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Diet-related factors in the conservation of kiwi

(*Apteryx mantelli*)

A thesis presented in partial fulfilment of the requirements for the degree of

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“An infinity of forest lies dormant in the dreams of one acorn.”

Wayne W. Dyer

ABSTRACT

Captive management is a valuable tool in the conservation of endangered species. The subtleties of a species' nutrient requirements are increasingly recognised as fundamental to their ability to thrive in captivity. This study focuses on the nutrient composition of diet and factors related to diet that can influence the conservation management of captive species. North Island brown kiwi (*Apteryx mantelli)* were used as a case study but the implications are applicable across species. A variety of techniques were used to determine the natural diet of kiwi. The fatty acid composition of gizzard fat was not a reliable predictor of dietary intake for kiwi. A synthetic diet was formulated to closely match the nutrient composition and apparent digestibility of a near-natural diet. Ingredients used in the synthetic diet were readily available worldwide, and the physical form of the diet was tailored specifically for kiwi, hence long, moist, 'worm-like' items were produced.

The unusual ecology of kiwi, coupled with a lack of data relating to digestibility values, led us to question whether the physiological processes of kiwi fit within known/common parameters for avian species. Thus, a common, domesticated, omnivorous species for which substantial digestibility and physiological data exist (roosters (*Gallus gallus*)) was also fed the kiwi near-natural and synthetic diets, and direct comparisons of apparent digestibilities were made with kiwi. Apparent digestibilities of macronutrients were higher in kiwi fed the synthetic than the near-natural diet. They were also higher in kiwi than roosters fed either diet. Inconsistencies in macronutrient digestibilities in both kiwi and roosters fed the near-natural versus the synthetic diets preclude using roosters to predict macronutrient digestibilities of a diet fed to kiwi. For both species the source of the macronutrients in the diet appears to have a marked influence on apparent digestibility.

Equally important to health as a nutritionally adequate diet is the community structure of enteral bacteria. Captive kiwi, brown teal (*Anas chlorotis*) and takahe (*Porphyrio* [Notornis] *mantelli*) had a greater diversity of intestinal bacteria than wild birds of the same species. The acquisition and colonisation of enteral bacteria in kiwi and chickens varied over the first three weeks of life. By three weeks of age the enteral bacteria of kiwi chicks was similar to that of adult birds. Acquisition and colonisation of intestinal bacteria in chickens was not influenced by bacteria adherent to their eggshells. Diet affected enteral bacterial diversity; as kiwi consumed a greater proportion of the

wild than the captive diet the number of dominant enteral bacterial genera decreased. Takahe fed both a captive and wild diet had enteral bacterial communities intermediate to that of birds eating either a solely captive or a solely wild diet.

The relevance of the work in this thesis to the captive management of other wildlife is discussed, and recommendations are presented on how to address a range of diet-related issues including palatability, neophobia, obesity, and preparation of captive-held animals for release into the wild.

Table of Contents

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All photos are taken by me unless source is given.

CHAPTER 1

General Introduction

The pervasive influence of human-kind has spread to every ecosystem on the surface of the Earth (Vitousek *et al.*, 1997). Current estimates of extinction rates for all taxa vary (Stork, 2010); the global loss per decade of Amazonian plant species has been estimated to be between 1% and 20% (Hubbell *et al.*, 2008; Feeley & Silman, 2009 respectively). Similarly, the number of species listed on the IUCN Red List of Threatened Species varies. For example, 30-40% of the species assessed on the IUCN Red List are currently classed as threatened (IUCN, 2010). Common reasons for the disparity in IUCN estimates include: newly available information on population numbers and/or rates of decline; taxonomic revisions that result in the splitting or merging of species; conservation measures that have improved the status of the species; or the development of new threats that cause a deterioration in status (IUCN, 2010). The anthropocene era is experiencing accelerated rates of extinction, and there is little doubt that human activities are the main cause (Rockstrom *et al.*, 2009). Given the huge effect humans have on species loss, we have an ethical obligation to actively reduce the current rate of extinction.

Often taxa that are targeted for conservation are those that are perceived to have benefits for human society. For example, those of benefit to medical research, or with the potential to be production animals, or those that are aesthetically pleasing to humans, tend to be favoured. This selectivity in conservation effort is likely to skew biological diversity.

Until recently, the general aim of conservation has been to prevent the extinction of as many species as possible (Soulé, 1985; Simberloff, 1998). While this is a desirable goal, it is becoming increasingly clear that pragmatic decisions need to be made on which species to manage intensively. The management of North Island brown kiwi (*Apteryx mantelli*) is such a case. The bird's status as the national icon of New Zealand has led to its intensive management, for example, Operation Nest Egg (ONE) is a programme where kiwi eggs are sourced from the wild, hatched in captivity and then released again into the wild when they are of a sufficient weight to defend themselves from stoats. However, despite kiwi having been held in captivity for over 100 years, little is known about their dietary needs, nutrient requirements, the manner in which nutrients are acquired or utilised, or optimal conditions for reproduction.

Whilst this thesis focuses on improving the survival and reproductive capacity of captive kiwi, it has much broader relevance to the issue of managing animals in

captivity. In all cases, it is important to also investigate reasons for the decline of a species in the wild.

Captive management: New Zealand and Internationally

Large numbers of animals are held captive in zoos and other captive institutions around the world. For example, at least 900 zoos and aquaria record information on over two million captive animals (15,000 taxa and 10,000 species) for the International Species Information System database (Pritchard *et al.*, 2011). While captive management is an important tool for species recovery across the globe, in New Zealand it is particularly important (McBride, 2011; Maciaszek, 2012). New Zealand has the highest number of endangered species per capita in the world (Garland & Butler, 1994; Craig *et al.*, 2000), and has proportionally more 'protected' species involved in captive management programmes than any other country. For example, captive management has been used as part of the species recovery plans for many species, including, brown teal, black stilt, shore plover, kiwi, tuatara and kokako (Department of Conservation, 2013). Given the increasing use of captive management in New Zealand, studies such as those carried out here are vital to ensure husbandry techniques are continually improved.

In situ versus ex situ management

One simple solution to the problem of species recovery could be to apply farming practices. However, not all species accept a confined environment, are willing to undergo forced pairings, or have the capacity to greatly increase reproductive output as do production animals, such as sheep, cows and pigs. The recovery of many threatened species has met with limited success because there is limited knowledge of the physiological and ecological factors that are necessary for their captive management.

It is not sufficient to judge species on their importance to man, as a seemingly insignificant species may be a keystone species to other taxa (Redford, 1992; Ives & Carpenter, 2007). For example, nectivorous and frugivorous avian species are important pollinators and seed dispersers, therefore the loss of taxa from these functional groups can have detrimental effects on whole ecosystems via population and community dynamics (Sekercioglu *et al.*, 2004).

Coupled with the issue of 'why' to conserve a species, is the question of 'where' to conserve it. Typically, conservation efforts focus on either *in situ* or *ex situ* management. *In situ* management involves managing species in their natural environment, whereas *ex situ* management is the removal of a population from the wild to a captive facility. A less frequently used management option is to preserve a part of the organism either by holding seeds in seed banks (Prosser *et al.*, 2007; Lennon & Jones, 2011) or by collecting, freezing and storing sperm, eggs or tissue for future use (Holt, 2008; Kouba & Vance, 2009). While this procedure ensures that DNA is preserved, it functions primarily as a safety net in the event of a failure of *in situ* and *ex situ* management.

The decision of whether to conserve a species *in situ* or *ex situ* is complex and the options are not mutually exclusive. A decision to manage animals *ex situ* is often driven by habitat availability, extent and consequence of predation pressures, existing genetic variation and how best to manage it, and what influence the removal of animals would have on the *in situ* ecosystem.

Habitat availability and in situ *management*

While the management of animals *in situ* is preferred over *ex* situ (Zimmermann, 2010) it is not always feasible. Often suitable habitats for *in situ* management no longer exist or are scarce, and may only become available following a reduction in the human population (Soule *et al.*, 1986; Zimmermann, 2010). Thus, habitat loss can make the management of species in the wild untenable. For example, a significant reduction in the number of wild Amur tigers (*Panthera tigris altaica*), due to habitat destruction and intensive hunting, has necessitated some populations to be managed in captivity (Sharma *et al.*, 2009).

'Captive' conditions generally differ from those in the wild and the link between an animal and its habitat is severed (McPhee & Carlstead, 2010). The long term effects of this phenomenon are unknown.

Extent and consequence of predation pressures

Predation pressure is a common cause of species decline in the wild and *in situ* management is often impeded by high predation rates. In captivity, predation can be monitored and generally eliminated. In the absence of intensive predator control within

in situ populations, a short spell of captive management may be beneficial. For example, stoat predation is the primary cause of the high mortality rates reported for juvenile kiwi in the wild (McLennan, 1996); however, if kiwi are hatched and reared in captivity until they large enough to defend themselves from predators, then their survival rate following release back into the wild is drastically increased (Department of Conservation, 2004; Colbourne *et al.*, 2005). Decisions regarding 'best practice' for conservation efforts are not always clear-cut and a combination of techniques may be required to achieve management goals.

Effect on genetic variation

Genetic diversity is often greater within wild populations than in captive ones because wild populations tend to be larger. *Ex situ* captive management often focuses on a subset of the wild population, thus the very act of capturing representatives of a wild population influences subsequent genetic diversity. However, in some cases careful management of genetic factors through planned pairings can result in greater genetic variability in captive than wild populations. For example, genetic diversity in captive Amur tigers is higher than in their wild counterparts (Henry *et al.*, 2009). Of concern for release programmes is that genetic variants that benefit survival in a captive population may differ from those favoured in the wild (Montgomery *et al.*, 2010). Thus, genetic considerations are not only applicable to *ex situ* management, because the removal of part or all of a population can also impact on the genetic diversity of the remaining population.

Effect of captive management on in situ *ecosystems*

In addition, removing a subset of the wild population may have consequences for the *in situ* ecosystem. Thus, the effect of captive management is not limited to the target species. The role of the target species in the environment will dictate whether the ecosystem is seriously affected by its depletion or absence. For example, the loss of a keystone species may have more impact on the ecosystem than the loss of a non-keystone species (Simberloff, 1998).

Ex situ *management*

An increasing number of species recovery programmes are favouring *ex situ* over *in situ* management (Frankham, 2008). Approximately 10,000 species are currently

kept on farms, zoos, conservation breeding centres and research laboratories around the world (Mason, 2010). For terrestrial vertebrates alone, it is estimated that approximately 4000-6000 species may have to be captive-bred in the future to ensure their survival (Lacy, 2006). There is, however, limited space available for *ex situ* management.

In addition to breeding for release into a natural or new environment, there are a variety of reasons why animals are held in captivity, including advocacy, research, production and conservation education (Ballou *et al.*, 2010; Hancocks, 2010; Kreger & Hutchins, 2010). Increasingly, zoos are actively involved in conservation efforts and have to balance this role against providing research opportunities and remaining economically viable (Zimmermann, 2010).

Agriculture versus conservation

The agricultural sector has taken full advantage of the ease with which some species reproduce under captive conditions and has markedly increased the reproductive output of these species for economic gain. While it would seem favourable to 'farm' endangered animals in a similar manner, selective breeding for desirable traits (to maximise production in agriculture) is not an option for the conservation field. This practice decreases genetic variation among individuals and can make small populations more susceptible to disease or stochastic events (Lande, 1998). Genetically depauperate 'bottleneck' populations are common for endangered species, so gene pools for colonising populations are often small. Despite this difference in focus, the agricultural industry has considerably more experience in livestock production than exists within the conservation sector. Thus, a more collaborative approach that combines knowledge and expertise from the production industry with conservation goals has the potential to improve captive husbandry techniques and increase the success of conservation programmes.

From a conservation perspective, once the decision is made to manage animals in captivity, success is not assured and is not equal across species. Ring-tailed lemurs (*Lemur catta*) (Jolly *et al.*, 2006), snow leopards (*Panthera uncia*) (Clubb & Mason, 2003), salmon (Heath *et al.*, 2003) and American kestrels (*Falco sparverius*) (Bardo & Bird, 2009) thrive in captivity, whereas mongoose lemurs (*Eulemur mongoz*) (Hearn *et al.*, 1996), forest duikers (*Cephalophus* spp.) (Barnes *et al.*, 2002), giraffe (*Giraffa cameleopardalis*) (Clauss *et al.*, 2007) and kakapo (*Strigops habroptilus*) (Lloyd &

Powlesland, 1994) do not reproduce well out of their natural environment. Thus, in some cases, even if *ex situ* management is possible it may not always be the best strategy.

Effect of captivity on social interactions

Social interactions between captive animals are altered and often contrived with the aim of maximising reproductive output and manipulating genetic crosses (McPhee & Carlstead, 2010). For example: captive animals no longer have to compete with conspecifics for reproductive success; the choice of dietary items is often restricted or dramatically altered; their home range is reduced in most cases; and their young are often removed to a 'safer' environment before normal adult-young interactions can be established.

Changes of physiology and behaviour in captivity

The long term consequences of captivity can be dire (Kleiman *et al.*, 2010). The removal of an animal from its environment can alter its physiology and behaviour, sometimes irreversibly (McPhee, 2003). This induces a change so profound that survival post-release can be severely affected, particularly for species whose behaviour is not instinctive, species lower down the food chain, or species released into areas that have not undergone extensive predator control (Snyder *et al.*, 1996). Some species, such as domestic cats (*Felis catus*), are able to establish feral populations from captivity relatively easily (Snyder *et al.*, 1996). Some species of turkeys (*Meleagris gallopavo*) are unable to form wild populations after a few generations in captivity (Snyder *et al.*, 1996). The mortality rate of captive golden lion tamarins (*Leontopithecus rosalia*) reintroduced into the wild was high due to a failure to recognise natural foods and non-avian predators (Kleiman, 1990). Whether these behavioural changes in themselves are problematic can be disputed; some changes in behaviour are not necessarily good or bad, they are simply changes. However, once behavioural changes induced by captivity impinge on the welfare of animals, then there is an ethical obligation to reconsider the choice of *ex situ* management.

Nutrition and captivity

Nutrition is critical for survival (Dierenfeld, 1997). The subtleties of a species' nutrient requirements are increasingly recognised as being fundamentally important to whether it will thrive in captivity (Kirk Baer *et al.*, 2010). Dietary deficiencies are difficult to identify, particularly in threatened species where sample sizes can be limited and the risks associated with dietary trials may be unacceptably high. Deficiencies in diet can influence health at any stage of life, including that of future generations, for example, diets can influence epigenetic regulation of gene expression in the foetus (Zeisel, 2009). This study focuses primarily on the nutrient composition of diet and factors related to diet that can influence the conservation management of captive species.

Nutrient composition of diet

The importance of macronutrients in the management of captive animals was first recognised in the nineteenth century (Dierenfeld, 1997). For example, protein quality and quantity was found lacking in the diets of cebid monkeys at Philidelphia Zoo (Corson-White, 1932). Similarly, the importance of micro and trace elements became apparent in the twentieth century (Wackernagel, 1966). The under or over supply of both macro and micronutrients can impinge on an animal's health (Barboza *et al.*, 2009). For example, in birds the nutrient composition of food consumed by the female prior to egg formation is imperative to breeding success (Cooper *et al.*, 2005), with nutrient excesses or deficiencies being potentially fatal to the embryo (Wilson, 1997). The correct macro and micronutrient balance is equally important in mammals (Santos, J. *et al.*, 2008; Van Saun, 2008), reptiles (Craven *et al.*, 2008), fish (Izquierdo *et al.*, 2001) and amphibians (Wright & Whitaker, 2001).

The manner in which nutrients are absorbed, transported, metabolised and stored differs widely across taxa (Cheeke & Dierenfeld, 2010). These differences may be related to ecological factors such as volancy, migration, reproductive strategy, and climate. Hence, it is important to explore dietary adequacy on a species-by-species basis (Kleiman *et al.*, 2010).

A recently recognised problem in many species is oversupply of nutrients leading to obesity, which engenders other health problems (Leigh, 1994; Bellisari,

2008; Clubb *et al.*, 2009). It is thus important to tailor dietary intake with weight management, i.e. to determine nutrient requirements with limits in regard to over and under supply.

Fertility

Diet is an important factor in reproduction as most food deficiencies, especially fatty acid deficiencies, affect fertility (Zaniboni *et al.*, 2006). Specific fatty acids are involved in the main functions and events that lead to fertilization (Zaniboni *et al.*, 2006). Inadequate levels of polyunsaturated fatty acids (PUFA) in spermatozoa have been linked to infertility in humans (Aksoy *et al.*, 2006), rabbits (Gliozzi et al., 2009), turkeys (Zaniboni et al., 2006), chickens (Cerolini et al., 2006) and cattle (Mattos et al., 2000). Levels of specific fatty acids in the phospholipids of sperm cells differ across species; for example, docosahexaenoic acid (DHA) concentrations are high in the sperm of sheep, cattle and humans, low in rabbits and dogs, and absent in avian semen (Gliozzi *et al.*, 2009). Furthermore, PUFA profiles of semen vary within avian species (Zaniboni *et al.*, 2006). For species for which little is known of the fatty acid requirements for reproduction it is generally not possible to extrapolate from other species, thus this is a topic that should be investigated further in kiwi.

Roughage/Fibre

Food items are often included in the diet for reasons other than the provision of nutrients; for instance, roughage influences the nutrient and energy densities of a diet (Galyean, M. L. & Defoor, P. J., 2003). Roughage or fibre refers to substances that are relatively resistant to digestion, for example ingredients high in cellulose, hemicellulose and lignin, however they still contribute some nutrient value to the diet. Their inclusion in the diet dilutes nutrient and energy densities by increasing viscosity in the gut, slows digestion and absorption of carbohydrate and fat, induces satiation through the addition of bulk (Burton-Freeman, 2000), and reduces the incidence of some digestive disorders (Galyean, M. L. & Defoor, P. J., 2003; Uwituze *et al.*, 2010). Roughage can also be added to high nutrient density diets to allow enteral bacteria to adapt to concentrated diets (Berry *et al.*, 2004).

Furthermore, items such as shells on nuts or seeds and stones in fruit, that provide relatively indigestible material, aid the physical process of digestion. Fruit stones, for example, may be used as substitute gizzard stones (Clout & Hay, 1989).

In some cases, roughage is also a source of nutrients. For example, chitin is a type of fibre found in the exoskeleton of arthropods or in the cell walls of fungi and yeast (Rinaudo, 2006) and its breakdown releases nutrients in some animals. Chitin degradation requires chitinase and chitobiase activity (Bhattacharya *et al.*, 2007). Digestion of chitin varies widely across taxa (Jackson *et al.*, 1992). The importance of chitin in general nutrition remains a point of debate in the literature (Gooday, 1990; Jackson *et al.*, 1992; Han *et al.*, 1997; Deblauwe & Janssens, 2008; Park *et al.*, 2010).

Diet palatability

Palatability is an important consideration when developing diets for captive-held animals (Clauss *et al.*, 2001). The definition of palatability is a much debated subject (Kissileff, 1990; Yeomans, 1998). The definition used in this study is comprised of two components, first, how readily the diet can be picked up, manipulated and placed into the mouth, and second, once in the mouth, how acceptable the smell, taste and texture of the diet is to the animal. The food offered to captive animals generally differs from items in their natural diet and has different characteristics of smell, taste and feel (Yo *et al.*, 1997). Neophobic reactions to novel food often impede the success of husbandry techniques and can extend to feeding problems when animals are released into the wild. It is thus important to monitor food intake and reduce habituation to diets, for example by supplementing synthetic diets with a range of natural food items.

Importance of intestinal microflora on nutrition

While the provision of a nutritionally adequate diet is important, an equally important aspect of nutrition that has been little studied in relation to wild animals is the effect of intestinal microflora on nutrition and general health. Enteral microflora contribute to a plethora of physiological processes that are essential to life, for example digestion (Hooper *et al.*, 2002), development of the immune system (Gabriel *et al.*, 2006; Kelly *et al.*, 2007) and cell repair (Hooper & Macpherson, 2010). Enteral bacteria also aid in the breakdown of carbohydrate to short-chain fatty acids (SCFAs) (Lunn & Buttriss, 2007). The presence of cellulolytic bacteria allows nutrients to be acquired from cellulose. For example, the presence of cellulolytic bacteria allows cellulose to be

broken down in mainly propionate and butyrate. These SCFAs are nutrients but have also been shown to induce apoptosis in human cancer cells, thus having an anticancer effect (Tang *et al.*, 2011). Hosts also benefit from the ability of enteral bacteria to competitively exclude pathogens (Lee *et al.*, 2010).

Digesta provides most of the required carbon and energy needed by bacteria in the gastrointestinal tract, therefore the chemical composition and structure of the digesta is an important determinant of bacterial community composition (Apajalahti *et al.*, 2004). The disruption of a normal suite of microflora in the intestinal tract can lead to intestinal dysbiosis. For example, an imbalance in the proportion of harmful and protective bacteria in the gut may promote chronic intestinal inflammation (Tamboli *et al.*, 2004). An unsuitable suite of microflora can also lead to problems of obesity through interference with the bacteria-host signalling pathway that regulates energy storage (Backhed *et al.*, 2004; DiBaise *et al.*, 2008). The issue of obesity can also lead to fertility problems (Waller *et al.*, 2007), and thus negatively impact on captive breeding programmes.

Dietary intake has been shown to effect a change in bacterial community structure at any period of an animal's life (Knarreborg *et al.*, 2002), but the most important stage in which to influence the diversity of enteral bacteria is at birth (Mandar & Mikelsaar, 1996; Ouwehand *et al.*, 2002; Kelly *et al.*, 2007) or hatch (Van der Wielen *et al.*, 2002; Scupham, 2007). In humans, delivery by caesarean, including routine prophylactic antibiotic use by the mother, decreases the exposure of newborns to bacteria along the birth canal, as well as general bacterial exposure from the mother post-parturition (Kelly *et al.*, 2007). The hygiene hypothesis states that the use of aseptic practices on newborns increases the chance of asthma and allergic reactions later in life (Strachan, 1989; Huffnagle, 2010). While substantial effort has been made to understand bacterial acquisition and colonisation in human young (Fanaro *et al.*, 2003; Langhendries, 2006; Palmer *et al.*, 2007), little research has focussed on captive or wild animals.

In the wild, animals interact with their natural environment from hatch or birth, and thus have the opportunity to recruit appropriate micro-organisms. In captivity, this link is often disrupted or severed completely (Schneitz, 2005). Eggs are often removed, sterilised and incubated artificially (Burnham, 1983; More, 1996; Maxwell & Jamieson, 1997; Department of Conservation, 2004), and young mammals are often raised

separate from their parents (Voelkl & Huber, 2006; Morimura & Mori, 2010). Given the importance of the host-enteral bacteria interaction (Alverdy *et al.*, 2005), animals should ideally be given the opportunity to acquire a suitable suite of microflora. There are a number of management techniques that may be effective in influencing intestinal bacterial communities. While it is impossible to translocate a wild animal into captivity with all of the micro-organisms that it encounters during its life, it is possible to facilitate access to a typical suite of microflora found in the wild, particularly while the animal is young. The provision of young and growing animals with faecal material from healthy, wild individuals is likely to promote normal enteral bacterial colonisation (Hume *et al.*, 2003; Schneitz, 2005; Gabriel *et al.*, 2006).

To determine the effect of *ex situ* management on the composition of enteral bacteria, comparisons of intestinal bacterial community structure between captive and wild individuals of a species are needed.

TRADITIONAL METHODS USED IN THE STUDY OF ANIMAL NUTRITION

This section outlines approaches that have been taken to develop captive diets that are representative of what animals eat in the wild. Also outlined are methods for determining the diversity of intestinal bacterial communities.

Gold standard of dietary determination

Ideally, the diet of an animal would be calculated according to the recommended daily allowance (RDA) (Alpers *et al.*, 1995) of each nutrient. RDAs are based on optimal intake, i.e. the amount needed before the target nutrient appears in the urine (Zeisel, 2009). Due to the potential effects of health of such feeding trials this technique is not feasible for many threatened species. Instead, broad-scale, less invasive methods are needed. Typical techniques include: direct observations of dietary intake; observations that infer diet; crop, gizzard or stomach content analyses of animals found dead in the wild; or faecal analyses. Observations of feeding are difficult for cryptic, nocturnal or marine animals, and faecal or digestive tract content analyses are often hampered by decomposition of digesta, or the complete digestion of soft-bodied prey. In

light of these problems, quantitative fatty acid signature analyses of body fat have been used to predict diets of endangered animals.

Determination of diet through fatty acid analyses

The capability of birds to store and use triglycerides as an energy reserve exceeds that of any other class of vertebrate (Blem, 1976). Fatty acids, the main constituent of most lipids, are released from ingested fat molecules, such as triglycerols, during digestion, but are not degraded before storage (Iverson *et al.*, 2002). Thus, samples of body fat have been shown to be an indicator of digested fat (Bradshaw *et al.*, 2003). Thus, with knowledge of both the dietary items digested and the proportion of fat in each item, the relative quantity of fats ingested can be calculated and used to predict dietary intake. However, this technique needs to be approached with caution as some taxa utilise and store fats in a different manner to others (Iverson *et al.*, 2004).

From a wild to a synthetic diet

Even once the wild diet has been determined it may not always be practicable to feed these items to captive animals. For instance, sourcing appropriate dietary items for insectivorous, nectivorous or planktivorous species can be particularly problematic. In such cases, a synthetic diet that provides a similar nutrient composition to that of the wild diet is needed. Therefore, the nutrient composition of the wild diet needs to be determined, along with the items of the diet that are not solely nutrient related but are important components of the diet, such as cellulose, lignin, pectin (Burton-Freeman, 2000) and chitin (Razdan & Pettersson, 1994). It is not sufficient, however, to stop at this stage of dietary formulation. As mentioned previously, different animals digest nutrients in different ways, thus the digestibility of the synthetic diet needs to be such that it provides a pattern of overall nutrient assimilation similar to that of the wild diet. Thus, whilst the synthetic diet may have a different digestibility to that of the wild diet, it must ultimately provide similar acquisition of nutrients. This can be achieved by adjusting nutrient concentrations to compensate for differences in digestibility.

Historically, once the dietary requirements have been determined for a species, it has been assumed that this is sufficient to maintain animals in a healthy state. However, recent advances in microbiology have highlighted the importance of intestinal bacteria

to digestion and overall health. Thus, it is no longer acceptable to ignore the importance of enteral bacterial communities to health.

Methods of enteral bacterial detection

Ideally, intestinal content is used for analysis of enteral bacterial populations, however this involves killing the animal or putting it under threat by endoscopic sampling that generally requires anaesthesia. Instead, for endangered species, indirect methods, such as faecal matter analyses, tend to be favoured. Faecal samples are easy to collect, non-invasive and provide temporal and locational 'averaging' of the colonic microbiota (Flint *et al.*, 2007).

In the past, identification of enteral bacterial communities has involved cultivation-based techniques. However, due to the dependence upon bacteria-specific media during cultivation, up to 80% of species may not have been identified using this technique (Suau *et al.*, 1999; Vaughan *et al.*, 2000). Similarly, this technique only identifies live bacteria in the sample. To overcome these biases, a DNA-based technique for identifying microbial communities is now increasingly used (Li *et al.*, 2003). Denaturing Gradient Gel Electrophoresis (DGGE) uses 16S rDNA as a molecular fingerprint to identify microbial communities (Tannock, 2001). Sections of DNA fragments are amplified using polymerase chain reactions (PCR), then loaded into a gel containing a chemical gradient (Hill *et al.*, 2008). Depending upon the sequence of base pairs in the fragment, the DNA will 'melt' (denature) at a specific gradient causing a band to appear on the gel (Myers *et al.*, 1985; Muyzer *et al.*, 1995). Each band generally corresponds to a different bacterial species, thus providing a profile of the bacterial community for that sample (Fromin *et al.*, 2002). Both quantitative and qualitative methods can be used, however this thesis used primers that allow counts of the number of genera present.

Ideally, real-time PCR is used to quantify the bacterial communities within a faecal samples. This technique measures PCR amplification as it occurs and results in a quantitative measure; data is collected when the quantity of the PCR product is directly proportional to the initial amount of nucleic acid in the sample (Hermansson & Lindgren, 2001). In contrast, traditional methods (as used in this study) measure the amount of accumulated PCR product at the end of the PCR cycle, therefore, as the initial concentration of nucleic acid is unknown, quantitative measurements are not

possible (Smith & Osborn, 2009). Access to real-time PCR equipment was not possible when DGGE analyses were conducted in this study, hence non-quantitative methods were used. Additional studies are needed to build on the work presented here, for example, work that both quantifies avian enteral microbial communities (real-time PCR) and identifies the bacterial species present (DNA sequencing).

FOCAL SPECIES FOR THIS THESIS

North Island brown kiwi are used as a case study for this thesis. The following section outlines aspects of their ecology.

Kiwi

Taxonomy

Kiwi belong to the Apterygidae family and are usually placed in the ratite group (Struthioniformes) along with emu, ostrich, cassowary and rhea (Sales, 2009). Five species of kiwi, all endemic to New Zealand, are recognised: one in the North Island - North Island brown kiwi (*Apteryx mantelli*); and four in the South Island - rowi (*A. Rowi*), tokoeka (*A. australis*), great spotted kiwi/roroa/roa (*A. haastii*) and little spotted kiwi (*A. owenii*) (Burbidge et al., 2003).

Ecology

Kiwi are flightless and nocturnal (Sales, 2005), and are believed to be primarily insectivorous birds that supplement their diet with other invertebrates and fruit (Kleinpaste, 1990), however a complete natural diet for kiwi has not been established. They generally live in native forests, although in some areas they live in pine forests (Taborsky & Taborsky, 1995). They have also been observed foraging in grasslands and on beaches (Colbourne & Powlesland, 1988). They locate food items through both olfactory and remote sensing cues (Cunningham *et al.*, 2009). They have long-term pair bonds and produce up to two clutches of eggs (1-2) per year (Sales, 2005). Each egg weighs up to one fifth of the female's body weight (Calder *et al.*, 1978), and incubation in the North Island brown kiwi (~80 days) is carried out solely by the male. Chicks are precocious and able to fend for themselves within a few days of hatch.

Conservation status

All five species of kiwi are on the verge of extinction on the mainland (Holzapfel *et al.*, 2008; Blue & Blunden, 2010). Whilst population numbers of North Island brown kiwi are the highest of the five species, their rate of decline is still 6% per annum (McLennan, 1996). In an attempt to halt and reverse this decline, species recovery plans have been developed and implemented since 1991 (Holzapfel *et al.*, 2008). To date, captive breeding programmes for kiwi have focused mainly on North Island brown kiwi, however skills and techniques can be transferred to the rarer species (Renwick *et al.*, 2009). North Island brown kiwi are listed as endangered (IUCN, 2010) with a total population of approximately 25,000 (Department of Conservation, 2006). The main agents of decline are habitat loss and predation by introduced mammalian predators, such as dogs, cats, stoats, ferrets, and pigs (Holzapfel *et al.*, 2008).

Current recovery methods and captive holdings

North Island brown kiwi have been held in captivity for at least 150 years; one of the first recorded examples was a female sent to the Zoological Society of London in 1851 (Peat, 1990). From 1891 kiwi were transferred to offshore islands (Young, 2004) and by 1897 they were classified as a protected species (Star & Lockhead, 2002). The first North Island brown kiwi chick to hatch in captivity was from a game farm near Napier in 1945 (Robson, 1947). Historically, kiwi were held in captive institutions for advocacy reasons, resulting in generation after generation bred in captivity. More recently, kiwi eggs have been sourced from the wild, incubated, hatched and reared in captivity until they are of a size presumed sufficient to fight off predation by stoats (McLennan, 1996). This practice, named Operation Nest Egg (ONE), has been successful in increasing the number of kiwi in the wild (12.5% population growth per annum (Robertson *et al.*, 2011)). However, ONE is solely a way to buy time for kiwi conservation as it does not solve the primary issue of high predation rates in the wild. In order for kiwi to survive in the wild and for populations to increase, effective predator control is required. Unfortunately, few populations have received intense predator control in the wild (Colbourne et al., 2005).

The removal of eggs from the wild followed by hatching in captivity creates a number of problems: young are hatched in a highly sterile environment compared to that of wild-hatched chicks, thus normal interactions between young and bacteria and

other parasites are severed; dietary intake differs considerably from the natural diet; natural foraging behaviour may be affected by being held in a confined space and food provided *en masse* in one location; predator avoidance behaviours may be reduced due to an absence of predators in captivity; and social contact with other kiwi is often limited.

There are additional problems with the *ex situ* management of kiwi. Despite effective predator control, captive-held kiwi suffer higher mortality and lower fertility rates than wild kiwi: for example, adult female kiwi are four to five times more likely to die in captivity than in the wild; eggs laid in captivity are about four times as likely to be infertile compared with wild laid eggs (Holzapfel *et al.*, 2008) and of those eggs that are fertile their chance of hatching is 33% less likely than a wild egg (McLennan, 1996; Holzapfel *et al.*, 2008). Furthermore, captive-reared birds (and their eggs) are often smaller than wild birds (Department of Conservation, 2004). These figures show that despite an absence of predators, the major cause of population decline in the wild, captive kiwi are struggling to survive and reproduce in captivity. Inadequacies with captive diets may be a major contributing factor (Potter *et al.*, 2010). This should not be surprising given that the current diet was formulated over forty years ago with little regard to nutrient requirements (Reid, 1970; Johnson, 1996; Potter *et al.*, 2010). No single diet exists for kiwi held in captivity, and the range of diets offered and their respective nutrient compositions differ markedly across institutions (Potter *et al.*, 2010).

Despite these issues, captive management provides a useful tool to assist with kiwi conservation, but a range of issues need to be addressed including diet and genetic factors. For example, removal of a subset of the wild population for the creation of a new captive population has the potential to reduce total genetic diversity and increase the presence of specific genes. This is particularly problematic as regional populations of the same species are bred separately. Hence small founder populations are used to breed and rear birds for release into the wild. As the overall number of kiwi in the wild continues to fall, this technique raises the question of whether it is more important to preserve regional characteristics with the potential for inbreeding, or to focus on the species as a whole and maximise genetic diversity. At present, while there remain tens of thousands of North Island brown kiwi in the wild (Department of Conservation, 2006), this question can be vigorously debated and/or ignored. However, the time may

not be far off when population numbers have declined to such a degree that questions of provenance will be of little relevance.

AIMS OF THESIS

While the North Island brown kiwi (*Apteryx mantelli)* is the focal species for this thesis, several other species (brown teal (*Anas chlorotis*) and takahe (*Porphyrio* [Notornis] *mantelli*)) are also investigated within specific sections. Thus, this thesis investigates the importance of diet, digestion and nutrition on the health of captive animals with the aim of improving conservation management for these species.

The acquisition and storage of nutrients in relation to diet among avian taxa is investigated in chapter 2, using the digestion of fat as an example.

In chapter 3, a near-natural diet consisting of items known to be consumed by kiwi is researched and developed. The nutrient composition and apparent digestibility of this diet is determined in both kiwi and roosters. Thus comparisons of apparent digestibility in kiwi were made with a species of which substantial digestibility and physiological data exist.

A synthetic diet for kiwi is developed in chapters 4 and 5, using readily available ingredients and based on the nutrient composition and apparent digestibility of the near-natural diet (from chapter 3). The apparent digestibility of the synthetic diet is determined in both kiwi and roosters.

In chapter 6, enteral bacterial communities from captive birds are compared with those from wild birds of the same species. Three endemic New Zealand avian species are used: North Island brown kiwi, takahe and brown teal. Also, a possible relationship between enteral microfloral communities and captive or wild location is investigated in kiwi.

Chapter 7 explores the influence of bacteria on the surface of the egg on the subsequent composition of microflora that colonise the caeca in young poultry chicks. Also, the ontogeny of intestinal bacteria of newly-hatched to three-week-old kiwi chicks is examined.

In chapter 8, the effect of dietary change on enteral bacterial community structure in two endemic New Zealand avian species (kiwi and takahe) is determined.

The final chapter provides a synthesis of the major findings of the research chapters and outlines the systematic approach taken here to better understand dietary requirements of kiwi, the role of intestinal microflora in wildlife health, and factors that affect microfloral acquisition and gut colonisation. The relevance of this work to the captive management of other wildlife is discussed, and recommendations are presented

on how to address a range of diet-related issues including palatability, neophobia, obesity, and preparation of captive-held animals for release into the wild.

CHAPTER 2

The fatty acid composition of depot fat in kiwi (*Apteryx mantelli*) and other wild avian species is a poor predictor of diet

ABSTRACT

The fatty acid composition of depot fat in the insectivorous North Island brown kiwi (*Apteryx mantelli*) was compared with published data of depot fat composition for a range of other avian species of differing dietary intake. For kiwi, as well as the other species considered, oleic (C18:1) and palmitic (C16:0) acids were the two most abundant fatty acids found in storage fats. With the exception of the insectivorous red-eyed vireo (*Vireo olivaceous*), proportions of palmitoleic (C16:1), stearic (C18:0) and linolenic (C18:3) acids are consistently low across species. Avian marine carnivores have a distinctly different fatty acid profile with markedly lower values of linoleic acid (C18:2) in depot fat than the herbivorous, insectivorous and omnivorous species analysed. North Island brown kiwi showed the highest degree of variation in fatty acid content of body fat of the species analysed. Dendrographic analysis was only able to cluster taxa with either a narrow range in diet, such as herbivores, or those that consume food items with a distinctive fatty acid signature, such as avian marine carnivores. Thus, analysis of the fatty acid composition of depot fat of kiwi is not a reliable indicator of dietary intake. As a broader technique it seems capable only of distinguishing broad-scale differences in avian dietary intake and relies on the presence of distinctive fatty acids in the diet, particularly those that are essential and cannot be synthesized by the body.

INTRODUCTION

The capability of birds to store and use triglycerides as an energy reserve exceeds that of any other class of vertebrate (Blem, 1976). Fatty acids, the main constituent of most lipids, are released from ingested fat molecules, such as triacylglycerols, during digestion but are not degraded before storage (Iverson *et al.*, 2004). This relative resistance to degradation has led to the use of fatty acid analyses of fat depots to infer dietary intake. The merit of this technique has been, and continues to be, widely debated in the literature. The majority of studies, based largely on mammalian species, conclude that the fatty acid composition of body fat may be a useful indicator of diet (Grahl-Nielsen & Mjaavatten, 1991; Walton *et al.*, 2000; Grahl-Nielsen *et al.*, 2003; Iverson *et al.*, 2004; Meynier *et al.*, 2010; McMeans *et al.*, 2012). This phenomenon also occurs in avian species, for example, the fatty acid composition of the adipose tissue of red grouse *(Lagopus lagopus scotica)* reflects that of its main food plants (Lynen, 1980). Again, relative proportions of fatty acids in the depot fat of Adelie penguins (*Pygoscelis adeliae)* correspond to that of their normal diet (Watkins & German, 1998). The fatty acid composition of the depot fat of pigs can be manipulated by a change in dietary constituents (McPherson & Spiller, 1996). Similarly, the fatty acid composition of depot fat in the rat is regulated by the relative proportions of the various fats in the diet (Hilditch, 1956). Taken together, these studies show that in birds and mammals at least, there is a relationship between the fatty acid profile of fat depots and that of the diet.

There is, however, a body of evidence indicating that the composition of depot fat differs in some respects from that of the diet. For example, depot fats in the muscle of some domestic vertebrates, such as beef and mutton, differ from those in their diet (Shorland, 1950). Such differences may extend to the fat composition of the whole body. Lovern (1938) reported that the unsaturation indices (index of unsaturated fatty acids) of whole body fat contents of herring gulls *(Larus argentatus)*, skua gulls *(Megalestris catarrhactes)*, gannets *(Sula bassana),* and fulmar petrels *(Fulmarus glacialis),* differ from those of their diets. In whole-bird carcasses of white crowned sparrows (*Zonotrichia leucophrys gambelii*), linoleic and linolenic acids were found in lower proportions than in the diet whilst the saturated and monounsaturated 16 and 18 carbon fatty acids were found in relatively greater proportions (Turunen, 1974). Similarly, the relative proportion of myristic acid is reported to be greater in the depot

fat of redpolls (*Acanthis flammea*) than in their natural diet (Khani *et al.*, 2007). Taken together these results indicate that differences in the composition of body fat from that of the diet may arise from differences in the dynamics of deposition, utilisation and metabolism of fats.

The degree of unsaturation of fatty acids may influence the site at which fat is stored or utilised (Sanz *et al.*, 2000). Relatively higher proportions of monounsaturated fat and lower proportions of polyunsaturated fats are found in the abdominal fat than in the muscle fat of chickens (Crespo & Esteve-Garcia, 2001). Unsaturated fats have a higher metabolizable energy, thus preferential deposition of unsaturated fats could allow greater amounts of energy to be stored (Shimomura *et al.*, 1990). It is also known that the primacy of deposition and utilisation of fats varies with site. In avian species, fats are deposited initially in subcutaneous tissues and latterly in the furcular (clavicocoracoid), interfurcular and abdominal regions (Blem, 1976). A similar phenomenon has been recorded in mammals. Koch et al. (1968) found greater fluctuations in the fatty acid composition of back fat compared to intramuscular fat in pigs with a change in diet. Similarly, in humans the turnover of dietary fats varies with the site of deposition (Phinney *et al.*, 1994; Garaulet *et al.*, 2001) with subcutaneous fat stores turning over rapidly to reflect short-term differences in dietary intake (<4 weeks) whilst intra-abdominal fat stores turn over more slowly and reflect long-term (>4 weeks) differences in dietary intake (Johnston, 1973). Rates of turnover of depot fats are also likely to vary with physiological demands and with differences between species such as degree of volancy, season and reproductive status (West & Meng, 1968a).

While many studies have been conducted on the influence of diet on the fatty acid composition of depot fat in single species, no data are available on whether this technique holds for comparisons of fatty acid composition across taxa. For example, is it possible to take a fatty acid profile from the depot fat of an unknown animal and accurately predict its general dietary intake, i.e. whether it is an omnivore, herbivore, carnivore or insectivore? If proven useful, this less conventional approach could be used to predict diets of species that to-date have proven difficult to ascertain due to their nocturnal or cryptic behaviour, for example, wild North Island brown kiwi (*Apteryx mantelli*).

Here, I surveyed the body of published work regarding the relative proportions of commonly metabolised fatty acids in storage fats and compared them with data

obtained by us from the gizzard fat of 14 wild North Island brown kiwi. In addition, I investigated whether the relative proportions of these fatty acids allow separation of various other species on a basis of their diet.

METHODS

The fatty acid content of gizzard fat from the carcasses of 14 adult North Island brown kiwi (*Apteryx mantelli*) was determined. The causes of death of these birds included getting caught in traps set for introduced and pest species, such as the Australian brushtail possum (*Trichosurus vulpecula*), vehicle accidents, predation, and other misadventure. The carcasses were received in varying states of decomposition, the abdominal cavities of the birds having been opened on receipt and fat sampled from the serosal surface of gizzard fat as part of a previous investigation (Pindur, 2004). The tissues were dried at 105 ºC in a convection oven (Watvic) and fats extracted with 60/80 petroleum spirit. The fats were methylated and relative proportions of fatty acids were determined following separation by gas chromatography as areas under the curve relative to a standard (West & Meng, 1968b). Analyses were conducted at the Nutrition Laboratory, Massey University, Palmerston North, New Zealand.

The relative proportions of four of the most common fatty acids palmitic, palmitoleic, stearic, oleic, and two essential fatty acids linoleic and linolenic acid in depot fat of nine avian species of known dietary habit was sourced from the literature (for references see Table 1). Data on the fatty acid composition of kiwi depot fat obtained from this study was combined with data from two kiwi reported by Shorland and Gass (1961) and Shorland and Jessop (unpublished results reported in Shorland & Gass, 1961).

Statistical comparison of results between species was complicated by the fact that a number of the publications did not provide individual data and sample size varied greatly between surveys, there being generally insufficient data to determine distribution and homogeneity of variance (see Table 1). Further, the various methodologies differed. Consequently I compared data by hierarchical cluster analysis (McGreal & Farner, 1956) computed on a basis of Euclidian distance (root mean squared distances); using SYSTAT version 12 (Systat Software Inc, 2004). Divisions between groups were calculated on a basis of unweighted pair group means (UPGMA) using Ward's algorhythm (Ward, 1963). Ward's method is a UPGMA algorhythm in which the Euclidean distances between all of the pairs of objects in different clusters is averaged to determine the distance between the respective clusters (Jobson, 1992). Euclidean distance is considered most suitable for separation based on quantitative variables (Chavent, 1998).

I carried out two hierarchical cluster analyses; the first using the mean from each study, the second incorporating all available data from individual birds. I then compared the clustering hierarchies obtained from the two methods noting similarities between the trees and the spread of results from individuals of a given species across the tree.

RESULTS

Direct quantitative comparison

The mean proportions of oleic and palmitic acids were generally higher than palmitoleic, stearic, linoleic and linolenic in all species (Table 1). In kiwi, the ratio of linoleic to linolenic acid was 5:1. The relative proportions of stearic and linolenic acids were generally lower (relative to the other fatty acids analysed) in all avian species analysed. Some differences were apparent between species on a basis of their diet. The relative proportion of linoleic acid was lower in the depot fat of marine carnivore species than in that of other avian species. Some differences were apparent between individual species. The relative proportion of palmitoleic acid was higher in red-eyed vireos (*Vireo olivaceus*) than in other species.

Dendrographic analyses

Dendrographic analysis based on means separated the 10 avian species into two main clusters (Figure 1). Thus, a group comprising a passerine insectivore (vireo) and three marine carnivores (sandpiper (*Calidris pusilla*), petrel (*Puffinus pacificus* or *Pterodroma macroptera*) and fulmar (*Fulmarus glacialis*) were most dissimilar (Euclidean distance between groups 28) from the remaining species. These two groups were further divided, the vireo being separated from the remainder of the first group at a Euclidean distance of 16, the petrel and kiwi being separated from the remaining species in the second group at a distance of 13. The remaining species in the second group were separated by a Euclidean distance of 10.

The dendrographic analysis of the relative proportions of palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acids in body fat incorporating the results of individual birds (Figure 2) separated the species into broadly similar clusters as those seen in the dendrographic analysis of the means. However, there was a wide spread of individuals of a given species across the hierarchical groups; those species with the greater number of individual results being spread more broadly than those for which fewer results were available. There was some variation in the degree of spread within species. Hence the results from individual kiwi were spread across the principal division of the dendrogram whilst those of individual mallards (*Anas platyrhynchos*) were not.

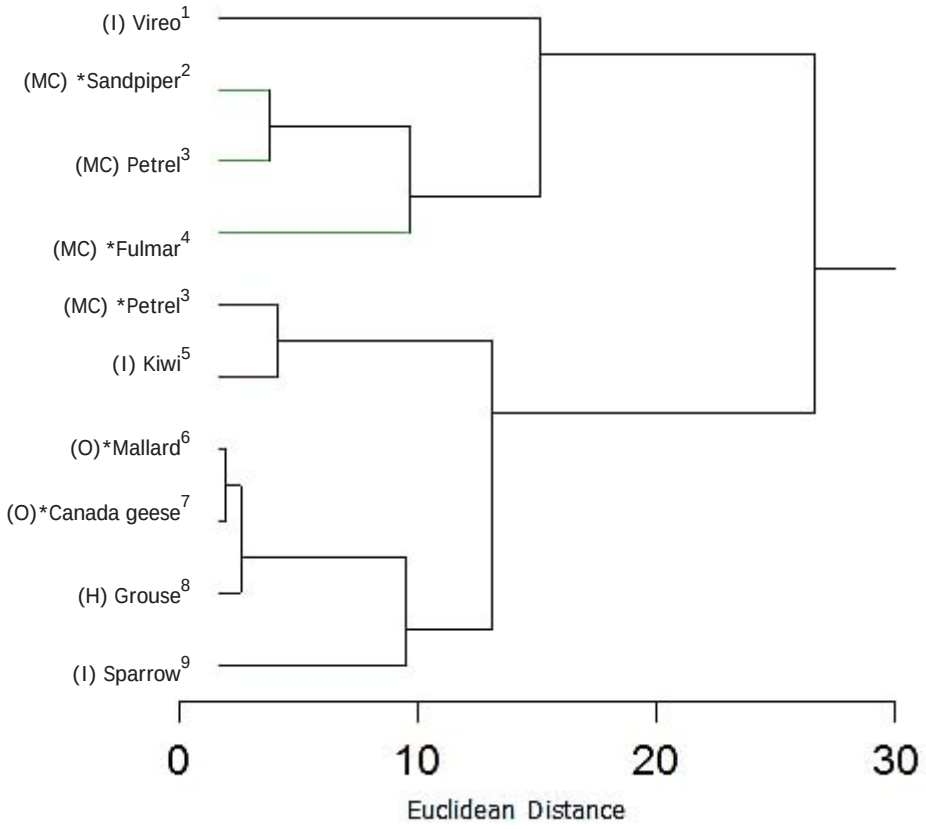
Table 1. Quantitative comparison of the mean proportions of various fatty acids (% of total fatty acid content) in the body fat of various avian species of differing dietary habit.

Dietary Habit	Species	Fatty Acid					
		Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	Linolenic
Herbivore	Black grouse[1]	19 (16-22)	3 (2-3)	7 (6-8)	42 (34-51)	21 (14-29)	4 (2-6)
Omnivore	Canada geese* [2]	22 (17-25)	3 (3-4)	5 (4-5)	46 (34-55)	20 (13-30)	4 (1-12)
	Mallard* [3]	20 (16-24)	3 (2-4)	7 (6-9)	48 (42-57)	19 (15-24)	2 (1-3)
Insectivore	Kiwi [4]	20 (13-28)	2 (1-4)	10 (4-20)	43 (24-58)	10 (5-19)	2 (0.3-8)
	Red-eyed vireo[5]	18	28	3	33	16	2
	White-throated sparrow[6]	16 (12-22)	3 (2-5)	6 (6-7)	35 (34-37)	33 (24-40)	4 (3-4)
Marine carnivore	Fulmar*[7]	9 (9-10)	4 (3-4)	3 (3-3)	17 (10-22)	1 (1-1)	0.4 (0.2-1)
	Pacific petrel[8]	25 (23-27)	5 (5-5)	7 (6-7)	21 (19-24)	2 (2-2)	2 (2-2)
	Petrel*[8]	15 (13-18)	5 (4-6)	8 (2-6)	41 (40-42)	2 (2-3)	2 (0.01-0.3)
	Semi-palmated sandpiper*[9]	27	7	8	23	4	2

Ranges are given in brackets after value. Sources are given in the footnotes below.
The sources of fat and sample sizes are indicated in brackets after each reference.
Species classified as insectivores may also consume seeds and/or crustaceans.
* Denotes migratory species. Adult birds used unless otherwise stated.

[1] Moss & Lough (1968) (crop, heart and gizzard fat) (2)
[2] Thomas & George (1975) (visceral fat (posterior to the gizzard)) (22)
[3] Heitmeyer & Fredrickson (1990) (peritoneal fat) (75)
[4] This study (gizzard fat) and Shorland & Gass (1961) (subcutaneous fat; breast fat) (16)
[5] Pierce et al., (2004) (subcutaneous fat) (22)
[6] Klaiman et al., (2009) (adipose fat) (68)
[7] Wang et al., (2007) (adipose fat) (101)
[8] Cheah & Hansen (1970) (adipose fat) (2 adults) (2 chicks)
[9] Napolitano and Ackman (1990) (subdermal depot fat)

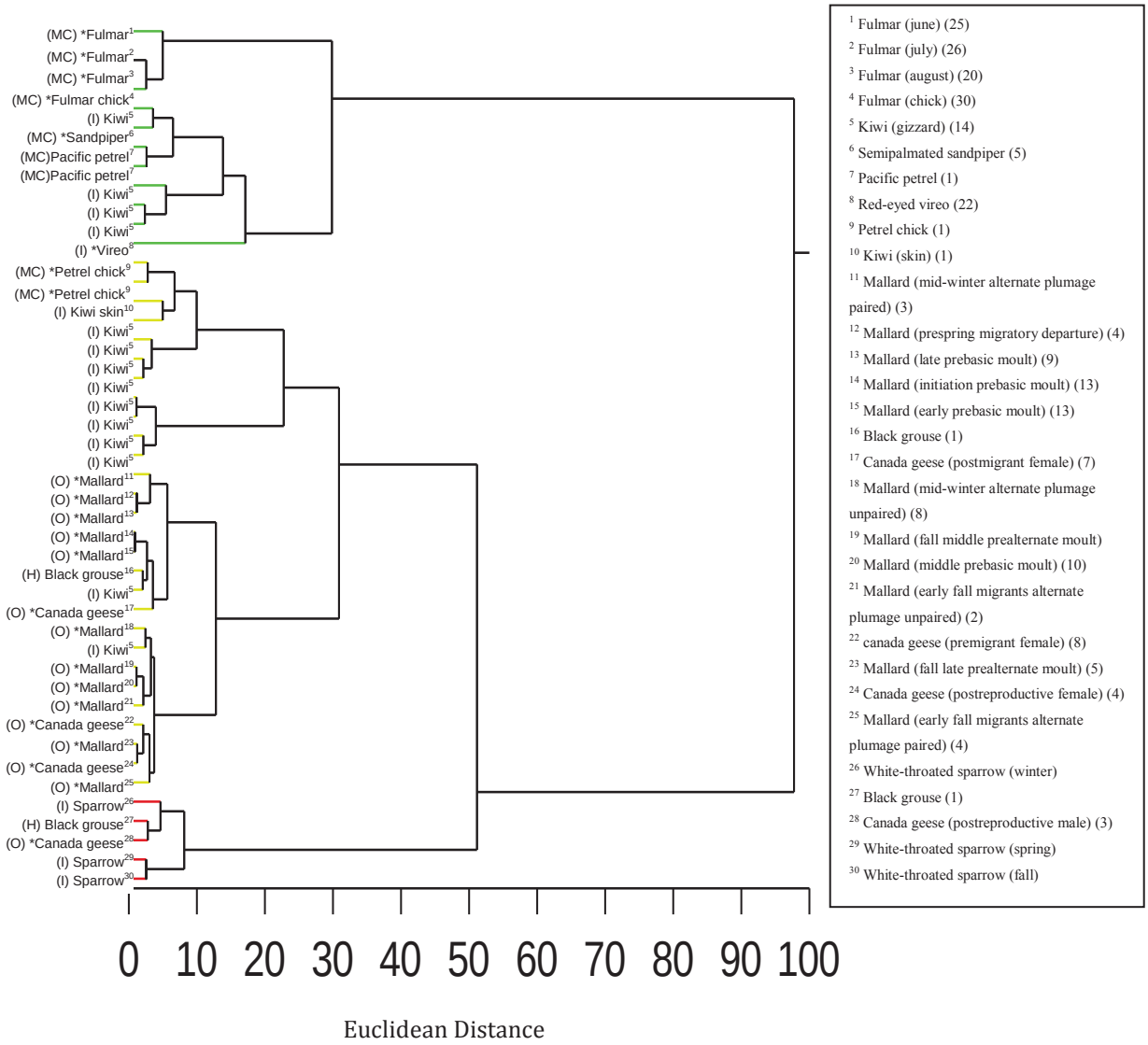
Figure 1. Dendrogram of the mean palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acid content of body fat of various avian species.



The sources of fat and sample sizes are indicated in brackets after each reference.
Adult birds used unless otherwise stated.
MC = marine carnivore; I = insectivore (may also consume seeds and/or crustaceans);
G = granivore; O = omnivore; H = herbivore; * = migratory

[1] Pierce *et al.,* (2004) (subcutaneous fat) (22)
[2] Napolitano & Ackman (1990) (subdermal depot fat) (5)
[3] Cheah & Hansen (1970) (adipose fat) (2) (2)
[4] Wang *et al.,* (2007) (adipose fat) (101)
[5] This study (gizzard fat) and Shorland & Gass (1961) (subcutaneous fat; breast fat) (16)
[6] Heitmeyer & Fredrickson (1990) (peritoneal fat) (75)
[7] Thomas & George (1975) (visceral fat (posterior to the gizzard)) (22)
[8] Moss & Lough (1968) (crop, heart and gizzard fat) (2)
[9] Klaiman *et al.,* (2009) (adipose fat) (68)

Figure 2. Dendrogram of the palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acid content of body fat of individual birds or species mean of various avian species.



Adult birds used unless otherwise stated. The source of fat is indicated in brackets after each reference. Sample sizes are given in brackets after the bird type in the legend of the figure. MC = marine carnivore; I = insectivore (may also consume seeds and/or crustaceans); G = granivore; O = omnivore; H = herbivore; * = migratory species.

[1-4] Wang et al., (2007) (adipose fat)
[5] This study (gizzard fat)
[6] Napolitano and Ackman (1990) (subdermal depot fat)
[7, 9] Cheah and Hansen (1970) (adipose fat)

[8] Pierce *et al.,* (2004) (subcutaneous fat)
[10] Shorland and Gass (1961) (subcutaneous fat)
[11-15, 18-21, 23, 25] Heitmeyer and Fredrickson (1990) (peritoneal fat)
[16, 27] Moss and Lough (1968) (crop, heart and gizzard fat)
[17, 22, 24, 28] Thomas and George (1975) (visceral fat (posterior to the gizzard))
[26, 29-30] Klaiman *et al.,* (2009) (adipose fat)

DISCUSSION

North Island brown kiwi showed the greatest degree of variation in the fatty acid content of body fat of the species analysed, and varied so much across the 14 individuals that it spanned the entire fatty acid profile range reported for species as diverse as marine carnivores to strict herbivores. This suggests that kiwi have a highly varied dietary intake. The two most abundant fatty acids across all species were oleic (C18:1) and palmitic (C16:0) acids. With the exception of red-eyed vireos, proportions of palmitoleic (C16:1), stearic (C18:0) and linolenic (C18:3) acids were consistently low across all species. Linoleic acid (C18:2) was found in similar amounts as palmitic acid in the herbivorous, omnivorous and insectivorous species but was markedly lower in the depot fat of the marine carnivores. Both for kiwi and the other species considered here the depot fat composition appeared to provide only broad-scale indications of dietary intake. Species of a narrow dietary range, or those that consumed prey with a distinctive fatty acid profile, were more suited to this method of predicting dietary intake than those that consumed a wide range of dietary items.

Most common fatty acids

The consistency of relatively high proportions of both palmitic and oleic acid found across a range of species differing in dietary intake was expected as these are two of the most common fatty acids found in animals. Palmitic acid is the most abundant fatty acid in eukaryotes (Lynen, 1980). It is the primary product of fatty acid synthetase in both plants and animals, thus it can easily be desaturated or elongated into a variety of fatty acids (Watkins & German, 1998). Oleic acid is the most common unsaturated fatty acid and is the precursor for the production of most other polyunsaturates (Watkins & German, 1998). Absorption of both palmitic and oleic acid is enhanced when eaten and digested together (Scott *et al.*, 1976).

With the exception of palmitoleic acid in the depot fat of vireos, both stearic acid and palmitoleic acids were found in low levels irrespective of diet. Stearic acid is generally found in low proportions in depot fat because it is rapidly desaturated into oleic acid (McPherson & Spiller, 1996). Ruminants, however, have characteristically stearic-rich fats (Hilditch, 1956). Palmitoleic acid is generally a minor component of animal and vegetable fats, although some fish and seed oils contain substantial quantities (Watkins & German, 1998). Certain invertebrates have relatively high

amounts of palmitoleic acid, for example, larval grass grubs and maggots have been found to have considerably higher palmitoleic acid levels compared with a range of both adult and larval/juvenile Orthoptera, Tenebrionidae, Hemiptera and Annelida (Potter, unpublished data). The relatively high level of palmitoleic acid found in red-eyed vireo fat is likely to be due to diet which consists of adult and larval beetles, caterpillars, fly larvae, and seeds (Williamson, 1971). Consumption of a quantity of a particular seed or invertebrate high in this fatty acid will increase proportions of palmitoleic acid in depot fat. Depending on dietary intake of palmitoleic-rich items, the level of this fatty acid in fat stores will vary.

Herbivores, omnivores and insectivores

Fatty acid compositions of the depot fat of the herbivorous, omnivorous and insectivorous species were generally similar. A difference in the proportions of linoleic and linolenic acids was expected due to differing intakes of plant material. The essential fatty acid composition of depot fat of the herbivorous black grouse (*Tetrao tetrix*) was not markedly different from that of the omnivorous or insectivorous species. The wide range of leaves, flowers, fruits and seeds consumed by black grouse (Starling-Westerberg, 2001) provides a variety of both linoleic and linolenic sources similar to those obtained by more omnivorous species. Most insects cannot synthesize linoleic or linolenic acid (Turunen, 1974), therefore the proportion of these fatty acids in insect lipids depends on the dietary supply, the stage of development, the age and sex of the insect (Turunen, 1974). Insects can also modify their fatty acid composition in response to changes in environmental conditions (Khani *et al.*, 2007). The highly variable fatty acid composition of insect fat means animals which prey upon these species will also have variable proportions of fat in their depot stores. This is shown by highly variable fatty acid composition results between individuals for both omnivorous and insectivorous species.

Migration

Migratory birds are known to vary not only the quantity of stored fat, but also the composition of their fat, as a possible adaptation for improved lipid utilization during long distance flights (Blem, 1990). Migratory birds that store large amounts of fat, store more oleic than linoleic acids, but the reverse is true for nonmigrants that do not store as much fat (Blem, 1976). This suggests a pattern of decreasing proportions of

essential fatty acids and replacement by fatty acids such as oleic acid in preparation for migration (Egeler & Williams, 2000). Our results do not show this pattern. The nonmigratory marine carnivorous species had both oleic and linoleic acid levels within the range of migratory species. The nonmigratory insectivorous species had generally higher oleic acid levels than those of the marine carnivores.

Strongly migratory birds also have been reported to have greater proportions of unsaturated fatty acids (Bairlein & Gwinner, 1994; Price *et al.*, 2008; Weber, 2009) which due to their increased mobility may be of advantage during the metabolic demands of migration (Johnston, 1973). This study did not find increased levels of unsaturated fats in migratory birds. These findings are consistent with other work that found no change in the proportion of unsaturated acids leading up to, during and post migratory phases (McGreal & Farner, 1956; Hicks, 1967).

Essential fatty acids

Most fatty acids across a range of chain lengths and degrees of saturation are able to be synthesized in animal tissue by various elongation and desaturation reactions (Ward, 1963), however two fatty acids, linoleic and linolenic acids, cannot be synthesized in animals and must be sourced from the diet (Spector, 2000). These essential fatty acids can only be obtained from plant material. Linoleic acid is found in quantity in most seeds and seed oils (Simopoulos, 1996) and linolenic acid is the main fatty acid in leaf material (Hartman & Shorland, 1968), algae, and phytoplankton (Ward, 1963). In general, the linolenic acid level in depot fat of most animals is relatively low (Wood & Enser, 1997).

Linoleic acid can be desaturated into arachidonic acid (C20:4), the main substrate for the synthesis of eicosanoid mediators (Spector, 2000). These mediators are some of the immunoregulatory molecules found in macrophages (Hand & Novotny, 2002) and their primary response is to promote inflammation (Spector, 2000). In contrast, linolenic acid has an opposing function to that of linoleic acid. It can be desaturated into eicosapentaenoic acid (EPA) (C20:5) and docosahexanenoic acid (DHA) (C22:6) and through EPA it may prevent the excessive production of eicosanoid from arachidonate, thereby reducing eicosanoid-mediated responses such as inflammation (Spector, 2000). Linolenic acid may also be required for optimum vision and central nervous system development (Connor *et al.*, 1992; Uauy *et al.*, 1992).

Avian marine carnivores

As both linoleic and linolenic acids are essential fatty acids, that is, they are not synthesized by the body, they are more likely to reflect dietary intake than other synthesizable fats such as oleic, palmitic, palmitoleic and stearic acids. The avian marine carnivores analysed in this study had relatively low levels of linoleic acid in their depot fat compared to the herbivorous, omnivorous and insectivorous species. This difference in the fatty acid profile of marine carnivores can be attributed to dietary intake. Palmitic, oleic, EPA and DHA were generally the most abundant fatty acids found in adipose tissue in a range (28 spp) of marine fish and invertebrates sourced from eastern Canada (Budge *et al.*, 2002). Marine fish also tend to have lower linoleic acid levels than freshwater species (Hilditch, 1956). The diet of sea birds contains little or no carbohydrate (excluding chitin) so they have little opportunity to synthesize fat, therefore the source of their depot fats is almost wholly exogenous (Hilditch, 1956).

The link between the fatty acid composition of sea bird adipose tissue and that of their major prey items is highlighted by a similar pattern of high palmitic and oleic acid and low linoleic and linolenic acid levels in the depot fat of seabirds compared to that found in the marine prey items that make up the majority of their diet. Eighty to ninety percent of all fatty acids analysed in the depot fat of western sandpipers (*Calidris mauri*) were made up of palmitic, palmitoleic, stearic and oleic acids (Egeler & Williams, 2000). The fats of gannets, fulmars, skua gulls and also herring gulls (*Larus argentatus*) are similar to the fatty acids found in the average marine fish fat (Lovern, 1938). As the fatty acid composition of seabird fat closely mirrors that of their diet, any change in dietary intake should effect a change in the fatty acid signature of depot fat.

Changes in the diet of a fish can result in a significant change in the fatty acid signature of its depot fat in as little as three weeks (Kirsch *et al.*, 1998). The fatty acid composition of fish varies with size (Iverson *et al.*, 2002) and geographic location (Budge *et al.*, 2002), therefore the fatty acid composition of the birds that prey upon these species will also change depending on age, season and feeding location. Despite the variability in fatty acid composition of depot fat of avian marine carnivores they can still be distinguished from herbivorous, omnivorous and insectivorous species by a relatively low level of linoleic acid.

Dendrographic analyses

The dendrographic analyses highlight the effect that the arrangement of data has on the results obtained, specifically, when species are represented by a mean value they group quite differently to when values for several individuals within each species are used.

The dendrogram of the *mean* palmitic, palmitoleic, stearic, oleic, linoleic and linolenic acids of body fat of various avian species separated into broadly similar groups as did the dendrogram using *individual* birds. Both these dendrograms separated three of the four marine carnivores and an insectivore into one of the main divisions, and the remaining marine carnivore and the other avian species into the second division. Even though the insectivorous vireo separated with the main group of marine carnivores it then separated again into a solo group. The high palmitoleic acid content of the depot fat of vireo compared with the other insectivorous species is likely to have effected this separation. The reason for the separation of the marine carnivores across the main division, even in the less rigorous cluster analysis of means, shows that these species have a widely varied dietary intake.

In the dendrogram of means, the herbivorous grouse was separated from the omnivorous species at a Euclidean distance of only three, whereas when using individual bird data these same species separated at a distance of 50. These results strongly suggest that using solely mean data gives an overall indication of separation on the basis of dietary intake but it is a relatively crude indication.

The cluster analysis using individual results gave similar groupings to mean data which indicates that there are real distinctions underlying the hierarchy but confirms the high degree of variation between individuals in a given species. Even black grouse, with perhaps the most limited restricted dietary intake of those species analysed here, showed a considerable level of variation in the fatty acid composition of depot fat. Thus, this method of distinguishing between avian taxa of differing dietary habit through adipose fat composition may not be equally accurate for every species. If dietary variations between species are not able to be distinguished using this method then it is unlikely that fat depot analyses will successfully distinguish differences within species.

Kiwi showed the highest degree of variation in fatty acid content of body fat of all the species analysed as they spanned both the main divisions. The sample size for kiwi was higher than for any of the other species, thus there was a greater chance for

kiwi to be present across distant locations on the dendrogram. The kiwi diet is highly varied with at least 90% of dietary intake from invertebrates and approximately 10% from seeds and fruit (Kleinpaste, 1990). The fatty acid composition of insect fat changes with age, season and in some areas location (Turunen, 1974), and that of fruit is highly variable between species (Wharemate, 2003). The highly variable dietary intake of kiwi in the wild and their tendency to feast on dietary items when abundant (Kleinpaste, 1996), as well as high variation in the fatty acid composition of individual food items, means that the fat content of adipose tissue of kiwi is extremely varied. While this renders their depot fat a poor indicator of dietary intake, their highly varied diet is of benefit when it comes to their maintenance in captivity. Their ability to survive on such varied diets suggests that kiwi should be amenable to a range of captive diets.

While Shorland and Gass (1961) present data on a single kiwi, this study gives the first comprehensive insight into the fatty acid profile of the depot fat of North Island brown kiwi. However, it does not provide conclusive evidence into dietary intake. This technique of determining a diet is more suited to taxa with less varied diets, or ones with a distinctive fatty acid signature. Thus, a different approach is needed to establish the diet of North Island brown kiwi, specifically a more direct technique; one focussed on identification of digesta or faecal material.

CHAPTER 3

Comparison of the apparent digestibilities of macronutrients in an invertebrate-based near-natural diet in kiwi (*Apteryx mantelli*) and roosters (*Gallus gallus*)

Blue worms

Tiger worms (not palatable to kiwi)

Freeze dried blue worms

Imported grasshoppers

Near-natural diet (without worms)

Homogenised near-natural diet

Trial enclosures

Kiwi in nest box

Inside trial enclosure

Weigh bucket

Trial location

ABSTRACT

This study provides data on the nutrient composition and apparent digestibilities of macronutrients in an invertebrate-based near-natural diet in kiwi (*Apteryx mantelli*) that was also fed to roosters (*Gallus gallus*) for comparison. The digestibility of organic matter, ash, crude protein, gross energy, hemicellulose, cellulose, neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin/chitin was higher in kiwi than roosters. The digestibility of fat was similar in roosters and kiwi.

Protein digestibility in kiwi (91%) was similar to that in other insectivorous species (range of 83-97%) and within the range of protein digestibilities (56-97%) of a variety of herbivorous, carnivorous and omnivorous species with markedly different protein intakes. Fat digestibility in kiwi (91%) is lower than that of other insectivores (97-98%) but within the range of protein digestibilities (87-97%) of a variety of carnivorous and omnivorous species. As kiwi digest fat to a similar level as they digest protein and their wild diet contains considerably more protein than fat, it appears that protein is the dominant energy source. The digestibility of NDF (33%) and ADF (26%) was generally lower in kiwi than in a range of herbivorous and omnivorous species (range of 45-71% (NDF) and 18-57% (ADF)). The high digestibility of lignin by kiwi (33%) and roosters (15%) suggested that some other digestible substance was included in this portion of the diet. The presence of chitinase activity in other animals that consume diets of relatively high chitin content (arthropods make up 60% of the near-natural diet of kiwi) suggests that kiwi may be able to digest chitin.

INTRODUCTION

The subtleties of a species' nutrient requirements are increasingly recognised as fundamental to their ability to thrive in captivity (Kirk Baer *et al.*, 2010). Ideally, diets for captive-held animals would be calculated according to recommended daily allowances (RDAs) (Alpers *et al.*, 1995) of each nutrient. However, the invasive technique used to calculate RDAs (feeding diets deficient and in excess of requirements) means that this method is often not feasible for threatened species. Instead, less invasive methods are used, such as: direct observations of dietary intake; observations that infer diet; crop, gizzard, stomach or faecal analyses; or the relatively new technique of diet determination through fatty acid analyses of depot fat (Meynier *et al.*, 2010). For most species, at least one of these techniques is suitable for determining dietary intake and thus a diet can be formulated based on this information. However, some species, for example cryptic or nocturnal ones, diets are difficult to formulate as the components that make up the natural diet are often not known (Piggott & Taylor, 2003). Insectivorous species are particularly challenging as they often consume large quantities of invertebrates that are difficult to source. Being insectivorous, nocturnal and cryptic, the identification of dietary items and the formulation of a natural diet for North Island brown kiwi (*Apteryx mantelli*) is particularly challenging. Furthermore, kiwi are also unusual in that they have an abnormally large egg relative to body size, are unable to fly, have a relatively low metabolic rate, forage in an atypical manner, and have male-only incubation (in some species) with associated changes to their reproductive endocrinology (Calder, 1990; Potter & Cockrem, 1992; Cunningham *et al.*, 2007). As a consequence, we cannot assume as a starting point that kiwi will be 'typical' with regard to any physiological processes.

Despite a long history of being held captive (Robson, 1947; Peat, 1990), kiwi bred or reared in captivity have a lower survival rate than those in the wild (McLennan, 1996; Department of Conservation, 2004; Holzapfel *et al.*, 2008). Dietary deficiencies are thought to be a major cause of this failure of kiwi to thrive in captivity (Potter *et al.*, 2010). Diet-related issues in captivity may include smaller eggs, lower hatching rates and higher adult mortality (Reid, 1972, 1981; McLennan, 1996; Department of Conservation, 2004). As current diets for captive kiwi are not based on nutrient requirements but on diets that kept birds alive in the initial stages of holding kiwi in

captivity (Reid, 1970; Johnson, 1996; Potter *et al.*, 2010), there is an urgent need for deficiencies in dietary intake to be addressed.

The reported success of using fatty acid analyses of depot fat to determine dietary intake (Herman, 2005; Chen *et al.*, 2008; Nordin *et al.*, 2008; Meynier *et al.*, 2010), as well as published work on the fatty acid composition of the depot fats of wild adult kiwi (Shorland & Gass, 1961); chapter 2) highlighted the possibility of using this technique to identify the natural diet of kiwi. Thus, I attempted to determine dietary intake by combining Shorland and Gass's (1961) results with our own data on the fatty acid composition of gizzard fat, however, the fat content of adipose tissue of wild kiwi proved to be a poor predictor of dietary intake (chapter 2). This technique is more suited to taxa with less varied diets, or diets with a distinctive fatty acid signature.

Other studies have taken a more direct route of reporting dietary items for kiwi, for example, details of the content of gizzard/stomach and/or faecal material (Gurr, 1952; Bull, 1959; Watt, 1971; Reid *et al.*, 1982; Miles *et al.*, 1997; Pindur, 2004). Only one, however, has attempted to estimate a complete natural diet (Kleinpaste, 1990). I used Kleinpaste's (1990) study, the most comprehensive information available on the natural diet of kiwi, to formulate a diet using natural ingredients. While identification of specific dietary items, i.e. nutrient intake, is vital for the formulation of a diet, so too is a thorough understanding of nutrient assimilation. Thus far, no attempts have been made to look at the apparent digestibilities of macro and micro-nutrients in kiwi. Furthermore, the unusual ecology of kiwi (as described above) means that physiological processes may not fit within known/common parameters for avian species. Hence it would be useful to run parallel studies with a bird of similar size of which more is known.

The purpose of this study was to determine the digestibilities of macronutrients in an invertebrate-based diet in kiwi, and to duplicate this method using Hyline brown roosters (*Gallus gallus*), thus allowing direct comparison with a common, domesticated, omnivorous species for which substantial digestibility and physiological data exist.

METHODS

Apparent digestibility trials

The apparent digestibility of a near-natural diet consisting of representative invertebrates and fruit in proportions similar to that consumed by kiwi in the wild was determined in North Island brown kiwi (*Apteryx mantelli*) and roosters (*Gallus gallus*) for comparison. The macronutrient composition of the near-natural diet on a g/100g dry matter basis was: ash 8; crude protein 53; gross energy 24 kJ/g; fat 16; total fatty acids 15; carbohydrate 23; neutral detergent fibre 6; and acid detergent fibre 10.

Kiwi

Seven captive, North Island brown kiwi (3 female, 4 male) that had been maintained in captivity at Westshore Wildlife Reserve, Napier, for a minimum of one year were used. During the ten days prior to confinement the kiwi were housed in their normal outdoor pens (5 x 12 m) with a natural light cycle (10/14 light/dark regime) and at ambient temperature (5-17 ºC). During this period they were progressively introduced to the natural diet in the proportions outlined in Table 1.

Table 1. Daily diet regime for acclimation period and digestibility trial (wet weight, grams)

Day	Near-natural diet[2]	Usual diet[4]	Total diet fed
	Acclimation Period		
1-9	44	156	200
10[1]	55	125	180
11	68	82	150
12	85	50	135
13	100	20	120
14-15	110	0	110[5]
	Digestibility Trial		
16-21[3]	110	0	110

[1] Birds moved from outside pens to inside enclosures.
[2] From Potter et al., unpublished
[3] Titanium dioxide (0.3%) was added to the near-natural diet on days 16-21 as a marker of intestinal transit. Faecal samples for apparent digestibility analyses were collected on days 20, 21 and 22.
[4] Composition details of the usual kiwi diet can be obtained from Potter et al. (2010; captive facility #6) and a standard commercial poultry maintenance diet was used as the usual diet of roosters.
[5] 110g of near-natural diet was equivalent to 200g (wet weight) of the usual diet (near-natural diet was partially dried to facilitate binding). These weights were equivalent to 90g dry matter of the near-natural diet and 90g dry matter of the usual diet.

The kiwi were then moved to individual indoor enclosures (1.2 x 1.5 x 1.2 m) with large windows, allowing natural light cycles and ambient temperatures to be maintained (7 - 15 ºC). Individual enclosures each had a nest box attached at one end. Non-slip, non-absorbent linoleum flooring was used in both the main chamber and the nest box to prevent kiwi slipping and to facilitate faecal collection.

Each day, food was placed into the enclosures in bowls identical to those normally used. Titanium dioxide (0.3%) was added to the near-natural diet for the period of the digestibility trial (days 16-21) as an indigestible marker. Water was available *ad libitum* from both a bowl attached to a wooden base to prevent tipping, and from a bottle (1.5 litre soft drink bottle) attached with wire (1.5 cm off the floor) to an internal corner of each enclosure. Each morning, all faecal matter was removed from the enclosures and the birds weighed (Super Sanson Salter Spring Balance 5 kg +/- 25 g).

Roosters

Four adult, male, Hyline Brown roosters were housed in individual grower cages (80 x 80 x 80 cm) equipped with faecal collection trays at the Poultry Unit, Massey University, Palmerston North, and maintained at 22°C under a 16/8 light/dark regime. The feeding regime was identical to that for the captive kiwi (Table 1). Each morning all excreta were collected, roosters were weighed, and their daily allowance of food provided.

All animal procedures were approved by Massey University Animal Ethics (permit 07/17) and the Department of Conservation (ECHB-21465-RES, DOC File NHS-03-04-01).

Nutrient Analyses

Dry matter contents of food were determined by drying freeze-dried samples to a constant weight in a convection oven at 105˚C (AOAC 391.02B) (Horwitz, 2000). Ash was determined following combustion at 550˚C for 8 hours in an electric furnace (AOAC 942.05) (Horwitz, 2000). Gross energy was determined by bomb calorimetry. Protein was determined by the total combustion method (AOAC 986.06) (Horwitz, 2000). Amino acids were determined by hydrochloric acid hydrolysis followed by HPLC separation (AOAC 994.12) (Horwitz, 2000). Neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin analyses were determined according to Robertson and Van Soest (1981). Fatty acid content was determined by the formation of methyl esters

and subsequent gas chromatography separation. Minerals were analysed through plasma emission spectrometry. Uric acid content of the excreta was determined by the Spectrophotometric method (Marquardt, 1983). All nutrient analyses were carried out in triplicate by staff at the Nutrition Laboratory, Massey University, Palmerston North.

Statistical Analyses

Dietary intake was first calculated on a wet weight basis, then converted to a dry matter basis. Dietary intake equalled weight of food fed minus refusals. None of the birds ate all of the diet supplied, and feeding occurred over several hours, meaning that dehydration of any refusals was inevitable. This was addressed by placing an equivalent amount of food in a bowl next to the enclosures and reweighing it in the morning. Dehydration of food was factored into calculations of intake.

Faecal output was determined on a basis of dry matter. Daily faecal collections were weighed, freeze-dried and subsequently dried again at 105˚C to a constant weight to give dry matter values.

The percentage of indigestible marker (titanium dioxide (TiO2)) recovered in the faecal material was calculated by the Indicator Method:

(Total excreta out (g) * %TiO2 in excreta) / (Total diet intake (g) *%TiO2 in diet)

A recovery of more than 95% of the TiO2 ingested was required for TiO2 to be used as an indigestible marker for apparent digestibility calculations; for values below this rate a Total Collection Method (Bourdillon *et al.*, 1990) was used as follows:

([(% Nutrient in diet * Total dry matter intake (g)) – (% Nutrient in excreta * Total dry matter in excreta (g))] / (% Nutrient in diet * Total dry matter intake (g))

Where intake varies from day to day the transit time of a meal must be established to relate intake with output. As both feeding and faecal collection were performed once per day (12 hours apart), it was possible to relate faecal output data to intake with daily intervals of 12, 36 and 60 hours. I therefore determined the correlations between intake and output over lags of 12, 36 and 60 hours.

The presence of uric acid in the excreta confounded protein content calculations. Therefore, the uric acid content of the excreta was calculated first and the associated nitrogen was removed prior to apparent protein digestibility calculations. The following equations were used (all calculated on a dry matter basis):

- **g/day of uric acid in excreta** = % uric acid in excreta/100 * g excreta per day

- **g/day uric acid N in excreta**
  = g/day uric acid in excreta * (atomic weight N * 4 / atomic weight of uric acid)
- **g/day total N in excreta** = % total N in excreta/100 * g excreta per day
- **g protein N/day in excreta** = g/day total N in excreta – g/day uric acid N
- **g protein/day in excreta with uric acid removed** = g protein N/day * 6.25
- **Mass (g) of excreta with uric acid removed**
  = g/day excreta – (% uric acid in excreta * g/day excreta/100)

I examined the effect of bird and day on the apparent digestibilities of organic matter, ash, protein, fat, neutral detergent fibre, acid detergent fibre, chitin/lignin and gross energy through analyses of variance using SAS 9.1.3 (SAS, 2004). Data were checked for normality using the Kolmogorov–Smirnov Test and transformed using the Johnson Transformation if not normally distributed. Data were back-transformed to produce means and standard errors.

RESULTS

The macronutrient composition of the near-natural diet is within 5% of that of the natural diet (Table 2).

Daily dietary intake varied significantly among both kiwi (n = 7; d.f. = 1, 6; F value = 12.68; P = 0.001; range 24.09g ±2.48 to 37.34g ±0.25 dry matter per day) and roosters (n = 4; d.f. = 1, 3; F value = 6.69; P = 0.002; mean intake 34.87g ±0.31 to 36.26g ±0.22 dry matter per day) but not between days for either species. No significant difference was found in daily intake between kiwi and roosters.

Daily faecal output varied significantly (n = 7; d.f. = 1, 6; F value = 20.40; P = 0.0001) among kiwi (8.95 ±0.62 to 13.87 ±0.62 g dry matter per day) but not between days. For roosters, daily faecal output did not differ significantly among individuals or between days. The daily faecal output of roosters was significantly (n = 6 (kiwi), n = 4 (roosters); d.f. = 1, 8; F value = 17.81; P = 0.003) greater than that of kiwi (rooster = 18.07 ± 1.11 g, kiwi = 11.99 ± 0.91 g dry matter per day).

The percent recovery of titanium dioxide in the faecal material of kiwi (61%) and roosters (71%) peaked on day one of the digestibility trials and then remained relatively constant. The percent recovery of titanium dioxide in both roosters and kiwi was below the 95% recovery limit required for use as an indigestible marker in apparent digestibility calculations (Table 3). Thus, apparent digestibilities were calculated using the Total Collection Method.

Nitrogen from uric acid made up 53% and 74% (average) of total nitrogen in the faecal material from kiwi and roosters, respectively.

The highest correlation between successive dietary intake and faecal output was found at 12 hours (R-squared = 0.88; P value = 0.002). Accordingly, digestibility was assessed by comparing dietary intake and faecal output over a 12 hour period.

Despite differences in body mass, the daily dry matter intake of adult kiwi (pers comm. NZ captive institutions) and adult roosters (Lessire *et al*., 1982; Coon *et al*., 1990) is similar. As the body mass of both kiwi and roosters changed by less than four percent of pre-trial body mass (less than 60 g) during the digestibility trial (Table 4) daily intake was sufficient to maintain body weight.

Comparison of apparent digestibilities

With the exception of neutral detergent and acid detergent fibres, the apparent digestibilities of macronutrients were significantly different between roosters and kiwi (Table 5). With the exception of fat, kiwi had consistently higher mean percent apparent digestibilities of macronutrients (organic matter, ash, protein, carbohydrate, neutral detergent and acid detergent fibres, lignin/chitin and gross energy) than roosters. While statistically different, the apparent digestibility of protein and fat in kiwi and roosters differed by only ~3%.

Table 2. Comparison of the nutrient composition of the natural and near-natural diets of kiwi (g/100 g, dry matter basis).

Diet	Ash	Crude Protein	Gross Energy (kJ/g)	Fat	Total Fatty Acids	Carbohydrate[2]	Neutral Detergent Fibre	Acid Detergent Fibre	Lignin & Chitin
Natural diet[1]	8.0	52	23	17	13	24	N/A	N/A	N/A
Near-natural diet	8.0	53	24	16	15	23	21	15	5

[1] Sourced from Potter *et al.*, unpublished

[2] Calculated by difference
N/A not available

Table 3. Comparison of the percent recovery of the indigestible marker titanium dioxide in faecal material from roosters and kiwi fed the near-natural diet.

Species	**Titanium dioxide recovered** (%)
Rooster (n=4)	71
Kiwi (n=6)	61

Table 4. Pretrial body weight of kiwi and roosters fed the near-natural diet.

Species	Bird	Body weight (kg)
Kiwi	1	1.4
	2	1.6
	3	1.5
	4	1.6
	5	1.6
	6	1.2
	7	1.4
Rooster	1	2.7
	2	2.7
	3	2.3
	4	2.8

Table 5. Analysis of variance of the percent apparent digestibilities of the macronutrients in the natural diet by kiwi and roosters.

Nutrient	Mean ± SE	F value	P value
Organic matter	Kiwi = 69 ± 2 Rooster = 51 ± 4	25.03	***
Ash	Kiwi = 39 ± 4 Rooster = 17 ± 6	11.40	**
Protein	Kiwi = 91 ± 1 Rooster = 88 ± 1	11.90	**
Fat	Kiwi = 91 ± 1 Rooster = 94 ± 1	9.67	*
Neutral detergent fibre	Kiwi = 33 ± 2 Rooster = 23 ± 8	4.43	NS
Acid detergent fibre	Kiwi = 26 ± 3 Rooster = 17 ± 7	3.22	NS
Hemicellulose	Kiwi = 51 ± 3 Rooster = 37 ± 6	22.43	***
Cellulose	Kiwi = 28 ± 2 Rooster = 18 ± 4	12.49	**
Lignin/chitin	Kiwi = 33 ± 4 Rooster = 15 ± 6	6.64	*
Metabolisable Energy	Kiwi = 75 ± 1 Rooster = 65 ± 2	27.54	***

n=6 (kiwi), n=4 (roosters). d.f. = 1, 8
*= P<0.05; **=P<0.01; ***=P<0.001; NS=P>0.05

DISCUSSION

A diet was formulated comprising representative invertebrates and fruit in similar proportions to a typical diet consumed by kiwi in the wild (Kleinpaste, 1990). The macronutrient composition of this diet fell within two percent of the predicted composition for crude protein, ash, gross energy, lignin/chitin, neutral detergent fibre and acid detergent fibre (Potter et al., unpublished). Feeding trials using this diet provide the first determination of macronutrient digestibility for kiwi. The diet was also fed to roosters to allow comparison with a species for which considerably more is known of nutrient uptake and gut physiology.

The discussion that follows looks first at the underlying experimental approach, including the assumptions relating to dietary intake, digesta transit times and the use of indigestible markers. Second, the similarities and differences in apparent digestibilities between kiwi and roosters are discussed, including the findings that kiwi had higher apparent digestibilities of organic matter, ash, crude protein, gross energy, hemicellulose, cellulose and lignin/chitin. These apparent digestibility results are also compared and discussed in relation to digestibility values from other avian and mammalian species.

Body mass versus dietary intake

All birds in this trial were offered the same amount of food, but body mass varied across individuals; on average roosters weighed a third more than kiwi. The decision on how much food to offer was based on data from the literature (Lessire *et al.*, 1982; Coon *et al.*, 1990); and from captive institutions. The amount fed was increased slightly from these values to exceed the predicted daily intake and all birds maintained their body weight over the trial period.

Digesta transit time and indigestible markers

Indigestible markers are commonly used to assess gut transit time and to aid in determinations of nutrient digestibility (Phinney *et al.*, 1994). No empirical data exist on either the retention time of digesta or the efficacy of indigestible markers in kiwi, but in chickens 100% recovery has been reported (Sales & Janssens, 2003). Thus, before I undertook digestibility calculations in kiwi, I determined the temporal relationship between dry mass intake and dry mass faecal output, as well as the recovery rate of the

indigestible marker titanium dioxide in kiwi. While the tightest correlation between intake and faecal output was found to be 24 hours, actual transit time may be shorter than this because feeding and faecal collection occurred only once every 24 hours. If the interval is shorter, the calculated digestibility values would not have been affected because we were still operating on full recovery of excreta. To minimise the possibility of some components being retained for longer periods (Moss, 1989; Vergara *et al.*, 1989), thus causing a carrier-over from the previous diet, the trial diet was fed for six days prior to the first faecal collection.

A number of indigestible markers are available, but their efficacy varies widely. For example, recovery rates of chromium oxide in poultry ranges from 88% (Dansky & Hill, 1952) to 101% (Wharemate, 2003), of acid insoluble ash in ostrich chicks of 127% (Nizza & Di Meo, 2000), and of titanium dioxide in poultry from 97-100% (Peddie *et al.*, 1982; Crespo & Esteve-Garcia, 2001; Myers *et al.*, 2004). Titanium dioxide has proven reliable as an indigestible marker in poultry, however, it is routinely used in conjunction with standard, commercial, highly processed, diets. No data are available on its efficacy with a wholly invertebrate-based, chitin-rich, diet. It is possible that the high level of relatively indigestible components of the diet, such as chitin and lignin, would not be distinguished and separated from the indigestible titanium dioxide, and instead all three might be retained in the caeca. This phenomenon has the potential to decrease the recovery rate of titanium dioxide and affect digestibility values. This potential issue meant that relying on a single method of measuring intake and output may have resulted in inaccuracies; thus, as well as using titanium dioxide as an indigestible marker, I used the Total Collection Method (Bourdillon *et al.*, 1990) for digestibility trials.

The recovery rate of titanium dioxide was lower in kiwi (61%) than in roosters (71%). These results were both lower than that reported in poultry studies (98%, (Peddie *et al.*, 1982); 97%, (Myers *et al.*, 2004); 100%, (Crespo & Esteve-Garcia, 2001)). Kiwi are known to retain fine particulate matter in the caeca (Potter *et al.*, 2006), possibly for prolonged fermentation. Thus, it is likely that the low recovery of titanium dioxide seen here is due to these fine, dense particles being diverted into the caeca. Furthermore, the lower rates of recovery of titanium dioxide seen here in roosters, compared with those reported in other studies of poultry, suggest that a similar phenomenon may have happened in roosters (fed an invertebrate-based diet) as in the

kiwi. Without further trials that quantify the retention of titanium dioxide in the caeca, which would require highly invasive and potentially fatal methods that would not be approved for use on an endangered species, the fate of almost 40% of the ingested marker remains unknown. After ruling out laboratory error, it is likely that this dense, particulate matter has been mixed with chitin and lignin and remained in the caeca. Consequently, titanium dioxide was not considered to be a suitable marker to be used in kiwi, or in roosters fed a wholly invertebrate-based, chitin-rich, diet; instead digestibility trials should be conducted using the total collection method, as was used in this study.

Nutrient Digestibilities

Studies on the digestive efficiencies of insectivorous species are sparse (Stannard & Old, 2012), and these data that exist tend to focus on small-sized, predominantly mammalian, insectivores. The small number of studies of insectivorous birds focus on very small-bodied passerine species (Johnston, 1993; Levey & Karasov, 1994; Weiser *et al.*, 1997; Wright *et al.*, 2010). Kiwi, as medium-sized, highly opportunistic foragers, consume not just a range of invertebrates but fruit as well (Kleinpaste, 1990). Perhaps the best candidate for comparison would be wild fowl (Red Jungle Fowl, *Gallus gallus*; Lafayette's Jungle Fowl, *G. lafayettei*; Green Jungle Fowl, *G. varius*; or Grey Jungle Fowl, *G. sonneratii* (Kanginakudru *et al.*, 2008)); however no data exist on the digestive efficiencies of these species. Thus, no obvious avian or mammalian insectivorous species are available to use as a model to predict digestibilities in kiwi.

Protein digestibility

Protein, fat and carbohydrate provide energy from the diet (Lewis *et al.*, 1987). The proportions of each of these nutrients, coupled with their respective digestibilities, indicate their contributions to dietary energy intake (Noblet & Perez, 1993). Kiwi consume a diet relatively high in protein (53%) compared to a range of other insectivorous species, including juvenile iguanas (23%, (Troyer, 1984)), hedgehogs (34-40%, (Webb *et al.*, 1993), bats (39%, (Graffam *et al.*, 1998), and anteaters (50-58%, (Oyarzun *et al.*, 1996)). This high protein intake suggests that kiwi either have a poor ability to digest protein, so need to consume large amounts to obtain sufficient quantities for normal physiological processes, or that they use protein as a major source of energy. The high apparent digestibility of protein by kiwi (91%) suggests the latter.

The digestibility of protein by kiwi was significantly higher than in the roosters, but the difference was small (91% and 88% respectively). Similar apparent digestibility values (75-83%) have been reported for roosters (Carré *et al.*, 1991; Bonnet *et al.*, 1997; Carré *et al.*, 2002; Huang *et al.*, 2005) fed a conventional diet of approximately 19-25% protein (Bonnet *et al.*, 1997; Wohl *et al.*, 1998; Langhout *et al.*, 2000; Carré *et al.*, 2002; Buchanan *et al.*, 2007). The efficiency with which the roosters from this study digest the comparatively high levels of protein in the near-natural diet is not surprising considering wild fowl consume a wide variety of invertebrates, fruit, seeds and young shoots (Klasing, 2005) and domestication is relatively recent (Fumihito *et al.*, 1996). Furthermore, an increase in the protein content of the diet from 0 to 30% (casein added as protein) did not significantly affect protein digestibilities in roosters (Kamisoyama *et al.*, 2009). These results suggest that poultry are capable of digesting diets considerably higher in protein content than are fed commercially, and that, in poultry at least, protein digestibility is independent of the protein content of the diet. Whether this holds true for kiwi is yet to be determined.

Despite consuming a diet relatively high in protein, the protein digestibility of kiwi is within the range of protein digestibilities of other insectivorous species. For example, hedgehogs and bats fed a diet of approximately 39% protein have a protein digestibility of 83% (Graffam *et al.*, 1998) and 97% (Webb *et al.*, 1993) respectively, juvenile iguanas fed a diet of 23% protein have a protein digestibility of 88% (Troyer, 1984) and phascogales and kultarrs fed a diet of 46% protein have a protein digestibility of 91% (Stannard & Old, 2012).

Similarly, the digestibility of protein by kiwi (91%) is within the range (56-97%) of protein digestibilities reported for non-insectivorous species fed diets with a variety of protein contents. Species with lower protein digestibilities than kiwi include ocelots fed a 65% protein diet (protein digestibility = 56% (Bennett *et al.*, 2009)), geese fed a 9% protein diet (protein digestibility = 61-80% (Buchsbaum *et al.*, 1986)), wolves and domestic dogs fed a 19% protein diet (protein digestibility = 71-75% (Childs-Sanford & Angel, 2006)), seals fed a 52% protein diet (protein digestibility = 78%, (Trumble *et al.*, 2003)), and foxes and minks fed a 30% protein diet (protein digestibility = 83% (both species) (Vhile *et al.*, 2005)).

Species reported to have similar or higher protein digestibilities as kiwi include rats fed a 17% protein diet (protein digestibility = 89% (Wisker & Knudsen, 2003)), pigs fed a diet of 17% protein (protein digestibility = 92% (Wisker & Knudsen, 2003)),

and the domestic cat fed a diet of 51% protein (protein digestibility = 97% (Vester *et al.*, 2010)). Thus, it appears that kiwi are able to digest protein to a similar level as that of other insectivorous species, and to a relatively high level to that of a range of herbivorous, carnivorous and omnivorous species with markedly different protein intakes.

Feather growth is an important factor to consider when conducting digestibility trials on avian species. Both protein and amino acid requirements increase during feather growth (Leeson & Walsh, 2004). Cysteine is one amino acid that is especially important for keratin synthesis (Murphy & King, 1987; McWilliams, 2008). Thus, either the availability of these nutrients in the diet must increase or the efficiency with which they are digested must increase. Kiwi appear to moult over long periods rather than in an intense brief period (Murray Potter, pers comm., 2013), so there is unlikely to be a need for a specific 'moult' diet for kiwi. Even so, the impact of moult on nutrient requirements in kiwi needs to be investigated further.

Dietary protein is not only a source of energy; it is also an important source of both essential and non-essential amino acids. Ideally, dietary protein provides sufficient levels of essential amino acids to meet requirements, with enough left over to provide a nitrogen source for non-essential amino acids (Klasing, 2005). To ensure protein and amino acid requirements in dietary formulations are met, essential amino acids must be bioavailable. The most common method of determining protein requirements, and thus the bioavailability of protein contained within diets, is through response trials with increasing dietary levels of specific amino acids (Lessire *et al.*, 1982). This approach is impracticable for endangered species such as kiwi because it would require feeding diets with intentionally imbalanced quantities of essential nutrients. Priority should, thus, be given to monitoring the long term health of kiwi fed a diet with 53% protein. The 53% protein diet here falls within the mid-range of protein content (43-63%) of current captive diets (Potter *et al.*, 2010), indicating that kiwi can survive long term on a diet of this protein content.

Fat digestibility

The fat content of the diet fed to kiwi (15%) is similar to that consumed by anteaters (11-15% fat (Oyarzun *et al.*, 1996)) but lower than that fed to other insectivores, such as: bats (31% fat (Webb *et al.*, 1993)); phascogales (27% fat

(Stannard & Old, 2012)), and hedgehogs (29-41% fat (Graffam *et al.*, 1998)). The digestibility of fat in kiwi (91%) is lower than that reported for other insectivores, for example, hedgehogs fed a diet of 29-41% fat (fat digestibility = 97% (Graffam *et al.*, 1998)), bats fed a diet of 31% fat (fat digestibility = 98% (Webb *et al.*, 1993)), and kultarrs fed a 27% fat diet (fat digestibility = 98% (Stannard & Old, 2012)).

Apparent digestibility of fat on the near-natural diet was similar in both kiwi (91%) and roosters (94%). These results are consistent with digestibility values of fat in other studies using roosters (11% fat diet, 85% fat digestibility (Maisonnier *et al.*, 2001); 10% fat diet, 95% fat digestibility (Honda *et al.*, 2009)). Furthermore, the fat content of the diet does not affect the digestibility of fat in roosters; the digestibility of fat (85-95%) was similar in diets ranging from 3-10% fat (Honda *et al.*, 2009). Whether the digestibility of fat by kiwi varies with the fat content of the diet needs to be determined.

The apparent digestibility of fat by kiwi falls within the range of fat digestibilities (87-97%) of other non-insectivorous species fed diets with a variety of fat contents. Species with lower fat digestibilities than kiwi include ocelots fed an 11% fat diet (fat digestibility = 10% (Bennett *et al.*, 2009)) and pigs fed a 9% fat diet (fat digestibility = 87% (Wisker & Knudsen, 2003)). Species with higher fat digestibilities than kiwi include ostriches fed a 7% fat diet (fat digestibility = 93% (Angel, 1993)), rats fed a 9% fat diet (fat digestibility = 93% (Wisker & Knudsen, 2003)), polar foxes fed a 23% fat diet (fat digestibility = 96% (Burlikowska *et al.*, 2003)), mink, domestic dogs and blue foxes fed a 20% fat diet (fat digestibilities = 96%, 97% and 97%, respectively (Vhile *et al.*, 2005)), and domestic cats fed a 30% fat diet (fat digestibility = 97% (Vester *et al.*, 2010)).

Thus, it appears that kiwi not only consume a diet with a lower fat content but their apparent digestibility of fat is also lower than that of a range of other insectivores. The digestibility of fat by kiwi is within the range reported for a variety of carnivorous and omnivorous species of differing fat intakes.

Kiwi digest fat to a similar level as protein. As the diet of wild kiwi contains considerably more protein (59%) than fat (22%) (Potter *et al.*, 2010), protein appears to be the dominant energy source in kiwi.

Carbohydrate digestibility

Direct comparisons among published studies of the digestibility of carbohydrate are difficult because different studies focus on different forms of carbohydrate. Here, I reported on the digestibilities of neutral detergent fibre (NDF) comprising hemicellulose, cellulose, soluble sugars and lignin, and on acid detergent fibre (ADF) comprising cellulose and lignin. Chitin was almost certainly represented in what I call the lignin fraction.

The NDF (21%) and ADF (15%) content of the kiwi diet was within the range of values for insectivorous and herbivorous species (ranges are NDF = 20-39% (ostrich = 20% (Nizza & Di Meo, 2000), emu = 26% (Herd & Dawson, 1984), anteater = 30% (Oyarzun *et al.*, 1996), and hoatzin = 39% (Grajal, 1995); ADF = 3-19% (hedgehog = 3% (Graffam *et al.*, 1998), anteater = 3% (Oyarzun *et al.*, 1996), emu = 12% (Herd & Dawson, 1984), ostrich = 13% (Nizza & Di Meo, 2000), and hoatzin = 19% (Grajal, 1995)).

Apparent digestibilities of both NDF (33±2%) and ADF (26±3%) in kiwi were markedly higher than in the roosters (23±8% and 17±7%, respectively) fed a diet containing 6% NDF and 10% ADF. However, due to considerable variation in digestibilities among the roosters in this trial, a significant difference was not seen. Kiwi had significantly higher digestibility values of both hemicellulose (51%) and cellulose (28%) than the roosters (37% and 18%, respectively). The digestibility values obtained here are markedly higher than those reported elsewhere for roosters. For example, hemicellulose and cellulose digestibility values for Leghorn roosters are 9.2±14% and 0%, respectively (Coon *et al.*, 1990, dietary values not reported).

The apparent digestibility of NDF in kiwi (33%) was lower than that in a range of herbivorous and omnivorous species, including emu fed a 26% NDF diet (NDF digestibility = 45% (Herd & Dawson, 1984)), ostrich fed a 20% NDF diet, (NDF digestibility = 47% (Nizza & Di Meo, 2000)), hoatzin fed a 39% NDF diet (NDF digestibility = 71% (Grajal, 1995)). Thus, kiwi appear to be able to digest less of the NDF portion of the diet than species that consume similar or greater quantities of this carbohydrate.

Similarly, with the exception of emu (ADF digestibility of 18% when fed a 12% ADF diet (Herd & Dawson, 1984)), the apparent digestibility of ADF in kiwi (26%)

was lower than that in a range of insectivorous, herbivorous and omnivorous species, such as, hedgehogs fed a 3% ADF diet (ADF digestibility = 57% (Graffam *et al.*, 1998)), hoatzin fed a 19% ADF diet (ADF digestibility = 58%, (Grajal, 1995)), ostrich fed a 13% ADF diet (ADF digestibility = 40% (Nizza & Di Meo, 2000)).

Comparison of NDF and ADF digestibilities among species of different dietary habit has highlighted considerable variation in the ability of each species to utilise these carbohydrates. With the exception of roosters, the apparent digestibility of NDF and ADF in kiwi is generally lower than that in medium and large bodied avian species. While it would seem logical that strictly herbivorous species, such as hoatzin, are able to digest greater quantities of plant material than insectivorous or omnivorous species, the availability of digestible carbohydrate in the diet may be a contributing factor to efficiency of digestion. That is, differences in carbohydrate digestibility across species may be related to the nature of carbohydrate in the diet rather than to an ability to break down NDF and ADF (Van Soest, 1996). The proportion of different carbohydrates within animal forage differs depending on plant species (Knudsen, 1997). Furthermore, within the categories of carbohydrate, for example NDF and ADF, there can be significant differences in hemicellulose, cellulose and lignin content. For example, two diets can have similar carbohydrate contents, yet the ratio of relatively indigestible to digestible carbohydrate can differ greatly (Choct & Kocher, 2000; Edwards *et al.*, 2001). To definitively determine differences in the digestibility of carbohydrate among species, a digestibility trial using the same diet for each bird is needed. This study conducted such a trial on kiwi and roosters and found no significant difference in carbohydrate digestibilities. Further research is needed to determine whether the apparent digestibility of carbohydrate in kiwi is in fact relatively low compared to other avian species, or if the differences reported are a factor of variations in dietary carbohydrate content.

Effect of Dietary Energy Source

The main energy source of a diet, whether it be protein, fat or carbohydrate, affects metabolism differently across species. For example, in chickens, diets with a high fat content reduced *de novo* lipogenesis compared with diets high in carbohydrate (Yeh & Leveille, 1971), but in turkeys the source of energy did not affect energy acquisition (Plavnik *et al.*, 1997). In chickens, when protein is the main energy source,

feed intake and assimilation increase resulting in weight gain (Rosebrough *et al.*, 1999). How energy source affects metabolism in kiwi needs to be investigated.

Faecal output

Roosters (18-19 g dry matter) had a higher faecal output than kiwi (9-14 g dry matter). No comparative data exist on the faecal output of kiwi with which to compare these results. However, the faecal output of roosters in this study is consistent with that reported elsewhere for roosters that were fed similar quantities of food (Garaulet *et al.*, 2001) (intake = 93-95 g per day (dry matter), faecal matter = 27-28 g (dry matter)). Lower apparent digestibilities of neutral and acid detergent fibres, and lignin/chitin in roosters compared with kiwi may have contributed to the greater bulk of faecal material produced by roosters.

The role of plant material in the diet of kiwi, specifically its preferential or incidental ingestion, has long been debated (Gurr, 1952; Bull, 1959; Watt, 1971; Reid *et al.*, 1982; Colbourne & Powlesland, 1988; Shapiro, 2006). The frequent occurrence of fruit pips found in the excreta of kiwi (Reid *et al.*, 1982; Kleinpaste, 1990) suggests that fruit is an important component of their natural diet. However, the results from this study suggest that digestive efficiency of carbohydrate in kiwi is relatively low. This apparent contradiction in intake versus digestibility values raises the question of why kiwi consume plant material at all. It is conceivable that they ingest fruit for the energy gained from simple sugars (fructose, glucose) contained within the flesh, rather than from the complex carbohydrate contained within the skin or seed coating. While this study did not assess the digestibility of simple sugars in the near-natural diet, it needs to be determined.

Chitin

In this study, the apparent digestibility of chitin was indirectly estimated through lignin analyses. The apparent digestibility of lignin in kiwi (33%) was significantly higher than that in roosters (15%). Given that lignin is typically considered to be indigestible, and hence is often used as an indigestible marker (Buchsbaum *et al.*, 1986; Stein *et al.*, 2007), this result indicates that some fraction of what I have identified as lignin is some other digestible matter. The greater proportion of chitinous material (arthropods made up 60% of diet) than lignin (fruit made up 10% of diet) in the near-natural diet, suggests that the most likely candidate for this other substance is chitin.

While chitinolytic enzymes have been found in the chyme and the cell lining of the gizzard (Han *et al.*, 1997), and a reported chitin digestibility level from 20-50% (Han *et al.*, 2000) to 88% (Hirano *et al.*, 1990), chitin digestibility in chickens is still controversial (Khempaka *et al.*, 2006; Hossain & Blair, 2007). Chitinase activity has also been reported in seabirds (Jackson *et al.*, 1992), raptors (Akaki & Duke, 1999) and mammals (Allen, 1989). Given the presence of chitinase in poultry, and in other avian and non-avian species that consume a diet containing chitin, as well as the absence of a significant difference in chitin digestibilities between kiwi and roosters (lignin analyses from this study), it seems almost certain that both roosters and kiwi are able to digest chitin. However, the extent of this ability, particularly in kiwi, has yet to be established and needs to be investigated further by assaying specifically for chitin and chitinase.

Soil ingestion and micronutrients

Dietary ingredients were thoroughly washed before inclusion in the reconstituted diet; therefore tilth from soil was absent. It is likely that kiwi in the wild incidentally ingest a considerable amount of soil while foraging, adherent to or contained within the digestive tract of prey items. The amount of soil ingested by kiwi is not known; however omnivorous wild turkeys (*Meleagris gallopavo*) consume soil at a rate of 9% of their diet (Beyer *et al.*, 1994). As ingested soil may be the principal source of certain minerals (Beyer *et al.*, 1994), mineral shortages may be present in the diet. Therefore, animals fed diets that preclude soil ingestion may be predisposed to mineral deficiencies. Micronutrient analyses and digestibilities were not assessed in this study but should be investigated in future studies. It is advisable, therefore, that micronutrient supplements be included in captive diets to counter this.

The comparative approach to kiwi nutrition used in this study, hence the use of roosters to validate nutrient digestibility values for kiwi is of considerable value for future dietary formulations for this endangered species. However, despite the development of the first diet for captive kiwi based on the nutrient composition of the natural diet, it is neither practical nor feasible to collect and process the quantities of invertebrates and fruit needed for daily consumption. This factor is a considerable limitation to the practical use and commercial value of the near-natural diet. Therefore,

a diet with a similar nutrient composition to that of the near-natural diet, but composed of readily available ‘synthetic’ ingredients is needed.

CHAPTER 4

Formulation, digestibility and palatability of a synthetic diet for North Island brown kiwi (*Apteryx mantelli*)

Trial enclosure

Kiwi eating its usual diet

Synthetic diet

ABSTRACT

A synthetic diet was formulated for North Island brown kiwi (*Apteryx mantelli*) with a macronutrient composition (crude protein, fat, ash, gross energy, neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin) that is within five percent of the composition of a near-natural diet comprised of ingredients known to be consumed by kiwi in the wild. The ingredients used in the synthetic diet were readily available worldwide and the form of the diet was tailored specifically for kiwi; hence long, thin, moist, robust 'worm-like' lengths were produced. The apparent digestibility of macronutrients was higher in kiwi fed the synthetic compared with the near-natural diet. The synthetic diet was also fed to roosters (*Gallus gallus*) to allow comparison with a species of which considerably more is known of nutrient uptake and gut physiology. Roosters were also fed the synthetic diet to assess the possibility of using roosters as a model to predict digestibility outcomes in kiwi in future formulations of the captive diet. Thus apparent digestibility results obtained here were compared with those from roosters fed the near-natural diet. With the exception of gross energy, apparent digestibilities of macronutrients were significantly different in roosters fed the synthetic compared to the near-natural diet. Furthermore, the apparent digestibility of macronutrients was consistently higher in kiwi than roosters when fed the synthetic diet. Inconsistencies in macronutrient digestibilities in both kiwi and roosters fed the near-natural versus the synthetic diets preclude using roosters to predict macronutrient digestibilities of a diet fed to kiwi. For both species the source of the macronutrients in the diet appears to have a marked influence on apparent digestibility.

INTRODUCTION

Captive management has become a valuable tool in the conservation of endangered species. An important factor in maintaining species in captivity is the provision of daily nutrient requirements; failure to do so may compromise the health of the animal, and thus affect the success of breeding programmes. For example, the reproductive output of kiwi held in captivity is significantly lower than for kiwi in the wild (McLennan, 1996; Department of Conservation, 2004). A major contributing factor to this low reproductive success in captivity is thought to be an inadequate diet (Potter *et al.*, 2010). I have begun to address this issue by formulating a near-natural diet based on the nutrient composition of the natural diet and assessing its apparent digestibility (chapter 3). The apparent digestibility of most macronutrients was higher in kiwi than in roosters when fed the near-natural diet (chapter 3). Whether this holds true for all types of diets is not known.

In order to produce a diet that closely emulated the natural diet of kiwi, the near-natural diet was largely composed of wild-caught food items, many of which are difficult to obtain. Thus, as for many other species (Dierenfeld, 1997), the logistic challenges of sourcing large quantities of natural food items on an ongoing basis makes it impractical to feed a mainly natural diet to captive kiwi. Therefore, a synthetic diet that closely matches the nutrient composition and apparent digestibility of the near-natural, and thus natural, diet is needed. The synthetic diet also needs to be comprised of ingredients that are readily available everywhere that kiwi are held captive. Vertebrate protein and fat is much more readily available than invertebrate protein and fat. However, the form of some macronutrients in vertebrate-based ingredients are different to that contained within invertebrate-based ones (McHargue, 1917; De Foliart, 1991), thus a diet based on invertebrates, such as the near-natural diet, will have markedly different macronutrient sources than a synthetic, vertebrate-based diet. The effect of radically different sources of macronutrients on the apparent digestibility of the synthetic diet in kiwi must be investigated. Thus, digestibility trials are needed to determine if kiwi assimilate nutrients from widely different sources in a similar manner. If this is the case, then an additional digestibility trial using roosters will determine whether this phenomenon is specific to kiwi.

The purpose of this study was, first, to formulate a synthetic diet for captive North Island brown kiwi (*Apteryx mantelli*) based on the nutrient composition of the near-

natural diet; second, to compare the apparent digestibility of this diet in kiwi and roosters; and third, to compare the digestibility of the synthetic diet with that of the near-natural diet in kiwi and roosters.

METHODS

A synthetic diet for North Island brown kiwi (*Apteryx mantelli*) was formulated based on the nutrient composition of the natural (Kleinpaste, 1990; Potter *et al.*, 2010) and near-natural diets (chapter 3). Data on the macro and micro-nutrient composition (protein, fat, dry matter, ash, and carbohydrate, fatty acid and amino acid levels of a range of meats (beef heart and liver, chicken muscle), grains (wheat bran), oils (soybean, corn), seeds (sunflower, sesame) and fruits (raisins, apricots, apples) (all of human grade quality)) were sourced from the literature. These data were entered into a spreadsheet and proportions of ingredients adjusted in an effort to mimic the nutrient composition of the near-natural diet as described in chapter 3. Seventeen recipes were developed using this technique, and all consisted of nutrient compositions that fell within 10% of that of the near-natural diet. The proportion and specific items of fruit contained within in each recipe were identical. A hierarchical cluster analysis in SYSTAT 12 (Systat Software Inc, 2004) was used to compare the nutrient compositions of the 17 recipes with that of the near-natural diet. The four recipes that clustered closest to the near-natural diet were chosen for further nutrient adjustments, including both the 13:1 linoleic to linolenic acid ratio found in the near-natural diet, and the 2:1 calcium to phosphorus ratio that is recommended for avian diets (Robbins, 1993). Again, a hierarchical cluster analysis was performed on these recipes and the one clustered closest to that of the near-natural diet was selected as the diet to synthesise and feed experimentally to kiwi.

In a parallel (unpublished) study, a series of palatability trials were carried out to provide guidance on the form and odour of food suitable for kiwi. Experiments included presenting kiwi with food items of various shapes and odours, as well as the inclusion of a range of binding agents. These trials were observation-based and results were on an acceptance or avoidance basis.

The synthetic diet was homogenised, calcium carbonate powder added (at 1% of wet weight of diet) and mixed in an industrial food mixer for five minutes.

Synthetic diet was thoroughly mixed in this mixer

Maize starch was added (at 8% of wet weight of diet) to attain the required consistency. The diet was then forced through a 6 mm gauge sausage maker into a solution of 0.5% sodium alginate and water (immersed for one minute). The addition of these ingredients did not affect the macronutrient composition of the diet. The long worm-like pieces were cut into three centimetre lengths, weighed into plastic retort bags (150 g of diet per bag), and vacuumed sealed at 60 ºC, 140 MPa for 8 seconds. Diet was stored at -20 ºC pending digestibility trials.

The final synthetic diet

Four captive adult North Island brown kiwi (2 females and 2 males) that had been maintained in captivity at Westshore Wildlife Reserve, Napier, for a minimum of one year, and 12 adult male Hyline Brown roosters (*Gallus gallus*) were used in the trial. The feeding regime used in chapter 3 (Table 1) for the digestibility trials was again used here, except the synthetic diet (ingredients Table 1) was fed instead of the near-natural diet. Nutrient analyses were carried out on samples of the diet and faecal material as stated in chapter 3. In addition, fatty acid and amino acid (hydrochloric acid hydrolysis followed by HPLC separation (AOAC 994.12)) analyses were determined for the synthetic diet.

The percentage of indigestible marker (titanium dioxide (TiO2)) recovered in the faecal material was calculated by the Indicator Method:

(Total excreta out (g) * %TiO2 in excreta) / (Total diet intake (g) *%TiO2 in diet)

Excreta were pooled per bird over the five day digestibility trial for TiO2 analyses. A recovery of more than 95% of the TiO2 ingested was required for TiO2 to be used as an indigestible marker for apparent digestibility calculations; for values below this rate a Total Collection Method (Bourdillon *et al.*, 1990) was used as follows:

[(% Nutrient in diet * Total dry matter intake (g)) – (% Nutrient in excreta * Total dry matter in excreta (g))] / (% Nutrient in diet * Total dry matter intake (g))

In chapter 3, a correlation between dietary intake and faecal output was found at 12 h, thus digestibility was assessed by comparing dietary intake of the preceding 12 h with faecal output of the following 12 h. Faecal samples for the last five days of the digestibility trials were pooled per bird. Levels of uric acid nitrogen in the excreta were determined and removed from protein calculations (method given in chapter 3).

All animal procedures were approved by Massey University Animal Ethics (permit 07/17) and the Department of Conservation (ECHB-21465-RES, DOC File NHS-03-04-01).

RESULTS

Four recipes clustered closely to the near-natural kiwi diet and these were selected for further refinement of nutrient composition (Figure 1). Following these adjustments, the recipe clustered closest to the near-natural diet was chosen as the synthetic diet.

The components of the selected synthetic diet are presented in Table 1. The ash, protein, gross energy, fat, fatty acid and carbohydrate (including neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin) composition of the synthetic diet was within 5% of the near-natural diet (Table 2). In order to produce the required consistency, maize starch was added to the diet. This addition of starch had little effect on the carbohydrate content (NDF, ADF and lignin) of the diet. Comparisons of the mineral contents of the near-natural and synthetic diets are presented in Table 3. Calcium content was markedly higher in the synthetic than the near-natural diet. Potassium and iron levels were markedly lower in the synthetic compared with the near-natural diet.

The total percent of fatty acids in the synthetic diet (11%) was slightly lower than in the near-natural diet (15%) (Table 4). The four most dominant fatty acids (palmitic, stearic, oleic and linoleic) were similar in both diets, as were the linoleic to linolenic acid ratios.

Of the 19 amino acids detected in the synthetic diet, six were present in levels greater than 2 mg/100mg: glutamic acid (5.85 mg/100mg), leucine (4.20 mg/100mg), aspartic acid (3.81 mg/100mg), lysine (3.69 mg/100mg), alanine (2.44 mg/100mg) and arginine (2.38 mg/100mg) (Table 5). The levels of amino acids were similar in the near-natural and synthetic diets.

Body weight of kiwi and roosters did not change markedly during the digestibility trials (Table 6, Figures 2 and 3). The greatest change in weight (6%) was a 125g gain in a 2kg kiwi.

The percent recovery of titanium dioxide in the faecal material of kiwi was 22% and that of roosters was 123% when fed the synthetic diet (excreta were pooled per bird). The percent recovery of titanium dioxide (TiO2) in kiwi (22%) was below the 95% recovery limit required for use as an indigestible marker in apparent digestibility calculations (Table 7). The roosters appeared to acquire TiO2 during the digestibility trial as the percent recovery was greater than 100%. This unusual result for roosters, coupled with a low recovery for kiwi, meant that TiO2 was not effective as an

indigestible marker, thus apparent digestibilities were calculated using the Total Collection Method (Bourdillon *et al.*, 1990) (equation given in Methods).

Dietary intake of the synthetic diet varied significantly (n = 4; d.f. = 1, 3; F value = 9.34; P = 0.005) between kiwi (24.38 ±7.66 g to 57.84 ±5.88 g dry matter) but not between days. Dietary intake varied significantly (n = 12; d.f. = 1, 11; F value = 33.48; P = 0.0001) between roosters (34.87 ±0.31 g to 78.03 ±0.67 g dry matter) but not between days. Dietary intake of the synthetic diet varied significantly between kiwi and roosters (n = 12 (roosters), 4 (kiwi); d.f. = 1, 14; F value = 38.88; P = 0.0001). Roosters consumed a significantly greater amount of the synthetic diet than kiwi (68.59 ±1.31 g, 40.02 ±3.48 dry matter, respectively). The average dietary intake in roosters was 80%, and in kiwi 60%, of the synthetic diet offered (dry matter).

Dietary intake of the two diets was not significantly different in kiwi. However, roosters ate significantly (n = 12; d.f. = 1, 10; F value = 22.50; P = 0.0001) more of the synthetic (69.00 ±0.11, g dry matter) than the near-natural (35.40 g ±0.19) diet.

Faecal output on the synthetic diet varied significantly (n = 4; d.f. = 1, 3; F value = 13.55; P = 0.002) between kiwi (1.87 ±0.44 g to 7.97 ±0.56 g dry matter) but not between days. Faecal output on the synthetic diet was not significantly different between roosters or between days. Faecal output varied significantly between kiwi and roosters (n = 12 (roosters), 4 (kiwi); d.f. = 1, 14; F value = 27.03; P value = 0.0001) fed the synthetic diet. Faecal output was significantly greater in roosters (34.61 ±0.63 g) than kiwi (4.40 ±1.08 g dry matter). Significantly (n = 4 (synthetic diet), 6 (near-natural diet); d.f. = 1, 8; F value = 29.14; P = 0.0006) more excreta were produced by kiwi fed the near-natural (11.99 ±0.89 g) than the synthetic (4.40 ±1.09 g dry matter) diet. Conversely, significantly (n = 12 (synthetic diet), n = 4 (near-natural diet); d.f. = 1, 14; F value = 25.97; P = 0.0002) more excreta were produced by roosters fed the synthetic (34.61 ± 0.63 g) than the near-natural (18.07 ± 1.44 g dry matter) diet.

Apparent digestibilities of ash, organic matter, protein, fat, neutral detergent and acid detergent fibres, lignin and gross energy were significantly higher in kiwi fed the synthetic than the near-natural diet (Table 8).

Apparent digestibilities of ash, organic matter and neutral detergent fibre were significantly higher in roosters fed the synthetic than the near-natural diet (Table 9). The apparent digestibilities of protein, fat, acid detergent fibre and lignin/chitin were significantly lower in roosters fed the synthetic than the near-natural diet. No significant

difference was found in the apparent digestibilities of gross energy in roosters fed either the synthetic or near-natural diets.

Apparent digestibilities of all the macronutrients (ash, organic matter, protein, fat, neutral detergent and acid detergent fibres, lignin and gross energy) were significantly higher in kiwi than roosters when fed the synthetic diet (Table 10).

Figure 1. Comparison of the gross nutrients (crude protein, fat, ash, carbohydrate), fatty acids (including 13:1 linoleic to linolenic acid and 2:1 calcium to phosphorus ratios) and amino acids of seventeen formulated diets with that of the near-natural 'kiwi diet'.

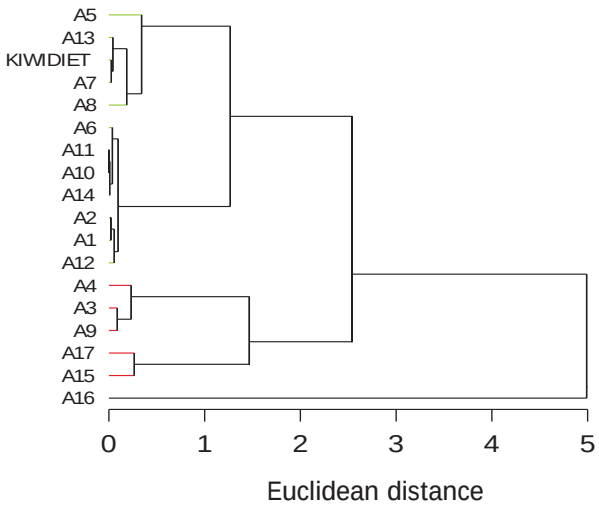


Table 1. Percent contribution of ingredients to the selected synthetic kiwi diet (on wet and dry matter bases).

Ingredient	% contribution to diet (wet matter basis)	% contribution to diet (dry matter basis)
Beef heart (fat cut off)	27	14
Beef liver	21	14
Chicken breast (fat cut off)	20	10
Soybean oil	3	7
Corn oil	3	6
Wheat bran	7	13
Seedless raisins	1	2
Dried raw apricots	1	2
Dried raw apples	1	1
Dried whole sesame seeds	1	1
Dried sunflower seed kernels	1	1
Calcium and phosphorus premix	1	1
Kiwi premix[1]	1	2
Limestone	2.5	6
Maize starch	8	17
Calcium carbonate	1	2
Sodium alginate	0.5	1
Total	100 %	100 %

[1] Kiwi premix (BoMix) sourced from Bomac Laboratories Ltd (Manukau City, NZ) (for ingredients see Appendix 2)

Table 2. Comparison of the nutrient composition of the near-natural and synthetic diets of North Island brown kiwi (g/kg, dry matter basis).

Diet	Ash	Crude protein	Gross energy (kJ/g)	Fat	Total fatty acids	Carbohy-drate[2]	Neutral detergent fibre	Acid detergent fibre	Lignin
Near-natural [1]	0.8	5.3	24	1.6	1.5	2.3	0.6	0.1	0.05
Synthetic	0.8	4.8	20	1.3	1.3	2.7	0.6	0.1	0.03

[1] Potter *et al.,* unpublished
[2] Calculated by difference

Table 3. Mineral content of the synthetic diet (g/100 g, dry matter basis).

Diet	Ca	Mg	K	Na	P	Fe	Cu	Mn	Zn
Near-natural [1]	0.24	0.19	1.25	0.55	0.70	0.10	0.002	0.009	0.01
Synthetic	1.80	0.10	0.69	0.35	0.60	0.03	0.003	0.007	0.01

[1] From Potter *et al.,* unpublished

Table 4. Comparison of the fatty acid content of the near-natural and synthetic diets (g/100 g, dry matter basis).

Fatty acid	Near-natural diet[1]	Synthetic diet
C6:0 Caproic	0.03	ND
C8:0 Caprylic	ND	ND
C10:0 Capric	<0.0001	ND
C11:0 Undecanoic	<0.0001	ND
C12:0 Lauric	0.06	ND
C13:0 Tridecanoic	0.02	ND
C14:0 Myristic	0.20	0.05
C14:1n5 - cis-9-Myristoleic	0.01	ND
C15:1n5 - cis-10-Pentadecenoic	ND	ND
C16:0 Palmitic	3.42	1.58
C16:1n7 - cis-9-Palmitoleic	0.41	0.18
C17:0 Margaric	0.06	0.07
C17:1n7 - cis-10-Heptadecenoic	0.02	ND
C18:0 Stearic	0.82	1.34
C18:1n9t Elaidic	0.03	0.01
C18:1n7t Vaccenic	ND	0.06
C18:1n9c Oleic	5.15	2.79
C18:1n7c Vaccenic	0.09	0.13
C18:2n6t Linolelaidic	ND	ND
C18:2n6c Linoleic	2.97	3.33
C20:0 Arachidic	0.06	0.03
C18:3n6 - cis-6,9,12-Gamma linolenic	0.02	0.02
C20:1n9 - cis-11-Eicosenoic	0.02	0.03
C18:3n3 - cis-9,12,15-Alpha linolenic	0.19	0.30
C21:0 Heneicosanoic	0.03	0.02
C20:2n6 - cis-11,14-Eicosadienoic	0.04	0.01
C22:0 Behenic	0.03	0.04
C20:3n6 - cis-8,11,14-Eicosatrienoic	0.03	0.06
C22:1n9 - cis-13-Erucic	ND	ND
C20:3n3 - cis-11,14,17-Eicosatrienoic	ND	ND
C20:4n6 - cis-5,8,11,14-Arachidonic	ND	0.01
C23:0 Tricosanoic	0.11	0.21
C22:2n6 - cis-13,16-Docosadienoic	ND	0.03
C24:0 Lignoceric	ND	0.02
C20:5n3 - cis-5,8,11,14,17-Epa	0.12	0.11
C24:1n9 - cis-15- Nervonic	ND	0.02
C22:5n3 - cis-7,10,13,16,19-DPA	ND	0.09
C22:6n3 - cis-4,7,10,13,16,19-DHA	ND	0.04
% Total fatty acid in diet	14.96	10.57

[1] Potter *et al.,* unpublished

ND = not detected

Table 5. Comparison of the amino acid content of the near-natural and synthetic diets (mg/100 mg, dry matter basis).

Amino acid	Near-natural diet[1]	Synthetic diet
Aspartic acid	4.34	3.81
Threonine	1.91	1.66
Serine	2.13	1.40
Glutamic acid	5.51	5.85
Proline	2.74	1.68
Glycine	2.55	1.75
Alanine	3.71	2.44
Valine	2.88	1.92
Isoleucine	2.01	1.61
Leucine	3.30	4.20
Tyrosine	2.48	1.26
Phenylalanine	1.80	1.69
Histidine	2.01	1.20
Lysine	2.89	3.69
Arginine	2.93	2.38
Taurine	0.11	0.04
Cysteine*	0.45	0.55
Taurine*		0.06
Methionine*	0.84	1.21

[1] From Potter *et al*., unpublished

* From performic acid oxidation

Table 6. Body weight of kiwi and roosters on day 1 of the digestibility trial of the synthetic diet (see chapter 3 for weights when fed the near-natural diet).

Species	Bird	Body weight (kg)
Kiwi	1	2.3
	2	2.2
	3	2.0
	4	1.4
Rooster	1	2.6
	2	2.4
	3	2.6
	4	2.3
	5	2.3
	6	2.5
	7	2.5
	8	2.4
	9	2.3
	10	2.6
	11	2.6
	12	2.4

Figure 2. Percent change in body weight from day 1 of the digestibility trial of kiwi fed the synthetic diet.

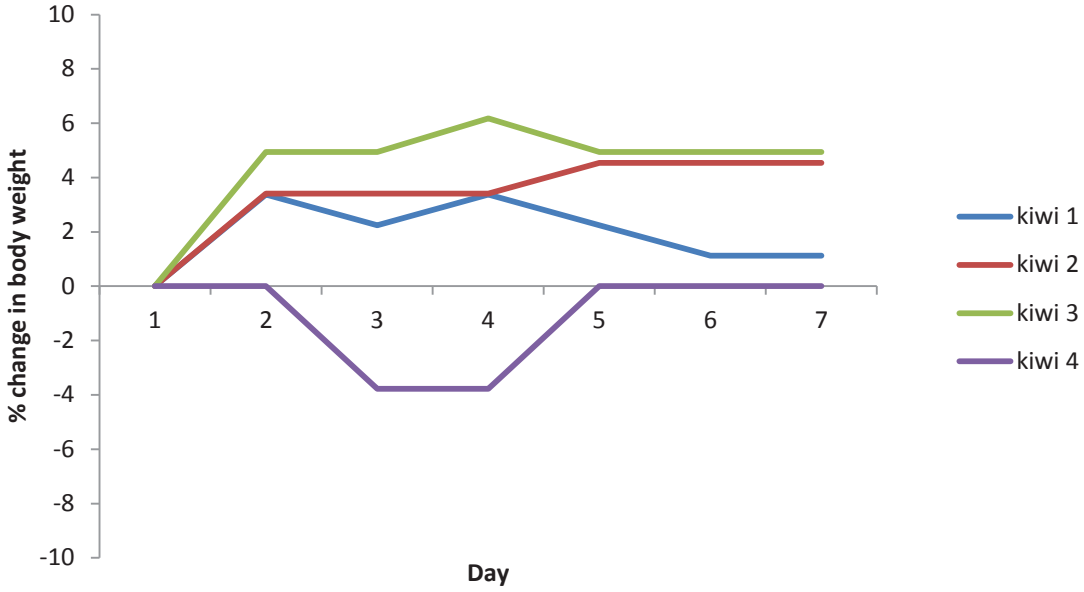


Figure 3. Percent change in body weight from day 1 of the digestibility trial of roosters fed the synthetic diet.

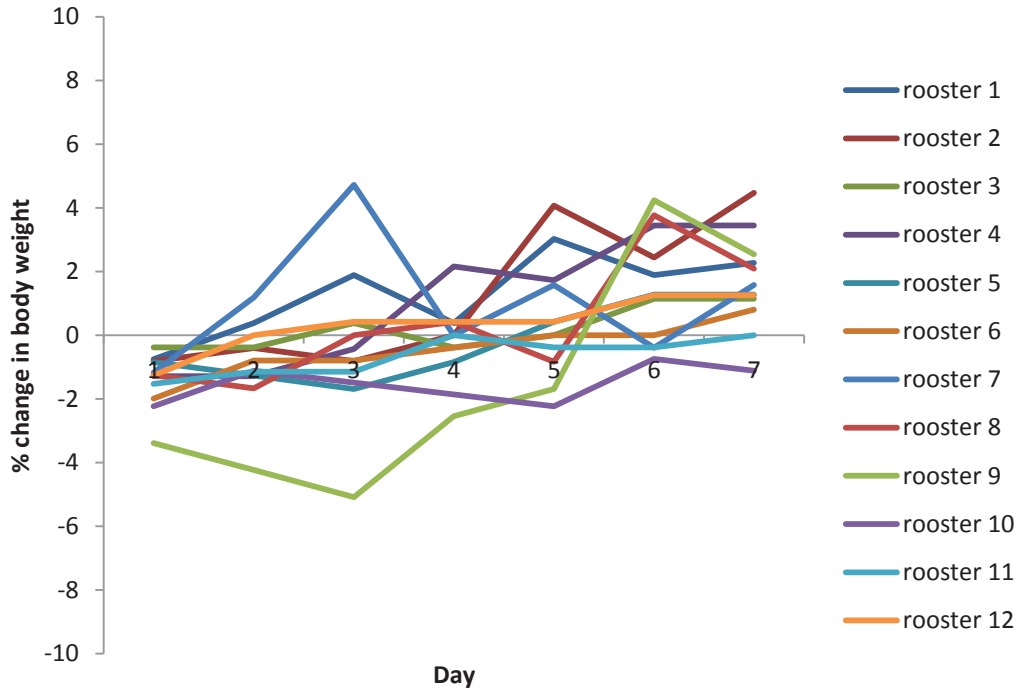


Table 7. Comparison of the percent recovery of the indigestible marker titanium dioxide in faecal material from roosters and kiwi fed the synthetic diet.

Species	Titanium dioxide recovered (%)
Rooster (n=12)	123
Kiwi (n=4)	22

Table 8. Analysis of variance of the apparent digestibility of macronutrients in kiwi fed either the near-natural or synthetic diets (%).

	Diet (Mean ± SE)			
Nutrient	**Near-natural[1]**	**Synthetic**	**F value**	**P value**
Organic matter	69 ± 2	96 ± 1	21.57	**
Ash	39 ± 4	73 ± 3	42.40	***
Protein	91 ± 1	98 ± 1	56.81	***
Fat	91 ± 1	98 ± 1	74.32	***
Neutral detergent fibre	33 ± 2	86 ± 2	19.95	**
Acid detergent fibre	26 ± 3	69 ± 10	25.65	**
Hemicellulose	51 ± 3	88 ± 2	17.94	***
Cellulose	28 ± 2	70 ± 14	32.70	***
Lignin	33 ± 4	61 ± 5	19.75	**
Gross energy	75 ± 1	94 ± 1	22.76	**

[1]Data from chapter 3
n=6 (near-natural diet); n=4 (synthetic diet). d.f.= 1, 8
**=P<0.01; ***=P<0.001

Table 9. Analysis of variance of the apparent digestibility of macronutrients in roosters fed either the near-natural or synthetic diets (%).

	Diet (Mean ± SE)			
Nutrient	**Near-natural[2]**	**Synthetic**	**F value**	**P value**
Organic matter	51 ± 4	65 ± 1	22.56	***
Ash	17 ± 6	55 ± 2	17.80	***
Protein	88 ± 1	78 ± 1	22.12	***
Fat	94 ± 1	85 ± 2	9.47	**
Neutral detergent fibre	23 ± 5	34 ± 2	9.22	**
Acid detergent fibre	17 ± 5	-51 ± 6	37.03	***
Hemicellulose	37 ± 6	49 ± 1	8.36	**
Cellulose	18 ± 4	-47 ± 11	33.18	***
Lignin/chitin[1]	15 ± 6	-84 ± 9	38.46	***
Gross energy	65 ± 2	69 ± 1	2.85	NS

[1]Chitin contained within ingredients of invertebrate origin
[2]Data from chapter 3
n=4 (near-natural diet); n=12 (synthetic diet). d.f.= 1,14
**=P<0.01; ***=P<0.001; NS=P>0.05

Table 10. Analysis of variance of the percent apparent digestibility of the synthetic diet by kiwi and roosters (%).

Nutrient	Mean ± SE	F value	P value
Organic matter	Kiwi = 96 ± 1 Rooster = 65 ± 1	27.03	***
Ash	Kiwi = 73 ± 3 Rooster = 55 ± 2	24.04	***
Protein	Kiwi = 98 ± 1 Rooster = 78 ± 1	23.45	***
Fat	Kiwi = 98 ± 1 Rooster = 85 ± 2	19.70	***
Neutral detergent fibre	Kiwi = 86 ± 2 Rooster = 34 ± 2	22.21	***
Acid detergent fibre	Kiwi = 69 ± 10 Rooster = -51 ± 6	20.83	***
Hemicellulose	Kiwi = 88 ± 2 Rooster = 49 ± 1	17.94	***
Cellulose	Kiwi = 70 ± 14 Rooster = -47 ± 11	32.70	***
Lignin	Kiwi = 61 ± 5 Rooster = -84 ± 9	18.90	***
Gross energy	Kiwi = 94 ± 1 Rooster = 69 ± 1	24.55	***

n=4 (kiwi), n=12 (roosters). d.f.= 1, 14
***=P<0.001; NS=P>0.05

DISCUSSION

A synthetic diet was formulated for North Island brown kiwi based on the nutrient composition of the near-natural diet (discussed in chapter 3). The macronutrient composition of this diet fell within five percent of the composition of the near-natural diet for crude protein, fat, ash, gross energy, neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin. Feeding trials using this diet provide the first determination of macronutrient digestibility for kiwi fed a wholly synthetic diet, allowing comparison of the digestibility of this diet with that of the near-natural diet that was comprised of quite different sources of fat and protein, i.e. vertebrate meat versus whole-bodied invertebrates.

As in chapter 3, the digestibility trials were conducted on both kiwi and roosters, thus facilitating both comparison with an extensively studied species and assessment of the validity of the kiwi results. While comparisons of apparent digestibilities between these two species are informative, poultry have been highly selected to maximise growth on minimal food intake, meaning that their ability to assimilate nutrients exceeds anything we would likely see in wild birds. Hence, caution needs to be taken when comparing apparent digestibilities of kiwi and roosters.

One of the aims of this chapter was to assess whether there is a consistent relationship between macronutrient digestibility in roosters and kiwi that could enable new kiwi diets to be tested first on roosters and results modelled to predict outcomes in kiwi. However, this study found that dietary ingredients affect kiwi and roosters differently in the manner in which they digest a diet. Thus, this type of modelling of digestibilities in kiwi and roosters was not feasible.

The discussion that follows looks first at the underlying experimental approach, including dietary composition, dietary intake, faecal output and the recovery of the indigestible marker titanium dioxide. Second the similarities and differences in apparent digestibilities between the synthetic and near-natural diets in kiwi and roosters are discussed.

Composition of near-natural vs. synthetic diet

The synthetic diet was formulated to provide a similar macronutrient composition as the near-natural diet but there were differences in mineral composition. Calcium content (1.8 g/100 g) was higher, and potassium (0.69 g/100 g) and iron (0.03

g/100 g) lower, in the synthetic diet than in the near-natural (0.24 g/100 g, 1.25 g/100 g and 0.10 g/100 g respectively (all units on a dry matter basis)). In terms of calcium, broilers require about 0.87 g/100 g (Vieira *et al.*, 2010) in their diet. All animals are thought to require 0.6-1.0 g/100 g of iron in their diet (Dorrestein *et al.*, 2000). The calcium content of the synthetic diet was markedly higher and iron content lower than these guidelines. High variation in the trace mineral content of commonly used ingredients, including those from the same source (Bao & Choct, 2009), make it difficult to formulate a diet with exact and consistent mineral values. Ingredients with consistent mineral content are needed to predict dietary mineral composition. In addition, the source of mineral, for example, organic or inorganic, has an impact on digestibility. In general, organic minerals that can be added to the diet in a chelated form have a higher bioavailability than inorganic minerals (Nollet *et al.*, 2007), i.e. being chemically bound to other compounds allows them to be more easily broken down and available for digestion.

The lack of tilth in the near-natural diet, due to washing ingredients prior to inclusion in the diet, may result in mineral deficiencies (see chapter 3). Furthermore, farmed worms were used in the near-natural diet, thus their digestive tracts were free of mineral-rich soil. Thus, the mineral content of the near-natural diet is likely to be lower than what wild kiwi actually ingest, but at this stage our estimates are the best available. The National Research Council has published recommended mineral intakes for poultry, however these recommendations are based on research carried out over 40 years ago (Leeson, 2005) and are now considered relatively inaccurate (Pierce *et al.*, 2009). Given these issues, it is difficult to determine the required mineral intake for kiwi. Ideally, trials to determine minimum and maximum mineral requirements would be conducted. However, these trials involve feeding diets that are both deficient in, and in excess of, mineral requirements. Such trials might adversely affect the health of kiwi and it is doubtful that they would be permitted given kiwi's threatened status. Further research is clearly required on this issue and the health of captive kiwi should be monitored carefully in case mineral deficiencies occur.

Dietary intake and output

Dietary intake of the synthetic diet was significantly greater in the roosters than in the kiwi (80% and 60% of diet offered, respectively). Direct observation of the birds

during the trial suggested that the roosters found the diet more palatable than the kiwi did. There may have been an element of neophobia in this; this possibility is discussed further in chapter 5. A significantly lower dietary intake by kiwi, as well as a higher apparent digestibility, meant that a lower faecal output was anticipated for kiwi than roosters, yet body weight did not vary markedly from pre-trial weights for either species.

Recovery of indigestible markers

In chapter 3 I found that the percent recovery of titanium dioxide in both kiwi (61%) and roosters (71%) fed a wholly invertebrate and fruit based diet was lower than values reported for roosters (range 97-100%; (Peddie *et al.*, 1982; Short *et al.*, 1996; Crespo & Esteve-Garcia, 2001; Myers *et al.*, 2004)). To determine if this low rate of recovery was due to the composition of the diet I compared the recovery of titanium dioxide in kiwi and roosters fed a wholly synthetic diet with that when fed the near-natural diet. Surprisingly, the recovery rate of titanium dioxide in kiwi was lower (22%) and in roosters higher (123%) than values obtained for the near-natural diet. In chapter 3 I argued that as kiwi retain dense, particulate matter in the caeca it was likely that the unrecovered titanium dioxide from the diet was retained in these chambers. A similar phenomenon is likely to have occurred with kiwi in this trial, but as the diet was more processed and digestible than the near-natural diet, a greater proportion of the indigestible marker might have been retained in the caeca. The greater than 100% recovery of titanium dioxide in the faecal material from roosters is puzzling. In chapter 3 I found that the amount of titanium dioxide in the excreta peaked on day one and remained relatively constant for the remainder of the trial, thus in the current trial using the synthetic diet I pooled the excreta per bird, resulting in a single value per bird for titanium dioxide recovery. This method meant that I was not able to determine if the recovery of the indigestible marker was constant over the trial period, or if a high value from a single day affected the results. Alternatively, this effect may have resulted from laboratory error during analysis or from the consumption of a diet in which the titanium dioxide might not have been mixed sufficiently. However, the likelihood of these issues occurring for each sample and/or bird is unlikely.

The unreliable rates of recovery of titanium dioxide meant that this indigestible marker was deemed unsuitable for digestibility trials. Therefore, digestibility trials were conducted using the Total Collection Method. (Bourdillon *et al.*, 1990).

Non-cross over trials

The experimental designs used in the digestibility trials here and in the previous chapter were intended to simulate a cross-over trial but the logistics of running both trials simultaneously on both kiwi and roosters on the same premises precluded a complete cross-over trial. Hence, the trials were not run in the same facilities nor concurrently and they varied in enclosure design, ambient temperature and season. A sample of the trial diet was analysed at the start of each trial to ensure nutrient composition remained constant over time. Different kiwi and roosters were also used in the two trials. Otherwise, the trials were as similar as possible.

Kiwi

Near-natural vs. synthetic diet

The apparent digestibilities of all macronutrients were higher in kiwi fed the synthetic than the near-natural diet, including ash, organic matter, fat, protein, gross energy, NDF, ADF and lignin. While the macronutrient composition of both diets was similar, the ingredients contained within each diet differed. These differences may have affected the digestibility of the diets. Amino acid ratios differ in some insects compared to that of beef and turkey meat (McHargue, 1917). The ratio of polyunsaturated fatty acids, such as linoleic and linolenic acids, is higher in insects than in poultry and fish (De Foliart, 1991). Furthermore, the carbohydrate content of the synthetic diet included considerable quantities of processed material compared to the whole invertebrates and fruit (some seeds were removed) included in the near-natural diet. Thus, variations in the micronutrient composition of the protein, fat and carbohydrate content of the near-natural compared with the synthetic diet may have led to significant differences in apparent digestibilities. A higher digestibility of fat by the kiwi fed the synthetic diet compared with the near-natural diet suggests a higher acquisition of energy on the synthetic diet. Greater efficiency of fat digestion and energy acquisition in kiwi fed the synthetic diet highlights the potential for over feeding and thus obesity problems for captive birds. Furthermore, reduced activity levels of kiwi held in captivity compared to

their wild counterparts may contribute to weight-related health issues as it does in other captive animals (Dierenfeld, 1997). Captive kiwi should be weighed regularly in order to detect changes in bodyweight and meal portions altered accordingly.

A significantly higher apparent digestibility of NDF was seen when kiwi were fed the synthetic diet compared with the near-natural diet. Excluding sugars, NDF is the most digestible component of the carbohydrate fraction of the diet (Gehman *et al.*, 2008). Wheat bran, fruit and seeds contained within the synthetic diet appeared to be more easily digested by kiwi than the fruit contained within the near-natural diet. This result is not surprising given that the carbohydrate fraction of the synthetic diet was processed to a higher degree; for example, the synthetic diet contained seedless raisins and apricots, seedless and skinless apples, highly processed bran and huskless sunflower seeds, compared to the whole fruit contained within the near-natural diet.

The higher apparent digestibility of both ADF and lignin in the synthetic than in the near-natural diet was not anticipated. In chapter 3 I argue that the relatively high digestibility of lignin was likely to be due to chitin contained within the lignin portion of the near-natural diet, a substance that was not distinguished from lignin in our analyses but which kiwi appear to be able to digest. However, the synthetic diet does not contain the invertebrates that provided dietary chitin in the near-natural diet, thus the lignin portion of the synthetic diet was thought to be indigestible. The reason for the high apparent digestibility of lignin by kiwi fed the synthetic diet remains unknown and should be investigated further.

Roosters

Near-natural vs. synthetic diet

Apparent digestibilities of organic matter, ash and NDF were higher, and of fat, protein, ADF and lignin were lower, in roosters fed the synthetic compared to the near-natural diets. ADF and lignin did not appear to be digested at all by roosters. The apparent digestibility of gross energy did not differ between diets. These results were not expected. The ingredients contained in the synthetic diet are not commonly fed to roosters, but they are more similar to components of standard poultry diets than the ingredients in the near-natural diet. Thus, it was expected that the ingredients of the synthetic diet were of a form more easily digestible by roosters than the natural ingredients contained within the near-natural diet. The higher digestibility of NDF in

roosters fed the synthetic compared with the near-natural diet is consistent with that of a diet containing a greater proportion of highly processed plant material. The lower apparent digestibility of fat and protein by roosters fed the synthetic than the near-natural diet was surprising. It appears that roosters are more able to break down the protein and fat contained within invertebrate matter than that contained within vertebrate matter. Given a lower digestibility of both protein and fat in roosters fed the synthetic diet, it was expected that gross energy values would reflect these results. However, acquisition of gross energy did not differ between diets. The higher digestibility of NDF in roosters fed the synthetic compared to the near-natural diet was not sufficient to account for the energy lost from the lower digestibilities of protein and fat. Thus, while both the near-natural and synthetic diets provided roosters with a similar gross energy content, the ingredients within each diet affected the overall macronutrient digestibility of the diets.

Synthetic diet – kiwi vs. roosters

Apparent digestibilities of all macronutrients were higher in kiwi than in roosters fed the synthetic diet. These results differ from those obtained when kiwi and roosters were fed a diet of similar nutrient composition but composed of less processed dietary ingredients (chapter 3). Thus, it appears that the composition of food items contained within a diet affects the way these species digest a diet. Surprisingly, significantly lower digestibility values for roosters fed the synthetic diet did not markedly affect body weights during the trial. Despite lower intake and a higher digestibility of macronutrients, the body weights of kiwi also remained constant throughout the trials. Therefore, lower intake and higher digestibility appear to have balanced each other out, thus not affected body weight.

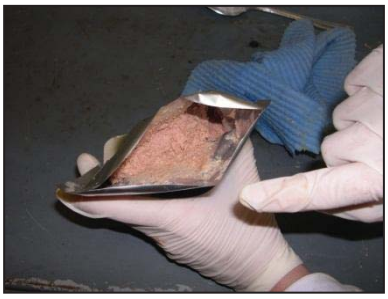
CHAPTER 5

Challenges in the production of a synthetic diet for kiwi

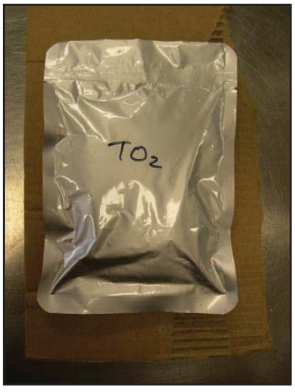
Mixer

Retort machine

Sealer

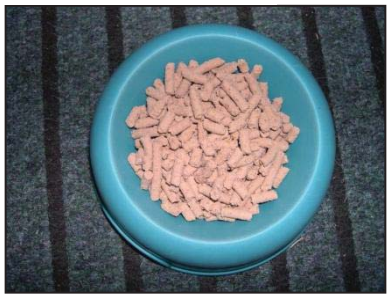


One formulation in a retort bag



Packaged synthetic diet

Formulations of the synthetic diet



Final synthetic diet

Palatable at last!

In the previous chapter I formulated and determined the digestibility of the first synthetic diet formulated to mirror the nutrient composition of what kiwi eat in the wild. In doing so, I encountered several challenges including producing a diet that kiwi find palatable and with physical properties that enable kiwi to pick it up, kiwi neophobia of new diets, maintenance of sterility of the diet, and how to package it for ease of use. These issues are discussed below starting with consideration of why I cannot simply feed kiwi an appropriate wild diet.

A synthetic diet

Ideally, once the natural diet of a species has been determined, this same diet would be fed to captive animals (Kirk Baer *et al.*, 2010). However, some animals consume dietary items that are difficult to source; this is especially true for kiwi. Most of the insect adults and larvae, worms, and fruit that kiwi consume in the wild are difficult to collect in bulk, so I was left with the reality of needing to feed them a diet containing readily available food items. Kiwi are not unique in this respect, as a variety of captive-held species present similar challenges (Dierenfeld, 1997). Furthermore, the food offered to captive animals generally differs from items in their natural diet and has different characteristics of odour, texture and taste (Yo *et al.*, 1997). Thus, the challenge of formulating a diet for captive animals is to construct a nutritionally adequate diet that is also palatable. The approach I took was to adjust combinations of ingredients until I had formulated a diet that when homogenised was of a similar nutrient composition to that of the near-natural diet (chapter 4). A homogenous diet is important in order to provide a consistent nutrient intake and prevent selection of preferred food items. The issue of palatability was addressed by formulating a diet with an odour and texture acceptable to kiwi (discussed below).

Consideration of feeding strategy

The manner in which an animal locates a food source is an important aspect to consider when formulating a diet. North Island Brown Kiwi (*Apteryx mantelli*) use two strategies to forage: olfaction and remote sensing (Cunningham *et al.*, 2007). Their sense of smell is enhanced by an extensively developed olfactory chamber and olfactory bulb (Bang, 1971), and a highly specialised sensory organ at the tip of their bill which allows them to detect vibro-tactile signals from soil-dwelling prey (Cunningham *et al.*, 2009). Of these two foraging strategies, smell is the primary method of food detection

in kiwi (Cunningham *et al.*, 2009). So the odour of the food is important when constructing a diet for captive kiwi.

Equally important in the formulation of a diet for captive animals is the physical form of the food. Typically, kiwi pick up a food item using the tip of their bill, shake it from side to side, throw it in the air and swallow it (Minson, personal observation). Thus, any diet that is too mushy or sticky, too runny, too dry, or consists of very large or very small items may reduce palatability.

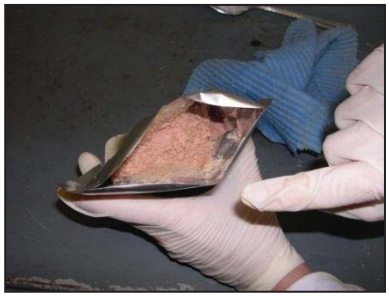
Palatability

There are a variety of definitions for palatability (Kissileff, 1990; Yeomans, 1998); here I consider three aspects to be vitally important. First whether kiwi find the odour attractive enough to motivate them to ingest the food. Second, whether kiwi can physically manipulate the food to get it in their mouth. Third, when in the mouth, whether the taste and texture of the food are acceptable.

Thus, palatability is a combination of taste, texture and smell. Taste was a difficult aspect to assess within the limitations of this study but it appeared that once I had addressed the first and second issues it was rare for kiwi to reject food once it was in their mouth. Further work could address the issue of taste. Thus the following discussion focuses on developing a diet with an odour and texture acceptable to kiwi.

Dietary odour

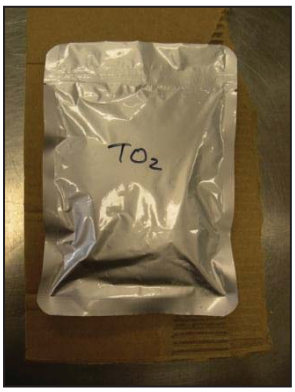
The synthetic diet was firstly sterilised/cooked and then presented to kiwi in their usual feed bowls as a number of rectangular pieces of food (20 x 5 x 5 mm).



Diet in pouches ready for sterilisation

Retort Machine: pouches of diet were sterilised by heat and pressure

Sealer: pouches of diet were heat and pressure sealed



The diet once sterilised

All kiwi went straight to the bowls and sniffed the new diet but few birds attempted to eat it. This initial willingness to feed then refusal following smelling the food suggested a problem with dietary odour. In order to determine whether a single ingredient was responsible for this inappetence, I presented the kiwi with four variations of the diet: without liver; without chicken; without fruit; and without either liver or chicken. The odour of cooked liver is strong, thus it was thought that this ingredient might have been the major issue.

The diet without liver

Again, having initially sniffed the food the kiwi refused to eat any of the formulations. As the removal of various ingredients didn't affect acceptance by kiwi I decided to mask the smell of the diet.

Typically, studies conducted on the effect of flavours or odours have focussed on bait attractants and deterrents for pest species. Flavours that have been added to baits to attract exotic mammalian pests in New Zealand, such as possums, include cinnamon, aniseed, almond, banana, clove, eucalyptus, raspberry and rose (Reid, 1986). In regards to kiwi, little is known about their response to different smells and flavours. Cinnamon was added to the usual diet of captive kiwi in an attempt to deter them from feeding,

however no change in intake was observed (McLennan *et al.*, 1992). In a later study, kiwi showed neither preference nor aversion to non-toxic baits flavoured with cinnamon or orange (Ward-Smith & Potter, 1999). Given these conflicting results on the effect of smell on intake, I used a range of flavours to encourage ingestion of the synthetic diet. Dilute concentrations of raspberry or caramel essence, vegemite, oxheart blood, or chicken, beef or garlic stock were sprayed onto the raw diet immediately before feeding. The birds would not eat any of the options, so I sprayed the same flavours onto samples of their usual diet to see if the odours would repel them. The kiwi ate all the samples of their usual diet despite the smell, and probably flavour, being considerably different. These results suggested that the added odours did not mask the smell of an unappetising diet.

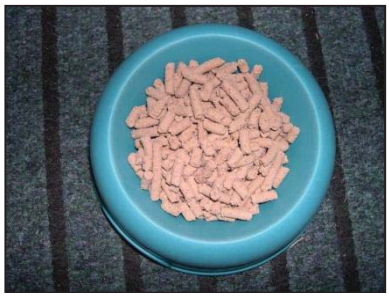
The complete refusal by kiwi to even attempt to consume the synthetic diet with or without added odours meant I had to rethink the whole process, that is, to consider the methods that weren't successful and try a different approach.

The process of sterilisation of the diet (discussed below) resulted in it being cooked and thus the odour of the ingredients, particularly the meat, was changed. To avoid the intense smell of the diet once sterilised, I decided not to sterilise it but rather to freeze it immediately after homogenization, and thaw it prior to use. It was anticipated that this technique of freezing the diet would not only reduce bacterial growth and spoilage, but also reduce the odour of the diet and encourage consumption. While synthesis of this diet was relatively straight forward, the process of homogenisation resulted in a diet with a porridge-like consistency that kiwi could neither pick up nor manipulate. To overcome this problem of consistency, gelatine was added to the diet in various concentrations. As gelatine has a high protein content, and may affect the nutrient composition of the diet, I added the minimum amount required to bind the ingredients. The gelatine coated the food in a smooth jelly-like layer and resulted in a firm, moist product that was readily cut into pieces of a size and shape typical of current captive dietary items. This technique enabled kiwi to easily pick up the food, however a strong smell was emitted from the gelatine and acceptance was low.

The diet coated with gelatine

Thus, kiwi found the physical form of the food was acceptable but the binding agent appeared unsuitable due to its odour. So the next step was to find a method that produced firm items of food without affecting the smell of the diet.

Dietary texture



The diet after extrusion – it appears to be an ideal form of food but is too sticky

Ideally, an extruder would be used to make the diet as it moulds food into various shapes and consistencies by adding water and/or heat immediately before the food exits the machine. Furthermore, a binding agent can easily be added if necessary. However, dry food is normally used as wet products are difficult to feed into the machine and exit as a sticky mess, thus not suitable for kiwi. Following the failure of the extruder to handle a wet diet, the only option appeared to be to use a Filler fitted with a narrow (7 mm diameter) tube. This hand-operated sausage machine produced lengths of 'worm-like' pieces. To ensure the outer surface of each piece was both firm and moist, sodium alginate (1% of wet weight) was added to the mixture and strands of 'worms' were immersed in a calcium carbonate water bath (0.5%) as they exited the Filler. Sodium alginate is commonly used as a thickening agent (Hong & Chin, 2010). Calcium ions form cross-links with the sodium alginate to bind the food and to form a non-odorous, gelatinous-type coating on the product (Butler *et al.*, 2006). This process created a strong, rubbery substance that remained moist on the inside and superficially resembled

earthworms.

The final synthetic diet

Palatable at last!

Kiwi were more accepting of this form of food than they had been for any of the previous formulations of the diet, but two out of six still showed signs of neophobia when first presented with it.

Neophobia

It is not uncommon for captive animals habituated to one diet to be hesitant to consume a new diet. Neophobic reactions to food vary among taxa (Addessi *et al.*, 2007), populations (Martin & Fitzgerald, 2005) and individuals (Visalberghi *et al.*, 2002). From a captive management perspective it is easier to have one constant diet per species (Morgan & Tromborg, 2007). A lack of variety, however, may incite neophobia when diets are modified. Even the occasional change of a portion of the diet for animals that naturally consume a range of items, for example, in-season fruit or vegetables, may be insufficient to diminish habituation. Furthermore, the movement of animals among captive facilities, for breeding, research or advocacy purposes, can often be hampered by reluctance to adjust to a new diet.

This problem is potentially fatal if captive birds that are released into the wild are not familiar with a natural diet. Brown teal (*Anas chlorotis*) released into the wild from captive institutions have been found starved to death, even when they have been observed to feed on supplementary food (Moore & Battley, 2003). Thus, even though these birds were receiving some supplementary food, they were not able to forage sufficiently in their natural environment to survive. Changes in the morphology of the digestive tract can also be caused by differences between captive and wild diets (Moore & Battley, 2006), so careful consideration has to be given to preconditioning birds destined for release into the wild. This is of direct relevance to kiwi, where a lack of

familiarity of foraging for wild food items for birds raised in captivity and released into the wild can lead to marked declines in body weight that may persist for four to five months (Grant, 2003).

Levels of neophobia can be reduced by introducing novel foods in familiar environments (Burritt & Provenza, 1997) or by adding a desirable colour, fragrance or taste. For example, high sugar content in novel foods significantly reduces neophobic responses in male rhesus macaques (Johnson, 2007). However, due to the effect on nutrient composition, the addition of comparable quantities of sugar is not appropriate for a kiwi diet. While a palatable product is wanted, one that encourages animals to gorge themselves is not desirable. In the wild, animals spend a large part of their day, and expend considerable energy, foraging for food. In contrast, in captivity food is often fed in the same location and the captive animal can consume the meal within minutes and with little expenditure in energy (Grant, 2003). Given the widespread problems with obesity in captive animals (Schwitzer & Kaumanns, 2001; Speakman, 2007; Bellisari, 2008), this issue needs to be addressed in management protocols.

Through the process of formulating a synthetic diet for captive kiwi I have found them to be neophobic towards novel foods. To investigate the level of neophobia in kiwi I put their usual diet through the sausage machine (Filler) and presented it to them. Again they were hesitant to eat it, showing that their neophobia extends from novel smells to novel shapes of food.

To increase acceptance of the novel diet, kiwi were initially fed a mixture of their usual and new diet, with a proportion of the usual diet being reduced through time (for quantities see Table 1, chapter3). There was individual variation in the acceptance of a new diet. Three weeks was sufficient time for some birds to fully adapt to a new diet, while others will need longer and may waiver between acceptance and refusal. This is a gradual process in which intake and body weight should be monitored closely, if necessary the proportion of usual diet added to the new diet should be adjusted until acceptance is achieved. Another approach of introducing a new diet to neophobic animals is to begin the process when they are young.

Early habituation to diet

Early habituation to a new diet may solve some of the problems encountered with kiwi and palatability. Newly-hatched wild chicks survive on nutrients absorbed from

their yolk sac for the first few days of life (Calder, 1979; Prinzinger & Dietz, 2002), after which they begin to forage. It is at this point that captive chicks are presented with the captive diet, the only food available to them. Newly hatched chicks have been observed to consume the synthetic diet formulated in this study (personal observation, Minson, 2010). If kiwi are habituated to this diet from an early age, palatability should cease to be a problem. However, the diet formulated here was developed specifically as a maintenance diet for adult kiwi. In other species, breeding females, incubating males, and growing chicks, typically require a different balance of nutrients to non-breeding adults (Martin, 1987; Krebs, 2001; Mitrus *et al.*, 2010). Diets should be developed for each specific stage but currently captive kiwi are fed a constant diet throughout their life. Thus the synthetic diet might be suitable for all stages, but this needs to be confirmed.

Sterility of the diet

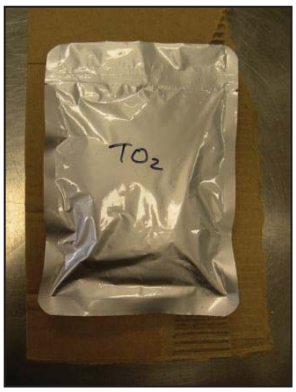
Formulated diets should ideally have a long shelf life; hence sterility of the product is an important consideration. Sterilisation is important for food products as it reduces bacterial and viral contamination and prolongs shelf life, however, it can also affect nutrient availability and digestibility by releasing some nutrients and destroying others. For example, the vitamin C content of fruit and vegetables is reduced following thermal treatment as heat increases the oxidation rate of ascorbic acid (Gregory, 1996), whereas the content of bioaccessible lycopene (a carotenoid and anti-oxidant) is increased after thermal processing (Dewanto *et al.*, 2002). Heat treatment can also affect the nutritive value of protein through changes in amino acid composition. Losses of cystine, lysine, arginine, threonine and increases in glycine have been reported following heat treatments (Papadopoulos, 1989). The effect of sterilisation techniques on the nutrient composition of a diet is difficult to predict and is dependent upon temperature and pressure values of treatments. Changes in the nutritive value of a diet following sterilisation treatment needs to be assessed, at a minimum, through analyses of the nutrient composition of the diet pre and post treatment, and preferably, in conjunction with digestibility trials.

A number of sterilisation processes were investigated for this study, including irradiation, heat, pressure, and a combination of heat and pressure. While irradiation seemed to be the easiest option, as the diet is sent away and returned sterile, there were issues with cost and perceptions about the acceptability of feeding an endangered

animal irradiated food. Therefore, I was left with the options of heat treatment, pressure treatment, or a combination of the two. The literature indicates that a combination of pressure and heat is required to kill bacterial spores (Patterson, 2005) while pressure is the most effective method to kill viruses (Grove *et al.*, 2006). In general, sterilisation involves heating the food to a high temperature for a short period or to a lower temperature for a prolonged period. The most pressure-resistant bacterial spores involved in food contamination and spoilage are *Clostridium botulinum* and *Bacillus amyloliquefaciens* (Margosch *et al.*, 2006). Thus, these were the species I aimed to kill with heat and pressure treatments. Following a review of the literature, a temperature of 121ºC and a pressure of 100kPa (50kPa overpressure) for 10 minutes (Margosch *et al.*, 2006) was deemed an appropriate combination to sterilise the diet. The diet was packaged in 150 gram portions in foil retort bags prior to sterilisation. The retort bags proved not to be suitable for such high temperatures and some bags leaked (discussed below). Microbiological tests for aerobic and anaerobic bacteria showed the compromised packages to have higher counts of colony-forming units than recommended by the International Commission on Microbiological Specifications for Foods (ICMSF) (McWilliams, 2008). Food in packages that passed sterility testing was fed to kiwi to assess palatability. The outcome was that kiwi refused to eat any of the sterilised formulations. Consequently, all subsequent trials proceeded using freezing as the sole method of preserving diets.

Packaging issues

Generally food sterilised to high temperatures and pressure is packaged in cans, as they are able to withstand intense temperature and pressure better than plastic or foil-type packaging. However, due to the ultimate form of food to be presented to kiwi – a flat-surfaced product - canned food was not suitable. Thus, a range of the most heat resistant plastic and foil retort bags commercially available were tested for durability in both the retort machine and the heat-aided vacuum sealer.



Sterilised and sealed pouch

Leakage was the main problem, both of nutrients out of and water into the bags. Furthermore, inspection of the sterilised product showed a lack of isostatic pressure and heat treatment; some parts were more 'cooked' then others. There are food technology solutions to this problem but the time required to pursue them was beyond the scope of this study. For the purpose of the trials conducted here the product was stored in these bags, heat sealed and frozen until required.

This study has highlighted challenges associated with developing and packaging a new diet that kiwi will eat. Worm-like pieces of food proved to be readily picked up and consumed by kiwi but due to neophobia the initial acceptance of this diet varied across birds. The level of neophobia varies widely between birds (Jenkins, 2001), and it does not appear to be a function of age, sex or time spent in captivity (personal observation, Minson). Captive diets for kiwi currently vary across institutes in composition (Potter *et al.*, 2010), and consequently also in odour and physical properties, so kiwi can learn to consume diets that vary in smell and texture. The length of time an animal has been fed a diet, and is thus habituated to a particular scent, will affect the ease at which a new diet can be introduced.

A gradual introduction of the synthetic diet, mixed with the normal diet, is recommended. For some birds, the acceptance of a new diet may take months and may initially waiver between partial and complete acceptance, therefore patience (by staff) in changing kiwi from one diet to another is advised.

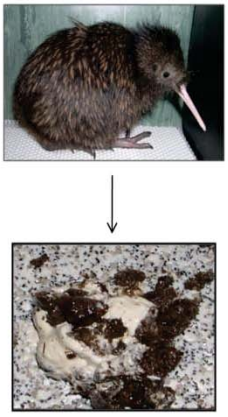
It is recommended that medium term studies be conducted on kiwi before the synthetic diet is released for commercial use. Close monitoring of the health and fecundity of the birds should be carried out during these trials. This is particularly important given the relatively high energy acquisition of kiwi on the synthetic diet and

the potential for obesity-related health problems. Once the validity of this diet is confirmed, further refinements can be achieved by incremental steps.

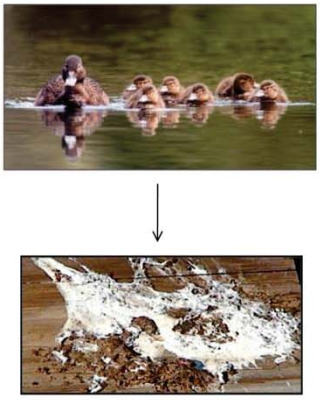
The synthetic diet was formulated as a maintenance diet for adult kiwi and further trials are required to assess the specific needs of growing juveniles and breeding adults.

CHAPTER 6

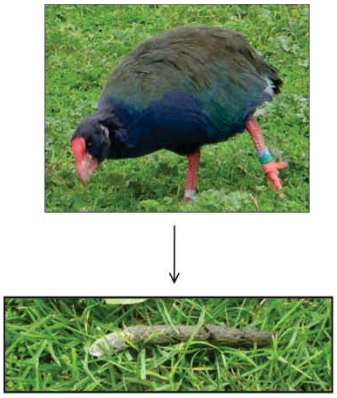
Enteral bacterial communities differ in captive and wild birds: implications for captive management as a conservation tool



North Island brown kiwi (*Apteryx mantelli*)



Brown teal (*Anas chlorotis*)



Takahe (*Porphyrio* [Notornis] *mantelli*)

A kiwi shaped kiwi poo!

Kiwi at Westshore Wildlife Reserve

Takahe on Mana Island

Brown teal on Tiritirimatangi

ABSTRACT

I compared the generic diversity of enteral microflora from three species of New Zealand birds with differing diet and ecology: the North Island brown kiwi (*Apteryx mantelli*); the brown teal (*Anas chlorotis*); and the takahe (*Porphyrio* [Notornis] *mantelli*) using Denaturing Gradient Gel Electrophoresis of faecal DNA with a generic primer. I found that the generic diversity of enteral microflora increased in captive compared with wild birds in the three species that I examined here, but not with diet. However, these differences were not associated with differences in diversity or evenness of enteral bacterial communities.

INTRODUCTION

The digestive tracts of vertebrates house amongst the most complex and diverse microbial communities known (Round & Mazmanian, 2009). Enteral microflora make a significant contribution to health and are considered 'essential' for life (Hooper *et al.*, 2002). They contribute to many physiological processes, degrade a number of non-digestible dietary components (Hooper *et al.*, 2002), and influence nutrient partitioning, such as lipid metabolism (Mulder *et al.*, 2009) and energy storage (Backhed *et al.*, 2005). They also influence intestinal immune development, cell repair (Hooper & Macpherson, 2010), and immune responses to pathogenic organisms (Round & Mazmanian, 2009).

It has become increasingly apparent that deviation from a suitable suite of enteral microflora, termed 'dysbiosis' (Neish, 2009), can predispose the host to a range of diseases, including chronic intestinal inflammation (Lepage *et al.*, 2008). Given the influence of enteral microbiota on health, it is important to acquire an appropriate suite of microflora (Qin *et al.*, 2010).

Mammalian young commonly acquire enteral microflora from the birth canal, rectum and breast (Adlerberth *et al.*, 2006; Kelly *et al.*, 2007). Due to the nature of their eggs, avian species are unable to directly acquire organisms by these routes. However, it is thought that newly hatched chicks may acquire enteral microflora indirectly during the breaching of the eggshell. Faecal contamination of the nest and communal areas with excreta from adult birds of the same species may provide innocula during the post-hatch period (Schneitz, 2005). Disruption of these processes is known to have a detrimental effect on the development of the avian immune system (Ouwehand *et al.*, 2002).

The chain of bacterial acquisition by avian young is particularly vulnerable to interruption when eggs of wild or captive species are removed from the nest for artificial incubation and rearing. The establishment of dysbiotic microflora in wholly captive species can be promoted by repeated cycles of incubation, hatching and rearing. Hence the passage of enteral microflora from generation to generation is interrupted. Captive rearing in overcrowded conditions may influence the acquisition of enteral microflora in avian species through psychological and physiological stress (Hawrelak & Myers, 2004) and through acquisition of inappropriate microflora from other species present in the captivity facility. These factors might be particularly problematic for

species of a solitary habit. Diet may promote greater enteral bacterial diversity in captive than occurs in wild birds, when nutrient content is more diverse and enteral niches are consequently expanded (Santos, A. A. J. *et al.*, 2008).

The diversity of enteral microflora has been widely studied in humans (Magalhaes *et al.*, 2007; Sokol *et al.*, 2008; Round & Mazmanian, 2009) and farmed livestock (Leser *et al.*, 2000; Zoetendal *et al.*, 2004; Metzler & Mosenthin, 2008) but has been little studied in wild animals, particularly non-mammalian species. Also, how being held captive versus living wild affects enteral microbial communities has been little studied. Given that captive management is often necessary for the conservation of threatened species and that successful species recovery requires the production of healthy animals, it is important to develop strategies promoting health in captive birds; for example, by the manipulation of enteral microbial communities to avoid dysbiosis.

Whilst the species distribution that constitutes dysbiosis has not been precisely defined, it is reasonable to assume that a loss of generic diversity would be significant and indicate likely dysbiosis.

Here I detail the results of a study of the generic enteral microbial diversity in three avian species, from both captive and wild environments, that differ in diet and social structure. All three species are classified as endangered (IUCN, 2010) and captive management plays an important part in conservation efforts (O'Connor *et al.*, 2007; Holzapfel *et al.*, 2008; Wickes *et al.*, 2009).

The three species were: the North Island brown kiwi (*Apteryx mantelli*), a flightless, nocturnal, primarily insectivorous, solitary species that inhabits mainly native forests; the brown teal (*Anas chlorotis*), a volant, diurnal, omnivorous, reputedly gregarious species of duck that prefers riparian habitats; and the takahe (*Porphyrio* [Notornis] *mantelli*), a flightless, diurnal, herbivorous, solitary species with a reputedly alpine grassland habitat (Higgins *et al.*, 2006).

METHODS

Faecal sample collection

Kiwi

Fresh, moist faecal samples were collected from adult wild kiwi in the Murchison Mountains (Fiordland), Moehau Wildlife Sanctuary (Coromandel) and Ponui Island (Hauraki Gulf), and from adult captive kiwi in Rainbow Springs Nature Park (Rotorua), Otorohanga Kiwi House (Otorohanga), Kiwi Birdlife Park (Queenstown), Auckland Zoo (Auckland), Willowbank (Christchurch), and Westshore Wildlife Reserve (Napier).

Brown Teal

Fresh, moist faecal samples were collected from adult wild brown teal from Egmont National Park (Taranaki) and from adult captive brown teal in Victoria Esplanade (Palmerston North), Brown Teal Conservation Trust (Carterton) and Peacock Springs Wildlife Park (Christchurch).

Takahe

Fresh, moist faecal samples were collected from adult wild takahe from Mana Island (Kapiti Coast) and from adult captive takahe in Te Anau Wildlife Centre (Te Anau).

Following collection all samples were immediately placed in a sealed, sterilised container to prevent cross contamination. Samples were frozen as practicable (within 24 h) and stored at -20^{o}C pending analysis.

DNA extraction and purification (removal of PCR inhibitors)

Bacterial DNA was extracted from 200 mg aliquots (wet weight) of each faecal sample according to the QIA amp® DNA Stool Minikit methods (Biolab- Cat # 51504, Qiagen, Valencia, CA, USA). The extracted DNA was cleaned according to the PowerClean^{TM} DNA Clean-Up Kit methods (Geneworks- Cat # 12877-50) and stored at -20^{o}C (for maximum of two weeks) pending analysis. Yield was checked by absorbance at 260/280 UV with a nanophotometer (Implen - IMP B-80-3004-31) according to Walter *et al.* (2000).

PCR Amplification

Extracted DNA was amplified using Universal Primers U968-GC-f (5′ CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC) and L1401-r (5′ CGG TGT GTA CAA GAC CC) (Marquardt, 1983; Burbidge *et al.*, 2003) which amplified V6 to V8 regions of bacterial 16S rRNA. The 3'end of the antisense primers each contained a 40 base pair G+C- rich sequence (Colbourne *et al.*, 2005). The generic primer used here identified bacteria to genus level. More specific primers that identify DNA to species-level are available for known bacterial species, but the lack of data on the enteral bacterial communities of the species analysed here meant that a primer able to target a wide range of bacterial taxa was needed.

PCR amplification was conducted in a Thermal iCycler (Bio Rad Cat # 170-8720) on 5 µl subsamples of each aliquot with a Go Taq® colourless Master Mix from Promega (Cat # M7121, Promega Go, Madison, WI, USA). Initial denaturation was conducted at 94^{o}C for 15 min, followed by 35 consecutive denaturation cycles each at 94^{o}C for 30 sec, with prior annealing at 57^{o}C for 1 min. Extension was conducted at 72^{o}C for 30 sec, with the final extension at 72^{o}C for 7 min. The post PCR concentrations of DNA were quantified by Nanophotometry (Implen - IMP B-80-3004-31). This process allowed appropriate volume adjustment to secure loading of DGGE slots with equal amounts of DNA.

Denaturing Gradient Gel Electrophoresis (DGGE)

The products of PCR were each subjected to DGGE according to the procedure outlined by Muyzer *et al.* (1993) and the Decode system manual (Bio-Rad Laboratories, Hercules, CA, USA) which enables determination of enteral bacterial community diversity (Hill *et al.*, 2008).

Gels were based on a 6% (vol/vol) solution of polyacrylamide (ratio of acrylamide- bisacrylamide 37.5:1) in 0.5x Tris-acetate-EDTA (pH 8.0) (TAE) buffer. The denaturing gradient was prepared using a solution of 6M urea and 55% formamide in the 6% polyacrylamide solution. Standardised gradient gels with denaturant gradients from 22- 55% formamide were prepared using a Gradient Delivery System (Model 475) (Bio Rad, Hercules, CA, USA) and a pump with an output of 5 ml per min. DGGE was run at 130V for 5h in 1.75x TAE buffer at a constant temperature of 60 °C in a

DCode™ System (Bio Rad, Hercules, CA, USA). The gels were subsequently stained with ethidium bromide (1:10,000) and scanned on DNR MiniBis PRO System (Biolab).

The number and intensity of bands in the 16 plates on each gel were quantified using Total Lab software (Phoretics 1D Advanced, Non-linear Dynamics, Newcastle, UK). The RF positions (i.e. the degree of migration from the origin) of the bands on each gel were standardised with reference to bands on a reference ladder containing DNA from *Lactobacillus* spp., *Staphyloccoccus* spp. and *Escherichia coli*.

The volume of amplified DNA, quantified as peaks on the graph, was determined by Gaussian fit using a rolling ball baseline and converted to percentage of total peak area per slot. Hence the percentages of total peak area were related to the relative proportions of the organisms in the sample as quantified by 16S DNA extractate.

Data processing

Total numbers of bands, i.e. bacterial genera, were determined by simple band counts. Indices of diversity, i.e. Shannon Weiner Diversity Index and evenness were determined on the basis of peak area. The Shannon Weiner Diversity Index is a measure of heterogeneity in a population and takes into account the degree of evenness in species/genera abundance (Magurran, 2004). The evenness index is based on the Shannon Weiner Diversity Index and is the ratio of observed diversity (H') to maximum diversity (H'max = all species have equal abundances) (Magurran, 2004). In addition to these commonly reported indices, I also report the number of bands of height greater than 50% of the largest peak expressed as a percentage of the total number of bands (Dominant Peak Index (DPI)). This index indicates the proportion of dominant genera present in a sample and provides more sensitive assessment of band dominance than does the evenness index. Only samples with a total of five or more bands were included in the DPI.

The indices were defined as (Aksoy *et al.*, 2006):

Shannon Weiner Index (H') = $-\sum P_i (\ln P_i)$

Evenness = H'/H'max

H' max = ln *s*

P_i is the percent Gaussian Volume (GV) of each genera.

s is the number of genera

N is the number of individuals identified (total GV per sample)

DPI = number of bands of height greater than 50% of the largest peak/total number of bands

Statistical Analysis

The distribution of band number, Shannon Weiner Diversity Index, evenness and the Dominant Peak Index were each assessed for normality using the Johnson algorhythm facility in the Minitab™ 15.1.0.0 software. Where necessary, data were transformed using the Johnson's algorithm.

Data were analysed using Analyses of Variance (ANOVA) (General Linear Model) in SAS 9.13 (SAS, 2004). Significance was taken to be $P<0.05$. Unless specified otherwise, results in the text are expressed as mean ± standard error.

I did not attempt overall dendrographic analysis as mean run distance varied significantly between gels. Visual assessment of band distributions within and between gels showed no obvious distribution by genus or captivity status.

RESULTS

Comparison of the enteral microfloral communities of captive and wild North Island brown kiwi

Captive kiwi had significantly greater numbers and diversity of enteral bacterial genera than wild kiwi but the evenness of the enteral bacterial community did not differ between these groups (Table 1).

Comparison of the enteral microfloral communities of captive North Island brown kiwi at four different captive institutions

No significant differences were found in the number of enteral bacterial genera present, evenness, DPI or diversity of enteral bacterial communities among kiwi from four captive institutions (Table 2).

Comparison of the enteral microfloral communities of wild North Island brown kiwi from three widely separated locations around New Zealand

No significant differences were found in the number of enteral bacterial genera, evenness or diversity of the enteral microfloral community of wild kiwi sourced from three locations (Table 3). There were insufficient data to conduct analyses on DPI.

Comparison of the enteral microfloral communities of captive and wild takahe

Captive takahe had significantly greater numbers and diversity of enteral bacterial genera than did wild takahe but neither the evenness nor the DPI index differed significantly between these groups (Table 4).

Comparison of the enteral microfloral communities of captive and wild brown teal

No significant differences were found between the enteral bacterial communities of captive and wild brown teal (Table 5).

Comparison of the enteral microfloral communities among captive and wild North Island brown kiwi, takahe and brown teal

No significant differences were found in the number of enteral bacterial genera present or in the diversity of these communities (as determined by community evenness, DPI and Shannon Weiner Indices) among kiwi, takahe or brown teal when captive and wild samples were combined per species (Table 6). When captive birds from all species were combined and compared with wild birds from all species, the enteral microfloral

communities in captive birds contained significantly greater numbers and higher diversity than did those of wild birds (Table 6).

Comparison of the enteral microfloral communities among captive North Island brown kiwi, takahe and brown teal

No significant differences were found in the number of enteral bacterial genera present or in the diversity of these communities among captive kiwi, captive takahe or captive brown teal (Table 7).

Comparison of the enteral microfloral communities among wild North Island brown kiwi, takahe and brown teal

No significant differences were found in the number of enteral bacterial genera present or in the diversity of these communities (as determined by community evenness, DPI and Shannon Weiner Indices) among wild kiwi, wild takahe or wild brown teal (Table 8).

Table 1. Comparison of the faecal bacterial communities (assessed by DGGE analyses) as an index of enteral bacterial communities of captive and wild North Island brown kiwi.

Analysis type[1]	F[3]	P	Mean ± SE (Data back transformed where necessary)
Number of Bands	4.08	0.050	captive 15.79 ± 1.52 wild 9.86 ± 2.51
DPI[2]	1.73	0.204	captive 17.78 ± 4.17 wild 23.00 ± 3.19
Evenness	0.29	0.600	captive 0.83 ± 0.02 wild 0.85 ± 0.04
Shannon Weiner Index	4.04	0.050	captive 2.16 ± 0.12 wild 1.69 ± 0.20

[1] Sample sizes: captive = 18; wild = 7
[2] Sample sizes for DPI: captive = 18; wild = 4
[3] Degrees of Freedom = 1, 23 for all analyses, except DPI (d.f. = 1, 20)

Table 2. Comparison of the faecal bacterial communities (assessed by DGGE analyses) as an index of enteral bacterial communities of captive North Island brown kiwi at four captive institutions around New Zealand.

Analysis type[1]	F[3]	P	Mean ± SE (Data back transformed where necessary)
Number of Bands	0.76	0.534	Otorohanga = 15.00 ± 3.97 Rainbow Springs = 10.67 ± 3.97 Willowbank = 16.33 ± 3.97 Westshore = 17.40 ± 2.17
DPI[2]	0.48	0.702	Otorohanga = 34.00 ± 23.3 Rainbow Springs = 13.50 ± 8.50 Willowbank = 14.33 ± 9.35 Westshore = 14.80 ± 2.24
Evenness	2.07	0.148	Otorohanga = 0.90 ± 0.04 Rainbow Springs = 0.86 ± 0.04 Willowbank = 0.77 ± 0.04 Westshore = 0.81 ± 0.02
Shannon Weiner Index	1.18	0.352	Otorohanga = 2.24 ± 0.27 Rainbow Springs = 1.75 ± 0.27 Willowbank = 2.02 ± 0.27 Westshore = 2.29 ± 0.15

[1] Sample sizes: Otorohanga = 3; Rainbow Springs = 3; Willowbank = 3; Westshore = 10
[2] Sample sizes for DPI: Otorohanga = 3; Rainbow Springs = 2; Willowbank = 3; Westshore =10
[3] Degrees of Freedom = 1, 17 for all analyses, except DPI (d.f. = 3, 14).

Table 3. Comparison of the faecal bacterial communities (assessed by DGGE analyses) as an index of enteral bacterial communities of wild North Island brown kiwi from three locations around New Zealand.

Analysis type[1]	F[2]	P	Mean ± SE (Data back transformed where necessary)
Number of Bands	F = 0.87	P = 0.485	Moehau = 8.00 ± 3.77 Murchison = 15.00 ± 4.62 Ponui = 7.5 ± 4.61
Evenness	F = 0.54	P = 0.620	Moehau = 0.80 ± 0.62 Murchison = 0.87 ± 0.08 Ponui = 0.89 ± 0.08
Shannon Weiner Index	F = 1.69	P = 0.294	Moehau = 1.38 ± 0.34 Murchison = 2.33 ± 0.41 Ponui = 1.53 ± 0.41

[1] Sample sizes: Moehau = 3; Murchison Mountains = 2; Ponui Island = 2
[2] Degrees of Freedom = 2, 4 for all analyses.

Table 4. Comparison of the faecal bacterial communities (assessed by DGGE analyses) as an index of enteral bacterial communities of captive and wild takahe.

Analysis type[1]	F[3]	P	Mean ± SE (Data back transformed where necessary)
Number of Bands	F = 12.87	P = 0.001	captive 16.00 ± 0.91 wild 10.00 ± 2.68
DPI[2]	F = 1.42	P = 0.287	captive 13.02 ± 3.27 wild 21.18 ± 6.75
Evenness	F = 0.69	P = 0.439	captive 0.82 ± 0.02 wild 0.62 ± 0.25
Shannon Weiner Index	F = 5.62	P = 0.050	captive 2.28 ± 0.06 wild 1.69 ± 0.36

[1] Sample sizes: captive = 4; wild = 4
[2] Sample sizes for DPI: captive = 4; wild = 3
[3] Degrees of Freedom = 1, 6 for all analyses, except DPI (d.f. = 1, 5).

Table 5. Comparison of the faecal bacterial communities (assessed by DGGE analyses) as an index of enteral bacterial communities of captive and wild brown teal.

Analysis type[1]	F[3]	P	Mean ± SE (Data back transformed where necessary)
Number of Bands	0.26	0.619	captive 12.54 ± 1.63 wild 11.14 ± 2.22
DPI[2]	1.55	0.233	captive 22.72 ± 3.21 wild 31.59 ± 8.18
Evenness	0.01	0.906	captive 0.81 ± 0.03 wild 0.81 ± 0.04
Shannon Weiner Index	0.65	0.432	captive 1.96 ± 0.13 wild 1.79 ± 0.17

[1] Sample sizes: captive = 13; wild = 7
[2] Sample sizes for DPI: captive = 12; wild = 5
[3] Degrees of Freedom = 1, 18 for all analyses, except DPI (d.f. = 1, 15)

Table 6. Comparison of the faecal bacterial communities (assessed by DGGE analyses) as an index of enteral bacterial communities of captive and wild North Island brown kiwi combined, versus captive and wild takahe combined, versus captive and wild brown teal combined, and of captive species (North Island brown kiwi, takahe and brown teal) versus wild species (North Island brown kiwi, takahe and brown teal).

Comparisons	Analysis type	$F^{5,6}$	P	Mean ± SE (Data back transformed where necessary)
combined species (captive + wild)[1]	Number of Bands	0.10	0.908	Kiwi = 14.20 ± 1.44 Takahe = 13.00 ± 1.73 Brown teal = 12.05 ± 1.29
	DPI[2]	2.18	0.125	Kiwi = 18.23 ± 3.52 Takahe = 16.52 ± 3.50 Brown teal = 25.33 ± 3.31
	Evenness	0.38	0.687	Kiwi = 0.84 ± 0.02 Takahe = 0.96 ± 0.30 Brown teal = 0.81 ± 0.02
	Shannon Weiner Index	0.13	0.880	Kiwi = 2.04 ± 0.11 Takahe = 1.99 ± 0.20 Brown teal = 1.92 ± 0.10
captive vs. wild species[3]	Number of Bands	5.89	0.019	captive = 14.64 ± 1.00 wild = 10.39 ± 1.42
	DPI[4]	3.47	0.07	captive = 18.64 ± 2.56 wild = 26.12 ± 3.89
	Evenness	0.53	0.47	captive = 0.83 ± 0.01 wild = 0.89 ± 0.13
	Shannon Weiner Index	6.79	0.012	captive = 2.11 ± 0.08 wild = 1.75 ± 0.12

[1] Sample sizes for combined species: kiwi = 25; takahe = 8; brown teal = 20

[2] Samples sizes for DPI for combined species: kiwi = 22; takahe = 16, brown teal = 7

[3] Sample sizes for captive vs. wild: captive kiwi = 18; captive takahe = 4; captive brown teal = 13; wild kiwi = 7; wild takahe = 4; wild brown teal = 7

[4] Sample sizes for DPI: captive: kiwi = 18; takahe = 4; brown teal = 13 and for wild: kiwi = 4; takahe = 4; brown teal = 3

[5] Degrees of Freedom for combined species = 2, 50 for all analyses, except DPI (d.f. = 2, 45)

[6] Degrees of Freedom for captive vs. wild = 1, 51 for all analyses, except DPI (d.f. = 1, 44)

Table 7. Comparison of the faecal bacterial communities (assessed by DGGE analyses) as an index of enteral bacterial communities of captive North Island brown kiwi, takahe and brown teal.

Analysis type[1]	F[3]	P	Mean ± SE (Data back transformed where necessary)
Number of Bands	1.17	0.324	Kiwi = 15.89 ± 1.49 Takahe = 16.00 ± 3.15 Brown teal = 12.54 ± 1.75
DPI[2]	1.98	0.156	Kiwi = 17.17 ± 4.24 Takahe = 13.02 ± 3.27 Brown teal = 22.72 ± 3.21
Evenness	0.25	0.780	Kiwi = 0.83 ± 0.02 Takahe = 0.82 ± 0.04 Brown teal = 0.81 ± 0.02
Shannon Weiner Index	0.89	0.422	Kiwi = 2.17 ± 0.11 Takahe = 2.28 ± 0.06 Brown teal = 1.96 ± 0.15

[1] Sample sizes: kiwi = 18; takahe = 4; brown teal = 13
[2] Sample sizes for DPI: kiwi = 18; takahe = 4; brown teal = 12
[3] Degrees of Freedom = 2, 32 for all analyses, except DPI (d.f. = 2, 31)

Table 8. Comparison of the faecal bacterial communities (assessed by DGGE analyses) as an index of enteral bacterial communities of wild North Island brown kiwi, takahe and brown teal.

Analysis type[1]	F[3]	P	Mean ± SE (Data back transformed where necessary)
Number of Bands	0.10	0.908	Kiwi = 9.86 ± 2.19 Takahe = 10.00 ± 2.90 Brown teal = 11.14 ± 2.19
DPI[2]	0.67	0.538	Kiwi = 23.00 ± 3.19 Takahe = 21.18 ± 6.75 Brown teal = 31.59 ± 8.18
Evenness	0.48	0.628	Kiwi = 0.85 ± 0.04 Takahe = 1.09 ± 0.64 Brown teal = 0.81 ± 0.05
Shannon Weiner Index	0.13	0.880	Kiwi = 1.69 ± 0.21 Takahe = 1.70 ± 0.28 Brown teal = 0.78 ± 0.02

[1] Sample sizes: kiwi = 7; takahe = 4; brown teal = 7
[2] Sample sizes for DPI: kiwi = 4; takahe = 3; brown teal = 5
[3] Degrees of Freedom = 2, 15 for all analyses, except DPI (d.f. = 2, 9)

DISCUSSION

Both kiwi and takahe living in captivity had a greater generic diversity of enteral bacterial genera than did those living in the wild and while not significant a similar trend was found in brown teal. However the excreta of these three phylogenetically distinct and ecologically disparate species could not be distinguished from each other on the basis of generic diversity of enteral microbial genera present when they were held captive. Similarly, the excreta of wild kiwi, wild takahe and wild brown teal could not be distinguished by enteral bacterial community structure. Thus, captivity appears to have a consistent influence on enteral bacterial diversity regardless of the host species.

Enteral microfloral diversity in kiwi did not differ between holding site despite their significant geographical separation over the North Island and South Island, nor did it differ between diets. This is surprising as the nutrient composition of diets fed to kiwi in captivity in New Zealand varies considerably, for example, crude protein ranges from 42 – 63%, crude fat ranges from 9 – 28% and starch ranges from 1 – 26% (for diets see Potter *et al.*, 2010). Similarly, the geographic location of generic diversity of wild kiwi did not vary geographically. Together these diversity results indicate that kiwi acquire a 'core set' of genera of enteral microflora, a concept that is in line with the current idea that the enteral microfloral genome contributes to that of the host (Akhter *et al.*, 2010; McKenney *et al.*, 2010). This is not to say that fluctuations in bacterial diversity do not occur at a species level with age, health, reproductive status, and changes in diet. Thus, further research is needed to determine whether enteral microflora vary at the species level.

Avifauna may be particularly prone to dysbiosis associated with captivity when eggs are taken from the wild and raised by hand. New-born mammalian young acquire their enteral bacteria from the birth canal, the rectum and the breast (Kelly *et al.*, 2007). Avian young are unlikely to do so, principally owing to the delay between the egg being laid and hatched. Instead the enteral microfloral community is thought to be derived from egg shells, adult and/or sibling faecal and other material in the nest (Kaldhusdal *et al.*, 2001; Schneitz, 2005). Thus the practice of removing eggs or young from the parents and raising them in a relatively sterile environment could sever the ongoing transfer of bacteria from generation to generation.

Conversely the introduction of faecal material from healthy adult birds into the environment of newly hatched chicks improves health, for example the treatment of

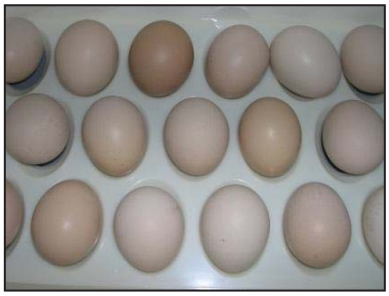
day-old broiler chicks with normal intestinal microflora from healthy adult chickens delayed the establishment of the pathogen *Clostridium perfringens* and reduced the frequency of necrotising enteritis (Kaldhusdal *et al.*, 2001).

It is also possible that the increase in the generic diversity of captive birds results from them encountering a wider range of nutrients than would be encountered in the wild or them encountering a wider range of bacteria from the excreta of greater numbers of cogenors. However, it seems likely that the encouragement of appropriate microfloral acquisition by captive young, for example the provision of nest material from adult birds, may be of benefit.

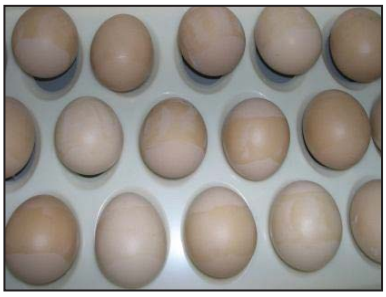
This study has highlighted the effect captivity has on the diversity of the enteral microfloral community in a number of native New Zealand avian species. Whether this phenomenon extends to other avian and non-avian native New Zealand species needs to be determined. Similarly, the effect of being hatched in captivity compared with being hatched in the wild on enteral bacterial community diversity needs to be established. In addition, methods of promoting a favourable suite of bacteria to colonise the intestinal tract are needed to ensure the development of a healthy immune system. From there, the effect of diet on the enteral bacterial environment needs to be determined, including the possibility of influencing enteral bacterial community diversity through manipulations of diet.

CHAPTER 7

Ontogeny and acquisition of enteral microflora in birds: implications for captive management



Control eggs (painted with distilled water)



Eggs painted with lactobacillus culture

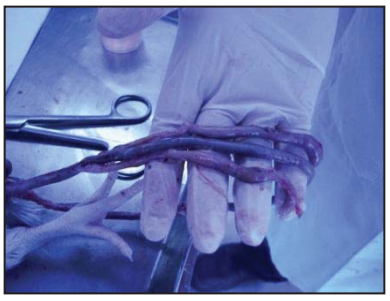
Eggs painted with hen excreta

Eggs in incubator

Chicks hatching in incubator



Chicks in trial cages

Ahmed Amerah and chicken caeca


Kiwi chicks

ABSTRACT

This study investigated the acquisition of generically diverse enteral bacteria by North Island brown kiwi (*Apteryx mantelli*) and chickens (*Gallus gallus*) over the first three weeks of life and the extent to which such acquisition can be manipulated. This was investigated in chickens by coating the external surface of the eggshells with either healthy adult faecal material, pure lactobacillus culture or distilled water.

The excreta of recently hatched kiwi and chickens contained significantly fewer enteral bacterial genera and lower generic diversity than at one week of age. By three weeks numbers of genera in the excreta of kiwi chicks did not differ significantly from those in captive adult kiwi. The application of adult faecal microflora, lactobacillus culture or distilled water to chicken eggs did not significantly influence the generic diversity of the newly hatched chicks. Hence, the acquisition of enteral microfloral genera appears to come from the environment rather than from ingesting the shell.

INTRODUCTION

The gastrointestinal tract of newly born or hatched young is relatively sterile (Pedroso *et al.*, 2005). Mammals acquire enteral microflora directly from the birth canal, rectum and breast of the mother (Kelly *et al.*, 2007). Avian young may acquire enteral microflora indirectly via contact with the surface of the eggshell, adult excreta, nest material or the surrounding environment (Schneitz, 2005). The acquisition of a suitable suite of enteral microflora is important for the development of elements of the immune system, as well as efficient digestion and nutrient partitioning (Qin *et al.*, 2010). Deviation from an appropriate set of microflora is termed dysbiosis and can lead to disease (Neish, 2009). It is now recognised that captivity increases numbers of genera of enteral microflora in a number of species, although it is not clear whether it is due to differences in diet, environment or adult/young transmission (chapter 6).

The difference in enteral microbial communities, i.e. dysbiosis, can affect cell repair, nutrient acquisition, energy storage and immune response to both pathogenic and non-pathogenic organisms (Turnbaugh *et al.*, 2006; Hill & Artis, 2010). Given the increasing reliance of species recovery programmes on captive management (e.g. Snyder *et al.*, 1996; Holzapfel *et al.*, 2008; Taylor & Parkin, 2010), as well as the importance of enteral bacterial colonisation on health (Sklan, 2005; Gabriel *et al.*, 2006; Kelly *et al.*, 2007; Fak *et al.*, 2008; Yegani & Korver, 2008), it is important to understand the mechanism that governs transmission from the mother or environment to the young.

Here I assess enteral microbial diversity in newly hatched, one, two and three week old captive North Island brown kiwi (*Apteryx mantelli*) chicks and in chickens (*Gallus gallus*). The enteral microfloral diversity of three week old captive kiwi chicks was also compared with that of captive adult kiwi. The effect on generic enteral bacterial diversity in newly hatched chicken chicks of the application of either a monoculture of lactobacillus or a suite of enteral microflora from adult chickens was also determined.

METHODS

Faecal sample collection

Kiwi

Recent, fresh, moist faecal samples were collected from adult wild North Island brown kiwi (*Apteryx mantelli*) in the Murchison Mountains (Fiordland), Moehau Wildlife Sanctuary (Coromandel) and Ponui Island (Hauraki Gulf). Faecal samples from wild chicks were collected from Ponui Island (Hauraki Gulf) and Moehau Wildlife Sanctuary (Coromandel). Faecal samples from captive adult kiwi were collected from Rainbow Springs Nature Park (Rotorua), Otorohanga Kiwi House (Otorohanga), Kiwi Birdlife Park (Queenstown), Auckland Zoo (Auckland), Willowbank (Christchurch), and Westshore Wildlife Reserve (Napier). Faecal samples from captive chicks (hatched from eggs sourced from the wild) were taken at hatch, week one, week two and week three post-hatch. Captive chicks were hatched and reared at Rainbow Springs Nature Park (Rotorua). All captive chicks were fed the same diet and water was available *ad libitum.* Following collection the samples were immediately placed in a sealed, sterilised container to prevent cross contamination. Samples were frozen as soon as practicable (within 24 hours) and stored at -20^{o}C pending analysis.

Chickens

Forty eight newly laid (one day old), fertilized Hyline Ross 308 eggs were obtained from a commercial hatchery (Tegel Hatcheries, New Plymouth). Eggs were removed from the packaging, cleaned in Virkon™ and immediately allocated randomly to one of three sterilised incubators (R.Com Digital Incubator 20 Pro PX20, Auto Elex Co. Ltd, Korea) to receive one of three treatments (shell painted with distilled water, pure lactobacillus culture or a suspension of fresh adult chicken excreta). In the incubator humidity was maintained with sterile distilled water at 61%. Temperature was maintained at 37 ^{o}C, natural light regimes (8/16 light/dark regime) were maintained with an egg rotation cycle (60 degrees per hour). Each treatment included twelve eggs and all eggs were painted on Day 19 of incubation.

Treatment groups:

(1) The control group was painted with distilled water.

(2) The lactobacillus group were painted with pure *Lactobacillus acidophilus* 52 culture. Cultures of *Lactobacillus acidophilus* were prepared by inoculating a seeding product (*Lactobacillus acidophilus* 52 culture (strain NZRM52 (ATCC 11975)) (ESR Limited NZ) in MRS media for 48 hours, at 37 ^{o}C under anaerobic conditions. Inoculation was carried out at Massey University and resulting culture was frozen prior to use.

(3) The faecal material group were painted with excreta from healthy adult chickens. Faecal material was collected from ten healthy adult chickens housed at the Massey University Poultry Unit. Excreta were collected over a period of a week, homogenised, suspended in sufficient sterile, distilled water to form a paste and frozen prior to use.

The three treatment solutions were applied in a broad band around the largest circumference of the egg, so that approximately half of the surface of the egg was coated thickly.

All eggs were hatched in incubators where chicks remained for a further 24 hours so as to give sufficient contact with discarded shells. Then all chicks were transferred, along with egg shells, to sterilised brooder cages (four birds per cage) in electrically heated battery brooders (Day 1). Three cages were used per treatment. At Day 14, the 24 remaining chicks were transferred to grower cages maintained under constant fluorescent illumination in an environmentally-controlled room. Ambient temperature was maintained at 31°C on Days 1 and 2, at 29°C on Days 3-6, at 28°C on Days 7-9, then a reduction of one degree Celsius every two days until Day 21 (22°C). Cages were 60 cm x 60 cm x 40 cm with a mesh size of 5 cm x 3 cm (2 cm x 3 cm on floor of cage) and raised 1.6 m off the floor. Chicks within the same treatment were housed in adjacent cages; sets of cages from each treatment were housed in the same room but separated by two metres.

Water was provided *ad libitum* regardless of treatment. Faecal material was allowed to drop to the floor. All treatment groups were given the same poultry starter diet *ad libitum*.

Twelve chicks (four from each treatment) were euthanized with an injection of Pentobarb 300 (delivered by intravenous injection) at Day 0, at Day 7, at Day 14 and at Day 21 (total of 48 chicks) and their caecal content collected (as described below).

Caecal digesta collection

A midline incision was made on the ventral surface of the abdomen of the chickens and both caeca exposed. A ligature was applied at the ileocaecal junction and tied off with sterile string. Caeca were removed by cutting on the side of the junction proximal to the ligature. Caeca were then placed into a sterile container and frozen at -20^{o}C. Sterile scissors and scalpels were used.

Sterility was maintained throughout the trials and during handling to prevent cross contamination.

Collection of caecal content for DNA extraction

Both caeca from each bird were thawed, the ligature removed and caecal contents expelled into a sterile collection tube.

DNA extraction and purification (removal of PCR inhibitors)

Bacterial DNA was extracted from 200 mg aliquots (wet weight) of each faecal sample according to the QIA amp® DNA Stool Minikit methods (Biolab- Cat # 51504, Qiagen, Valencia, CA, USA). The extracted DNA was cleaned according to the PowerClean^{TM} DNA Clean-Up Kit methods (Geneworks- Cat # 12877-50) and stored at -20^{o}C (for maximum of two weeks) pending analysis. Yield was checked by absorbance at 260/280 UV with a nanophotometer (Implen - IMP B-80-3004-31) according to Walter *et al.* (2000).

PCR Amplification

Extracted DNA was amplified using Universal Primers U968-GC-f (5′ CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC) and L1401-r (5′ CGG TGT GTA CAA GAC CC) (Marquardt, 1983; Burbidge *et al.*, 2003) which amplified V6 to V8 regions of bacterial 16S rRNA. The 3'end of the antisense primers each contained a 40 base pair G+C- rich sequence (Colbourne *et al.*, 2005). The generic primer used here identified bacteria to genus level. More specific primers that identify DNA to species-level are available for known bacterial species, but the lack of data on the enteral bacterial communities of the species analysed here meant that a primer able to target a wide range of bacterial taxa was needed.

PCR amplification was conducted in a Thermal iCycler (Bio Rad Cat # 170-8720) on 5 µl subsamples of each aliquot with a Go Taq® colourless Master Mix from Promega (Cat # M7121, Promega Go, Madison, WI, USA). Initial denaturation was conducted at 94^{o}C for 15 min, followed by 35 consecutive denaturation cycles each at 94^{o}C for 30 sec, with prior annealing at 57^{o}C for 1 min. Extension was conducted at 72^{o}C for 30 sec, with the final extension at 72^{o}C for 7 min. The post PCR concentrations of DNA were quantified by Nanophotometry (Implen - IMP B-80-3004-31). This process allowed appropriate volume adjustment to secure loading of DGGE slots with equal amounts of DNA.

Denaturing Gradient Gel Electrophoresis (DGGE)

The products of PCR were each subjected to DGGE according to the procedure outlined by Muyzer *et al.* (1993) and the Decode system manual (Bio-Rad Laboratories, Hercules, CA, USA) which enables determination of enteral bacterial community diversity (Hill *et al.*, 2008).

Gels were based on a 6% (vol/vol) solution of polyacrylamide (ratio of acrylamide- bisacrylamide 37.5:1) in 0.5x Tris-acetate-EDTA (pH 8.0) (TAE) buffer. The denaturing gradient was prepared using a solution of 6M urea and 55% formamide in the 6% polyacrylamide solution. Standardised gradient gels with denaturant gradients from 22- 55% formamide were prepared using a Gradient Delivery System (Model 475) (Bio Rad, Hercules, CA, USA) and a pump with an output of 5 ml per min. DGGE was run at 130V for 5h in 1.75x TAE buffer at a constant temperature of 60 °C in a DCode™ System (Bio Rad, Hercules, CA, USA). The gels were subsequently stained with ethidium bromide (1:10,000) and scanned on DNR MiniBis PRO System (Biolab).

The number and intensity of bands in the 16 plates on each gel were quantified using Total Lab software (Phoretics 1D Advanced, Non-linear Dynamics, Newcastle, UK). The RF positions (i.e. the degree of migration from the origin) of the bands on each gel were standardised with reference to bands on a reference ladder containing DNA from *Lactobacillus* spp., *Staphyloccoccus* spp. and *Escherichia coli*.

The volume of amplified DNA, quantified as peaks on the graph, was determined by Gaussian fit using a rolling ball baseline and converted to percentage of total peak area per slot. Hence the percentages of total peak area were related to the relative proportions of the organisms in the sample as quantified by 16S DNA extractate.

Data Processing

Total numbers of bands, i.e. bacterial genera, was determined by simple band counts. Indices of diversity, i.e. Shannon Weiner Diversity Index and evenness were determined on the basis of peak area. The Shannon Weiner Diversity Index is a measure of heterogeneity in a population and takes into account the degree of evenness in species/genera abundance (Magurran, 2004). The Evenness index is based on the Shannon Weiner Diversity Index and is the ratio of observed diversity (H') to maximum diversity (H'max = all species have equal abundances) (Magurran, 2004). In addition to these commonly reported indices, I also report the number of bands of height greater than 50% of the largest peak expressed as a percentage of the total number of bands (Dominant Peak Index (DPI)). This index indicates the proportion of dominant genera present in a sample and provides a more sensitive assessment of band dominance than does the evenness index. Only samples with a total of five or more bands were included in the DPI.

The indices were defined as (Aksoy *et al.*, 2006):

Shannon Weiner Index (H') = $-\sum P_i (\ln P_i)$

Evenness = H'/H'max

H' max = ln *s*

P_i is the percent Gaussian Volume (GV) of each genera.

s is the number of genera

N is the number of individuals identified (total GV per sample)

DPI = number of bands of height greater than 50% of the largest peak/total number of bands

Statistical Analysis

The distribution of band numbers, Shannon Weiner Diversity Index, evenness and the Dominant Peak Index were each assessed for normality using the Johnson algorithm facility in the Minitab™ 15.1.0.0 software. When necessary, data were transformed using the Johnson's algorithm.

Data were analysed using Analyses of Variance (ANOVA) (General Linear Model) in SAS 9.13 (SAS, 2004). Significance was taken to be $P < 0.05$. Similarly, repeated measures ANOVAs were used to compare data obtained from kiwi chicks at various ages. Unless specified otherwise, results in the text are expressed as mean ± standard error.

RESULTS

Ontogeny of enteral microbial development in North Island brown kiwi chicks

Newly hatched kiwi chicks had significantly fewer numbers and diversity of enteral bacterial genera than chicks of one or three weeks of age (Table 1). There were no significant differences in the evenness or DPI of enteral bacterial communities between cohorts.

Comparison of the enteral microfloral communities of three week old captive kiwi chicks with that of captive adult North Island brown kiwi

There were no significant differences in the number of enteral bacterial genera present, or in the indices of community diversity (determined by DPI, evenness and Shannon Weiner Indices) of three week old captive kiwi chicks compared to captive adult kiwi (Table 2).

Ontogeny of enteral microbial development in newly hatched, one week, two week and three week old chickens from eggs painted with either distilled water, pure lactobacillus culture, or adult faecal material

In the two-way ANOVA for treatment (distilled water, pure lactobacillus culture or adult chicken excreta) and day, neither treatment nor day*treatment were significant. Thus the model was run as a one-way ANOVA for the effect of day. No bands were detected for newly hatched chickens (Day 0), thus verifying the asepsis of our sampling techniques. No significant differences between cohorts were found in the number of enteral bacterial genera in the caecal content of chickens (Table 3). There were significantly more dominant peaks (DPI), i.e. dominant bacterial genera, in three week old chickens than in either one or two week old chickens.

Table 1. Comparison of faecal bacterial genera diversity (assessed by DGGE analyses) as an index of enteral bacterial diversity in captive North Island brown kiwi chicks at hatch, one week, two week and three weeks of age.

Analysis type[1]	F[2]	P	Mean ± SE[3] (Data back transformed where necessary)
Number of Bands	6.18	0.005	Hatch = 3.20 ± 1.86[a] Week 1 = 13.00 ± 2.09[b] Week 2 = 7.33 ± 1.70[ab] Week 3 = 12.50 ± 1.70[b]
DPI	1.21	0.338	
Evenness	0.63	0.608	
Shannon Weiner Index	4.32	0.020	Hatch = 0.96 ± 0.23[a] Week 1 = 1.98 ± 0.25[b] Week 2 = 1.54 ± 0.21[ab] Week 3 = 1.92 ± 0.21[b]

[1] Sample sizes: hatch = 5; week 1 = 4; week 2 = 6; week 3 = 6
[2] Degrees of Freedom = 3, 17 for all analyses, except DPI (d.f. = 2, 10).
[3] Within an analysis type, days with different letters ([a, b]) are significantly different at P < 0.05 as indicated by Least Square Means

Table 2. Comparison of the differences in the faecal bacterial genera diversity (assessed by DGGE analyses) in captive three week old and adult North Island brown kiwi

Analysis type[1]	F[2]	P
Number of Bands	1.16	0.293
DPI	0.47	0.634
Evenness	1.10	0.306
Shannon Weiner Index	0.98	0.333

[1] Sample sizes: chicks = 6; adults = 19
[2] Degrees of Freedom = 1, 23 for all analyses

Table 3. Comparison of the caecal bacterial genera diversity (assessed by DGGE analyses) among Day 0, Day 7, Day 14 and Day 21 chickens.

Analysis type	F[2]	P	Mean ± SE*[3] (Data back transformed where necessary)
Number of Bands	2.86	0.072	
DPI	19.86	0.001	Day 7 = 0.11 ± 0.02[a] Day 14 = 0.18 ± 0.02[a] Day 21 = 0.29 ± 0.02[b]
Evenness	1.23	0.799	
Shannon Weiner Index		0.306	

* No bands were detected for Day 0
[1] Sample size = 12 per day
[2] Degrees of Freedom = 2, 33 for all analyses, except DPI (d.f. = 2, 32)
[3] Within an analysis type, days with different letters ([a, b]) are significantly different at P < 0.05 as indicated by Least Square Means

DISCUSSION

The diversity of enteral bacteria changed rapidly over the first few weeks of life, with newly hatched kiwi and chickens having fewer enteral bacterial genera and lower generic diversity than week old kiwi and chickens. By three weeks of age, the enteral bacterial communities of kiwi chicks did not differ markedly to those of captive adult kiwi. Despite newly hatched chicks pecking at broken eggshells, the application of adult faecal material, pure lactobacillus culture or distilled water to the surface of their eggs did not markedly alter generic enteral bacterial communities. Hence, adequate numbers of bacteria appear to be acquired from the environment rather than from ingesting microflora that adhered to the eggshell.

Many studies have reported that the intestinal tract of newly hatched chicks is relatively sterile (Elsayed, 2002; Van der Wielen *et al.*, 2002; Pedroso *et al.*, 2005; Schneitz, 2005; Sklan, 2005; Neish, 2009) and that enteral bacterial colonisation begins during the hatching process (Nurmi & Rantala, 1973; Ellis *et al.*, 2001; Kizerwetter-Swida & Binek, 2008). This study found a low diversity of bacteria in the first faecal material of newly hatched kiwi chicks. Kiwi chicks acquired bacteria from an increasing number of genera over their first three weeks of life. Generic diversity was initially low, then fluctuated significantly before becoming relatively stable at week three, at which time it did not differ significantly from the enteral bacterial community of adult kiwi. The decrease in the number of genera of bacteria present in the excreta of kiwi chicks at week two is indicative of the changing environment within the digestive tract; the structure of the enteral bacterial community is fluctuating as bacterial genera compete for resources while the colonisation process occurs.

Variation in bacterial community dynamics is not uncommon in colonisation events (Zaniboni *et al.*, 2006). Initial colonisers encounter an environment with little competition for nutrients and space and are able to acquire niches and reproduce at a relatively high rate. Two groups of bacteria are found in the digestive tract, transient, allochthonous microbes that are ingested and expelled with the digesta, and resident, autochthonous bacteria that attach to the intestinal epithelium and live and reproduce within the intestinal environment (Sonnenburg *et al.*, 2004; Neish, 2009). Pioneer bacteria are able to create a favourable environment for themselves and can prevent the colonisation of other bacteria introduced later into the ecosystem (Guarner & Malagelada, 2003; Gabriel *et al.*, 2006). Therefore, the initial colonisation event is

important for the final composition of permanent 'adult' flora (Guarner & Malagelada, 2003). Thus, a period of episodic variation within the enteral bacterial community may be necessary for the establishment of a mature suite of intestinal microflora. This was clearly shown here during the first three weeks post-hatch. By three weeks of age, kiwi chicks appear to have established a more stable suite of genera that matches the enteral bacterial community structure of adult kiwi.

Enteral microflora change with development and age, and are influenced by the rearing environment (Hooper & Gordon, 2001). Kiwi chicks hatched and reared in captivity experience a different environment to those hatched in the wild. Husbandry practices vary across institutions but, in general, kiwi eggs are brought in from the wild, disinfected, placed into a sterile incubator until hatch and the chicks then placed into individual enclosures, thus the chicks have no contact with adults, siblings or conspecifics. Such contact would provide a continuous source of bacteria to aid in the acquisition and colonisation of a suitable suite of enteral bacteria (Apajalahti *et al.*, 2004). Bacteria common to the intestines of healthy adult poultry are often supplemented to young chickens to retard growth of pathogenic bacteria (competitive exclusion) (Flickinger *et al.*, 2003; Schneitz, 2005). Thus, the provision of nest material, including faecal material from healthy juvenile or adult birds provides chicks with suitable microbiota from which colonising bacteria can be sourced.

Here I attempted to influence colonisation and acquisition in poultry chicks by painting unhatched eggs with either pure lactobacillus culture or fresh excreta from healthy adult chickens. This technique did not modify the acquisition and colonisation of microflora by chicks at a generic level. Specific diversity may have been influenced by this technique but was not detected as generic primers were used to assess enteral bacterial diversity. In the poultry industry live coccidian vaccines (species specific) are used to reduce the incidence of coccidiosis in chicks (Gliozzi *et al.*, 2009). Thus, direct inoculation of chicks may increase the acquisition and colonisation of a suitable suite of enteral bacteria. Probiotics, live microbial feed supplements that beneficially affect the host animal's microbial intestinal balance (Patterson & Burkholder, 2003), are being used increasingly in the poultry industry as a replacement for prophylactic antibiotics (Chichlowski *et al.*, 2007). Probiotic bacteria are added to feed supplements of newly hatched chicks with the aim of reducing or inhibiting the colonisation of pathogens by competing for colonisation sites and nutrients, producing toxic compounds, and stimulating the immune system (Amit-Romach *et al.*, 2004; Sklan, 2005; Haghighi *et*

al., 2006). In this study, I used frozen monocultures and excreta as a source of colonising bacteria, thus I was unsure if the bacteria were alive or not. Further work is needed to determine the efficacy of the use of live versus dead bacteria on both unhatched eggs and newly hatched young.

Here, the lack of bacteria identified in the caeca of newly hatched poultry chicks, coupled with the low diversity of bacteria in the first faecal material of kiwi chicks, strengthens the hypothesis that the young of these two avian species are hatched with little, if any, enteral bacteria. Furthermore, these results suggest that between the time of hatch and the production of the first faecal matter some intestinal bacteria are acquired, and the colonisation process has commenced. The physical properties of the egg may affect the rate at which this colonisation takes place.

There is debate about the effect of the level of porosity of the egg shell on the ease of penetration by bacteria. Some studies have shown that porosity does influence penetration (Bruce & Drysdale, 1994; Mine *et al.*, 2003) and other studies state that eggshell characteristics, such as shell thickness and number of pores, does not affect the penetrability of eggshells by bacteria (Suzuki *et al.*, 2002). Whether this holds true for all avian species has not been established, however the porosity of the kiwi eggshell is low in relation to egg size (Delahunty, 2010). Research into the effect of different media applied to the surface of the eggshell of kiwi is needed to determine if this technique can be used to influence the microflora of newly hatched kiwi chicks. However, the protected status of kiwi and the high value of each egg limit the likelihood of approval of such studies.

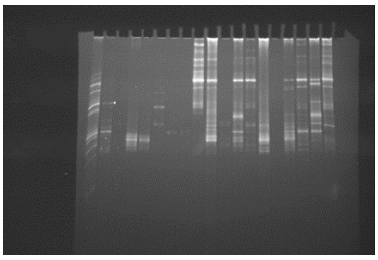
Not all bacteria are equally able to penetrate eggshells with motile and non-clustering bacteria achieving this most frequently (Suzuki *et al.*, 2002). Pathogenic bacteria, such as *Salmonella* species, are able to penetrate the egg shell, however once inside survival of the pathogen is not assured (Parry *et al.*, 2002) as anti-microbial factors in the albumen and vitelline membrane affect bacterial migration to the yolk (Yeomans, 1998). The newly hatched poultry chicks used in this study were observed to peck their own and other chick's eggshells. Despite this behaviour, the intestinal microflora of the day-old chicks was not influenced by bacteria present on the eggshells. Thus, either the chicks did not ingest any of the material adhering to the eggshells, or the bacteria had not migrated to the caeca by the time of sample collection.

While kiwi chicks appear to acquire enteral microbial bacteria quickly in the first few weeks of life, whether this can be subsequently influenced by diet has not been

investigated as it has in other species (Backus *et al.*, 2002; Apajalahti *et al.*, 2004; Bjerrum *et al.*, 2005; Blanco *et al.*, 2006; Flint *et al.*, 2007; Turnbaugh *et al.*, 2009; De Filippo *et al.*, 2010). Thus, the next step in understanding the complex dynamics of enteral microflora in kiwi is to determine whether food intake can be used to alter community diversity.

CHAPTER 8

The influence of diet on the community structure of intestinal bacteria in birds: implications for captive management



DGGE gel

Kiwi at Westshore Wildlife Reserve

Takahe on Mana Island

ABSTRACT

Intestinal bacteria are critical to digestion and the health of the host. The timing of colonisation of the intestinal tract by bacteria differs among taxa but typically starts during birth or hatch. Once intestinal bacteria have become established, it is primarily dietary intake that influences the microbial community within the digestive tract. In chapter 6 I found that the number of enteral bacteria genera present and the diversity of the communities were greater in both captive North Island brown kiwi (*Apteryx mantelli*) and captive takahe (*Porphyrio* [Notornis] *mantelli*) than in their wild counterparts. I found that while the numbers of bacterial genera present and overall diversity did not differ in the enteral bacterial communities of kiwi fed varying proportions of the captive and wild diets, the structure of these communities did vary. As kiwi consumed a greater proportion of the wild diet the number of dominant enteral bacterial genera decreased. This suggests that new enteral bacterial genera were not acquired with a progressive change in diet over three weeks, but rather the novel diet changed the overall bacterial community structure. Takahe held captive and fed a mix of the captive and wild diets developed a community of enteral bacteria that was not significantly different to that of birds that had consumed either solely a captive or solely a wild diet. Thus the enteral bacterial communities of these birds appeared to be intermediate between those consuming captive or wild diets.

INTRODUCTION

Intestinal bacteria exert an enormous influence on the nutritional and health status of the host (Ogue-Bon *et al.*, 2010). Enteral microflora are not only important for modulating the immune system (Laparra & Sanz, 2010), they also influence intestinal epithelial metabolism and proliferation (Hooper & Gordon, 2001), angiogenesis (Stappenbeck *et al.*, 2002), cell repair (Hooper & Macpherson, 2010) and digestion (Bauer *et al.*, 2006; Flint *et al.*, 2007; Turnbaugh *et al.*, 2009). The acquisition and maintenance of a suitable suite of intestinal bacteria is considered essential for life (Hooper *et al.*, 2002) and it is becoming increasingly apparent that a deviation from a suitable suite of enteral microflora, termed 'dysbiosis' (Neish, 2009) can predispose the host to a range of diseases including chronic intestinal inflammation (Lepage *et al.*, 2008). The timing of colonisation of the intestinal tract by bacteria differs among taxa. For example, mammalian young acquire intestinal microbiota from the birth canal, rectum and breast (Kelly *et al.*, 2007), whereas colonisation begins during the hatching process of avian young and bacteria are acquired from parental and sibling faecal material and the surrounding environment (chapter 7 ; (Ellis *et al.*, 2001; Kizerwetter-Swida & Binek, 2008). Two groups of bacteria inhabit the digestive tract: transient, allocthonous microbes that are ingested and expelled with the digesta; and resident, autocthonous bacteria that attach to the intestinal epithelium and live and reproduce within the intestinal environment (Sonnenburg *et al.*, 2004; Neish, 2009). Once intestinal bacteria have become established, it is primarily dietary intake, including allocthonous bacteria, that influences the microbial community within the digestive tract (Apajalahti *et al.*, 2004).

The influence of diet on the composition of intestinal bacterial communities has been established in a range of species, including kites (Blanco *et al.*, 2006), chickens (Apajalahti *et al.*, 2004), pigs (Hill *et al.*, 2005), cats (Backus *et al.*, 2002), dogs (Ogue-Bon *et al.*, 2010), mice (Ley *et al.*, 2005), fish (Ringø *et al.*, 2006) and humans (De Filippo *et al.*, 2010). Nutrient requirements of the bacteria are met by contact with the digesta. In order to acquire nutrients following enzymatic digestion, the host depends largely upon a suitable suite of microflora to break down and digest the diet. Furthermore, the presence of enteral bacteria can expand the range of dietary items that can be broken down and digested by the host (Neish, 2009). Every diet creates a slightly different intestinal environment. For example, increased viscosity of digesta changes

nutrient flow and reduces the potential for bacteria and digesta to mix (Metzler-Zebel *et al.*, 2010). A reduction in bacterial access to new substrate reduces their ability to break down food particles, and thus decreases the overall digestion of nutrients (Langhout *et al.*, 2000). Furthermore, an increase in undigested nutrients in the distal end of the digestive tract may promote microbial growth (Langhout *et al.*, 2000), potentially of harmful bacteria (Smith & Osborn, 2009).

While dietary intake has been widely reported as the primary driver of changes in the community structure of intestinal bacteria (Flint *et al.*, 2007; Lubbs *et al.*, 2009; Ogue-Bon *et al.*, 2010), the length of time needed for significant diet-related shifts to occur varies across taxa. These changes can occur in as little as one day (Turnbaugh *et al.*, 2009) or as long as two to six weeks (Leser *et al.*, 2000; Backus *et al.*, 2002; Engberg *et al.*, 2004) depending on the host species.

Here I investigate the effect of changes in diet on enteral bacterial community diversity in two endemic New Zealand avian species: the North Island brown kiwi (*Apteryx mantelli*) and the takahe (*Porphyrio* [Notornis] *mantelli*). In captive kiwi the effect of a change from a captive diet to a wild-type diet on the diversity of the intestinal microflora is investigated. In takahe, intestinal bacterial communities of captive-held birds fed either a captive diet, a mixed captive-wild diet, or a wild diet are investigated.

METHODS

Kiwi Feeding Trial

Seven captive North Island brown kiwi (*Apteryx mantelli*) from Westshore Wildlife Reserve were used in the trial. They were housed and fed in their normal, individual, outdoor enclosures from Days 0 – 7 and then transferred indoors to individual trial enclosures for Days 8 - 22. They were fed once a day (dietary regime given in Table 1) with water available *ad libitum.* Faecal samples were collected on Days 0, 6, 15 and 22. One sterile container was used per bird per collection then immediately frozen at -20^{o}C. Equipment was sterilised and gloves changed between collections.

Table 1. The dietary regime for kiwi based on a daily feed ration of 200 g per bird (as-fed).

Day	Near-natural diet[1] (%)	Captive kiwi maintenance diet[2] (%)
0	0	100
1 - 6	50	50
7 - 9	22	78
10	30	70
11	55	45
12	63	37
13	83	17
14 - 22	100	0

[1] Diet given in chapter 3
[2] Usual diet fed by Westshore Wildlife Reserve (Potter *et al.*, 2010)

Takahe Feeding Trial

Fifteen adult takahe (*Porphyrio* [Notornis] *mantelli*). were used in the trial; seven captive, four intermediate-wild (see below for description) and four wild birds. The seven captive takahe were from Te Anau Wildlife Park, Te Anau and were fed a captive diet (diet description given in Table 2). The four intermediate birds were from Burwood Bush Takahe Rearing Unit, Te Anau and were fed a wild diet with a twice

weekly supplement of the captive diet (diet description given in Table 2). The four wild takahe were from Mana Island, Kapiti Coast and consumed a wild diet (grass and seeds). All diets were fed for at least one year prior to faecal collection. Excreta from each bird were collected into a separate container, and samples were frozen as practicable (within 24 hours) and stored at -20°C pending analysis.

Table 2. Comparison of the captive, intermediate and wild diets fed to takahe.

Captive diet	Intermediate diet	Wild diet
• corn kernels • kumara • carrot • apple • banana • takahe maintenance pellets[1]	Birds foraged for their own food and received a supplement of the captive diet twice a week	Birds foraged for their own food - grass - seeds

[1] Poultry Unit, Massey University

DNA extraction and purification (removal of PCR inhibitors)

Bacterial DNA was extracted from 200 mg aliquots (wet weight) of each faecal sample according to the QIA amp® DNA Stool Minikit methods (Biolab- Cat # 51504, Qiagen, Valencia, CA, USA). The extracted DNA was cleaned according to the PowerClean™ DNA Clean-Up Kit methods (Geneworks- Cat # 12877-50) and stored at -20°C (for maximum of two weeks) pending analysis. Yield was checked by absorbance at 260/280 UV with a nanophotometer (Implen - IMP B-80-3004-31) according to Walter *et al.* (2000).

PCR Amplification

Extracted DNA was amplified using Universal Primers U968-GC-f (5′ CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC) and L1401-r (5′ CGG TGT GTA CAA GAC CC) (Marquardt, 1983; Burbidge *et al.*, 2003) which amplified V6 to V8 regions of bacterial 16S rRNA. The 3'end of the antisense primers each contained a 40 base pair G+C- rich sequence (Colbourne *et al.*, 2005). The generic primer used here identified bacteria to genus level. More specific primers that identify DNA to species-level are available for known bacterial species, but the lack of data on the enteral bacterial communities of the species

analysed here meant that a primer able to target a wide range of bacterial taxa was needed.

PCR amplification was conducted in a Thermal iCycler (Bio Rad Cat # 170-8720) on 5 µl subsamples of each aliquot with a Go Taq® colourless Master Mix from Promega (Cat # M7121, Promega Go, Madison, WI, USA). Initial denaturation was conducted at 94^{o}C for 15 min, followed by 35 consecutive denaturation cycles each at 94^{o}C for 30 sec, with prior annealing at 57^{o}C for 1 min. Extension was conducted at 72^{o}C for 30 sec, with the final extension at 72^{o}C for 7 min. The post PCR concentrations of DNA were quantified by Nanophotometry (Implen - IMP B-80-3004-31). This process allowed appropriate volume adjustment to secure loading of DGGE slots with equal amounts of DNA.

Denaturing Gradient Gel Electrophoresis (DGGE)

The products of PCR were each subjected to DGGE according to the procedure outlined by Muyzer *et al.* (1993) and the Decode system manual (Bio-Rad Laboratories, Hercules, CA, USA) which enables determination of enteral bacterial community diversity (Hill *et al.*, 2008).

Gels were based on a 6% (vol/vol) solution of polyacrylamide (ratio of acrylamide- bisacrylamide 37.5:1) in 0.5x Tris-acetate-EDTA (pH 8.0) (TAE) buffer. The denaturing gradient was prepared using a solution of 6M urea and 55% formamide in the 6% polyacrylamide solution. Standardised gradient gels with denaturant gradients from 22- 55% formamide were prepared using a Gradient Delivery System (Model 475) (Bio Rad, Hercules, CA, USA) and a pump with an output of 5 ml per min. DGGE was run at 130V for 5h in 1.75x TAE buffer at a constant temperature of 60 °C in a DCode^{TM} System (Bio Rad, Hercules, CA, USA). The gels were subsequently stained with ethidium bromide (1:10,000) and scanned on DNR MiniBis PRO System (Biolab).

The number and intensity of bands in the 16 plates on each gel were quantified using Total Lab software (Phoretics 1D Advanced, Non-linear Dynamics, Newcastle, UK). The RF positions (i.e. the degree of migration from the origin) of the bands on each gel were standardised with reference to bands on a reference ladder containing DNA from *Lactobacillus* spp., *Staphyloccoccus* spp. and *Escherichia coli*.

The volume of amplified DNA, quantified as peaks on the graph, was determined by Gaussian fit using a rolling ball baseline and converted to percentage of total peak area per slot. Hence the percentages of total peak area were related to the

relative proportions of the organisms in the sample as quantified by 16S DNA extractate.

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Total numbers of bands, i.e. bacterial genera, were determined by simple band counts. Indices of diversity, i.e. Shannon Weiner Diversity Index and evenness were determined on the basis of peak area. The Shannon Weiner Diversity Index is a measure of heterogeneity in a population and takes into account the degree of evenness in species/genera abundance (Magurran, 2004). The Evenness index is based on the Shannon Weiner Diversity Index and is the ratio of observed diversity (H') to maximum diversity (H'max = all genera have equal abundances) (Magurran, 2004). In addition to these commonly reported indices, I also report the number of bands of height greater than 50% of the largest peak expressed as a percentage of the total number of bands (Dominant Peak Index (DPI)). This index indicates the proportion of dominant genera present in a sample and provides a more sensitive assessment of band dominance than does the evenness index. Only samples with a total of five or more bands were included in the DPI.

The indices were defined as (Aksoy *et al.*, 2006):

Shannon Weiner Index (H') = $-\sum P_i (\ln P_i)$

Evenness = H'/H'max

H' max = ln *s*

P_i is the percent Gaussian Volume (GV) of each genera.

s is the number of genera

N is the number of individuals identified (total GV per sample)

DPI = number of bands of height greater than 50% of the largest peak/total number of bands

Statistical Analysis

The distribution of band numbers, Shannon Weiner Diversity Index, evenness and the Dominant Peak Index were each assessed for normality using the Johnson algorithm

facility in the Minitab™ 15.1.0.0 software. When necessary, data were transformed using the Johnson's algorithm.

Data were analysed using Analyses of Variance (ANOVA) (General Linear Model) in SAS 9.13 (SAS, 2004). Significance was taken to be $P < 0.05$. Unless specified otherwise, results in the text are expressed as mean ± standard error.

RESULTS

Comparison of the enteral microfloral community of captive North Island brown kiwi fed diets differing in the proportions of captive or wild ingredients (for diet descriptions see Table 1).

There were no significant differences in either the number of enteral bacterial genera present or the Shannon Weiner index of enteral bacteria sampled from excreta from adult North Island brown kiwi fed a varied diet (Table 3). However, the evenness values for the enteral bacterial communities of kiwi at Days 0 and 6 were lower than at Day 22 (Figure 1), i.e. there were significantly more dominant genera at Day 0 than at Days 15 and 22 (Figure 2).

Comparison of the enteral microfloral communities of captive, wild and intermediate takahe fed different diets (for diet descriptions see Table 2).

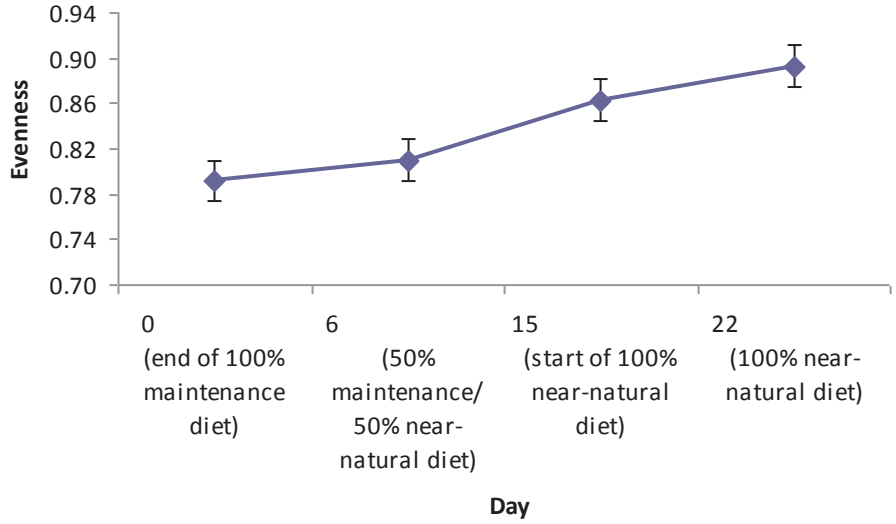
Significantly greater numbers and diversity of enteral bacterial genera were found in captive than in wild takahe (Table 4). The diversity of the enteral bacterial communities in intermediate birds was not significantly different to that of birds fed either the captive or wild diets. The evenness and the DPI of the enteral bacterial communities did not significantly differ among captive, intermediate or wild takahe.

Table 3. Comparison of the enteral bacterial communities (assessed by DGGE analyses) of North Island brown kiwi at Days 0, 6, 15 and 22 fed a progressively larger proportions of wild to captive diets (see Table 1 for diet regimes).

Analysis type[1]	F[2]	P
Number of Genera	0.59	0.630
Evenness	6.78	0.002
DPI	4.50	0.013
Shannon Weiner Index	0.26	0.860

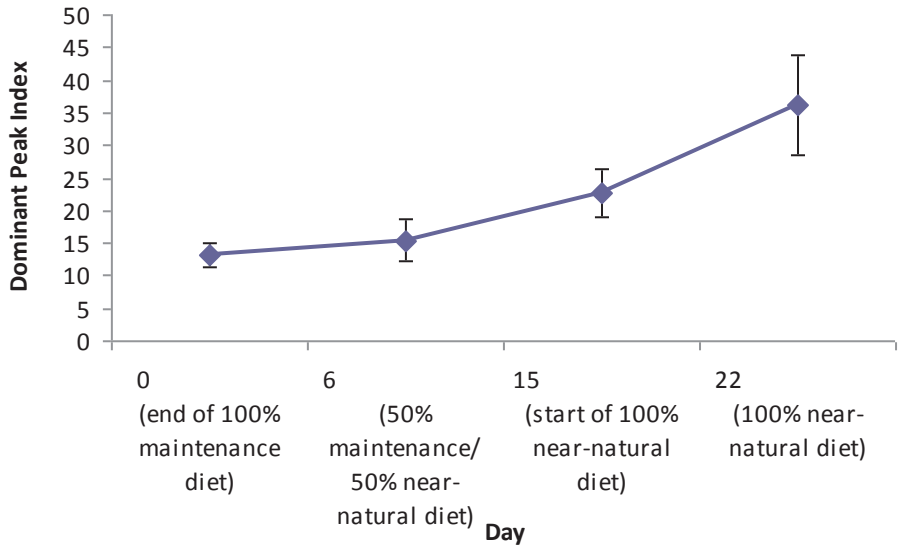
[1] Sample sizes = 7 per day
[2] Degrees of Freedom = 3, 24 for all analyses, except DPI (d.f. = 3, 22, Day 15 n=5)).

Figure 1. Comparison of the evenness (mean ± SE) of enteral bacterial communities (assessed by DGGE analyses) of North Island brown kiwi fed a progressively larger proportions of wild to captive diets (see Table 1 for diet regimes).



NB Evenness of 1 = all enteral bacterial genera have equal abundance

Figure 2. Comparison of the Dominant Peak Index (DPI) (mean ± SE) of the enteral bacteria communities (assessed by DGGE analyses) of North Island brown kiwi fed a progressively larger proportions of wild to captive diets (see Table 1 for diet descriptions)



DPI = mean number of bands of height greater than 50% of the largest peak expressed as a percentage of the total number of bands

Table 4. Comparison of the enteral bacterial communities (assessed by DGGE analyses) of adult takahe that had consumed one of three diets (captive diet, or mixture of captive and wild diet, or wild diet; see Table 2 for diet descriptions)

Analysis type[1]	F[2]	P	Mean ± SE[3] (Data back transformed where necessary)
Number of Bands	3.27	0.048	*captive 16.00 ± 1.71[a] intermediate 11.86 ± 1.29[ab] *wild 10.00 ± 1.71[b]
DPI	1.02	0.392	
Evenness	2.24	0.149	
Shannon Weiner Index	3.89	0.045	*captive 2.28 ± 0.11[a] intermediate 2.01 ± 0.35[ab] *wild 1.69 ± 0.38[b]

[1] Sample sizes: captive = 4; intermediate = 7; wild = 4

[2] Degrees of Freedom = 2, 12 for all analyses, except DPI (d.f. = 2, 11)

[3] Within an analysis type, days with different letters ([a, b]) are significantly different at $P < 0.05$ as indicated by Least Square Means.

* Data from chapter 6

DISCUSSION

Diet influenced the community structure of the enteral bacteria in two native New Zealand avian species: the North Island brown kiwi; and the takahe. As kiwi consumed a greater proportion of the wild diet the number of dominant enteral bacterial genera decreased, but the overall diversity of the community remained the same. This suggests that new enteral bacterial genera were not acquired with a progressive change in diet over three weeks, but rather the novel diet changed the overall bacterial community structure. The enteral bacterial community structure in takahe fed a mixed diet of captive and wild food items was not significantly different to that of takahe consuming either a solely captive diet or a solely wild diet. Thus, in takahe fed a mixed captive and wild diet it appears that the enteral bacterial community structure is a blend of both a captive and wild diet.

There is a large body of work indicating that dietary intake significantly influences intestinal microfloral community structure (Backus *et al.*, 2002; Apajalahti *et al.*, 2004; Bjerrum *et al.*, 2005; Blanco *et al.*, 2006; Flint *et al.*, 2007; Turnbaugh *et al.*, 2009; De Filippo *et al.*, 2010). Indeed, this is precisely what I saw in chapter 6 and what I observed here as a result of two different methods applied to kiwi and takahe. The first method, using kiwi, assessed the effect of a progressive change in diet on the structure of intestinal microflora. The second method, using takahe, assessed the effect of long term intake of a diet on the structure of intestinal microflora, where the birds consumed either a wild diet, a captive diet, or a mixture of the wild and captive diets. A gradual change in diet over a three week period did not affect the number or overall diversity of bacterial genera in kiwi, however it did increase the evenness of the communities, i.e. the number of dominant genera decreased. To determine what was driving this enteral bacterial community shift, from one of a small number of dominant genera to a more uniform community structure, an additional index was used, the Dominant Peak Index (DPI) where each peak represents a bacterial genus. The contribution of the area of each peak to the total area of all peaks indicates the contribution of each bacterial genus to the total. Thus, the higher the peak, the greater the number of that particular genus present in the sample. The DPI identifies the percentage of peaks of bacterial genera that are greater in height than 50% of the tallest peak. The DPI showed an increase in the number of dominant bacterial genera over time. That is, as kiwi ate more of the natural diet and less of the captive diet, the number of dominant genera decreased, thus

increasing the evenness of the community. Therefore, these results suggest that new enteral bacterial genera were not acquired with a change in diet over three weeks, but rather the novel diet changed the overall bacterial community structure.

Due to restrictions on prolonged manipulation of kiwi I restricted the feeding period to three weeks, however this period appears to be too short to show differences in enteral bacterial diversity due to diet. Given that significant differences in the community structure of enteral bacteria exist between captive and wild kiwi, and captive and wild takahe (see chapter 6), it is likely that with a longer acclimation on the wild diet the enteral bacterial diversity in captive kiwi would become increasingly similar to that of wild kiwi.

The length of time needed for a change in diet to effect a change in intestinal bacterial community diversity varies among taxa, for example, a day in mice (Turnbaugh *et al.*, 2009), three days to two weeks in pigs (Varel *et al.*, 1987) (Leser *et al.*, 2000), two weeks in cats (Backus *et al.*, 2002) and four to six weeks in poultry (Apajalahti *et al.*, 2001; Engberg *et al.*, 2004). How long the acclimation period needs to be to effect a significant change in enteral bacterial diversity of these species is unknown. In those studies the period targeted to influence intestinal bacteria for production animals, particularly poultry, was from birth or hatch until age of slaughter. For non-laying poultry, the average age at slaughter is about 45 days (Anthony, 1998; Rasschaert *et al.*, 2007), thus, there is neither need nor time to influence gut bacteria once the birds are mature. For laying poultry, the promotion of a suitable suite of enteral microflora is aimed at acquisition and gut colonisation of newly hatched chicks, not at manipulating the gut flora of older birds. It is therefore surprising that no data appear to have been published on the minimum period needed for dietary intake to affect the community structure of enteral bacteria in poultry.

The second method of assessing the effect of diet on enteral microfloral community structure builds on the results obtained in chapter 6. I determined the effect on enteral microfloral community structure of takahe consuming either a solely wild diet, a solely captive diet, or a mixture of the captive and wild diets. The results showed that the intestinal bacterial community structure of takahe that consumed a mix of the captive and wild diets appeared to be 'intermediate' between that of captive and wild birds. Thus the number of bacterial genera present and their overall diversity lay within the range I reported for takahe consuming either a captive or a wild diet (chapter 6). These results suggest that the magnitude of change in enteral microfloral community

structure and diversity depends on the time over which the individuals consume a particular diet.

One factor that influences the rate at which the enteral bacteria change is diet composition (Flint *et al.*, 2007). For example, in general, complex carbohydrate-based diets are more difficult to digest than protein based diets and this is reflected in the speed with which enteral bacteria change (Santos, A. A. J. *et al.*, 2008). An increase in available substrate increases the number of bacterial genera that the gut can support and a reduction in carbohydrate may result in a state of nutrient stress on the gut microbiome (Hildebrandt *et al.*, 2009). This effect is also seen in humans where a change in the quantity of non-digestible carbohydrates in a diet influences metabolic products and faecal bacterial populations (Flint *et al.*, 2007).

The type of protein and fat in a diet also affects intestinal bacterial diversity (Demeyer & van Nevel, 1995; Danicke *et al.*, 1999) (Turnbaugh *et al.*, 2009). Sources of both fat and protein in the captive and near-natural diets were considerably different (chapters 3 and 4); the captive diet consisted of vertebrate fat and protein, whereas the near-natural diet contained fat and protein from invertebrates. Vertebrate fat is different to that of invertebrate fat (De Foliart, 1991); terrestrial insects have markedly lower levels of polyunsaturated fats than vertebrates, and some species can form essential fatty acids *de novo* (Blomquist *et al.*, 1991). Dietary fat source (Knarreborg *et al.*, 2002) and the type of fat (Danicke *et al.*, 1999) included in the diet affect intestinal microbial composition in broilers. The effect, if any, of different fat and protein sources on the health of kiwi is not known. Similarly, whether vertebrate protein is of a different quality to invertebrate protein for kiwi is also not known. However, the difference in enteral bacterial diversity seen in kiwi fed a captive diet compared with a wild diet for prolonged periods (chapter 6) suggests that different sources of ingredients may affect enteral bacterial community structure. Here, I report that three weeks is not sufficient to induce significant changes in enteral bacterial diversities of kiwi due to a change in diet. Additional studies on enteral bacteria in kiwi would contribute to the understanding of the short and long term effect of the captive diet on the health of kiwi. This is also important for determining how long captive kiwi should be fed a more natural diet before they are released into the wild.

While the emphasis of this study has been on the effect of diet on the intestinal microflora of kiwi and takahe, this issue is important for all species that are held in captivity, whether they are to be released into the wild or not. The complexity and

importance of enteral microflora communities to the health of an animal cannot be overstated and needs to be addressed in captive management protocols. The symbiotic relationship that intestinal bacteria have with the host to acquire and utilise nutrients in response to the diet (Laparra & Sanz, 2010) is vital at all stages of life. For captive institutions that focus on breeding and rearing, management of the enteral bacterial health of an animal needs to start before birth/hatch as colonisation of the intestinal tract by bacteria generally starts immediately after hatch (chapter 7) or as young are born (Kizerwetter-Swida & Binek, 2008). From this point on diet is important for intestinal bacterial community structure and can be used to effect a change in community diversity, however the length of time needed to effect this changes varies with species and diet.

CHAPTER 9

General Discussion

Here, I have provided the first detailed investigation into the nutrient intake of a flightless, insectivorous avian species, the North Island brown kiwi (*Apteryx mantelli*). This is also the first study to investigate the enteral bacterial communities of three endemic New Zealand birds (North Island brown kiwi, brown teal (*Anas chlorotis*) and takahe (*Porphyrio* [Notornis] *mantelli*)). Detail is provided on the acquisition and storage of fat in relation to diet through comparison of the North Island brown kiwi with data published on depot fat in other avian species.

The discussion that follows highlights the thought processes, practical methods and changes that were involved during the course of this thesis. I also outline the importance of this work to conservation and finish with specific recommendations plus suggested directions for research that I consider both essential and beneficial to conservation in the future.

North Island brown kiwi diet

A number of studies have attempted to determine the diet of kiwi by using direct observations (Buller, 1888), gizzard content analyses (Buller, 1888; Gurr, 1952; Bull, 1959; Watt, 1971; Reid *et al.*, 1982; Pindur, 2004), or faecal analyses (Kleinpaste, 1990; Miles *et al.*, 1997; Shapiro, 2006). While these studies have noted the consumption of diverse taxa, the consensus is that kiwi forage primarily on invertebrates and to a much lesser extent on plant material. Due to the protected status of kiwi (Holzapfel *et al.*, 2008), fresh gizzards are seldom available and gizzard contents are often in an advanced state of decomposition by the time they can be examined. This, coupled with the tendency for soft-bodied invertebrates to be almost completely digested, and thus difficult to detect in faecal material (Oehm *et al.*, 2011), means that such studies have to be interpreted with caution. Furthermore, it has been suggested that leaf material is only ingested incidentally as a consequence of adhesion to intended food items (Reid *et al.*, 1982). Specific dietary intake varies through space and time (Kleinpaste, 1990), thus I wanted to develop a diet for captive kiwi that was based on the overall nutrient composition of a typical wild diet rather than focussing on specific food items eaten by individual kiwi.

Thus, I aimed to produce a diet based on dietary intake by kiwi over longer time frames, such as a year. Two approaches were considered: the first was analysis of body fat composition, as this has been reported to be a reliable indicator of diet in some

species (Meynier *et al.*, 2010); second was to make use of published data on annual variation in kiwi diets (Kleinpaste, 1990) to balance out short-term seasonal variation.

Fatty acid signature analyses used to determine diet

This is the first time the depot fat (specifically gizzard fat) of kiwi has been used to attempt to determine dietary intake (chapter 2). Oleic acid was consistently found to be the dominant fatty acid in their gizzard fat. This finding is not surprising given that oleic acid is the precursor for the production of most other polyunsaturated fatty acids (Watkins & German, 1998). Fatty acid profiles of the gizzard fat of kiwi highlighted the variation in composition between individuals. Again, this is not surprising as the gizzards came from birds foraging in markedly different locations and soil type, thus their specific dietary items are likely to have differed (Mattos *et al.*, 2000; Pindur, 2004). In addition, the fatty acid composition of insects is highly variable and depends on age, sex (Turunen, 1974) and environmental conditions (Khani *et al.*, 2007). Thus, due to variation in fatty acid composition between individuals, this method of determining the diet through analyses of gizzard fat appears to have limited value for kiwi.

To establish whether the results for kiwi are unusual and whether fatty acid profiling of depot fat could provide insights into gross dietary intake across species, I searched the literature and made comparisons of fatty acid profiles of storage fat in avian species with diverse diets. The results indicated that avian marine carnivores had a distinctly different fatty acid profile to herbivores, omnivores and insectivores, with linoleic acid being notably less prevalent than for the other groups. Within species, the insectivores showed the largest variation in fatty acid profiles, with relatively high palmitoleic acid levels in the depot fat of red-eyed vireos and high levels of linoleic acid in the storage fat of white throated sparrows. Thus the variation in fatty acid profiles across kiwi is consistent with them being primarily insectivorous.

While trends in fatty acid profiles were evident across herbivores, omnivores, insectivores and marine carnivores, I wanted to unravel this further to see whether individual species could be separated out on the basis of diet. Hierarchical cluster analyses were used to investigate this. The mean fatty acid profile data per species separated in a broadly similar manner to that of the data from individual birds within a species. However, using a mean value for a species obscured the high degree of

variation that exists between individuals within a species, particularly so for omnivores and insectivores.

Thus, dendrographic analysis was only able to cluster taxa with either narrow ranges in diet, such as strict herbivores, or those that consume food items with a distinctive fatty acid signature, such as avian marine carnivores. This technique relies on the presence of distinctive fatty acids in the diet, particularly those that are essential and cannot be synthesized by the animals themselves. Thus, the presence of these fatty acids, such as linoleic and linolenic acids, are directly attributable to dietary intake (Moss & Lough, 1968; West & Meng, 1968b; Blem, 1976; Cerolini *et al.*, 2006). For species that consume a more omnivorous diet, and thus possess fat stores lacking in a single or unique set of fatty acids, this technique of diet determination is neither robust nor particularly informative. Hence for kiwi, that are usually classified as primary insectivores, their wide dietary intake suggests that a classification of omnivore might be more appropriate. Thus, like with most omnivores, predictions of diet from fatty acid analyses of depot fat are not absolute, and cannot be relied upon to provide informative data about dietary intake. Given this predicament I progressed to the second option of assessing nutrient intake based on assessment of the annual dietary intake of wild kiwi.

Wild and current captive diets for kiwi

Only one study has examined in detail seasonal variation in the diet of wild kiwi (Kleinpaste, 1990). I used these data to formulate a near-natural diet (chapter 3). Kleinpaste's (1990) study was conducted in Waitangi State Forest in Northland so not all the species consumed by those kiwi were readily available for collection. Thus, the diet was formulated using closely related taxa to those consumed in the wild; ones that were available in relatively large quantities. Once formulated, the nutrient composition of an homogenised sample was determined allowing us the first direct assessment of the nutrient intake of wild kiwi.

The current range of captive diets for kiwi are based on a diet that was formulated in the 1970s without reference to the nutrient requirements of the birds (Reid, 1970; Johnson, 1996; Potter *et al.*, 2010). It is my view that this oversight may have led to a number of dietary related problems, such as a decline in survival, fertility, and size of eggs and chicks in captive birds. The effect of diet on health and reproductive success is no longer a matter of debate; many studies across diverse taxa and niche have concluded

that optimum nutrient intake is important for health and well-being (Dierenfeld, 1997; Hendriks *et al.*, 2000; Koutsos *et al.*, 2001; Kleiman *et al.*, 2010).

Kiwi digestibility

Prior to this study no information was available on the apparent digestibility of the natural diet in kiwi. Furthermore, there are no extant, flightless, avian insectivores of a similar weight that could be used to predict digestibility values for kiwi. Thus, the issue arose of how to validate results obtained from digestibility trials on kiwi. To address this problem I also fed the diets to roosters as they are of a relatively similar body weight to kiwi, were historically insectivorous or omnivorous (Klasing, 2005), are non-egg laying, and a wealth of information exists on their digestive physiology. As it transpired, kiwi had higher apparent digestibilities of all macronutrients except fat than roosters (chapter 3). Apparent digestibility values for roosters seen here were consistent with those reported elsewhere, thus I wase confident of the results I obtained for both kiwi and roosters.

Uric acid

Calculation of protein digestibility from avian faecal samples can be spurious; uric acid nitrogen is not accounted for because total protein is calculated from the amount of nitrogen in the excreta. Along with undigested nitrogen from dietary protein, avian excreta contain nitrogen from uric acid (Nahm, 2003), from enteral microflora and from mucosal cells sloughed off the wall of the intestinal tract (Ravindran *et al.*, 1999). If unaccounted for, these extra sources of nitrogen in the excreta erroneously decrease protein digestibility estimates. Thus I removed non-nitrogen protein from protein values before calculating protein digestibilities (chapters 3 and 4).

Protein and fat digestibility in the near-natural diet

Protein digestibility in kiwi (91%) was significantly higher than in roosters (88%) but the difference was small (chapter 3). Apparent protein digestibilities of kiwi was within the range of values (83-97%) reported for insectivorous species (Troyer, 1984; Webb *et al.*, 1993; Graffam *et al.*, 1998; Stannard & Old, 2012) and at the higher end of the range (56-97%) for a range of herbivorous, carnivorous and omnivorous species with markedly different protein intakes (Wisker & Knudsen, 2003; Vhile *et al.*, 2005; Vester *et al.*, 2010).

While the apparent digestibilities of fat were significantly lower in kiwi (91%) than roosters (94%) the difference again was minimal. These results are consistent with digestibility values of fat in other studies using roosters fed a diet of similar fat content (Maisonnier *et al.*, 2001; Honda *et al.*, 2009). The digestibility of fat in kiwi was lower than that reported for other insectivores (range = 97-98% (Webb *et al.*, 1993; Graffam *et al.*, 1998; Stannard & Old, 2012)) but within the range reported for a variety of carnivorous and omnivorous species with differing fat intakes (range = 87-97% (Angel, 1993; Burlikowska *et al.*, 2003; Wisker & Knudsen, 2003; Vhile *et al.*, 2005; Vester *et al.*, 2010)).

Here I found that kiwi digest both fat and protein with similar efficiency. As the wild diet of kiwi contains higher levels of protein (59%) compared to fat (22%) (Potter *et al*., unpublished) it appears that protein is the dominant energy source for kiwi (chapter 3).

NDF and ADF digestibility

Plant material is generally of little nutritional value (Grajal, 1995), difficult to digest, and bulky. The ability of birds to digest different forms of carbohydrate is not always dependent upon diet. For example, hoatzin, a strict folivore, is able to digest almost three quarters of the NDF contained in the diet (Grajal, 1995) whereas ostriches, a strict herbivore, digest less than half that in their diet (Nizza & Di Meo, 2000) and emu, an omnivore, has digestibility values similar to that of ostriches (Herd & Dawson, 1984). However, with the more indigestible ADF, the literature suggests that the greater the proportion of this fibre in the diet the greater the digestibility values. The problem with comparisons across studies is that carbohydrate content differs with plant species (Knudsen, 1997). Furthermore, the proportion of hemicelluloses, cellulose and lignin in the NDF and ADF categories can vary with diet. Thus, caution is needed when comparing carbohydrate digestibilities across taxa. This is especially true in species such as kiwi whose diet comprises carbohydrate of diverse forms, for example, carbohydrate from invertebrates and plant material.

Plant material and kiwi

Along with the longstanding debate about what kiwi consume in the wild, is debate over the role of plant material in their diet. Based on the frequency of fruit stones in their faecal matter (Kleinpaste, 1990), it is now accepted that kiwi deliberately

consume fruit. This study (chapter 3) has found that with the exception of roosters, the apparent digestibility of NDF and ADF in kiwi (33% and 26% respectively) is relatively low compared with medium and large bodied avian species with similar NDF and ADF intakes (range of NDF = 39-71% (Herd & Dawson, 1984; Grajal, 1995; Nizza & Di Meo, 2000), range of ADF = 40-57% (Grajal, 1995; Graffam *et al.*, 1998; Nizza & Di Meo, 2000)).

Diets high in roughage are also important to promote passage of digesta through the intestinal tract, particularly for species prone to colic. For example, a constant supply of high-fibre roughage is used as a dietary prophalytic measure against colic in captive elephants as it continuously stimulates gut peristalsis (Hatt & Clauss, 2006). In addition, increasing the content of roughage in a diet can help reduce obesity by filling the stomach with low energy feed (D'Eath *et al.*, 2009) and promoting satiety.

NDF and ADF contents of the wild diet of kiwi were surprisingly high (21% and 15% respectively) given their relatively low apparent digestibilities. This apparent contradiction in intake versus digestibility raises the question of why kiwi consume plant material at all. Here (chapter 3) I suggest that kiwi ingest fruit for the nutrients they gain from simple sugars (fructose, glucose) contained within the flesh rather than for nutrients extracted from the complex carbohydrates contained within the skin or seed coating.

Chitin

Another issue of debate is the extent to which kiwi use the high levels of chitin contained in their diet. Arthropods, which contain high levels of chitin in their exoskeleton (Galyean, M.L. & Defoor, P.J., 2003), make up 60% of the diet of kiwi (Potter et al., unpublished). For kiwi, neither the digestibility of chitin nor the presence of chitinase has been established, although the possibility that caeca may function in chitin digestion has been suggested (Potter *et al.*, 2006). Here I used titanium dioxide as an indigestible marker (instead of the indigestible lignin), providing me with the opportunity to evaluate whether the values obtained for lignin where comprised solely of lignin or an additional material, such as chitin. I did not directly measure chitin in this study as I did not have access to an assay specific to chitin. However, I did attempt to measure it indirectly using lignin assays.

The data obtained here suggested that some of what was assayed as lignin was actually being digested by kiwi. This seems improbable given that lignin is reported as

being indigestible. It is far more likely that this apparent digestion of lignin represents the digestion of chitin that was not distinguished from lignin in the lignin assay.

Chitinolytic activity has been reported in chickens (Han *et al.*, 2000), seabirds (Jackson *et al.*, 1992), raptors (Akaki & Duke, 1999) and mammals (Boot *et al.*, 2001), thus it is conceivable that kiwi are also able to digest chitin. Two methods of chitinase production have been proposed: gut bacteria break down chitin (Bhattacharya *et al.*, 2007); or chitinase is produced by glands in the mucosa of the proventriculus, intestine and liver (Stemmler *et al.*, 1984; Suzuki *et al.*, 2002). Chitinolytic enzymes can also be introduced by ingested food (Gooday, 1990). In taxa that are known to contain chitinases and break down a considerable amount of chitin, it has been suggested that chitin itself is probably not an important source of nutrients (Jackson *et al.*, 1992). Rather, it is the nutrients acquired from the soft tissue, protected by the chitinous exoskeleton, that are sought (Akaki & Duke, 1999). It is unlikely that this is the case for kiwi. A highly muscular gizzard is involved in the mechanical breakdown of food items until they are small enough to enter the intestine. If only small digesta particles are able to enter the caeca and *if* these organs are the main site of chitin digestion (Potter *et al.*, 2006), then the soft tissue of prey items has already been exposed further up the digestive tract. Thus in my view, the caeca in kiwi function to break down very small pieces of chitin which in turn expose nutrients trapped within the polysaccharide matrix. When a suitable assay becomes available, I predict that chitinolytic enzymes will be detected in the caeca of kiwi.

In chapter 3 I give the first assessment of the nutrient composition and apparent digestibility of a wild diet of kiwi. While it is preferable to feed animals held in captivity a natural diet (Dierenfeld, 1997; Kirk Baer *et al.*, 2010), for some species, such as kiwi, it is difficult to collect the required dietary ingredients in sufficiently large quantities. In these cases it is necessary to formulate a diet of similar nutrient composition but comprised of readily available ingredients, i.e. a synthetic diet. Chapters 4 and 5 detail the processes involved in developing a synthetic diet for captive kiwi that has a nutrient composition that closely matches that of the natural diet and is palatable to kiwi. The challenges faced in this process are outlined below.

Development of a synthetic diet

Palatability

A nutritionally adequate diet is not necessarily a palatable diet. The palatability of a diet has three aspects to it (chapter 5): whether the animal finds the odour attractive enough to motivate it to ingest the food; whether the animal can physically manipulate the food to get it into the mouth; and, when in the mouth, whether the taste and texture are acceptable. I encountered several challenges when developing a synthetic diet that was palatable to kiwi. Odour was the first hurdle to overcome as I needed the kiwi to at least want to pick up the food. Attaining an acceptable odour was complicated by the necessity to ensure that the diet was sterile. Initially we sterilised the diet by heat and pressure but the change in odour due to the heating process rendered the diet unappetising to kiwi. I therefore opted to use raw ingredients that had a milder odour than the cooked version and then I froze the diet immediately following homogenisation. Kiwi presented with this form of the diet were more willing to pick it up.

The other major challenge of formulating a palatable diet for kiwi was texture. Kiwi have an unusual feeding strategy; they pick up a food item using the tip of their bill, shake it from side to side, throw it in the air and swallow it (Minson, personal observation). Thus the texture of the diet must be such that it can withstand intense vibration, yet moist enough to slide down the throat. To obtain the required texture I added sodium alginate to the diet and then immersed worm-like pieces of this formulation into a calcium carbonate water bath. This process allowed the calcium carbonate ions to bind with sodium alginate to form a non-odorous, gelatinous-type coating on the product. This method of preparation proved to be more acceptable to kiwi that any of the previous formulations.

Thus, I finally succeeded in formulating a synthetic diet that was both nutritionally adequate and palatable to kiwi. Even then kiwi showed a surprisingly high level of neophobia to the new diet.

Neophobia and habituation to a diet

Neophobia to a new diet is not uncommon in animals habituated to a particular diet (Visalberghi *et al.*, 2002; Martin & Fitzgerald, 2005; Addessi *et al.*, 2007) and can be reduced by introducing novel foods in a familiar place or by adding a desirable

colour, fragrance or taste to the diet. Most of the kiwi used in the trials conducted here were neophobic to novel diets. To reduce this, I fed kiwi in their normal enclosures and added a range of flavours to the diets to disguise the odour. Kiwi were reluctant to consume the new diet even with flavours added, but enthusiastically ate their usual diet when the same flavours were added. However, when their usual diet was made into a similar form as the synthetic diet, i.e. worm-like strips, they were hesitant to eat it. Thus, for kiwi neophobia extends from novel smells to novel shapes of food.

To increase acceptance I initially fed a mixture of the synthetic and usual diets and progressively increased the proportion of the synthetic diet over time. For kiwi that are habituated to a particular captive diet, the acclimation period for the acceptance of a new diet may be extensive. It is recommended that captive facilities factor in neophobic reactions when planning to change a diet. Another approach is to introduce a new diet to chicks. While the synthetic diet formulated here is palatable to newly hatched kiwi chicks (Minson, personal observation), it has been formulated specifically as a maintenance diet for adult kiwi. Further research is therefore needed to determine the nutrient requirements of not only kiwi chicks but of all stages of life. In addition, as determined in chapter 4 for adult kiwi fed the synthetic diet, the apparent digestibility of any new diet fed to chicks, juveniles or reproducing females needs to be assessed.

Digestibility of the synthetic diet - kiwi

Knowledge of the apparent digestibility of the synthetic diet was necessary to ensure that the overall quantities of nutrients acquired by kiwi from this diet were similar to those acquired from the near-natural diet. This was particularly important as predictions of nutrient assimilation of the synthetic diet were difficult because dietary ingredients varied to those in the near-natural diet. Indeed the apparent digestibility of macronutrients was consistently higher in kiwi fed the synthetic diet compared to the near-natural diet (chapter 4). Thus as the nutrient composition of the two diets was similar, it appears that the composition of the food items contained within the diet affected its digestibility. The synthetic diet was comprised of vertebrate protein and fat and of highly processed carbohydrate, whereas the near-natural diet contained whole invertebrates and fruit. It is likely that the ingredients contained within the synthetic diet were more easily digested by kiwi than those in the near-natural diet. The greater efficiency with which kiwi digested both protein and fat, and thus acquired energy, from the synthetic diet indicates the potential for kiwi to overfeed which could lead to

obesity. This is a common problem with captive held animals fed diets comprised of human-grade, processed ingredients that are typically more easily digested than wild diets (McKenney *et al.*, 2010). The problem can be made worse because of reduced activity levels, therefore weight-related health issues need to be monitored carefully. Thus captive animals should be weighed regularly in order to detect changes in body weight and meal portions altered accordingly.

Digestibility of the synthetic diet – kiwi vs. roosters

Roosters were also fed the near-natural (chapter 3) and synthetic (chapter 4) diets and digestibility trials were conducted to provide a comparison with kiwi. In chapter 3 I reported that, with the exception of fat, NDF and ADF, kiwi fed the near-natural diet had higher apparent digestibilities of macronutrients than did the roosters. Similarly, kiwi fed the synthetic diet had consistently higher apparent digestibilities of all macronutrients compared with the roosters (chapter 4). Thus kiwi and roosters showed different responses to macronutrient digestibility depending upon dietary composition. This suggests that roosters are not ideal models for predicting how kiwi might respond to newly formulated diets.

Therefore, the most accurate method of determining apparent digestibilities of future modifications of the synthetic diet is to use kiwi, however gaining access to suitable numbers of birds for these trials may be difficult.

Problems with sample size

Small sample sizes have been a problem throughout this study. The captive population of kiwi is limited and only a small subset of this is available for research involving manipulation. The neophobic responses of kiwi to both the near-natural and synthetic diets made these limited initial samples sizes even smaller. Caution is therefore needed when extrapolating from the results; for example, for formulating diets for laying kiwi. Further trials with larger sample sizes are needed to improve the accuracy of digestibility estimates for kiwi.

A product of chapters 3, 4 and 5 was the formulation and development of a palatable synthetic diet for captive kiwi that is based on the nutrient composition of the wild diet of kiwi. The next step was to assess the role enteral bacteria play in the intestinal tract.

MICROFLORA

The gastrointestinal tract of all animals is populated by micro-organisms that play a vital role in the normal nutritional, physiological and immunological functions of the host (Mackie, 2002; Mrázek *et al.*, 2008; Xu *et al.*, 2010). Interactions between the host and their enteral bacteria are complex and bacterial community structure differs along the intestinal tract (Mackie, 2002; McKenna *et al.*, 2008). These communities are made up of resident autochthonous and transient allochthonous bacteria that can be both beneficial and/or pathogenic to the host (Sonnenburg *et al.*, 2004; Neish, 2009). Given the importance of intestinal microflora to health, it is important to understand the relationship between bacterial communities and their host, as well as factors that influence this association. There has been extensive research into the intestinal microbiota of humans (Backhed *et al.*, 2005; Kelly *et al.*, 2007; Neish, 2009; Hooper & Macpherson, 2010), 'higher' non-human primates (Xu *et al.*, 2010), production animals (Mulder *et al.*, 2009; Metzler-Zebel *et al.*, 2010) and poultry (Barnes, 1972; Edelman *et al.*, 2002; Hume *et al.*, 2003; Schneitz, 2005), but very limited research on other species, particularly endangered ones. Chapters 6, 7 and 8 give the first assessment of enteral bacterial diversity in three native New Zealand avian species. Here I consider the influence of captive status, diet, and acquisition and colonisation on the diversity of enteral bacteria.

Microflora of captive versus wild animals

Animals managed *ex situ* are physically removed from their natural habitat, and are consequently exposed to environments, including bacterial communities, that are not indigenous to their native range (Villers *et al.*, 2008). Hence, it is reasonable to anticipate that the composition of intestinal bacterial communities differs between captive and wild individuals of the same species. Supportive evidence for this was presented in chapter 6 where I provided the first documented evidence of differences in enteral bacterial communities due to captive status in three native New Zealand avian species; the kiwi, the takahe and the brown teal. The trend was consistent across these species, with captive birds having a greater generic diversity of intestinal bacteria than did wild birds. Similar differences have been reported in the major intestinal flora of captive and wild ring-tailed lemurs (*Lemur catta*) (Villers *et al.*, 2008). Potential drivers of these differences in generic enteral microfloral diversity between captive and wild

birds are diet and differences in the probabilities of encountering faecal material from other species.

Here (chapters 6, 7 and 8), male and female kiwi were analysed together, thus a difference in intestinal bacteria due to the sex of the bird was not investigated. Differences in enteral bacterial composition between males and females have been reported in other species, for example, rhesus macaques (*Macaca mulatta*) (McKenna *et al.*, 2008), mice (Schloss & Handelsman, 2006) and turkeys (Scupham *et al.*, 2008). Such sex-related differences in intestinal microfloral community structure should be investigated further.

Whether the observed differences in the generic intestinal microfloral diversity affects the health of captive kiwi, brown teal or takahe has not been directly ascertained. However, what is known is that kiwi held in captivity suffer lower fertility rates, lay smaller eggs and hatch proportionally fewer healthy chicks than wild kiwi (McLennan, 1996). Furthermore, some captive brown teal that were released into the wild were known to have starved post-release despite having sufficient natural food sources on which to forage (Moore & Battley, 2003). Many more studies have demonstrated the link between enteral microflora and health (Ferguson *et al.*, 2007) and assessment of whether there is a direct impact on health of captive-held kiwi, takahe or brown teal needs to be investigated. Some consequences of disruption to intestinal bacterial communities are discussed below.

Dysbiosis

A disruption in the normal balance of the intestinal ecosystem, termed dysbiosis, can cause minor, usually non-pathogenic bacteria to proliferate within their normal habitat or to move to new sites where the host is unable to accommodate or tolerate the new coloniser, resulting in an autoimmune response (Falk *et al.*, 1998; Mwangi *et al.*, 2010). A common form of inflammatory bowel disease, Crohn's disease, is thought to be caused by enteral microflora triggering the immune system, leading to the intestinal inflammation characteristic of this disease (Seksik *et al.*, 2006; Sokol *et al.*, 2008). Germfree mice have altered intestinal morphology and function, including slower epithelial cell regeneration, longer and thinner microvilli, and fewer crypts and lymphocytes (McCracken & Lorenz, 2001). The composition of intestinal bacterial communities in diseased rhesus macaques differed significantly from those of healthy animals (McKenna *et al.*, 2008).

Dysbiosis has been reported to be a useful biomarker and a potential trigger for disease (Hill & Artis, 2010). Thus, a difference in enteral bacterial communities between captive and wild birds (chapter 6) should be of concern to captive management. Adult birds were sampled in this study, thus it is unclear at what stage of life the bacterial communities deviated from a composition similar to that of wild birds. It is likely that this phenomenon commenced either at hatch (chapter 6) or with the introduction of a captive diet (chapter 6). The process of acquisition and colonisation of enteral bacteria differs across taxa.

Acquisition and colonisation in mammals and birds

Mammals

At birth or hatch the intestinal tract of young is relatively sterile (Pedroso *et al.*, 2005). For mammalian young, the immediate sources of bacteria are the birth canal, rectum and mammary glands (Kelly *et al.*, 2007). The normal development of the intestinal immune system depends largely on the colonisation of suitable microflora (Mwangi *et al.*, 2010). Caesarean delivery affects the colonisation process by decreasing the exposure of newborns to bacteria along the birth canal (Kelly *et al.*, 2007). Disruption of the development of the immune system can lead to chronic diseases later in life, such as asthma and allergies (Huffnagle, 2010).

Birds

Newly hatched avian young acquire their bacteria from eggshells and the excreta of adult birds (Schneitz, 2005). The first microflora that settle often hamper others from settling and colonising (Gabriel *et al.*, 2006), thus, it is vital that young are exposed to a range of bacteria common to healthy adults of their species.

Enteral bacterial colonisation in captive and wild animals

In wild birds, enteral bacteria are acquired from the surrounding environment (Van der Wielen *et al.*, 2002), including from pecking their own and conspecific's eggshells, faecal material from siblings and parents, nest material and dietary items. In captivity, as with the kiwi chicks used in this study (chapter 7), newly hatched chicks often have limited or no contact with conspecifics, siblings or adults. This practice aims is to reduce the incidence of disease in young animals, but it also prevents the 'normal'

colonisation of bacteria. While few studies have focussed specifically on the effect of husbandry practices on enteral microfloral colonisation in endangered species held in captivity, many studies have detailed the link between environmental exposure and intestinal microfloral composition of juvenile (Van der Wielen *et al.*, 2002) and adult (Mulder *et al.*, 2009) poultry. Furthermore, poultry chicks exposed to faecal bacteria from healthy adults have been shown to acquire early protection against pathogenic intestinal bacteria (Nurmi & Rantala, 1973).

Influencing enteral bacterial colonisation in birds

As the intestinal tract of newly-hatched chicks is germfree (Sklan, 2005) and bacteria are ingested through pecking the eggshell, the application of specific bacteria to the surface of the shell should influence colonisation (chapter 7). However, the methods used in this thesis did not result in the predicted enteral bacterial compositions. That is, the enteral bacterial communities of one day to three week old chicken chicks whose eggs had been painted prior to hatch with thick bands of either healthy hen excreta or pure lactobacillus culture did not significantly differ from those whose eggs had been painted with distilled water. Furthermore, no enteral bacteria were detected in the caeca of day-old chicks (thus confirming aseptic sampling methods). Similar results of sterility have been reported for the small and large intestine, and caeca of newly hatched chickens (Elsayed, 2002). The diversity of enteral microflora in poultry is most extensive in the caeca (Mead, 1997). The function of the gastrointestinal tract in the first hours post-hatch is likely to be low. Young chicks absorb nutrients from the yolk sac in the hours, and for some species days, after hatch, thus limiting the function of the intestinal tract. The post-hatch period in which enteral bacteria are first detected in the digestive tract varies across taxa. For example, in the experiments conducted here (chapter 7), no enteral bacteria were detected in the caeca of newly hatched poultry chicks, whereas a low diversity of bacteria was found in the first faecal material of kiwi chicks. With the ingestion of solid food and interactions with the immediate environment, bacterial acquisition and colonisation will commence in all animals, but the duration of this process will differ across taxa.

Colonising enteral bacteria

The pattern of colonisation, that is, the process involved when bacteria compete for niches and resources, is dynamic. Some enteral bacteria are specialised primary

succession species, that is, they are fast growing, well-dispersed and opportunistic, whereas others favour conditions specific to secondary succession, and are more competitive. Thus, throughout the enteral bacterial community development, the relative proportions of some bacteria remain stable, some decrease and some increase in density. For example, in poultry, populations of lactobacilli and proteobacteria decrease after the first week of colonisation, whereas bifidobacteria numbers increase (Amit-Romach *et al.*, 2004). The current study (chapter 7) also found a shift in the composition of caecal bacteria in poultry chicks over the first few weeks of life. Enteral bacterial community diversity was significantly higher in week-old than in day-old chicken chicks. From then on the diversity of enteral bacterial communities did not change but community structure did; there were more dominant bacterial genera in three week compared to one week old chicks. Similarly, the community structure of enteral bacteria in kiwi fluctuated over the first three weeks of life, by which stage the community structure did not differ markedly from that of captive adult kiwi. Thus, the first three weeks of a kiwi chick's and chicken chick's life are the most important to influence the colonisation, and ultimate structure, of the bacterial community within the intestinal tract. Following the initial colonisation events, the enteral bacterial community structure fluctuates until it stabilises into that of an 'adult' community, from this point the major influence on enteral bacterial community structure is likely to be diet (chapter 8).

Effect of diet on enteral microflora

The environment in the digestive tract of animals with different diet bases can differ markedly (Apajalahti *et al.*, 2004; Ringø *et al.*, 2006). The diet, and hence the quantity and quality of digesta, affects the structure of the intestinal bacterial community (Backus *et al.*, 2002; Apajalahti *et al.*, 2004; Bjerrum *et al.*, 2005; Blanco *et al.*, 2006; Flint *et al.*, 2007; Turnbaugh *et al.*, 2009; De Filippo *et al.*, 2010). In the current study (chapters 6 and 8), the ingestion of a captive diet by both kiwi and takahe resulted in an intestinal environment that had greater numbers and higher diversity of bacterial genera than kiwi and takahe that consumed a wild diet. Furthermore, takahe fed both a captive and wild diet possessed a suite of enteral bacteria that was intermediate to that of their captive and wild counterparts. Similar effects of diet on enteral bacterial composition have been found in pigs where a high fibre diet increased the numbers of fibrolytic bacteria (Varel *et al.*, 1987), in poultry where a whole-wheat diet resulted in lower numbers of enteral bacteria than a pelleted diet (Bjerrum *et al.*,

2005), in turkeys where corn-based diets resulted in lower diversities of intestinal bacteria than wheat-based diets (Santos, A. A. J. *et al.*, 2008). Enteral bacteria benefit from a stable nutrient supply (Neish, 2009), thus a sudden change in dietary intake and/or nutrient concentration may disrupt the balance of bacterial species in the intestinal tract and lead to either reduced acquisition of nutrients, or in severe cases, disease.

If the growth requirements of bacteria are species-specific, then it may be possible to alter microbial community structure by changing the diet, which in turn affects the intestinal environment, and thus, gut dynamics (Apajalahti *et al.*, 2004). The current study (chapter 8) attempted to influence the composition of enteral bacteria in kiwi by gradually changing their diet from a captive to a natural one. As kiwi consumed more of the natural diet and less of the captive diet, the number of dominant enteral bacteria decreased, thus the overall diversity of the bacterial community became more even. The length of time needed to significantly change the composition of enteral bacteria through dietary manipulation varies across species (Varel *et al.*, 1987; Leser *et al.*, 2000; Backus *et al.*, 2002; Turnbaugh *et al.*, 2009). Given a greater length of time on the near-natural diet, it is likely that the enteral bacteria in captive kiwi would show a greater deviation in diversity from that when they are fed solely the captive diet. These findings highlight the possibility of altering, and thus preparing, the bacterial communities within the digestive tract of captive-held animals for the digestion of a natural diet. Hence, in the lead up to the release of captive animals into the wild, it would be beneficial for their health, and thus their post-release survival, to slowly introduce a more natural diet. A similar technique will be required when wild animals are brought into captivity, that is, a mixture of the natural and captive diets need to be fed in the first instance, with the proportion of natural food progressively reduced over time.

RECOMMENDATIONS AND DIRECTIONS FOR FUTURE RESEARCH

The research undertaken for this thesis has highlighted specific issues relating to the management of animals in captivity. The following section provides recommendations for conservation management and identifies directions for future research.

Palatability

Palatability was a constant issue throughout the formulation of a synthetic diet for kiwi (chapter 4 and 5). Captive kiwi were neophobic to novel diets and this seemed to be triggered both by novel odours and by novel textures. Even an homogenous form of their usual diet markedly reduced acceptance. To reduce habituation to a diet and to minimise palatability issues with a novel diet, the following strategies are recommended:

- avoid feeding exactly the same diet each day by supplementing it with seasonal and natural food items as far as is practicable
- vary feeding locations daily
- slowly introduce a new diet over several months (or longer if needed) -
    - mix in greater proportions of the new diet and reduce the proportions of the normal diet every few days
    - if a preferred odour is identified, add this to the new diet and reduce levels over time
    - if possible, introduce the new diet to animals at a young age

Refinement of the synthetic diet

The synthetic diet formulated here (chapter 4) is a maintenance diet for adult, non-reproducing kiwi. Thus, it is a starting point from which further refinements can be made. Formulations of the synthetic diet and assessment of its digestibility was based largely on macronutrient analysis, so further research is needed into the micronutrient requirements of kiwi. Ideally, the diet should be reassessed and improved on an on-going basis.

Age and stage-specific diets

In general, kiwi are currently fed the same diet in captivity regardless of their age or life stage. For species in which extensive nutritional research has been conducted, namely production animals, each stage of life requires a specific dietary formulation. Thus, even though kiwi of all ages and reproductive stages survive on current captive diets, it is likely that specific nutrients are required in different proportions at different times in their lives. Therefore, additional research is needed into the nutrient requirements of chicks, juveniles, laying females and incubating males. If the maintenance diet is fed to all kiwi, close monitoring of weight and general health is recommended.

Obesity in captive animals

Obesity can be a major problem with captive animals, particularly due to reduced energy expenditure compared with free-roaming animals. Thus, dietary intake in captive animals needs to be monitored carefully and intake adjusted accordingly. In addition, research into possible seasonal influences on dietary intake and fat deposition in captive-held animals is needed.

Preparation for release of captive animals into the wild

While this thesis has focussed on the nutrient requirements of kiwi held in captivity, it is equally important to consider the management of birds that are to be released into the wild. Every effort has been made to formulate a diet for captive kiwi based on the nutrient composition and digestibility of the natural diet; however this resulted in a synthetic diet consisting of ingredients not sourced from the natural environment, i.e. ingredients that are foreign to kiwi in the wild. Thus, as captive kiwi need to be slowly introduced to this new synthetic diet, birds targeted for release into the wild also need to become accustomed to a wholly natural diet. Techniques to encourage a smooth transition from the captive to a natural diet include:

- including natural food items into the daily diet of all animals, regardless of whether they are to be released into the wild or not.
- over time, increasing the proportion of natural food items in the captive diet and decreasing the proportion of the normal captive diet fed to animals targeted for release into the wild.

Establishing suitable enteral bacterial communities in young

The importance of enteral bacteria for immune development and digestion has been continually highlighted throughout this thesis (chapters 6, 7 and 8). While the relevance of this field of research to human and production-animal health is widely acknowledged, its relevance to wildlife management is only now being recognised, and it is an entirely new concept when it comes to the captive management of New Zealand wildlife. Only superficial studies were possible in this thesis, but these studies should encourage further, more detailed research into the relevance of enteral bacteria to the health of wildlife in captivity. Some recommendations that derive from this thesis and that are applicable across species are:

- ensuring young have access to appropriate bacteria immediately after birth/hatch
- add nest litter and/or healthy adult faecal material to areas with young
- avoid placing young in disinfected and sterilised areas
- allow young to have contact with adults, siblings and conspecifics if possible
- avoid antibiotic use if possible

Future studies need to identify the normal structure of enteral microfloral communities in other native species. In addition, an understanding of if, how, and why these communities change throughout life is important for captive management programmes. Such data would be useful in establishing species-specific databases. These databases would be of benefit in comparisons of enteral bacterial communities of captive and wild animals, and could be a starting point to determine whether a deviation from a 'normal' enteral bacteria community structure could be useful as an indicator of ill health.

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APPENDIX 1

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Charlotte Minson

Name/Title of Principal Supervisor: Dr Murray Potter

Name of Published Research Output and full reference:

Potter, M. A., Hendriks, W. H., Lentle, R. G., Thomas, D. V., Minson, C. J., & Pindur, N. B. (2010). An exploratory analysis of the suitability of diets fed to a flightless insectivore, the North Island brown kiwi (Apteryx mantelli), in New Zealand. *Zoo Biology*, *29*(5), 537-550.

MASSEY UNIVERSITY
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In which Chapter is the Published Work:

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate:

and

/ or

- Describe the contribution that the candidate has made to the Published Work:

Candidate's Signature

Date

Principal Supervisor's signature

Date

APPENDIX 2

NEW KIWI VITAMIN PREMIX FORMULA

In early 2000 the Westshore Wildlife Reserve spearheaded the development and production of a new Kiwi Vitamin Premix.

Development of this dietary supplement was done in conjunction with Bomac Laboratories Ltd. and Animalz Veterinary Clinic. This new premix formula will replace the seriously outdated formula currently in use elsewhere in many Kiwi facilities. The old formula was originally designed 30 years ago, and ironically is no more that a modified cat formula!

Back then, the development of a Kiwi dietary supplement was basically a "shot gun" approach. The primary aim was to introduce calcium into an artificial diet and to provide other trace nutrients at "best guess" levels, based on poultry and carnivore data and in discussion with Otorohanga and Auckland Zoos.

In the development of this formula, the entire chemical spectrum was critically analysed and constructed, so that it is specifically more applicable to the dietary needs of Kiwis.

Bomac Laboratories studied the current kiwi supplement in the light of today's recommendations for poultry, game birds and ostrich/emu. None of these can be said to be close to Kiwi in aspects of physiology, diet or habitat, but it's probably the best comparison we had.

It was assumed that nocturnal life and forest habitat means kiwis have either very low vitamin D requirements or are super-efficient at synthesizing this vitamin.

Calcium demand for egg shell was also determined to be somewhat different to domestic species. Layer poultry have a constant daily calcium requirement for eggs shells over an 18 months period. Ostriches have a more intermittent calcium requirement for production of up to 100 eggs over a 6-8 month period.

Contrast this to Kiwis laying one very large egg every 3-8 weeks during the breeding season. This egg may have 20-25g of shell or 8-10g calcium. We were unsure as to how much calcium comes from current diet and how much comes from body reserves, or even if wild kiwis change their eating habits prior to egg lay in order to boost calcium intake.

Modern layers require around 4 grams of dietary calcium per day. This is 6-7 times more than the 600mg supplied by the current kiwi supplement. 600mg may be sufficient if it's to build body reserves between eggs.

On the trace nutrient side we needed to raise manganese, Zinc, B_{12}, B_2 and Niacin. Thiamine was reduced. The old level was the requirement of cats, on which the premix supplement was founded. Additional additives considered, which fringe the supplement side and are related to egg size, were Linoleic acid and Methionine.

Revised Kiwi Premix Formula

Recommended dose rate is 1 gram Premix per 100 grams of food.

Calcium	1.47	grams
Manganese	10	mg
Iodine	0.1	mg
Iron	5.0	mg
Copper	1.0	mg
Cobalt	0.1	mg
Zinc	10.0	mg
Selenium	0.03	mg
Vitamin A	1600	iu
Vitamin D3	250	iu
Vitamin E	12	iu
Thiamine	0.4	mg
Riboflavin	1.5	mg
Pyridoxine	0.5	mg
Vitamin B12	0.15	mg
*Ascorbic acid	10.0	mg
Vitamin K3	0.5	mg
Biotin	0.04	mg
Niacin	7.0	mg
Pantothenic acid	3.0	mg
Choline	75.0	mg
Folic acid	0.2	mg
* Inositol	20.0	mg
Antioxidant	2.0	mg

* May not be required but inclusion will do no harm.

Once the formula development was completed the next step was to produce and trial the new compound. The premix powder is now vacuum sealed in individual 500gm foil bags, for both ease of handling and extended viable shelf life.

Trials on all kiwi held at the Westshore Wildlife Reserve indicate that the new premix formula is more than successful. Issues such as palatability, ease of mixing into artificial diets and improved growth rates on chicks appear to be some of the primary benefits to date.

As we do not know for sure what a typical daily dietary regime is for Kiwis, (i.e. what proportion of the diet is worms, insects, vegetation, soil etc), it was difficult to accurately assess additive requirements and levels. This highlights the need for further research in order to ascertain this information.

This new formula, however, is a big improvement on the current old cat formula. As we gradually learn more on the Kiwi's typical daily dietary regime we can then easily modify this formula further.

Production and distribution of the new Kiwi Vitamin Premix formula is being co-ordinated by the Westshore Wildlife Reserve. Should anyone be interested in ordering a supply of the new formula or wishes to discuss this matter further please contact me on either:-

Phone: - 06 834 4136
0274 488 094 or

E-mail me at – tonyb@napier.govt.nz

TONY BILLING

WESTSHORE WILDLIFE RESERVE

"For as this appalling ocean surrounds the verdant land, so in the soul of man lies one insular Tahiti, full of peace and joy, but encompassed by all of the horrors of the half-lived life."

from Moby Dick by Herman Melville, 1851