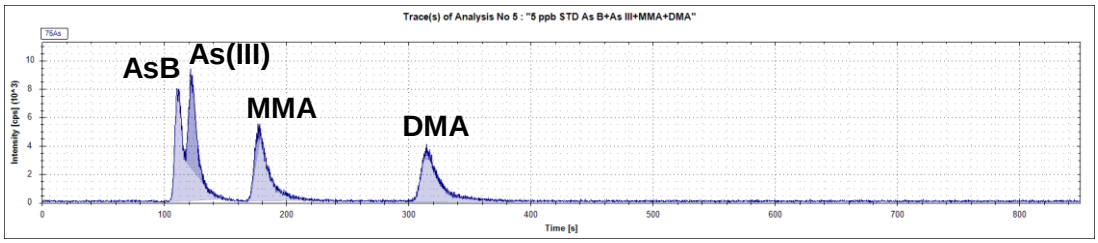


Figure 17. Chromatograms of standard solutions of **(a)** 5.0 µg/L arsenate (As V), **(b)** 5.0 µg/L arsenite, monomethylarsinic acid and dimethylarsinic acid (As III + MMA + DMA), **(c)** 5.0 µg/L arsenobetaine (As B), and **(d)** 5.0 µg/L arsenobetaine, arsenite, monomethylarsinic acid and dimethylarsinic acid (AsB + As III + MMA + DMA). Arsenic species were detected in the standards by high performance liquid chromatography coupled to inductively coupled plasma mass spectrometry (HPLC-ICP-MS; HPLC 5000 series, Thermo Scientific™) using a PRP-X100 anion exchange column (PS-DVB/Trimethyl ammonium exchanger; 5µm particle size; 4.6mm internal diameter, ID; 250mm length) in isocratic mode. Peaks indicating the presence of arsenobetaine, arsenite, dimethylarsinic acid, monomethylarsinic acid and arsenate appeared at retention times ~110s, ~125s, ~175s, ~310s and ~660s, respectively.

2.4. Discussion

2.4.1. Renal lesions occur more commonly and are more severe in domestic cats than in domestic dogs and Scottish wildcats

As expected, we found that a higher proportion of the domestic cats, than dogs and Scottish wildcats in the current study had histopathological evidence of renal interstitial inflammation (CIN) and fibrosis. Furthermore, a high proportion of these domestic cats had moderate-severe CIN and fibrosis, indicated by coverage of an appreciable proportion of the renal tissue section with mononuclear inflammatory cells or collagenous connective tissue, respectively. This corroborates prior work indicating that CKD, for which CIN is the most common renal lesion (Chakrabarti et al., 2013), is frequently observed in domestic cats, but not in dogs (Bartges, 2012). Similar to the current study, Chakrabarti et al. (2013) discovered a correlation between renal inflammation and fibrosis scores. However, McLeland et al. (2015) reported that scores for inflammation were generally greater than scores for fibrosis at each IRIS stage of feline CKD. This may suggest, as is commonly thought, that inflammation precedes fibrosis in the kidneys and other organs (Wynn, 2008). Therefore, analysing mineral content of kidney tissue and urine from cats and dogs, grouped by the presence or absence of interstitial inflammation (CIN), rather than fibrosis score, should have allowed identification of a greater number of potential cases of CKD in the current study; possibly at an earlier stage of progression, when inflammation is evident but fibrosis is not yet present.

The presence of inflammation in the renal interstitium has been associated with azotaemia in cats (McLeland et al., 2015) and dogs (Minkus et al., 1994). Azotaemia reflects a reduction in renal function and worsens with CKD progression (Polzin, 2013). Thus, while clinical parameters, such as serum creatinine concentration, used to assess the severity of azotaemia and thus stage CKD according to the IRIS system, were not measured in the current study, it is likely that our CIN scores 2 and 3 are indicative of CKD. However, there is conflicting evidence to suggest a direct correlation between the severity of renal interstitial inflammation and the degree of azotaemia

(McLeland et al., 2015, Chakrabarti et al., 2013, Alfrey, 1992). Therefore, it should be noted that the CIN scores used in the current study indicate differing degrees of CIN and not necessarily differing severities of CKD.

In the current study, only minimal inflammation and fibrosis were detected in the kidneys of Scottish wildcats. The Scottish wildcat (*Felis silvestris grampia*) is one subspecies of wildcat (*Felis silvestris*) (Corbett, 1979), whereas domestic cats are believed to descend from another wildcat subspecies, the Near-Eastern wildcat (*Felis silvestris lybica*) (Driscoll et al., 2007). Interbreeding between domestic- and wild-cats is possible, so a population of wild-living cats possessing an admixture of domestic- and wild-cat genes does exist (Beaumont et al., 2001). However, the Scottish wildcat is endemic to northern Scotland, is truly wild and has been a protected species under Schedule 5 of the Wildlife and Countryside Act since 1988 (Beaumont et al., 2001). Moreover, the Scottish wildcats collected in the current study were identified as such by an expert at National Museums Scotland. Thus, despite the difficulty in distinguishing these animals from feral tabby domestic cats (Driscoll et al., 2007), we are confident that our population of Scottish wildcats is distinct from the domestic cat group in the current study. Nonetheless, genetic differences between the domestic cat and the Scottish wildcat are likely to be only very subtle (O'Brien et al., 2002), therefore, it is likely that there is only a minor genetic contribution, if any, to the contrasting prevalences of renal disease detected in these distinct feline subspecies.

Aspects of domestication, including the diet, may also contribute to the higher prevalence of renal disease in domestic cats compared to Scottish wildcats. The Scottish wildcat is unlikely to have ever consumed a commercially available cat food; investigations into the feeding habits of Scottish wildcats have revealed a predominance of wild rabbits in the diet of these animals (Corbett, 1979). However, in other wildcats, including cheetahs, cougars, jaguars, leopards, lions, lynx, ocelots and tigers, renal lesions have been evidenced and appear to be relatively common (Junginger et al., 2015, Newkirk et al., 2011). Yet these wildcat species, whether captive or truly-wild, also consume a wild-type diet, consisting of raw, unprocessed, meat. Therefore, clearly, the absence of commercial pet foods in a cat’s diet is not

protective against renal disease. Stress has been proposed to cause renal disease in captive wildcats, but not in free-ranging wildcats, such as cheetahs (Mitchell et al., 2018). Therefore, perhaps other environmental factors, such as stress, may have contributed to the higher prevalence of renal disease in the domestic cats, compared to Scottish wildcats, in the current study.

Furthermore, we cannot ignore the known effect of age on the prevalence of renal disease in any population (Mitchell et al., 2018, Brown et al., 2016, Cianciolo et al., 2016, Newkirk et al., 2011). Indeed, cats and dogs with CIN in the current study were older on average than those without CIN. The exact age of the Scottish wildcats in the current study was unknown, although it was presumed that they were fully-grown adult cats due to the size of their whole kidneys (4–6 cm long). Nonetheless, the Scottish wildcats in our study were likely to be younger than the domestic cats, since they were often found dead at roadsides. This age difference may have been, at least in part, responsible for the lower, and negligible, occurrence of chronic renal lesions found in the Scottish wildcats.

In the current study, only a single tissue section from one kidney from each animal was assessed histologically. Scoring of renal lesions was based on this single slice of the kidney. Therefore, it is possible that some cases of CIN may have been missed, or scored lower or higher than if the whole organ had been evaluated. However, other, similar studies that have evaluated renal lesions histologically have also done so on a single tissue section per case (Chakrabarti et al., 2013, McLeland et al., 2015). Minkus et al. (1994) inspected kidney biopsies from cats and dogs for evidence of renal lesions and compared these findings to the lesions observed in larger sections of kidney tissue collected from the same animals at necropsy. They concluded that, providing at least ten glomeruli are present in a kidney tissue sample, a reliable diagnosis of diffuse renal disease can be made in over 90% of cases (Minkus et al., 1994). Despite observing only a proportion of the whole kidney, the tissue sections assessed in the current study contained many more than ten glomeruli. Therefore, based on the report from Minkus et al. (1994), our analysis should have been sufficient to identify renal lesions, where present, in most, if not all, cases.

2.4.2. Use of post-mortem material is sufficient for comparisons between species

In the current study, the tissue that was assessed both histologically and for mineral analysis was post-mortem material. Previously, Sanchez-Lara et al. (2015) suggested that kidney tissue degradation, by endogenous matrix metalloproteinases (MMPs), may occur between the time of euthanasia and collection of the tissue at necropsy, and further during frozen-storage of the tissue before analysis. Their concern was based on the degradation of tissue proteins; specifically an enzyme that they were measuring to assess its activity (Sanchez-Lara et al., 2015). While MMPs degrade ECM components and may thereby affect the assessment of interstitial lesions, as was carried out in the current project, Sanchez-Lara et al. (2015) did not propose this limitation in relation to the histological staining and scoring of renal lesions in their study. The same method of use of post-mortem tissue, tissue fixation, sectioning and staining was conducted in the current study as in similar studies that evaluated renal lesions histologically (Chakrabarti et al., 2013, Sanchez-Lara et al., 2015, McLeland et al., 2015). In these studies, again, there was no mention of a limitation regarding tissue degradation (McLeland et al., 2015, Chakrabarti et al., 2013).

There have been few investigations into the effect of using necropsy specimens on the histological appearance of tissues. In mice, it has been shown that the lumina of the renal tubules, particularly proximal and distal tubules in the outer cortex, may be wider when the kidneys are frozen immediately after death, compared to when freezing is delayed post-mortem (Hanssen, 1960). This suggests that immediate freezing of the tissue preserves the normal in vivo structure of the kidney when the tubules are distended with fluid, whereas fluid may drain from the tubular system and particularly rapidly from the proximal and distal tubules after death when renal function ceases (Hanssen, 1960). Indeed, it has been shown that the water content of the kidney is higher if the kidney is frozen immediately at the point of collection, than if freezing is delayed (Hanssen, 1960). This may suggest that some of the kidney content is lost if the tissue is allowed to rest for a period after death, as in the current study. However, it has been suggested that most

of the tubular fluid is reabsorbed post-mortem and a smaller proportion is gradually redistributed distally along the nephron (Hanssen, 1960). It should be considered that reabsorption of tubular fluid might increase the volume of the interstitium, thereby affecting the histological appearance of the kidney and the results of the current study. However, the interstitial compartment was in fact only marginally larger in mice kidneys that were frozen immediately after death, compared to mice kidneys that were frozen after a delay (Hanssen, 1960). To our knowledge, this is the only study of its kind that focuses on the kidney. However, if these findings also apply to cat and dog kidneys, they suggest that our histological examination of renal interstitium should not have been affected by a slight delay in tissue fixation.

The redistribution of tubular fluid within the kidney after death may also affect the location of the solutes that undergo glomerular filtration and tubular reabsorption or secretion in the functioning kidney (Hanssen, 1960). Mineral redistribution in the kidney was not measured in that study (Hanssen, 1960), however, post-mortem movement of mineral elements between intracellular and extracellular compartments has been evidenced within the thyroid gland of rats and pigs (Wroblewski, 1994) and in rat liver (Iyengar, 1981), pancreas and heart (Kuypers and Roomans, 1980). In all organs studied, it appears that the direction of movement of a particular mineral depends on whether it is normally found within the intracellular or extracellular environment (Kuypers and Roomans, 1980). If the same movement occurs in the kidney, the measurement of major and trace element concentrations in post-mortem kidney tissue, as was conducted in the current study, therefore would not enable localisation of mineral elements to a particular renal segment to reliably reflect the distribution of minerals in the kidney in vivo (Hanssen, 1960). However, in the current study, we aimed to compare the kidney mineral content between species, not between different cellular compartments within the kidney. Furthermore, we measured mineral concentrations in renal cortex, not within RPTECs specifically. From all animals in our study, we analysed a portion of kidney tissue which would have contained tubules and interstitium, therefore, any movement of mineral elements between compartments that does occur, should not have affected our conclusions. Moreover, in many of

the cats and dogs from which we obtained kidney tissue, we also analysed urine samples that were collected from the bladder. Therefore, if some solutes, along with the tubular fluid, are lost from the kidney via this route after death but prior to sample collection, they should still have been measured in our study. Indeed, our conclusions based on comparisons between cats and dogs are still valid.

Similar to the current study, post mortem tissue has been used previously for determination of kidney tissue mineral content (Passlack et al., 2014, Andreani et al., 2010, Passlack et al., 2015, Shimamura et al., 2013). While renal biopsy is possible, is suitable for assessing the severity of renal lesions (Maniaki and Finch, 2018) and would have allowed for immediate tissue fixation, it is not readily undertaken in cats or dogs (Murgia, 2014) and it would not have provided an adequate amount of tissue for mineral analyses. Furthermore, taking renal biopsies from animals with CKD may be contraindicated (Vaden, 2004, Maniaki and Finch, 2018). Therefore, the use of retrospectively-collected necropsy material was appropriate for the current study.

To our knowledge, whether post-mortem change affects the overall mineral content and composition of the kidney has not been investigated. It has been evidenced in the liver (Iyengar, 1981) however, that the total amounts of certain minerals in the tissues may increase or decrease if the organ is not removed from the body and preserved in a timely manner after death. Nonetheless, while the concentrations of minerals measured in necropsied kidneys may not equal the concentrations that were present in the live animals' kidneys, the timing between death and tissue collection and between collection and freezing was roughly the same for all domestic cats and dogs in our study. Therefore, our results still enable a valid comparison of kidney mineral content between domestic cats and dogs. On the other hand, although not known with certainty, a longer period between death and tissue collection may have occurred with the Scottish wildcats. Therefore, the results obtained from this study population should be interpreted with caution. Nevertheless, the interval between tissue collection and freezing was similar to that performed in the domestic species and the Scottish wildcat serves as a useful, novel control group in the current area of research.

2.4.3. Pet food mineral analyses are comparable to industry standards

Information detailing the specific methods used by pet food manufacturers to measure major and trace elements in pet foods, is not easily accessible or obvious. FEDIAF (2019) references EU commission regulation 152/2009 to guide the analysis of essential nutrients in feedstuffs. It suggests the use of photometric methods for phosphorus, and atomic absorption spectrometry (AAS) for iron, copper, manganese and zinc, analyses, with LoQ: 10-20 mg/kg. Regulation 2002/32/EC covers undesirable substances in animal feed but does not detail recommended methods for analysis of heavy metal elements, such as mercury and arsenic. The Direct Mercury Analyser used in the current study is an atomic absorption spectrophotometer that provides comparable results to ICP-MS in less time. It has been shown previously to enable precise measurement of mercury (Khan et al., 2015, Hong et al., 2019). Moreover, excellent recovery of mercury in the fish muscle ERM-BB422 CRM, purposed as a quality control for analytical methods, including AAS and ICP-MS, for trace element concentrations in fish, validated our method for mercury analysis. As in the current study, total arsenic is commonly measured by ICP-MS (Khan et al., 2015, Hong et al., 2019). Others have reported similar recoveries of other mineral elements using AAS and ICP-MS (Zhang et al., 1997), with improved reproducibility, sensitivity and detection limits and reduced matrix effects with the latter method (Tothill et al., 1990, Davies et al., 2017a). Thus, the methods used for pet food mineral analysis in the current project, are highly similar, if not more advanced than the current industry standard defined for most elements (Zhang et al., 1997).

Regulation 152/2009 states that, prior to analysis, samples must be homogenised to represent the entire pet food. First, food is usually dried to reduce the water content to 8-12%. Preparation of pet food samples by freeze-drying and grinding in the current study therefore emulates the industry standard protocol. Regarding storage of pet food samples, regulation 152/2009 only states that samples must be stored at a temperature at which their composition will not be affected. Although the timescale between purchase and analysis of the foods was longer than the amount of time pet

owners would typically store the foods for, studies have shown that the mineral content, and indeed mercury (De Boer and Smedes, 1997) and arsenic (Naito et al., 2015) concentrations, is relatively unaffected by storage conditions such as those used here, or even at higher temperatures, for up to a year or more. Particularly, sample quality and stability can be preserved for longer when freeze-dried (Bordas and Bourg, 1998, Fang and Bhandari, 2012). Thus the element concentrations measured in pet foods during this study should adequately reflect those contained within the foods at time of purchase and feeding.

2.4.4. Accumulation of pro-oxidant minerals in the kidney is not associated with CKD

In previous work, we (Davies et al., 2017a), and others (Squadrone et al., 2017), have shown that some commercially available, complete pet foods that contain fish have high levels of total arsenic. Fish and fish-containing pet foods have also been found to have high levels of mercury (Cheng et al., 2009, Luippold and Gustin, 2016). We suggested that domestic cats receive fish as part of their diet more often than dogs, due to a perceived preference for fish flavours (Houpt and Smith, 1981). Therefore, cats may be exposed to greater concentrations of arsenic and mercury. Particularly, the kidney, which has a major role in the excretion of these minerals from the body (Chang and Singh, 2018, Lewchalermvong et al., 2018, Rana et al., 2018), may accumulate greater amounts of these minerals in cats than in dogs. Yet, domestic cats descended from the Near Eastern wildcat living in a desert environment (Driscoll et al., 2007), where fish would not ordinarily be consumed. Therefore, domestic cats may not be physiologically able to handle the high levels of these minerals found in fish.

Moreover, arsenic and mercury are pro-oxidant minerals, meaning that they can increase the formation of ROS and, in the absence of sufficient anti-oxidant defences to combat this ROS-production, oxidative damage to tissues may be induced or increased (Antunes Dos Santos et al., 2018, Jomova et al., 2011). An imbalance between pro- and anti-oxidant factors in the kidney may

result in oxidative stress within the renal parenchyma (Locatelli et al., 2003, Agarwal, 2003). Renal oxidative damage may instigate or contribute to CIN and fibrosis and, ultimately, may result in CKD (Ratliff et al., 2016, Brown, 2008, Agarwal, 2003). CKD, characterised by CIN, is especially prevalent in domestic cats (Chakrabarti et al., 2013, Brown et al., 2016). We proposed that this might be attributed to greater renal accumulation and handling of pro-oxidant minerals, relative to antioxidant minerals, possibly due to the mineral composition of the diet.

We measured concentrations of pro- and anti-oxidant minerals, as well as other minerals, in the kidneys and urine of domestic cats and dogs, with and without CIN. This allowed us to test our hypothesis that cat kidneys and kidneys with CIN have greater accumulation of pro-oxidant mineral elements and lower concentrations of minerals with antioxidant functions, relative to dog kidneys and kidneys without CIN, respectively. While mineral concentrations in the kidney and urine may not directly relate to levels of ROS and anti-oxidants, they are a useful indicator. Free radicals have half-lives of only a few seconds, so they themselves are too unstable to be measured to assess the association of their accumulation with CIN in the kidney (Locatelli et al., 2003). Besides, it is already known that oxidative stress, resulting from an imbalance between ROS and antioxidants, contributes to the development and progression of CKD, as the levels of lipid-, protein- and DNA-oxidisation products, which are stable and thus can be measured, are increased in the kidney in cases of CKD (Locatelli et al., 2003, Ratliff et al., 2016). What remains unknown, is whether an imbalance between pro- and anti-oxidant mineral elements in the kidney is associated with the disease.

Due to the retrospective recruitment of animals into our study, we were unable to obtain dietary histories. Therefore, we could not assess whether the mineral composition of the kidney reflected that of the diet. Notably, it was not possible to associate fish consumption with kidney tissue arsenic or mercury content. However, our mineral analysis of kidneys from a population of Scottish wildcats allowed a further intra-species comparison. The feline genome is remarkably-well conserved between domestic and wild cat species (O'Brien and Yuhki, 1999, O'Brien et al., 2002). Additionally, the Scottish wildcat primarily eats wild

rabbits (Corbett, 1979) and is unlikely to have ever consumed a commercial diet, nor any wild fish. Therefore, any differences between the kidney mineral profiles of domestic cats and Scottish wildcats may suggest mineral differences between commercial and wild-type cat diets.

2.4.4.1. Arsenic

Most of the arsenic that is obtained through the diet is excreted by the kidney within 24 to 48 hours (Charbonneau et al., 1978, Mazumder et al., 2013, Diaz et al., 2015). Therefore, our measurements of arsenic concentration in spot-samples of urine that were collected from animals at necropsy probably reflect the dietary arsenic intake during the 24-48 hours preceding death, rather than indicating chronic exposure to the mineral. It is possible that the animals, from which samples were obtained in the current study, reduced their dietary intake in the days prior to death or euthanasia, therefore the urinary arsenic concentrations presented may not truly reflect the average daily intake. In humans, greater urinary arsenic excretion has been found to associate with increased dietary arsenic intake (Mazumder et al., 2013). Diaz et al. (2015) and Mazumder et al. (2013) report similar ranges of urinary total arsenic concentrations in humans living in arsenic-endemic areas and with excessive arsenic intake, while urinary arsenic concentrations in humans with low arsenic exposure are significantly lower (Mazumder et al., 2013). Accordingly, greater urinary arsenic concentrations in domestic cats, compared to dogs, in the current study may suggest that the domestic cats had greater daily dietary arsenic intake, at least in the few days prior to death.

To our knowledge, a normal range of urinary arsenic concentrations has not been defined for any population (Chen et al., 2011). However, in humans, there is higher odds of renal dysfunction with urinary arsenic concentrations >75 µg/g creatinine, versus ≤35 µg/g creatinine (Chen et al., 2011). In that study, like our own, arsenic was measured using ICP-MS (Chen et al., 2011). In the current study, although there was no significant effect of CIN on the urinary arsenic concentration, most animals with CIN and, indeed, almost all cats but not dogs, had urinary arsenic concentrations >75 µg/g creatinine, perhaps indicating a vulnerability to renal disease in domestic cats. The lack of

significant effect was likely due to the small number of urine samples available for the analysis in our study. Evidently, species-differences in renal handling and metabolism are likely. Indeed, arsenic appears to behave differently in cats, compared to other species, possibly due to an unusual high affinity for haemoglobin in RBCs (Buchweitz et al., 2019). High blood arsenic concentrations are expected and considered normal in domestic cats and captive wild cats, whereas in other species, such as domestic dogs, blood arsenic concentrations are usually below LoD (Buchweitz et al., 2019). Due to this accumulation of arsenic in the blood, arsenic excretion may be delayed in cats. This perhaps enables greater arsenic accumulation in the kidney of cats, compared to dogs, as was determined in the current study. While cats may tolerate higher levels of arsenic in circulation, without exhibiting signs of toxicity until much greater concentrations are reached (Buchweitz et al., 2019), it is unclear whether greater arsenic accumulation within the kidney can be tolerated as effectively.

To our knowledge, it is unknown whether the level of arsenic accumulated in kidney tissue relates to the amount of arsenic consumed. Rats fed different levels of arsenic through different varieties of rice had similar concentrations of total arsenic in their kidneys, suggesting that tissue arsenic concentrations do not correlate with dietary arsenic exposure (Lewchalermvong et al., 2018). However, the rats were studied over a period of only 28 days, therefore conclusions about chronic arsenic accumulation cannot be drawn. Nonetheless, similar to kidney arsenic concentrations measured in cows, sheep, pigs, horses (Bilandžić et al., 2010) and even humans living in an arsenic-polluted area in Spain (García et al., 2001), generally, the levels of total arsenic measured in kidney tissue in the current study were very low. Low kidney tissue arsenic concentrations may be the result of effective urinary arsenic excretion, preventing storage within the kidney, which could otherwise be damaging. However, in the aforementioned studies, arsenic concentration was recorded as per kilogram of tissue wet weight, rather than dry weight as in the current study, hence direct comparisons of tissue arsenic concentrations between cats and dogs and other species are not possible. Nonetheless, in accordance with urinary arsenic concentrations, kidney arsenic content was

significantly greater in domestic cats than in dogs and, although not significant, greater kidney tissue arsenic concentration was measured in domestic cats than in Scottish wildcats. Therefore, like increased urinary arsenic concentrations (Mazumder et al., 2013), greater kidney arsenic content in domestic cats might be due to greater arsenic consumption by domestic cats. Notably, increased consumption of fish-containing, commercial pet foods, which contain higher levels of arsenic (Davies et al., 2017a), may be responsible for the proposed elevated arsenic intake in domestic cats, compared to dogs and Scottish wildcats. However, we did not obtain dietary histories for the animals involved in this study and there is no existing data on the arsenic content of wildcat diets to support this contention. Nonetheless, our results suggest that domestic cats have greater dietary arsenic intake than dogs. Therefore, if high arsenic intake truly increases the risk of CKD, as suggested in humans (Chen et al., 2011), we could infer that elevated dietary arsenic intake contributes to the particularly high prevalence of CKD in cats, relative to dogs.

2.4.4.1.1. The high total arsenic content of fish-containing pet foods is predominantly relatively non-toxic arsenobetaine

Our results suggest that arsenic accumulates in domestic cat kidneys to a greater extent than in dog, or Scottish wildcat, kidneys and that domestic cats have greater dietary intake of arsenic compared to dogs, and possibly Scottish wildcats too. Prior to this project, we had measured total arsenic in pet foods and found that fish-containing foods had higher, and in some cases excessive, total arsenic concentrations (Davies et al., 2017a). Similar analyses of cat and dog foods in Italy (Squadrone et al., 2017) and dog foods in the USA (Kim et al., 2018) showed the same result. However, it is known that most arsenic in fish for human consumption is a relatively non-toxic organic form, arsenobetaine (Komorowicz et al., 2019, Sele et al., 2015, Kalantzi et al., 2017, Miyashita et al., 2009). To our knowledge, no prior analysis of pet food arsenic content has identified the species of arsenic that are present within these foods. Therefore, it was necessary to conduct an analysis of the arsenic species found within the fish-containing pet foods that are commonly fed to

domestic cats, to determine the risk of toxicity. As expected, the TAs in fish-containing cat foods was almost exclusively arsenobetaine. Other organic arsenic species, DMA and MMA, were detected only in minute quantities. The highly toxic inorganic forms of arsenic, As(III) and As(V), were undetectable in all of the cat foods analysed. Our results mirror those of previous analyses of the arsenic species in fish for human consumption (Komorowicz et al., 2019, Kalantzi et al., 2017). Therefore, although cats that consume fish-based pet foods may have high levels of dietary arsenic, this is mostly comprised of organic arsenic, which is unlikely to pose a risk of toxicity (Jomova et al., 2011).

The arsenic speciation method used in the current study has some limitations, which are necessary to consider. The TAs concentration measured in the CRM, after both acid-digestion and water-extraction, was 1.5-times higher than the certified value of TAs in the CRM. This could be due to contamination in the process and may mean that the TAs concentrations measured in the pet food samples are also 1.5-times greater than the actual concentrations. However, we did not adjust the TAs concentrations that were measured in the pet food samples by this 1.5 correction factor because our fundamental reason for measuring TAs concentrations was to compare the recovery of arsenic in water-extracted samples with that of samples prepared by acid digestion. Acid digestion is a widely accepted method for preparing samples prior to measuring total trace element concentrations by ICP-MS (Bolann et al., 2007). However, for arsenic species determination, we wanted to prepare samples using water-extraction, due to the success of this technique reported by others previously (Komorowicz et al., 2019). As water-extraction had not been used previously in our laboratory, we measured the total arsenic concentrations in pet food samples by ICP-MS after both acid digestion and water-extraction, in order to compare the recoveries of TAs using both methods. There was good agreement between the TAs concentrations measured by ICP-MS after water-extraction and after acid-digestion for all samples and the CRM. Therefore, we were confident in our ability to extract arsenic species from pet food samples, using preparation with water.

The sum of the arsenic species concentrations that were detected did not equal the TAs concentration measured in any sample. This may be because

TAs concentrations in the pet food samples were not reduced by the 1.5 correction factor, or due to imperfect efficiency of water to extract the arsenic species. Indeed, unless all the arsenic species are equally soluble in water, the ratio of arsenic species in the pet food samples might be affected by the extraction efficiency. Another possible explanation for the mismatched TAs and sum of arsenic species results is the existence of sugar-arsenic complexes in the samples. These, even if present, would not have been detected by the ICP-MS-coupled chromatography method used. Nonetheless, if other arsenic species or complexes were present in the samples, it is likely that they would only have constituted a small proportion of the total arsenic, since only a minor amount of the total arsenic measured in the samples was unaccounted for by the arsenic species that were detected.

Samples were spiked with an AsB standard solution in an attempt to separate AsB and As(III) peaks on the resultant chromatograms. However, this did not work. Nonetheless, based on comparisons between peak retention times in standards and samples, we concluded that large amounts of AsB and only minute amounts of As(III) were present in the pet food samples. Low As(III) concentrations in the samples would have been masked by the large AsB peak and were therefore undetectable. An isocratic anion exchange column was used in the current study, however, Watts et al. (2008) reported that an isocratic cation exchange system is needed in order to identify both AsB and As(III). Thus, it is probable that a second column and thus a separate, second run was needed in the current study in order to measure both AsB and As(III). Alternatively, it may be possible to measure all five arsenic species (AsB, DMA, MMA, As[III] and As[V]) on a single chromatogram if a gradient system, with a slow gradient switchover time at the beginning of the elution program, was employed (Watts et al., 2008). This was not possible in the current study with the equipment available in our laboratory.

2.4.4.2. Mercury

In our previous analysis of pet foods, we had not measured mercury concentration (Davies et al., 2017a). However, we proposed that, like arsenic (Davies et al., 2017a), mercury concentration might be higher in fish-flavoured

foods. Indeed, similar to others (Luippold and Gustin, 2016), we found this to be true. Most foods had low mercury concentrations and just a few foods, mostly wet and/or fish-flavoured foods, had high content of mercury and were responsible for increasing the range of mercury concentrations measured across all of the foods. Similar to the findings in the current study, higher and more variable mercury concentrations have been measured in wet versus dry cat and dog foods previously (Paulelli et al., 2018, Sakai et al., 1995, Luippold and Gustin, 2016). Additionally, some cat and dog foods have been found to contain levels of mercury higher than the maximum tolerable level for these species (Paulelli et al., 2018). In that study, however, the association of high mercury concentrations with a particular ingredient, such as fish, was not investigated (Paulelli et al., 2018). All foods included in our study, except one wet cat food that contained fish, complied with the European legal limit of mercury in pet foods. Nonetheless, we did not find significantly different mercury concentrations between cat and dog foods, although the two foods with the highest mercury concentrations were cat foods. Luippold and Gustin (2016) similarly found that the highest mercury concentrations were in cat foods, rather than dog foods. From the results of these investigations, it is possible to conclude that, while the level of mercury in most pet foods is below the legal maximum limit, wet foods and foods containing fish often have high mercury concentrations. Furthermore, mercury content of fish that is suitable for human consumption but which is often fed to pet cats, such as tuna (tinned and fresh) and sardines, has been shown to have greater mercury content than commercial cat foods (Sakai et al., 1995). These types of foods are probably fed to cats more often than to dogs, due to a particular preference for these foods among cats (Adamec, 1976, Houpt and Smith, 1981). Therefore, cats may be exposed to even higher mercury concentrations, compared to dogs, when their entire diet is considered.

Despite commercial cat and dog foods not having significantly different mercury contents, dogs appeared to have significantly higher concentrations of mercury in their kidneys, in both cortex and medulla, compared to domestic cats. Previously, mercury accumulation within the kidney has been shown to correlate positively with dietary levels of the metal element in chickens (Ma et

al., 2018). Furthermore, humans living in Greenland, with predicted high mercury intake due to high seafood consumption, reportedly have higher concentrations of mercury accumulated in their kidneys, than humans living in countries where seafood consumption is generally lower (Johansen et al., 2007). Moreover, kidney mercury content was higher in kittens receiving greater dietary concentration of mercury compared to kittens with lower mercury intake (Boyer et al., 1978). Therefore, contrary to our hypothesis that cats would have greater mercury intake than dogs due to more frequent consumption of fish, our results may suggest that dietary mercury intake was higher in the dogs, compared to the cats in our study. Furthermore, the mercury concentration measured in renal medulla from Scottish wildcats was not significantly different from that measured in domestic cats. This suggests that consumption of commercial pet foods and fish, which would be a rarity in the wildcat diet, does not necessarily cause higher dietary intake, or renal accumulation, of mercury. This differs from the conclusion by Serpe et al. (2012) who found higher mercury concentrations in the kidneys of household dogs, compared to stray dogs, and therefore suggested that companion animals may be exposed to mercury through the consumption of pet foods.

In cats, hair mercury concentration has been shown to reflect the level of mercury in the diet, and has been found to be greater than that in dogs (Sakai et al., 1995). This perhaps suggests that cats have greater dietary intake of mercury than dogs, contrasting the conclusions made from the kidney tissue mercury analyses in the aforementioned studies and our own study. However, mercury concentration in hair also reflects mercury accumulation in the body. Therefore, the results of Sakai et al. (1995) imply a greater body burden of mercury in cats compared to dogs. Furthermore, cat hair mercury concentration increased with age, suggesting chronic accumulation of mercury in the body in cats (Sakai et al., 1995). In the present study, in all dogs and cats, mercury concentrations were higher in the cortex than in the medulla, suggesting that mercury accumulates to a greater extent in the renal cortex than in the renal medulla. Indeed, this has been found in other species, including rabbits (Zalups and Barfuss, 1990) and rodents (Zalups, 1993). Moreover, in rats, dose-dependent mercury accumulation in renal cortex and

medulla has been observed, with primary deposition in the cortex under usual exposure levels but higher concentrations found in the outer medulla versus cortex when exposure levels are high. As found in our study, the mercury concentrations in the inner medulla are consistently lowest (Zalups and Diamond, 1987). The level of damage in the kidney has also been shown to increase with increasing renal accumulation of mercury (Ma et al., 2018). Although the prevalence of renal disease is greater in cats than in dogs (Brown et al., 2016), it appears that domestic cats do not have greater renal accumulation, or dietary intake, of mercury, compared to dogs. Therefore, it seems that consumption of mercury is unlikely to be responsible for the high prevalence of CKD in domestic cats.

2.4.4.3. Iron

The kidney has a role in iron homeostasis (Thevenod and Wolff, 2016). Although most iron in circulation is bound to proteins (Jomova and Valko, 2011), even under physiological conditions, it is able to pass through the glomerular filtration barrier and enter the renal tubules (Thevenod and Wolff, 2016). However, iron that does enter the renal tubules is mostly reabsorbed and only minimal iron is excreted into the urine (Martines et al., 2013). Nonetheless, in rats, urinary iron excretion has been shown to increase with increased iron intake from the diet (Smith and Thevenod, 2009, Wareing et al., 2003). In our study, although not significant at our more stringent level, urinary iron concentration appeared to be greater in domestic cats than in dogs. This might suggest that cats have greater dietary iron intake compared to dogs. In humans, urinary iron excretion may also increase due to renal disease that affects glomerular filtration and tubular reabsorption (van Raaij et al., 2019). This effect was not observed in the cats and dogs with CIN, versus those without CIN, in the current study. Perhaps the renal dysfunction associated with CIN in the current study was not severe enough to significantly alter renal iron handling. Nonetheless, greater urinary iron concentrations in cats compared to dogs, per se, may reflect greater risk of renal disease in the former species.

Inflammation and oxidative stress can cause upregulation of renal tubular iron reabsorption in humans (van Raaij et al., 2018). If the inflammation and/or oxidative stress is severe, as in CKD, excessive levels of iron may accumulate in the kidney tissue (Martines et al., 2013). Iron is involved in the development of oxidative stress as it catalyses the formation of ROS (Jomova and Valko, 2011). Therefore, iron accumulation in the kidneys may damage the renal tubules and contribute to CKD progression (Nankivell et al., 1994, Martines et al., 2013, van Raaij et al., 2018, Nakatani et al., 2018, Wang et al., 2001). Indeed, the amount of iron within the kidneys and urine has been shown to be increased in humans and rodents with CKD, compared to those without CKD (Shah et al., 2007, Nankivell et al., 1994, van Raaij et al., 2018). Furthermore, elevated iron content in kidneys and urine have been associated with increased severity of renal lesions in rats (Alfrey, 1992) and indicators of renal damage and dysfunction, including proteinuria, haematuria and haemoglobinuria in rats and humans (Wang et al., 2001, Nankivell et al., 1994, Martines et al., 2013). However, in our study, the domestic cats and dogs with CIN had lower concentrations of iron in their kidneys compared to animals without CIN, although this effect was not statistically significant at our stringent level. Furthermore, while urinary iron concentration was greater in domestic cats than in dogs, we found significantly higher kidney tissue iron concentrations in dogs than in domestic cats. Perhaps this reflects more effective urinary excretion of iron by cats, than by dogs.

Evidently, our method of measuring iron did not allow for the separate identification of protein-bound and free-iron. It is free-iron which is able to induce ROS formation and oxidative damage, while accumulation of protein-bound iron may have less pathological significance (Alfrey, 1992). It is unknown whether the higher iron content of dog kidneys was due to higher levels of free-iron or protein-bound iron. Nonetheless, in previous human studies, increased renal accumulation of iron, including protein-bound iron, has been associated with CKD (van Raaij et al., 2018). Renal handling of minerals, including iron, has not been studied specifically in companion animals to know whether it differs from the mechanisms in effect in other species. However, if the aforementioned findings in humans and rodents also apply to domestic

animal species, it would seem that our results do not imply that the high CKD prevalence in cats is due to iron accumulation within their kidneys.

2.4.5. Mineral elements with antioxidant functions are lower in cat versus dog kidneys

2.4.5.1. Copper and zinc

Oxidative damage to tissues is caused by an imbalance between pro-oxidants, such as the minerals discussed previously, and local antioxidant defences. Copper and zinc are two minerals with key antioxidant functions (Ratliff et al., 2016). In the current study, these minerals were both depleted in domestic cat versus dog kidneys. It has been shown that zinc concentrations in the kidneys of cats and dogs (Drinker et al., 1927a) and rats (Drinker et al., 1927b) increase in accordance with zinc intake from the diet. Therefore, the results of the present study may suggest that dogs obtain more zinc from their diet than cats. Unfortunately, as dietary histories were not obtained for the animals in our study, we cannot corroborate this assumption. However, our previous analysis of the mineral composition of commercially-available, complete pet foods revealed no significant difference in the zinc content of foods for cats and dogs and most foods complied with the FEDIAF-recommended minimum and maximum concentrations of zinc (Davies et al., 2017a). Thus, it is unlikely that cats and dogs consuming a complete commercial diet will be zinc-deficient. Dietary zinc deficiency has been associated with increased renal injury in mice models of diabetes, while ensuring adequate or high dietary zinc intake reduced the expression of pro-inflammatory factors and the occurrence of renal histopathological changes. These effects may be mediated by zinc-induced anti-oxidative pathways (Yang et al., 2017a). The same pathways have been shown to be affected in Sprague-Dawley rats with 5/6 nephrectomy, used as a model of CKD, resulting in progression of renal damage (Kim and Vaziri, 2010). Therefore, perhaps deficient zinc supply to the kidney could increase renal oxidative stress in cases of feline CKD too. Scottish wildcats had similar kidney content of copper and zinc compared to domestic cats, suggesting that the commercial diet does not provide a richer source of these antioxidant minerals, compared to the amounts that cats would obtain in the

wild by consuming prey. It is possible that our results suggest that cats, whether domestic or wild, have intrinsically lower levels of copper- and zinc-dependent antioxidant enzymes in their kidneys, compared to dogs. This could predispose cats to oxidative damage and consequently CKD in the long-term.

In human kidneys, reduced zinc reabsorption has been associated with increased tubular impairment (Satarug et al., 2018). However, no difference was observed between urinary or kidney tissue zinc concentrations in cats and dogs with versus without CIN in the current study. It is possible that lesions were not severe enough in the CIN groups, and/or the number of urine samples was too few, for any effect to be evident. Human studies on the association of copper and zinc with CKD are limited to serum, rather than kidney, concentrations. It has been shown that serum copper concentrations do not change significantly as CKD progresses, but zinc concentrations tend to be lower in late, versus early stage CKD (Shih et al., 2012). Furthermore, another study of feline kidneys found lower zinc, but not copper, concentration in the kidneys of cats with CKD, compared to cats without renal disease (Passlack et al., 2014). In contrast, as hypothesised, we found decreased copper concentrations in kidneys with CIN, compared to those without CIN, in both domestic cats and dogs. Nonetheless, Passlack et al. (2014) reported reduced copper and zinc concentrations in older (12-18 years of age [copper]; 4.5-18 years of age [zinc]), versus young (<1 year old), cats (Passlack et al., 2014). Interestingly, Passlack et al. (2015) found no significant difference in the copper and zinc concentrations in the kidneys between dogs with CKD and dogs without CKD. Furthermore, the oldest dogs in that study did not have the lowest renal tissue copper and zinc concentrations (Passlack et al., 2015). CKD is more common in older animals and we identified cats and dogs with CIN to be older than those without CIN. Therefore, reduced copper and/or zinc concentrations in the kidneys of older cats, as evidenced by Passlack et al. (2014) and our own study, may facilitate the development of CKD in older cats. Dog kidneys per se may have greater content of antioxidant minerals, which does not decrease with age (Passlack et al., 2015), conferring greater and continued protection against renal oxidative stress, contributing to a lower prevalence of CKD in dogs, compared to cats.

2.4.6. Further mineral differences between domestic cat, dog and Scottish wildcat kidneys and urine

No a priori hypotheses were made for the roles of any of the major elements or other trace elements, such as cadmium, rubidium and strontium, in relation to feline CKD. However, lower concentrations of all major elements were detected in domestic cat than in dog kidneys. Furthermore, calcium concentration was elevated in kidneys with CIN, compared to kidneys without CIN, in both domestic cats and dogs. Hypercalcaemia (Finch, 2016) and nephrocalcinosis, which is the deposition of calcium salts, often calcium phosphate or calcium oxalate, in the kidney (Markowitz et al., 2005), can be consequences or causes of renal disease. This might explain the higher concentration of calcium in kidneys with CIN than in kidneys without CIN in our study. However, unlike calcium, phosphorus concentration was not increased in kidneys with CIN compared to kidneys without CIN, which might suggest that calcium phosphate crystals were not present in the animals with renal disease in our study. Indeed, calcium phosphate deposition in the kidney may be more likely to indicate vitamin D toxicity, where calcium and phosphorus concentrations usually increase concomitantly, rather than reflecting CKD-induced hypercalcaemia (Finch, 2016, Crossley et al., 2017). Calcium oxalate crystals may form in the kidney due to hypercalcaemia or due to ethylene glycol poisoning, which is relatively common, and a known cause of CKD, in cats (Amoroso et al., 2017). Any kidneys with histological evidence of crystals, or otherwise known ethylene glycol toxicity were excluded from our study. Therefore, in the remaining cases that were included in the present study, calcium deposition in the kidney may have been insufficient to cause observable stone formation. It is unlikely that processing of the tissue sections would have removed any crystals that were originally present. Indeed, HE staining of feline kidney tissue sections, as was conducted in the current study, has been used previously to identify calcium crystals with success (Amoroso et al., 2017). Hence, it is more likely that such crystals were not present in the retained cases. While higher levels of calcium in the blood may be responsible for higher levels in the kidney, plasma ionised calcium concentrations would

have needed to be measured to determine whether the animals with CIN, with higher kidney tissue calcium content, were hypercalcaemic (Finch, 2016).

Reduced kidney content of magnesium in cats, compared to dogs, may be relevant to the increased prevalence of CKD in the former species, since reduced dietary magnesium intake has been associated with renal dysfunction (Rebholz et al., 2016), increased risk of CKD in cats (Hughes et al., 2002) and increased levels of fibroblast growth factor 23 (Matsuzaki et al., 2013, van den Broek et al., 2018), which is involved in the development of renal fibrosis (Zhu et al., 2018). However, to our knowledge, there is no data to suggest that the levels of magnesium in the kidney reflect the amount of magnesium consumed. Therefore, it is uncertain whether our measurements of lower magnesium concentrations in the kidneys of cats, relative to dogs, reflect lower dietary intake of magnesium. As a result, we cannot ascertain that cats, compared to dogs, have an increased risk of renal disease due to lower levels of magnesium in their kidneys. Nonetheless, low levels of magnesium in blood serum have also been associated with increased mortality risk in cats with CKD (van den Broek et al., 2018). The kidneys filter 70% of the total amount of magnesium in blood serum, of which most is reabsorbed by the renal tubules (Blaine et al., 2015). Hence, it is possible that the levels of magnesium in the diet, blood and kidney are all related and that lower levels of magnesium in the kidneys of cats indeed increase the risk of renal disease in this species. This would, however, require further investigation.

Cadmium is a non-essential trace element with no known biological function (Andreani et al., 2010). Despite not having a postulated hypothesis for cadmium's involvement in feline CKD, it has been shown that high tissue content of cadmium can induce mitochondrial dysfunction, leading to increased ROS formation and risk of oxidative damage (Thevenod and Lee, 2013). In the kidney, excess cadmium may induce apoptosis or necrosis of tubular cells, interstitial inflammation and fibrosis, reducing renal function and eventually resulting in ESRD (Jarup and Akesson, 2009, Hellstrom et al., 2001, Ferraro et al., 2010). Similar to others (Passlack et al., 2014), we found that kidney tissue cadmium concentration was higher in domestic cats with CIN compared to domestic cats without CIN. In contrast, we found largely similar

cadmium concentrations in dog kidneys with CIN as in those without CIN. Similarly, Passlack et al. (2015) found that kidney tissue cadmium concentrations were relatively unaffected by CKD in dogs. These findings may suggest that cadmium plays a role in the development of CKD in cats, but perhaps not in dogs.

Cadmium excretion from the body is limited (Kjellstrom, 1979), therefore, long-term exposure to cadmium over the animal's lifetime, especially if high, may lead to chronic accumulation of cadmium in the kidney (Thevenod and Lee, 2013, Andreani et al., 2010). Renal cadmium accumulation has been shown to occur primarily within the RPTECs (Thevenod and Lee, 2013). Unsurprisingly, older animals tend to have greater renal tissue concentrations of cadmium, compared to younger animals. This has been shown in cats (Passlack et al., 2014, Andreani et al., 2010), dogs (Passlack et al., 2015, Lopez-Alonso et al., 2007) and humans (Kjellstrom, 1979, Phuc et al., 2017). In our study, cats and dogs with CIN tended to be older, on average, than cats and dogs without CIN. However, while there was little overlap of age between the cats with and without CIN, the dogs with CIN displayed a wide range of ages including very young dogs and old dogs over 10 years of age. In dogs, renal tissue cadmium concentration has been shown to increase up to the age of 10 years old and then decrease thereafter (Passlack et al., 2015). Therefore, age may be a confounding variable contributing towards the lower cadmium concentration observed in dog kidneys with CIN. Moreover, in cats (Passlack et al., 2014), dogs (Passlack et al., 2015, Lopez-Alonso et al., 2007) and humans (Kjellstrom, 1979), kidney cadmium concentrations are generally higher in females than males. Nearly all of the dogs with CIN in the current study were male, which might also explain the lower kidney tissue cadmium concentrations measured in this group of animals. Nonetheless, overall, kidney cadmium content in domestic cats, dogs and Scottish wildcats was similar, suggesting that cadmium exposure and thus the risk of cadmium-induced renal oxidative damage (Ferraro et al., 2010, Hellstrom et al., 2001, Thevenod and Lee, 2013) are similar among domestic cats and dogs and wild-living cats.

To our knowledge, the functions and toxicity of rubidium and strontium in the body are largely unknown (Yang et al., 2019). However, in rats, it has been

shown that increased dietary intake of rubidium results in greater rubidium accumulation in kidney tissue (Yokoi et al., 1996). Therefore, the lower levels of rubidium in domestic cat, compared to dog and Scottish wildcat kidneys might suggest that dietary intake of rubidium is also comparatively low in domestic cats. Furthermore, lower dietary rubidium intake has been found to associate with higher blood urea nitrogen (BUN) in rats (Yokoi et al., 1996). An elevated BUN level, or azotaemia, is often due to considerable renal dysfunction (Jepson et al., 2009) and has been associated with shorter survival time in cats with CKD (Kuwahara et al., 2006, Syme et al., 2006). Therefore, lower rubidium concentration in the diet and, consequently, in the kidney may contribute to renal disease. Moreover, greater rubidium concentration in urine has been suggested to confer preservation of renal function in humans (Yang et al., 2019). Indeed, in our study, urinary rubidium levels were higher in dogs, the species less prone to renal disease, than cats, however, these results were not significant at our stringent level. Furthermore, the presence of CIN did not cause cats or dogs to have reduced rubidium concentrations in their kidneys compared to cats and dogs without CIN. Nonetheless, lower levels of rubidium in the domestic cat's kidney, in comparison to dogs and Scottish wildcats may mean that the domestic cat's kidney is less protected, compared to other domestic species, such as the dog, and to wild-living felids.

In contrast to rubidium-associated renal protection, high plasma concentrations of strontium have been associated with increased risk of renal disease and reduced renal function in humans (van den Berkhof et al., 2018). Similarly, we found that higher strontium concentration in kidney tissue was associated with the presence of CIN, in both domestic cats and dogs. Other studies that have investigated the content of certain mineral elements in the kidney tissue of dogs (Passlack et al., 2015) and cats (Passlack et al., 2014) have also found higher strontium concentration in kidneys with, versus without, CKD, although, in cats, this finding was not significant (Passlack et al., 2014). In our study, kidney tissue strontium concentration was significantly higher in domestic cats than in Scottish wildcats. As strontium is believed to enter the body mainly through ingestion of contaminated food (Melnyk et al., 2019), this may suggest that domestic species are exposed to greater amounts of

strontium through their diet, compared to wildcats. Furthermore, our results suggest a role for strontium in renal disease development in domestic species. Therefore, perhaps the strontium level within cat foods could be reduced, and the rubidium level increased, in order to reduce the risk of renal disease in domestic cats. Further investigations are warranted in order to establish the effects of rubidium and strontium on renal disease development.

2.4.7. Limited urinalysis

A limitation of the current study is that full urinalysis was not completed on any of the samples. Broader urinalysis data could have given a greater insight into the renal, and overall, health of the animals from which samples were obtained (Piech and Wycislo, 2019). Moreover, such data could have explained certain results. For example, higher urinary concentrations of iron, a key component of haemoglobin (Jomova and Valko, 2011), could be explained by the presence of RBCs in the urine sample. Additionally, as arsenic has a high affinity for haemoglobin in RBCs, especially in cats (Buchweitz et al., 2019), the presence of RBCs in urine might also increase urinary arsenic concentrations. Although the finding of higher urinary arsenic concentrations in cats compared to dogs was not statistically significant, it may have been impacted by blood contamination of urine samples. Such contamination would be expected to cause higher arsenic levels in cat, versus dog, urine.

2.4.8. Conclusion

In conclusion, we have shown that the prevalence of chronic inflammation and fibrosis in the renal interstitium is greater in domestic cats than in domestic dogs. For the first time, we included the non-domesticated Scottish wildcat to provide an insight into the effect that domestication, including aspects such as feeding of a commercial pet food, might have on the occurrence of renal disease in cats. CIN was uncommon in our study population of wild-living cats. Reduced age and stress in the Scottish wildcats, compared to domestic cats, and a contribution of genetics may be partially responsible. Furthermore, Scottish wildcats are unlikely to have ever consumed commercial pet food, or

fish, as part of their diet. On the other hand, fish-based diets are often fed to domestic cats, probably more commonly than to pet dogs. Fish-based foods have higher arsenic and mercury content compared to foods that do not contain fish. However, we showed that the arsenic in fish-containing cat foods is predominantly arsenobetaine, which is relatively non-toxic. Therefore, although domestic cats do appear to accumulate higher concentrations of arsenic in their kidneys and urine than dogs and Scottish wildcats, this alone probably does not exert a major effect on the cat's propensity to renal injury. Indeed, greater renal tissue content of pro-oxidant mineral elements, including arsenic and iron, was not associated with CIN. Furthermore, the pro-oxidant heavy metal element, mercury, appears to be more concentrated in dog, compared to cat, kidneys.

On the other hand, our comprehensive analysis of the mineral composition of cat and dog kidneys did show that the concentrations of mineral trace elements with antioxidant functions, such as copper and zinc, or those with other protective functions, for example rubidium, are depleted in domestic cats relative to dogs. Furthermore, reduced copper concentration in the kidney associated with the presence of CIN. Thus, an overall pro-oxidant environment, underpinned by excess pro-oxidant mineral accumulation relative to antioxidant minerals, may be present in the cat's kidney. Such an environment may enhance ROS formation, particularly within the highly metabolically active RPTECs. CKD occurs more commonly with increased age. Therefore, chronic, and particularly lifetime-, exposure to such imbalances of pro- and anti-oxidant mineral elements, establishing a negative redox-state in the kidney, may contribute towards the development of this highly prevalent disease in the domestic cat.

Further studies would be necessary to investigate the effect of excess pro-oxidant mineral accumulation relative to antioxidant minerals on the development of renal lesions and the decline in renal function that are associated with CKD. The animal tissues used in the current study were collected retrospectively at necropsy. Thus, it was not possible to obtain clinical data for these animals to confirm the presence, or assess the severity, of CKD. Prospective recruitment of cats and dogs with and without CKD, based

on known clinical data, would have improved the current study. Tissue sample collection at the time of clinical parameter assessment would also be ideal. However, this would mean collecting renal biopsies, rather than necropsy material. Renal biopsy is possible but is not usually undertaken in cats or dogs; it is contraindicated in some cases of CKD and it would not have provided suffcient tissue for mineral analyses. Therefore, the ethical issues surrounding this would be numerous and it would not have fulfilled the aims of the current project. Necessary future studies might involve controlled prospective feeding trials to establish whether the mineral concentrations found within the kidney reflect those obtained through the diet. Long-term follow-up to determine the effect on the kidney and disease outcome would also be useful. However, such an investigation would have extended beyond the duration of this PhD study and, with a known probable negative outcome, it would also have ethical implications.

Chapter 3. Study 2: Lipid content of domestic cat, dog and Scottish wildcat kidneys and the relationship with CKD

3.1. Introduction

3.1.1. Lipid droplets are a normal finding in the renal tubular cells of cats

In humans and most other mammalian species, renal intracellular lipid deposition is an adverse finding associated with metabolic syndrome and obesity (Bobulescu et al., 2014). It can induce renal damage through lipotoxic processes and may eventually result in CKD (Escasany et al., 2019, Kang et al., 2015, Muller et al., 2019, Izquierdo-Lahuerta et al., 2016). However, in cats, the presence of a large quantity of lipid droplets within the renal tubular epithelial cells is accepted as normal (Lucke, 1968) and not associated with body weight (Modell, 1933). In cats, these intracellular lipid droplets are predominantly found within the proximal convoluted tubules of the renal cortex (Lobban, 1955) but some lipid is also found in the proximal straight tubule (Foote and Grafflin, 1938). Intracellular lipids have also been observed in the RPTECs of wild cats such as lions and tigers (Hewer et al., 1949, Newkirk et al., 2011). Conversely, in a small number of adult dogs, Foote and Grafflin (1938) found that lipid was only in the proximal straight tubule and there was none within the convoluted portion. However, subsequently, Macnider (1945) observed lipid in the proximal convoluted renal tubules of adult dogs, in quantities that increase with age, with young adult dogs under one year of age exhibiting only a sparse, granular density of lipid in these segments of the nephron. Lipid was not found in the proximal convoluted tubule epithelial cells of puppies aged between 6 weeks and 4 months (Macnider, 1945). Thus, in dogs, it has been suggested that the extent of renal intracellular lipid deposition is related to the age of the animal (Macnider, 1945). A similar event is seen in cat kidneys. However, even at birth, kittens already have a few intracellular lipid droplets towards the end of their proximal convoluted tubules and by 10 days old, the lipid is distributed throughout the proximal tubule, with a granular appearance. Once kittens have grown to around half of their adult size, their renal tubule intracellular lipid distribution is similar to that seen in fully-grown

adult cats (Modell, 1933). In contrast to dogs, even foetal cats, whose renal tubules have not yet developed, have particulate lipid material around the corticomedullary junction. This suggests a departure from the normal age-associated development of intracellular lipid in dog kidneys, as lipid appears to be present intracellularly in the kidneys of cats at all ages. In part, the increased kidney lipid content in foetal cats, compared to young dogs, may be due to the fact that the pregnant cat has the greatest renal lipid concentration. In pregnant cats, the lipid is distributed throughout the loop of Henle, as well as extensively in the proximal tubules. In the non-pregnant adult cat, lipid is only occasionally found in the loop of Henle and any droplets here are much smaller than those in pregnant females (Modell, 1933). However, it is unclear why lipid in the kidney of the pregnant cat would spill over into the offspring's kidneys.

3.1.2. What are the lipids in cat kidneys and why are they there?

The identity of the intracellular lipids in the feline kidney is unclear. Many lipids look identical histologically. Haematoxylin and eosin stained kidney tissue sections show lipid droplets as vacuoles due to dissolution of the fat by the alcohol used during the processing of the sections (Modell, 1933). To visualise the lipids, Oil Red O (ORO) stain and haematoxylin counterstain is often used (Martino-Costa et al., 2017). Alternatively, non-specific fat colourants, such as Scarlet Red, Sudan III, Sudan Black or Nile Blue, can be used (Lobban, 1955, Koopman et al., 2001). Although certain stains may have a preference for certain lipid classes (Lobban, 1955, Koopman et al., 2001), it is not possible to determine a single specific species of lipid contained within the cytoplasmic droplets by staining (Mehlem et al., 2013). Moreover, it is unknown whether larger vacuoles represent lipid droplets containing several lipid types or whether they have formed through amalgamation of several smaller lipid droplets within the cells of the renal tubules (McLeland et al., 2015).

It has been postulated that the lipid in feline kidneys originates from that which is circulating in the blood (Mottram, 1916). It may therefore have a dietary origin. Indeed, Mottram (1916) showed that the fatty acids in cats' kidneys were more saturated than those stored in adipose tissue and the liver. The cats in

that study consumed cow's milk, which also contains saturated fatty acids, thus Mottram (1916) concluded that these dietary fatty acids may enter the kidney as blood is filtered. Furthermore, rodents fed a high fat diet have been shown to accumulate more lipid within their kidneys compared to rodents fed a normal, lower fat diet (Muller et al., 2019, Decleves et al., 2014). However, others have reported that the amount of lipid within the renal tubular epithelial cells is unrelated to dietary composition in cats (Modell, 1933, Bargmann et al., 1977) and dogs (Macnider, 1945). Studies in healthy cats have shown that the fatty acid content of blood in the renal artery is greater than that in the renal vein (Bargmann et al., 1977). This might suggest that circulating lipid that reaches the kidney becomes stored within the renal tissue, is used for metabolic processes, and/or is excreted. Thus, the intracellular lipid deposits in the cat's kidney may be present physiologically, rather than due to degeneration (Bargmann et al., 1977). Indeed, lipid droplets have been identified within the lumina of the renal tubules along the entire length of the nephron in tigers (Hewer et al., 1949) and in domestic cats, especially in pregnant female cats, where intracellular lipid droplets are most abundant (Modell, 1933). The presence of lipid within tubule lumina suggests that lipid is excreted in the urine, probably after secretion from the tubular epithelial cells (Hewer et al., 1949).

Histochemical experiments have revealed the presence of cholesterol, triacylglycerides and phospholipids in the lipid droplets within cats' RPTECs (Bargmann et al., 1977). It was suggested that cholesterol is taken up into the RPTECs by pinocytosis of the glomerular filtrate, as a means of recycling this compound (Bargmann et al., 1977). Thus, cholesterol in the kidneys may have a dietary origin. In earlier histochemical experiments on the cat's kidney, the reaction that would be expected to occur if cholesterol or even its androgen esters had been present, did not. Instead, it was suggested that, while the intracellular lipids in the kidney are probably partly steroidal in nature, they are more likely related to endogenous oestrogens than to androgens or cholesterol alone (Lobban, 1955). This conclusion was based upon early findings that renal lipid content is greatest in female cats during pregnancy (Modell, 1933), but is also high during the luteal phase of oestrus (Lobban, 1955).

Furthermore, in male cats, renal lipid content increases after castration or when sexual activity is lost in older age (Lobban, 1955). In contrast, Martino-Costa et al. (2017) concluded that interstitial lipid deposition, which arises from the degeneration of tubular epithelial cells, affects male and female, neutered and non-neutered cats equally. Therefore, they suggested that renal interstitial lipid deposition is not determined by reproductive hormones. However, they observed greater deposition of lipid in the interstitium, more commonly in female, than in male, cats, which conflicts their conclusion (Martino-Costa et al., 2017). It seems that female cats may have greater deposition of lipid within their RPTECs, which leads to greater accumulation of lipid in the interstitium if the tubules rupture. Thus, hormones, such as oestrogen, may influence renal lipid accumulation.

3.1.3. All lipids are not created equally

There are many different classes of lipids, which each have different biological roles. For example, triacylglycerides are considered a storage form of lipid (Bobulescu et al., 2014) and phospholipids are a structural component of biological membranes (Galván, 2018). Lipotoxicity is often attributed to other lipid classes such as diacylglycerides and ceramides (Guebre-Egziabher et al., 2013) or to free, or non-esterified, fatty acids (Schaffer, 2003). Each lipid class has a different general structure, which may comprise a different number of fatty acids. Fatty acids, even within a lipid class or within a single lipid molecule, can also vary in structure due to chain length, linearity and saturation, which consequently determines the specific properties of the compound lipid (Galván, 2018). They can be short (<5 carbons), medium (6-12 carbons), long (13-19 carbons), or very long chain (≥20 carbons); straight or branched chain (e.g. a methyl-, or other alkyl-, group is attached to the fatty acid carbon chain); saturated (no double bonds), monounsaturated (one double bond) or polyunsaturated (>1 double bond) fatty acids. Polyunsaturated fatty acids (PUFAs) are further categorised as omega (Ω) 3 or Ω6 PUFAs, where the number relates to the position of the double bond at the end of the chain farthest away from the carboxyl group (formula R-COOH, where R is an alkyl group/chain) (Sassa and Kihara, 2014). Additionally, the structure and

properties of different lipid compounds are determined by the bonding of fatty acids within the molecule. For example, in glycerolipids (lipids containing a glycerol moiety), such as the acylglycerides (mono-, di- and tri-acylglycerides), glycerophospholipids and ether lipids, the component fatty acids may be linked to the glycerol backbone by an ester bond, making them an acyl chain, or an ether bond, making them an alkyl chain. The nature of ester, versus ether, bonds can affect lipid properties, including solubility and membrane fluidity and permeability (Guler et al., 2009, Balleza et al., 2014).

Lipotoxicity in cardiomyopathy and in diabetic pancreatic dysfunction has been attributed to long chain fatty acids and a greater degree of carbon saturation in the fatty acid chains (Khan et al., 2018, Finck et al., 2003, Riedel et al., 2006, Riedel and Light, 2005). Evidence for this in regard to the kidney is limited but increased plasma levels of saturated, long-chain (C16-C20) fatty acids have been measured in humans with advanced renal disease, compared to those in earlier stages of CKD (Afshinnia et al., 2018). If lipids in circulation accumulate in the kidney, it is possible, therefore, that saturated long chain fatty acids in the kidney may be involved in CKD progression. On the other hand, short chain fatty acids (C2-C4) may inhibit oxidative stress and inflammation in the kidneys, although this effect is likely to be concentration-dependent and requires further investigation (Huang et al., 2017). Certain PUFAs are essential to health. These include the Ω-3 PUFAs, α-linolenic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are obtained through fish and fish oil in the diet (Gai et al., 2019); and Ω-6 PUFAs, such as arachidonic acid, an essential nutrient for cats that is abundant in animal tissues (MacDonald et al., 1984). Omega-3 fatty acids have roles in immune, inflammatory and metabolic pathways, signal transduction, cell membrane formation, blood pressure regulation and reducing hypertriglyceridaemia by downregulating lipid synthesis and upregulating β-oxidation (Davidson, 2006, Gai et al., 2019). Indeed, Ω-3 fatty acid supplementation in the diet of nephrectomised rats, an experimental model of CKD, has been found to improve tubulointerstitial damage and fibrosis in the remnant kidney (An et al., 2009). Additionally, Ω-3 fatty acid supplementation is able to reduce oxidative stress and inflammation in damaged blood vessels

in CKD (Zanetti et al., 2017). In fact, it is widely accepted that normal or high levels of Ω-3 PUFAs are protective to kidney function and reduce the risk of CKD progression (Turolo et al., 2018).

Both Ω-3 and Ω-6 PUFAs appear to mediate most of their effects through their metabolites. As well as essential roles, Ω-3 and Ω-6 PUFAs can have detrimental effects in the body, which may relate to the particular metabolites that are acting (Turolo et al., 2018, Jung et al., 2010). For example, Ω-6 PUFAs have a role in health, maintaining normal glomerular permeability and blood flow within the kidney (Williams et al., 2007). Higher dietary intake of Ω-6 PUFAs has been associated with reduced risks of CKD (Yuzbashian et al., 2015) and progression to ESRD (Malhotra et al., 2016). However, during disease or at lower levels, metabolites of Ω-6 PUFAs can exert pro-inflammatory effects, may cause vasoconstriction, reduce the selectivity of the glomerular filtration barrier and contribute towards CKD progression (Wiktorowska-Owczarek et al., 2015, Turolo et al., 2018). The ratio between Ω-3 and Ω-6 PUFAs, as well as the absolute levels of each of these fatty acids, is important in order to strike a balance between their anti- and pro-inflammatory effects, in favour of health rather than disease (Turolo et al., 2018, Wiktorowska-Owczarek et al., 2015). It is clear that, in order to establish a role for the high intracellular lipid content in the feline kidney, particularly in relation to renal disease development, the full lipid composition of these lipid droplets, including the lipid molecular classes and the component fatty acids, should be studied. To our knowledge, such a study has not been conducted previously.

3.1.4. Aims and hypotheses of Study 2

The presence of lipid within cells of non-adipose tissues is usually associated with obesity and/or disease development through a mechanism of lipotoxicity (Bobulescu et al., 2014, Bobulescu, 2010). Yet, in healthy cats, even from a young age, the lipid content of the kidney is deemed to be abnormally high for a mammal (Lobban, 1955). Despite a high prevalence of CKD in domestic cats, an association between the high intracellular lipid content of the kidney and feline CKD has not been investigated. However, intracellular lipid released into

the tubulointerstitium when renal tubules degenerate in the diseased or aged kidney may trigger, or contribute to, the hallmark interstitial inflammation of feline CKD (Lucke, 1968, Martino-Costa et al., 2017). The increased content of lipid within the feline kidney, compared to other species, may therefore increase the risk of progressive renal injury and contribute to the relatively higher prevalence of CKD in domestic cats.

The role of intracellular lipid droplets within the kidneys remains to be established. In other species and other tissues, intracellular lipid droplets have been shown to contain predominantly triacylglycerides, thus, in the kidney, they may serve as a store of energy substrate for the high metabolic activity of the renal tubular epithelial cells (Escasany et al., 2019, Bobulescu, 2010). Limited studies have reported that the intracellular lipid droplets in the cat's kidney contain triacylglycerides, phospholipids and cholesterol (Bargmann et al., 1977). However, the full composition and proportions of lipids within these intracellular lipid droplets in cats has not been determined. We aim to add to the existing, relatively scant data on feline renal intracellular lipids, to increase the knowledge surrounding this relatively under-investigated, but potentially important normal physiological and/or pathological feature of the feline kidney. We propose that the high content and composition of lipids within the feline kidney creates an environment that predisposes cats to renal injury and CKD.

Aims

1) In this chapter, I will quantify the renal lipid content of domestic cats, dogs and Scottish wildcats.
2) Alongside the evaluation of CIN in these kidneys as per the analyses conducted in Study 1 of this thesis, I aim to identify whether an association between the amount of lipid and the presence of lesions in the kidney exists.
3) A primary aim of this study is to elucidate the types of lipids that are present in the intracellular lipid droplets in domestic cat, dog and Scottish wildcat kidneys, to establish whether there are differences between different species and between domestic and wild-living felids,

which might underpin the increased prevalence of CKD in domestic cats.

Hypotheses

1) Domestic cat kidneys contain more lipid than the kidneys of another domesticated species, the dog, and of a non-domesticated feline subspecies, the Scottish wildcat (*to address Aim 1*).
2) The lipid content of the domestic cat's kidney is increased during renal disease (*Aim 2*).
3) The composition of intracellular lipids within the domestic cat's kidney differs from that within the kidneys of dogs and Scottish wildcats, in a manner that contributes to the common occurrence of CKD in domestic cats (*Aim 3).*

3.2. Materials and Methods

3.2.1. Tissue collection

Kidney tissue from domestic cats and dogs and Scottish wildcats was collected as described in Study 1 (Section 2.2.2). All kidneys were stored at -80°C upon collection, from 1 month to 2 years until processing for this study.

3.2.2. Histological evaluation of renal lipids

3.2.2.1. Haematoxylin and eosin staining

Kidney tissue from domestic cats, dogs and Scottish wildcats was prepared and stained with H&E as described for Study 1 (Sections 2.2.3.1 and 2.2.3.2), then viewed using a light-microscope (20x objective). Tag Image File Format (TIFF) images were obtained using a Nikon Digital Sight DS-U1 camera and computer software. A visual assessment to compare the presence of vacuoles, indicating lipid droplets, in domestic cat, dog and Scottish wildcat kidneys was conducted.

3.2.2.2. Oil Red O staining

Kidneys from n=21 domestic cats, n=22 dogs and n=8 Scottish wildcats were used in this study. Sections of frozen kidney tissue containing cortex, with or without medulla, were cut at 10 µm using a microtome at -20°C. Sections were mounted onto glass polylysine slides before staining with Oil Red O (ORO) according to standard protocols in the pathology department at The University of Nottingham. In brief, sections were immersed in 1% ORO in 70% industrial methylated spirit (IMS) for 30 minutes, rinsed in 30% IMS, washed in running tap water, rinsed in distilled water, then counterstained in haematoxylin for 30 seconds and washed in water until the sections were blue. Sections were finally mounted with aqueous mountant and a coverslip was applied. To check that the staining method would positively stain lipids and allow them to be identified, sections of adipose tissue (n=3), leftover from a previous study not associated with this project, were mounted onto slides and stained with ORO as described.

3.2.2.3. Lipid quantification

Under the light-microscope and 20x objective and using Nikon Digital Sight DS-U1 camera and computer software, TIFF images of ten random fields of view of the cortex of each kidney section from each species, and the adipose tissue sections, were taken. At this stage, the researcher was blinded to all information (e.g. species, sex, neutered status, age, CIN score) about the animal from which each tissue section came. Randomisation was achieved by selecting ten areas, roughly evenly spaced, working from left to right of the cortex in the image. Image J computer software was used to quantify the coverage of each of the ten images from each tissue section with lipid. The area positively stained red with ORO was calculated, together with the total area of the image that was tissue. The percentage of ORO positive (ORO^{+ve}) tissue was then calculated as a proportion of the total area. Hue, saturation and brightness parameters were adjusted to identify the red area correctly. This was done first on an adipose tissue section and then the same parameters were applied to every image. The mean percentage red area was calculated from all ten images of each tissue section and used as an estimation of the proportion of the kidney cortex in that section, that contained lipid.

3.2.3. Lipid identification

After lipid quantification, to qualitatively evaluate the kidney intracellular lipids, we first isolated lipids specifically from the cytoplasm (Section 3.2.3.1), then purified samples further by extracting all lipids from non-lipid substances, producing total lipid extracts (TLEs; Section 3.2.3.2). Lipid classes within TLEs were then separated into bands, according to biochemical properties, by thin layer chromatography (TLC; Section 3.2.3.3). Over time, three separate total lipid extractions were undertaken and slightly altered to produce more concentrated TLEs to improve upon the clarity of the separated bands on the TLC plates from the previous set of TLEs. Experiment 1 (Section 3.2.3.2.1) used TLEs from domestic cat (n=12), dog (n=12) and Scottish wildcat (n=6) kidneys without CIN. For experiment 2 (Section 3.2.3.2.2), total lipid was extracted again from the same n=12 domestic cat kidneys used in experiment 1, to verify the presence of an additional band of lipid on TLC that appeared to

be present in the domestic cat samples. TLEs from healthy domestic cat (n=1), dog (n=1) and Scottish wildcat (n=1), extracted by the experiment 1 procedure, were also used for a preliminary analysis with ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS; Section 3.2.3.5). This confirmed differences between domestic cats, dogs and Scottish wildcats. Experiment 3 (Section 3.2.3.2.3) generated kidney TLEs from domestic cats with CIN (n=14), and again from n=2 healthy dogs and n=2 Scottish wildcats used previously in experiment 1, for TLC and UPLC-MS/MS, to establish whether domestic cat kidneys with CIN also contained the additional lipid class that was found in healthy cat kidneys. Lipid bands were scraped (Section 3.2.3.4) from the TLC plates from experiment 3 and analysed by UPLC-MS/MS to aid identification of the additional lipid molecular class in domestic cat, versus dog and Scottish wildcat, kidneys. Scraped lipid bands and TLEs from experiment 3 were also transmethylated (Section 3.2.3.6), to enable gas chromatography (GC) identification of the fatty acid components within each lipid molecular class and compare the overall intracellular fatty acid profiles between domestic cats, dogs and Scottish wildcats. Gas chromatography-mass spectrometry (GC-MS; Section 3.2.3.7) was used first, to guide identification of fatty acids on the gas chromatography-flame ionisation detector (GC-FID; Section 3.2.3.8) profiles. Therefore, GC-MS methods are reported but data not shown.

3.2.3.1. Isolation of kidney cytoplasmic lipids

A 1 litre stock solution of microsomes isolation buffer containing 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 11.92 g), 10 mM potassium chloride (0.746 g), 62.5 mM potassium acetate (6.14 g), 5 mM EGTA (ethylene-bis(oxyethylenenitrilo)tetracetic acid; 1.91 g), 5 mM DTT (1,4-dithiothreitol; 0.772 g), 1 mM magnesium chloride (0.0952 g) and 5 mM EDTA (ethylenediamine tetraacetic acid; 1.86 g) was prepared. The pH was adjusted to 7.5 with potassium hydroxide (KOH). Samples of kidney tissue (~1 g), were homogenised on ice (~4°C), in 2 ml of extraction buffer (0.6 M protease-free sucrose in isolation buffer plus 1% w/v bovine serum albumin, BSA), using the gentleMACS^{TM} Dissociator. The homogenate was then centrifuged at 3000 g

at 4°C for 5 minutes to remove cell debris. A sucrose step gradient was prepared in a 5 ml polyallomer tube by overlaying 1 ml of the extraction buffer containing 0.6 M sucrose with the top 2 ml layer of the centrifuged homogenate, then 2 ml of 0.25 M protease-free sucrose in isolation buffer plus 1% w/v BSA on top. During the whole process, everything was kept close to 4°C as much as possible. The layered samples were centrifuged at 100,000 g, at 4°C, for 1 hour in a P55ST2 rotor (Hitachi CP80NX ultracentrifuge), forming a turbid layer of cytoplasmic lipid at the top of the polyallomer tube, indicated in Figure 18. This was collected using a Pasteur pipette and stored at -20°C until required for further analysis at Rothamsted Research Institute.

Figure 18. Cytoplasmic lipid isolation from kidney tissue.

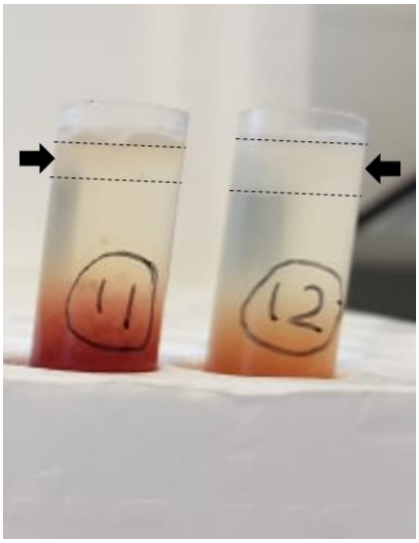


Figure 18. Cytoplasmic lipid was isolated from homogenised kidney tissue samples using a sucrose concentration gradient and centrifugation at 4°C. The resultant layer of cytoplasmic lipid (areas outlined with black dashed lines and indicated by black arrows) was collected using a Pasteur pipette and frozen for further lipid extraction for chromatography analyses.

3.2.3.2. Total lipid extraction

3.2.3.2.1. Experiment 1

Isolated renal cytoplasmic lipid samples from 12 dogs, 12 domestic cats and 6 Scottish wildcats, all without renal disease, were further purified (Table 7 shows animal details, including breed, age, sex, neutered- and CIN-status). In brief, 2 ml of 2:1 (v/v) chloroform:methanol was added to 1 ml of each sample which was subsequently vortexed twice for 15 seconds, before adding 500 µl of 1% sodium chloride (NaCl), vortexing again for 15 seconds until homogenised and centrifuging at 1000 g for 2 minutes. Two layers could then be visualised and the lower organic phase, comprising the sample lipids, was collected with a Pasteur pipette and transferred into a new vial. Another 2 ml of 100% chloroform was added to the sample in the original vial, which was then vortexed twice for 15 seconds and centrifuged at 1000 g for 2 minutes, resulting in separation of the contents of the vial into two layers. Again, the lower layer was transferred, using a Pasteur pipette, into the second vial and then its contents were dried under nitrogen gas. Once dried, the lipid samples were re-suspended in 100 µl 100% chloroform. This extraction method was repeated to obtain TLEs of the same samples for UPLC-MS/MS analysis.

3.2.3.2.2. Experiment 2

Total lipid was extracted again from isolated renal cytoplasmic lipid samples (Section 3.2.3.1) from the same 12 healthy domestic cats used in experiment 1, which had been stored frozen since. The extraction method used here was the same as in experiment 1 but volumes of 2:1 chloroform:methanol, isolated lipid samples and NaCl were increased 2-fold, while the final volumes of 100% chloroform were unchanged, to produce TLEs that were two times more concentrated than the TLEs produced in experiment 1.

3.2.3.2.3. Experiment 3

In this experiment, the lipid extraction method used previously in experiments 1 and 2, was adapted to follow Folch's method more closely and achieve a total lipid extraction (Folch et al., 1957). Using a Gilson pipette, 1 ml of isolated

cytoplasmic lipid samples from the kidneys of 14 domestic cats with CIN, as well as two healthy dogs and two Scottish wildcats previously used in experiment 1 (Table 7 details breed, age, sex, neutered-status and disease characteristics), was transferred into glass tubes. 5 ml of 2:1 (v/v) chloroform:methanol was added to each sample. The mixture was vortexed twice for 15 seconds, then 1 ml of 1% NaCl solution was added. The mixture was vortexed again until homogenised, then centrifuged at 1000 g for 2 minutes, separating the mixture into two layers. The bottom fraction was transferred to a glass vial using a Pasteur pipette. The solution remaining in the original tube was re-extracted with 3 ml of 100% chloroform, then vortexed twice for 15 seconds and centrifuged at 1000 g for 2 minutes. Again, good separation was achieved and the bottom fraction was added into the glass vial. The resultant TLE samples were dried under nitrogen at 35°C, then re-suspended in 200 µl of 100% chloroform. Each TLE sample was split into a 150 µl portion and a 50 µl portion. The 150 µl portion was used for TLC. The 50 µl portion was diluted with 40 µl of 100% chloroform, centrifuged and further split into 30 µl and 60 µl portions for UPLC and GC analyses, respectively.

Exp. 1 TLC position	Exp. 2 TLC position	Exp. 3 TLC position	Species	Breed	CIN	Age (months)	Sex	Neutered
1			Dog	Labradoodle	N	11	M	Y
2	**2**		Cat	DSH	N	9	M	Y
3			Dog	Labrador	N	12	F	N
4	**8**		Cat	DSH	N	17	F	Y
5			Wildcat		N			
6			Dog	English Pointer	N	84	M	Y
7	**4**		Cat	DSH	N	108	M	Y
8			Dog	Golden Retriever	N	32	M	Y
9	**3**		Cat	DSH	N	36	M	Y
10			Wildcat		N			
11			Dog	Cocker Spaniel	N	117	F	Y
12	**5**		Cat	DLH	N	126	F	Y
13			Dog	German Spitz	N	112	F	Y
14	**7**		Cat	DSH	N	134	F	Y
15		**18**	Wildcat		N			
16			Dog	Labrador	N	60	M	Y
17	**11**		Cat	DSH	N	60	M	Y
18		**1**	Dog	Jack Russel Terrier cross	N	132	M	Y
19	**9**		Cat	DSH	N	154	M	Y
20			Wildcat		N			
21		**10**	Dog	Greyhound	N	89	M	Y
22	**1**		Cat	DSH	N	17	F	Y
23			Dog	Crossbreed	N	72	M	Y
24	**12**		Cat	DLH	N	97	M	Y
25			Wildcat		N			
26			Dog	Yorkshire Terrier	N	100	F	Y
27	**10**		Cat	DSH	N	103	F	Y
28			Dog	Scottish Terrier cross	N	34	F	Y
29	**6**		Cat	DSH	N	27	F	Y
30		**9**	Wildcat		N			
		2	Cat	DSH	Y	252	F	Y
		3	Cat	Birman	Y	216	F	Y
		4	Cat	Burmilla	Y	192	M	Y
		5	Cat	DSH	Y	180	M	Y
		6	Cat	Persian	Y	159	F	Y
		7	Cat	DSH	Y	139	M	N
		8	Cat	Bengal	Y	191	F	Y
		11	Cat	Oriental shorthair	Y	107	M	Y
		12	Cat	British shorthair	Y	48	M	Y
		13	Cat	DSH	Y	101	M	Y
		14	Cat	Maine coon	Y	136	F	Y
		15	Cat	DLH	Y	189	F	Y
		16	Cat	DSH	Y	122	M	Y
		17	Cat	DSH	Y	97	M	Y

Table 7. Characteristics (breed, age, sex, neutered status, CIN status) of the domestic cats, dogs and Scottish wildcats from which renal lipid was extracted. The position of each animal's total lipid extraction sample on the thin layer chromatography plate for each experiment (Exp.) is also detailed. Key: DSH; domestic shorthair; DLH, domestic longhair; M, male; F, female; Y, yes; N, no.

3.2.3.3. Thin layer chromatography

Silica plates were left in lidded glass boxes containing 70:30:1 (v/v/v) hexane: diethyl ether: acetic acid until completely coated in the solvent by capillary action, then left to dry. Using a Hamilton syringe, TLEs (experiment 1, 2: 100 µl; experiment 3: 150 µl) were loaded in several layers onto the solvent-soaked silica plates, 3 cm up from the bottom, in an area labelled appropriately for the corresponding sample. Each layer was dried rapidly under nitrogen before the next was applied. In the same way, standards were loaded onto the silica plates to run alongside the renal lipid samples. In experiment 1, standards of aldehydes, ketones, hydroxyl-fatty acids (FAOH), free fatty acids (FA), triacylglycerides (TAGs), diacylglycerides (DAGs), free sterols (FS) and sterol esters (SE), individually and combined, were used. In experiment 2, one standard solution of DAGs, FA, TAGs and alkanes, and one standard solution containing FS, FA, fatty acid methyl esters (FAMEs) and SE were used. In experiment 3, a single standard solution containing DAGs, TAGs, FS and SE was loaded in the same way at both the left and right sides of the silica plate to run alongside the renal TLE samples. Seven TLC plates were prepared in experiment 1, alternating healthy dog, domestic cat and Scottish wildcat samples. Two plates were prepared in experiment 2, which used TLEs from only healthy domestic cat kidneys. In experiment 3, two TLC plates were prepared with one dog and one Scottish wildcat sample included on each, alongside 7 samples from domestic cats with CIN.

Meanwhile, chromatography paper was left in the lidded glass box containing 200 ml of 80:20:1 (v/v/v) hexane: diethyl ether: acetic acid solvent. Once the solvent had diffused to the top of the paper, the loaded silica plate was placed into the box, ensuring that the solvent did not splash above the sample loading line. The lid was replaced and weighted down to increase the vapour pressure in the tank to improve and accelerate solvent migration at the top of the plate, where, otherwise, the solvent would evaporate faster than it migrates. When the solvent had diffused up to ~1 cm from the top, the plate was removed from the box, left to dry at room temperature, then sprayed with 10 mg/l (0.0001%) primuline yellow dye in 80:20 (v/v) acetone: H_2O and dried under nitrogen. Bands of different lipid molecular classes separated according to biochemical

properties, essentially solubility in the solvent, were then visualised under ultraviolet light and photographs were taken. Bands of lipids observed in the samples could be matched up with bands of standards at the same level on the plate, allowing tentative identification of the molecular classes of lipids present in the different samples. On the TLC plates from experiment 3, bands of polar lipids, SE, TAGs and an unknown band above the TAG band, for all samples; and the FA band from the two dogs, the two Scottish wildcats and four of the domestic cats with CIN, were outlined in pencil. All traced bands were subsequently scraped from the plates and collected into individual, appropriately labelled, glass tubes.

3.2.3.4. Extracting lipids from scraped silica bands

Lipid was extracted from the silica of the scraped TLC bands from experiment 3, by adding 1 ml of 100% chloroform to the tubes containing the bands, shaking, then heating at 35°C in an oven for 20 minutes. Using a Pasteur pipette, the chloroform was transferred to a new, labelled vial, avoiding the silica settled at the bottom of the tube. A second 1 ml of 100% chloroform was added to the original tube containing the silica, which was then shaken. Again, the chloroform, but not the silica, was transferred to the new vial, using a Pasteur pipette. The 2 ml of chloroform in the vial was then dried under nitrogen, leaving just the extracted lipid from the scraped band. This lipid was then re-suspended in 90 µl of 100% chloroform and centrifuged. The resultant 90 µl samples were subsequently split into 30 µl and 60 µl portions for UPLC and GC analyses, respectively.

3.2.3.5. Ultra-performance liquid chromatography-tandem mass spectrometry

Lipid molecular species composition were analysed by high resolution/ accurate mass (HR/AM) lipidomics using a Vanquish–Q Exactive Plus UPLC-MS/MS system (Thermo Fisher Scientific) at Rothamsted Research Institute. A preliminary UPLC-MS/MS analysis of TLEs from one healthy domestic cat, one dog and one Scottish wildcat from experiment 1 (Section 3.2.3.2.1) directed further analysis of all TLEs (Section 3.2.3.2.3) and prepared samples

of TLC bands A, B, C, D and FA (Section 3.2.3.4), from experiment 3. Any fine particulate silica matter remaining in the TLC band samples (30 µl) was first removed by adding 1 ml of 100% chloroform, filtering the entire volume through a 0.45 µm non-sterile syringe-driven filter unit with a hydrophobic fluoropore™ (PTFE) membrane (Millex®), drying under nitrogen, then re-suspending in 50 µl of 100% chloroform. Samples were then transferred into appropriately labelled 300 µl fixed insert vials (Thermo Scientific™ Chromacol™). A 20 µl aliquot of each sample was injected into the UPLC-MS/MS system. A blank was added after every 4–5 samples. Lipids were separated on an Accucore C18 column (2.1 x 150 mm, 2.6 mm; Thermo Fisher Scientific) at 35°C with autosampler tray temperature at 10°C and 400 µl/minute flow rate. The mobile phase used in the column was A= 10 mM ammonium formate in 50% acetonitrile + 0.1% formic acid and B= 2 mM ammonium formate in acetonitrile/propan-2-ol/water (10/88/2) + 0.02% formic acid. The elution gradient ran for 28 minutes from 35% B at the start to 100% at 24 minutes. The Thermo Q Exactive heated electrospray ionisation (HESI II) probe was used in position C to accommodate the relatively high flow rate and maximise the LC-MS assay sensitivity. Conditions were adjusted for separate positive and negative runs. Running samples in a single polarity enabled identification of a greater number of molecular species. LC/MS full scan at a mass resolution of 140,000 and data-independent higher energy collisional dissociation tandem mass spectra (HCD MS2) experiments (35,000 mass resolution) were performed in positive and negative ion modes. The top 15 most abundant peaks resulting from the LC/MS full scan for *m/z* 150-1200 were used for fragmentation. The HCD MS2 experiment used stepped collision energies, 25, 30, 40 eV; replacing 25 with 30 eV in negative ion mode. Settings were as follows: sheath gas 60, Aux gas 20, sweep gas 1, spray voltage 3.2 KV in positive ion mode, with small adjustments in negative ion mode, capillary temperature 320°C and aux gas heater 370°C. Lipidsearch 4.2 (Thermo Fisher Scientific) experimental workflow was used for lipid characterisation and potential lipid species were identified separately from positive or negative ion adducts. The data for each replicate were aligned within a chromatographic time window by combining the positive and negative ion annotations and merging these into a single lipid annotation. The UPLC-MS/MS methods and

workflow, developed at Rothamsted Research Institute and as followed here but applied to an alternative project, has recently been published (West et al., 2020).

Detectable lipids in the samples were represented by peaks on UPLC total ion chromatograms (TIC). Where major differences were noted (retention time ~12–18 minutes) between the TICs of domestic cat, dog and Scottish wildcat TLEs from experiment 1 and preliminary UPLC analysis, the mass spectra were inspected. The m/z of fragment ions were used to elucidate potential structures of the lipid compounds that were responsible for the peaks that were relatively abundant within this area in the domestic cat, versus dog and Scottish wildcat samples. The UPLC-MS/MS data from three domestic cat and one Scottish wildcat TLEs from experiment 3 were excluded as numerous lipids within these datasets were undetectable, while levels of the same lipids in the other samples from the particular species were much higher. The percentage of each individual lipid in the total amount of lipid was calculated for each domestic cat (n=2), dog (n=1) and Scottish wildcat (n=1) TLE sample from experiment 3, for which the dataset was retained. The sum of individual lipids within each lipid structural class (ceramides, diacylglycerides, lysophospholipids, glycerophospholipids, sphingomyelin and triacylglycerides) was calculated and the percentage of the total lipid in each sample that was made up of each lipid class was determined.

3.2.3.6. Transmethylation of samples for gas chromatography

Transmethylation is a technique generally used to extract fatty acyl chains from biological samples or lipid fractions and convert them into FAMEs which are less polar, more volatile and therefore more suitable for analysis by GC. TLEs and scraped TLC band samples (60 µl) from experiment 3 were dried under nitrogen. Next, 1 ml of transmethylation mixture, comprising 70% methanol; 28% toluene, to increase the solubility of the fatty acids in the transmethylation mixture; 2% concentrated sulphuric acid (v/v/v); and 2,2-dimethoxypropane (2,2-DMP), used as a water quencher, thus shifting the reaction towards completion, was added. The methylated sample was heated at 85°C for 1 hour to speed up the reaction, then 1 ml of 1% NaCl and 1 ml of heptane were

added, shaking well after each addition. This caused separation of the mixture into two layers. The top layer was transferred into a GC vial using a Pasteur pipette, then dried under nitrogen. Using a Pasteur pipette, two drops of heptane were added to the dried sample, used to wash the sides of the vial, then the heptane-re-suspended sample was transferred to another GC vial with a glass insert, allowing analysis of small sample volumes.

3.2.3.7. Gas chromatography-mass spectrometry

A complete set (TLEs and individual scratched TLC lipid bands: A, polar lipids; B, TAGs; C, unknown lipid; D, sterol esters; FA, fatty acids) of representative domestic cat with CIN, dog and Scottish wildcat samples, all from experiment 3 and subsequently transmethylated, were run on the GC-MS. The GC-MS used helium carrier gas and a HP-1 column. Fatty acid molecules were separated largely based on their volatility, as well as according to their interactions with the particular non-polar column. Roughly, fatty acids with higher boiling points come off the column later, when the column oven temperature is higher. Thus, unsaturated fatty acids eluted before saturated fatty acids with the same carbon chain length in this analysis. After GC separation of the different fatty acids, they passed into the mass spectrometer, where electrons from an electron ionisation source fragmented them into ions. All major peaks, representing fatty acid molecules, on each GC-MS profile were selected in turn and their fragmentation patterns were studied, to identify the particular fatty acid molecule. The positions of the peaks on the chromatogram, compared to known compounds whose MS spectra allowed unequivocal identification, and the mass-to-charge ratio (m/z) of the molecular ions, gave an indication of the size and structure of the compounds.

3.2.3.8. Gas chromatography-flame ionisation detector analysis of fatty acid methyl esters

All TLEs and scraped TLC bands from domestic cat with CIN (n=14), dog (n=2) and Scottish wildcat (n=2) transmethylated samples from experiment 3, were analysed using GC-FID. Each sample (1.0 µl) was injected into the GC-FID, which used helium as the carrier gas in an Agilent J&W DB-23 column (length

30m, inner diameter 0.25mm, film/stationary phase thickness 0.25µm), specifically designed for the high-quality separation of FAMEs. The DB-23 column (50%-Cyanopropyl)-methylpolysiloxane matrix has a high polarity, therefore separation of unsaturated fatty acids was improved using GC-FID, versus GC-MS (Section 3.2.3.7). Compounds were initially separated based on boiling point; then on their interaction with the stationary phase, which is determined by chemical and physical properties. Hence, more polar (unsaturated and branched chain) molecules interact more strongly with the stationary phase and come off the column later than saturated molecules with the same carbon number, as higher temperatures are required to desorb them from the column. The initial oven temperature, set at 70°C, was held for 1 minute before increasing at a rate of 10°C per minute, reaching 240°C, which was held for a further 2 minutes.

For data analysis, first, the samples were assigned to one of two batches based on the average concentration of lipids detected in the samples (Batch 1, low concentration, ~≤ 500 pA; Batch 2, high concentration, ~>500 pA). Batch is a function of the Agilent HPCHEM GC software that allows automated peak integration, annotation using a calibration table, and data extraction. Using retention times of known fatty acid molecules in straight chain saturated (C12:0–C22:0) and polyunsaturated (fish oil) fatty acid standards and data extracted from the GC-MS analysis of cat and dog samples (Section 3.2.3.7), known fatty acids (e.g. C12:0, C14:0, C16:0…etc.) were identified on the GC-FID chromatograms of TLE samples (example shown in Appendix 1) and were added to a calibration table with their retention times. Major peaks corresponding to lipids that could not be identified were labelled as unknown peaks (UP) and added to the calibration table with their retention times. The calibration table was applied to all samples within the batch, then each sample was manually checked to ensure that peaks were identified correctly. The same procedure was carried out for the other batch of samples.

The data obtained were the areas under each lipid peak on the chromatograms. Although the weights of all tissue samples from which lipid was first isolated (Section 3.2.3.1) were approximately equal, internal standards were not included throughout the methods, so loss of tissue lipid

during extraction could not be calculated. Therefore, the data were not absolutely quantitative. To enable comparisons between samples, the peak areas for individual fatty acids were converted to percentages of the total area of all peaks in the sample. The mean percentage of each fatty acid in each sample was calculated for domestic cats (n=14, total lipid, polar lipid, triacylglyceride, unknown lipid band and sterol samples; n=3 for fatty acid samples), dogs (n=2, all samples) and Scottish wildcats (n=2, all samples). Graphs of each species' average percentage fatty acid composition for total lipid, polar lipid, fatty acid, triacylglyceride, unknown lipid band and sterol samples are presented in this thesis (Figure 32) as a concise presentation of the data. However, as all individual samples within each species group come from individual animals with different specifications (e.g. age, sex, disease severity etc.), there may be large variation in fatty acid compositions within groups. Therefore, graphs of the peak areas and percentage areas of identified known fatty acids and unknown fatty acids in all individual domestic cat, dog and Scottish wildcat samples are presented separately in the appendices for completeness (known fatty acids [C12:0, C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, C18:3n3, C18:3n6, C18:4, C20:0, C20:1, C20:2, C20:3, C20:5]: peak areas, appendix 2; percentage areas, appendix 3; unknown fatty acids: peak areas, appendix 4; percentage areas, appendix 5).

3.2.4. Statistical analysis

General ANOVA (GenStat v19; VSNi Ltd., Rothamsted, UK) was used to statistically evaluate the effect of species (domestic cat, dog and Scottish wildcat) on the amount of ORO^{+ve} staining. Since ten random fields of view of each section from a particular animal were obtained, 'Animal ID' was used as a blocking factor if all images were used in the analysis. Residual plots, generated to assess the applicability of the model, were improved when using the percentage Red area of all ten images with blocking (% Red Area; Animal ID as a blocking factor) compared to using the mean % Red Area of the ten images for each tissue section (Figure 19). A Bonferroni post-hoc multiple comparisons test assessed which groups (domestic cat, dog, Scottish wildcat) were significantly different at $P<0.05$.

Due to missing covariate data for Scottish wildcats (e.g. age and sex were unknown; all were likely entire as opposed to neutered), Scottish wildcat data were excluded when comparing domestic cat and dog data, with these variables included as covariates, to determine any influence of covariates on ORO^{+ve} staining. Additionally, CIN (Yes/No) was included as a fixed effect with Species x CIN as a pre-specified interaction. After checking the residuals, data were log_{10}-transformed to normalise the non-normal distribution prior to statistical analysis. The percentage of ORO^{+ve} tissue was plotted against the percentage of CIN occupying the kidney tissue and, on a separate graph, against the age of the cat or dog to which the tissue section belonged. Two, separate linear regression analyses were conducted on these data. Due to occasional missing information (age, neutered status) for some domestic cats and dogs and unbalanced datasets (e.g. varying n-numbers), REML linear mixed models were used in preference to ANOVA, so that estimation of the significance of fixed effects would not be skewed nor data lost inappropriately. Sex information was known for all domestic cats and dogs so ANOVA was used in this case.

Figure 19. Distribution and residual checks.

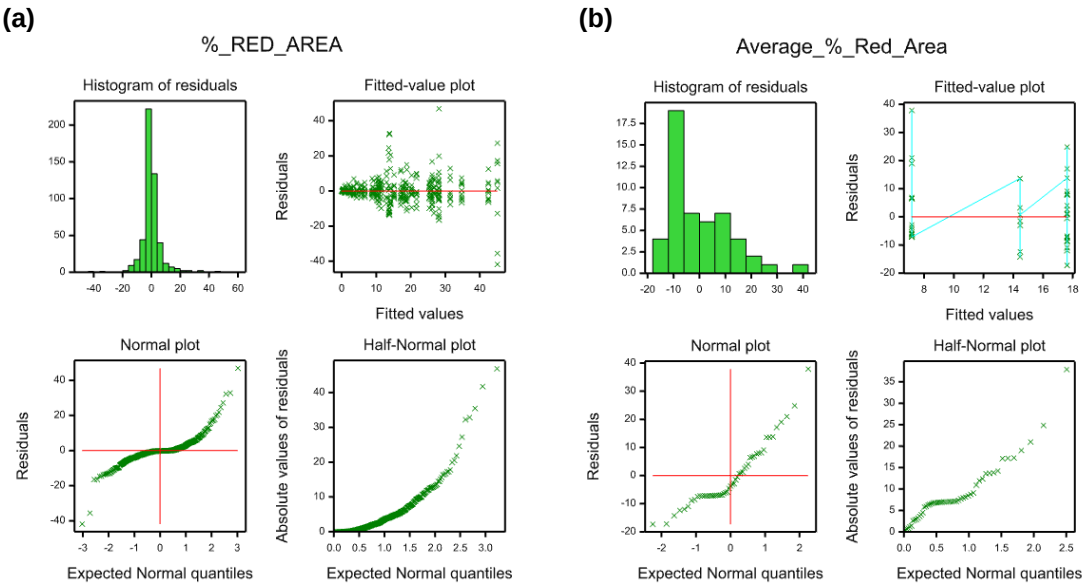


Figure 19. Residual plots from running **(a)** Percentage of tissue section stained red in each of ten fields of view of each kidney (%_RED_AREA) versus species, with 'Animal ID' used as a blocking factor **(b)** The percentage of each kidney tissue section stained red as an average of the staining across ten fields of view (Average_%_Red_Area) versus species, showing that model **(a)** was more appropriate for statistical analysis than **(b)**.

3.3. Results

3.3.1. Amount of lipid in domestic cat, dog and Scottish wildcat kidney tissue

Numerous vacuoles were visualised in domestic cat and Scottish wildcat, but not in dog, H&E-stained kidney tissue sections (Figure 20). The proportion of kidney tissue that stained positively with ORO and thus deemed to contain lipid was significantly (P=0.018) greater in domestic cats (n=21; median [IQR]: 18.48 [9.069, 25.66] %) than in dogs (n=22; 1.002 [0.0917, 13.63] %). The lipid content of Scottish wildcat kidneys (n=8; 14.09 [6.756, 22.90] %) was not statistically different from that of domestic cat kidneys. Compared to all species' kidney sections, adipose tissue (n=3) stained more extensively with ORO (54.76 [53.18, 63.92] %), as expected (Figure 21). Representative examples of ORO-stained sections from each species and from the adipose tissue positive control are shown in Figure 22.

In both dogs and domestic cats, the amount of lipid in kidneys with CIN was significantly lower than in kidneys without CIN (P=0.032; Species*CIN interaction, P=0.158; Figure 23a). The percentage of kidney tissue stained positively with ORO decreased by roughly the same amount in both domestic cats and dogs (Cat vs. Dog, negative gradients, P=0.353; linear regression analysis), with increasing percentage of mononuclear inflammatory cell infiltration in the renal interstitium (% CIN). The intercepts of the line of best fit for cats and dogs were significantly different (P=0.002) however, suggesting that, on average, with the same amount of renal interstitial inflammation, cats have more fat in their kidneys than dogs (Figure 23b).

In addition, as cats and dogs age, it is well established that they lose functional renal mass, a phenomenon which is termed cellular senescence. Here, we show that, on average, cats and dogs also lose a proportion of lipid droplets in renal cells, consistent with the loss of, primarily, RPTECs. A linear regression analysis showed that the amount of lipid in the kidney decreases by roughly the same amount with increasing age in both domestic cats and dogs (cat vs. dog, negative gradients, P=0.8182). The intercepts of the lines of best fit for cats and dogs were highly significantly different (P=0.0008), suggesting that,

on average, cats have more fat in their kidneys than dogs at all ages studied (Figure 24).

Figure 20. Haematoxylin and eosin stained kidney tissue sections from dog, domestic cat and Scottish wildcat.

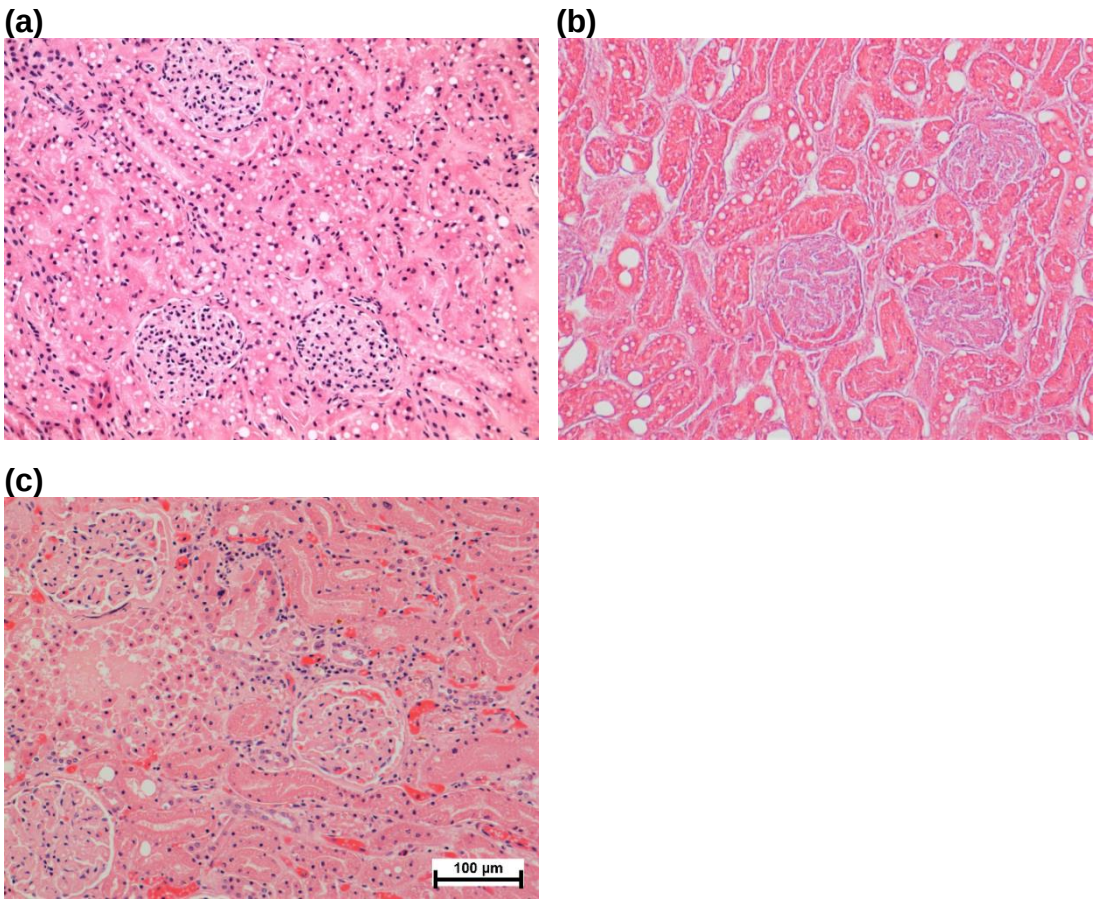


Figure 20. Representative images (all at 20x resolution) of **(a)** dog, **(b)** domestic cat and **(c)** Scottish wildcat kidney tissue sections (5 µm) stained with haematoxylin and eosin (H&E). H&E-stained kidney tissue sections were viewed using a light-microscope (20x objective). Tag Image File Format images were obtained using Nikon Digital Sight DS-U1 camera and computer software. White vacuoles represent lipid droplets, which have been dissolved during tissue processing.

Figure 21. Proportion of dog, domestic cat and Scottish wildcat kidney tissue and adipose tissue stained positively with Oil Red O.

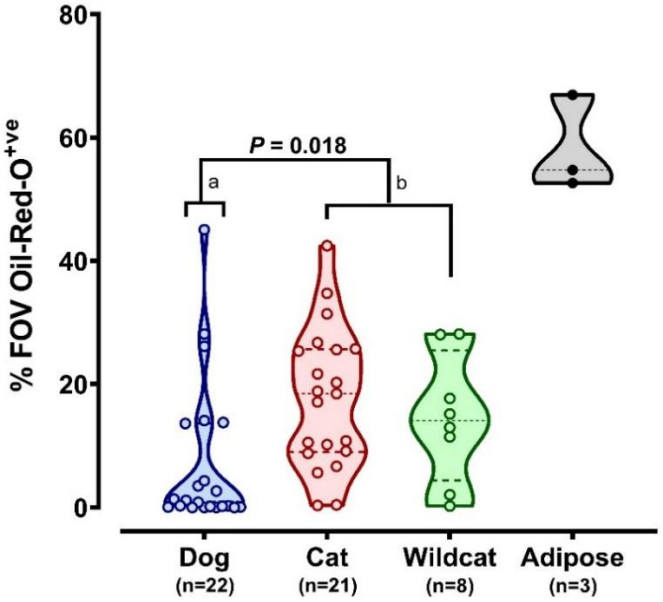


Figure 21. Violin plot showing all data points (represented as circles) for average percentage of ten random fields of view stained positively with Oil Red O, for dog, domestic cat and Scottish wildcat kidneys and adipose tissue positive control. The shape of the plots shows the distribution of the full range of data. Dashed lines represent the lower and upper quartiles and the median. Bonferroni multiple comparisons test showed that lipid content of cat and wildcat kidneys was similar (b) but was significantly greater than that of dogs (a; P=0.018, ANOVA).

Figure 22. Oil Red O-stained dog, domestic cat and Scottish wildcat kidney tissue and adipose tissue.

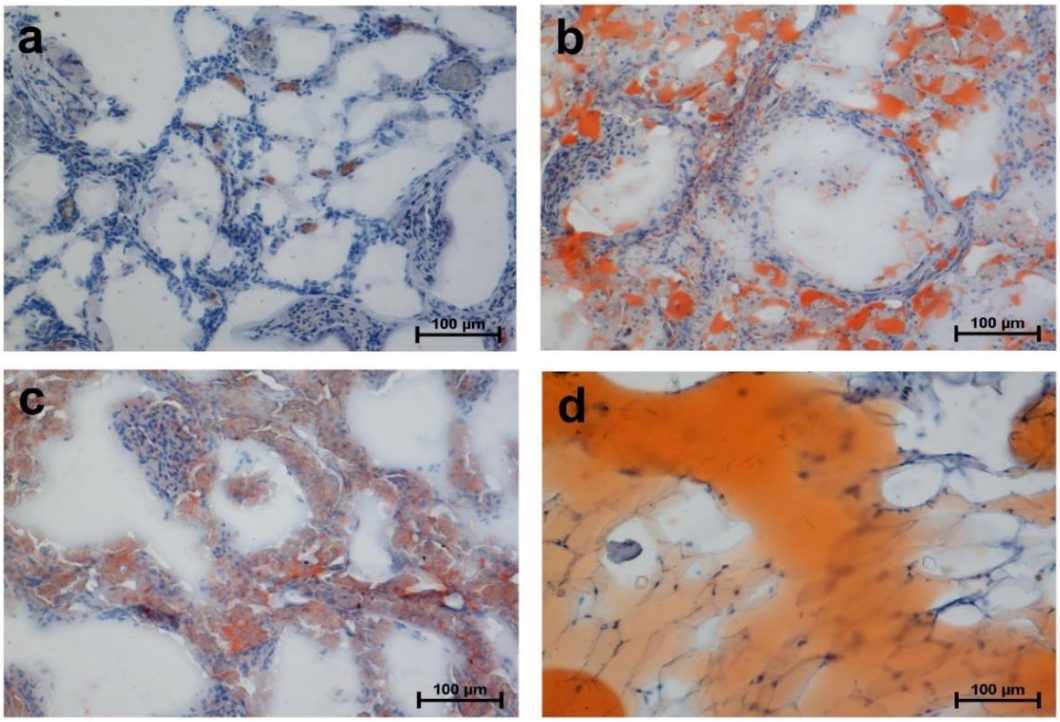


Figure 22. Representative images of Oil Red O-stained kidney tissue from **(a)** dog, **(b)** domestic cat and **(c)** Scottish wildcat and **(d)** adipose tissue positive control. Images were taken with a Nikon Digital Sight DS-U1 camera and computer software when tissue sections were viewed under the 20x objective of the light microscope.

Figure 23. Relationship between chronic interstitial nephritis (CIN) and the proportion of kidney tissue stained positively with Oil Red O in dogs and domestic cats.

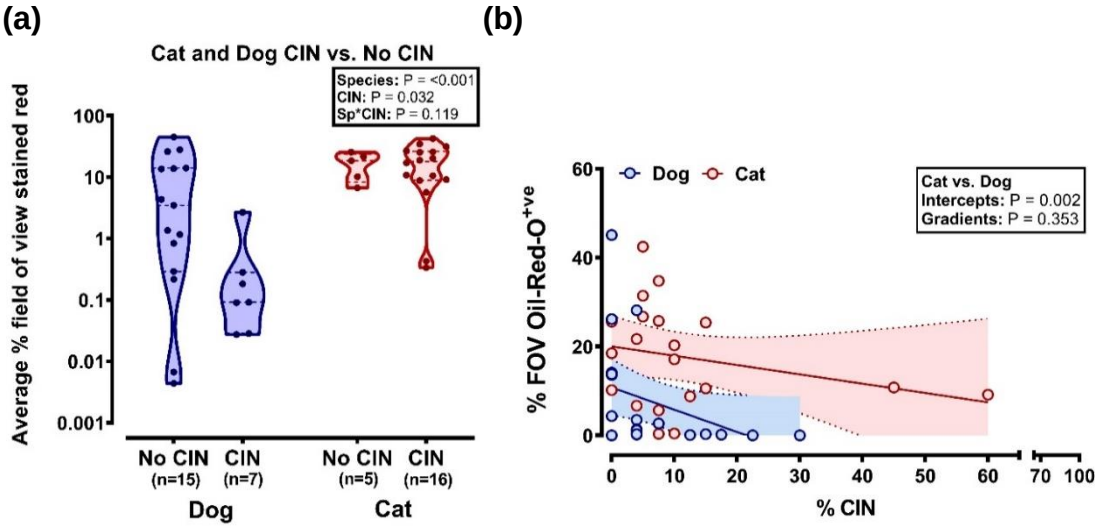


Figure 23. (a) Violin plot showing all data points (represented as circles) for average percentage of ten random fields of view stained positively with Oil Red O (ORO), for dog and domestic cat kidneys, either with or without CIN. The shape of the plots shows the distribution of the full range of data. Dashed lines represent the lower and upper quartiles and the median. Data were log_{10}-transformed to normalise the distribution and then analysed by a linear mixed model. Data are presented on a logarithmic scale. **(b)** Scatter graph showing the relationship between the average percentage of 10 random fields of view of kidney cortex stained positively with ORO and the percentage of kidney tissue occupied by CIN, in cats and dogs. Solid lines are lines of best fit. The dotted lines indicate the 95% confidence interval around the mean. A linear regression analysis was conducted on the data. Statistical significance was accepted at P<0.05.

Figure 24. Proportion of kidney tissue stained positively with Oil Red O in dogs and domestic cats of a range of ages.

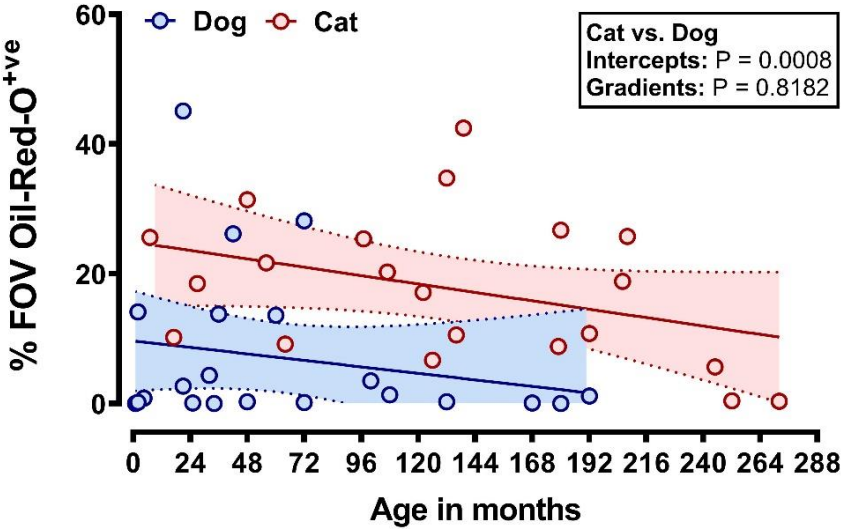


Figure 24. Scatter graph showing the relationship between the average percentage of ten random fields of view of kidney cortex stained positively with Oil Red O (represented as circles) and age in months of cats and dogs. Solid lines are the lines of best fit. Dotted lines indicate the 95% confidence interval around the mean for age. A linear regression analysis was conducted on the data and statistical significance was accepted at P<0.05.

In general, the amount of lipid (ORO^{+ve} stained tissue) was greater in males than females (mean ± standard deviation: 15.83 ± 14.32 % vs. 7.30 ± 8.06 %, respectively; P=0.006) in both domestic cats and dogs (Species*Sex interaction; P=0.374; Figure 25). Further sub-group analyses, albeit with reduced numbers per group, showed that mean kidney lipid content was greater in both dogs and cats that were entire compared to those that had been neutered (mean ± standard deviation, dog: 10.06 ± 15.26 % vs. 4.63 ± 8.33 %; cat: 42.47 ± 0 % vs. 17.46 ± 10.06 %). However this effect was not significant, probably due to only n=1 entire cat (Neutered [Yes/No], P=0.072; Species*Neutered interaction, P=0.115; Figure 26).

Figure 25. Proportion of kidney tissue stained positively with Oil Red O in male vs. female dogs and domestic cats.

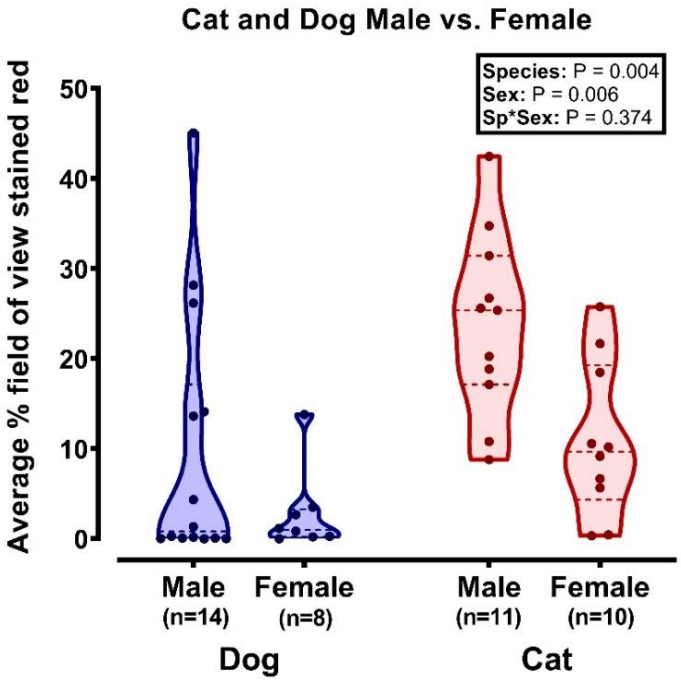


Figure 25. Violin plot showing all data points (represented as circles) for average percentage of ten random fields of view of kidney tissue stained positively with Oil Red O, in dogs and domestic cats, split according to sex (male/female). The shape of the plots shows the distribution of the full range of data. Dashed lines represent the lower and upper quartiles and the median. Statistical significance was tested by ANOVA and accepted at P<0.05.

Figure 26. Proportion of Oil Red O-positive-stained kidney tissue in neutered and entire dogs and domestic cats.

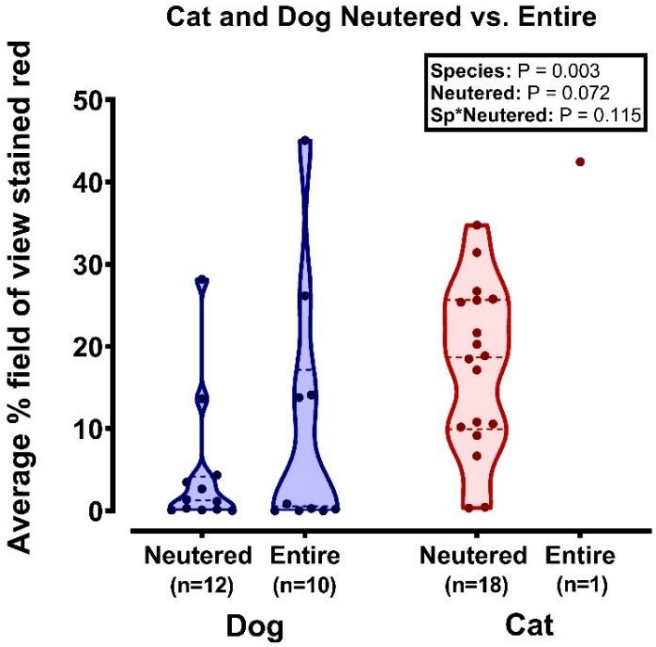


Figure 26. Violin plot showing all data points (represented as circles) for average percentage of ten random fields of view of kidney tissue stained positively with Oil Red O, in dogs and domestic cats, split according to neutered status (neutered/entire). The shape of the plots shows the distribution of the full range of data. Dashed lines represent the lower and upper quartiles and the median. Data suggest that lipid content of kidneys is greater in entire, versus neutered, cats and dogs, although more samples would be needed to be able to make this conclusion.

3.3.2. Thin-layer chromatography revealed an additional band of lipid in cats but not dogs

A lipid band that did not align with any of the lipid standards run in the thin-layer chromatography (TLC) experiments was identified in all of the domestic cat kidney samples (healthy cats, Figure 27 and Figure 28; cats with CIN, Figure 29) and some of the Scottish wildcats but in none of the dog samples (Figure 27 and Figure 29). This band of unidentified lipid eluted up the TLC plate, just above the TAG band. The ratio of the amount of lipid in the TAG band, to the amount of lipid in the additional unidentified band appeared to be inverted in the Scottish wildcat compared to domestic cat samples (Figure 29).

Figure 27. Separation of neutrolipids from healthy dog, domestic cat and Scottish wildcat kidney intracellular total lipid extracts.

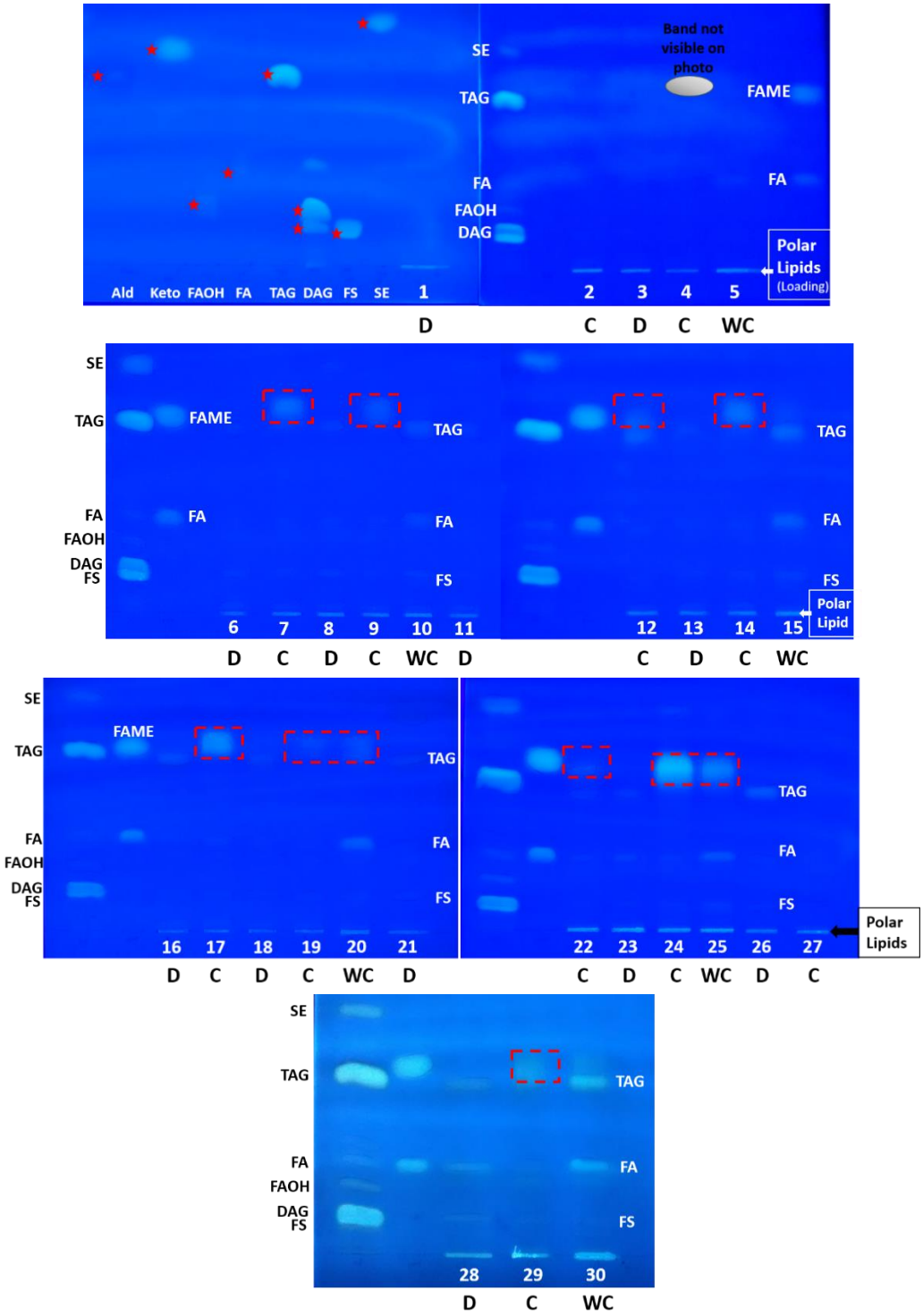


Figure 27. Total lipid extracts from healthy kidneys of 12 dogs (**D**; 1, 3, 6, 8, 11, 13, 16, 18, 21, 23, 26, 28), 12 domestic cats (**C**; 2, 4, 7, 9, 12, 14, 17, 19, 22, 24, 27, 29) and six Scottish wildcats (**WC**; 5, 10, 15, 20, 25, 30) were loaded onto silica thin layer chromatography plates. Neutrolipids were separated based on solubility in the solvent (hexane: diethyl ether: acetic acid, 80:20:1, v/v/v). Standards of aldehydes (Ald), ketones (Keto), hydroxyl-fatty acids (FAOH), free fatty acids (FA), fatty acid methyl esters (FAME), triacylglycerides (TAG), diacylglycerides (DAG), free sterols (FS) and sterol esters (SE) were also loaded (individually and combined) onto the silica plate and run alongside the renal lipid samples. Bands of lipids were visualised under ultraviolet light after plates were sprayed with primuline yellow dye in 80:20 (v/v) acetone: H_2O and dried under nitrogen. An additional band of lipid (outlined by red dashed box), which did not align with lipid standards, was identified in most domestic cat and some Scottish wildcat samples. Red stars and annotations on the plates indicate locations of lipid bands.

Figure 28. Separation of neutrolipids from healthy domestic cat kidney total lipid extracts.

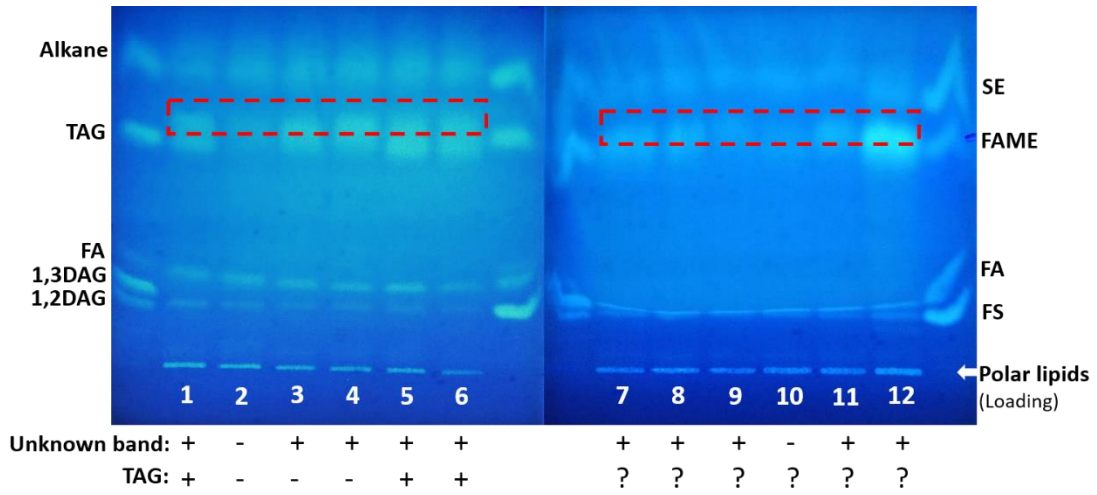


Figure 28. Total lipid extracts from 12 healthy domestic cat kidneys (1-12) were loaded onto silica thin layer chromatography plates. Neutrolipids were separated based on size and solubility in the solvent (hexane: diethyl ether: acetic acid, 80:20:1, v/v/v). One standard solution of diacylglycerides (DAG), free fatty acids (FA), triacylglycerides (TAG) and alkanes, and one standard containing free sterols (FS), free fatty acids (FA), fatty acid methyl esters (FAMEs) and sterol esters (SE) were loaded on each plate, to run alongside the renal lipid samples. Bands of lipids were visualised under ultraviolet light after plates were sprayed with primuline yellow dye in 80:20 (v/v) acetone: H_2O and dried under nitrogen. Presence (+), absence (-) or ambiguous detectability (?) of TAG and unknown lipid bands in each sample are indicated below the image.

Figure 29. Separation of neutrolipids from kidney total lipid extracts from domestic cats with CIN, and healthy dogs and Scottish wildcats.

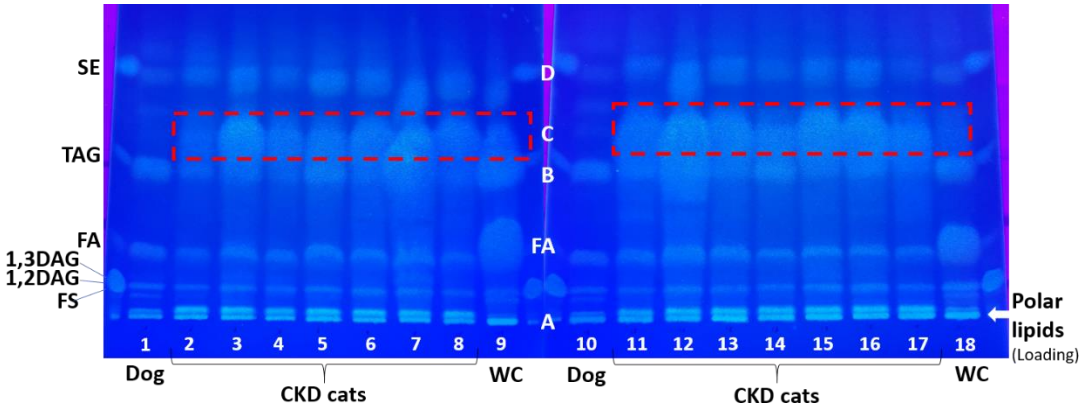


Figure 29. Total lipid extracts from kidneys from 14 domestic cat with CIN (CKD cats; 2-8, 11-17), two healthy dogs (1, 10) and two healthy Scottish wildcats (WC; 9, 18) were loaded onto silica thin layer chromatography plates. Neutrolipids were separated based on size and solubility in the solvent (hexane: diethyl ether: acetic acid, 80:20:1, v/v/v). A standard solution of free sterols (FS), diacylglycerides (DAG), free fatty acids (FA), triacylglycerides (TAG) and sterol esters (SE) was loaded on each plate, to run alongside the renal lipid samples. Bands of lipids were visualised under ultraviolet light after plates were sprayed with primuline yellow dye in 80:20 (v/v) acetone: H_2O and dried under nitrogen. An additional band of lipid (band C; outlined by red dashed box), which did not align with any of the lipid standards, was observed in domestic cat and Scottish wildcat, but not dog, samples. Bands of lipids at the levels marked A (polar lipids), B (triacylglycerides), C (unknown lipid band), D (sterol esters) and FA (free fatty acids) were subsequently traced and scratched from the plate.

3.3.3. UPLC-MS/MS analysis of total lipids in domestic cats versus dogs and Scottish wildcats

The quality of the data obtained through UPLC-MS/MS analysis of bands of lipids separated by TLC and extracted from the silica plate, was too poor to enable further identification of the lipids in these samples. Data from TLEs from two domestic cats, one dog and one Scottish wildcat from experiment 3 was of adequate quality to be used. Most of the lipid in domestic cat samples was triacylglycerides, while in dogs and Scottish wildcats, the predominant lipid class was glycerophospholipids. Triacylglycerides constituted a greater proportion of the total amount of lipid in domestic cat kidney samples, compared to dog and Scottish wildcat kidneys. The proportions of ceramides, diacylglycerides, phospholipids (lyso- and glycero-phospholipids) were lower in total lipid samples from domestic cat kidneys, compared to dog and Scottish wildcat kidneys (Figure 30). The analytical software did not distinguish between TAGs and monoalkyldiacylglycerols (MADAGs). However, preliminary analysis of the raw data from the UPLC-MS/MS analysis of one healthy domestic cat, one dog and one Scottish wildcat kidney TLE from experiment 1, revealed additional lipid peaks, with m/z ~700–800, between retention times of 12 to 18 minutes on the UPLC-MS/MS profiles from the healthy domestic cat, that were not present in the dog or Scottish wildcat samples. The fragmentation patterns of the ions in the mass spectra, based on m/z data, showed that the domestic cat kidney, but not dog or Scottish wildcat kidneys, contained MADAGs as well as a relative abundance of triacylglycerides (Figure 31).

Figure 30. The proportions of ceramides, diacylglycerides, lysophospholipids, glycerophospholipids, sphingomyelins and triacylglycerides in aliquots of total lipid extracts from domestic cat, dog and Scottish wildcat kidneys.

Lipid class	The total amount of lipids within a class, as a proportion of the total lipid in the sample (%)			Fold difference		
	Dog (n=1)	Scottish wildcat (n=1)	Domestic cat (n=2)	Wildcat vs. dog	Cat vs. dog	Cat vs. wildcat
Ceramides	0.832	2.115	0.199	2.544**	0.239*	0.094*
Diacylglycerides	4.935	2.183	0.281	0.442*	0.057*	0.129*
Lysophospholipids	3.837	4.236	3.570	1.104	0.931	0.843
Glycerophospholipids	63.12	51.78	19.59	0.820	0.310*	0.378*
Sphingomyelins	0.391	0.715	0.129	1.830**	0.331*	0.181*
Triacylglycerides	26.89	38.97	76.24	1.449**	2.835**	1.956**

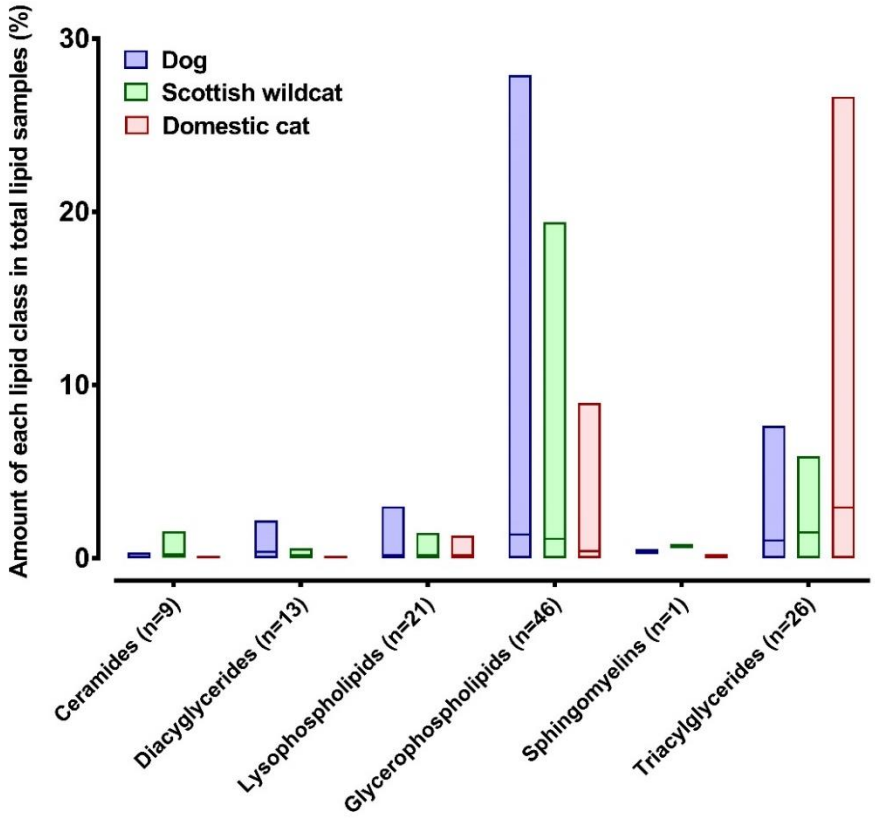


Figure 30. Kidney cytoplasmic lipids were isolated from homogenised kidney tissue using a sucrose concentration gradient and HEPES microsomal isolation buffer. Total lipid was extracted from isolated lipid samples using 2:1 (v/v) chloroform:methanol and 1% sodium chloride. Total lipid extracts (20µl) were analysed by UPLC-MS/MS in positive and negative ion modes. Lipids were characterised using Lipidsearch 4.2. Lipid species that were detected and identified were grouped into lipid classes (ceramides, diacylglycerides, lysophospholipids, glycerophospholipids, sphingomyelins and triacylglycerides). Peak area data was used to calculate the proportion of each lipid class within the total lipid detected in each domestic cat (n=2), dog (n=1) and Scottish wildcat (n=1) sample. Data are presented in a table and graph format. The fold difference in the total amount of each lipid class as a proportion of the total lipid in the sample was calculated for Scottish wildcat vs. dog, domestic cat vs. dog, and domestic cat vs. Scottish wildcat. Asterisks indicate a considerably lower (*) or higher (**) amount of the particular lipid class in the former, compared to the latter, species listed in the fold-difference column subheading of the table.

Figure 31. Preliminary UPLC-MS/MS analysis of lipid extracted from healthy domestic cat, dog and Scottish wildcat kidneys.

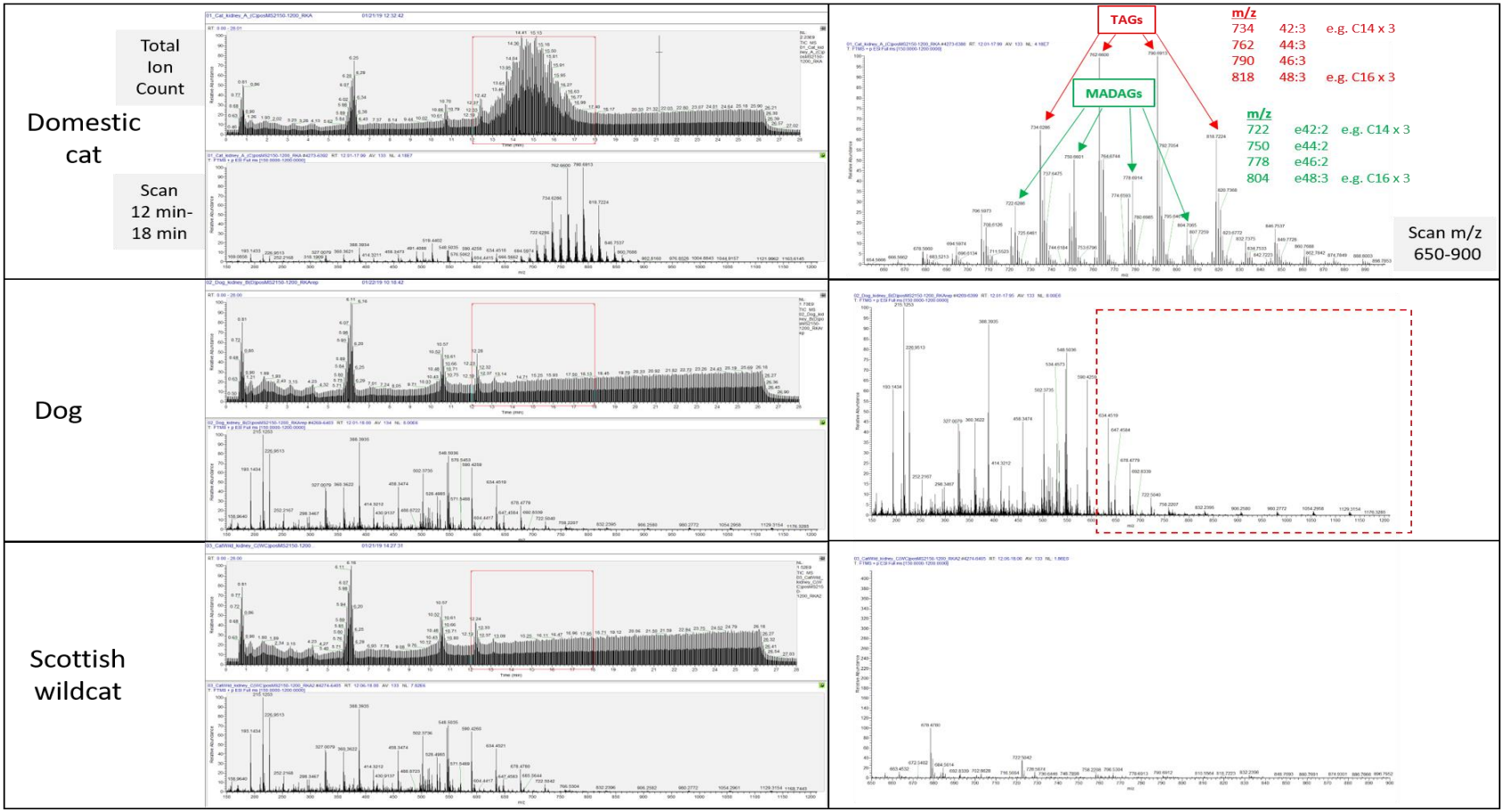


Figure 31. Preliminary UPLC-MS/MS analysis of intracellular lipid extracted from kidneys of one domestic cat, one dog and one Scottish wildcat, all without evidence of renal disease (CIN), from experiment 1. The UPLC-MS/MS was run with a reverse phase column in positive ion mode. The left panel shows a total ion chromatogram (TIC) above the mass spectrum of the 12-18 minute scan area outlined by a red dashed line on the TIC, for domestic cat, dog and Scottish wildcat. The panel to the right contains a zoom (scan area: m/z 650-900) of the mass spectra shown in the left panel, for the domestic cat, dog and Scottish wildcat. Peaks with the highest relative abundance, that were present on the domestic cat MS spectrum but were absent from the dog and Scottish wildcat MS spectra, were determined to represent triacylglycerides (TAGs) and monoalkyldiacylglycerols (MADAGs), based on m/z. The most likely TAG and MADAG series (number of carbons: number of double bonds) that the peaks could represent, based on m/z data, are detailed in red and green text, respectively, on the domestic cat MS spectrum zoom in the right hand panel.

3.3.4. GC-FID analysis of fatty acid methyl esters

On the whole, the profiles of identifiable known fatty acids (e.g. C12:0, C14:0, C16:0 etc.; Table 8), as percentages of the total amount of all fatty acids detected in the samples, did not show major differences between domestic cats, dogs and Scottish wildcats (Figure 32). However, shorter chain known fatty acids, especially C12:0, were more abundant in the domestic cat samples (Figure 32a) and were particularly concentrated in domestic cat 'unknown lipid band' (TLC band C) samples (Figure 32e; Appendix 3a,e). The known fatty acids, especially longer chain fatty acids (e.g. C16:0, C18:0, C18:1, C18:2), were particularly abundant in Scottish wildcat samples; mostly in the triacylglyceride (TAG) and fatty acid samples (Figure 32c,d; Appendix 2a,c,d). In domestic cats, the known fatty acids were most abundant in 'unknown lipid band' (Figure 32e) samples.

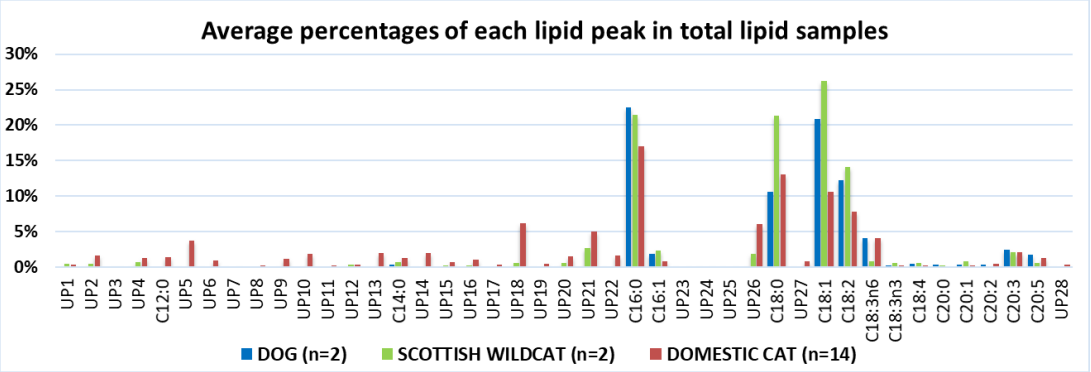
Twenty-eight peaks corresponding to unknown fatty acids were detected in the GC-FID profiles. The peaks of most of these unidentifiable fatty acids eluted at retention times between those of C12:0 and C14:0 (n=9; UP5-13) or between C14:0 and C16:0 (n=9; UP14-22). Most of these were found primarily in the domestic cat samples (Figure 32a) and were especially concentrated in the 'unknown lipid band' samples (Figure 32e; Appendix 4a,e). Other peaks corresponding to unknown fatty acids eluted before C12:0 (n=4; UP1-4), between C16:1 and C18:0 (n=4; UP23-26), between C18:0 and C18:1 (n=1; UP27) or after C20:5 (n=1; UP28).

Formula	Name	Chain length	Saturation
C12:0	Lauric acid	Medium	Saturated
C14:0	Myristic acid	Long	Saturated
C16:0	Palmitic acid	Long	Saturated
C16:1	Palmitoleic acid	Long	Monounsaturated
C18:0	Stearic acid	Long	Saturated
C18:1	Oleic acid	Long	Monounsaturated
C18:2	Linoleic acid	Long	Polyunsaturated
C18:3n6	Omega-6 gamma linolenic acid	Long	Polyunsaturated
C18:3n3	Omega-3 alpha linolenic acid	Long	Polyunsaturated
C18:4	Stearidonic acid	Long	Polyunsaturated
C20:0	Arachidic acid	Very long	Saturated
C20:1	Eicosenoic acid	Very long	Monounsaturated
C20:2	Eicosadienoic acid	Very long	Polyunsaturated
C20:3	Eicosatrienoic acid	Very long	Polyunsaturated
C20:5	Eicosapentaenoic acid	Very long	Polyunsaturated

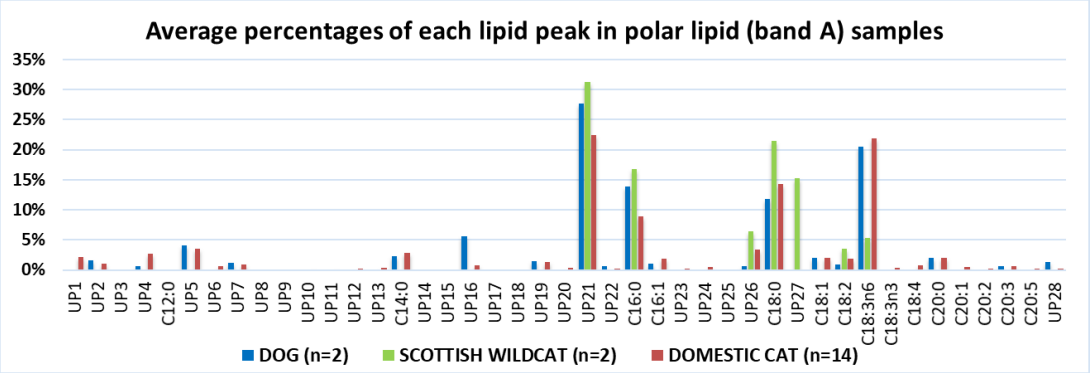
Table 8. The names of the known fatty acids that were detected in domestic cat, dog and Scottish wildcat kidney lipid samples analysed using GC-FID.

Figure 32. Fatty acid profiles of dog, Scottish wildcat and domestic cat kidney intracellular lipids.

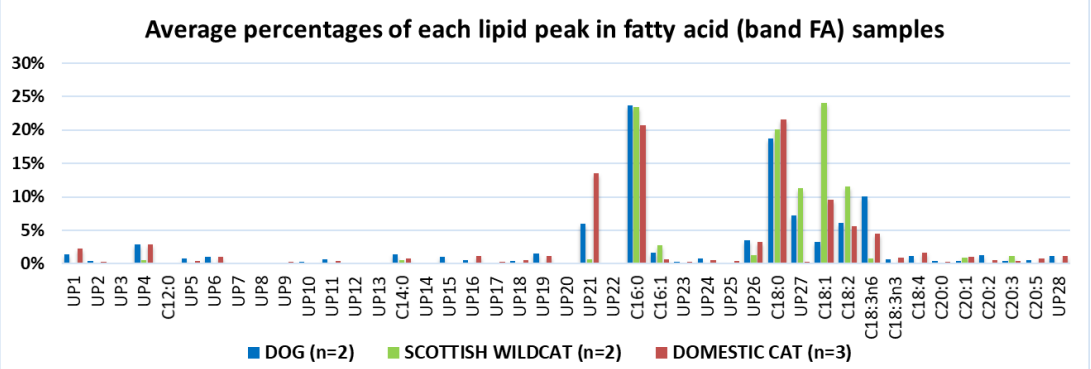
(a)



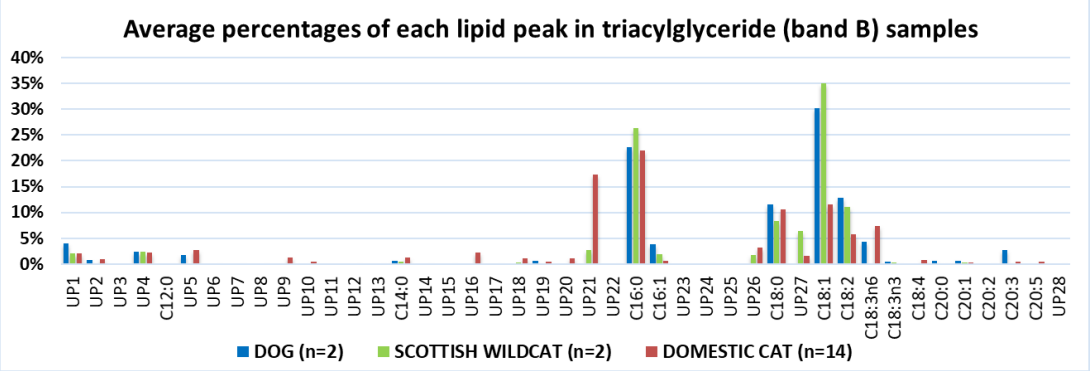
(b)



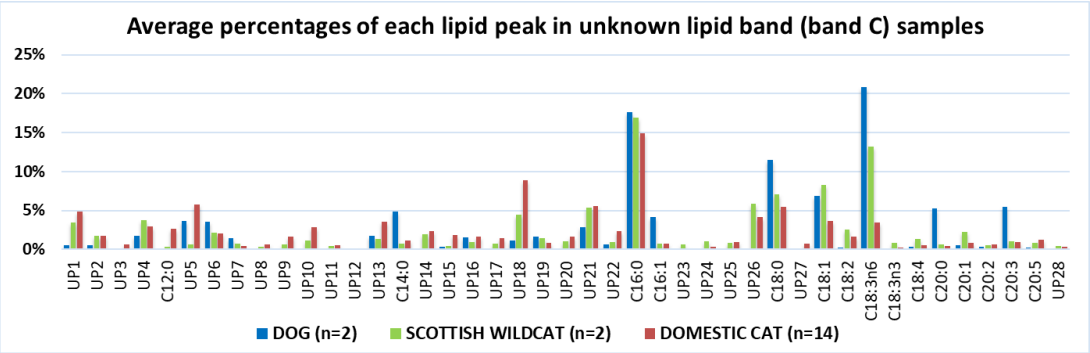
(c)



(d)



(e)



(f)

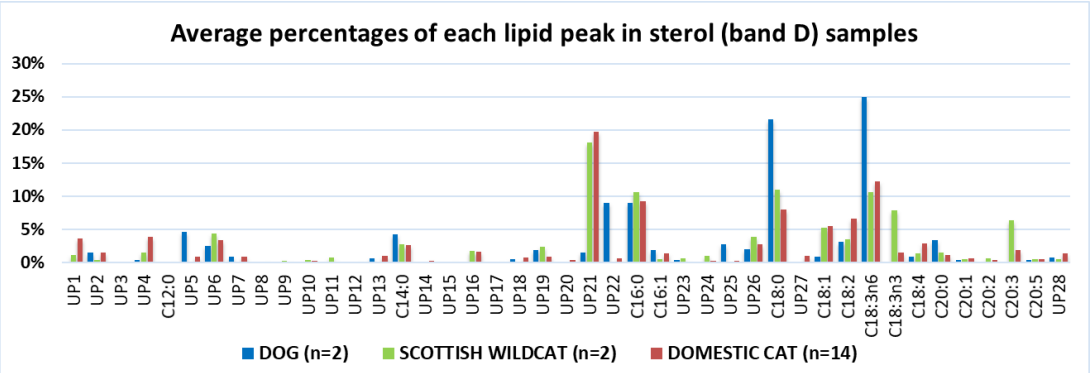


Figure 32. The percentage amounts of each lipid peak in the total lipid profile detected by GC-FID analysis of FAMEs in dog (blue), Scottish wildcat (green) and domestic cat (red) samples of **(a)** total lipid (an aliquot of the original total lipid extract, incorporating all lipid types), **(b)** polar lipid (lipid extracted from TLC band A), **(c)** fatty acids (lipid extracted from TLC band FA), **(d)** triacylglycerides (lipid extracted from TLC band B), **(e)** unknown lipid band (lipid extracted from TLC band C) and **(f)** sterols (lipid extracted from TLC band D). Lipids along the graph x-axes are in order of retention time as they appeared on GC-FID profiles. UP = unknown peak.

3.4. Discussion

3.4.1. Cats have more lipid in their kidneys than dogs

From our study, we conclude that domestic cats have more lipid in their kidneys, compared to dogs. In general, this appears to be true for the full range of ages in these species. Previously, it has been reported that the lipid content of the feline kidney is lowest in neonatal kittens, then increases with age (Lobban, 1955) at least up until adulthood (Modell, 1933), exhibiting the same distribution at four to five months of age, as in the adult cat (Lobban, 1955). In our study, the youngest cat was seven months old, thus all cats in the current study would be expected to have this 'adult' distribution of lipid within their kidneys. However, the youngest cat in our study had greater kidney lipid content than most of the older cats. Modell (1933) concluded that the amount of lipid within the kidney is generally similar among adult cats. Once adult age is reached, the intracellular lipid content of the kidney may only increase further during pregnancy (Lobban, 1955, Modell, 1933) or with loss of sexual maturity and after castration in male cats (Lobban, 1955). This has led others to suggest that lipid accumulation within the feline kidney is associated with higher levels of oestrogenic hormones (Lobban, 1955). However, our analysis did not include pregnant animals and we found greater lipid content in male, compared to female, cat and dog kidneys, which does not support this notion. Furthermore, we could not deduce an effect of neutering on lipid accumulation within the kidney as only one domestic cat in our study population was entire. Rodents are known to exhibit sex-related differences in their kidneys, including RPTEC cytoplasmic lipid, predominantly in males, especially after castration (Zabel and Schiebler, 1980). Given the similar observation in cats in our study, rodents may provide an interesting model to investigate sex-related differences in renal lipid accumulation.

While others have suggested that renal intracellular lipid content increases into adulthood (Modell, 1933), we observed a trend of decreasing lipid content with increasing age, indicating that the kidney does not accumulate lipid throughout the life of the animal. However, increasing proportions of the kidney affected by CIN similarly reduced the tissue lipid content. Since CIN prevalence

increases with age and will gradually progress, in time expanding the renal interstitial space as tubules are pushed apart by infiltrating leukocytes and increased ECM deposition (Zeisberg and Kalluri, 2015), these two consistent results may be confounding. That is, it is uncertain whether aging or CIN primarily underpins the observed reduction in kidney tissue lipid content. The presence of increased CIN in tissue sections may have reduced the density of tubules within the observed field of view, consequently reducing the amount of observable lipid, which is primarily found within the cells of the renal tubules (Lucke, 1968, Bargmann et al., 1977). To overcome this issue, lipid-staining across the whole of each kidney tissue section, rather than just ten random fields of view, could have been quantified. Nonetheless, domestic cats had more lipid in their kidneys than dogs with the same level of CIN, which is particularly interesting considering that renal disease is more prevalent in domestic cats than in dogs. Furthermore, while CKD is mostly diagnosed in aged animals, due to the progressive nature of the disease and the accumulation of renal injury over time (Brown et al., 2016), we have shown that there is considerable lipid present within the domestic cat's kidney from at least as early as 7 months old. Therefore, perhaps the relatively high lipid content of the domestic cat's kidney potentiates the renal disease that is particularly prevalent in domestic cats.

Scottish wildcats, like domestic cats, have lipid within their kidneys. Our statistical analysis of ORO^{+ve} staining showed that, on average, Scottish wildcat kidneys contain the same amount of lipid as domestic cat kidneys. Only a few of the domestic cats had higher kidney lipid content than all of the Scottish wildcats. Thus, perhaps greater lipid content in the kidney is a common feature among cats, and one which separates them from other species such as the dog.

Oil Red O is used to stain neutral lipids, including triacylglycerides and diacylglycerides (Koopman et al., 2001), as well as cholesterol esters (Kruth, 1984). ORO does not stain unesterified cholesterol (Fowler and Greenspan, 1985), sphingolipids, ceramides and phospholipids (Mehlem et al., 2013), therefore, it does not bind readily to biological membranes (Koopman et al., 2001). A non-specific lipid soluble stain, such as Nile Blue, would be required

to identify these additional lipids (Koopman et al., 2001). ORO is an appropriate stain to use for assessing intracellular, but not membrane, lipid (Koopman et al., 2001). As the lipid droplets that exist in the feline kidney are found intracellularly (Lucke, 1968), ORO was suitable for use in the current study. Evidently, phospholipids and free fatty acids that may exist within the intracellular lipid droplets may not have been positively identified by the ORO stain. However, the intracellular lipid droplets within the cat's kidney likely contain a variety of different lipid classes (Bargmann et al., 1977). Moreover, intracellular lipid droplets usually comprise predominantly triacylglycerides (Escasany et al., 2019). Therefore, ORO should have identified the presence of most, if not all, of the intracellular lipid droplets nonetheless. Indeed, ORO has been used previously to facilitate the quantification of lipid droplets within muscle cells using image analysis software (Goodpaster et al., 2000), as was conducted for kidney tissue in the current study. Clearly, ORO cannot be used to quantify individual lipids within lipid droplets (Mehlem et al., 2013), but this was not an aim of histological staining in the current project.

Frozen tissue sections were used for histological evaluation of intracellular lipids. This was important because tissue fixation with alcohol or embedding in paraffin would have extracted most of the lipids from the tissue sections (Mehlem et al., 2013). Ideally, tissue should be frozen as soon as possible after collection, to avoid freezing artefacts, including cell shrinkage alongside ice crystal formation in the extracellular space, which are larger when tissue is frozen slowly (Schäfer and Kaufmann, 1999). If such artefacts are severe, cell-to-cell connections are disrupted, which can affect the histological appearance of the tissue structure (Schäfer and Kaufmann, 1999). It may have been advantageous to flash-freeze kidney tissue in liquid nitrogen at collection (Mehlem et al., 2013), however, the tissue used in the current study was initially collected for mineral analysis in Study 1, where freezing artefacts were not a concern, and it was not possible to collect additional kidney tissue for this study. Despite slower freezing, the kidney structure, with characteristic glomeruli, can be recognised from the images obtained. Attempts were made to keep tissues frozen during sectioning, to prevent any further loss of tissue integrity by thawing (Schäfer and Kaufmann, 1999); the microtome was kept

at -20°C. Moreover, the amount of lipid and the fatty acid composition in the tissue should be unaffected by freezing (Chakanya et al., 2017). Therefore, while our experiment would not have allowed specific localisation of lipid within the kidney, it was adequate for quantification of the lipid in domestic cat, versus dog and Scottish wildcat, kidneys, especially as tissues from all animals were subject to the same preparation methods.

3.4.2. Renal proximal tubule intracellular lipid in other species

Lipids are important in the kidneys of all species. Indeed, as studies in dog (Gold and Spitzer, 1964) and human (Nieth and Schollmeyer, 1966) kidneys have shown, FAO is the primary means of energy production. This is especially true in the RPTECs, as demonstrated in-vitro in rabbit RPTECs (Balaban and Mandel, 1988), which have particularly high metabolic activity and thus, high energy requirement (Bobulescu, 2010). It is an imbalance between FAO, uptake and synthesis of fatty acids, as is often seen in humans and rodents with metabolic syndrome or obesity, that results in excess lipid accumulation (Bobulescu et al., 2014, Kang et al., 2015). Pathological renal lipid accumulation is universally recognised in many types of kidney disease, with lipotoxicity being implicated in renal inflammation, oxidative stress, fibrosis (Kang et al., 2015, Escasany et al., 2019), tubular atrophy (Khan et al., 2014, Li et al., 2009) and reduced renal function (Muller et al., 2019) in humans and rodents. However, such evidence does not exist for companion animal species. The domestic cat is known to have a high prevalence of CKD, as well as an abundance of RPTEC intracytoplasmic lipid, however, these phenomena have not been linked. Yet, a physiological role for abundant intracellular lipid has not been established either. In fasted mice, free fatty acids present in blood are stored as triacylglycerides within renal intracellular lipid droplets (Scerbo et al., 2017). Opposite to FAO in renal disease states, in fasted mice, renal FAO is upregulated, suggesting that this physiological lipid accumulation serves to provide essential energy to the kidney when supply is limited (Scerbo et al., 2017). It is unknown whether the high lipid content of the feline kidney is due to increased uptake, to satisfy greater energy consumption, or whether it is due to decreased FAO as in the pathological kidney.

3.4.3. Adding to early research on the lipid composition of the feline kidney

Previously, based upon histological staining and histochemistry experiments, it has been reported that the intracellular lipid droplets within the cat's kidney contain triacylglycerides, phospholipids and cholesterol (Bargmann et al., 1977, Hewer et al., 1949). A full lipid profiling of the cat's renal intracellular lipid droplets has not been completed previously. As well as triacylglycerides, phospholipids and sterol esters, we also identified diacylglycerides, ceramides, sphingomyelin and free fatty acids in the renal intracytoplasmic lipid droplets of domestic cats, dogs and Scottish wildcats. Furthermore, we detected an abundance of an additional class of lipid, which contains molecular species that have masses consistent with MADAGs, in the domestic cat's kidney, distinguishing them from the dog and Scottish wildcat. To our knowledge, this is the first comparative, and most complete, analysis of renal intracellular lipids in companion animals and wild felines. Our work goes some way to increase the understanding of renal intracellular lipid droplets in domestic cats, towards the level of knowledge that exists for lipid droplets in other species, such as humans.

3.4.4. The profile of several major lipid classes within the kidney differs between the domestic cat, dog and Scottish wildcat

Our UPLC-MS/MS analysis revealed a greater proportion of triacylglycerides and lesser proportions of phospholipids, diacylglycerides and ceramides in the total renal intracellular lipid from two domestic cats with CIN, versus one dog and one Scottish wildcat. Phospholipids, specifically glycerophospholipids, comprised the largest proportion of the total intracellular lipid in the dog and Scottish wildcat kidneys whereas, in the domestic cats, triacylglycerides accounted for most of the intracellular lipid content. It is possible that CIN, rather than species, was responsible for the increased proportion of triacylglycerides and decreased proportion of diacylglycerides in the domestic cats, versus the healthy dog and Scottish wildcat kidneys. Indeed, plasma triacylglyceride concentrations have been shown to be elevated in humans

with CKD, compared to healthy individuals (de Gómez Dumm et al., 2001). These triacylglycerides may then accumulate ectopically in non-adipose tissues (Guebre-Egziabher et al., 2013). However, others found no association between human kidney cortex triacylglyceride content and markers of renal function, including estimated GFR (eGFR) and serum creatinine concentration (Bobulescu et al., 2014). Although, in that study, eGFR and creatinine measurements did not identify CKD in any of the study subjects (Bobulescu et al., 2014). Therefore, it is unclear whether kidney triacylglyceride content is related to CKD. Reduced levels of diacylglycerides in human blood serum have been associated with the progression of CKD to ESRD (Afshinnia et al., 2016). However, it is unknown whether the cats in our study progressed to ESRD and whether kidney intracellular, rather than blood, diacylglyceride content is reduced in cats with CKD, compared to healthy cats, and further with CKD progression. A similar analysis of total lipid composition in healthy cat kidney intracellular lipid droplets would have helped to conclude whether species, or disease, is responsible for the greater contribution of triacylglycerides and lesser contributions of phospholipids, diacylglycerides and ceramides to the total intracellular lipid content of domestic cat, relative to dog and Scottish wildcat, kidneys.

Lipotoxicity is not induced by excess triacylglycerides as such, but rather by their component free fatty acids, or the metabolites of these fatty acids, including fatty acyl-CoAs, diacylglycerides and ceramides, which are toxic (Chavez and Summers, 2012, Schaffer, 2003, Guebre-Egziabher et al., 2013, Bobulescu, 2010). For example, intracellular ceramides have been shown to be involved in apoptosis signalling pathways, including in the kidney (Lee and Thévenod, 2008). Thus, an increased proportion of triacylglycerides at the expense of reduced proportions of toxic lipid species, such as ceramides and diacylglycerides, may be an attempt to protect the kidney from lipotoxic damage (Listenberger et al., 2003, Bobulescu, 2010). The high prevalence of renal disease in domestic cats undermines this as an explanation for such findings in this species. Indeed, it is perhaps surprising that the kidneys of domestic cats with CIN, versus the healthy dog and Scottish wildcat kidneys, contained smaller proportions of certain toxic lipid species. However, it is

important to note that our results are not quantitative and only show that the proportions of total intracellular lipid in the kidney that are ceramides, phospholipids and diacylglycerides are lower, in domestic cats with CIN, compared to the healthy dog and Scottish wildcat. The amounts of ceramides, phospholipids and diacylglycerides are not necessarily lower in the domestic cat and could in fact be similar in the three groups of animals; the lower proportion of these lipids being due to higher total lipid content, resulting from increased levels of triacylglycerides. Indeed, in chicks with fatty liver and kidney syndrome, a disorder in which the kidney is particularly susceptible to lipid accumulation, the proportion of triacylglycerides in the kidney is increased, with a concomitant decrease in tissue phospholipid content (Whitehead, 1975). In humans (Bobulescu et al., 2014) and mice (Scerbo et al., 2017), higher levels of triacylglycerides in the kidney have been associated with lower levels of ceramides. The increased proportion of triacylglycerides and lower proportions of phospholipids and ceramides in the diseased domestic cat kidneys, relative to the proportions of these lipid classes detected in dogs and Scottish wildcats, might similarly be related. Further work could include a quantitative analysis to determine whether the absolute amounts of each lipid class are different between species.

3.4.5. Domestic cat kidneys contain additional lipid compounds that are rarely present in domestic dog and Scottish wildcat kidneys

Rather than increased triacylglyceride content of the domestic cat, versus dog and Scottish wildcat, kidney, as suggested by the full UPLC-MS/MS data described, it is more likely that the increased proportion of triacylglycerides detected in the domestic cat samples is due to the presence of an additional type of lipid. The unknown lipid band in our TLC experiments aligns with bands concluded to contain MADAGs in other studies (Bartz et al., 2007, Yamazaki et al., 1981, Ma et al., 2017). Moreover, the masses of some of the additional domestic cat-specific lipid compounds observed in our preliminary analysis of UPLC-MS/MS raw data were consistent with MADAGs. Yet, MADAGs were not detected in the full UPLC-MS/MS analysis of kidney TLEs. Rather than

negating the suggestion that MADAGs are present in these samples, this likely shows that the MADAGs present in domestic cat kidneys contain branched chain fatty acids. Branched chain MADAGs have an odd number of carbons and, therefore, the same mass as similar TAGs. For example, there is a 14 Dalton (Da) difference between 48:0 TAG (m/z 824.8, M+NH4) and e48:0 MADAG (m/z 810.7, M+NH4) but, if the MADAG contained a branched-chain fatty acid, such as 17:0 (15-methyl-16:0) for example, it would have an odd number of carbons (e.g. e49:0) and a mass of 824, just like 48:0 TAG. Hence, some of the compounds categorised as TAGs by the UPLC-MS/MS software may in fact be MADAGs containing one branched chain fatty acid and thus, an odd number of carbons (+14 Da). Therefore, it is likely that the greater proportion of total lipid classed as TAGs in the domestic cats, compared to dogs and Scottish wildcats, in the full UPLC-MS/MS analysis actually comprises both TAGs and MADAGs, which probably contain some branched chains.

The conclusions drawn here are based on very small group sizes. However, the n=1 domestic cat, n=1 dog and n=1 Scottish wildcat were taken to represent all animals within each study population, based upon prior consistent findings; that is, the presence of the additional lipid band on TLC profiles of domestic cat renal intracellular lipid, irrespective of the cats' CIN-status, and the absence of the same band in all dog samples. Nonetheless, it is pertinent to recognise that individual differences, including dietary history and concurrent disease, information that was not obtained, may have contributed to the observed differences in renal lipid between the three study groups.

The band of unknown lipid was larger and more opaque on domestic cat, than Scottish wildcat, TLC profiles, suggesting more unusual lipids, such as MADAGs, in the domestic cat kidney. Furthermore, no evidence of lipid compounds with masses compatible with MADAGs were found in the Scottish wildcat UPLC-MS/MS raw data. It is likely that the additional lipid band that was present in Scottish wildcat TLC profiles contains TAGs from the band below. The poor quality data obtained from UPLC-MS/MS analysis of extracted TLC bands corroborates this assumption of incomplete separation of different lipid classes by TLC. Thus, Scottish wildcat kidneys may not contain MADAGs.

An important consideration is that the Scottish wildcats are likely to be younger than the domestic cats in the current study. While young and adult domestic cats have the same distribution of lipid within their kidneys (Lobban, 1955), perhaps age affects the accumulation of specific lipid types, such as MADAGs. This would corroborate the reduced appearance of the unidentified band in Scottish wildcats, versus domestic cats. However, the current study cannot confirm such an effect. Nonetheless, the absence of the unknown band from the dog TLC profiles implies an effect of species and that certain lipid compounds, which may be MADAGs, within the kidney are a cat-specific finding.

MADAGs are glycerol-based ether neutral lipids (Magnusson and Haraldsson, 2011). Similar to triacylglycerides, which have three fatty acid chains each esterified to a glycerol backbone via an acyl group (Karupaiah and Sundram, 2007), MADAGs have two fatty acids attached to a glycerol backbone via acyl groups in the stereospecific positions *sn* (stereospecific number) -2 and *sn*-3 but a third fatty acid with an alkyl group is ether-bonded to the glycerol molecule in position *sn*-1 (Magnusson and Haraldsson, 2011). Figure 33 shows the general structure of a triacylglyceride and a MADAG.

Figure 33. The general structure of a triacylglyceride and a monoalkyldiacylglycerol.

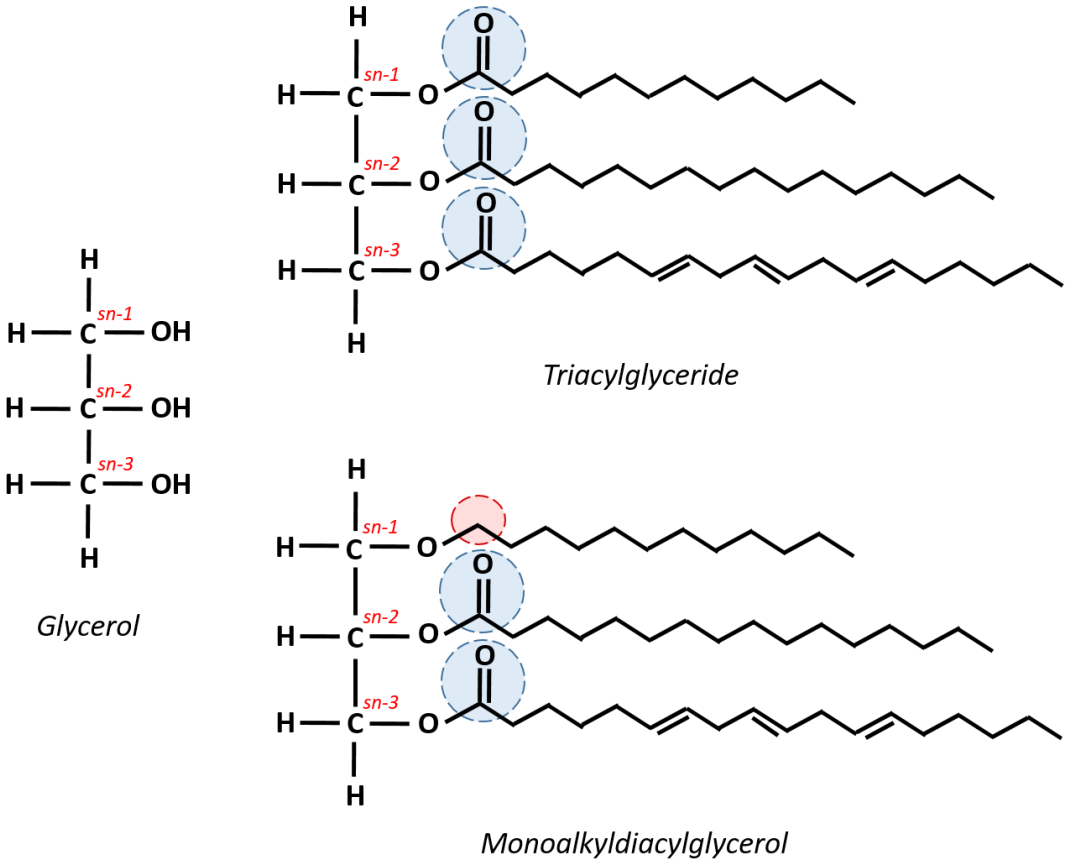


Figure 33. The general structure of a triacylglyceride and a monoalkyldiacylglycerol. Stereospecific numbering (*sn-1, sn-2, sn-3*) of the three carbon atoms of the glycerol backbone is shown. In triacylglycerides, fatty acid chains with acyl groups (blue circle) are esterified to the glycerol backbone in positions *sn-1, sn-2 and sn-3.* In monoalkyldiacylglycerols, fatty acids are esterified to the glycerol backbone in positions *sn-2 and sn-3* but position *sn-1* is occupied by a fatty acid with an alkyl group (red circle), attached to the glycerol backbone via an ether bond. Fatty acids depicted in this example are C12:0 (lauric acid), C16:0 (palmitic acid) and C18:3n6 (gamma-linolenic acid) in positions *sn-1, sn-2 and sn-3*, respectively. Double lines indicate double bonds.

In mammals, MADAGs are synthesised endogenously from dihydroxyacetone phosphate (DHAP) in a series of steps catalysed first by peroxisome-associated enzymes (Hajra et al., 2000, de Vet and van den Bosch, 2000) and finally by enzymes associated with the endoplasmic reticulum. Tissue, including kidney, concentrations of MADAGs may also be increased by greater dietary intake (Blank et al., 1991), and effective intestinal absorption (Magnusson and Haraldsson, 2011), of alkylglycerol ether lipids. Alkylglycerols then undergo acylation reactions to become MADAGs in body tissues (Ma et

al., 2017). Lipid droplets within different in vitro cell types contain varying levels of MADAGs, according to the specific functions of the cells and the density and activity of peroxisomes (Bartz et al., 2007). For example, lipid droplets in liver and kidney cells, which have a high density of peroxisomes (Vasko, 2016), contain MADAGs, while adipose tissue does not (Bartz et al., 2007). In humans, the MADAG content of the kidney exceeds that of other tissues, including the brain and testes (Snyder and Wood, 1969). However, generally, ether lipid, particularly MADAG, content of mammalian tissues is low (Magnusson and Haraldsson, 2011); ether lipids are more abundant in teleost fish, including oily fish species, and marine crustaceans and shellfish (Karupaiah and Sundram, 2007). Thus, perhaps the relatively high level of MADAGs in the kidney of domestic cats, among mammals, is obtained through the carnivorous diet and increased consumption of fish (Mangold, 1983).

Excessive dietary supply of ether lipids increases MADAG synthesis and storage in lipid droplets (Ma et al., 2017). When ether lipid levels are low, lipase enzymes break down stored MADAGs to release the alkylglycerols (Ma et al., 2017). Alkylglycerols have signalling functions or may be converted to ether phospholipids, suggesting a role for MADAG-containing lipid droplets in phospholipid recycling (Ma et al., 2017, Bartz et al., 2007). Indeed, our finding of a reduced proportion of phospholipids in the domestic cat, compared to dog and Scottish wildcat, renal intracellular total lipid, alongside the presence of MADAGs, supports this supposition. Our evidence suggests that domestic cats accumulate MADAGs in their kidney, while Scottish wildcats may to a lesser extent and domestic dogs do not. It is possible that a) domestic cats consume greater amounts of ether lipids than dogs and Scottish wildcats, resulting in increased storage of MADAGs in the kidney of domestic cats and b) from an early age, cats have a propensity for lipid storage within the kidney to provide a supply of phospholipids, perhaps fulfilling some role related to being obligate carnivores and uncertain or interrupted supply of meat and fat.

3.4.6. The MADAGs in cat kidneys, unusually for mammals, contain shorter- and may contain branched-chain fatty acids

As we found in domestic cat, dog and Scottish wildcat kidneys, the predominant fatty acids in mammalian tissues tend to be C16 and C18 chains (Magnusson and Haraldsson, 2011, Muller et al., 2015). The main difference of note, with regard to the known fatty acids, was a greater proportion of C12:0 in domestic cat, compared to dog and Scottish wildcat, kidneys. C12:0 only constituted a minor portion of the total lipid in domestic cats but this finding is of particular interest because C12:0 was particularly concentrated in the unknown lipid, or MADAG, band in domestic cats. The absence, or reduced amount, of this additional lipid band in dog and Scottish wildcat kidneys may explain the lower proportion of C12:0 in these species, compared to domestic cats. C12:0 (lauric acid) and, to a lesser extent, C14:0 (myristic acid) (Monserrat et al., 2000), are abundant in coconut oil (Sankararaman and Sferra, 2018). Additionally, C12:0, along with C16:0 (palmitic acid) and C18:1 (oleic acid), is highly concentrated in other plant oils, seeds and fruits of the palm tree family (Silva et al., 2015, Sankararaman and Sferra, 2018). These foods would not ordinarily be consumed by an obligate carnivore in the wild. Therefore, the domestic cat may be maladapted to metabolise these fatty acids. However, incorporation of plant-based oils, including coconut and palm oils, into commercial pet foods to provide other, including essential, fatty acids for nutrition, food preservation and palatability, may increase the domestic cat's exposure to these fatty acids (Weisman, 1992, Thompson, 2008). Commercial diet coupled with a lack of physiological adaptation to metabolise such fatty acids may lead to their accumulation within intracellular lipid droplets in the cat's kidney.

Alongside the usual known fatty acids, our GC-FID analysis revealed a number of fatty acid compounds that we were unable to identify. Many of these were specific to the domestic cat and were absent from dog and Scottish wildcat kidneys, likely due to their predominance in the unknown lipid band which was reduced in dogs and Scottish wildcats compared to domestic cats. In addition to higher content of shorter-chain identified fatty acids, such as C12:0, the majority of the unknown fatty acids that were detected in the domestic cat

kidney appeared to be shorter-chain since they eluted between C12:0 and C16:0. While the exact identities of these unknown fatty acids were undetermined, the masses of these molecules (data not shown) suggested that they may contain odd numbers of carbons, proving their separation from triacylglycerides (Murphy and Axelsen, 2011) and that they were likely to be branched-chain compounds. Additionally, this supports the non-detection of MADAGs in our full UPLC-MS/MS analysis being attributed to identical masses of triacylglycerides and branched-chain MADAGs as explained above.

It has been reported previously that the MADAGs in animal cells predominantly contain saturated and monounsaturated C16 and C18 alkyl chains (Magnusson and Haraldsson, 2011). MADAGs containing alkyl chains shorter than C16 or longer than C18 are apparently uncommon in mammals (Magnusson and Haraldsson, 2011). Furthermore, while MADAGs containing branched acyl and/or alkyl chains have been found in mammalian cellular lipid droplets previously (Bartz et al., 2007), these too are uncommon in mammals (Magnusson and Haraldsson, 2011). MADAGs containing short- and branched-chain fatty acids are more common in marine organisms and teleost fish, although still comprise only a minor proportion of the lipids in these species (Magnusson and Haraldsson, 2011). Our results suggest that domestic cats are unusual among mammals, due to a relatively higher proportion of MADAGs, with greater content of short, branched-chain fatty acids, in their renal intracellular total lipid.

With a renal MADAG profile more similar to that of aquatic species than other mammals, it is suggestive that domestic cats are exposed to higher levels of short, branched-chain ether lipids through their diet, potentially through fish consumption. Another theory to consider is greater content of branched amino acids, such as valine, leucine and isoleucine, in the diet of domestic cats, as these amino acids give rise to the primers that are used to synthesise branched-chain fatty acids (Oku and Kaneda, 1988). Indeed, the primary dietary source of branched-chain amino acids tends to be meat (Górska-Warsewicz et al., 2018), which constitutes the majority of the cat's diet. It is known that nitrogen metabolism is unusual in cats, compared to other mammals (Morris and Rogers, 1982). Idiosyncratic nitrogen metabolism is

perhaps responsible for higher levels of branched amino acids and, therefore, branched-chain fatty acids, in the feline kidney, compared to other species. The synthesis of branched-chain fatty acids, in general, may be upregulated in domestic cat kidneys, relative to other mammals, which could be an area for future study.

Additionally, the peroxisomal or endoplasmic reticulum enzyme systems involved in the generation of MADAGs may be upregulated in the domestic cat kidney, relative to other species, accounting for the increased lipid content of the domestic cat kidneys. Peroxisomes are also involved in energy production by β-oxidation of long-chain fatty acids, but not shorter-chain fatty acids with 12 to 16 carbons (Lazarow, 1978) as the unique MADAGs in the domestic cat kidney seem to primarily contain. Mitochondrial β-oxidation would carry out this function (Lazarow, 1978). MADAG accumulation within domestic cat kidney cytoplasmic lipid droplets may be caused not only by storage of excess ether lipids but also by a reduced ability to metabolise these compounds for energy production. Indeed, Boren and Brindle (2012) reported that cytoplasmic lipid droplets may form due to reduced β-oxidation and increased de novo lipogenesis. Similarly, increased triacylglyceride and free fatty acid content in renal tubular cells has been associated with downregulation of enzymes involved in mitochondrial and peroxisomal β-oxidation (Weinberg, 2006). However, ACOX2, a gene involved in peroxisomal metabolism of branched-chain fatty acids and possibly also regulation of triacylglyceride levels, is reportedly upregulated in the feline genome, relative to other mammalian genomes (Montague et al., 2014). This is perhaps surprising since higher levels of the encoded protein, branched-chain acyl-CoA oxidase, would be expected to result in lower levels of branched long-chain fatty acids in the feline kidney. Indeed, few of the unidentified fatty acids detected in our study were long chains, although this was comparable among domestic cat, dog and Scottish wildcat samples. Domestic cat, versus dog and Scottish wildcat, renal intracellular lipid droplets contained more shorter-chain unknown fatty acids, which might arise from increased degradation of branched long-chain fatty acids. However, although we suggest the presence of branches in these unidentified fatty acids, they are not all necessarily branched; the exact

structures remain to be established. In any case, it is unknown whether the relatively high expression of ACOX2 in the overall genome translates co-ordinately to the kidney. A study of the expression of several lipid metabolism and biosynthesis genes, specifically within the kidney, may be better suited to establishing the role of genetics in the high content of certain lipids or specific fatty acids within the feline kidney. Further work, including evaluation of the mitochondrial genome in the domestic cat kidney, could be useful to establish whether β-oxidation is inherently reduced in domestic cats, compared to other species that have less lipid within their kidneys.

3.4.7. Further work

Despite UPLC-MS/MS revealing a higher proportion of triacylglycerides in the total lipid from domestic cats, versus dog and Scottish wildcat, renal intracellular lipid droplets, when evaluated alongside UPLC-MS/MS raw data, TLC and GC-FID data, it seems that this proportion actually comprises both triacylglycerides and MADAGs. Branched-chain MADAGs were probably categorised as triacylglycerides due to having the same masses as triacylglyceride species. Thus, MADAGs, which appear to be more abundant in domestic cats than in dogs and Scottish wildcats, would have contributed to the higher proportion of total lipid that was detected as triacylglycerides in domestic cat kidneys. Indeed, a greater proportion of triacylglycerides per se might be expected to confer some protection from lipotoxic damage. However, it is unlikely that cats, but not dogs, possess a protective mechanism such as this in their kidneys, since renal disease is so common in the former species. To determine whether intracellular lipid droplets in domestic cat kidneys indeed have greater content of triacylglycerides as well as additional lipid species, such as MADAGs, compared to dogs and Scottish wildcats, a quantitative analysis should be conducted.

Unfortunately, the quality of UPLC-MS/MS data for the additional, unidentified lipid band that we extracted from the TLC plates was too poor and the concentration of lipid within the samples was too low to allow for characterisation of the lipids within these samples. The poor quality of the samples was probably due to incomplete separation of bands of lipids of

different molecular classes, for instance, triacylglycerides versus MADAGs, hence resulting in contamination of the triacylglyceride band (band B) with MADAGs, and contamination of the MADAG band (band C) with triacylglycerides. Nonetheless, we concluded that the additional lipid in domestic cats, which is less pronounced or absent in Scottish wildcats and dogs, is MADAGs based on the alignment of the unknown lipid band on our TLC plates with the position occupied by MADAGs in TLC experiments conducted by others. Furthermore, the masses of lipid compounds in raw data from UPLC-MS/MS analysis of TLEs, which would include the lipid contained within the separate TLC bands, showed evidence of MADAGs in domestic cat, but not dog or Scottish wildcat, kidneys. To confirm the presence of MADAGs in the additional unknown lipid band that was unique to cats, improved separation of bands of different lipid molecular classes, such as TAGs and MADAGs, would be required. This could be achieved using high resolution TLC or HPLC. The identity of the lipid molecular class in the additional lipid band in domestic cats could then be established using UPLC-MS/MS, which could confirm its identity as MADAGs. Alternatively, the identification could be attempted using a combination of techniques such as atmospheric pressure chemical ionisation-tandem mass spectrometry (APCI-MS/MS), matrix-assisted laser desorption-time of flight-mass spectrometry (MALDI-TOF-MS) and electrospray ionisation-mass spectrometry (ESI-MS), as applied by others (Bartz et al., 2007).

Further work should also include identification of the fatty acids corresponding to the unknown peaks that were detected in kidney samples, particularly from domestic cats and in the unknown lipid, or MADAG, band. The masses of the compounds represented by the unknown peaks can be acquired from our existing GC-FID and GC-MS data and used to elucidate the potential structures. This should enable a more precise identification of the unknown fatty acids. Our current conclusion that the additional lipids in domestic cat, but absent or reduced in dog and Scottish wildcat, kidneys are mostly comprised of shorter (C12-C16), branched-chain fatty acids is based on an inspection of the GC-MS peak masses during data acquisition, but, due to unforeseen circumstances during this project, the data was unavailable for the necessary,

more thorough interpretation. Our conclusion is also supported by the m/z values of the most abundant MADAG and triacylglyceride peaks in the UPLC-MS/MS raw data; the triacylglyceride peaks may indeed relate to branched-chain MADAGs with the same masses. However, while we suggest that the MADAGs in cat kidneys contain branched-chain fatty acids, this could not be evidenced by our TLC experiment. This is because branched and linear MADAGs would have co-migrated in the normal phase TLC that was used. To improve on this, a combination of normal and reverse phase TLC or HPLC could be conducted prior to further analyses: normal phase to separate the molecular classes, e.g. triacylglycerides from MADAGs, followed by reverse phase to separate the linear and branched-chain molecular species within these two classes (Mangold, 1961, Kroumova and Wagner, 1995). In summary, better separation of triacylglycerides and MADAGs would remove cross-contaminations of the triacylglyceride and MADAG samples for subsequent UPLC-MS/MS analysis and facilitate the confirmation of the presence of MADAGs and identification of the specific fatty acid profiles within the MADAGs versus triacylglycerides using GC-MS/FID analysis.

3.4.8. Conclusion

Ectopic lipid accumulation, as we have demonstrated in the kidneys of domestic cats, relative to dogs and Scottish wildcats, is known to have a toxic effect on cells, including those within the kidney (Izquierdo-Lahuerta et al., 2016, Herman-Edelstein et al., 2014). Renal lipotoxicity may induce renal disease (Izquierdo-Lahuerta et al., 2016, Gai et al., 2019). We demonstrated that this high lipid content of the domestic cat kidney is present prior to the age normally associated with the diagnosis of feline CKD. Thus, we suggest that high lipid content of the feline kidney predisposes the cat to CKD.

Our further analyses revealed the presence of additional lipid species in healthy and diseased domestic cat kidneys, that may be less abundant in Scottish wildcats and are absent from domestic dog kidneys. We propose that these additional lipids are MADAGs and are composed of several branched-chain fatty acids with between 12 and 16 carbons. MADAGs and such fatty acids are unusual in mammals (Magnusson and Haraldsson, 2011). The

higher content of these fats in domestic cats, compared to dogs and Scottish wildcats, may be related to increased dietary intake of fish and plant oils added in commercial pet foods. Collection of dietary information or, better still, long-term feeding trials using diets with only animal- or only plant-based fats, to assess the extent and type of lipid accumulation in the kidney that results, would be useful. Additionally, upregulation of MADAG synthesis pathways and/or impaired mitochondrial β-oxidation of short-chain fatty acids may be involved in the accumulation of MADAGs within the kidneys of domestic cats (Weinberg, 2006). Kidney and mitochondrial transcriptomics may be important to explore these premises. Indeed, discovering the impetus for the high lipid content of the domestic cat's kidney, could provide a foundation for investigating whether this can be interrupted, for example with dietary or medical interventions; and if such an intervention would be beneficial for kidney function.

Despite consuming a different diet to domestic cats, the Scottish wildcats in the current study also had intracellular lipid deposits within their kidney. Therefore, the feline species, rather than diet is likely to be the primary explanation for this feature. However, since the MADAGs and specific fatty acids that were uniquely identified in the domestic cats' kidneys are likely to be of fish and plant origin, we suggest that consumption of commercial pet foods may contribute to the increased intracellular MADAG content in the kidney of the domestic cat, but not the Scottish wildcat. Dietary trials may be required to validate this contention. However, in the meantime, we suggest that it might be beneficial for pet food manufacturers to avoid incorporating certain fish-based ingredients and plant-based fats, such as coconut oil or palm oil, which the cat is not evolutionarily adapted to consume, into commercial cat foods.

Unfortunately, we are unable to conclude whether the composition of the total lipid or particular lipid classes within the kidney intracellular lipid droplets of domestic cats with renal disease, with respect to their component fatty acids, is altered from the profile of lipids within the healthy domestic cat kidney. Nonetheless, we conclude that, atypically for mammals, even healthy cats have increased total lipid content and additional lipid species, such as

MADAGs with branched chains, within their kidneys. Coupled with a reduced proportion of phospholipids in the domestic cat, compared to dog and Scottish wildcat, renal intracellular lipid droplets, increased MADAG content might suggest greater phospholipid metabolism in domestic cats. Additionally, this extra lipid could promote a lipotoxic environment within the RPTECs, predisposing the domestic cat to CKD (Izquierdo-Lahuerta et al., 2016). Establishing such a role for the unusual lipid content of the domestic cat's kidney in the development of CKD, or even a physiological role, could be an interesting and important area for further research (Gai et al., 2019).

Chapter 4. Study 3: Is feline hyperthyroidism associated with dietary iodine intake?

4.1. Introduction

4.1.1. The role of changing levels of iodine supply in hyperthyroidism development

Thyroid hormone synthesis is not only controlled by TSH, whose own production is adjusted according to levels of thyroid hormone exerting negative feedback on the HPT axis (Mooney, 2017). Iodine is a key constituent of thyroid hormones, thus, it is fundamental to thyroid hormone synthesis (Kopp, 2013b). The level of iodide in circulation also controls the function of the thyroid gland (Dumont et al., 2016). When circulating levels of iodide are high, the thyroid gland is less responsive to TSH; gene expression of the TSH receptor, TPO, thyroglobulin and the sodium-iodide symporter are reduced, and thyroid hormone secretion is inhibited (Dumont et al., 2016). This prevents excessive production of thyroid hormone and growth of the thyroid gland, when iodine is abundant (Gärtner, 2009). On the other hand, when iodide levels are low, many thyroidal processes are upregulated to augment the incorporation of iodine into thyroid hormones when iodine supply is limited (Laurberg et al., 2010). Ultimately, this leads to increased T3 secretion from the thyroid gland and T3 conversion from T4, maintaining a euthyroid state (Kopp, 2013b, Abrams and Larsen, 1973), without an increase in TSH (Laurberg et al., 2010). Negative feedback through the HPT axis, to increase the production of thyroid hormone via increased TSH, occurs later, only if upregulation of the intrathyroidal systems to maintain normal thyroid hormone levels has been ineffective (Laurberg et al., 2010).

However, long-term, mild to moderate iodine deficiency results in chronic upregulation of the systems for thyroid hormone synthesis (Laurberg et al., 2010). For example, the generation of H_2O_2, which is required for thyroid hormone synthesis, is upregulated during iodine deficiency (Corvilain et al., 2000, Song et al., 2007). H_2O_2 is a ROS, requiring neutralisation by antioxidant enzymes; otherwise, in excess, H_2O_2 may cause damage, and indeed

mutations, to the DNA of thyroid cells (Song et al., 2007). The chronic increased activity of the thyroid gland, as well as mutations, results in the formation of nodules of autonomously growing and functioning follicular cells (Laurberg et al., 2010, Watson et al., 2005). The autonomy depicts an independence from TSH control; another adaptation to maintain normal levels of thyroid hormone in the absence of sufficient iodine (Laurberg et al., 2010). However, problems can arise if iodine supply suddenly increases. While elevated iodine supply would normally downregulate the mechanisms involved in thyroid hormone production (Dumont et al., 2016), the adaptations to chronic iodine deficiency render the thyroid gland unable to respond quickly enough to the sudden increased and excessive iodine supply, which provides additional substrate for the hyper-functioning thyroid follicular cells (Laurberg et al., 2010). Therefore, thyroid hormone production continues, thyroid hormone secretion increases and hyperthyroidism may ensue (Stanbury et al., 1998). This is the mechanism by which TNG develops in humans, particularly in the elderly population living in iodine deficient areas (Stanbury et al., 1998).

4.1.2. Similarities between human toxic nodular goitre and feline hyperthyroidism

The pathology in the human thyroid gland affected by toxic nodular goitre, i.e. the presence of nodules comprising autonomously functioning follicular cells, also occurs in feline hyperthyroidism (Hoenig et al., 1982). Yet, unlike human TNG, the pathogenesis of feline hyperthyroidism is largely unknown. Certain aspects of the development of human TNG have also been discovered in cats with hyperthyroidism, for example mutations in the TSH receptor (Watson et al., 2005). Additionally, downregulation or mutation of some of the G-proteins involved in the TSH receptor signal transduction pathway have been noticed in some hyperthyroid cats (Peeters et al., 2002). Thus, as in human TNG, mutations may play a role in the aetiology of the condition in cats. Goitrogens, such as the environmental contaminants previously described (Chapter 1, section 1.5.2.4.1), may induce these mutations. Chronic exposure to these goitrogens may therefore cause an accumulation of mutations in thyroid cells, increasing the risk of hyperthyroidism. This notion may be the reason behind

the increased risk of hyperthyroidism in older cats (Wakeling et al., 2009b). In contrast, the known implication of dietary iodine in the development of TNG has not yet been proven in the pathogenesis of feline hyperthyroidism.

4.1.3. Dietary iodine variation may be a risk factor for feline hyperthyroidism

Feeding canned commercial pet foods to cats has been implicated with an increased risk of developing feline hyperthyroidism (Kass et al., 1999, Wakeling et al., 2009b, Scarlett et al., 1988, Edinboro et al., 2004). Complete cat foods that are commercially available outside of the UK have been shown to have highly variable iodine contents, with some foods even containing excessive levels of iodine with respect to the nutritional recommendations (Ranz et al., 2002, Johnson et al., 1992, Edinboro et al., 2013). Therefore it appears plausible that domestic cats may experience wide fluctuations in their iodine intake over time, especially if their diet is changed. However, pet foods that are commercially available within the UK have not been analysed to determine whether the same risk may apply to domestic cats in the UK.

Fish consumption has also been associated with higher risk of hyperthyroidism in cats (Martin et al., 2000, Wakeling et al., 2009b). Fish for human consumption is known to contain high levels of iodine (Nerhus et al., 2018), however, whether fish-containing pet foods have higher iodine content than foods that do not contain fish is less clear (Edinboro et al., 2013) and requires further investigation. We propose that as cats are generally more likely to eat fish more frequently than dogs (Sakai et al., 1995), they probably experience high, and perhaps excessive, iodine intakes more often. Coupled with a wide variation in the iodine content among commercial pet foods in general, high consumption of fish by cats, relative to dogs, may increase the likelihood of excess iodine consumption and larger fluctuations in iodine intake when the diet is changed. Consequently, this may impart greater risk of hyperthyroidism development in cats, via a mechanism similar to iodine-induced TNG in humans.

4.1.4. The effect of changing dietary iodine levels in cats

While it is unclear whether changing dietary iodine levels can induce hyperthyroidism in cats, a few studies have shown that thyroid hormone concentrations in circulation may be altered by dietary iodine changes. Serum free thyroxine (fT4) levels in cats, aged 2 years old, have been shown to respond rapidly to fortnightly changes in dietary iodine intake, decreasing significantly when switched from a control diet to one with high or medium iodine concentration, and increasing back to baseline when switched back. However, serum fT4 levels did not change significantly when switched from the control diet to one with lower iodine content (Tarttelin et al., 1992). Cat urine iodine concentrations measured in that study (Tarttelin et al., 1992) and others (Ranz et al., 2002, Paetau-Robinson et al., 2017) were found to respond concordantly to increases and decreases in dietary iodine intake, corroborating reports that urinary iodine concentrations reflect dietary iodine intake (Vought and London, 1967, Nath et al., 1992). When cats aged 1-2 years were fed either a high or low iodine diet longer-term, serum fT4 levels were not significantly different between the two diet groups and did not change significantly over a five month period (Kyle et al., 1994). However, in the studies conducted by Tarttelin et al. (1992) and Kyle et al. (1994), serum TSH and T3 concentrations were not measured so the impact of dietary iodine on the hypothalamic-pituitary-thyroid axis could not be assessed.

Paetau-Robinson et al. (2017) investigated the effect of feeding a limited iodine diet to healthy, young to middle-aged adult cats for two years, and found that serum thyroid hormone (fT4, total T4, fT3, total T3) and TSH concentrations, on average, were maintained within the normal reference ranges. It might be that iodine deficiency over a period longer than two years, and/or a subsequent excessive iodine intake as in human TNG, is required for functional thyroid changes to become apparent. Furthermore, as hyperthyroidism most commonly presents itself in older age, such changes may not be evident until cats enter the senior or geriatric age categories. These factors may explain why such changes were not observed by Paetau-Robinson et al. (2017) and Kyle et al. (1994). Longer term feeding trials are obviously necessary to determine the effect of dietary iodine concentration changes on

hyperthyroidism development in cats. Nonetheless, the results of these studies suggest that cats are able to maintain normal levels of serum fT4 during chronically occurring iodine deficiency or excess, but that thyroid function is affected when iodine intake changes dramatically in a short space of time. In light of these findings, the relationship between iodine and TNG and the similarities between TNG and feline hyperthyroidism, the importance of consistent dietary iodine provision for cats needs to be considered.

4.1.5. Aims and hypotheses of Study 3

Due to the clinical and histopathological similarities between feline hyperthyroidism and human TNG, it is necessary to investigate whether the aetiologies of these two conditions may be similar. Human TNG is caused by excessive dietary iodine intake after a period of iodine deficiency but the cause of feline hyperthyroidism is unknown. Many risk factors for feline hyperthyroidism have been identified, including the feeding of commercial wet, or canned, foods and the consumption of fish. Teleologically, domestic cats would never have consumed fish as part of their natural diet. However, domestic cats are often fed fish-based diets. Fish are known to be a rich source of dietary iodine. Therefore, fish-based pet foods may provide cats with a high dietary intake of iodine and contribute to the pathogenesis of feline hyperthyroidism.

Aims

1) The first aim of this study is to determine the iodine content of a range of commercially-available complete pet foods fed to cats and dogs.
2) A further aim is to determine the variability in iodine content across multiple different batches of otherwise-identical complete cat foods, with high or low iodine content.
3) To assess the compliance of all the measured, commercially available pet foods with the recommended nutritional minimum and legal maximum iodine content, as defined by FEDIAF (2019).
4) In this chapter, I will also obtain information from owners of cats about what they feed their cats most often and how often they change those

foods, in order to estimate how variable the domestic cat's intake of iodine may be.

5) Finally, in this chapter, using urine iodine concentration as a biomarker of recent dietary iodine intake and hair iodine content as a marker of long-term iodine intake, I will investigate whether dietary iodine intake is different between cats and dogs and between cats with, and cats without, hyperthyroidism.

Hypotheses

1) Similar to pet foods that have been analysed in the USA, New Zealand and Germany, the iodine content of complete cat and dog foods that are commercially-available in the UK varies widely (*to address Aim 1, 2, 3*).
2) The iodine content of commercially-available complete cat foods is more variable and more commonly low or high in relation to FEDIAF guidelines, than that of commercially-available complete dog foods in the UK (*Aim 1, 3*).
3) Commercially-available complete cat and dog foods with fish as the main flavour have higher iodine content than complete foods that do not have fish as the main flavour (*Aim 1, 3)*.
4) Pet cats are fed fish-based foods regularly (*Aim 4*).
5) Cat owners vary the commercial pet foods (by type [wet/dry], brand and/or flavour) that they feed to their cats regularly (*Aim 4*).
6) Cats have greater short-term dietary iodine intake than dogs, as indicated by urinary iodine concentrations (*Aim 5*).
7) Hair iodine content suggests that long-term iodine intake differs between euthyroid and hyperthyroid cats (*Aim 5*).

4.2. Materials and Methods

4.2.1. Ethics

This study was approved by the University of Nottingham School of Veterinary Medicine and Science Research Ethics Committee (REC 2074 170721) and carried out in accordance with good laboratory practice, as in Study 1 (Chapter 2; section 2.2.1).

4.2.2. Source of animal material

A population of healthy cats (n=38) was recruited via a process of informed consent from veterinary students at The University of Nottingham who owned a cat without a known history or symptoms of hyperthyroidism. Owners collected hair from their cats by grooming, usually over several days to gather enough fur. Hair from hyperthyroid cats (n=32) was obtained by clipping when patients at Pride Veterinary Centre were having blood sampled for assessment of their thyroid function. Thus, these cats were known to have elevated thyroid hormone (T4) levels and a veterinary diagnosis of hyperthyroidism. The veterinarians undertaking this procedure obtained owner consent for collection of the hair clippings for research purposes, beforehand. Information, such as the ages and breeds of the euthyroid and hyperthyroid cats, was collected.

Urine was obtained from the bladders of domestic cats (n=57) and dogs (n=54) submitted to UoN-VPS for necropsy as in Study 1 (Chapter 2, section 2.2.2). Consent to take samples from these animals was obtained from the owners by veterinarians not associated with the research.

4.2.3. Selection of pet foods

A range of commercially available, wet (n=105) and dry (n=79), cat (n=109) and dog (n=75) foods that we had analysed previously (Davies et al., 2017a) and in Study 1 of this thesis for their content of other minerals, were selected for determination of their iodine content. These foods were purchased from local supermarkets and pet shops and comprised a range of familiar brands sold in the UK. Foods of a variety of main flavours, determined by the

ingredient listed on the packaging as comprising the highest proportion of the diet, were included. The foods were packaged in a range of sealed tins, cans, pouches or bags. All of the foods were labelled as “complete” as opposed to “complementary”. The majority of the foods analysed in the current study were intended for consumption by adult cats or dogs, although some were designed for young or senior animals.

After initial iodine concentration determination of all foods, four foods with high levels of iodine (Foods 1 to 4) and three foods with low iodine concentration (Foods 5 to 7) were selected. These were all complete wet foods for adult cats. All of the high iodine foods happened to contain seafood as the main flavour (Food 1, tuna; Food 2, haddock; Food 3, Prawn; Food 4, salmon), while none of the low iodine foods were seafood-flavoured. Foods 5, 6 and 7 contained beef, turkey and chicken as the predominant flavour, respectively. Between four and six different, consecutive batches of each of these seven foods were purchased over time and prepared in the same way as had been done previously (section 4.2.4.1) for iodine content determination by ICP-MS (section 4.2.4.3). This was to assess the inter-batch variability in iodine concentration in the same foods over time.

4.2.4. Iodine analysis of pet foods, urine and hair

4.2.4.1. Sample preparation

Hair was stored in sealed plastic bags in the dark at room temperature until processing. This method of storage has been reported to avoid compositional change in the hair over time (Forte et al., 2005). At all times throughout preparation of the hair samples, gloves were worn to prevent contamination. Each hair sample was washed, as previously described (Forte et al., 2005, Davies et al., 2017b). In brief, this involved immersion of ~0.5-1 g of hair in 3:1 (v/v) ethyl ether-acetone, stirring the sample in this solution for 5 minutes to eliminate the sebaceous film coating the hair; followed by soaking in 5% ethylenediaminetetraacetic acid (EDTA) for 1 hour to chelate out any chemical elements on the hair surface and, finally, rinsing the sample 3 times for 15 minutes each time with deionised milli-q water. Between each rinse, the

previous water was discarded before fresh water was added. Washed hair samples were subsequently transferred to 250 ml solvent-resistant containers (Sarstedt, UK) labelled with the specific allocated ID, and dried in an oven at 50°C for 48 hours. Urine samples were prepared as described in section 2.2.4.1.

Pet foods that were analysed previously for their content of other minerals (Davies et al., 2017a) and that were sampled, freeze-dried, homogenised and stored as described in section 2.2.5.1, were selected for iodine concentration determination. Subsequent batches of the seven foods that were purchased during the current study period were sampled, freeze-dried and homogenised in the same way. After freeze-drying and homogenisation, these foods were also stored in sealed containers in the dark at 4°C, but for a maximum of one month prior to analysis.

4.2.4.2. TMAH digestion

After drying, duplicate, 100-200 mg samples of each pet hair or pet food specimen were weighed into 50 ml inert PTFE-TFM-lined digestion vessels and digested in an alkali matrix of 10% tetramethylammonium hydroxide (TMAH). Hair samples were pushed right down to the bottom of the digestion vessels to ensure full coverage with the 5.0 ml of 10% TMAH solution that was added. Samples were then left to incubate for 1 hour, after which, the digestion vessels were transferred to the microwave reaction system described in section 2.2.4.2, and the TMAH110 programme was selected to accelerate the digestion process. For every 11 samples digested in duplicate, two digestion vessels were left empty (blank; with no hair sample) and only 5.0 ml of 10% TMAH was added. This was to control for any accidental contamination of the solvents and equipment used throughout the procedure; the iodine concentrations measured in the samples would be corrected by subtracting the average iodine concentration measured in the blanks (Bulska and Wagner, 2016).

4.2.4.3. Inductively-coupled plasma-mass spectrometry

TMAH-digested pet food and hair samples were decanted from the digestion vessels into 50 ml centrifuge tubes and diluted 1 in 10 to a total volume of 50 ml by addition of 45 ml deionised milli-q water. This resulted in a 1% TMAH matrix. The solutions were then centrifuged for 30 minutes at 2000xg. An aliquot (5-10 ml) of each solution was subsequently transferred into appropriately labelled 14 ml (105mm x 16.8mm) Polypropylene tubes (Sarstedt, Leicester UK) and capped ready for iodine analysis by ICP-MS without further dilution. Urine samples were not digested in the TMAH matrix but, after thawing and centrifugation, 500 µl of each neat urine sample was pipetted into an appropriately labelled 14 ml (105mm x 16.8mm) Polypropylene tube (Sarstedt, Leicester UK) and diluted 1 in 20 by addition of 9.5 ml of 1% TMAH. Per ≤60 samples and blanks, two Polypropylene tubes containing a CRM of SERO210705 Seronorm Trace Elements Urine L-2 (LGC Standards), with known iodine content of 295 µg/L, were submitted for iodine analysis by ICP-MS in a 1% TMAH matrix. The ICP-MS conditions for iodine concentration measurement were as described in Chapter 2, Study 1; section 2.2.4.3. The only modification of the procedure outlined previously was the use of 5 ppb rhenium in 1% TMAH as the internal standard.

The reproducibility of iodine measurements in cat hair was assessed by inter- and intra-assay variability. Intra-assay CV was determined based upon eight repeats of two samples and six repeats of another one sample, which were digested simultaneously and analysed on the same ICP-MS run. Iodine concentrations of 12 hair samples measured in (at least) duplicate, at two different time-points, were used to calculate inter-assay CV. Inter-assay variability in iodine measurements was assessed. Recovery of the certified level of iodine in seronorm CRM was calculated as an average of 30 repeats over n=15 ICP-MS runs for pet food analyses; inter-assay CV was also calculated. Intra-assay CV of pet food iodine concentrations was calculated from eight replicates of one sample and six replicates of another.

4.2.4.4. Human hair iodine

To our knowledge, the iodine content of cat hair has not been previously measured or published. Due to the lack of an accepted normal range of cat hair iodine contents, we would therefore not be able to validate our method of measuring iodine in hair by assessing the iodine content of cat hair alone. Previous studies that have measured iodine concentration in hair have been conducted on human hair samples (Momčilović et al., 2013). Therefore, to test that our method of measuring iodine in hair could produce similar results as those obtained through an alternative method (Momčilović et al., 2013), we first collected human hair samples for analysis. Healthy humans without thyroid disease (n=9) were recruited via a process of informed consent and collected their hair either by brushing, over several days, or cutting. Human hair samples were prepared in the same way as the cat hair samples, by washing, drying and digesting with TMAH, and then the iodine content was determined by ICP-MS.

4.2.4.5. Measurement of urine creatinine concentration to correct for different volumes and dilutions among the spot samples

The concentration (in µg/L) of iodine measured by ICP-MS in each urine sample was corrected to the creatinine concentration measured in the particular urine sample as described in Chapter 2, Study 1; section 2.2.4.4. Iodine concentrations in urine samples are reported as µg/g creatinine.

4.2.5. Survey of cat feeding habits

To determine whether cats are commonly fed the same diet or whether it is changed regularly, and in what way, a survey (Appendix 6) was created. Closed-ended questions were included in the survey to collect information about the identity of the cat, such as breed, sex and neutered-status, date of birth and health status. Free-text answer boxes were provided to obtain extra information about any specific diseases or signs of illness affecting the cats in this study, as well as the specific prescription diet where these were fed. A question regarding how many cats lived within the household was also

included. A series of multiple-choice questions were used to determine the proportions of different types of food (commercial diet: dry, wet [canned, pouches, foil trays, other packaging], flavours [fish, red meat, white meat]; non-commercial diet: home-prepared diet, leftovers, table-scraps, other]) that each cat was fed and the frequencies of dietary changes (by type [wet vs. dry], flavour, brand) and provision of treats. Free-text answer boxes were provided to obtain additional details of the types of treats offered to cats. Questions regarding the amount of time spent outside on average within a 24-hour period, the frequency of prey-consumption and the prey species consumed were also included. At the end of the survey, respondents could select whether they lived in the UK or outside of the UK. If within the UK, a further multiple-choice question became available for the respondent to specify the region (East Midlands, East of England, London, North East, North West, Northern Ireland, Scotland, South East, South West, Wales, West Midlands and Yorkshire and the Humber). If respondents lived outside of the UK, they could specify their country of residence in a free-text answer box. A notes section was included at the end of the survey for survey respondents to make any additional comments.

The survey was first drafted as a word document and reviewed by two academics. Alterations and additions were made accordingly and then the document was submitted to the Clinical Ethical Review panel at the University of Nottingham School of Veterinary Medicine and Science (UoN-SVMS). Four members of the panel reviewed the survey proposal and no ethical issues were raised. Upon ethics approval, the survey was designed on Jisc Online Surveys. The survey was then piloted. Based on feedback (n=4), some questions in the survey were adapted to make them more understandable and the responses easier to analyse. The survey was then redistributed online, via social media and cat owner forums, and shared, by email, among staff and students at UoN-SVMS. Respondents could answer the survey for up to three cats that they owned. If they owned more than three cats, they could access the survey again to complete it for any additional cats.

4.2.6. Statistical analysis

Results for the iodine content in euthyroid and hyperthyroid cats' hair were compared using Mann-Whitney U (GenStat v19, VSNi Ltd, Rothamsted UK), due to the non-normal distribution of the data. Cat and dog urine iodine concentrations corrected to urine creatinine concentration were analysed using linear mixed models after normalisation of the distribution by log-transformation of the raw data. Statistical significance was accepted at $P<0.05$. Data are reported as median (IQR: 1st to 3rd quartiles).

Pet food iodine concentration data was log-transformed prior to statistical analysis (GenStat v19; VSNi Ltd., Rothamsted, UK) to normalise the distribution of the data. General ANOVA was used to test whether the iodine concentration was significantly different between cat and dog foods and between wet and dry foods. The interaction between the iodine contents of cat versus dog foods and wet versus dry foods was also tested. Due to unequal group sizes, linear mixed models were used to assess the difference in iodine concentration between wet foods in different packaging types (plastic trays, foil trays, pouches and cans) and to determine any significant difference in the iodine content of foods containing different fish derivatives (fish meat and fish oil). Statistical significance was accepted at $P<0.05$.

Survey responses for cats within the UK and cats outside of the UK were grouped separately for an initial statistical analysis using linear mixed models to test whether geographical location had an effect on feeding habits. Cats within the UK were grouped further, according to their geographical location within the UK (East Midlands, East of England, London, North East, North West, Northern Ireland, Scotland, South East, South West, Wales, West Midlands and Yorkshire and the Humber), and their data were compared using linear mixed models. The survey question regarding the proportion of the commercial diet fed which was wet food, received the most responses and a relatively even distribution of cats over the multiple-choice answer categories, therefore, this data was used for these initial assessments. Respondent ID was applied in a random model to account for cats within each group that may have a shared owner and thus, potentially more similar feeding habits, compared to

cats from single-cat households. Statistical significance was accepted at $P<0.05$.

Pearson chi-square test was used to determine any effect of multi-cat versus single cat households on the feeding habits data, using the survey responses concerning wet food proportions for this model. Additionally, for multi-cat households, Pearson chi-square was used to assess if each cat was more likely to have the same dietary pattern as the other cats in that household. Linn's coefficient of concordance was used to evaluate the correlation between the proportions of wet food fed to two cats within the same household. Kendall's coefficient of concordance, adjusted for ties, was used to assess the degree of agreement between the proportions of wet food fed to three different cats residing within the same household. For these multi-cat analyses, there were n=136 owners that had two cats and n=33 of these owners had a third cat. There were four cats in n=5 households, a fifth cat in n=4 of these households, a sixth cat in n=3 households and 8 cats in n=1 of these. Where there were four or more cats within a household, only responses for up to the first three cats, for which the owner completed the survey, were formally analysed due to insufficient numbers of owners with four or more cats. Statistical significance was accepted at $P<0.05$. To check that the percentage of total cats for which each multiple-choice answer was selected was not skewed by potentially identical feeding practices for multiple cats within a single household, data from only the first cat of each owner was used and the percentages of these cats that represented each multiple choice answer were compared to the percentages obtained when all cats were included. Data from the survey questions regarding the proportion of the commercial diet that is wet food, and the frequency of changing the diet by flavour, were used for these sample investigations.

For further analyses of the categorical survey data, where there was a roughly even distribution of cats among the multiple-choice answer options, rather than a clear 'most common' answer, Pearson chi-square test was used. This was to assess whether the small differences in the number of cats between the multiple-choice answer categories were significant or due to chance.

4.2.7. Power calculation and sample size

Retrospective power calculations were conducted after one year of sample collection. Iodine and creatinine concentrations had been measured in 26 cat and 25 dog urine samples. In order to detect a response of 400.0 (the difference between the two groups' means), at a two-sided significance level of 0.05 with 80% power, 350 urine samples, with iodine concentration adjusted to creatinine, would be required in each study group. This was not feasible in the current study.

For cat hair samples, after one year, iodine content of hair from 12 hyperthyroid cats and 38 euthyroid cats had been measured. In order to detect a response of ~50% of the median, with the accepted two-sided significance level of 0.05 and a power of 80%, each study population would require 32 samples. Therefore, hair samples from a further 20 hyperthyroid cats were required to determine whether the results were likely to be true of the entire populations.

4.3. Results

4.3.1. Human hair iodine concentration

The median (interquartile range) iodine concentration measured in the human hair samples in the current study was 0.364 (0.276–1.632) µg/g DM. The mean hair iodine concentration was 1.21 µg/g DM, with a range of 0.098–4.21 µg/g DM among the individuals.

4.3.2. Iodine content of hair from hyperthyroid and euthyroid domestic cats

Average intra- and inter-assay variation were both 11%, showing good repeatability of iodine measurement in cat hair using our method. The (median [interquartile range] µg/g DM) iodine content of hair from euthyroid cats (0.847 [0.632–1.130]) was significantly greater than that from hyperthyroid cats (0.156 [0.0682–0.703], P=<0.001; Figure 34). Median (interquartile range) ages were 78 (37–96.75) months and 146 (131–163.5) months for euthyroid and hyperthyroid cats, respectively. The majority of euthyroid (n=28; 73.7%) and hyperthyroid cats (n=19; 63.3%) were domestic short-haired. Other breeds included domestic longhair (euthyroid, n=1; hyperthyroid, n=3), Ragdoll (euthyroid, n=4; hyperthyroid, n=1), British shorthair (euthyroid, n=1), Siamese (euthyroid, n=1) and Maine Coon-cross (euthyroid, n=2). The breed was unknown for n=1 (2.6%) euthyroid cats and n=7 (23.3%) hyperthyroid cats.

Figure 34. Iodine concentration in hair from euthyroid and hyperthyroid cats.

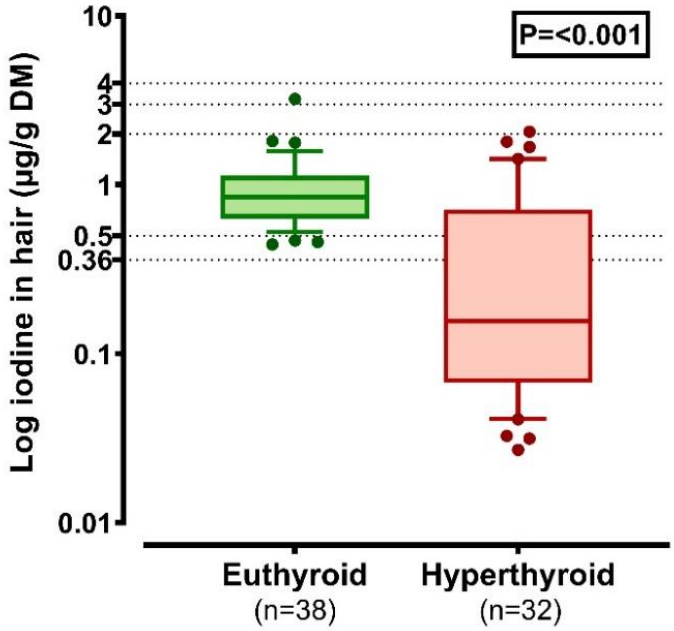


Figure 34. Euthyroid and hyperthyroid cat hair iodine content. Hair was washed consecutively with 3:1 (v/v) ethyl-ether:acetone, 5% EDTA, then purite water and was then dried at 50°C for 48 hours. Washed and dried hair samples (100-200mg) were digested with TMAH and then the iodine concentration was measured using ICP-MS. Data were log_{10}-transformed to normalise the distribution prior to statistical analysis using the Mann-Whitney U test. Statistical significance was accepted at $P<0.05$. Log_{10}-transformed data are presented as box and whisker plots on a logarithmic scale. Boxes indicate the median, lower and upper quartiles and whiskers show the 10^{th} to 90^{th} percentiles. Data outside of this range are presented as individual dots.

4.3.3. Urinary iodine concentration of domestic cats versus dogs

A much wider range of iodine concentrations was measured in dog (1.736–12906 µg/g creatinine), compared to cat (7.220–1594 µg/g creatinine), urine. This was likely due to a few, high outliers in dogs. Overall, urine iodine concentration was not significantly different between domestic cats and dogs (median [interquartile range]: 296.8 [109.9–683.7] and 306.0 [81.59–545.9] µg/g creatinine, respectively; P=0.604; Figure 35). Many more samples would be needed in order to fulfil the requirements determined by the retrospective power calculation. So far, however, it appears that there will not be a statistically significant difference in the urinary iodine concentration between cats and dogs.

Figure 35. Urinary iodine concentrations in domestic dogs and cats.

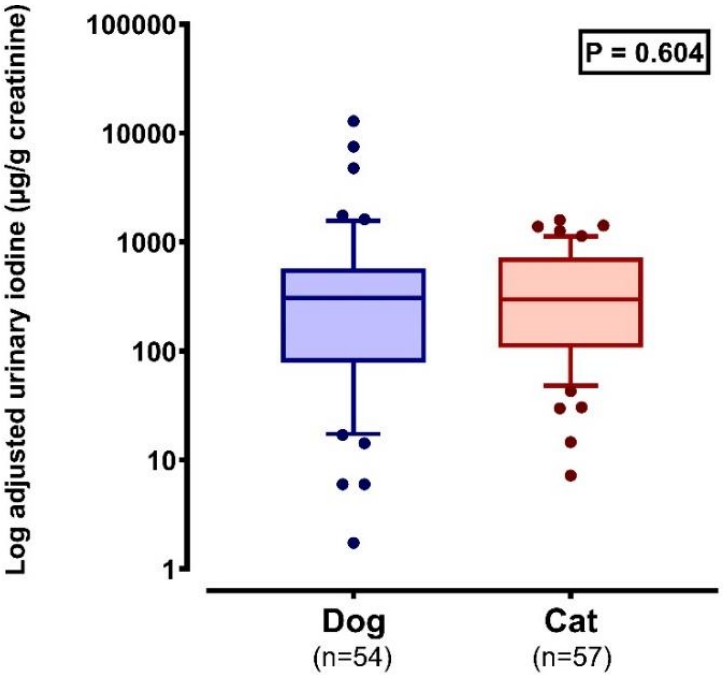


Figure 35. Iodine concentration was measured in neat urine samples (500µl), diluted 1-in-20 with 1% TMAH, from dogs and cats using ICP-MS and adjusted to the creatinine concentration measured in the particular urine sample using RX-Imola. Data were log_{10}-transformed to normalise the distribution prior to statistical analysis using linear mixed models. Statistical significance was accepted at $P<0.05$. Log_{10}-transformed data are presented as box and whisker plots on a logarithmic scale, where boxes indicate the median, lower and upper quartiles and whiskers show the 10^{th} to 90^{th} percentiles. Data outside of this range are presented as individual dots.

4.3.4. Pet food iodine content

Average recovery of the CRM certified level of iodine was 91.2%, with 10.5% inter-assay variation. Intra-assay CV of iodine concentrations measured in eight replicates of one pet food sample and six replicates of another, were 3% and 5%, respectively. Iodine content (mean [minimum–maximum] mg/100g DM) of all foods analysed (dry and wet combined) was significantly (P=0.008) higher in dog foods (0.567 [0.089–4.927]) than in cat foods (0.379 [0.093–1.942]). The dog food with the highest iodine content and responsible for increasing the range of iodine contents across all canine foods, relative to the cat foods, was a wet food for young dogs. The mean (minimum–maximum) of iodine concentrations measured in complete diets for adult dogs and cats was 0.530 (0.089–1.63) mg/100g DM and 0.383 (0.093–0.990) mg/100g DM,

respectively. Both dog and cat wet foods had significantly greater mean iodine content and a wider range of iodine concentrations than dry foods (0.571 [0.089–4.927] and 0.302 [0.093–0.918] mg iodine/100g DM, respectively; P=<0.001. The iodine concentrations of wet and dry pet foods showed the same patterns for both dog and cat foods (P=0.332; Figure 36a).

74.7% (59/79) of dry and 84.8% (89/105) of wet foods analysed complied with recommended levels of iodine in cat or dog food (e.g. minimum for adult cat and dog foods: 0.17 and 0.12 mg/100g DM, respectively; minimum for foods for growth and reproduction: 0.18 (kitten) and 0.15 (puppy) mg/100g DM; EU legal maximum: 1.1 mg/100g DM (FEDIAF, 2019)). All dry foods that were non-compliant (n=20; 25.3%) had iodine levels below the recommended nutritional minimum. Only four (20.0%) of these non-compliant dry foods were for dogs, whereas 16 (80.0%) were for cats. Five (4.76%; 2 dog, 3 cat) wet foods failed to provide the nutritional minimum and eleven (10.5%; 8 dog, 3 cat) wet foods exceeded the nutritional maximum guidelines (Figure 36b). Overall, 81.3% (61/75) of dog foods and 79.8% (87/109) of cat foods analysed complied with both the minimum and maximum level of iodine in feed as recommended by FEDIAF (Figure 36c).

Overall, there was no significant difference in the iodine content of wet foods packaged in plastic trays, foil trays, pouches or cans (P=0.070; Figure 36d). No association was found between any particular main flavour (listed as the main ingredient by weight) and the iodine content of the pet foods (Figure 37a). Investigated further, pet foods containing fish and/or fish oil did not contain higher levels of iodine than those without fish derivatives (P=0.633; Figure 37b). Moreover, pet food iodine content did not correlate strongly with the percentage of fish in the feed (Figure 37c).

4.3.5. Pet food batch-to-batch variation in iodine content

The iodine content of a particular cat food appears to vary between its batches. There was greater variation in the amount of iodine between the batches of foods with high iodine concentration, compared to those with low iodine content (Figure 38). The iodine concentration in batches of high iodine foods

(n=4) had between 14.5 and 31.2 % variation around the mean iodine content. This was similar to 13.8–26.2 % variation around the mean for low iodine foods (n=3). Overall, the average batch-to-batch percentage variation in iodine content for the four high iodine foods and that of the four low iodine foods, was similar (Table 9). Some batches of some foods had levels of iodine above the legal maximum or below the recommended minimum level that cat foods should contain, according to FEDIAF (Figure 38).

Figure 36. Iodine content of complete, wet and dry, dog and cat foods.

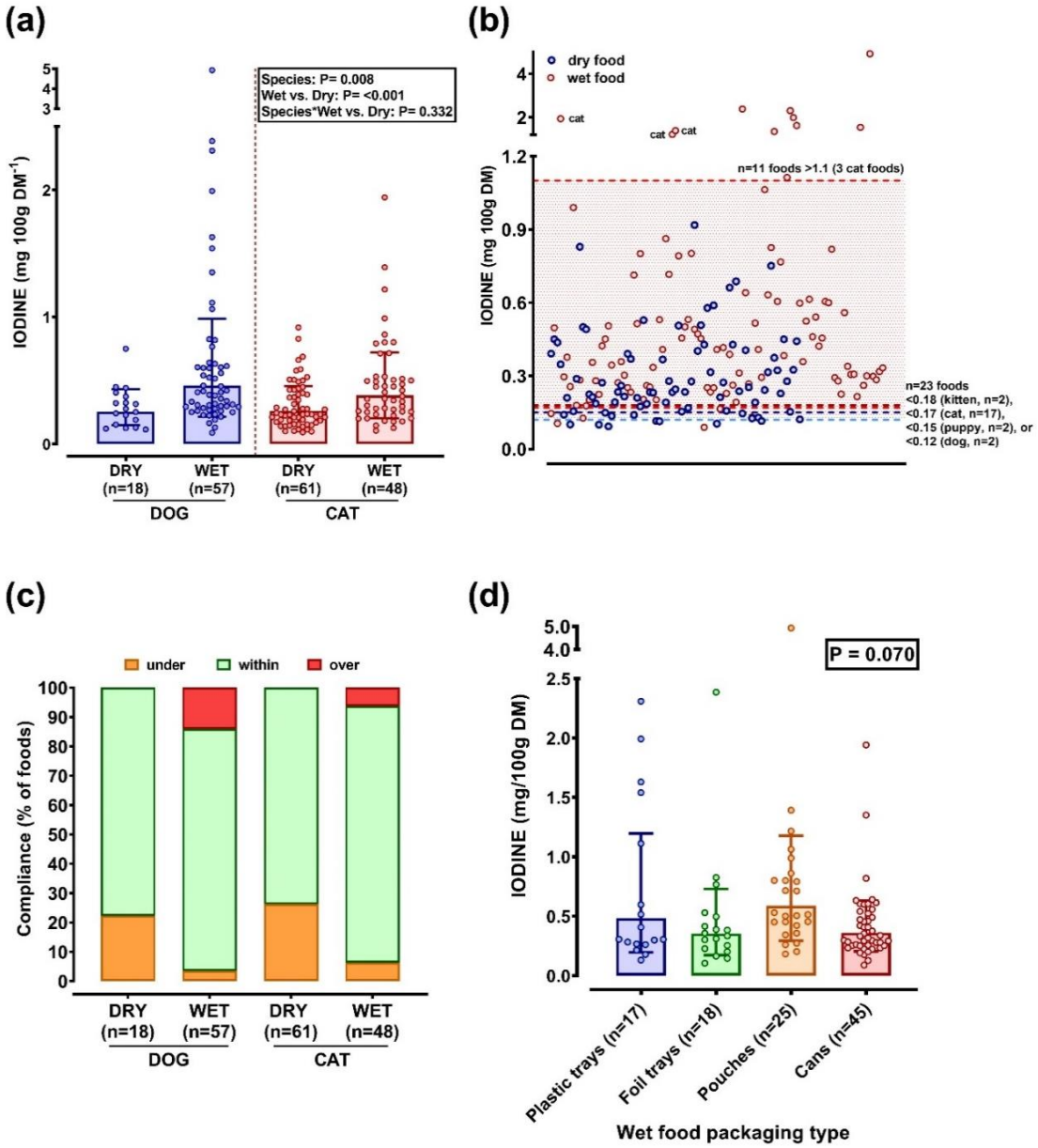


Figure 36. Iodine content of complete, wet and dry, dog and cat foods. Freeze-dried wet and dry pet foods were homogenised and duplicate samples (100-200mg) were digested with TMAH for subsequent analysis of their iodine content by ICP-MS. **(a)** Iodine content of wet and dry, dog and cat foods where each food is represented by an individual dot, boxes show the geometric mean and whiskers show the geometric standard deviation of the data. Data were log_{10}-transformed prior to statistical analysis to normalise the distribution. General ANOVA was used to evaluate the difference in iodine content between cat and dog foods (Species), between all wet and all dry foods (Wet vs. Dry) and between wet versus dry foods for cats and dogs separately (Species*Wet vs. Dry). **(b)** Assessment of compliance of individual wet (red dots) and dry (blue dots) pet foods with FEDIAF recommendations for the maximum (1.1 mg/100g DM) and minimum (0.18 [kitten], 0.17 [cat], 0.15 [puppy] and 0.12 [dog] mg/100g DM) iodine content of pet foods, represented by dotted lines. **(c)** Breakdown of the percentages of the wet and dry, cat and dog foods analysed with iodine contents within the range recommended by FEDIAF, under the minimum recommendation and over the legal maximum. **(d)** Iodine content of wet foods grouped according to packaging type (plastic trays, foil trays, pouches and cans). Dots represent individual foods, boxes show the geometric mean and whiskers show the geometric standard deviation of the data. Data were log_{10}-transformed to normalise the distribution then linear mixed models were used to evaluate the difference in iodine content between the wet foods in various packaging types. Statistical significance was accepted at $P<0.05$.

Figure 37. Iodine content of complete, wet and dry, dog and cat foods, grouped according to main ingredients.

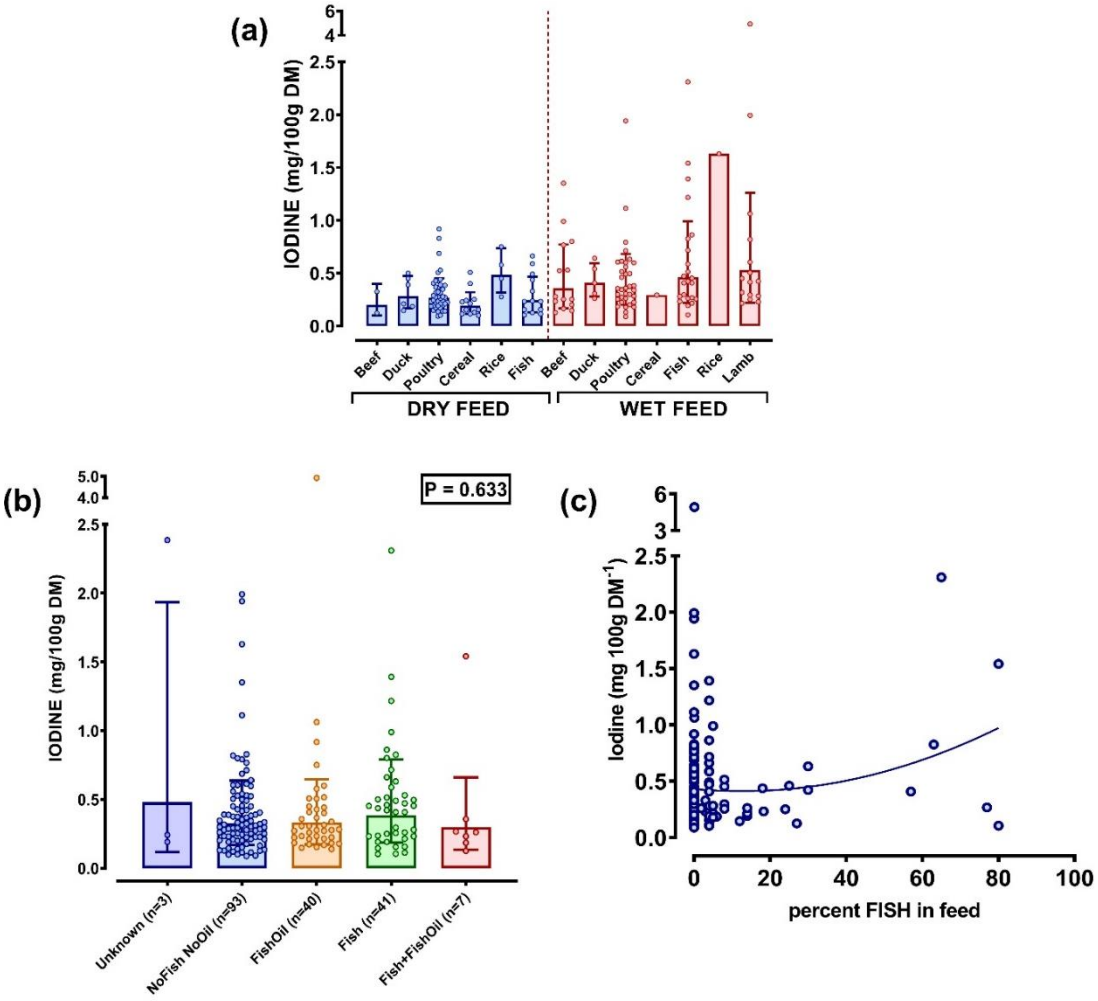


Figure 37. Iodine content of complete, wet and dry, dog and cat foods, grouped according to main ingredients. Freeze-dried pet foods were homogenised and duplicate samples (100-200mg) were TMAH-digested for subsequent analysis of their iodine content by ICP-MS. **(a)** Iodine content of dry and wet foods, separated by main ingredient, as listed by weight on the packaging. Each dot represents a single pet food; boxes and whiskers represent the geometric mean and standard deviation, respectively. **(b)** Iodine content of all wet and dry, cat and dog foods combined, separated by incorporation of fish-derivatives (fish meat [Fish], fish oil [FishOil], neither [NoFishNoOil], or both [Fish+FishOil]). Inclusion of fish derivatives in three pet foods was unknown. Each dot represents a single pet food; boxes and whiskers represent the geometric mean and standard deviation, respectively. Data were log_{10}-transformed to normalise the distribution, then analysed using linear mixed models. Statistical significance was accepted at $P<0.05$. **(c)** Scatterplot of the percentage incorporation of fish derivatives against the food iodine content. Each food is represented by a separate dot.

Figure 38. Batch-to-batch variation in the iodine concentration of complete wet foods for pet cats.

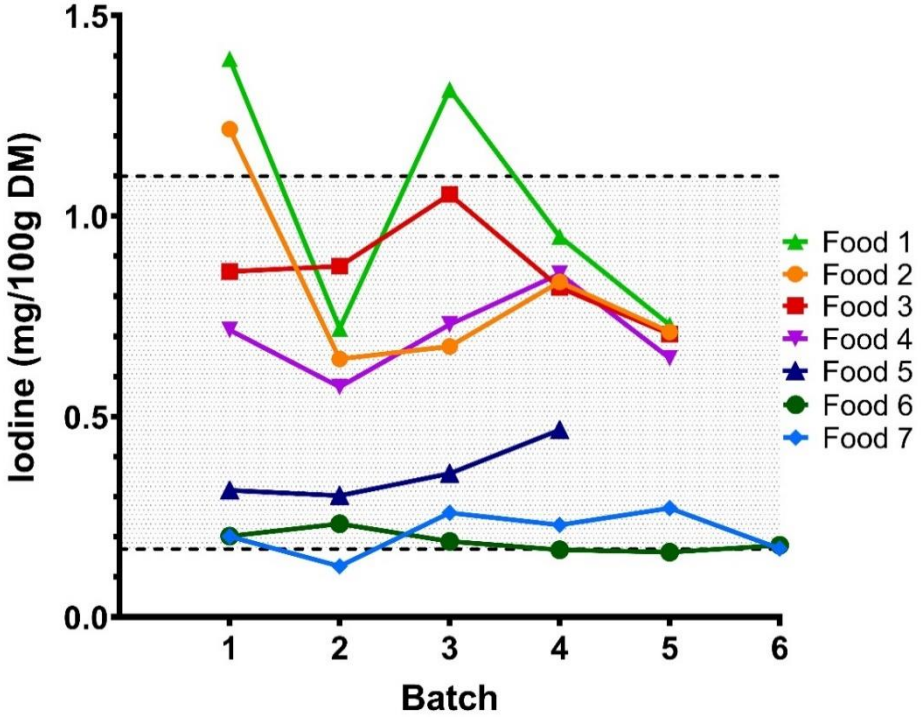


Figure 38. Between four and six subsequent batches of seven different complete, wet foods for adult cats were purchased over time. Iodine was measured by ICP-MS in duplicate samples (100-200mg) of the freeze-dried, homogenised, TMAH-digested foods and corrected to the iodine concentration measured in duplicate TMAH-digested blank samples and certified reference material (CRM; SERO210705 Seronorm Trace Elements Urine L-2; LGC Standards). Dashed lines show the legal maximum and recommended minimum level of iodine that complete foods for adult cats should contain, according to FEDIAF.

Food	Iodine content	Iodine range among batches (mg/100g DM)	Coefficient of variation, CV (%)	Average CV (%)
1	High	0.573	28.8	22.4
2	High	0.348	14.5	
3	High	0.673	31.2	
4	High	0.282	14.9	
5	Low	0.145	26.2	20.2
6	Low	0.071	13.8	
7	Low	0.165	20.7	

Table 9. Variation in the iodine concentration among subsequent batches of wet cat foods (n=7) with either high or low iodine concentration. Data presented here are the range and coefficient of variation (CV) of iodine concentration across the batches of each food, and the average CV of iodine concentration among all batches of all foods with high iodine content and among all batches of all foods with low iodine content. Iodine concentration was measured by ICP-MS in duplicate samples (100-200mg) of freeze-dried, homogenised, TMAH-digested foods and corrected to the iodine concentration measured in duplicate TMAH-digested blank samples and duplicate certified reference material (CRM; SERO210705 Seronorm Trace Elements Urine L-2; LGC Standards).

4.3.6. Survey of cat owner feeding habits

The survey was completed by a total of 362 cat owners for 421 cats in the UK and 129 cats which reside outside of the UK. Nearly half (59 cats; 45.7%) of the latter group were from the USA, with smaller proportions coming from Australia (9 cats), Belgium (4 cats), Brazil (4 cats), Canada (20 cats), Denmark (1 cat), Dominican Republic (1 cat), Egypt (1 cat), France (4 cats), Germany (1 cat), Ireland (5 cats), Lithuania (1 cat), Mauritius (1 cat), Mexico (1 cat), Netherlands (7 cats), New Zealand (1 cat), Philippines (1 cat), Singapore (1 cat), Switzerland (3 cats) and UAE (2 cats). Of the cats from the UK for which the region was specified, 88 were from the East Midlands, 14 from the East of England, 35 from London, 10 from the North East, 18 from the North West, 9 from Northern Ireland, 16 from Scotland, 76 from the South East, 76 from the South West, 7 from Wales, 40 from the West Midlands and 28 from Yorkshire and the Humber. No significant difference in the proportion of wet commercial cat food fed was found between cats residing within the UK and those outside of the UK (P=0.773). Similarly, feeding habits did not differ significantly between cats in different geographical regions within the UK (P=0.419).

Most (n=381; 69.4%) of the cats for which the survey was completed, were domestic shorthaired cats; 57 (10.4%) were domestic longhaired cats and the rest were other purebred cats and cross breeds. Owners of 530 cats provided information about the sex and neutered status of their cats (spayed female: n=263, 49.6%; entire female: n=11, 2.08%; castrated male: n=246, 46.4%; entire male: n=10, 1.89%). The mean (± standard deviation) age of the cats was 7.28 (±4.87) years (median [range]: 7.02 [0.033–23.2] years). According to cat life-stages, there were 44 kittens (<1 year), 220 adults (1-7 years), 134 senior cats (>7-11 years), 110 geriatric cats (>11-16 years) and 24 cats older than 16 years of age (>16-24). Eighty-four (15%) cats were reported to be ‘unhealthy’ (i.e. had a disease recognised by their vet, and/or signs of illness). In cats with ill-health, the most common diseases reported were hyperthyroidism (n=16; 19.0% cats), CKD (n=13; 15.5% cats), arthritis (n=12; 14.3% cats), inflammatory bowel disease (n=7; 8.33% cats) and diabetes (n=6; 7.14% cats). There were 60 other reports of 39 different conditions or

symptoms. Prescription diets were fed to 77 (14%) cats, which included 32 cats with reported ill-health and 45 healthy cats.

The survey was completed for 222 cats that did not live with another cat and 327 cats that lived in multi-cat households (n=138 households). Most (n=104; 75.4%) owners that completed the survey for multiple cats had two cats, 27 (19.6%) owners had three cats, two (1.45%) multi-cat owners completed the survey for four, five, and six cats in each case and one (0.725%) owner had eight cats. The proportion of wet food that constituted the commercial diet fed to cats was not significantly different between cats in multi-cat households and cats that did not live with other cats (χ^2 [4 d.f., n=535] =2.89, P=0.576; Table 10). For cats in multi-cat households, there was no significant difference in the proportions of wet food fed to cat 2 or cat 3, versus cat 1 (χ^2 [8 *d.f.*, n=33] =2.4, P=0.96). Indeed, Linn's coefficient of concordance showed that, in households with at least two cats, the proportion of wet food fed to cat 2 was highly concordant with that fed to cat 1 (Cb 0.89, n=136 households, n=272 cats; Table 10). In households with three or more cats, cats 2 and 3 were highly likely to be fed the same proportion of wet food as cat 1 in that household (Kendall's coefficient of concordance, adjusted for ties: 0.72; χ^2 [32 *d.f.,* n=33] =69, P=<0.001; Table 10). For the five owners with ≥4 cats that completed the survey question concerning the proportion of wet food in the commercial diet, responses for cats within these households were 100% concordant. However, the distribution of cats over the selection of multiple-choice answers for the questions regarding 'proportion of wet food' and 'frequency of changing the diet by flavour' was unaffected by including all data for all cats in single- and multi-cat households, or just data for the first cat in each household (Table 11). Thus, for further data presentation and analyses, we retained responses for all cats and the results of this survey are expressed as 'number of cats' rather than 'number of owners'.

Proportion of commercial diet that is wet food	Single cat household	Multi-cat household	Cat 1	Cat 2	Cat 3
	n=217 owners n=217 cats	n=140 owners n=318 cats	n=353 owners n=353 cats	n=136 owners n=272 cats	n=33 owners n=99 cats
'All'	11 (5.06)	17 (5.35)	20 (5.67)	7 (5.19)	1 (3.03)
'More than half'	43 (19.8)	68 (21.4)	76 (21.5)	27 (20.0)	5 (15.1)
'Half'	72 (33.2)	84 (26.4)	105 (29.7)	39 (28.9)	9 (27.2)
'Less than half'	42 (19.3)	69 (21.7)	69 (19.5)	30 (22.2)	9 (27.2)
'None'	49 (22.6)	80 (25.2)	83 (23.5)	32 (23.7)	9 (27.2)
P-value	0.576		0.960		

Table 10. The proportion of the commercial diet that is wet food, for cats in single cat households and cats in multi-cat households. A survey of cat feeding habits was created using Jisc Online Surveys and data were collected from n=362 owners of a total of n=550 cats. A multiple-choice question concerning the proportion of wet food (all, more than half, half, less than half, none) in the cat's commercial diet was completed by n=357 respondents for n=535 cats. The data presented here are number of cats (% of total cats for that column). The Pearson chi-square test was used to determine whether the proportion of wet food in the cat's commercial diet was different for cats that lived in multi-cat households and cats that did not live with other cats (χ^2 [4 *d.f.,* n=535] =2.89, P=0.576) and for individual cats within the same household where there were up to 3 cats (χ^2 [8 *d.f.*, n=33] =2.4, P=0.96).

Proportion of wet food	All cats	1st cat only	Frequency of flavour changes	All cats	1st cat only
	n=535 cats	n=353 cats		n=535 cats	n=353 cats
'All'	28 (5.2)	20 (5.7)	**'Daily'**	179 (34.2)	117 (33.6)
'More than half'	111 (20.7)	76 (21.5)	**'A few times a week'**	96 (18.3)	63 (18.1)
'Half'	156 (29.0)	105 (29.7)	**'Weekly'**	36 (6.9)	23 (6.6)
'Less than half'	111 (20.7)	69 (19.5)	**'Monthly'**	35 (6.7)	25 (7.2)
'None'	129 (24.1)	83 (23.5)	**'Less than monthly'**	60 (11.5)	44 (12.6)
			'Never'	118 (22.5)	76 (21.8)

Table 11. Does inclusion of all data from all cats, including those that live in the same house, affect the result compared to only using data from one cat per household? The 'proportion of wet food' and the 'frequency of flavour changes' in the commercial diet fed to cats, when data from all cats for which the survey was completed, including those within the same household, was analysed, versus when data for just the first cat reported by each owner were analysed. The survey of cat feeding habits was created using Jisc Online Surveys. The multiple-choice questions concerning the proportion of wet food ('All', 'More than half', 'Half', 'Less than half', 'None') in the cat's commercial diet and the frequency of flavour changes ('Daily', 'A few times a week', 'Weekly', 'Monthly', 'Less than monthly', 'Never') were completed by n=353 respondents for n=535 cats. The data presented here are number of cats (% of total cats for that column) when data from all cats are considered and when just the first cat reported by the owner in each survey was used.

The majority (463 of 549; 84.3%) of cats for which the survey was completed, were fed only commercially available cat food as their main diet. For 9.3% of cats, commercial cat food was said to make up more than half of the cats' diet. Half, less than half, or none, of the diet was made up of commercial cat food for very few cats (1.8%, 2.4% and 2.2%, respectively) (Figure 39a). Where commercial cat food did not comprise the entirety of the cats' diet, the remainder was a mixture of home-prepared diets (30 of 86 [34.9%] cats), table scraps (31 of 86 [36.0%] cats), leftovers (29 of 86 [33.7%] cats), or other items such as other human foods (21 of 86 [24.4%] cats) or prey items (16 of 86 [18.6%] cats).

The number of cats fed wet food as 'all' or 'half' of the commercial diet that they received varied significantly (χ^2 [4 d.f., n=535] =85.59, P=<0.001) from the number that would be expected if all the cats in the study were evenly distributed among the groups. The biggest contribution to this significant observed effect was feeding exclusively wet food ('all'), which was much less common than expected; only 28 (5.2%) of 535 cats were fed in this way. In contrast, feeding only commercial dry food (i.e. no wet food) is relatively common (129 of 535; 24.1% cats). Feeding 'half' of the commercial diet as wet food, meaning the remaining half is dry food, was significantly more common than expected and the most common way cats that ate commercial food in this study were reported to be fed (156 of 535; 29% cats). Feeding more than half or less than half of the commercial diet as wet food, with the remainder being commercial dry food, were also reasonably common (111 of 535; 20.7% cats) but the observed result was not different from that which was expected (Figure 39b).

Figure 39. Commercial cat food constitutes the entire diet for most cats and a combination of wet and dry commercial cat foods are often fed.

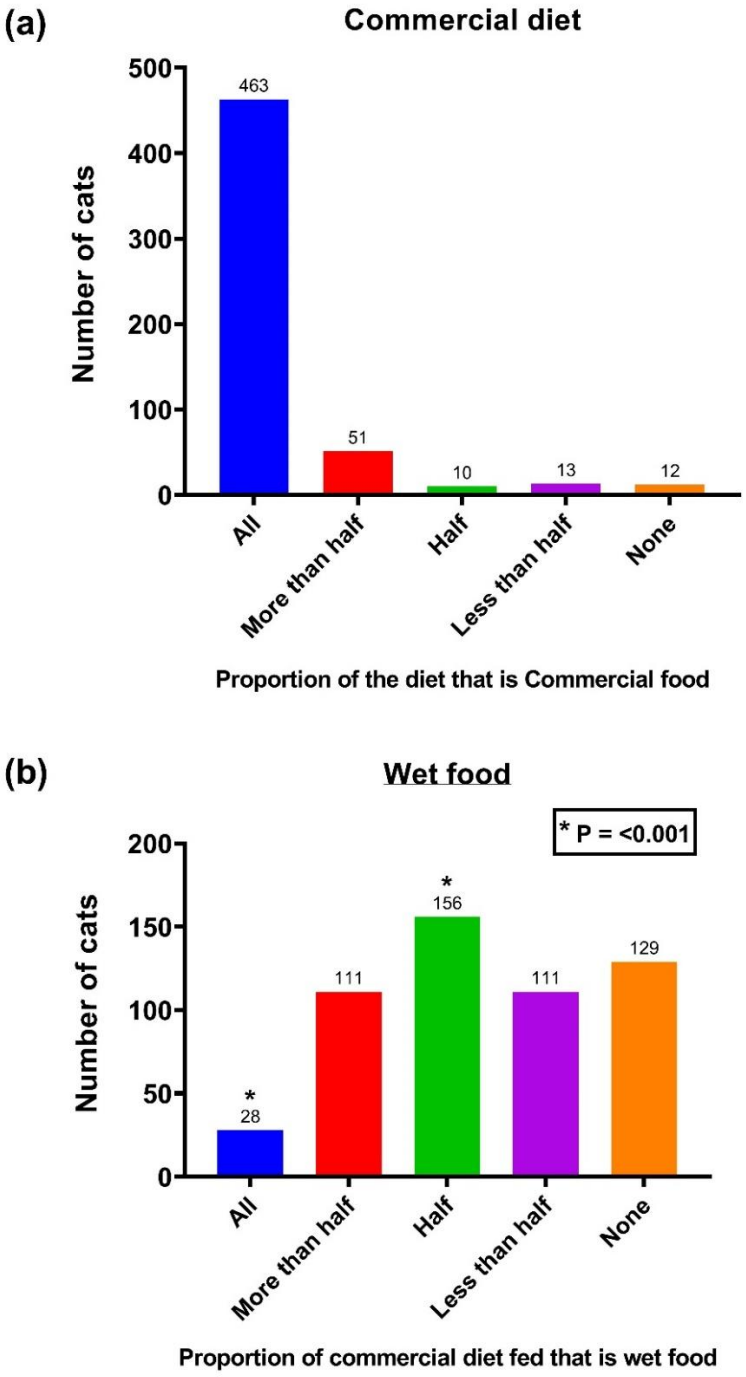


Figure 39. (a) Survey responses from n=362 cat owners about how much of their cats' (n=549) diet is made up of commercially available cat food. **(b)** Proportion of the cat's commercial diet that is wet, as opposed to dry, cat food. This multiple-choice question was completed by n=355 cat owners of a total of n=535 cats. The survey of cat feeding habits was created using Jisc Online Surveys. The data collected are presented as bar graphs where bars represent the multiple-choice answer options ('All', 'More than half', 'Half', 'Less than half' and 'None') and the numbers above the bars indicate the number of cats for which the particular response option was selected. Data were analysed using Pearson chi-square (χ^2 [4 d.f., n=535] =85.59, P=<0.001) and the multiple-choice responses that were identified as significant contributions to the chi-square statistic are indicated with an asterisk (*) above the relevant bars.

If wet food in only one packaging type was fed to cats, this would most likely be in pouches (210 of 394; 53.3% cats), followed by cans (71 of 394; 18% cats). It appears uncommon for cats to be fed wet food only in foil trays (7 of 394; 1.8% cats). For wet food packaged in cans and foil trays, it was most common that cats did not receive any food of this type (236 of 394 [59.9%] and 331 of 394 [84%] cats receive no wet food in cans and foil trays, respectively), whereas only 88 (22.3%) cats did not receive any wet food packaged in pouches. Very few cats (9 of 394; 2.3%) were fed wet food in another packaging type, which was plastic packaging in most of these cases (Figure 40). 105 (26.6%) cats were fed wet food in a combination of packaging types.

Meat-flavoured foods were fed more commonly than fish-flavoured foods. It appears most common for fish to make up less than half, none, or half of a cat's diet with 168 (31%), 161 (29.8%) and 155 (28.7%) cats receiving these proportions of their diet as fish-flavoured foods, respectively. In each of these cases, the remaining proportion of the diet would be meat-flavoured foods. Only 14 of 541 (2.6%) cats were said to eat only fish-, and no meat-, flavoured foods, whereas 161 of 541 (29.8%) cats ate only meat-, and no fish-, flavoured foods. Likewise, more than half of the diet comprised fish-flavoured foods for only 43 (7.9%) cats, but meat-flavoured foods made up more than half of the diet for 168 (31%) cats. Of the meat that is fed to cats, this is more commonly white meat (e.g. chicken, turkey), as opposed to red meat (e.g. beef, lamb, pork) flavours. White meat-flavoured foods constituted all, or more than half, of the meat-flavoured foods that 145 (29.8%) and 141 (29.0%) cats received, respectively. In these cases the lesser proportion of meat consumed would be red meat. While it was common for cats not to receive any red meat, and all white meat, flavoured foods (n=145 [29.8%] cats), it was uncommon for cats to receive no white meat-, and all red meat-, flavoured foods (n=4 [0.82%] cats). A third (164 of 486; 33.7%) of cats were said to receive half white and half red meat as their meat intake (Figure 41).

Figure 40. Proportions of commercially available wet cat food fed to cats that is packaged in cans/tins, pouches, foil trays, or other packaging.

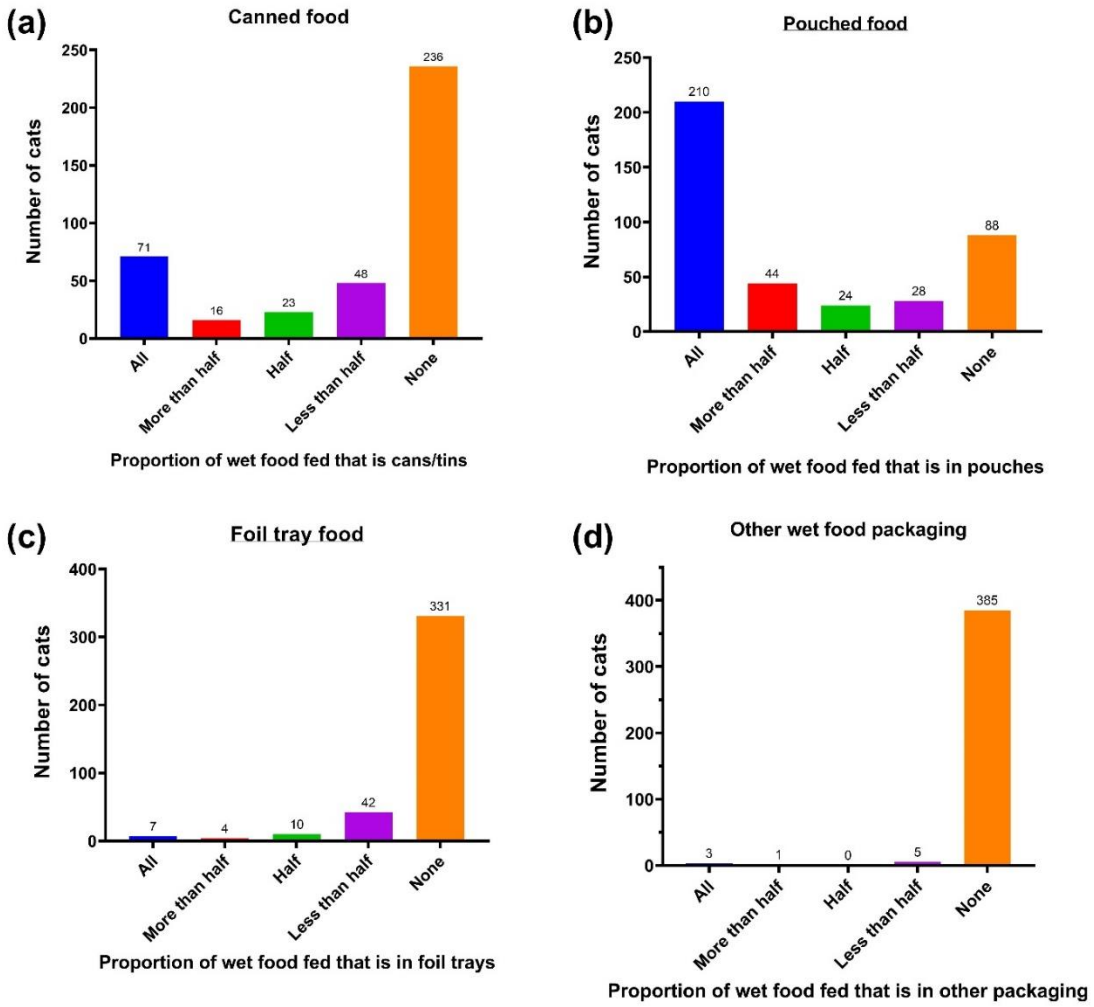


Figure 40. Proportions of commercially available wet cat food fed to cats that is packaged in **(a)** cans/tins, **(b)** pouches, **(c)** foil trays, or **(d)** other packaging. The survey was created using Jisc Online Surveys and this multiple-choice question was completed for n=394 cats. Bars represent the multiple choice answer options ('All', 'More than half', 'Half', 'Less than half', 'None'), with the numbers above the bars indicating the number of cats for which the particular response option was selected.

Figure 41. Proportions of fish, red meat and white meat-flavoured foods fed to cats.

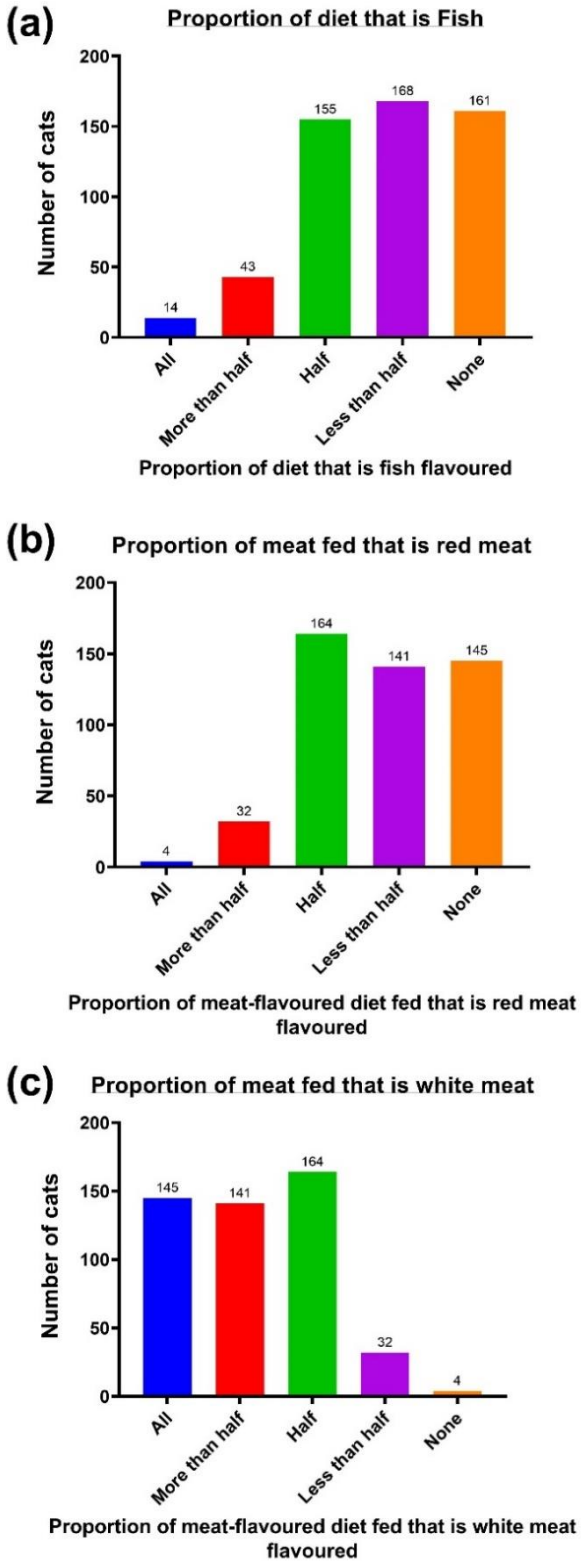


Figure 41. Proportions of food fed to cats, that is primarily **(a)** fish-, as opposed to meat-flavoured and, of the meat-flavoured food that is fed, the proportion that is **(b)** red meat (beef, lamb, pork) and **(c)** white meat (chicken, turkey) flavoured. The survey was created using Jisc Online Surveys and these multiple-choice questions were completed for n=541 cats (fish) and n=484 cats (red vs. white meat). Bars represent the multiple choice answer options ('All', 'More than half', 'Half', 'Less than half', 'None'), with the numbers above the bars indicating the number of cats for which the particular response option was selected.

Only 83 of 524 (15.8%) cats were reported to never experience dietary changes of any form. There was significant variation in the frequency at which the brand of cat food was changed for the cats in the current study (χ^2 [5 d.f., n=524] =424.89, P=<0.001). The brand of pet food was claimed to never change for nearly half (240 of 524 [45.8%]) of cats. If the brand of pet food was changed for a particular cat, this was done less than monthly in most cases (141 [26.9%]). It appears less common to change the brand of food on a more regular basis (daily, 7.6% cats; a few times a week, 7.1% cats; weekly, 4.2% cats; monthly, 8.4% cats; Figure 42a).

The type of food (wet/dry) that a cat receives was never changed for 175 (33.4%) cats and most commonly changed on a daily basis, with 266 of 524 (50.8%) cats being given both wet and dry food each day. In contrast, only 3.8%, 1.3%, 2.5% and 8.2% of cats had the type of cat food (wet/dry) changed a few times a week, weekly, monthly and less than monthly, respectively. The number of cats for which each multiple-choice answer, relating to the 'frequency of changing the diet by type', applied differed significantly from the expected outcome, as tested by chi-square (χ^2 [5 d.f., n=528] =674.32, P=<0.001; Figure 42b).

It was also most common to change the flavour of pet food on a daily basis (179 of 524 [34.2%] cats) and least common to change it weekly (6.9% cats) or monthly (6.7% cats). These frequencies of flavour changes contributed significantly to the variation from the expected distribution, tested by chi-square (χ^2 [5 d.f., n=524] =177.93, P=<0.001). It was not uncommon to vary the flavour of food a few times a week (18.3% cats) or less than monthly (11.5% cats). The flavour of food was never changed for 118 (22.5%) cats. (Figure 42c).

Figure 42. Frequency of changing the cat's commercially available cat food by brand, type (wet vs. dry diets) and flavour.

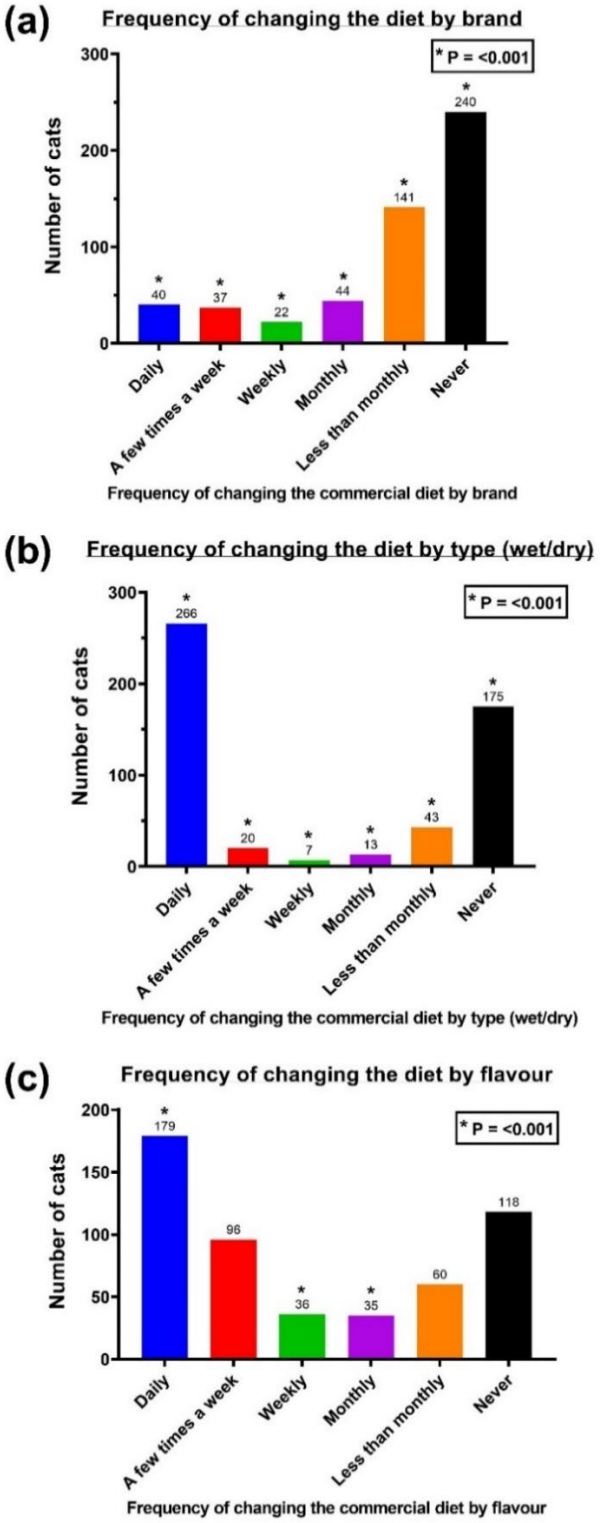


Figure 42. Frequency of changing the cat's commercially available cat food by **(a)** brand, **(b)** type (wet vs. dry diets) and **(c)** flavour.The survey was created using Jisc Online Surveys and these multiple choice questions were completed for n=524 cats. Bars represent the multiple-choice answer options ('Daily', 'A few times a week', 'Weekly', 'Monthly', 'Less than monthly', 'Never'), with the numbers above the bars indicating the number of cats for which this response option was true. Data were analysed using Pearson chi-square (χ^2 [5 d.f., n=524] =424.89 [**(a)** brand]; χ^2 [5 d.f., n=528] =674.32 [**(b)** type]; (χ^2 [5 d.f., n=524] =177.93 [**(c)** flavour]; P=<0.001) and the multiple choice responses that were identified as significant contributions to the chi-square statistic are indicated with an asterisk (*) above the relevant bars.

There was significant variation in the frequency at which cats are fed (χ^2 [4 d.f., n=542] =221.43, P=<0.001). Most cats (n=188; 34.7%) were said to be fed twice a day, however, some respondents that said that they fed their cats once, twice, three times, or more frequently per day specified in the survey notes section that their cats (n=103; 19%) were also provided with dry food ad libitum. Therefore, these responses were grouped together, alongside the 'food is always freely available' multiple-choice answer, and categorised as 'ad-lib'. When this was done, ad-lib feeding (n=189; 34.9% cats) was as common as feeding twice a day. Very few cats (n=17; 3.1%) were fed only once per day and feeding more than 3 times a day was also significantly less common than expected if the distribution of cats among the multiple-choice categories had been even (χ^2 [4 d.f., n=542] =221.43, P=<0.001; Figure 43a). When asked if their cat always eats all of the food provided, owners of 239 (44.9%) cats responded with 'most of the time', while fewer cats always (28.6%), sometimes (18.2%) or never (8.3%) eat all of the food provided. The multiple-choice answers 'most of the time' and 'no, never' contributed most significantly to the variation among the groups (χ^2 [3 d.f., n=532] =156.50, P=<0.001), while the effect of 'sometimes' was less significant (χ^2 [3 d.f., n=532] =156.50, P=0.025). The number of cats for which the response 'yes, always' was selected, did not differ from the expected outcome (Figure 43b).

Most (435 of 539 [80.7%]) cats receive treats. These treats are given at a frequency of more than 5 a day (36 of 435 [8.3%] cats), 2-5 a day (78 of 435 [17.9%] cats), 1 a day (54 of 435 [12.4%] cats), 4-6 per week (67 of 435 [15.4%] cats), 1-3 per week (109 of 435 [25.1%] cats) or less than 1 per week (91 of 435 [20.9%] cats). A fifth (104 of 539 [19.3%]) of cats never receive treats (Figure 44a). Where cat owners specified the type of treats that they fed to their cats, the most common was commercially available complimentary dry food treats, fed to 331 of 410 (80.7%) cats (Figure 44b); in the majority of cases (204 of 331 [61.6%] cats) these complimentary dry treats were Dreamies (Figure 44d). Human food was fed as treats to 103 of 410 (25.1%) cats (Figure 44b) and was most commonly meat (70 of 103 [68.0%] cats), although fish (28 of 103 [27.2%] cats) and dairy products (29 of 103 [28.2%] cats) were also common human food treats (Figure 44c). Complimentary semi-moist treats,

such as “meat sticks” were the third most common category of treat mentioned; fed to 50 of 410 (12.2%) cats (Figure 44b).

Figure 43. Frequency of feeding and how often cats eat all of the food provided.

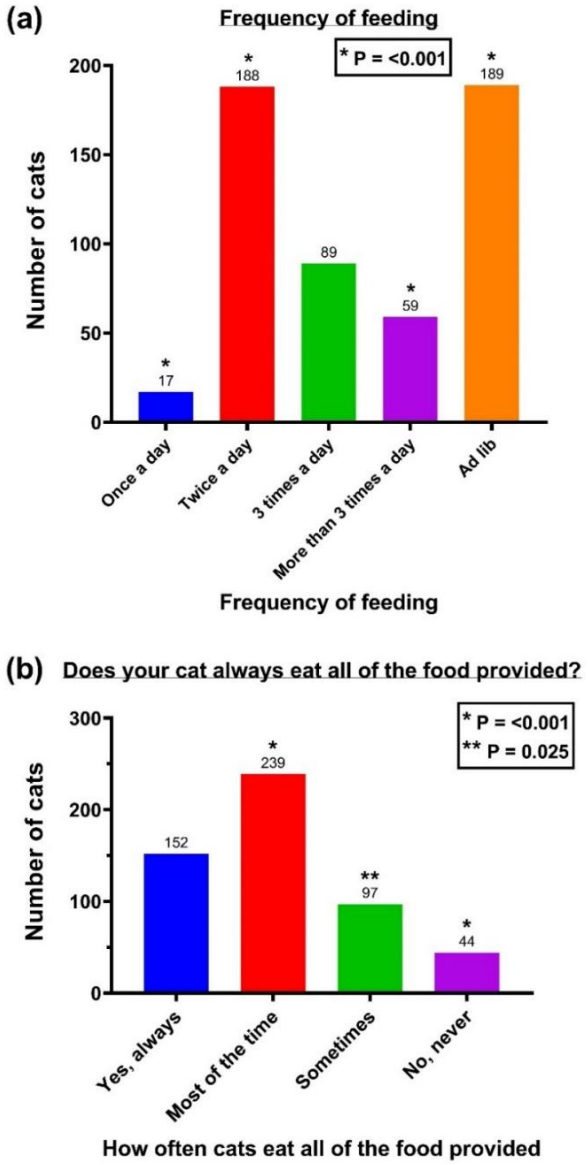


Figure 43. (a) Frequency of feeding and **(b)** how often cats eat all of the food provided. A survey of cat feeding habits was created using Jisc Online Surveys. The data collected are presented as bar graphs, where bars represent the answers given (**(a)** multiple-choice answers ‘Once a day’, ‘Twice a day’, ‘3 times a day’, ‘More than 3 times a day’ and ‘Ad lib’, where the latter category comprised all cats for which their owners had selected the ‘food is always freely available’ multiple-choice answer or had specified giving dry food ad libitum **(b)** multiple-choice answers ‘Yes, always’, ‘Most of the time’, ‘Sometimes’, ‘No, never’). The numbers above the bars indicate the number of cats for which the particular response option was selected. Data were analysed using Pearson chi-square (χ^2 [4 d.f., n=542] =221.43, P=<0.001 [**(a)**]; (χ^2 [3 d.f., n=532] =156.50, P=<0.001 or P=0.025 [**(b)**]). A single asterisk (*) above a bar indicates a multiple-choice response that contributed to the chi-square statistic at a significance level of P=<0.001; two asterisks (**) indicates a multiple-choice response that contributed to the chi-square statistic at a significance level of P=0.025.

Figure 44. Treats given to cats.

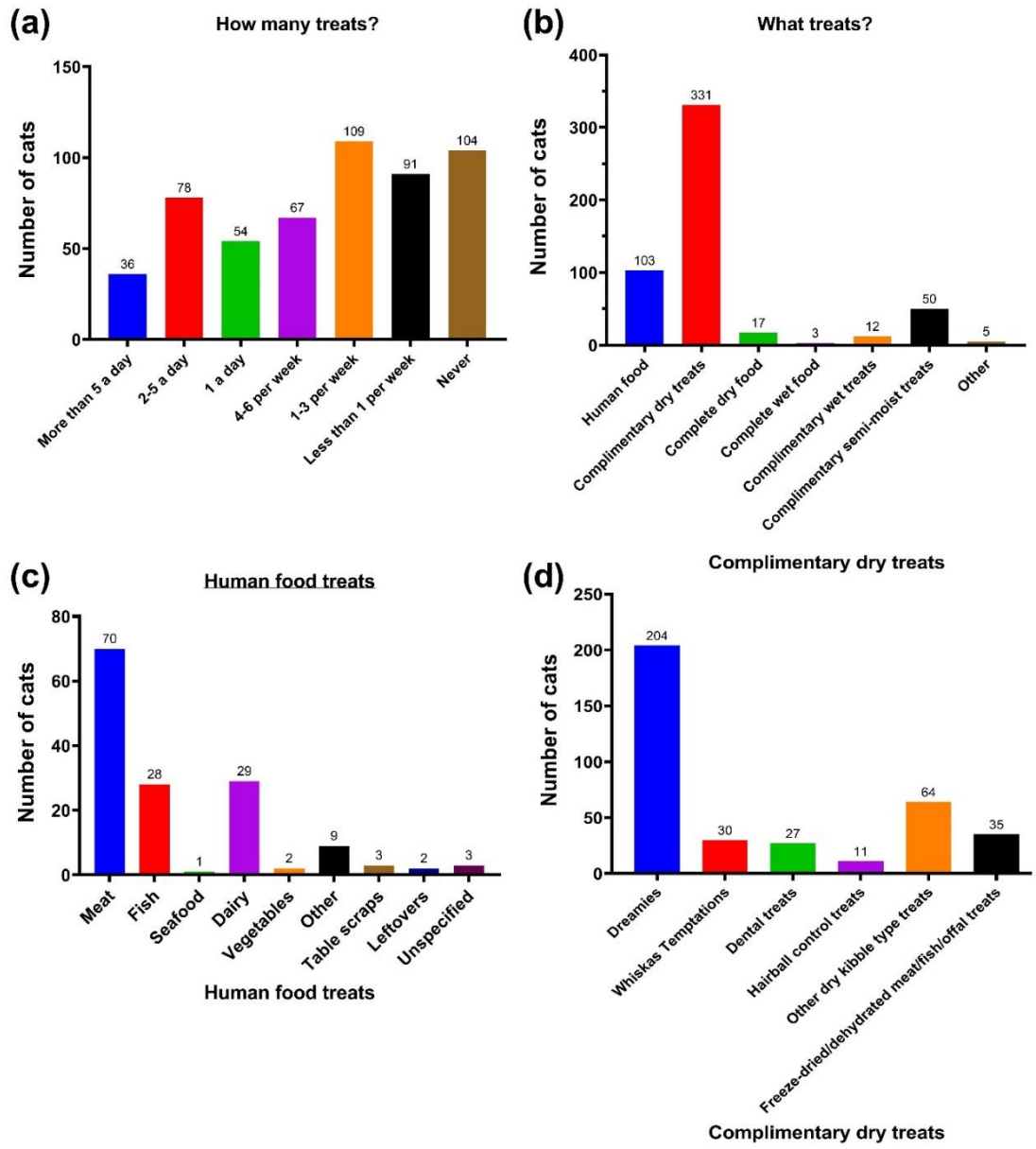


Figure 44. The **(a)** amount and **(b)** type of treats given to cats and the particular types of **(c)** human food treats and **(d)** complimentary dry treats that are given. A survey of cat feeding habits was created using Jisc Online Surveys. Graph **(a)** was created from a multiple-choice question where the bars represent the response options, while graphs **(b)**, **(c)** and **(d)** were created from free-text answers where all responses were subsequently grouped into the categories depicted by the bars. The numbers above the bars indicate the number of cats for which each particular response was given.

A survey question regarding the number of hours within a 24-hour period that cats spent outdoors, was completed for 528 cats. Of these, 147 (27.8%) were reported to be indoor cats, spending no time outdoors. For the rest (n=381; 72.2% cats), a wide range (0.1–24 hours) of time spent outside was reported. It was most common for cats to spend between 0.1 and 6 hours outside (n=183; 34.7% cats) and relatively common for cats to spend between 6.1 and 12 hours outside (n=149; 28.2% cats; Figure 45). Owners of 537 cats completed the survey question about how often their cats caught and ate prey items. Over half (n=302; 56.2%) of cats never ate prey items, 33 (6.15%) cats ate prey daily, 64 (11.9%) cats ate prey weekly, 45 (8.38%) cats ate prey monthly, 77 (14.3%) cats ate prey a few times a year and 16 (2.98%) cats ate prey once a year (Figure 46a). Mice and birds were the most common prey item eaten by domestic cats (80.9% and 65.1% of cats that ate prey, respectively). Other prey, including voles, insects, amphibians and rabbits were eaten less commonly and even fewer cats ate rats, reptiles, shrews, moles, bats, squirrels and goldfish (Figure 46b).

Figure 45. The amount of time spent outdoors by cats.

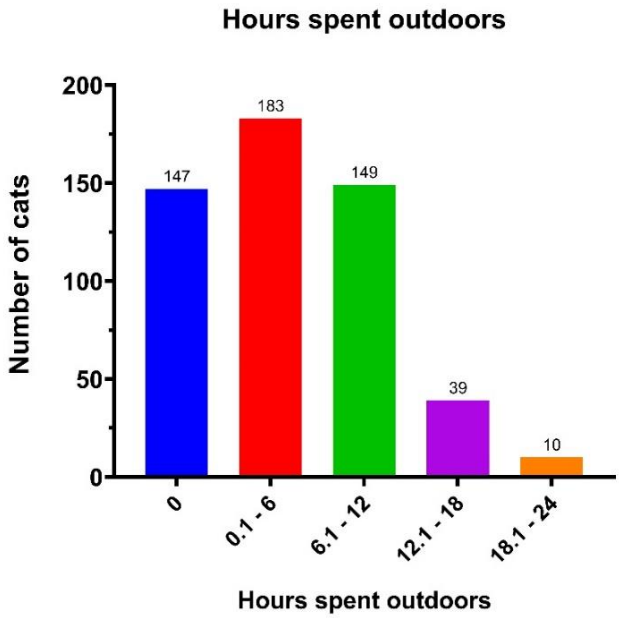


Figure 45. The amount of time spent outdoors by cats. Each bar represents a free-text answer that was given to the survey question regarding the length of time cats spent outside, on average, within a 24-hour period. The survey was created using Jisc Online Surveys. The heights of the bars and the numbers above them indicate the number of cats (out of 528 cats) for which this response was given.

Figure 46. How often cats eat prey and the common prey items eaten by cats.

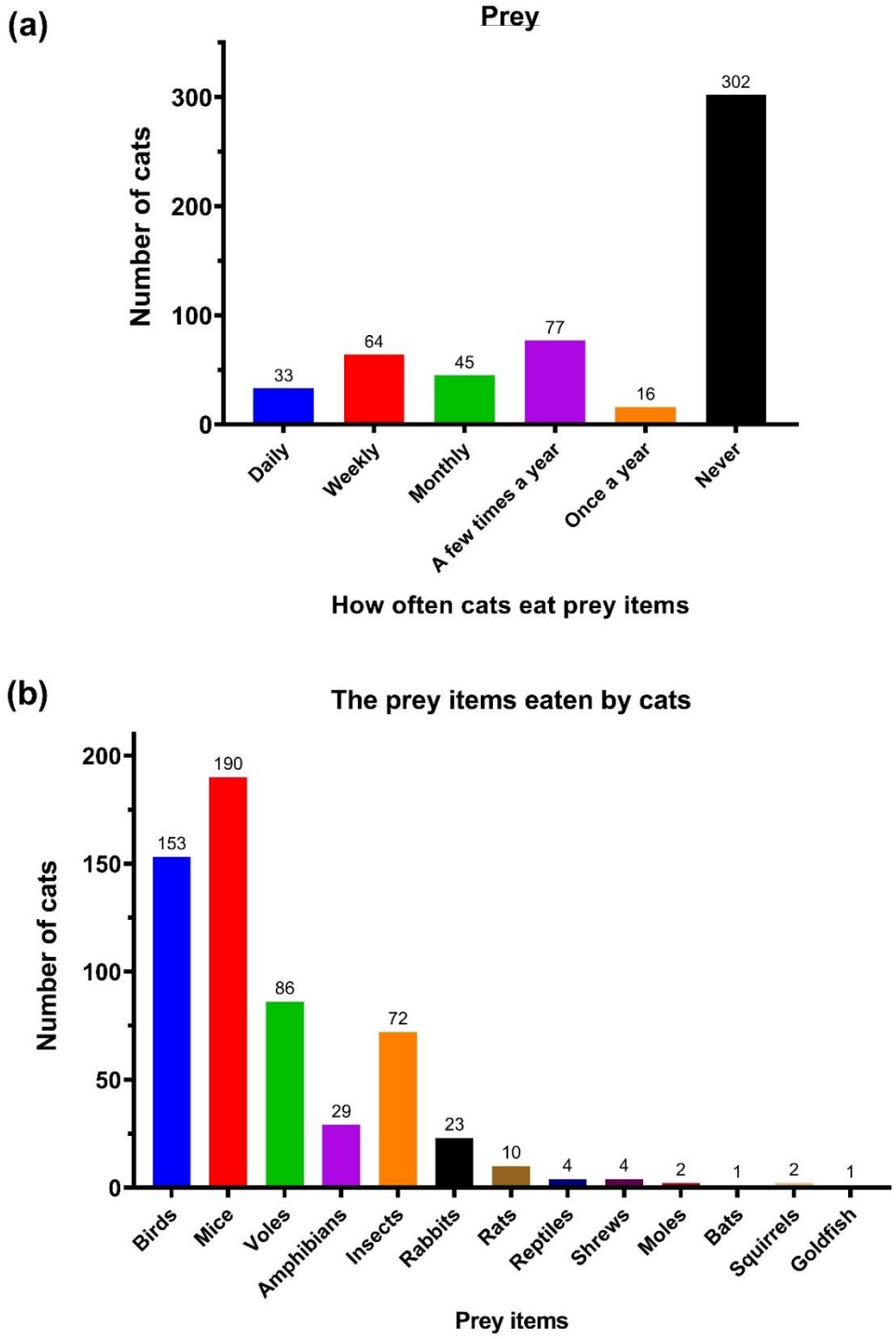


Figure 46. (a) How often cats eat prey and **(b)** the common prey items eaten by cats.A survey of cat feeding habits was created using Jisc Online Surveys. The data collected are presented as bar graphs, where bars represent the answers given (**(a)** multiple-choice answers 'daily', 'weekly', 'monthly', 'a few times a year', 'once a year' and 'never'. **(b)**) multiple-choice answers 'birds', 'mice', 'voles', 'amphibians', 'insects' and 'rabbits' and the free-text answers (rats, reptiles, shrews, moles, bats, squirrels and goldfish) given by cat owners that selected the 'other' multiple choice answer option for prey items consumed. The heights of the bars and the numbers above them indicate the number of cats for which the particular response was given.

4.4. Discussion

Dietary iodine, particularly excessive intake after a prolonged period of iodine deficiency, is involved in the aetiology of a form of hyperthyroidism in humans (TNG) (Laurberg et al., 2010, Stanbury et al., 1998) that clinically and histopathologically resembles the disease in cats (Hoenig et al., 1982). The definitive cause(s) of feline hyperthyroidism are unknown, although numerous risk factors have been identified (McLean et al., 2014). Among these, the feeding of wet, or canned, cat foods (Scarlett et al., 1988, Kass et al., 1999, Edinboro et al., 2004) and the feeding of fish are included (Martin et al., 2000, Wakeling et al., 2009b). These types of foods are known to have high or variable iodine contents (Johnson et al., 1992, Edinboro et al., 2013, Ranz et al., 2002). Therefore, as in human TNG, we suggested that variable iodine intake is also responsible for the development of hyperthyroidism in cats. We proposed that, as domestic cats probably eat more fish than dogs, cats might often experience high dietary iodine intake. We compared urinary iodine concentrations between euthyroid cats and dogs to determine whether cats have greater short-term iodine intake than dogs. We also measured hair iodine content in euthyroid and hyperthyroid cats to establish whether long-term iodine intake differed between these two groups of cats. We aimed to determine the iodine content of a variety of complete cat and dog foods that are commercially available in the UK and to assess the compliance of these foods with the FEDIAF-recommended minimum and legal maximum concentrations of iodine that complete pet foods should contain. The iodine concentrations measured in fish-, versus other, flavoured pet foods were compared, to test our hypothesis that pet foods containing fish have the highest iodine content. We also measured the iodine concentration in successive batches of a selection of wet foods for cats to establish whether cats fed the same food over time would receive consistent or varying iodine from their diet. We proposed that cats are more likely than dogs to receive excessive or deficient iodine concentrations from their commercial diet. To supplement this, we conducted a survey to find out how cat owners most commonly feed their cats and, particularly, whether they change the

commercial complete food by type (wet/dry), flavour or brand and how frequently this is usually done.

4.4.1. Urine iodine concentration as a marker of recent iodine intake in cats and dogs

In the current study, domestic cats and dogs did not have significantly different urinary iodine concentrations, suggesting that their short-term dietary intake of iodine was not different. The kidneys have a major role in maintaining iodide homeostasis by excreting >90% of dietary iodine, with iodine excretion being linearly proportional to iodine intake (Nath et al., 1992, Vought and London, 1967). This has been shown to be true in cats (Ranz et al., 2002). Furthermore, the urinary iodine concentration of healthy cats has been shown to respond to dietary iodine changes, reducing significantly following dietary iodine restriction (Paetau-Robinson et al., 2017) and increasing significantly when dietary iodine is increased (Tarttelin et al., 1992, Kyle et al., 1994). On the other hand, it has been shown that cats fed a consistently normal level of iodine experience little change in urinary iodine concentration (Paetau-Robinson et al., 2017). Thus, urinary iodine concentrations are a useful indicator of dietary iodine intake (Hambidge, 2003, Kyle et al., 1994). However, measurement of iodine concentration in the urine can only be used to determine an individual's dietary iodine intake over the preceding day or so (Hambidge, 2003, World Health Organization and International Council for Control of Iodine Deficiency Disorders, 1994). In the current study, analysis of spot samples of urine collected at necropsy, would realistically only reflect the iodine intake of the individual animal over the day or so prior to death or euthanasia. Thus, while we proposed that hyperthyroidism might develop in cats due to excessive iodine intake after a period of iodine deficiency, it is impossible to determine whether cats and dogs in the current study experienced fluctuations in their dietary iodine intake over time or whether they received consistently high, or low, dietary iodine long-term.

Wide ranges of urinary iodine concentrations were observed in both dogs and cats, in the current study. This perhaps reflects the variation in iodine intake among dogs and cats over the day prior to death. Of course, this may be due

to the differing mix of reasons for death/euthanasia in each species. During the 24 hours prior to death or euthanasia, overall food, and therefore iodine, intake by the cats and dogs in this study may have been reduced due to malaise. Thus, while our results imply that the cats and dogs, on average, had similar daily iodine intakes, they are unlikely to adequately reflect the daily dietary iodine intakes in the general, healthy domestic cat and dog populations. However, urinary iodine excretion was reported not to be affected by appetite in a study comparing hyperthyroid and euthyroid cats (Wakeling et al., 2009a). In that study, a wide range of urinary iodine concentrations was also found (Wakeling et al., 2009a), supporting our finding that urinary iodine excretion is highly variable between individuals within a species. Furthermore, there appears to be great variability in urine iodine concentrations even within individual cats, as occurs in individual humans (Andersen et al., 2008), when measured at different time points (Wakeling et al., 2009a). This may reflect variability in dietary iodine intake by individual cats over time, as we hypothesise, however, Wakeling et al. (2009a) did not obtain dietary histories for the cats in their study, or evaluate the iodine content of diets commonly fed to pet cats. Furthermore, it highlights the importance of obtaining many more urine samples before conclusions about the iodine status of cat and dog populations can be deduced from our current data (Andersen et al., 2008).

While we proposed that hyperthyroidism may develop in cats exposed to excess dietary iodine following a period of iodine deficiency, interestingly, hyperthyroid cats, prior to treatment for hyperthyroidism, were found to have lower urinary iodine excretion compared to euthyroid cats (Wakeling et al., 2009a). This might signify lower dietary iodine intake by hyperthyroid cats at the time of diagnosis, however, no dietary analysis was conducted to corroborate this (Wakeling et al., 2009a). Furthermore, hyperthyroid cats may excrete a greater proportion of total iodide in their faeces, rather than in urine, compared to euthyroid cats (Wakeling et al., 2009a). Therefore, while it is accepted that urinary iodine concentration accurately reflects dietary iodine intake in healthy animals, it is unknown whether this is also true in cases of hyperthyroidism (Wakeling et al., 2009a). Thus, based on urinary iodine concentrations alone, without dietary analysis, Wakeling et al. (2009a) could

only conclude that the euthyroid cats in their study did not have a dietary iodine deficiency. In the current study, urinary iodine concentrations were measured exclusively in euthyroid animals and a comparison between two different species was made. Not only may this allow comparison of dietary iodine intake between the two species, but it is also relevant for the identification of dietary iodine as a potential risk factor for hyperthyroidism, since cats are a species in which hyperthyroidism is common, and dogs are a species in which hyperthyroidism is rare. Nonetheless, we did not observe a difference between the urinary iodine concentration of cats and dogs, which may mean that their average dietary intake of iodine is not different. However, analysis of a greater number of samples is needed in order to make this, or the converse, conclusion.

4.4.2. Hair iodine content as a marker of long-term iodine intake in euthyroid and hyperthyroid cats

Iodine content of hair from euthyroid human volunteers was determined as an ancillary measure to validate our method for iodine concentration measurement in cat hair. Previously, a wide range of iodine concentrations (0.01–114 µg/g DM) has been measured in human hair samples, although, in that particular study, the normal range of hair iodine concentrations in healthy human subjects was suggested to be much tighter, between 0.09–2.0 µg/g DM (Momčilović et al., 2013). Prior to that study, reference values for iodine concentrations in human hair had been reported as 0.65–9.0 and 0.65–8.0 µg/g for men and women, respectively (Momčilović et al., 2013). Another study found the median (interquartile range) hair iodine concentration in healthy humans to be 0.385 (0.209–0.535) µg/g (Ochi et al., 2011), which is almost identical to our finding. Furthermore, the range of human hair iodine concentrations determined in our study falls within the parameters defined previously, validating that the methods for measuring hair iodine content, in our hands, were appropriate and robust.

Very few studies have been conducted to assess the relationship between hair iodine content and dietary iodine intake. Even fewer studies have assessed hair iodine concentrations in relation to hyperthyroidism. The studies that have

been completed in this area have primarily involved humans. Certainly, to our knowledge, the iodine concentration of hair from companion animal species, such as the cat and the dog, has not been measured previously. While such data would allow the best comparison with our own results, hair iodine concentrations measured in elderly humans with, versus without, TNG, may also allow a useful comparison with the novel data we present here. However, it appears that hair iodine content has not been investigated in that population either. The only human hair iodine data in relation to hyperthyroidism seems to be from children. In the current study, euthyroid cats had significantly greater hair iodine content than hyperthyroid cats. This contrasts findings in humans: children with goitre had 66% higher iodine concentration in their hair compared to healthy children of the same age and gender (Kudabayeva et al., 2016). Furthermore, the size of the thyroid gland correlated significantly with hair iodine concentration (Kudabayeva et al., 2016); such an increase in thyroid gland volume in children is believed to arise from chronic, excessive iodine intake (Zimmermann et al., 2005). In a study of healthy children, the hair iodine content was slightly higher in children receiving dietary iodine supplementation than that of children without dietary iodine supplementation (Kirichuk et al., 2019). However, this effect was not statistically significant, which may be due to the grouping of children into high and low iodine groups only according to the iodine content of their school meals, rather than taking their total dietary iodine intake into account (Kirichuk et al., 2019). It is possible that children in the iodine-supplemented group did not actually have greater total iodine intake. Therefore, a valid conclusion about the relationship between dietary iodine intake and hair iodine content cannot be made from this data (Kirichuk et al., 2019). Nonetheless, it has been suggested previously that hair iodine concentration is a useful biomarker of long-term dietary iodine intake; not just long-term iodine status in the body (Momčilović et al., 2013). Therefore, the results of the present study may suggest that the euthyroid cats had greater long-term dietary intake of iodine in comparison to the hyperthyroid cats. However, as dietary histories were not obtained in the current study, it is not possible to exclude the feeding of a low iodine therapeutic diet, by way of controlling the condition in the hyperthyroid cats, as an explanation for this result.

While hair iodine content can be used to reflect long-term dietary iodine intake (Momcilovic et al., 2014), hair samples were only collected once in the current study so the average iodine intake over only one 'long-term' preceding period can be inferred. Moreover, the specific duration of this 'long-term' period is unknown. The hyperthyroid cats in our study may have experienced a substantial iodine deficiency prior to an increased iodine intake, causing them to have lower hair iodine content, reflecting lower 'long-term' average dietary iodine intake, compared to the euthyroid cats whose iodine consumption over the same period may have remained more consistent. This would fit with the proposed aetiology of hyperthyroidism. However, without dietary history information for the cats in the current study, this is purely speculative. It would have been useful to have collected a second sample from each of these cats, perhaps several months after the first, to establish whether the hair iodine content and thus long-term average dietary iodine intake had changed since the first sample analysis. In future studies, it would perhaps be even more beneficial to prospectively study the effect of long-term changes in dietary iodine on hair iodine content to confirm that hair iodine is a useful biomarker of dietary iodine intake and establish the period of time over which this might be valid.

Hair is a useful biomarker of long-term mineral status because elements are permanently deposited in the hair matrix during the period of hair formation, and then removed from the site of active metabolism as the hair grows (Hopps, 1977). Mineral elements are primarily incorporated into the hair via the hair follicle (Combs et al., 1982). Therefore, the present study is limited by the different methods of hair collection from euthyroid, versus hyperthyroid, cats. Collecting hair from euthyroid cats by grooming would have enabled the analysis of iodine in the whole hair, including the portion within the follicle, which would not have been obtained by clipping, as was conducted for hyperthyroid cats. This may explain the lower levels of iodine measured in hyperthyroid, compared to euthyroid, cats' hair. Moreover, the rate of mineral uptake into hair may decline with age (Combs, 1987). Hyperthyroidism is more common in older animals and, indeed, in the current study, the hyperthyroid

cats were older than the euthyroid cats. Hence, slower iodine uptake into the hair may be responsible for the lower iodine content of hyperthyroid cat hair.

4.4.3. Pet food iodine content

4.4.3.1. Pet foods contain a wide range of iodine concentrations, but this is not associated with any particular flavour

Analysis of complete pet foods revealed wide variability in the iodine content of both cat and dog foods. To our knowledge, this is the first time that the iodine content of pet foods that are commercially available in the UK has been investigated, but the results are congruous with those from similar studies in the USA (Edinboro et al., 2013), New Zealand (Johnson et al., 1992) and Germany (Ranz et al., 2002).

Despite storing the samples for a year prior to analysis, freeze-drying and cold-storage should have preserved the iodine content of the foods (Iyengar et al., 1978, Bordas and Bourg, 1998), in line with EU regulation 152/2009. Unlike for arsenic and mercury, measured in study 1, FEDIAF (2019) provides guidance for analytical methods appropriate for determining iodine concentration in foodstuffs; commonly, ICP-MS with alkaline-digestion, as was performed in the current study, is used (Rose et al., 2001, Shelor and Dasgupta, 2011). Moreover, although the seronorm CRM had a urine matrix, it was prepared in a 1% TMAH matrix, synonymously with pet food sample preparation, and the percentage recovery of CRM iodine content was used to correct pet food iodine concentrations measured by the same analytical procedure. Excellent recovery of the certified level of iodine, as well as inter- and intra-assay variation, validated our method of iodine analysis and indicated excellent precision of the analytical procedure for measuring iodine concentration in pet food samples. Consequently, iodine concentrations measured in pet foods in the current study should be reflective of those measured by pet food manufacturers.

Pet food manufacturers are responsible for conducting regular analyses of their foods to ensure that they meet the FEDIAF recommendations. However, some foods failed to meet the minimum iodine content recommended by

FEDIAF, or exceeded the maximum legal limit (FEDIAF, 2019). Cats may be at greater risk of receiving excess or deficient levels of iodine from their diet, compared to dogs, as we found more of the cat than the dog foods that we analysed to be non-compliant. The iodine-deficient cat foods in the present study were only very mildly deficient with respect to the FEDIAF recommended minimum iodine content. However, in humans, TNG occurs mostly in people with mild to moderate, not severe, iodine deficiency (Laurberg et al., 2010). Therefore, if the pathogenesis of feline hyperthyroidism is indeed akin to that of human TNG, the iodine-deficient foods that we analysed may pose a risk for hyperthyroidism in cats fed these diets.

Greater variability in iodine content was found among wet foods, than among the dry foods, and all foods that exceeded the legal maximum were wet foods. This is similar to previous findings of higher concentrations of other minerals in wet foods than in dry foods (Paulelli et al., 2018, Davies et al., 2017a), and greater iodine content of canned foods compared to wet foods packaged in pouches or dry foods (Ranz et al., 2002, Edinboro et al., 2013). In the current study, the iodine content of canned wet foods was not significantly different from wet foods in other forms of packaging and, in fact, the highest iodine concentration measured was that of a pouched wet food. Regardless, it is clear that iodine deficient and iodine excessive ‘complete’ diets for cats do both exist. Thus, it would be possible for a cat to experience wide fluctuations in its iodine intake and, potentially, excessive or deficient iodine intakes over time if switched from one particular food to another. Such dramatic changes in dietary iodine intake have been shown to affect thyroid hormone levels in cats (Tarttelin et al., 1992). Therefore, the routine feeding of cats with commercially available cat foods could inadvertently result in feline hyperthyroidism by a mechanism similar to that involved in the pathogenesis of human TNG. Feeding trials would be needed to determine the effect of changing diet and iodine intake on the hypothalamic-pituitary-thyroid axis in cats, to establish whether feline hyperthyroidism may be induced by this mechanism.

In contrast to reports of high iodine content in fish (Nerhus et al., 2018), in the current study, pet foods containing fish and/or fish oil did not have higher iodine content than those without fish derivatives. Therefore, the results of the current

study refute the suggestion that cats that consume more fish would have higher intakes of iodine compared to cats that consume less fish. This is in agreement with the conclusion from another study conducted in America (Edinboro et al., 2013). Therefore, while some epidemiological studies have reported an increased risk of feline hyperthyroidism with consumption of fish-containing foods (Martin et al., 2000, Wakeling et al., 2009b), if this is indeed the case then it may not be due to greater iodine consumption. Furthermore, higher iodine concentration in foods did not associate with any particular main flavour in the pet foods. In fact, there appears to be a greater risk of developing hyperthyroidism in cats that are fed a variety of flavours of canned foods (Olczak et al., 2005). Together, these results suggest that changing the diet is detrimental, perhaps due to the associated widely fluctuating iodine intake, with iodine deficiencies and excesses, which cats may experience as a result of consuming a variety of wet foods, as opposed to a particular food flavour.

4.4.3.2. Iodine concentration of pet foods is not consistent across batches

Previous studies have focused on determining the iodine content of a selection of different pet foods. To our knowledge, only one prior study has measured the iodine concentration in separate samples of these foods purchased at different time points (Johnson et al., 1992). Johnson et al. (1992) found that the iodine concentrations in two samples each of six canned cat foods, purchased two months apart, were similar. Yet, whether the two samples of each food were defined by different batch codes, as in our study, is unknown. Thus, for the first time, we report clear batch-to-batch variation in the iodine concentration of some commercially available complete cat foods. The results of the current study suggest that if a cat is fed the same wet food over a long period of time, it will receive variable iodine concentration from its diet, likely every time a new batch of the food is purchased. This is similar to previous work that highlighted discrepancies between the concentrations of other mineral elements in different batches of a selection of wet pet foods (Davies et al., 2017a). Moreover, the current research implies that a cat that is fed the same diet over time may also receive insufficient *or* excessive iodine intake

periodically. Toxic nodular goitre, the form of hyperthyroidism in humans that resembles feline hyperthyroidism, is induced by excessive iodine intake after a period of iodine deficiency (Stanbury et al., 1998). However, no wet food that was analysed in the current study was found to have both iodine deficient *and* iodine excessive batches, with respect to the FEDIAF guidelines (FEDIAF, 2019). Therefore, consistently feeding one particular pet food may not pose a risk for feline hyperthyroidism if the aetiology is akin to that of TNG. It may be of greater detriment to change the food that a cat is fed from one diet to another if this results in both inadequate and excessive iodine intakes. Nonetheless, considering that subsequent batches of the same food are identically labelled, the consumer should be able to expect an identical product. More importantly, as is recognised in humans (Laurberg et al., 2010), consistency of dietary iodine content should be achieved in order to reduce any potential associated risk of hyperthyroidism development in cats, a species that anyway seems particularly prone.

4.4.4. Common feeding practices for pet cats

4.4.4.1. Owners change their cat's commercial diet on a daily basis

The primary objective of our survey was to determine whether cats commonly experience changes in their given commercial diet and, if so, the type and frequency of these changes. Information about non-commercial diet, treats and outdoor lifestyle, including hunting habits, was also collected, to provide an overview of the most common feeding practices for pet cats. Previously, in similar surveys, owners of more than one cat have provided information about only one of their cats (Laflamme et al., 2008, Michel et al., 2008, Delgado et al., 2020) or a maximum of two cats in their household (Fennell, 1975). By collecting information about every cat owned by each survey respondent, we were able to assess whether cats within the same household are fed in the same way as each other. Indeed, cats within multi-cat households tend to receive the same proportion of wet food as each other, suggesting that other aspects of their diet may also be similar. However, there was not 100% agreement of wet food feeding between all cats in each multi-cat household, indicating that even cats sharing a single multi-cat household may be fed

differently. On the whole though, cats living in multi-cat households appeared to be fed similarly to cats that do not live with other cats.

We proposed that variability in iodine intake from commercial diets may contribute towards hyperthyroidism development in cats. Since hyperthyroidism affects cats throughout the world and as variability in cat food iodine content has also been found in other countries, we retained survey responses from cat owners residing in countries other than the UK. Although the majority of survey respondents were from the UK, this gave us a reasonable overview of the feeding habits of cat owners internationally. Furthermore, we found that the proportion of wet food that is fed did not differ significantly between cats in the UK and those outside of the UK, nor between cats living in different regions of the UK. Thus, for further analyses, data for all cats from all regions were analysed simultaneously.

Feeding information was acquired for cats of the full range of cat ages in the current study, with the majority being adults and slightly fewer being senior or geriatric cats. A small proportion of these cats had health conditions, but most were declared to be healthy. A roughly equal number of male and female cats were included in this study, with the majority being neutered. The survey was completed for cats covering a range of breeds, however, the majority were domestic shorthair cats. All things considered, this study sample is probably reflective of the overall domestic cat population.

4.4.4.2. Domestic cats are most commonly fed a combination of wet and dry, commercially-available pet food

As expected and as others have suggested (Laflamme et al., 2008, Michel et al., 2008, Robertson, 1999), we found that commercial pet foods make up most, if not all, of the diet for nearly all domestic cats. Interestingly, the proportion (95.4%) of cats that were reported to consume commercial food as at least half of their dietary intake in our survey was similar to that (98.8%) found by Laflamme et al. (2008). Dog owners were not surveyed in the current study, but it has been shown previously that significantly fewer dog than cat owners feed commercial food as the major proportion of the diet (Laflamme et

al., 2008, Michel et al., 2008). Therefore, if commercial diets are varied, any associated consequences may be more pertinent to cats than to dogs.

We discovered that, most commonly, half of the total amount of commercial pet food that is provided to cats is wet food and the other half is dry food. Furthermore, half of cats were said to receive both wet and dry foods on a daily basis. Similarly, another survey identified that over half of cats were fed both canned and dry cat foods every day within a one week period (Robertson, 1999). Similar to another online survey conducted recently in America (Delgado et al., 2020), we found that dry food is the most common type fed and it is relatively common for cats to receive only dry food, but rare to receive only wet food. A decade prior to the aforementioned study, another American survey reported that a higher proportion (>40%) of cats eat only commercial dry food (Laflamme et al., 2008). In stark contrast, an even earlier survey conducted in Great Britain found that only 39% of cats received any dry food at all, and only 2% of cats were fed dry food exclusively (Fennell, 1975). While the use of different methods for recruiting survey respondents, such as online surveys, shared via social media and veterinary networks (Delgado et al., 2020), telephone interviews of a randomised population (Laflamme et al., 2008) and systematic selection of cat owners across a geographical area (Fennell, 1975) should be taken into account, these results suggest that feeding practices have changed over time, irrespective of geographical location. As the iodine content of wet foods is more variable than that of dry foods, a shift towards feeding increased amounts of dry foods may help to ensure more consistent iodine nutrition. Nonetheless, as a large proportion of cats are fed a combination of wet and dry foods, it is probable that iodine intake will vary from meal to meal within a single day. However, the association between iodine and hyperthyroidism, if such an association exists in cats, is probably related to longer-term fluctuations in dietary iodine intake, rather than daily fluctuations, as it is in human TNG. Therefore, cats that eat both wet and dry foods in a single day may not have a greater risk of developing hyperthyroidism.

4.4.4.3. The wet foods fed to cats are most often in pouches and uncommonly in cans

With regard to wet food, the early study in Great Britain (Fennell, 1975) and another in Australia (Robertson, 1999) showed that 85% of cats received tinned commercial cat food. We found that 40% of cats receive canned foods as part of their wet food intake. Our results are more similar to those from the more recent American survey, where around 30% of cat owners fed canned food but less than 10% of cats received canned food exclusively (Laflamme et al., 2008). Taken together, these results suggest again that feeding practices have changed over time. As with wet food, as opposed to dry food, feeding canned cat food appeared to be common in 1975 but became substantially less common between 1975 and 2008 and has since remained relatively stable. We and others have found that many cats receive none or less than 5% of their diet as canned commercial cat food (Laflamme et al., 2008). Yet, it seems that even fewer dogs consume canned foods (Laflamme et al., 2008). Since canned food consumption has been linked with increased risk of hyperthyroidism (Scarlett et al., 1988, Kass et al., 1999, Wakeling et al., 2009b, Edinboro et al., 2004), perhaps greater consumption of canned food by cats, compared to dogs, is involved in the higher prevalence of the disease in the former species. However, it appears that iodine concentration is not necessarily more variable among canned foods than among wet foods in other packaging types. Therefore, if increased feeding of canned foods does contribute to increased risk of hyperthyroidism, it may be due to high content of goitrogenic contaminants, such as BPA and PBDEs (Edinboro et al., 2004, Dye et al., 2007), rather than iodine.

It is noteworthy that the only commercial wet cat food described in previous studies was ‘tinned’ (Fennell, 1975) or ‘canned’ (Laflamme et al., 2008, Robertson, 1999) and consumption of wet foods in other packaging such as pouches or foil trays was not assessed. It may be that ‘tinned’ or ‘canned’ descriptors were taken to represent all wet foods in general, although this is not stated in these reports. Additionally, as we found that around a quarter of cats received wet food in a variety of packaging types and that pouched foods are, by far, the most common wet foods to be fed to cats, this may highlight

that a significant portion of the cats' diet was not evaluated in these prior studies. On the contrary, the current study gives a more comprehensive insight into the variety of foods commonly fed to cats.

4.4.4.4. Most cats are regularly given different flavoured foods, but meat-flavours are given more commonly than fish-flavoured foods

Although cats are believed to prefer fish-flavoured foods (Houpt and Smith, 1981, Hullar et al., 2001, Van den Bos et al., 2000), our survey showed that fish-flavoured foods comprised a smaller proportion of the cat's diet than meat flavours. If only one flavour of cat food was fed, it was uncommon for this to be fish. It appears that cats are most commonly fed white-meat flavoured foods. Previous studies have not examined the flavours of commercial diets fed to cats but our survey highlighted that most cats experience regular changes in dietary flavour, with many cats even receiving different flavours every day. Evidently, pet food labelling can be confusing; pet foods commonly contain a variety of meats, fish or other animal and vegetable products, despite being labelled as a single flavour, such as chicken, for example. Most often, the flavour of the food is also the main ingredient in the food. Therefore, the term 'flavour', instead of 'ingredient', was specifically used so that cat owners would answer the question according to the main meat or fish flavour that the food is marketed as, rather than getting confused by the list of ingredients on the packaging. Michel et al. (2008) found that cat owners tend to have neutral opinions regarding the clarity of the information on pet food labels or they generally find it easy to understand rather than misleading. This lends reassurance to the flavour data collected in the current survey. Although we hypothesised that fish-containing pet foods had highest iodine content, we showed that this was not the case. Instead, we found equally wide variation in iodine concentrations in foods of all flavours. Our results show that cats will not necessarily have higher dietary iodine intake by consuming a single flavour of complete pet food. Similarly, by consuming a variety of different flavours of pet foods, as appears to be the situation for most cats, cats will not necessarily have greater variation in their iodine intake.

4.4.4.5. The brand of pet food given to cats is never, or rarely, changed

In contrast to daily dietary flavour changes being common, we found that for nearly half of cats, the brand of commercial cat food is never changed. Just over half of cats will receive a different brand of food at some point, however. Another study investigated the number of different brands of pet foods provided to cats within a week, assessing wet and dry foods separately (Robertson, 1999). They found that 78% cats that ate dry food received only one brand but just over half of cats that ate canned pet food were fed a single brand, while 27% of cats received two brands and 18% of cats ate three or more brands of canned cat food (Robertson, 1999). The brand changes reported in that study occurred over a single week and evidently may not reflect the general changes in the brands of pet foods fed to cats in the long-term. Nonetheless, it appears that cat owners are generally loyal to a particular brand of pet food, particularly if they feed dry food, but they are likely to vary the brand more often if they feed wet food. We measured iodine in complete pet foods of a variety of brands. However, we did not compare the iodine contents of foods according to brand. Therefore, it is unknown whether, by consuming foods of a single brand, cats will have more consistent iodine intake, than if the brand is often changed.

4.4.4.6. Other aspects of the pet cat's feeding routine

4.4.4.6.1. Food availability

We, and others, have found that cats are most commonly fed ad-libitum or twice a day (Laflamme et al., 2008, Sapowicz et al., 2016). An earlier survey of cat owners in Australia found that most cats were fed twice a day but only 13% of cats were fed ad-libitum (Robertson, 1999). Other studies have found ad-lib feeding to be associated with dry food and meal feeding to be associated with wet food (Fennell, 1975, Delgado et al., 2020). This association was not investigated in the current study, however, many owners that selected the twice or three times daily multiple-choice option for feeding frequency then specified in their notes that they meal-fed wet food at the selected frequency but that dry food was freely available throughout the day. In these cases, the

results were adjusted to ‘ad-libitum’. Although this may have introduced bias into the results, evidently, some of the owners who declared meal feeding but whose cats actually had food available throughout the day because they didn’t eat it all in one sitting may not have provided such a statement to explain this. Therefore, the number of cats fed ad-libitum may realistically be even higher. Other surveys have not established whether cats eat all of the food provided but we found that most cats eat all of the food provided most of the time. Only 28.6% of cats were said to always eat all the food provided, suggesting that many more cats that were fed ‘meals’ actually had food freely available throughout the day, unless the owner removed the uneaten portion. Further studies would be required to gather this information. In relation to the hypotheses of the current study, if indeed feline hyperthyroidism can be triggered by dietary iodine fluctuations, it is unclear over what duration a large change in dietary iodine intake would be necessary. Ad-lib feeding may dissipate iodine intake over the course of the day, avoiding wide swings in iodine intake, which might occur due to meal feeding, especially if the diet is changed from one meal to the next.

4.4.4.6.2. Non-commercial pet foods, treats and prey species make up only a minor portion of the domestic cat’s diet

We and others (Laflamme et al., 2008, Michel et al., 2008) have found that few cats receive home-prepared diets or foods that are suitable for humans as part of their main diet. Similar to Robertson (1999), we have shown that more cats receive meat, fish and/or dairy products as treats. The current study showed that most cats are fed treats but these are usually complimentary dry food treats, such as ‘Dreamies’. Commercial treats were found to be the most common type given to cats in other studies too. However, these studies reported that fewer cats received treats (Robertson, 1999, Laflamme et al., 2008, Sapowicz et al., 2016) and, if treats were given, they were given more frequently (at least once a day) (Sapowicz et al., 2016), compared to our study. Of course, owners may report feeding fewer treats than they actually do feed. Nonetheless, dogs seemingly receive a much wider variety of treats and more frequently, compared to cats (Sapowicz et al., 2016, Laflamme et al., 2008).

The iodine content of complementary pet foods and foods intended for humans but which are given to cats, either as treats or as part of the main diet, was not measured in the current study. However, these foods form only a minor part of the diet for most cats, so their contribution of iodine to the cat's overall intake of this mineral, is also likely to be minimal.

In the current study, there was a large proportion of indoor cats, however, most cats had access to the outdoor environment. Fennell (1975) found a similar ratio of indoor to outdoor cats in Great Britain. In contrast, surveys of cat owners in America and Australia (Laflamme et al., 2008) and just in America (Delgado et al., 2020) found that over half and 82% of cats, respectively, lived exclusively indoors. Nonetheless, even indoor cats may have the opportunity to catch prey species, such as insects, which they may continue to eat. Owners were asked how often their cat caught *and* ate prey; it was not sufficient for cats to simply catch the prey and not proceed to eat it. Nevertheless, some owners may have incorrectly reported prey consumption by cats that only caught prey. On the other hand, it is likely that some owners never witness their cats consuming prey items so assume that it doesn't happen. Even so, in agreement with Fennell (1975), we found that prey items, most commonly mice and birds, are an additional food source for just less than half of cats. Additionally, very few cats eat prey daily. Therefore, it seems that prey items are only a minor contributor to the domestic cat's diet in general. Consequently, although the iodine contents of prey species were not analysed in the current study, they probably only contribute a small amount of iodine to the domestic cat's total iodine intake.

4.4.4.7. Limitations

In contrast to the current study, previous investigations into cat feeding habits have primarily focused on the reasons behind owners' feeding choices. The consensus from previous studies is that diets are chosen based on cost, availability, convenience, the cat's preference, the cat's health needs, ingredients and nutritional quality of the food, and the pet food manufacturer's reputation (Sapowicz et al., 2016, Michel et al., 2008). Apparently, most owners obtain nutritional information from their cat's veterinarian (Laflamme et

al., 2008, Sapowicz et al., 2016) but 16% of owners also acquire information on pet nutrition from the media, including the internet (Michel et al., 2008, Laflamme et al., 2008). The same factors may govern the decision-making regarding the dietary changes implemented by cat owners surveyed in the current study. Michel et al. (2008) found that, generally, owners believe, somewhat, that cats need a variety of different foods. Indeed, it seems that nearly all owners vary their cat's diet in some way and most cats experience frequent changes to the commercially-available cat food that they receive.

Evidently, the survey data represent a snapshot of feeding practices around the time of the survey completion, rather than indicating the cats' diets over their lifetime. Although questions about dietary changes (by brand, type or flavour) were included, the most long-term multiple-choice answer that respondents could select was 'less than monthly'. The survey did not collect information about past dietary histories. Therefore, the results indicate short-term dietary changes and do not show lifetime variations in the cat's diet. This is perhaps most pertinent for the cats that were fed a prescription diet due to a specific medical condition, since they would almost certainly have been fed a different diet prior to this. In these cases, and particularly for hyperthyroid cats, it may have been interesting to provide additional questions regarding the cat's previous diet to determine any major, or subtle, lifetime dietary changes prior to their diagnosis. However, none of the 16 hyperthyroid cats for which our survey was completed were reported to receive the Hill's y/d low iodine diet, which is the only dietary intervention available for managing feline hyperthyroidism.

4.4.5. Conclusion

Similar to studies conducted in the USA, New Zealand and Germany, we conclude that there is wide variation in iodine content among commercial complete cat and dog foods in the UK. However, contrary to our hypothesis, iodine content does not appear to be more variable among cat foods than among dog foods. Most cat and dog foods contain at least the recommended minimum and less than the legal maximum iodine concentration, however, some iodine deficient and excessive foods do exist. It is deficient, followed by

excessive, iodine intake, rather than simply variation in the iodine intake, that may induce the development of hyperthyroidism in cats, as in humans. Therefore, while the iodine content of different batches of a particular cat food may vary, it may be better to feed cats a single complete food consistently, rather than changing the diet, to reduce the risk of deficient and excessive iodine intakes.

The survey in the current study highlighted that commercially-available complete pet food makes up most of the domestic cat's diet and cat owners regularly change the commercial diet that they feed to their cats. While the brand of pet food that a cat is fed is rarely changed, cats are usually offered both wet and dry foods each day and a variety of flavours of pet foods. Wet foods have particularly variable iodine contents, although feeding some dry foods could also result in deficient iodine intake.

Unsurprisingly, we found that over two thirds of cats eat fish-based foods, although fish makes up a lesser proportion of the diet for most cats, compared to meat. However, fish-containing foods generally do not have higher iodine content than foods of any other main flavour. In fact, it seems possible that a cat could experience a range of iodine intakes, regardless of the flavour of pet food that they are fed; cats are not necessarily more likely to have excessive iodine intake than dogs, due to greater consumption of fish. If the short-term dietary changes reported in this study were made over the cat's lifetime, the absolute amount of iodine that the cat consumes would probably change regularly too. Certainly, iodine concentration varies widely across a large number of wet and dry commercially-available, complete cat foods of different flavours and brands. Therefore, we conclude that most cats will experience changes in their dietary iodine intake and possibly even dietary iodine deficiencies or excesses from time to time.

Variable dietary iodine intake is a suggested, but not proven, risk factor for feline hyperthyroidism. In general, daily iodine intake appears not to differ between dogs and cats, but a difference in longer-term iodine intake between euthyroid and hyperthyroid cats is suggested and may underpin the occurrence of the condition. However, our use of urine and hair iodine

concentrations as markers of iodine intake was not appropriate for identifying fluctuations in dietary iodine intake over time. Prospective dietary trials would be required to demonstrate a cause and effect relationship for iodine consumption and hyperthyroidism. However, there would be issues regarding the duration, cost and ethics of these kinds of studies (Wakeling et al., 2009a). Furthermore, interviews with owners of hyperthyroid and euthyroid cats could help to identify any common differences in the lifetime dietary histories between these two groups of cats, although recall of such long-term information could be difficult for some owners. Alternatively, Wakeling et al. (2009a) suggested that the influence of dietary iodine intake on plasma TSH concentration could be studied to establish the role of iodine intake in the development of hyperthyroidism. However, there is not yet a cat-specific TSH test and the canine TSH assay, which may be used to diagnose feline hyperthyroidism, may not be specific enough for this purpose (Peterson et al., 2015).

Despite not being able to prove an association between dietary iodine intake and feline hyperthyroidism in the current study, the fact remains that the iodine content of pet foods, that are commercially available in the UK, is highly variable. Moreover, according to labelling, these foods should be ‘complete’, however, some are even iodine-deficient or excessive. In humans, it has been recognised that the iodine intake should be kept within a relatively narrow range, avoiding deficient and excess intakes of this essential mineral, in order to reduce the risk of iodine-induced hyperthyroidism (Laurberg et al., 2010). As the highly prevalent feline hyperthyroidism displays striking similarities to human TNG, ensuring adequate but not excessive iodine content in foods seems a sensible precaution for cats too. If this is achieved and providing accurate iodine content is clearly indicated on the pet food label, owners can then make informed decisions when selecting foods for their cats. In this way, and possibly aided by veterinary advice, owners could potentially reduce the risk of their cat developing hyperthyroidism in the long-term.

Chapter 5. General Discussion

The studies presented within this thesis have focused on two chronic diseases, chronic kidney disease and hyperthyroidism, which are particularly prevalent in domestic cats relative to other species (Brown et al., 2016, Mooney and Peterson, 2012). The general aim of these studies was to investigate potential underlying reasons for the apparent predisposition to these two conditions in domestic cats. Since both conditions are most commonly diagnosed in aged animals (Bartges, 2012, Peterson et al., 1994, Bree et al., 2018), we suggested that lifetime exposure to an ill-suited commercial diet might contribute to the pathogeneses. CKD is irreversible and treatment focuses on managing the symptoms and slowing the progression of the disease (Brown et al., 1997). Treatment options for feline hyperthyroidism have varying degrees of success (Peterson and Eirmann, 2014). The intention of our research was to provide an insight into how the diseases could perhaps be prevented. Hence, the potential outcome of our work and the further work that is necessary could benefit many cats, increasing their disease-free longevity and improving their quality of life in older age.

A particular area of interest in the current project, with regard to the domestic cat's apparent propensity to develop CKD and hyperthyroidism, was the consumption of fish. Teleologically, domesticated or wild felines would rarely, if ever, consume fish as part of their natural diet. Yet, through our survey conducted in Study 3, we showed that most domestic cats receive fish as part of the diet, even if this only constitutes a minor proportion of the total food intake. Fish consumption has been implicated as a risk factor for feline hyperthyroidism (Wakeling et al., 2009b, Martin et al., 2000) and as a significant contributor to iodine (Nerhus et al., 2018), arsenic (Bae et al., 2017) and mercury (Maulvault et al., 2015, Cheng et al., 2009, Luippold and Gustin, 2016) intake. Iodine is implicated in the pathogenesis of a form of hyperthyroidism in humans, toxic nodular goitre (Stanbury et al., 1998, Laurberg et al., 2010), which bears similarities to feline hyperthyroidism (Hoenig et al., 1982). Hence, we proposed that variable dietary iodine, likely due to fish consumption, contributes to hyperthyroidism in cats. While we disproved our hypothesis that iodine concentration is highest in commercial

pet foods containing fish, we found that there is wide variation in the iodine content among cat foods that are commercially available in the UK, as has been shown in other countries (Ranz et al., 2002, Edinboro et al., 2013, Johnson et al., 1992). While this study (Study 3) did not allow for determination of the effect of varying iodine intake on the thyroid gland, others have shown that thyroid function in cats may be impacted by changes in dietary iodine intake (Tarttelin et al., 1992). Further research is required to establish whether a causative link exists between dietary iodine and feline hyperthyroidism. In the meantime, we suggest that it is a necessary precaution that cats receive consistent iodine from their diet. However, our study indicated that owners cannot be reassured that they are providing consistent dietary iodine to their cat simply by purchasing the same pet food over time. Our work has implications for pet food manufacturers, to ensure that their products supply adequate, but not excessive, levels of essential minerals such as iodine, in accordance with the most up to date nutritional guidelines.

Previously, we demonstrated that fish-containing pet foods contain increased levels of arsenic relative to foods that do not contain fish (Davies et al., 2017a). In Study 1 of this thesis, we also showed that mercury concentration in pet foods associates with the inclusion of fish muscle. As the kidneys are involved in the excretion of waste substances, such as the pro-oxidant trace elements, arsenic and mercury, which serve no obvious biological role (Bridges and Zalups, 2010, Lewchalermvong et al., 2018), we suggested that high intake of these elements, possibly through consumption of fish, could induce renal disease in cats, as has been shown in humans (Park and Zheng, 2012, Chang and Singh, 2018). In fact, since oxidative stress is often involved in the progressive cycle of renal damage (Locatelli et al., 2003, Ratliff et al., 2016), we proposed that an imbalance between trace element minerals with pro-oxidant activity versus those with anti-oxidant functions, in favour of increased ROS generation, might exist within the feline kidney. We hypothesised that such an imbalance would be greater still when renal disease is evident. In Study 1, while we found that renal accumulation of arsenic was higher and anti-oxidant minerals such as copper and zinc were lower in cats than in dogs and Scottish wildcats, overall there was no clear association between a pro-

oxidant environment in the kidney and the presence of renal lesions. Concentrations of mercury and iron, the other two pro-oxidant minerals of interest in the current study, due to the proposed elevated dietary exposure in domestic cats, relative to dogs and Scottish wildcats, were in fact higher in dog than in domestic cat kidneys. Furthermore, we, like others (Komorowicz et al., 2019, Kalantzi et al., 2017), have shown that arsenobetaine predominates in foods that contain high levels of arsenic. Thus, the primary arsenic species that accumulates in the domestic cat's kidney is likely to be arsenobetaine and therefore, of little toxicological concern (Jomova et al., 2011). While we could not associate mineral imbalances with renal disease, our study has revealed that there are some differences in the renal mineral profile between cats and dogs and, perhaps more interestingly, between domestic cats and Scottish wildcats. We suggest that the imbalance between pro- and anti-oxidant mineral elements may be greater in domestic cats than in dogs and Scottish wildcats. However, to establish diet as a true source of such mineral differences, the next research in this field should involve long-term, controlled feeding trials. Furthermore, to determine the impact of such mineral imbalances on the kidney, long-term prospective studies, with the opportunity to collect dietary information and clinical data to monitor kidney function and detect CKD, would be an important consideration.

During histological assessment of kidneys, we noted a prevalence of cytoplasmic lipid droplets in the domestic cat's kidney, compared to the dog or Scottish wildcat kidneys. After reviewing the literature, it was apparent that, while intracellular lipid droplets have been frequently observed in the domestic cat's kidney and are known to be more abundant in cats than in other species, the contents of the lipid droplets was, for the most-part, unknown (Modell, 1933, Bargmann et al., 1977). Thus, the role of these lipid droplets is also unclear. Intracellular lipid accumulation is associated with lipotoxicity in other organs and in other species. Additionally, the current body of evidence suggests that CKD can develop and progress through a mechanism of lipotoxicity (Bobulescu, 2010, Izquierdo-Lahuerta et al., 2016). Surprisingly, despite the high prevalence of renal disease in domestic cats, the intracellular lipid content of the kidney has not been studied in relation to CKD in this

species. We aimed to determine the composition of the intracellular lipid droplets within the kidneys of domestic cats, dogs and Scottish wildcats. Indeed, using sensitive and specific lipidomic methods, we found an additional lipid class in the domestic cat's kidney, which was less abundant in Scottish wildcat kidneys and completely absent from dog kidneys. We suggest that this additional lipid in domestic cat kidneys is predominantly monoalkyldiacylglycerols (MADAGs), in particular those containing medium-long (i.e. C12–C16) and branched-chain fatty acids. Since these lipids are rarely found in mammalian tissues (Magnusson and Haraldsson, 2011), we propose that they arise in the domestic cat's kidney through dietary intake. A hypothesis that we had not considered at the outset of Study 2 is that incorporation of fish in the domestic cat's diet contributes to the additional lipid that is observed in the cat's kidney, compared to other species such as dogs. Our work has highlighted the need for further research to determine whether increased feeding of fish to cats increases MADAG accumulation in the kidney. Likewise, incorporation of plant-based oils, such as palm, palm-kernel or coconut oils, in the diet should be investigated to establish whether the constituent fatty acids of these oils accumulate in the domestic cat's kidney. We make these suggestions based on the fact that the cat, as an obligate carnivore, may have lost the physiological mechanisms to metabolise such plant-based ingredients, or fish, which they would not normally consume in the wild. A corollary is that cats have lost the ability to synthesise arachidonic acid (C20:4 Ω6) as it is readily available in sufficient quantities from eating other animals (Bauer, 2006, Montague et al., 2014). Kidney transcriptomic studies, including a more specific analysis of the mitochondrial genome, would be useful to determine whether cats, compared to other species, have a reduced capacity for metabolism of the lipids that are found in plant and fish ingredients and/or upregulation of the pathways that are involved in synthesis of lipid storage compounds such as MADAGs.

Methylmercury, the primary form of mercury found in fish (Maulvault et al., 2015), is lipid soluble (Simmons-Willis et al., 2002). In Study 1, given that cats have relatively lipid-laden kidneys, we expected that the renal tissue mercury concentration would be greater in cats than in dogs. However, we found that

this was not the case. Indeed, whilst the cortex of all animals had more mercury than the medulla, dogs appeared to have more mercury per se in their kidneys, relative to cats. This was surprising, but we take it to indicate that there is relatively constant turnover of lipid within the intracellular droplets in the cat's kidney. The contents therefore, may not reflect a gradual accumulation with time. This may reflect the gradual loss of tubular cells that contain the lipid, which occurs as a result of an age-related progressive increase in interstitial fibrosis. Others have similarly shown that intracytoplasmic lipid droplets are dynamic organelles, formed as a store of lipid which is moved within the cell and released when required (Olofsson et al., 2009, Bartz et al., 2007). It might be that subcellular lipid metabolism pathways are regulated differently, and renal intracellular lipid turnover is more rapid, in cats compared to dogs. This could be an area for further research using comparative genomics studies as discussed above.

While the studies presented within this thesis are limited by the use of post-mortem tissue, which prohibited the collection of dietary history and clinical information, our work provides a novel angle with the inclusion of the Scottish wildcat as an intra-species comparator for the domestic cat. Since the feline genome is relatively well conserved (Murphy et al., 2007, O'Brien et al., 2002, Wurster-Hill and Centerwall, 1982), genetics are likely to play only a minor role, if any, in the particular susceptibility to CKD in domestic cats compared to Scottish wildcats. Evidently, wild-living cats are unlikely to live as long as domestic cats, therefore the occurrence of age-related diseases, such as CKD, in this population is naturally lower. If minerals and certain lipids accumulate in the kidneys with age, the younger age of the Scottish wildcats, compared to the domestic cats in the present studies, could reasonably explain the reduced content of certain minerals and MADAGs in the kidney of Scottish wildcats versus domestic cats. However, while we cannot exclude an effect of age on the accumulation of minerals and specific lipids in the kidney, we demonstrated in Study 2 that kidney content of total lipid, at least, does not increase with age. Hence, in studies 1 and 2, we are able to make some broad assertions regarding the effect of domestication, such as commercial diet and fish consumption, on renal health. Indeed, cats in the wild, like Scottish wildcats,

would not ordinarily consume fish (Corbett, 1979). Our studies have shown that the inclusion of fish oil in commercial pet foods, for the supply of the essential omega-3 PUFAs, EPA and DHA, is not associated with higher levels of minerals which might be of detriment to health, such as arsenic, mercury and iodine. However, whether fish oil consumption contributes to the unique lipid profile in domestic cat kidneys requires further investigation. Our finding of reduced levels of shorter and branched-chain fatty acids, that are commonly found in fish or plant oils (Sankararaman and Sferra, 2018, Karupaiah and Sundram, 2007, Magnusson and Haraldsson, 2011), in Scottish wildcats, compared to domestic cats, implies that consumption of commercial food, and fish in particular, may play a part. Dogs, compared to cats, may eat less fish and have virtually no MADAGs in their kidneys. Nevertheless, we have not been able to establish with certainty that fish consumption is accountable for increased CKD in domestic cats relative to other domestic species and wild cats that do not consume as much fish. Further studies are warranted to confirm an association between fish muscle consumption and CKD in cats. For example, it would be of interest to measure MADAGs by TLC and mass-spectrometry in certain types of fish, fish oils and, ultimately, pet foods with fish as the main ingredient. However, since cats can obtain all of the essential nutrients by consuming other animal tissues (Morris, 2002), it may be worthwhile considering whether incorporation of fish in the cat's diet is really necessary; avoidance of fish may be an appropriate precaution in the meantime.

In summary, we have shown that differences exist between domestic cats, domestic dogs and Scottish wildcats, with regard to the mineral and lipid content of their kidneys. As a result, we conclude that a species and environmental interaction may be responsible for the more common occurrence of renal disease in domestic cats compared to domestic dogs and Scottish wildcats. We suggest that lipid metabolic pathways in the kidney should be studied to establish whether cats have a genetic susceptibility to store lipid in the kidney and to elucidate the purpose that this may serve. In the long-term, perhaps such pathways could be targeted in order to reduce the potential for lipotoxic events and the risk of renal disease in domestic cats.

Further, we have established that some commercially available pet foods in the UK are not complete with regard to the micronutrient composition. Thus, our work has highlighted the importance of continued and more targeted exploration within this field of research, to finally establish the impact of such mineral imbalances on the health of the animal, with a specific focus on their role in the development of chronic diseases such as CKD and hyperthyroidism. For domestic cats, largely dependent on their owners for their diets, it is therefore incumbent on those owners to feed an appropriate, balanced and healthy diet to their cats. In particular, our studies provide a foundation for further research to determine whether feeding fish to cats may be harmful and should be avoided. In conclusion, we recommend that pet food manufacturers review their products to ensure that they meet the nutritional requirements, and support the health, of the domestic cat. Not only may this reduce the risk of certain diseases which may be promoted by dietary inadequacies, but it could also help to reassure cat owners that they are providing consistently adequate nutrition to their pets.

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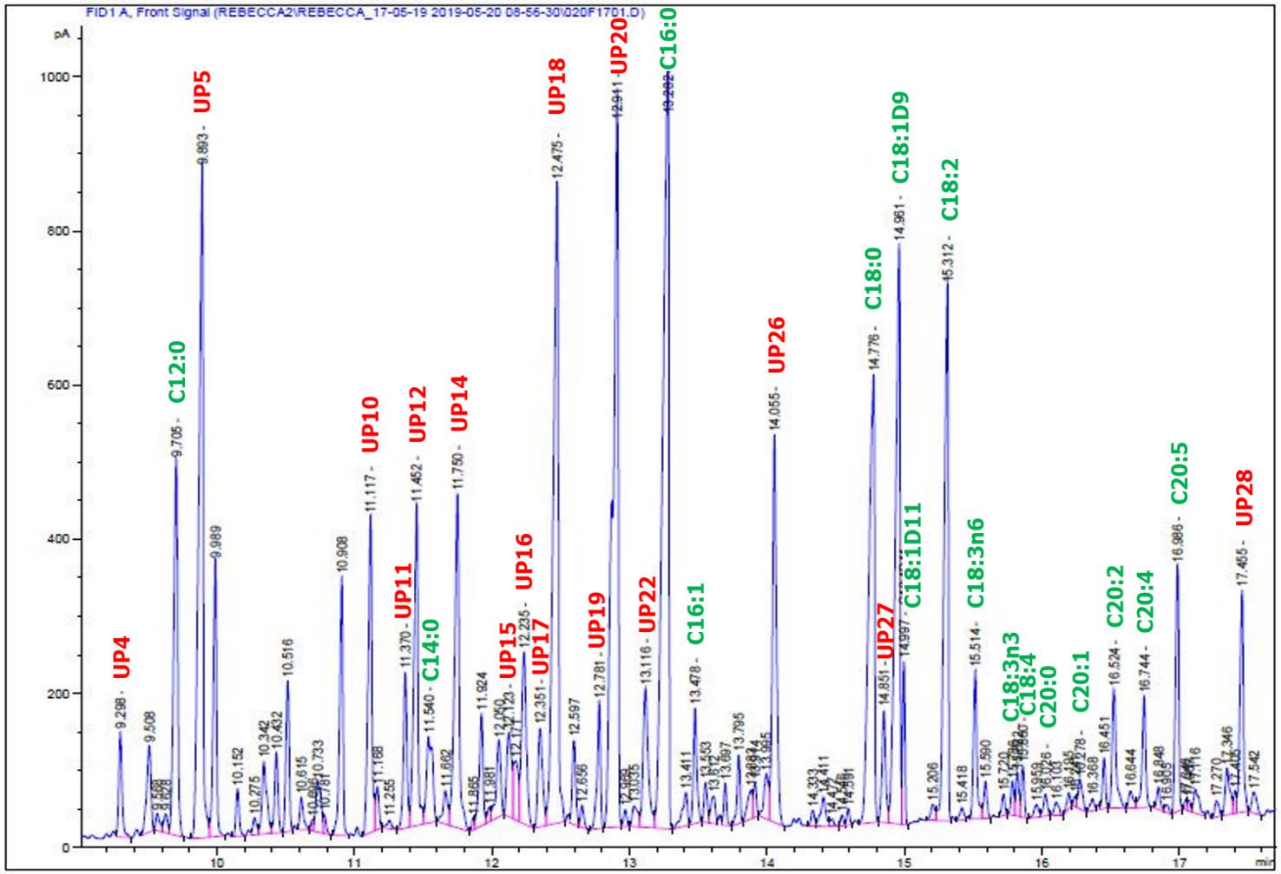
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7. Appendices

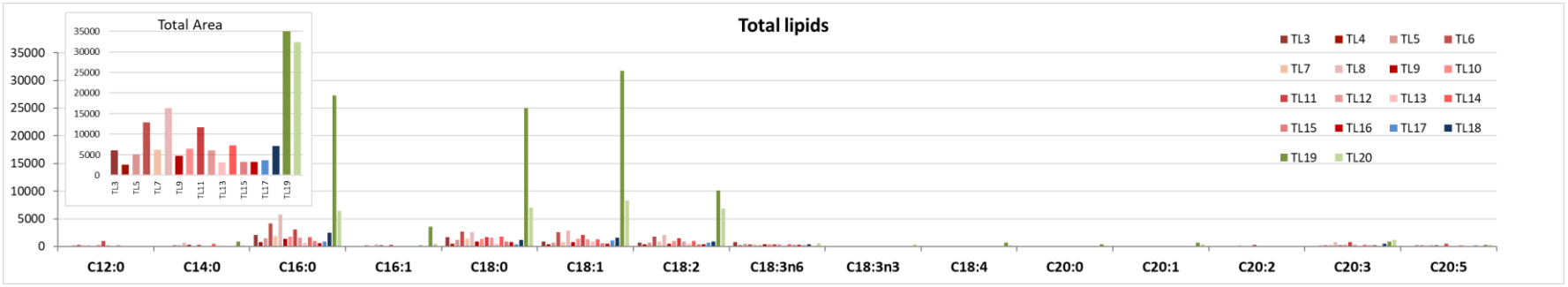
Appendix 1. A labelled chromatogram of a domestic cat kidney intracellular total lipid sample analysed by GC-FID.



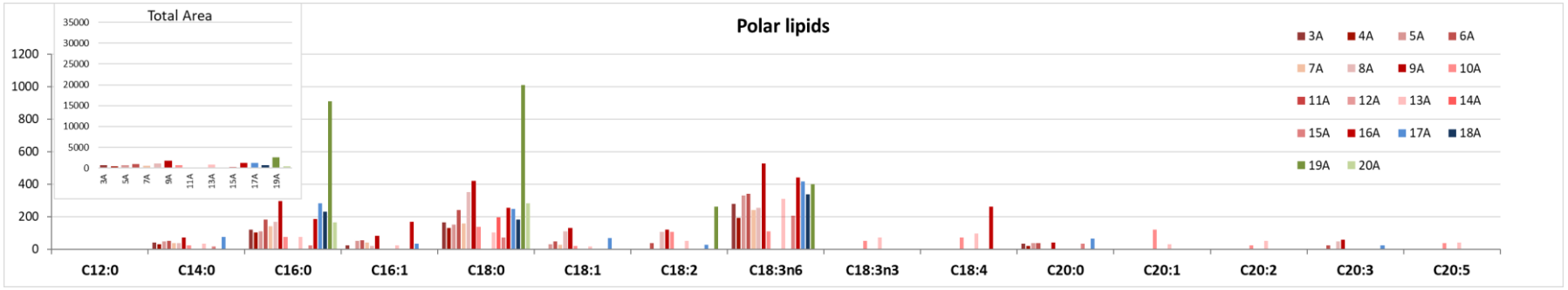
Appendix 1. A labelled chromatogram of a domestic cat kidney intracellular total lipid extract analysed by gas chromatography-flame ionisation detector (GC-FID)**.** The total lipid extract was transmethylated for analysis of constituent fatty acid methyl esters (FAMEs) by GC-FID, using helium carrier gas in an Agilent J&W DB-23 column and Agilent HPCHEM GC software. Peaks on the gas chromatogram were identified as known fatty acids (green), based on known masses (m/z) and/or matching with peaks in known fatty acid standards, or labelled as unknown lipids (UP1-28; red) if they could not be identified as known fatty acids.

Appendix 2. Peak areas of identified known fatty acids in total lipid, polar lipid, fatty acids, triacylglycerides, unknown lipid band and sterols samples from domestic cats, dogs and Scottish wildcats.

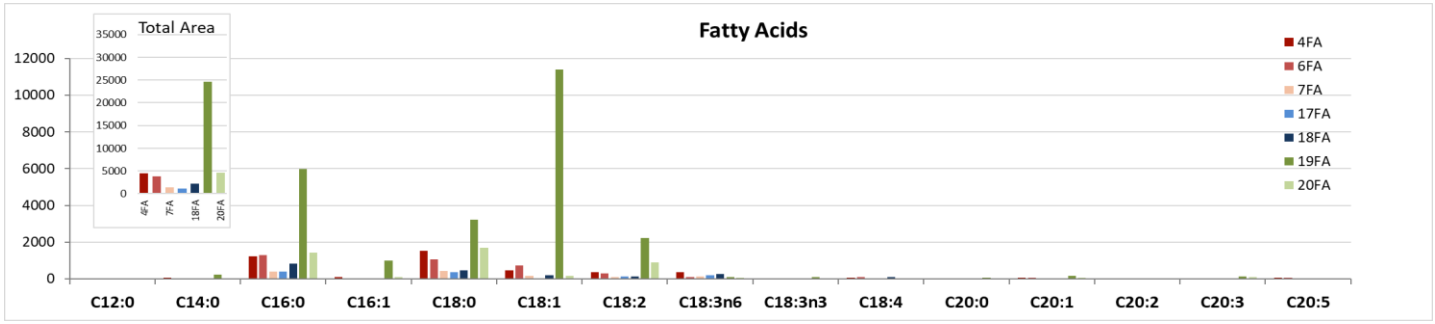
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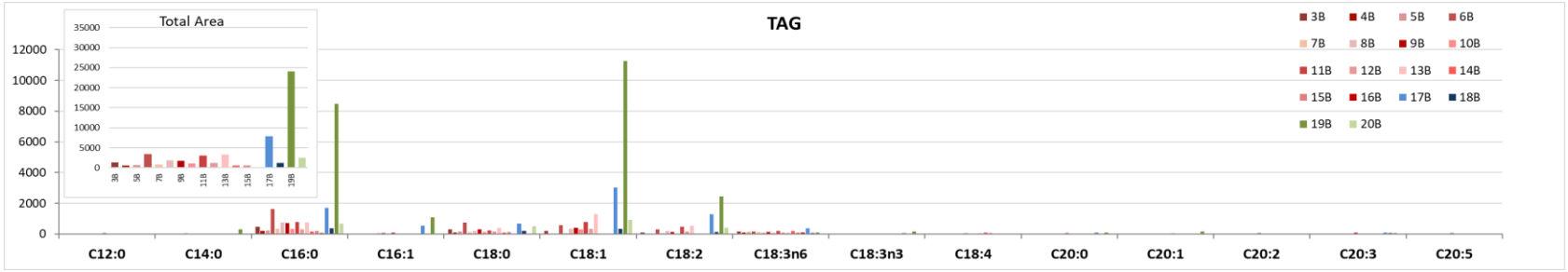
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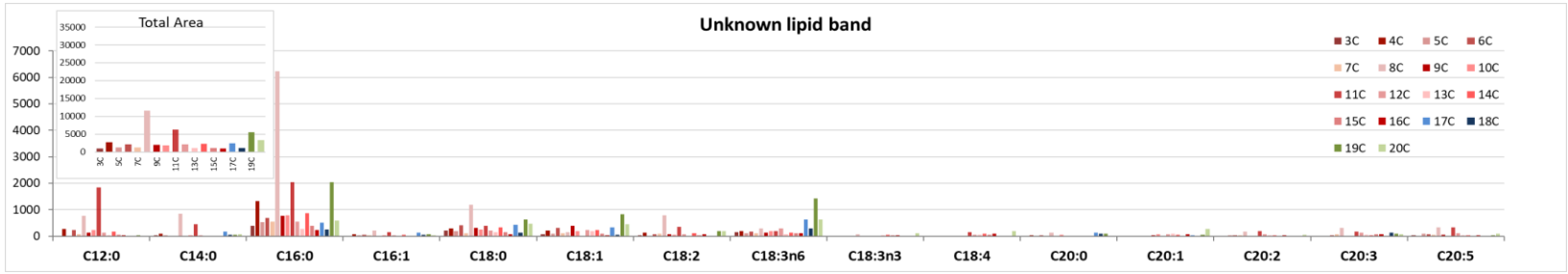
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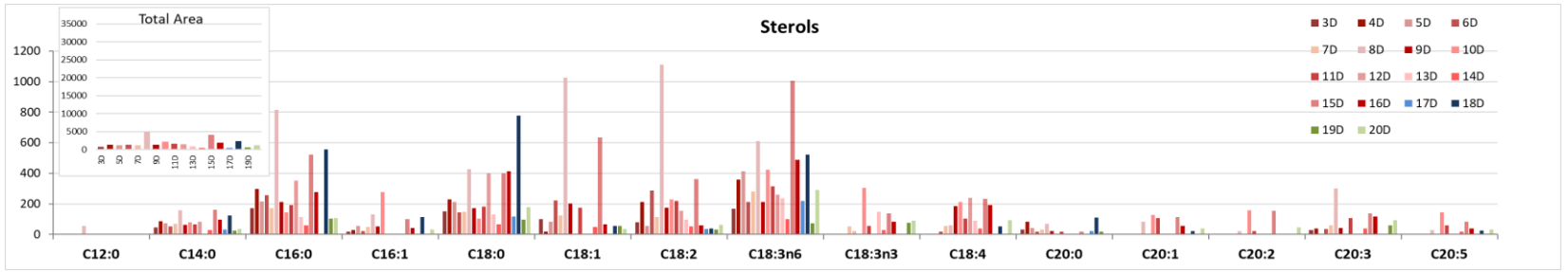
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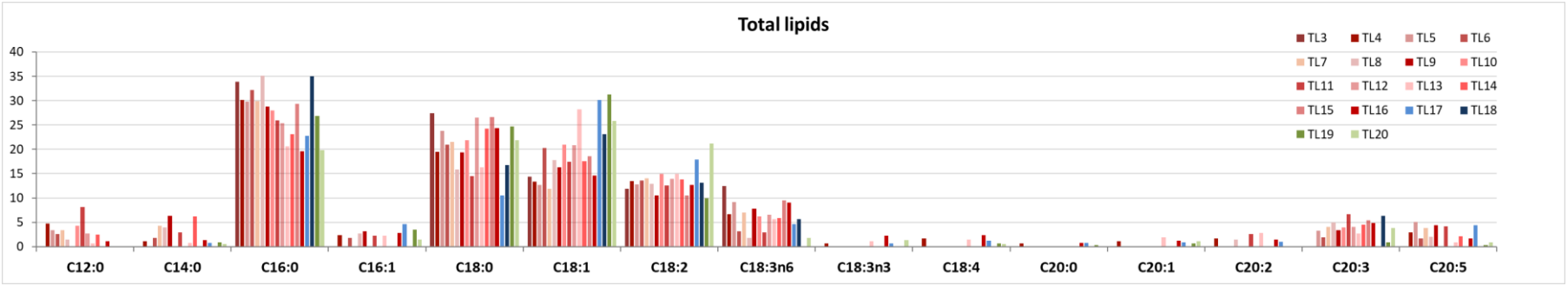
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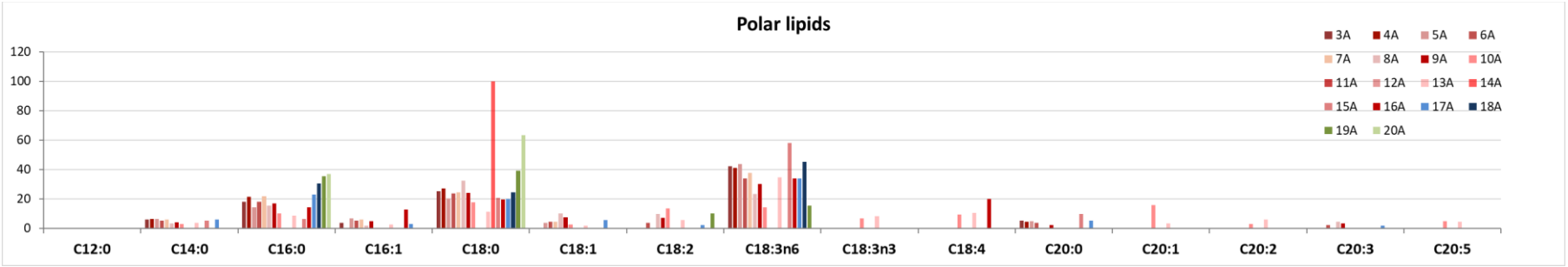
Appendix 2. Peak areas of identified known fatty acids in **(a)** total lipid, **(b)** polar lipid, **(c)** fatty acids, **(d)** triacylglycerides (TAG), **(e)** unknown lipid band and **(f)** sterols samples from domestic cats (red; n=14), dogs (blue; n=2) and Scottish wildcats (green; n=2). Graph inserts show the total area of all identified known fatty acid peaks in each sample. Transmethylated total lipid extracts and samples of different lipid molecular classes, separated by TLC and subsequently extracted from the silica TLC plate, were analysed by GC-FID, using helium carrier gas in an Agilent J&W DB-23 column and Agilent HPCHEM GC software. Peaks on the gas chromatograms that were identified as known fatty acids, based on known masses (m/z) and/or matching with peaks in known fatty acid standards, were labelled. The areas under the peaks were integrated and the area of each identified known fatty acid (main graphs) as well as the total area of all identified known fatty acids (graph inserts) was plotted for each individual animal for each lipid sample type.

Appendix 3. Identified known fatty acids in total lipid, polar lipid, fatty acids, triacylglycerides, unknown lipid band and sterols samples from domestic cats, dogs and Scottish wildcats as percentages of the total amounts of all identified known fatty acids in the samples.

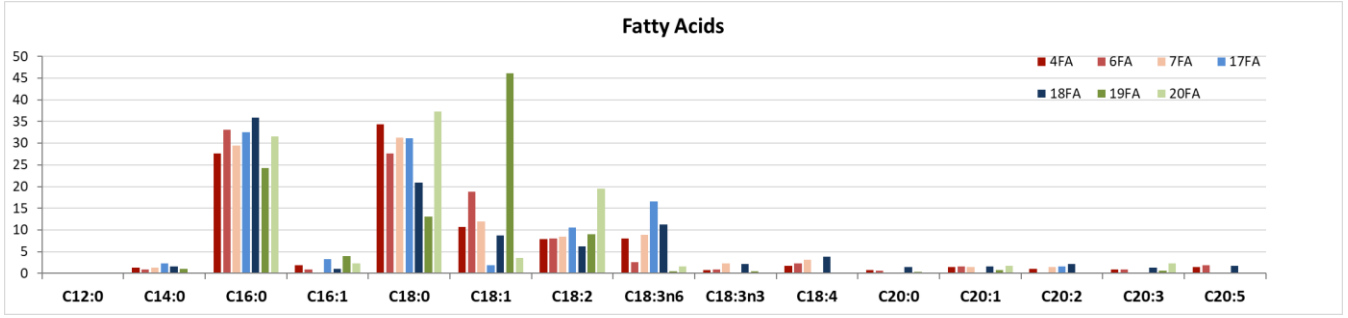
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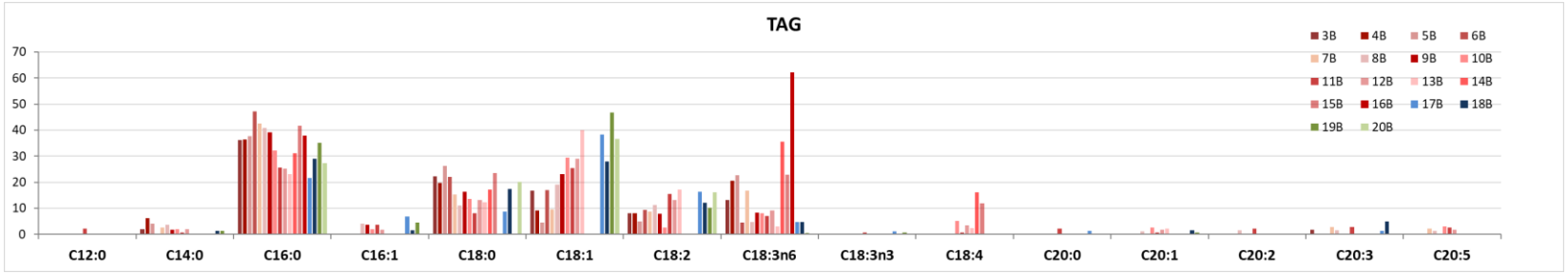
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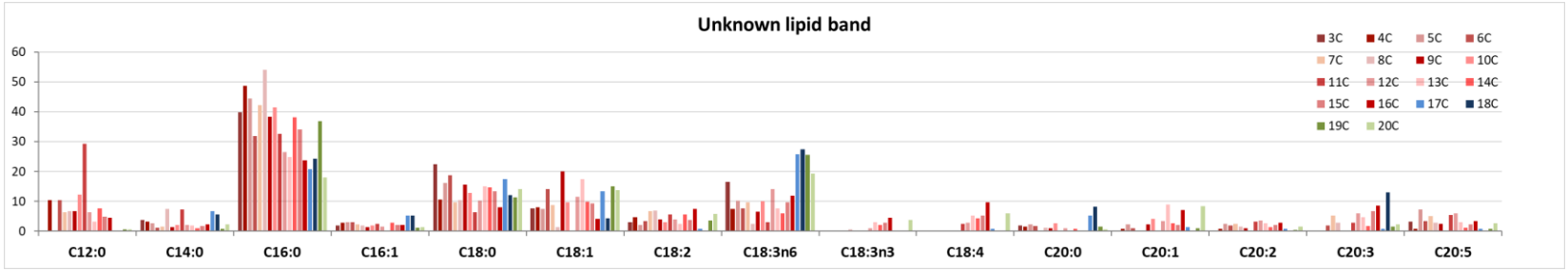
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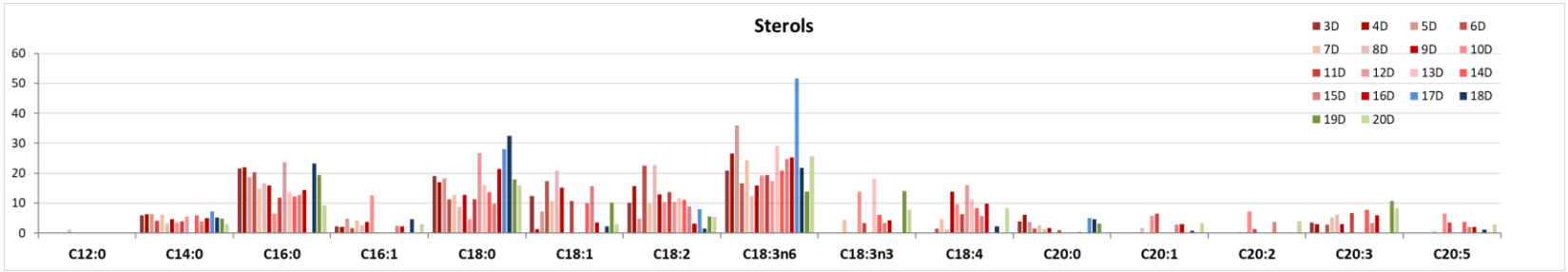
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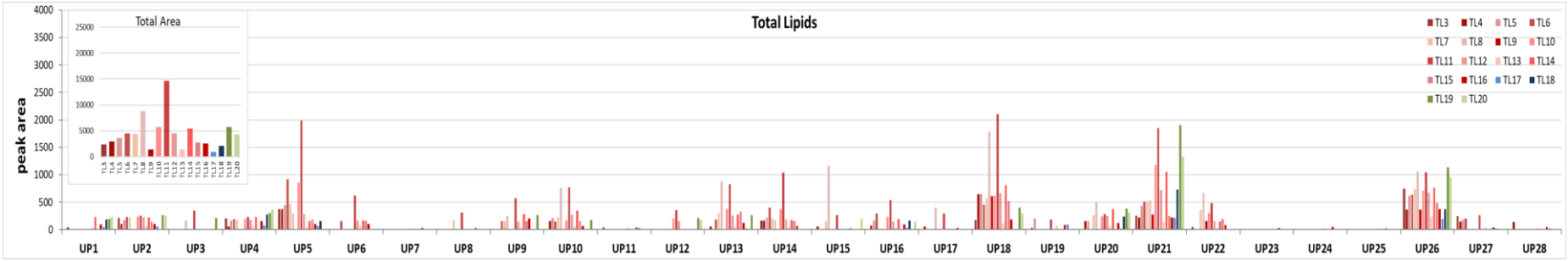
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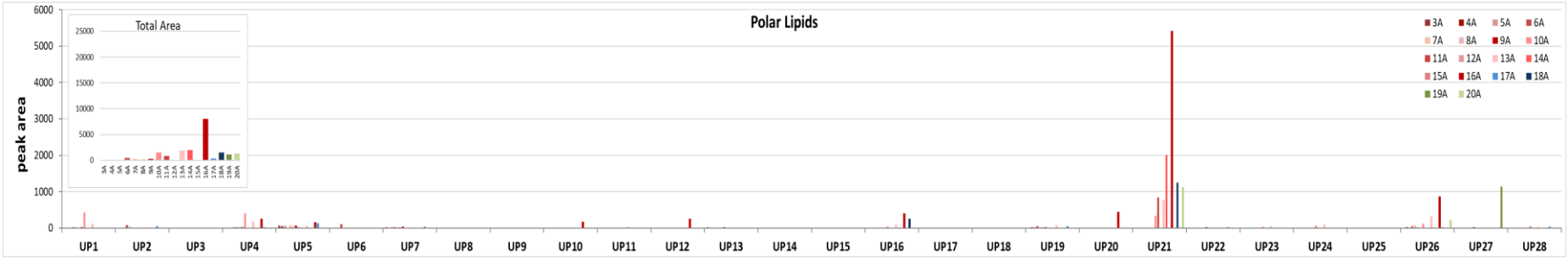
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Appendix 4. Peak areas of unknown fatty acids in total lipid, polar lipid, fatty acids, triacylglycerides, unknown lipid band and sterols samples from domestic cats, dogs and Scottish wildcats.

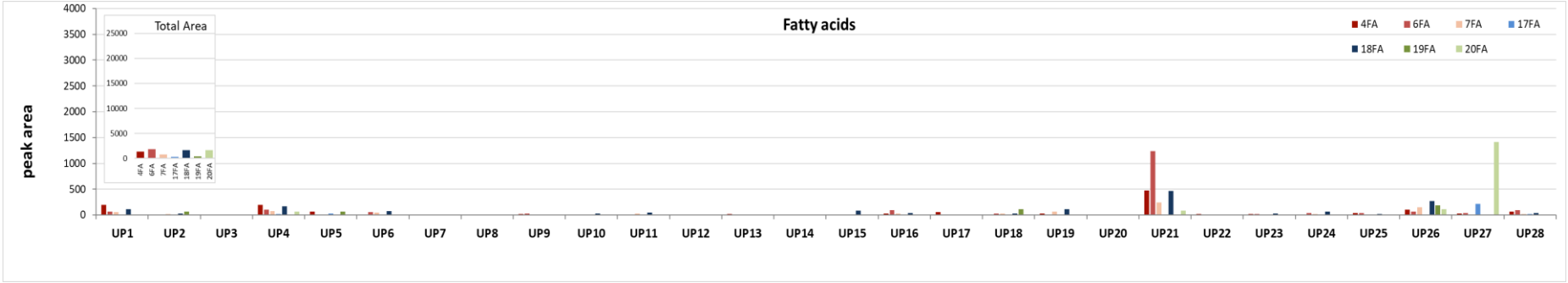
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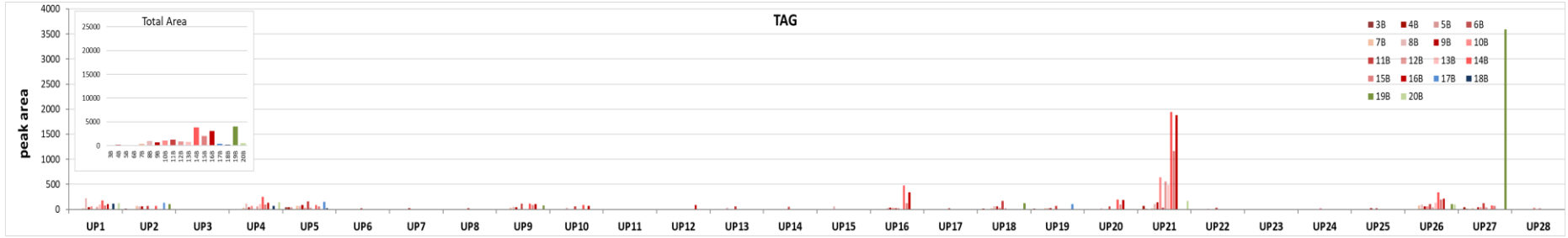
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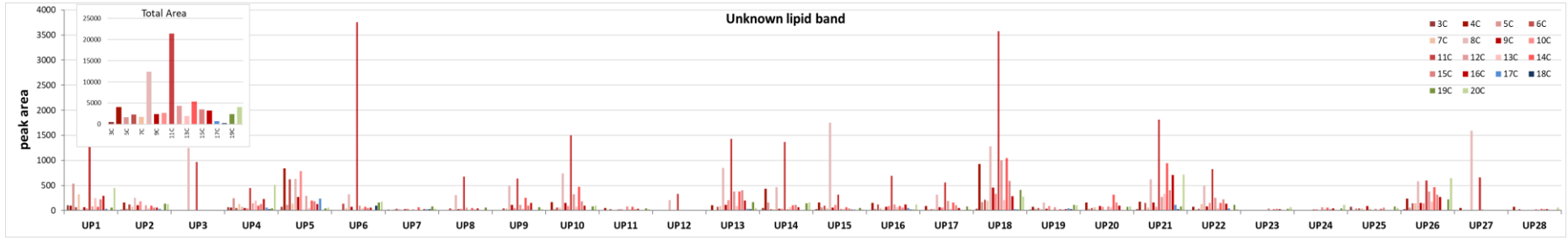
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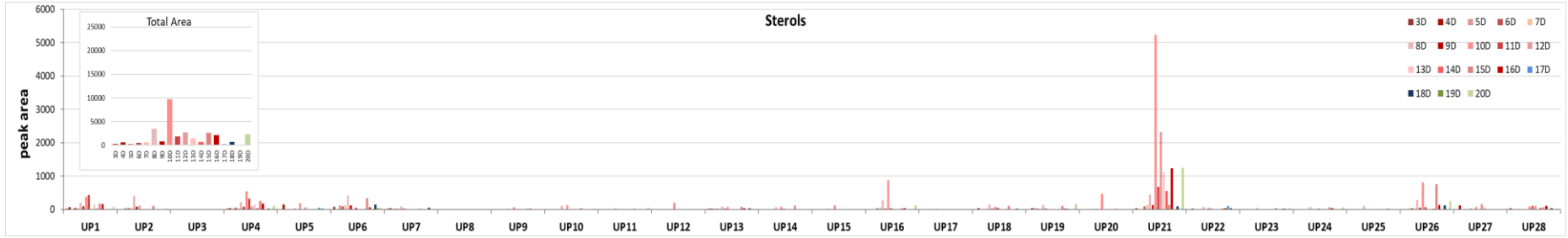
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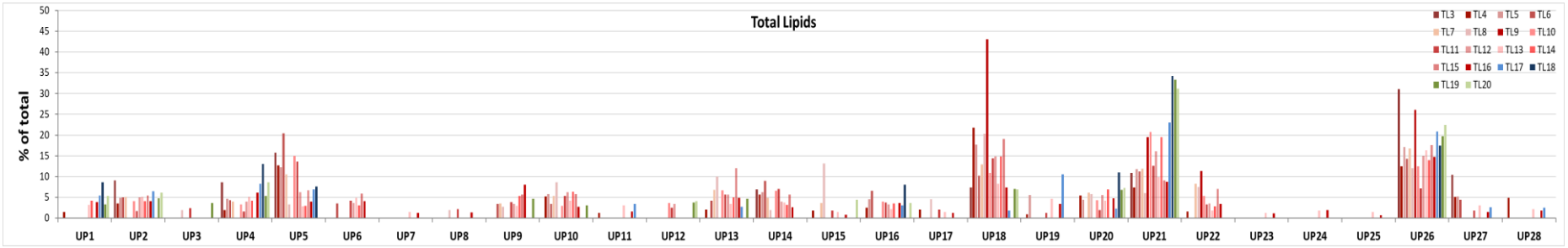
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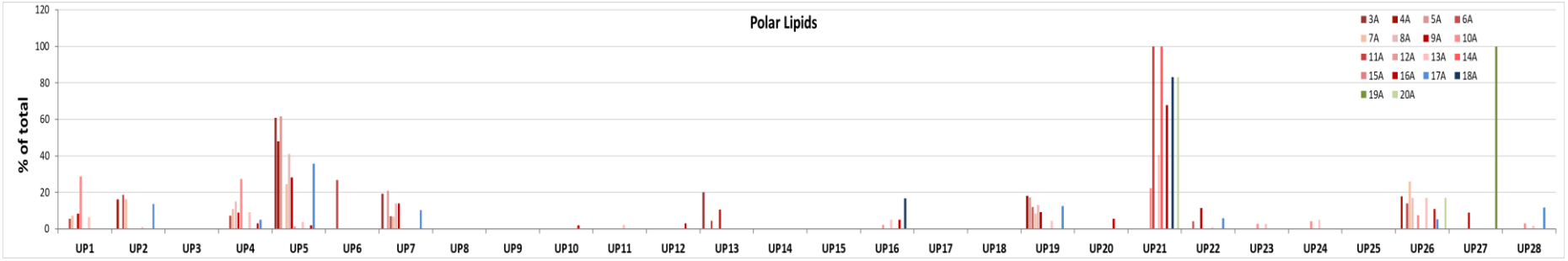
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Appendix 5. Unknown fatty acids in total lipid, polar lipid, fatty acids, triacylglycerides, unknown lipid band and sterols samples from domestic cats, dogs and Scottish wildcats as percentages of the total amounts of all detected unknown fatty acids in the samples.

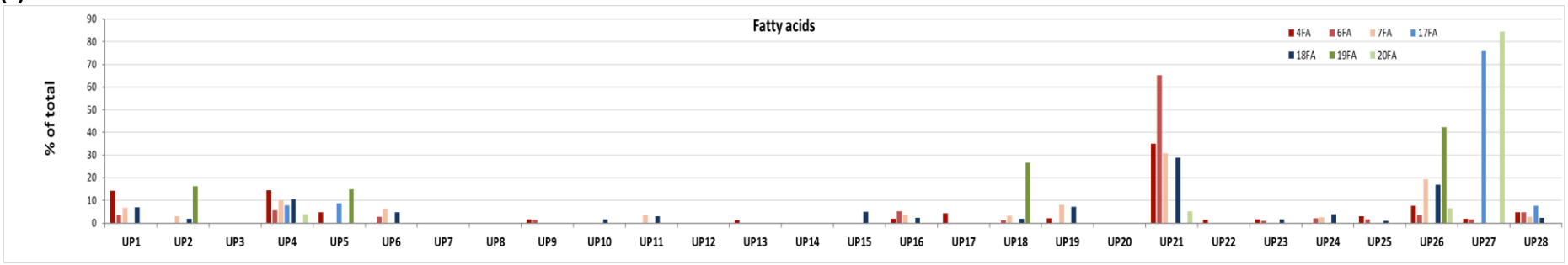
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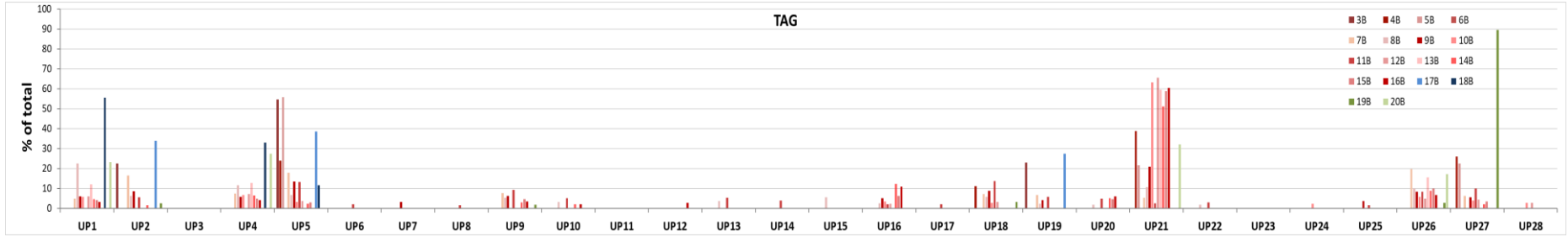
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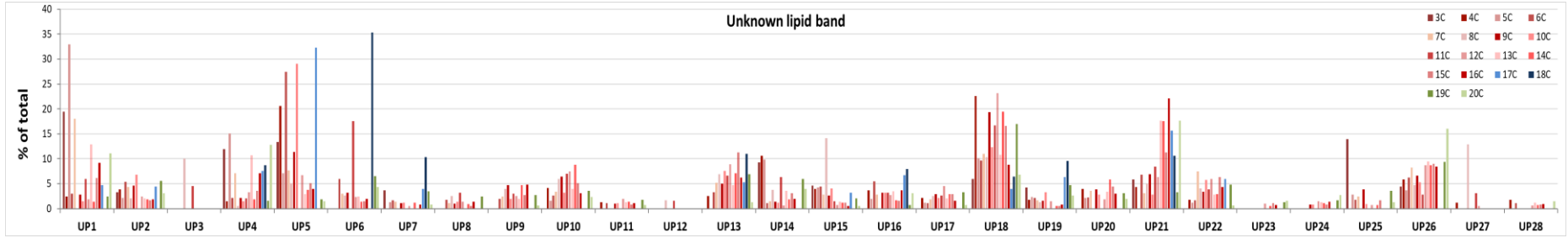
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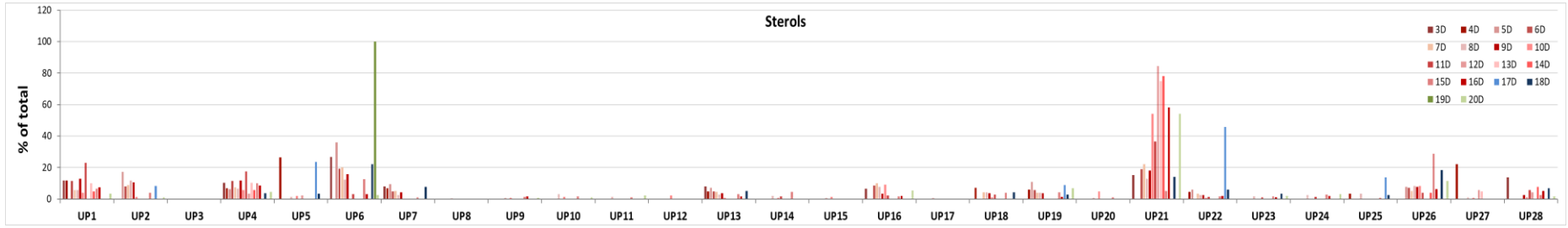
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Appendix 6. Survey of cat owner feeding habits structure.

Cat feeding habits

A request for your help

This survey aims to gather information about the lifestyle, and particularly the feeding habits, of domestic cats.

There are many different options available for feeding our pet cats. We hope that the information collected here will help to better understand how this is actually done and provide an insight into the domestic cat's diet.

This survey will allow you to answer for up to 3 cats and will take around 5 minutes to complete for each cat. If you own more than 3 cats, click on the survey link again to complete the survey for any additional cats.

No personal information (name, email, etc.) will be collected as part of this survey. All responses will remain anonymous and will not be kept for longer than the study period, which ends in September 2020.

By completing this survey you are consenting to our use of the information you provide, as described.

Thank you for your time and interest in taking our survey. Your help will be greatly appreciated.

Cat details

What is the breed of your cat? * *Required*

- ○ Domestic Shorthair (DSH)
- ○ Domestic Longhair (DLH)
- ○ Maine Coon
- ○ Persian
- ○ Ragdoll
- ○ Bengal
- ○ Birman
- ○ Burmese
- ○ Siamese
- ○ Scottish fold
- ○ Sphynx
- ○ Other

If you selected Other, please specify:

What is your cat's date of birth? *(If the exact date of birth is unknown, please estimate as closely as possible.)* * *Required*

Dates need to be in the format 'DD/MM/YYYY', for example 27/03/1980.

(dd/mm/yyyy)

Is this Date of Birth exact or estimated? * *Required*

- Exact
- Estimated

What is your cat's sex? * *Required*

- Male
- Female

Is your cat neutered? * *Required*

- Yes
- No

How much, in kilograms (kg), does your cat weigh?

Is this body weight exact or estimated?

- Exact
- Estimated

Is your cat considered healthy? (i.e. no disease recognised by your vet, and no signs of illness?) * *Required*

- Yes
- No

What disease or signs of illness does your cat have?

Is your cat fed a prescription diet? (i.e. a diet designed to treat a specific disease process - e.g. renal/kidney, urinary, liver, weight management, joint care, sensitivity diet etc.) * *Required*

- ○ Yes
- ○ No

What prescription diet is your cat fed?

How many other cats does your cat live with? * *Required*

Commercial diets

Definitions:

- **Commercial pet food:** Food that you buy that is ready to feed to your cat as their main diet. This is not food that you prepare or cook yourself for your pet.
- **Dry food:** Commercial pet food that is usually sold in bags or boxes in the form of small "biscuits". Not treats.
- **Wet food:** Commercial pet food that is usually sold in cans or foil trays or pouches, looks meaty and is wet. Not treats.

What proportion of your cat's diet is made up of commercial pet food? * *Required*

- ○ All
- ○ None
- ○ Half
- ○ More than half
- ○ Less than half

Of this, what proportion is **dry food**?

- All
- None
- Half
- More than half
- Less than half

What proportion of your cat's commercial diet is **wet food**?

- All
- None
- Half
- More than half
- Less than half

Of the commercial wet food that your cat consumes, what proportion is packaged in **cans/tins**?

- All
- None
- Half
- More than half
- Less than half

Of the commercial wet food that your cat consumes, what proportion is packaged in **pouches/sachets**?

- All
- None
- Half
- More than half
- Less than half

Of the commercial wet food that your cat consumes, what proportion is packaged in **foil trays**?

- All
- None
- Half
- More than half
- Less than half

Of the commercial wet food that your cat comsumes, what proportion is packaged in **another packaging type** not previously mentioned?

- ○ All
- ○ None
- ○ Half
- ○ More than half
- ○ Less than half

If the wet food fed to your cat is "in other packaging", what packaging is this?

How often do you vary the **BRAND** (e.g. Felix, Sheba, Whiskas etc.) of commercial food that you feed to your cat?

How often do you vary the **FLAVOUR / MAIN INGREDIENT** (e.g. beef, chicken, fish etc.) of commercial food that you feed to your cat?

How often do you vary the **TYPE (e.g. wet/dry)** of commercial food that you feed to your cat?

Non-commercial pet food

Definitions:

- **Commercial pet food:** Food that you buy that is ready to feed to your cat as their main diet. This is not food that you prepare or cook yourself for your pet.
- **Non-commercial pet food:** Food that is NOT commercial pet food. Includes home-prepared diets, table-scraps and leftovers.

Concerning the proportion of your cat's main diet that is not commercial pet food, how much is:

	** Required*
Home-prepared diets?	Please select
Table scraps?	Please select
Leftovers?	Please select
Other?	Please select

If 'other' non-commercial pet foods are fed, please give details of these. *Optional*

Pet food flavours

What proportion of your cat's diet is **fish**? ** Required*

What proportion of your cat's diet is **meat**? ** Required*

Of the meat that your cat consumes, what proportion is **red meat (e.g. beef, lamb, pork)**?

Of the meat that your cat consumes, what proportion is **white meat (e.g. chicken, turkey)**?

Frequency of feeding

How frequently do you feed your cat? * *Required*

- Once a day
- Twice a day
- 3 times a day
- More than 3 times a day
- Food is always freely available

Does your cat eat all of the food that you provide? * *Required*

- Yes, always
- No, never
- Most of the time
- Sometimes

Treats

How frequently does your cat receive a treat? * *Required*

- More than 5 treats a day
- 2 to 5 treats a day
- 1 treat a day
- 4 to 6 treats a week
- 1 to 3 treats a week
- Less than 1 treat a week
- Never

Please give details of the treats your cat receives.

Outdoors

How many hours a day (24 hour period) does your cat spend outdoors, on average? * *Required*

How frequently does your cat catch AND eat prey? * *Required*

- ○ Daily
- ○ Weekly
- ○ Monthly
- ○ A few times a year
- ○ Once a year
- ○ Never

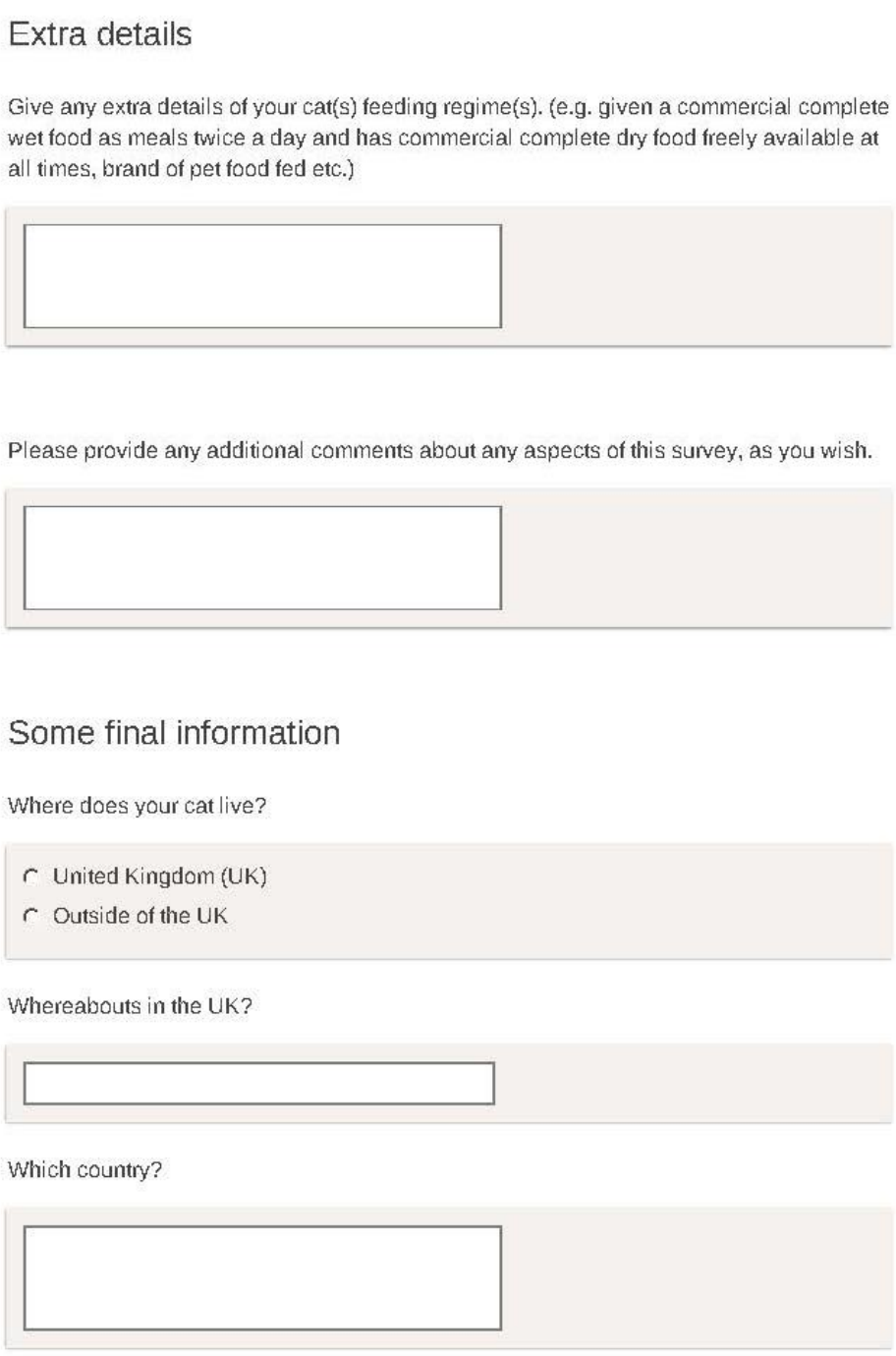
If your cat eats prey, what typical prey items does your cat eat? (Select all that apply)

- ☐ Birds
- ☐ Mice
- ☐ Voles
- ☐ Rabbits
- ☐ Frogs
- ☐ Insects
- ☐ Other

If you selected Other, please specify:

Another cat?

Do you have another cat? * *Required*



Appendix 6. Survey of cat owner feeding habits, created using Jisc Online Surveys, showing the default route through the survey for respondents with one cat. The full set of questions would become available again, through custom routing, for owners that answered 'yes' to the question 'Do you have another cat?'. Similarly, respondents that answered 'All' to the question regarding the proportion of commercial pet food in the diet would bypass the questions on 'non-commercial pet food'.