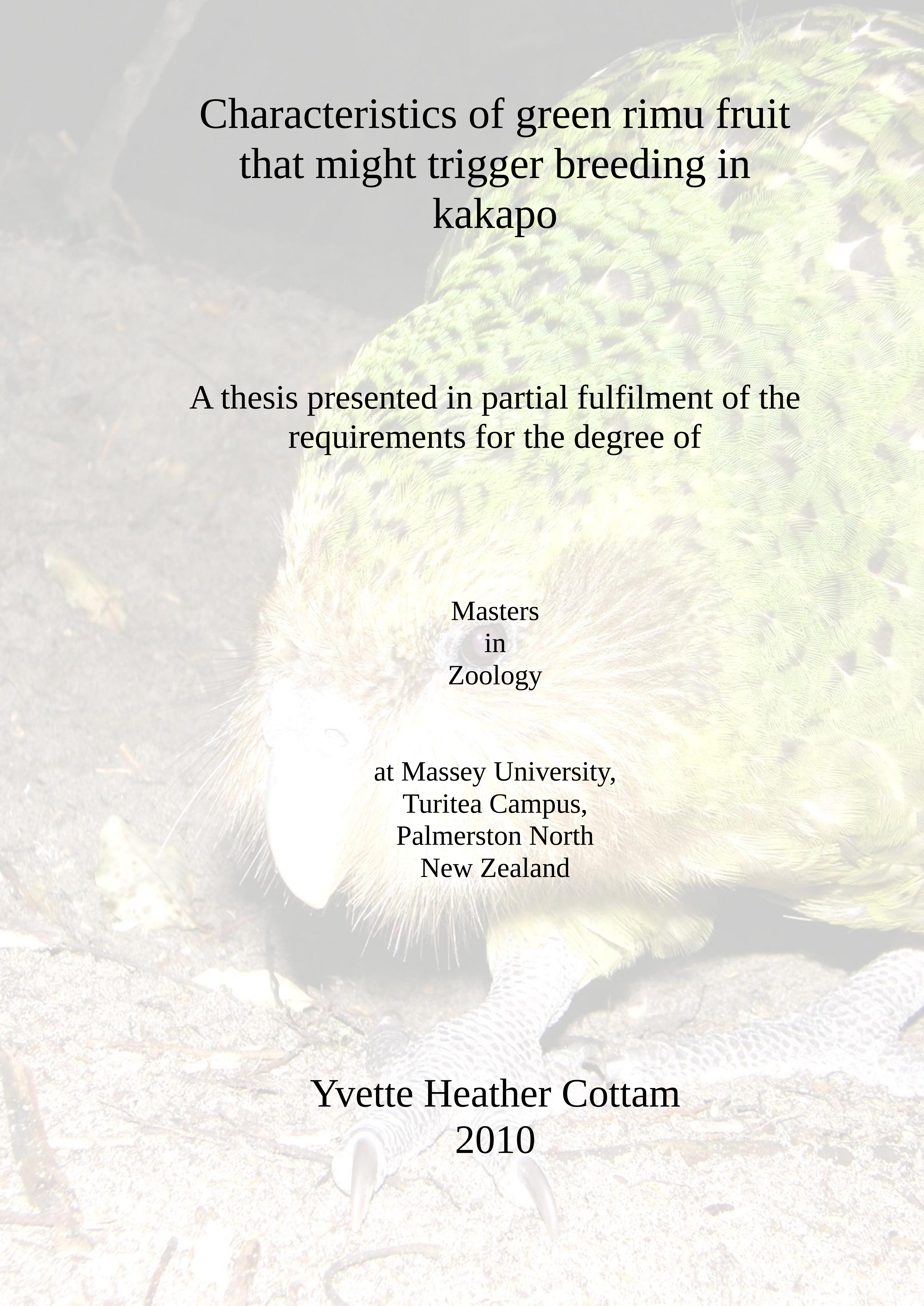

Characteristics of green rimu fruit that might trigger breeding in kakapo

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ABSTRACT

Species living in unpredictable habitats cannot prepare for a regular reproductive year, and must reproduce when conditions are favourable. The timing of reproduction of the seed predators involves anticipating an abundant food supply by tracking cues or triggers which initiate reproduction. These cues are poorly understood. The most supported theory is that a cue or cues is the size or abundance of the developing fruit crop, and possibly involves nutritional compounds in the crop.

The rimu (*Dacrydium cupressinum*) is a masting podocarp tree strongly linked to reproduction in kakapo (*Strigops habroptilus*). On Codfish Island kakapo only breed in rimu mast years and nesting is timed so chicks hatch when the rimu fruit crop (the sole food source for chicks) is due to ripen. This thesis investigates the theory that some component in unripe rimu fruit is involved in timing of nesting in kakapo. I characterised seasonal changes (over one year) in nutritional components (gross nutrients), polyphenolic compounds and other potential target polyphenolic substances with possible hormonal activity, including isoflavones and antioxidants, during rimu fruit development.

A number of the compounds found in rimu fruit are known to affect reproduction in other species and could be potential triggers for reproduction in kakapo. In non-mast years insufficient fruit is available to trigger reproduction and there is no breeding, supporting the idea that the abundance of the fruit crop is also involved in the timing of reproduction. The trigger for breeding in these birds could be ingestion of a threshold level of a trigger substance or substances, or, as in the antbird, it could be entirely visual. The most likely contenders for the trigger in green rimu fruit include increasing levels of crude protein, crude fat, individual fatty acids, dietary fibre (changing the digestibility of other nutrients), calcium, phosphorus, calcium, total polyphenolic content and antioxidant levels, some flavenoids or a combination of these.

It is possible that the physiological changes necessary for reproduction are triggered by the abundance of the unripe fruit crop but that nesting itself is triggered by changes in the green, undeveloped fruit associated with ripening.

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BOG
RIMU
LOOP
JEM

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SECTION 1: COMPARATIVE OVERVIEW OF TRIGGERS TO BREEDING

1. Introduction

Species living in unpredictable habitats, such as those prone to drought or those with an intermittent food supply, cannot prepare for a regular reproductive year, and must reproduce when conditions are favourable. An example of an unpredictable habitat is plant species that mast, producing intermittent very heavy crops of seeds, and thereby providing a food source for seed predators. The timing of reproduction of the seed predators is often determined precisely to maximise reproductive success, and involves tracking cues or triggers which initiate reproduction e.g. blue tits (*Parus caeruleus*) and wild canaries (*Serinus canaria*) (Leitner *et al.*, 2003).

Many organisms initiate reproduction before regular seasonal resource peaks, but irregular resource pulses, such as provided by masting species, represent a unique challenge because future resources are often not correlated with past or current availability. Nonetheless, several avian seed predators appear able to anticipate mast years and initiate reproduction before masting events. Examples of birds exhibiting anticipatory reproduction include the red crossbill (*Loxia curvirostra*), the piñon jay (*Gymnorhinus cyanoephalus*) (Ligon, 1978) and the great tit (*Parus major*) (Nager & van Noordwijk, 1995). Anticipating mast years would require both the perception of reliable cues indicating the upcoming mast event and the capacity to increase reproductive investment at times of limited food supply (i.e. so the peak food supply appears when the chicks are in the nest). The animal needs to track environmental cues to provide information on when successful reproduction is most likely. While it is unclear what cues birds use to fine-tune sexual development and trigger reproduction, it is likely that a complex suite of inter-related cues is involved.

The rimu (*Dacrydium cupressinum*) is a masting podocarp tree species strongly linked to reproduction in kakapo (*Strigops habroptilus*). On Codfish Island kakapo only breed in rimu mast years and nesting is timed so that the chicks hatch when the rimu fruit crop (the sole food source for chicks) is due to ripen. Kakapo have also bred in the presence of other food species. For example, Reischek noted in 1890 that "the Maoris maintain that kakapo breed only once every 5 years, when fruits of *Taphyra trycinetta* are ripe" (*Taphyra trycinetta* was identified as kiekie, *Freycintia banksii*) (Westerskov, 1981). As in other avian species, it is unclear what triggers reproduction in the kakapo. An investigation into the possible identity of nutritional triggers to breeding in kakapo could aid the recovery of this endangered species, if this trigger could be identified and provided.

A number of authors have investigated possible triggers for reproduction in a number of different species. A summary of the latest scientific investigations are presented in the following sections. These investigations have focussed mainly on avian species, and have lead to the conclusion that, although there are several kinds of environmental cues that may serve as triggers, there is probably no generalised trigger for breeding across all species.

2. Bird Reproduction

Photoperiod is the initial predictive cue which initiates (or terminates) reproduction in many non-tropical birds (Ball & Ketterson, 2009). As photoperiod increases in spring, the hypothalamo-pituitary gonadal axis (HPG axis) is stimulated leading to the secretion of reproductive hormones and growth of reproductive organs, preparing the bird for reproduction and initiating the process which later results in regression of the gonadal portion of the HPG axis (Hau *et al.*, 2004). This can be a function of how many long days the bird has experienced as well as lengthening daylight. Gonadotropin-releasing hormone (GnRH) regulates the release of leutinising hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland. There is a decrease in the amount of GnRH with the onset of photorefractoriness, and an increase in LH with photostimulation. The state of photosensitivity is when the avian reproductive system responds to long days with a dramatic increase in reproductive physiology components like gonadal size and hormone secretion. Photorefractoriness is the state when there is no response to different day length. Photorefractoriness discontinues with short days, when photosensitivity is acquired. Short days reset the system, so birds can respond to stimulatory photoperiod and other cues.

The sensor for the photoperiodic response in birds is thought to be in the hypothalamic region in the brain. It is thought that the response of the supplementary cues is mediated by sensory receptors in the visual and auditory systems, totally separate from the photosensory receptors in the brain (Ball & Ketterson, 2009).

Initiation of avian reproduction is timed to maximise reproductive success while minimising risk to survival. Birds must start their breeding cycle when food supplies are limited, well in advance of the time when food is most abundant, which generally coincides with when young are in the nest (Perrins, 1996). The female must assess the state of nest sites, and the availability of resources to sustain her during incubation and while her young are dependent. She must then get into breeding condition, which involves developing the reproductive organs from the regressed non-breeding state into the egg production stage at a time of limited food supply. This is challenging for birds living in unpredictable habitats as these cannot prepare for a regular reproductive year and must respond opportunistically to reproduce when conditions are favourable (Hau *et al.*, 2004). It is also important that the attainment of breeding condition in males and females is in synchrony so that the sexes are ready to reproduce at the same time.

Little is known about whether the sexes differ in their response to environmental cues controlling the timing of seasonal breeding (Ball & Ketterson, 2009). However, because the measure of reproductive fitness differs between males and females the sexes are expected to differ in the cues they use to time reproduction. In males, reproductive fitness varies as a function of fecundity, but in females it varies as a function of sexual selection. Females have more at stake in reproductive timing and they make the final reproductive decision (Ball & Ketterson, 2009). Males need cues to trigger the recrudescence of reproductive organs (i.e. to reduce the metabolic cost of carrying around non-functioning organs), and the accompanying territory establishment and courtship. Females need cues to acquire the resources required for egg production and incubation. Female gonads also increase in size considerably prior to egg formation (Perrins, 1970), with associated metabolic costs of carrying them around. There is evidence in a number of avian species that males need only photoperiodic stimulation for full gonadal development, whereas females need both long photoperiods and supplementary factors for follicles to develop (Ball & Ketterson, 2009). Therefore, supplementary cues are more important in regulating reproductive endocrine responses in females than males. In the kakapo, photoperiod alone does not trigger breeding. Male kakapo boom (the mating display) most years but boom much more intensively in nesting years, so are obviously picking up proximate cues. There is evidence that kakapo respond to photoperiod just like other birds (Cockrem, 2006) but nesting is still contingent on ultimate factors from a range of plant species.

It is possible that the sexes may respond differently to secondary cues, with one sex stimulated and the other inhibited, or the degree of response to the same cue could differ (Ball & Ketterson, 2009). Species in which the sexes exhibit similar roles in reproduction have greater similarity in their response to supplementary cues to time onset, prolongation and termination of reproduction. Birds such as the kakapo in which the sexes have very divergent breeding behaviour probably respond to less similar cues, or have differential sensitivity to these cues.

Excess fat (energy storage) is known to interfere with normal regulation of the reproductive axis in mammals (Caprio *et al.*, 2001). Leptin is a protein hormone produced by white fat cells (adiposites) and circulates in the blood stream at levels correlated with the amount of fat in the body, linking the peripheral energy deposits with the central nervous system (Lõhmus & Bjõrklind, 2009). It was discovered in 1994 (Duggal *et al.*, 2000), and stimulates the release of reproductive hormones GnRH from the hypothalamus (Caprio *et al.*, 2001) and LH and FSH from the anterior pituitary (Lõhmus & Bjõrklind, 2009). Its effects on the HPG axis involve multiple biochemical pathways and various tissues (Caprio *et al.*, 2001). Leptin plays a role in regulating food intake and reproduction (Duggal *et al.*, 2000), acts on all levels of the reproductive axis and plays a critical role in the allocation of resources between metabolism and reproduction.

Reports concerning the biological role of leptin in birds are scarce (Paczoska-Eliasiewicz *et al.*, 2006) and the actual existence of an avian leptin gene remains controversial (Ohkubo & Adachi, 2008). A number of authors have found that leptin administration decreases food intake in chickens (Denbow *et al.*, 2000) and wild birds

(Lõhmus *et al.*, 2003). Leptin administration significantly advanced puberty in immature female chickens (Paczoska-Eliasiewicz *et al.*, 2006). Treating female passerine birds with leptin resulted in these being significantly more likely to have a second clutch of chicks (Lõhmus & Bjõrklind, 2009). This suggests that leptin can alter a female's perception of her energy status, making her think she is in better condition than she actually is. In contrast, systemic leptin administration inhibits ovulation in rats but the mechanism remains unclear (Duggal *et al.*, 2000).

Leptin is thought to act as a signal of energy supply status, regulating reproductive hormones to coordinate reproduction with times of sufficient nutrients (Lõhmus & Bjõrklind, 2009). It is thought that leptin provides information on fat and energy reserves in migrating birds (Kochan *et al.*, 2006). Leptin seems to have a direct effect on the ovary, as well as on the amount of circulating gonadotropin and other growth factors (Duggal *et al.*, 2000). Although a threshold level of fat is needed for normal fertility (Lõhmus & Bjõrklind, 2009), either too much or too little fat can result in reproductive dysfunction, and elevated leptin levels exert an inhibitory effect on reproduction (Duggal *et al.*, 2000). A specific narrow range of leptin concentrations are necessary in mammals to maintain normal reproductive function, with concentrations above or below this level interfering with reproductive function (Caprio *et al.*, 2001). This range is not known in birds.

Kallander and Karlson (1993) summarise two main hypotheses of how birds time laying. As proposed by Lack in 1954, birds may lay eggs in response to an external cue so that the young coincide with a time of food abundance (see Kallander & Karlsson, 1993). The second hypothesis is that timing of laying is determined by the date at which the female can find enough food and accumulate enough energy to form eggs (Perrins, 1970).

Anticipatory reproduction, where animals invest heavily in reproduction before the maturation of food crops, has been shown to occur in a number of species. For example, female American red squirrels (*Tamiasciurus hudsonicus*) and Eurasian red squirrels (*Sciurus vulgaris*) adjust their reproductive investment according to future, rather than past, seed crops (Boutin *et al.*, 2006). This ensures that their maximum reproductive output coincides with the seed crop maturing in autumn (Boutin *et al.*, 2006). In this case reproductive investment is correlated with the 'future crop' as seed production in the current year is not available for consumption until females have already invested heavily in reproduction. American red squirrels usually have one litter per year, and depend on stored food to raise this litter. However, in anticipation of a heavy seed crop, female American red squirrels breed earlier, produce larger faster-growing litters than in low-seed years, and produce a second litter while still feeding the first, circumventing the normal physiological inhibition of ovulation by lactation. An explanation for this breeding strategy is that food is normally limiting for juveniles, but in mast years there is abundant food and females therefore produce more offspring because these have a greater probability of survival than they would in average seed years (Boutin *et al.*, 2006). In Eurasian red squirrels, the proportion of females producing spring litters was positively correlated with future food abundance, and the summer litter was initiated when seeds of the precious year's crop were exhausted, 5-6 weeks before the new seed crop is available for harvest.

Kaka (*Nestor meridionalis*) have been observed to produce two broods in the same season during major beech masts; the fledglings from the first brood were left to feed on abundant beech seed while the parents began the second nest (R. J. Moorhouse, pers com).

It has been common knowledge since 1926 (Rowan, see Perrins, 1970) that birds time laying so that food will be abundant for their young, which means that they must begin reproduction when food is less abundant. Producing eggs requires a lot of resources, and depending on the species, can comprise a large proportion of the female's body weight. Larger species produce comparatively small eggs that comprise a smaller proportion of the female's weight, representing proportionately less maternal investment (Houston, 1997). The amount of nutrient resources invested in eggs relative to the mother's mass is variable: for example, a clutch of Manx shearwater (*Puffinus puffinus*) eggs are equivalent to 15% of adult weight whereas a clutch of blue tit eggs are 150% of the adult female's weight (Perrins, 1970). The nutritional demands of egg-laying can therefore be considerable; passerines need to find enough food to form the equivalent of one egg every 24 hours (with up to four eggs being made concurrently). Eggs require adequate levels of fat, protein, water, and high levels of minerals such as calcium. For example, a domestic hen lays down calcium at 125 mg/hr, with 25 mg circulating in the total blood volume at any one time. To do this, hens must withdraw calcium from special stores in the bone medulla (Perrins, 1996). The bone medulla in smaller birds has limited storage capacity during egg formation so the birds must ingest supplemental calcium (Patten, 2007).

The resources for egg production come from three routes: increased food intake, body reserves and metabolic changes in the female i.e. reallocation of resources from maintenance into egg production (Houston, 1997). Bird muscle contains certain proteins, rich in essential amino acids, which if limited in the diet, can be mobilised to assist egg production. Out of twenty-nine studies on body composition changes during egg laying, twenty-one found significant muscle loss at egg formation time (Houston *et al.*, 1995a). For example, zebra finches (*Taeniopygia guttata*) provided with extra food did not increase their food intake during egg production, with the decline in female body reserves accounted for most of the nutrients in the clutch of four eggs. There was significant loss of water-soluble and alkali-soluble proteins in the pectoral muscle of zebra finches as egg laying progressed, with a 14% loss of pectoral tissue (Houston *et al.*, 1995b). Pectoral muscle contains a sarcoplasm protein which declines rapidly during the laying cycle, possibly functioning as a reserve for egg production without impairing normal muscle function (Houston *et al.*, 1995a).

The pre-breeding diet has a profound effect on egg production and body condition (Houston, 1997). A diet with high quality protein in zebra finches resulted in twice the weight of eggs than those on low quality protein, higher hatching success and less muscle loss (Selman & Houston, 1996)

In summary, the information reviewed above suggests that most temperate birds primarily use short-term changes in photoperiod (i.e. environmental information) as a proximate cue to initiate gonadal development and change reproductive behaviour in anticipation of the breeding season (Hahn, 1998; Ligon, 1978; O'Brien & Hau, 2005). Changes in photoperiod physiologically prepare birds for breeding by stimulating gonadal development and other physiological processes. Birds then rely on supplementary cues or triggers to provide information about when successful reproduction is most likely. These triggers are discussed in the following sections. There are a number of ways in which these cues may trigger reproduction in birds, with most authors agreeing that inhibition or enhancement of gonadal development and alteration of the levels of sex hormones are responsible for initiating or terminating egg production. Some types of supplementary cues undergo complex neural processing which regulates the GnRH system and associated endocrine system. Both types of cues, photoperiodic and supplementary are thought to act on the GnRH system, and probably influence different areas in the brain (Ball & Ketterson, 2009).

3. Triggers

In most seasonally variable environments animals must time their reproduction precisely to maximise reproductive success, thus requiring that they can predict when reproduction will be most successful. They must rely on some flexible mechanism to track environmental cues that can provide information to synchronise their reproduction precisely to seasonal patterns in food abundance (Nager & van Noordwijk, 1995). These environmental cues are proximate or ultimate: the proximate factors act to stimulate the endocrine system and are the predictors (such as photoperiod) which initiate breeding at an appropriate time (Ligon, 1974), and the ultimate factors determine the actual onset of breeding (fine-tuning), such as food (Ball & Ketterson, 2009). The identity of these external cues that birds use to fine-tune sexual development and timing of breeding remain unknown (Bourgault *et al.*, 2006), and it is likely that there are a number of cues, many of which may be strongly inter-related (Nilsson & Kallander, 2006).

To time breeding successfully, birds must integrate proximate cues, such as photoperiod with ultimate cues that provide supplementary information about finer-scale variation in environmental conditions such as food supply. The more predictable the local conditions are, the fewer cues are thought to be needed, or these cues are integrated with the initial predictive cues to a lesser extent (Ball & Ketterson, 2009). Some birds do not use supplementary cues for timing breeding. For example, if there is a very short breeding window, such as in the Arctic, birds are unresponsive to supplementary cues (Ball & Ketterson, 2009).

A ‘complex suite’ of proximate factors, including temperature, food availability and social interactions may influence the onset of blue tit reproduction, and fine-tune laying date (Bourgault *et al.*, 2006). The use of multiple cues is thought to increase the precision of birds’ ability to evaluate environmental conditions (Hau *et al.*, 2004) and precisely predict the best time to reproduce. The absence of these triggers leads to a complete shutdown of the reproductive processes in some species, such as Darwin’s finches (Hau *et al.*, 2004). The causes of this shutdown are deteriorating environmental conditions (such as drought), a decrease in food abundance or exhaustion of body reserves. Food scarcity is a potent inhibitor of reproduction, possibly related to the lack of high protein food (Hau *et al.*, 2004). Adult birds can be induced to lay another egg if one is lost or removed (Monaghan & Nager, 1997), so once triggering has occurred, a further trigger may not be needed.

The possible proximate cues thought to initiate breeding can be divided into the following categories: masting, climatic cues, food cues, chemical cues and visual cues. Many of these cues are linked, and difficult to separate. For example, rainfall leads to green grass growth which leads to increasing insect numbers. Since it is difficult to identify which of these are single cues, some have been included in related categories. I found no references on olfactory cues specifically, so these have not been included. Other possible proximate cues not discussed in this literature review are social cues, such as the territorial behaviour of conspecifics and nest building.

3.1. Masting:

Mast seeding is the intermittent production of a large seed crop by certain plants. Plants that produce occasional large crops of seeds rather than a number of smaller ones benefit from an “economy of scale” (Norton & Kelly, 1988). There has been much debate over the causes of mast seeding (Kelly & Sork, 2002). Prior to 1987, there were two main theories: resource matching (if more resources such as warm temperatures are available then more reproduction occurs) and predator satiation (the bigger the seed crop the more seeds escape predation) (Kelly, 1994 ; Norton & Kelly, 1988). By 1994, weather and resource matching were considered the proximate cause, with a number of theories postulated on the ultimate cause including environmental prediction (predicting the most favourable time for seedlings), animal pollination, dispersal of fruit by predators, accessory cost of reproduction (i.e. high 'start-up' cost so small reproductive efforts are energetically inefficient) and selection for large seed size (Kelly, 1994). Boutin *et al.,* (2006) suggested that the evolution of masting is driven by the survival prospects for plants' progeny (seeds); i.e. an adaptive response by the plant rather than simple resource matching. Kelly & Sork (2002) suggested that masting is an adaptive trait overlaid on the influence of weather. Growing evidence suggests that masting is likely to be induced by a combination of these causes, with most supporting evidence for wind pollination, predator satiation and environmental prediction. The association of weather and masting has been shown in New Zealand (e.g. King, 1983; Schauber, 2002), Europe (e.g. Pucek, 1993; Stenseth, 2002) and in North America (Kon *et al.*, 2005). The association of seed crops and seed predators has been noted in a number of species, including mice (Ostfeld *et al.*, 1996) and red squirrels (Rusch & Reeder, 1978). See Falls (2007) for further examples.

A New Zealand example of a masting species is rimu, the developing fruits of which are strongly linked to breeding in kakapo (on Codfish Island). There is strong evidence that masting in rimu is driven by wind pollination or predator satiation. Rimu masts are negatively correlated with cool temperatures two years prior to the mast and warm temperatures in the summer of the seedfall (Norton & Kelly, 1988). The reproduction of rimu takes place over three seasons (24-30 months). The reproductive phases overlap, so single trees can be in more than one stage of reproduction at any one time. The negative correlation between seedfall and cool temperatures could be a result of competition between cohorts of reproductive structures on the same tree (competition for resources), and/or low temperatures affecting cone initiation and seed ripening (Norton & Kelly, 1988). Schauber *et al.,* (2002) also suggested that bud initiation could involve a temperature sensitive process, which is shared by many taxa. In the 18 masting New Zealand taxa they reviewed, all of them (including rimu) responded to warm temperatures the summer before seedfall. In Norton and Kelly's (1988) study of rimu reproduction 15 of 33 years were mast years, six of which occurred in successive years. In mast years the tree switches from vegetative (wood) to reproductive tissues (cone production), with less wood produced in mast years compared with non-mast years (Monks & Kelly, 2006). Reproductive effort is limited by resource availability: plants can only reproduce when they have accumulated enough resources (Kon *et al.*, 2005). Even when many ovules are initiated and become visible in late spring, this does not always lead to a good crop of fruit 18 months later. Two possible explanations for this are that the young ovules fail to develop due to lack of fertilisation (leading to a large proportion of empty seeds) or pollination failure (due to wet weather) (McEwen, 1983). Rimu trees are long-lived so occasional failed reproductive efforts are of no great consequence to a tree's overall fitness.

All basic population models explain the rate of populations increase as a function of current, or past, rather than future resource availability; the 'boom and bust' model of population dynamics (Boutin *et al.*, 2006). This model postulates that low resource availability before masting prevents seed predators from increasing their reproductive investment, creating a delayed population response in successive trophic levels once the resource becomes available. Therefore, seed predators increase reproduction and population size only after the mast crop becomes available, resulting in starvation once this crop is exhausted (Boutin *et al.*, 2006). A number of species peak in numbers in the year following a mast, which leads to increasing numbers of predators and a population crash in the following year (Ostfeld & Keesing, 2000). For example, a sudden increase in food resources is a precursor to mouse population eruptions (Ruscoe *et al.*, 2004). House mouse (*Mus musculus*) population dynamics have been shown to follow the mast seeding of southern beech trees, which implies that changes in populations are a direct result of food supply (Fitzgerald *et al.*, 2004). Two hypotheses that could explain this are that mice increase their reproductive output by eating the abundant beech seed directly, or by eating lepidopteran larvae, which are feeding on the abundant beech crop.

A good summary of research on the effects of pulsed food resource on a community is provided by Ostfeld and Keesing (2000). As resource pulses are episodic with long inter-pulse intervals, few herbivores are expected to specialise on masting species, with most being trophic generalists. Some species, such as the edible dormouse (*Glis glis*) and the fat dormouse (*Myoxus glis*), have the flexibility to adjust their life history to local mast conditions, and may skip entire breeding seasons if a major food source is absent, such as in non-mast years (Pilastro *et al.*, 2003; Ruf *et al.*, 2006). These animals reproduce only once before they die, so it is advantageous to wait for a good breeding year before breeding (Ruf *et al.*, 2006). Their relative longevity and intermittent breeding make the life histories of these dormouse species unique.

If masting evolved as a ‘swamp and starve’ adaptation against seed predators, anticipatory reproduction and population growth are counter-adaptations by the predator, with increased reproductive output coinciding with low current but future high resources (Boutin *et al.*, 2006). It is unlikely that the trigger for breeding in seed eating bird species is the same as that which triggers masting.

3.2. Climatic Cues (temperature, rainfall):

Some insects use environmental cues to emerge in synchrony with seed masts (e.g. McKone *et al.*, 2001) and there are a few rare examples of vertebrates using unknown cues to reproduce in mast years only (Ruf *et al.*, 2006; Wilson *et al.*, 1998).

Seasonality in photoperiodic species is mediated by increased day-length in spring when plasma testosterone levels start increasing (Leitner *et al.*, 2003). The exact timing of the breeding window is determined by critical day length (minimum photoperiod), the development of photorefractoriness, and the integration of a number of environmental cues (proximate factors) such as food, temperature, rainfall and behavioural interactions. This seasonality shows temporal flexibility in some species, with several examples of breeding outside the ordinary ‘breeding window’ e.g. autumn breeding. These species must be responding to cues other than the ‘usual’ climatic cues.

Weather is certainly known to indirectly affect reproduction in birds and other vertebrates (e.g. bats) through its impact on food availability (Arlettaz *et al.*, 2001).

3.2.1. Rainfall

There are a number of species in which breeding is initiated by rainfall, especially in climates which include irregular droughts. In Australia, a number of birds breed after rain, suggesting that water stress might inhibit reproduction and rehydration may reverse this (Perrins, 1996). For example, wild canaries with high levels of plasma testosterone and widely abundant food responded to a period of heavy rainfall by beginning breeding six weeks earlier than usual (Leitner *et al.*, 2003). Clearly the critical stimulus in this case was not photoperiod or temperature. Substantial rainfall, predictive of an increase of plant growth, was a possible cue for reproduction in these birds. This response to rainfall was also seen in small ground finches in the Galapagos, which responded to rainfall by initiating gonadal development and increasing the production of sex hormones such as LH (Hau *et al.*, 2004). Hau *et al.,* (2004) concluded that rainfall (or related factors such as humidity and barometric pressure) was the proximate factor and food availability (increased arthropod abundance following plant growth) the ultimate factor. Further support for rainfall being a proximate factor is provided by Vleck and Priedkalns (1985), who found that high humidity and green vegetation following rainfall were stimulated reproduction in zebra finches in Australia. In favourable conditions zebra finches are frequent breeders, but cease breeding during periods of drought. Compared to wild white-crowned sparrows (*Zonotrichia leucophrys gambelii*) which take two months to respond to breeding cues, zebra finches respond much more quickly to favourable conditions for reproduction (Vleck & Priedkalns, 1985).

Illera and Diaz (2006) concluded that variability in annual fecundity in the Canary Islands stonechat (*Saxicola dacotiae*) is a food-mediated process driven by rainfall, with clutch size and number (i.e. reproductive investment) correlated with arthropod abundance. Although there may be no reproduction in dry seasons there, may be up to two clutches in wet seasons. Once again, rainfall is suggested as the proximate cue because it is a good predictor of future food availability.

3.2.2. Temperature

Many studies show correlation between the timing of breeding and temperature in early spring. For example, in great tits, spring temperature, caterpillar abundance, low temperatures at expected laying date and the presence of birds in the breeding territory during the previous winter all explained within-population variation in the timing of reproduction (Nager & van Noordwijk, 1995). There have been a number of theories proposed to explain this, such as low temperature increasing females' maintenance costs at the expense of egg formation, low temperature affecting food abundance, or food availability in spring is too low to support both maintenance and eggs formation. An alternative theory is that birds are not limited by energy in egg formation but that nesting depends on a signal indicating that a favourable period for rearing young is approaching (Nager & van Noordwijk, 1995). This probably depends on the size of the egg: if it is relatively small then it is more likely that the limiting factor is finding sufficient food to raise young.

The relationship between mast seed crops in conifers, beeches, and oaks, and rodent numbers has been well established (e.g. (King, 1983; Pucek *et al.*, 1993; Stenseth *et al.*, 2002). In deer mice (*Peromyscus maniculatus*), weather-driven pulses of resource abundance caused spikes in population abundance (Falls *et al.*, 2007). Population growth was influenced strongly by autumn seed crops mediated by temperature during the previous summer and snowfall in the previous winter, with population peaks following heavy seed crops. These rodents depend on stored food to survive the winter. Annual variation in summer temperature result in massive fluctuations in food abundance (seed crops) for this generalist rodent (Falls *et al.*, 2007). Falls *et al.,* (2007) cite three similar examples in New Zealand and Central Europe, where summer temperatures in the year when flower primordia cued masting a year later. The extra food available in mast years led to intense winter breeding in rodents (both in mice and bank voles), an occurrence not seen in non-mast years (Pucek *et al.*, 1993).

Reproduction in three species of Finnish grouse (capercaillie: *Tetrao urogallus*, black grouse: *T. tetrix*, hazel grouse: *Bonasa bonasia*) fluctuates in synchrony from year to year (average 6-year cycle) (Lindstrom *et al.*, 1997). A number of possible causes of this synchrony have been discounted, namely, climate, predators switching prey, parasites and diseases and changes in food quality (including plant chemical defences).

3.3. Food Cues (abundance, nutritional, energetic):

The use of these short-term cues can increase the precision of the timing of reproduction thereby improving reproductive success. The relative importance of photoperiod and food cues varies between species using different breeding strategies, with all flexible seasonal breeders possessing the ability to respond to either of these cues (O'Brien & Hau, 2005). Kelly *et al.,* (1997) concluded from numerous food supplement experiments that food availability is the primary proximate factor affecting the timing of reproduction in birds. Birds make marked shifts in diet at specific times of annual cycles (Houston, 1997).

The number of eggs produced may be adjusted not due to food supply but because they are responding to cues in the environment which tells them how large the food supply will be later in the season (Houston, 1997).

3.3.1. Abundance

The best-known example of a bird synchronising its breeding with that of a tree species is the piñon jay. The piñon jay and piñon pine (*Pinus edulis*) have a mutualistic relationship, with the birds having influenced the reproductive strategy of the pine and in turn have evolved a behavioural and physiological dependence on the pine (Ligon, 1978). The synchronised and large-scale breeding of the jays occurs in years when a major piñon pine crop is maturing (Ligon, 1978). The presence of large quantities of green cones triggers late summer-autumn reproductive activity in piñon jays; accelerating gonadal growth, inhibiting testis regression and triggering sexual activity. Jays assess piñon seeds using visual, tactile and auditory cues (Ligon & Martin, 1974), and collect and store vast quantities of seeds; a flock of 250 birds were estimated to have collected 30,000 seeds per day, equivalent to 4.5 million seeds over a 5-month period (Ligon, 1978). It is thought that it is the abundance of the green piñon cone crop, rather than the nutritional quality of the cones, that is the trigger for nesting (Ligon, 1974; Ligon, 1978; Ligon & Martin, 1974), indicating that an abundant crop of ripe seeds will be available for nestlings.

A similar situation is seen in the great tit, with the abundance of caterpillars, an important food for nestlings, along with variations in temperature, modifying the timing of egg laying (Nager & van Noordwijk, 1995). Breeding in kakapo on Codfish Island is closely correlated with rimu fruit abundance; nesting has only occurred when more than 10% of rimu branches bore fruit (Harper *et al.*, 2006). As with the piñon jay, green fruit comprises only a small proportion of the diet so it is more likely that breeding is triggered by the abundance of green fruit rather than its nutritional quality. Furthermore, kakapo eat more rimu in breeding than non-breeding years (Harper *et al.*, 2006).

The red crossbill is an opportunistic breeder that is commonly thought to breed whenever food is abundant (Hahn, 1998). In fact, like other birds, reproductive development occurs in spring (prior to the discovery of new conifer cones) and ends in autumn when maximum seed is available. Crossbills anticipate the discovery of new cones in summer by preparing the reproductive system in advance; LH levels increase in both sexes and testes enlarge in males. Crossbills regulate changes in reproductive physiology and behaviour in response to initial predictive and supplementary cues, as seen in other temperate birds (Hahn, 1998). White-footed mice also show an increase in fecundity in response to seed crop abundance (Merson & Kirkpatrick, 1981), and reproductive capacity in female edible dormice was linked to the natural abundance of beech nuts rather than to the nutritional content (Fietz *et al.*, 2009). Breeding in edible dormice was also linked with sufficient seed abundance; the mice are thought to respond primarily to the information content in energy-rich seeds (using the high abundance of seeds to signal later food availability) rather than the energy content itself (Lebl *et al.*, 2010).

Timing of parturition in mouse-eared bats (*Myotis myotis* and *M. blythii*) is controlled by seasonal food availability (Arlettaz *et al.*, 2001). These bat species prey on different insect species, with the preferred prey of *M. blythii* (cockchafers) only present every fourth year. Both species of bats mate and deposit enough fat reserves to see them through their winter hibernation. In spring, the mean parturition date was the same in both species if the preferred prey of both species were present, but in years with no cockchafers, parturition was 10 days later in *M. blythii*. The presence of cockchafers therefore advances parturition in this species (Arlettaz *et al.*, 2001).

Supplementary food may be a proximate factor which provides the extra energy which augments endogenous reserves, or may contain micro- or macro-nutrients required for egg formation (Reynolds *et al.*, 2003). It may also act at the ultimate level as a cue for the female to breed when food resources are abundant. The quality of supplementary food may influence the degree to which the laying date is advanced. Most food supplementation studies in birds have found that supplemented females lay earlier than controls, but also that the nutritional state of the female is not the only cue used to time laying, and that the effects of food supplementation are weaker when there is abundant natural food (Kallander & Karlsson, 1993). For example, supplementary feeding in blue tits resulted in advanced laying date by 9-10 days in territories with low natural food levels, with no effect on high quality territories (Svensson & Nilsson, 1995).

Schultz (1991) suggested the existence of a threshold energy level below which breeding cannot occur. Above this threshold, parental fitness and environmental cues such as ambient temperature, bud burst or invertebrate activity initiate breeding. The presence of a threshold may explain why some supplementary feeding experiments have failed to advance the date of breeding; if the birds in question were above the saturation point extra food would have no effect. This appears to be the case in the kakapo. Supplementary feeding had no effect on breeding frequency on Southern Islands but did on Little Barrier Island where birds were generally in poor condition before supplementary-feeding began (Elliott *et al.*, 2001). On the other hand, overweight female kakapo were less likely to breed than those within the ‘average’ weight range.

The provision of supplementary food to birds has had mixed results; it did not advance the laying date in some species (Nilsson & Kallander, 2006), but did so in others, such as the Lesser Kestrel (*Falco naumanni*) (Aparicio & Bonal, 2002). Many of the studies in which supplementary food did not advance laying date used nutritionally complete diets and focussed on the absolute energy in the diet, rather than the relative importance of different macronutrients (Reynolds *et al.,* 2003). A number of authors have examined how short-term cues affect egg-laying, rather than if they can trigger the initiation of gonadal growth (O'Brien and Hau, 2005).

3.3.2. Nutrient Composition

Many authors have investigated the nutrient composition of foods and have concluded that nutritional content is a potential trigger for reproduction in birds and other vertebrate taxa, such as marine iguanas (*Amblyrhnchus cristatus*) (Rubenstein & Wikelski, 2003), the edible dormouse (Ruf *et al.*, 2006), house mouse (Tann *et al.*, 1991) and voles (*Clethrionomys sp.*) (Selas *et al.*, 2002). In white-footed mice (*Peromyscus leucopus*), the restriction of food intake by as little as 10% of *ad libitum* for 2-3 weeks resulted in reduced oestrus and reduced production of young (Merson & Kirkpatrick, 1981). The nutritional value of food is usually linked to its gross energy content and quality (chemical composition) but the relationship between food preferences and nutritive value is poorly understood (Bucher *et al.*, 2003). Nutrients stored in seeds are readily used by animals, whereas lignin and cellulose are difficult for either plants or animals to use (Smith, 1970). The quality of plant foods is determined by the relative amounts of nutritive compounds and deterrents (including fibres and phenols).

For example, in plantcutters (*Phytotoma rutila*) Bucher *et al.,* (2003) found that the protein content of the leaves of its four most preferred food species was highest in spring, whereas phenols were highest in summer. The protein content was relatively high, ranging from 25-32% (average 29%), higher than is recommended for poultry (NRC, 1994). This study correlated diet composition with nutritional value, with only protein and phenols showing a significant relationship.

It is possible that seasonal changes in the nutritive content of food are a trigger for reproduction. The results from a study of marine iguanas (Rubenstein & Wikelski, 2003) indicate that the median copulation date coincided with the peak in nutritional quality of their preferred food (*Gelidium* spp. algae). This suggests that these animals use subtle changes in food quality, rather than quantity, to initiate breeding. The nutritional quality of the iguanas' food was assessed by measuring its carbon (a crude measure of energy) and nitrogen content and calculating the C:N ratio. The nitrogen content of the preferred algae increased throughout the season, whereas its carbon content and C:N ratio peaked then declined. Marine iguanas are more sensitive to food availability prior to breeding compared with land iguanas which accumulate and store nutrient reserves in their tails for several months prior to breeding (Rubenstein & Wikelski, 2003).

A study of the Florida scrub jay (*Aphelocoma coerulescens*) (Reynolds *et al.*, 2003) was the first to demonstrate that the nutritional quality of the pre-breeding diet can simultaneously influence laying date, clutch size, egg size and composition in birds. The provision of either high (32%) or low (3%) protein and high fat (19.7%) supplementary food advanced the laying date by 12-14 days over the control birds. The birds on the high protein/high fat diet allocated more water and protein to the third egg and maintained the same egg mass over the entire clutch, while those on the low protein diet laid progressively smaller eggs (Reynolds *et al.*, 2003). Directing resources at increasing egg quality, rather than quantity, is a less costly investment when food is abundant. Florida scrub jays fed high protein/high fat supplementary food laid eggs significantly earlier than those fed high protein/low fat (Schoech *et al.*, 2004). This was the first study to differentiate between protein and energy in reproductive timing.

Food cues were shown to be the proximate factor in the regulation of avian seasonal breeding in neotropical spotted antbirds (*Hylophylax n. naevioides*) (Hau *et al.*, 2000; O'Brien & Hau, 2005). This species is an insectivore which breeds only in the rainy season when insect abundance increases. Males allowed to see, but not eat, live crickets significantly increased their song activity whereas those fed nutritionally equivalent dead crickets did not. This indicates that proximate cues can be cognitive rather than nutritional (Hau *et al.,* 2000). This was the first study to show that the visual stimulus of food could act as a breeding cue. In the same species, O’Brien and Hau (2005) also found that an increase in food quantity and quality initiated gonadal development independent of photoperiod stimulation. However, these authors could not determine if nutritional or non-nutritional factors, such as an increase in body mass, triggered breeding. They postulated that food cues might act via the GnRH-II (gonadotropin-releasing hormone), stimulating reproductive behaviour directly when food availability is limited (O'Brien & Hau, 2005).

Female capercaillie tend to switch from their winter food plants to nitrogen and phosphorus-rich foods (catkins, flower buds, berries, stems, new leaf growth) in the early spring following snow melt, a phenomenon not seen to the same extent in males (Pulliainen & Tunkkari, 1991). The capercaillie commences breeding as early as possible in the season to ensure the young are big enough to survive the following winter. Their spring diet contains over 17.5% crude protein, enough to attain a positive nitrogen balance (Pulliainen & Tunkkari, 1991).

The red crossbill is a specialist predator of conifer seed and the availability of this ‘special food’ is an important integrative cue for breeding activity (Hahn, 1998). Seasonal changes in sensitivity to external cues may also be involved in the regulation of the annual cycle in these birds. In absolutely refractory species, critical day length inhibits this (Hahn, 1998). In canaries, if day length is shorter than a minimum threshold, food or rainfall becomes the primary stimulus of the GnRH system (Leitner *et al.*, 2003).

The protein in unripe grass seeds eaten by Australian grass finches (zebra finches, *Taeniopygia guttata*) has a similar amino acid profile to the protein content of their egg (Allen & Hume, 1997). Unripe and ripe grass seeds exhibit significantly different amino acid profiles, with levels of histidine, lysine, phenylalanine and threonine the most different. The level of lysine in unripe seeds was much higher (150%) than in ripe seeds. Lysine is the most limiting essential amino acid in ripe seeds, so timing of reproduction so that the nestlings coincide with unripe seeds is advantageous. Associated with the higher lysine in the unripe seeds, a further benefit of eating unripe seeds is that they are easier to digest, with consequence of greater extraction of limiting amino acids such as lysine, threonine and methionine (Allen & Hume, 1997). The trigger for breeding in the house mouse in Australia is thought to be the presence of ripening and maturing seeds (but not ripe seeds) (White, 2002). This food source contains high levels of soluble amino acids compared with ripe seeds, and may provide energy or act as a signal of energy levels high enough for reproduction. Conversely, amino acids were discounted as the critical factor triggering reproduction in edible dormice, as the proportion of amino acids does not differ between beech nuts and leaves, which are an important constantly-available resource (Lebl *et al.*, 2010).

Red grouse (*Lagopus lagopus scoticus*) mainly eat heather in spring, selecting those parts of the plant that are particularly rich in nitrogen and phosphorus. Both nitrogen and phosphorus are known to be limiting in poultry and are thought to be limiting in red grouse (Moss, 1972). Moss (1972) analysed crop samples (in the crop for 15-20 minutes) and plant material collected from the plants where the birds were feeding. Their results confirmed that red grouse select heather that is particularly rich in nitrogen and phosphorus prior to egg laying. In a previous study, digestibility of dry matter increased up to a maximum intake (71 g/day) after which digestibility decreased (Moss & Parkinson 1972). Moss and Parkinson (1972) suggested that the same could apply for individual nutrients, such that beyond an optimal intake the amount of nutrient actually available to the bird decreases. The only way to increase levels of these nutrients would be to select food items that contain more of them. The preference for foods higher in nitrogen and phosphorus increased from winter to spring but there was no difference in the fat, carbohydrates or crude fat content of the heather the birds ate. Similar results were found in a later study in which the nitrogen and phosphorus content of the plants eaten in spring was correlated significantly with the birds mean breeding density (Moss, 1972). The birds have a strong preference for new growth in spring, and it was concluded that this may have some "virtue" not related to its nitrogen or phosphorus content (Moss, 1972). In other words, there is an unidentified factor in the plant material that stimulates egg laying, or that the actual quantity of the nutrients absorbed by the bird (rather than the amount in the food source) may be leading to breeding success. It was also suggested that new growth is more digestible than old, allowing a greater absorption of some nutrients. It should be noted that some of the experimental birds lost weight on a diet comprised exclusively on heather, however this was attributed to them not being sufficiently used to this diet rather than its low protein content (Moss & Parkinson, 1972).

Ruffed grouse (*Bonasa umbellus*) were also found to select male aspen buds highest in protein but also in potassium (Doerr *et al.*, 1974). They compared crop samples with plant material, and concluded that since the crop contained a high content of protein and potassium levels that the bird was selecting plant material higher in these nutrients. There was a difference in protein levels between trees that the birds were seen feeding in (11.7 %) compared to those in which they weren't seen feeding (9.2 %). If the plant parts eaten are lower in some nutrients but are relatively easy to collect then the actual amount consumed may be important (Doerr *et al.*, 1974). In Galliformes, there is seasonal variation in the size of the gut and caeca, with these reaching their greatest development when birds are eating a more fibrous diet. This may also affect digestion and the amounts of nutrients absorbed in other species.

It is thought female Sage grouse (*Centrocercus urophasianus*) eat ‘forbs’ in the pre-lay period to improve their nutritional status and egg quality (Barnett & Crawford, 1994). Although forbs contain 25-30% protein, they were found to be lower in protein than any other plant consumed. They were possibly eating the forbs to get nutrients other than protein. In a two-year study, the protein, calcium and phosphorus content of forbs was lower in the second year than the first, and this was associated with a decrease in reproductive productivity.

The gonads of male edible dormice regress during non-mast years during which time they are sexually inactive (Ruf *et al.*, 2006). Reproduction in these animals is thought to be triggered solely by the availability of food resources, postulated to be either the presence or absence of seed buds in spring or the energy-richness of the beechnuts (42% lipids).

Tann *et al.,* (1991) investigated the diet of the house mouse in Australian wheat fields by sampling at approximately 6-weekly intervals. There were major shifts in the diet immediately preceding and following the start of breeding. During the 2 years of this study the onset of breeding coincided with the period when the highest proportion of invertebrates was found in mouse stomach contents, and the peak of breeding coincided with the availability of fresh grains. It is possible that the pulse of invertebrate material just before breeding indicated the availability of high quality food and triggered breeding. They concluded that possible triggers for mice reproduction were invertebrates, grass seeds or possibly even green plant material, and proposed that nutritional factors were responsible for the onset of breeding.

Protein supplementation in young wild deer mice resulted in breeding and a dramatic increase in nestling growth rates when there was limited natural food available (McAdam & Millar, 1999). In good years, with sufficient natural food available, and when growth rates were already good, there were limited effects of additional protein. The addition of high energy but low protein had no effect on growth or maturation of young. The conclusion of this study was that it was the protein not the energy that was the component limiting breeding and nestling growth. Protein limitation is also implicated in the reproduction in the Florida scrub jay, with supplementary food advancing laying date (Schoech *et al.*, 2004). The lack of a breeding response to the addition of supplementary food when there was adequate natural food available has also been seen in birds e.g. great tits (Nager *et al.*, 1997) and blue tits (Svensson & Nilsson, 1995). The addition of mealworms or sunflower seeds, similar energy but different protein content, advanced laying date by 5-6 days, discounting the theory that low protein diet restricts laying in great tits (Nager *et al.*, 1997).

A study by Ramsay & Houston (1998) is believed to be the first with evidence that egg production might be constrained by certain amino acids, rather than overall protein level. They supplied five amino acids in a balance close to that found in eggs, and found that the females laid 18% larger clutches than those fed imbalanced amino acids. A previous study by these authors (Ramsay & Houston, 1997) showed that timing of laying was influenced by energy but egg production was constrained by other nutrients.

It is well documented that mouse populations increase in response to high levels of beech seedfall. This has now also been shown to occur after rimu seedfalls of up to 2100 nuts per m^2 (Ruscoe *et al.*, 2004). Protein is a limiting nutrient for mice in grain-growing areas of Australia (Bomford, 1987). Mice switched to eating weed seeds after rains and a month later started to breed. Beech seeds are high in nitrogen, major minerals and energy, nutrients thought to be directly responsible for the increase in mouse numbers. Following a review of a number of studies, Knapka (see Ruscoe *et al.*, 2004) concluded that 12-14% protein was adequate for mouse growth, and 17-19% for reproduction. Mountain beech and rimu seed contain 33% and 23% protein respectively (Ruscoe *et al.*, 2004). Previous analyses done on various potential kakapo foods gave much lower protein levels, between 2.64 to 12.8% (D.V. Merton, pers. comm.). Individual results include red beech seed 11.9% protein, silver beech seed 12.8%, entire unripe rimu fruit (collected in November) 7.43% protein, developed but green rimu (June) 5.4%, ripe entire kahikatea fruit 7.16% protein, unripe (Sept) yellow silver pine (*Dacrydium intermedium*) 2.64% protein. Some of the discrepancies may be the part of the fruit or seed which was analysed, and whether the analyses included the seed coat.

In contrast to the findings by Ruscoe (2004), Fitzgerald *et al.,* (2004) also found that mouse populations reached their highest densities in beech mast years but discounted beech seed as the food responsible for this increase because they could find no evidence that wild mice ate either beech seeds or flowers. They suggested that mouse populations increased due to an increase in the invertebrates that feed on fallen beech flowers. This corroborated the results of King (1983) who found a strong correlation between beech seedfall and populations of mice, but were not sure if mice ate beech seed or were responding to another food source. An increase in the amount of invertebrates eaten by breeders versus non-breeders was also noted by Tann (1991).

The Norwegian field bilberry (*Vaccinium myrtillus*) and *Clethrionomys* vole populations fluctuate cyclically, with one year between mast bilberry production and an increase in vole number (Selas *et al.*, 2002). Selas *et al.,* (2002) tested a number of possible nutritional or chemical mechanisms and found that neither increased levels of soluble carbohydrates and amino acids, nor the nutrients provided by the berries, explained the relationship. They concluded that improved food quality after the mast is the most probable explanation (Selas *et al.*, 2002). It is possible that masting provides a good source of nutrients until the plants chemical defence is re-established the following year (Selas *et al.*, 2002). This explanation is supported by the fact that some field bilberry browsing deterrents are nitrogen based, and the level of nitrogen fell with increasing crop size. Increased levels of chemical defence compounds in masting food plants after population outbreaks have been reported in insect herbivore species (Selas *et al.*, 2002).

The timing of breeding in the Parea (Chatham Island pigeon, *Hemiphaga novaeseelandiae chathamensis)* was related to both food abundance and quality (Powlesland *et al.*, 1997). The fruits of the most common food plants, matipo (*Myrcine chathamica*) and hoho (*Pseudopanax chathamicus*), were rich in lipids and available carbohydrates compared to the foliage. Breeding coincided with an abundance of fruit, maybe due to their relatively high levels of lipids and carbohydrates and greater digestibility compared to foliage (Powlesland *et al.*, 1997). Some frugivores consume leaves and other plant parts if fruit is in short supply, but this also occurs when fruit is abundant, implying that the vegetative plant may provide some important dietary components found in fruit (Oliveira, 2002).

The mixing of different fruits in the diet of birds may either produce 'resource complementary' diets (where one component improves the nutrition of other components) or antagonistic 'diets' (one component reducing the nutritional benefit of others) (Whelan *et al.*, 1998). Digestibility is an important aspect of palatability and can vary with season (Karasov 1993). Some fruits may contain nutritional attributes important for specific parts of the life cycle (Dijkgraaf, 2002). The consumption of fleshy fruits is thought to be either antagonistic (resulting in reduced fitness), complementary (resulting in increased fitness) or perfectly substitutable (Whelan *et al.*, 1998). Complementary diets are thought to be the most common type among frugivorous birds. With complementary resources, nutrients in some foods may neutralise toxic components of others (Whelan *et al.*, 1998). As shown by many authors, the relationships between nutritional composition of foods and reproduction are extremely complex. Optimal foraging should lead to nutritional and calorific optimisation (Greenstone, 1979). For example, it is well known that animals can enhance their diet (i.e. eat to meet their nutritional requirements) by selecting appropriate foods containing complementary nutritional resources, such as sugar-rich, lipid-rich, protein-rich, vitamin- or mineral-rich (Dijkgraaf, 2002). Birds seek calcium-rich foods when needed for egg shell formation (Houston, 1997; Houston *et al.*, 1995b) and travel to seek it out (Wilkin *et al.*, 2009). For example, Ramsay & Houston (1999) found low numbers of snails in the field but the gizzards of female breeding blue tits were full of snail shells. Another function of selecting certain foods is to dilute, or neutralise, secondary toxins or deterrent substances ingested from other foods. By consuming several types of fruit a bird may obtain a more balanced diet (Whelan *et al.*, 1998). Species differ in their digestive capacities (Whelan *et al.*, 1998), and may differ seasonally. It is possible that birds seeking buds are actually focussing on certain nutrients available in the buds (Houston, 1997).

Raubenheimer & Simpson (2006) used geometric analysis to explore the relationships between different foods and reproduction in kakapo. Their aim was to identify the ratios of different nutrients in kakapo food (both natural and supplementary) and thereby possible conflicts in nutrient requirements. One of their findings was that protein and lipid concentrations were much lower, and calcium higher, in natural food (green rimu fruit) than in supplementary food (Harrisons' pellets). This means that if a kakapo ate similar amounts of supplementary food and rimu fruit they would ingest 2.7 times as much protein and 12.2 fold more fat from the supplementary food than the fruit. If they ate to meet their fat requirements they would only get 22% of the protein they needed. Conversely if they ate to meet their protein requirements they would get 2.2 times of the amount of fat they needed. As kakapo are strict herbivores they must obtain their calcium by eating plant material. The authors hypothesised that the calcium might be the key limiting factor in kakapo reproduction (Raubenheimer & Simpson, 2006), and that providing supplementary foods containing low macronutrient:calcium ratios may be effective in supporting increased reproduction.

Little is known about protein digestion, nutrient absorption and conversion efficiency into egg production except in poultry (Ramsay & Houston, 1998). It is likely that there are major changes to digestive physiology at the time of egg laying (Ramsay & Houston, 1998), so actual levels of nutrients in the food may not reflect what is digested by the bird, may be masking breeding triggers.

3.3.3. Energetic

There are conflicting results from studies focussing on energy as a potential trigger, most have ruled out energy as the cue for reproduction. The trigger to reproduce is probably not solely energy, as foods providing just energy (such as glucose) would not have enough essential nutrients (such as amino acids) to support reproduction. It is more likely that a certain level of energy must be present before reproduction is triggered by a different cue/s. As mentioned previously (see section 3.3.1.) the energy content of food was discounted as a direct limiting factor in edible dormice (Lebl *et al.*, 2010). It is thought that supplementary food influences the reproductive axis either through an internal perception of energy availability or maybe via some stimulatory sensation of the food itself (Ball & Ketterson, 2009). Total dietary energy intake was more important than nutrient composition for the maintenance of reproduction in white-footed mice (Merson & Kirkpatrick, 1983), Merson (1983) concluded that the secretion of gonadotropins (and consequent effects on reproductive tissues) was more dependent on energy than protein intake.

In crossbills, sufficient food for the nestling stage is the ultimate factor limiting reproduction (Benkman, 1990). Crossbills initiate egg formation whenever conifer seed availability (energy) increases to levels sufficient to raise nestlings. If seed abundance subsequently decreases birds cease breeding. Intake (net intake and rate of change) is thought to be a proximate cue for nesting, but many other factors, including maturing cone crops, affect breeding readiness. When forming eggs, crossbills required greater intakes to meet estimated energy requirements than to meet protein requirements i.e. they over-eat protein to get enough energy. This overeating to obtain the required nutrients has support from other authors. Plant-eating bats (e.g. *Cynopterus sphinx)* eat fruit rich in soluble carbohydrates and low in crude protein (Ruby *et al.*, 2000). Therefore to get enough protein it is suggested that these bats overeat fruit and 'dump; carbohydrates to meet their protein requirements (Ruby *et al.*, 2000). Captive birds adjusted to a diet of fruit pulp (low protein) are thought to compensate for this low protein by a compensatory increase in food intake (Bairlein, 1996).

Results from a study in blue tits by Ramsay & Houton (1997) suggest that the food constraint on the onset of laying is actually nutrient availability, either as a cue or a proximate constraint. Experimentally provisioning blue tits with either fat or cooked eggs resulted in advancement of laying date by 2.5 days (Ramsay & Houston, 1997). Eggs from birds provisioned with cooked egg were 7% heavier than those from fat provisioned. From this result they concluded that there is a nutrient or nutrients present in the cooked egg which is usually missing from the natural diet. This limiting factor is thought not to be fat, energy, and probably not protein, but possibly amino acids (sulphur amino acids or lysine) (Ramsay & Houston, 1997). A slightly higher increase in egg size (10%) resulted from feeding lesser black backed gulls (*Larus fuscus*) a supplement of egg but not from fish (Bolton *et al.*, 1992), but with no advancement in laying date in either supplement. This study suggests that egg production may be limited by specific nutrients found in eggs but not in fish.

In the two species of squirrel studied by Boutin *et al.,* (2006) energy alone was ruled out as the trigger for reproduction, as food supplement experiments failed to increase litter size or to produce a second litter. This situation is the same as in the kakapo, with energy ruled out as the factor limiting egg production. Food supplementation with nuts failed to increase egg production, whereas pellet supplementation resulted in an increase in clutch size (Houston *et al.*, 2007). It was postulated that the squirrels may be following a resource tracking strategy, tracking an alternative energy source correlated with the future seed crop. Good candidates for this were reproductive structures (e.g. buds, flowers, pollen cones) as these were consumed in advance of the forthcoming seed crop. Boutin *et al.,* (2006) ruled out these immature plant reproductive structures as triggers for reproduction because they contain the same amount of energy as vegetative buds which are abundant throughout the year.

Belted kingfishers (*Ceryle alcyon*) were supplementary fed to calculated energy requirements, resulting in earlier nesting than when they were supplementary fed with less energy (no change in nesting time) (Kelly & van Horne, 1997). This supported the hypothesis that providing more energy leads to earlier nesting, but also supports the 'habitat selection model', that food availability influences the location of the reproductive individual but does not alter timing. The addition of supplementary food in the wild may increase the quality of some territories, leading to earlier breeding in these patches. Of the 27 avian studies reviewed in Kelly *et al.,* (1997), with 53 food manipulations, 41 showed significant advances in nesting date, but most authors could not distinguish whether the energy constraint or habitat selection models were supported.

Wilson *et al.,* (1998) supplied high energy food to kaka to study the effect of energy, but most of the nests were predated by stoats. Kaka only attempted to breed in years with beech seed available (high protein and energy) even though honey dew (high energy, high carbohydrates, low protein) was available.

One species of Darwin's finch (*Geospiza fuliginosa*) has varying levels of fat depots from year to year (Hau *et al.*, 2004). It is thought that these deposits may function as energy reserves for increased energetic demands and unpredictable environmental conditions, although this was ruled out as a potential trigger for reproduction.

3.4. Non-nutrient Chemical Cues

There are a number of possible non-nutrient, chemical cues, such as tannins, which may be involved in the regulation of consumption of the plant material, and could trigger reproduction. Included in this section are other naturally occurring chemicals (pollutants and phytoestrogens) which may affect reproduction, albeit not necessarily as triggers. Secondary metabolites (such as tannins) are considered to act as feeding deterrents, affecting nutrient metabolism and metabolism (Bairlein, 1996). These secondary metabolites appear to be less deterrent in avian frugivores than in other herbivores. Birds seem able to counteract detrimental effects in food, with these secondary metabolites may even be acting to stimulate food intake and metabolism in birds (Bairlein, 1996).

Reproductive disruption in wildlife may be caused by environmental pollutants, specifically endocrine-disrupting compounds (EDC), mainly obtained through the diet (Weber, 2002). The emphasis to date has been on naturally-occurring plant oestrogens (phytoestrogens), which mimic oestrogenic activity and interfere with the normal functioning of the endocrine system (and reproduction). If an EDC mimics or competes with exogenous steroids during embryonic and post-hatch development this may impact the reproductive axis, affecting normal reproduction (Ottinger *et al.*, 2001). Some EDC's bioaccumulate in animal tissues. Many are lipophilic, and get deposited in egg yolk, with the potential to affect the embryo. It is well established that the oestrogenic activity of any phytochemicals ingested may be enhanced by metabolic modification in the animal (Fidler *et al.*, 2000).

Diadzein is one of the two principal isoflavones commonly found in plants. Phytoestrogens have been reported to have adverse effects on reproductive function in ruminants and avian species, and to have both agonistic and antagonistic effects under different conditions (Zhao, 2005). In poultry, diadzein may have a negative influence on reproductive functions before the onset of lay and early in the lay period, but can improve laying performance after the peak of lay. Shaoxing ducks treated with diadzein in the early lay period had a significantly lower egg mass (significantly lower egg-laying rate and lower egg weights) compared to a control group. The egg mass of the diadzein treated group increased by 9-11% a day after the peak of laying had passed, however, this was accompanied by decreased fertility and hatch rate. In a third experiment, the egg weight of the diadzein treated group remained similar to that of the control, but the composition of the eggs significantly changed, with the albumin increasing and the yolk weight decreasing significantly.

Fidler *et al.,* (2008) hypothesised that low levels of dietary phytochemicals may affect reproduction in the kakapo. In brief, in non-mast years low levels of phytochemicals result in the ovarian follicles failing to develop, whereas in mast years the increased exposure to low levels of phytochemicals results in egg production. As previously mentioned, the hypothalamo-pituitary gonadal axis (HPG axis) is stimulated leading to the secretion of reproductive hormones and growth of reproductive organs, preparing the bird for reproduction (Hau *et al.*, 2004). The production of FSH and LH promote the growth of small ovarian follicles which release steroidal hormones including oestrogens into the bloodstream (Fidler *et al.*, 2008). These are transported to the liver where they activate the expression of genes for egg yolk proteins (EYP), including vitellogenins which transport fatty acids to the growing oocyte. The expression of EYP genes is influenced by oestrogens, which have a cumulative effect, with any subsequent exposure to oestrogens resulting in a more rapid response by the genes (Fidler *et al.*, 2008).

A number of authors have investigated the theory that the ingestion of buds and other plant material might provide a chemical signal to trigger reproduction. Most have concluded that this is unlikely. Blue tits are small passerine birds with a breeding schedule tightly linked to the phenology of oak trees (Bourgault *et al.*, 2006). Bourgault *et al.,* (2006) tested the theory that these birds use chemical substances in leaf and bud tissue as a predictor of the increased insect abundance that follows spring leaf growth. They concluded that this was unlikely because buds were present in only 30% of gizzards (at <1% of gizzard content) in the critical pre-breeding period. A similar conclusion was reached by Vleck and Priedkalns (1985) who found that there was little evidence that zebra finches selected key plant material during the critical pre-breeding period.

Conversely, there is evidence to suggest that increased testis length in Australian zebra finches may be caused by a chemical trigger contained in the green grass shoots which appear soon after rain (Vleck & Priedkalns, 1985). This breeding response has also been seen in microtine rodents (Berger *et al.*, 1981). It is also possible that grass shoots stimulate reproduction in the finches because it is nest building material. It is unlikely that a single chemical is triggering reproduction in kakapo, as they can be triggered to breed on a variety of foods.

The fat dormouse makes the decision to breed in any given year approximately 2 months before beech seeds are mature (Pilastro *et al.*, 2003). These rodents feed on floral buds in spring and summer, so it is possible that these contain specific compounds that trigger reproduction. Similar anticipation of food crops has been reported in the meadow vole (Berger *et al.*, 1981). These are clear examples of a mammalian breeding cycle synchronised with a single variable food source.

The addition of leaf tannins to mealworms in the diet of captive blue tits resulted in slower weight increase compared to birds fed tannin-free mealworms (Perrins, 1975). It is possible that tannins reduce the nutritive quality of insects to birds. Removing an insect's gut before consuming them may be an adaptation by tits to mitigate the negative effect of tannins in the insect's diet (Perrins, 1975). Secondary plant compounds were discounted as possible triggers in the edible dormouse as relatively unrelated plant species (sunflower seeds and beech nuts) led to a reproductive response (Lebl *et al.*, 2010).

A chemical compound in plant material has been found to trigger reproduction in the montane meadow vole (*Microtus montanus*) (Berger *et al.*, 1981; Sanders *et al.*, 1981). This compound was subsequently isolated and identified as 6-methoxybenzoxazolinone, a nonestrogenic compound. Samples from non-breeding winter populations of the meadow vole that were experimentally fed this compound had a high incidence of pregnancy than those that were not fed it (Berger *et al.*, 1981). The uterine weights of female house mice also increased after being fed this compound so it may be a reproductive cue for other mammals in addition to the montane meadow vole. This compound could be an ultimate cue for this species because it indicates that a high quality food supply, mature grasses, is available to support reproduction.

3.5. Visual Cues

There are few studies of visual cues as triggers to reproduction and these have conflicting conclusions.

As previously described, the neotropical spotted antbird is known to be stimulated by visual cues; being able to see live crickets elicited intermediate song activity in male birds even though freshly-dead crickets did not (O'Brien & Hau, 2005).

The breeding schedule of blue tits is closely linked to oak tree phenology. Blue tits may respond to oak phenology by a visual assessment of bud development (Bourgault *et al.*, 2006), however, this was found not to be an absolute cue for the start of breeding in great and blue tits (Nilsson & Kallander, 2006). Ruffed grouse may select aspen buds higher in protein and potassium on the basis of visual cues such as colour and/or size (Doerr *et al.*, 1974). Colour was discounted (low light intensities), but bud size is a possible cue, as the trees used by birds had larger buds compared to the trees not used for feeding.

4. Conclusions

In order to time breeding so that there is sufficient suitable food for young, female birds often need to start egg production before food is abundant, a transition that can require profound changes in their reproductive organs. Birds living in unpredictable habitats must breed opportunistically whenever suitable food supplies are available. The ability to track environmental cues to provide information on when reproduction is likely to be successful would clearly be advantageous under such conditions.

The initial predictive cue to reproduction in birds is photoperiod. In many males, this is the only cue needed to initiate reproduction, whereas in females supplementary cues are needed. Kakapo males boom every year, although much more intensively in breeding years, so like the females they need supplementary cues to initiate breeding.

These supplementary cues fine-tune reproduction so the offspring are in the nest when abundant suitable food is available. The main putative types of triggers are climatic (e.g. temperature, rainfall), food (abundance, nutritional composition, energy content), chemical and visual cues. It is also likely that males and females either respond to the same cues at different times, or respond to different cues. It is likely that animals use a complex suite of multiple inter-related cues to fine-tune sexual development and trigger reproduction, and there is likely no single generalised trigger for all species.

The provision of supplementary food has had variable effects on reproduction in different bird species. In a number of studies, supplementary food failed to advance breeding date, whereas in others it succeeded. This may be because, in some species, there is a minimum threshold of energy below which breeding cannot occur. A number of studies have focussed on seasonal changes in food quality. A number of species are known to reproduce in response to a switch to nitrogen- and phosphorus-rich foods in spring. Reproduction has also been linked with calcium and potassium. Amino acid profiles are different in unripe and ripe seeds, so it is possible that some deficit or excess in some amino acids could be involved in reproduction initiation. Food quality was discounted in the reproduction of neotropical antbirds, in which the visual stimulus of live crickets alone was sufficient to induce males to sing.

There is some evidence that chemical defences, such as secondary metabolites or toxins, in plant reproductive structures may deter predators. Most authors have concluded that the ingestion of buds and other plant parts are unlikely to provide a chemical signal for reproduction, although there is some evidence to the contrary. The isolation of a chemical compound which triggers reproduction in the meadow vole suggests that the possibility of a chemical signal should not be discounted, at least in species with a very limited diet.

Masting in plants leads to a reproductive response in seed predators, but it is unlikely that the cues for masting (thought to be temperature related) are also the cues used by seed eaters to time their reproduction. The main conclusion of this review is that it is likely that some aspect of the mast itself, such as some aspect of the reproductive structures, is involved in triggering reproduction in seed predators, as seen in some species. The most likely candidates for the cue/s in rimu fruit is the actual abundance of the green rimu fruit, or some chemical aspect of it, such as nutrient content or secondary plant compounds, or interactions between these. As kakapo are known to breed in the presence of other food species, it is very unlikely that a single chemical is responsible for triggering breeding,

The focus of this study is the kakapo, and how rimu fruit is involved in triggering breeding in this endangered bird. Based on the main conclusion of this review, changes in the reproductive structures of masting plants (specifically abundance, seasonal changes in nutrient composition, secondary metabolites or other non-nutrient chemicals) are the most likely candidate triggers of reproduction. To investigate this I carried out a study into the nutrient composition/quality and the non-nutrient chemical composition of rimu fruit over the breeding season, and whether any of the most likely triggers in other avian species have the potential to induce breeding in the kakapo.

SECTION 2: RESEARCH RESULTS

1. Introduction

The kakapo is a flightless, nocturnal parrot endemic to New Zealand. Once widespread, kakapo are extremely vulnerable to introduced mammalian predators and now survive only on a few offshore islands that are completely free of these pests. Kakapo are exclusively herbivorous (eating fruits, seeds, leaves, stems and rhizomes) and are the only parrot species with a lek breeding system (Merton *et al.*, 1984). The only flightless parrot, kakapo have an unusually slow metabolic rate and are longer lived than any other parrots (Linstedt & Calder, 1981; Lloyd & Powlesland, 1994). Their daily energy expenditure is the lowest recorded for any adult wild bird (Bryant, 2006) and they can survive, but not breed, on a low quality diet for years. Adults may increase in weight by as much as 100% before the breeding season. Kakapo have one of the lowest reproductive rates of any bird, but as they are very long-lived they probably produce a similar number of young over their life-span as more fecund birds.

In southern New Zealand, breeding is known to be dependent on infrequent mast fruit crops of two native trees; rimu and pink pine (*Halocarpis biforme*) (Podocarpaceae). On Codfish Island, there is virtually no pink pine so nesting only occurs in conjunction with rimu masts. In our recently published study on the nutrient composition of the diet of parent-raised kakapo nestlings (Cottam *et al.*, 2006) we found that female kakapo were feeding almost exclusively 100% rimu fruit to their chicks. Rimu is also part of the adult diet, although not exclusively. Close monitoring of kakapo in the Stewart Island region over the last 25 years revealed that breeding has been synchronous with every rimu and pink pine mast that has occurred over that period (i.e. 1981, 1985, 1992, 1997, 1999 and 2002) (Kelly, 1994 ; Merton, 2002; Powlesland *et al.*, 1992; Wilson, 2004). In 2002, an exceptionally heavy rimu fruiting year, 95% of the adult females on Codfish Island bred. Interestingly, young females with no experience of rimu (since they were nestlings) are able to identify the green rimu fruit as a food source for nestlings and breed in the very first mast they experience as adults (R. J. Moorhouse, pers. comm.).

Reproduction in rimu spans three growing seasons (Norton & Kelly, 1988). Cone initiation occurs in late summer of season 1, pollination in season 2, fertilisation in early spring-summer of season 3 and ripening and seedfall in autumn of season 3 (Norton *et al.*, 1988). The ovules are held erect on the ends of upturned branchlets from the time of pollination, with the tips of the branchlets turning upwards by 90^{o} (McEwen, 1983). The ultimate leaves form a fleshy receptacle, which develops succulence as it matures, and the entire reproductive unit is shed at seed-fall. The ripe fruit comprises a single ovule (seed) supported on the fleshy receptacle. The proportion of branch tips bearing fruit is closely correlated with breeding on Codfish Island; nesting has only occurred when more than 10% of rimu branches bore fruit (as manually counted early in the year) (Harper *et al.*, 2006). Female kakapo begin to feed on green rimu fruit in late October/early November (Harper *et al.*, 2006), Podocarp fruits were more prevalent in diets of both male and female kakapo in breeding seasons compared with non-breeding seasons (Wilson *et al.*, 2006). In female kakapo, the percentages of podocarp fruit in the diet increased with time; 20% in December, 80% in January, and 100% in both February and March. In males the percentages were 40, 50, 100 and 60% (Wilson *et al.*, 2006).

As previously mentioned, ripe rimu fruit is the predominant food provided to kakapo nestlings on Codfish Island. When there is insufficient rimu fruit kakapo do not attempt to nest, and if a crop large enough to induce nesting subsequently fails to ripen chicks invariably starve. Females start nesting about a month before the rimu crop is due to ripen, which causes hatching to coincide with the ripening of the crop. Male kakapo can begin booming as early as December, two months before the fruit ripens. This indicates that kakapo ‘anticipate’ the availability of a quantity of ripe rimu fruit sufficient to raise chicks. The most obvious predictor of ripe rimu fruit is unripe, green rimu fruit (Harper *et al.*, 2006). Green rimu fruit precedes ripe fruit by an entire year thereby giving kakapo plenty of time to “decide” whether or not to breed. The actual mechanism by which kakapo assess the size of the rimu crop is unknown. It could be visual but, since kakapo eat green rimu fruit, it is also possible that substances present in the unripe fruit or leaves trigger reproduction. For example, Fidler *et al.,* (2008) hypothesised that low levels of dietary phytochemicals may affect reproduction in the kakapo. However, attempts to induce kakapo to breed with food supplements, including harvested podocarp fruits, have so far been unsuccessful. In 2004 kakapo were fed a combination of ripe freeze-dried rimu and another more easily harvested podocarp fruit, kahikatea (*Dacrycarpus dacrydiodes*), but failed to nest (Harper *et al.*, 2006). Although supplementary foods in the past have increased the body weights of female kakapo they have not induced breeding. It is possible that the female kakapo release reproductive hormones in response to the size of the green rimu crop. In the past the larger rimu fruit crops have been linked with larger clutches of kakapo chicks (Harper *et al.*, 2006).

This kind of *anticipatory reproduction* has been documented in a variety of bird and mammal taxa. Examples of these include: female American and Eurasian red squirrels (Boutin *et al.*, 2006), piñon jay (Ligon, 1978), great tits (Nager & van Noordwijk, 1995), crossbills (Hahn, 1998), white-footed mice (Merson & Kirkpatrick, 1981), edible dormice (Fietz *et al.*, 2009).

In each case animals are breeding in anticipation of an essential food supply for their young. In most cases, the cues they use to anticipate these food sources are, however, poorly understood. It is possible that nutritional compounds may play a role in triggering reproduction. However, although a number of authors have investigated the theory that the ingestion of buds and other plant material triggers reproduction, most have concluded that this is unlikely. Nonetheless, the possibility that chemical cues, such as tannins, could trigger reproduction cannot be ruled out. Tannins and other naturally occurring chemicals (pollutants and phytoestrogens) are known to affect reproduction, albeit not necessarily as triggers. Some phytoestrogens (flavenoids) have been reported to have adverse effects on reproductive function in ruminants and avian species, and can have both agonistic and antagonistic effects under different conditions (Zhao, 2005).

Polyphenolic compounds in plants are known to affect reproduction in some mammalian species, increasing reproductive efficiency by increasing fecundity and reducing embryonic loss (Min *et al.*, 2001). Oxidation produces energy to fuel biological processes, and is essential to many living organisms (Barros *et al.*, 2007). These biological processes produce free-radicals as by-products. Free radicals are an integral part of normal physiological functions in humans (and in other mammals), but their overproduction can cause cellular injury and death (Wong *et al.*, 2006). Many organisms possess antioxidant defences but these are insufficient to counteract the overproduction of free-radicals. Plant products (e.g. leaves, fruits, seeds) contain antioxidants, including flavonoids and polyphenols, which are effective in reducing the effects of the free-radicals. Polyphenols and other antioxidants may have a role in reproduction in the kakapo.

This thesis investigates the theory that some chemical component(s) (nutritional or non-nutritional) in unripe rimu fruit acts as a 'reproductive trigger' in the kakapo. Kakapo are critically endangered so it was not possible to perform experiments on them. Therefore, my approach was to characterise seasonal changes in nutritional components (gross nutrients), polyphenolic compounds and other potential target polyphenolic substances with possible hormonal activity, including isoflavones and antioxidants, during rimu fruit development. Samples of rimu fruit were collected from a number of trees over one calendar year. These samples were analysed for gross nutrients, polyphenolic compounds, antioxidant activities and flavenoids. The object of this study was to elucidate possible changes in these compounds (increases or decreases) which could potentially trigger breeding in kakapo.

2. Materials and methods

2.1. Study location

This study was carried out in Whenua Hou Nature Reserve (on Codfish Island) (*Figure 1*) from May 2007 to February 2008. Only female rimu trees have fruit, therefore only female trees were used in this study. Female trees were initially identified using binoculars and the identification confirmed by collecting a small sample of foliage.

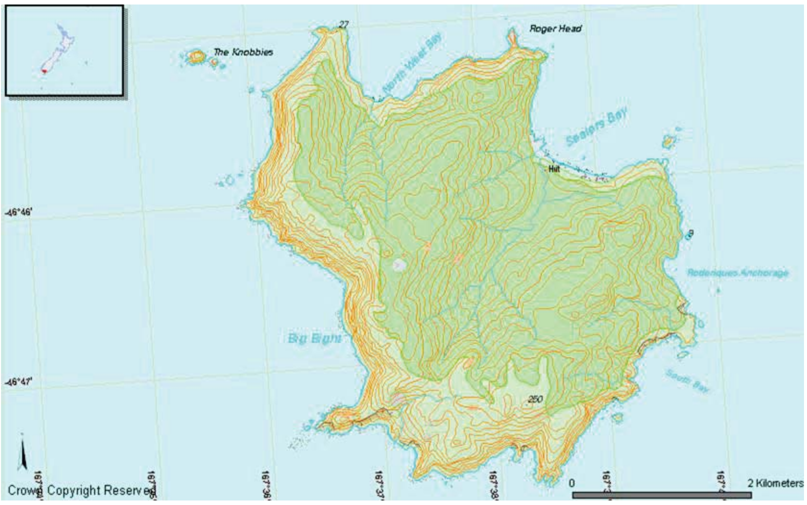


Figure 1. Codfish Island.

2.2. Sample collection

Twenty-one female rimu trees were identified and numbered 1 to 21 (*Figure 2*). Trees 1-8 were located on the south-eastern (loop) side of the island, and trees 9-21 located on the north-western (summit) side of the island, where there are more rimu trees.

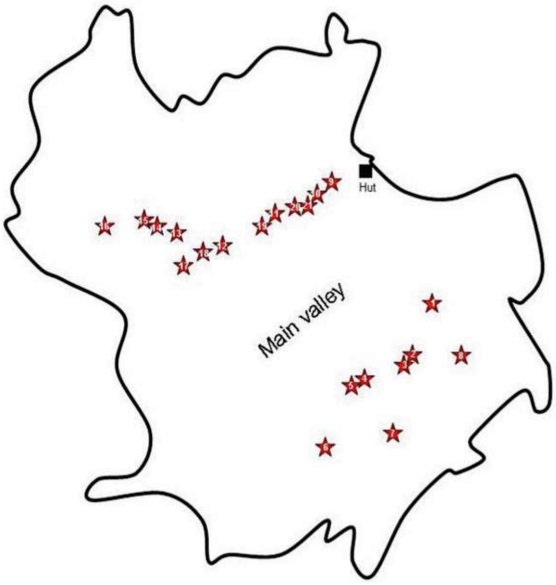


Figure 2. Approximate location of each of the rimu trees (numbered red stars) from which fruit was collected, on Codfish Island.

Samples of rimu fruit were collected from these 21 trees at 3-monthly intervals for one year, in mid-May 2007, mid-Aug 07, late-Nov 07 and mid-Feb 2008. There was insufficient fruit on four of these trees at the May sampling (trees 9, 12-14), so these trees were dropped from the sample. One tree was added (tree 5a) to increase sample size. To investigate possible changes over the kakapo breeding period, extra samples from six of these trees (trees 1, 4, 7, 10, 16, 18) were collected between Nov-07 and Feb-08 on 9 Jan, 21 Jan and 6 Feb 2008.

To investigate possible within- and between-tree variation, a subset of six trees was selected (trees 1, 7, 10, 18, 20, 21) and an extra four samples were collected from each of these, one from each of the four cardinal points, i.e. branches facing north, east, south and west (n,e,s and,w) around the tree. These 'aspect samples' were collected at the same time as the Feb-08 samples. A summary of all the samples collected is presented in *Table 1*.

Table 1. Summary of samples collected over the year from individual, marked female rimu trees. Sample codes in bold are the 3-monthly samples.

	2007			**2008**				Aspect samples
Sample code:	**May-07**	**Aug-07**	**Nov-07**	9-Jan	21-Jan	6-Feb	**Feb-08**	(n,e,s,w)
Tree #								
1	✓	✓	✓	✓	✓	✓	✓	✓
2	✓	✓	✓				✓	
3	✓	✓	✓				✓	
4	✓	✓	✓	✓	✓	✓	✓	
5	✓	✓	✓				✓	
5a			✓				✓	
6	✓	✓	✓				✓	✓
7	✓	✓	✓				✓	✓
8	✓	✓	✓	✓	✓	✓	✓	
9	✓							
10	✓	✓	✓	✓	✓	✓	✓	
11	✓	✓	✓				✓	
12	✓							
13	✓							
14	✓							
15	✓	✓	✓				✓	
16	✓	✓	✓	✓	✓	✓	✓	
17	✓	✓	✓				✓	
18	✓	✓	✓	✓	✓	✓	✓	✓
19	✓	✓	✓					
20	✓	✓	✓					✓
21	✓	✓	✓					✓
Total trees	21	17	18	6	6	6	18	6
Date collected	9-16 May	12, 13, 16, 17 Aug	28, 30 Nov, 2 Dec	9 Jan 08	21 Jan 08	6 Feb 08	16, 18 Feb	16, 18 Feb

From each tree, branches were harvested by shooting them from the tree with a 12 gauge shotgun firing number 2 or 3 size stainless steel shot in 1.5 inch (38.1 mm) cartridges. Trees were checked visually and by telemetry for kakapo and other birds in the tree or nearby before firing. The aim was to collect a 'supermarket bag' full of vegetation (*Figure 3*), which was bagged and taken back to the hut for hand-picking. The sample from each tree was hand-picked, with each individual fruit picked into a bowl, taking around an hour per tree and yielding approximately 10-15 g of fruit. The hand-picking was fiddly and time consuming, especially earlier in the season when the fruit were tiny. For example, the fruit was smallest in May-07, when a sample of 36 individual fruit weighed 0.1827 g, and a sample of 4.22 kg of foliage yielded 75.5 g of fruit. The 169 g total fruit collected on this trip represented approximately 33,300 individual tips. A total of 1408 g of fruit was collected over the course of the study.

A number of attempts were made to follow female kakapo at night to locate rimu trees in which they were foraging, but this was unsuccessful as they were only found foraging on the ground. Therefore, although I cannot be sure if females foraged in the actual trees sampled, I have assumed that the trees sampled were similar to those that the females forage in.

Figure 3. An example of the amount of vegetation collected from one tree.

A ripe rimu fruit has two parts: a seed and a fleshy aril upon which the seed rests. My aim was to collect for analysis only the parts of the fruit that are eaten by kakapo. What these were was confirmed by two methods. A branch of rimu fruit was offered to Hoki, a female kakapo (*Figure 4*), and observation confirmed that she ate the entire fruit, both the seed and aril. To provide further confirmation, a branch of rimu was placed at the supplementary feed hopper of another female kakapo and the bird's feeding behaviour recorded by remote digital video. A visual assessment of faecal samples confirmed that the seed was digested and did not pass straight through. In mid-January 169 individual seeds from tree #6 were examined for soundness, with an average of 87 % sound (with a juicy kernel as opposed to a dry brown seeds). The unsound seeds were visually defective, so were not included in the samples. *Figure 5* shows an example of a branch of vegetation and what was actually collected (the fruit), both unripe fruit from the start of collection (May-07) and almost ripe fruit from Feb-08. The fruits were picked by breaking them from the branch at the point where the tip starts to bend upwards, which is a point of natural weakness. *Figure 6* shows the fruit collected from one tree over the entire season, and *Figure 7* is an example of fruit development over the collection period. *Figure 8* is an example of the 'aspect samples'; the fruit collected from each of the four cardinal points to investigate possible within-tree variation.

Figure 4. Hoki sampling a branch of rimu fruit. Photo taken from video footage.

Figure 5. Rimu fruit on branches in May 2007 (left) and February 2008 (seed mostly developed) (right). The circles are examples of the fruits as collected, and inset are samples actually collected.

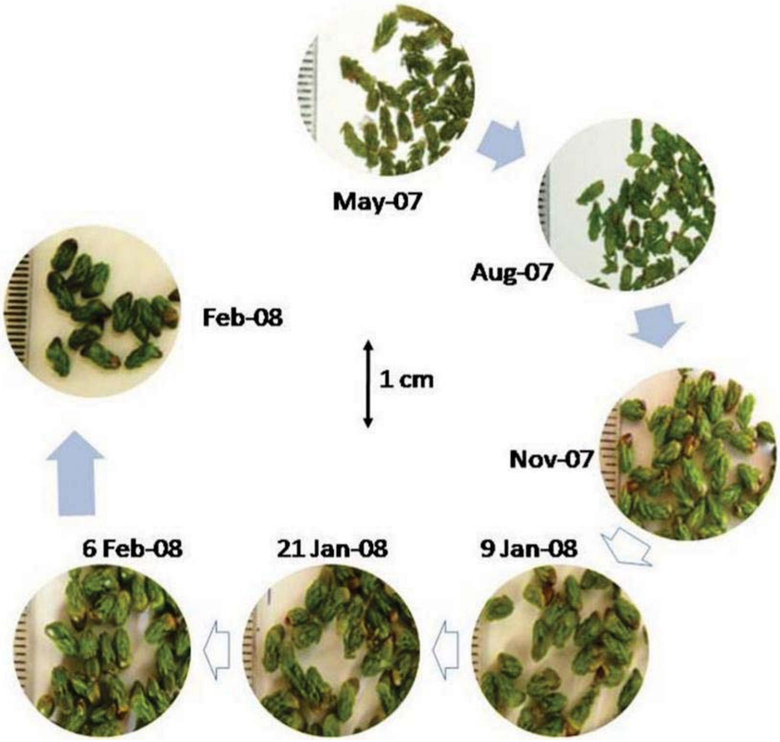


Figure 6. Rimu fruit collected from tree 1 over one year, with the least developed fruit in May 2007 to the most developed fruit in February 2008. Note that the bottom three samples were taken at 2-weekly intervals, whereas the other samples were taken at 3-monthly intervals.

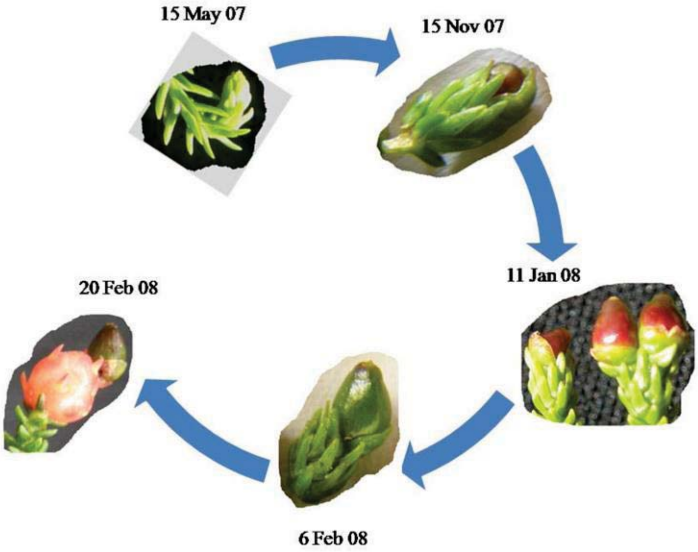


Figure 7. Rimu fruit development from May 2007 (least developed) to February 2008 (most developed). Upturned shoot tips in May, the ovule is emerging in November, is bigger in the January and 6 February samples, and the ripe red receptacle had developed in some fruit by 20 February.

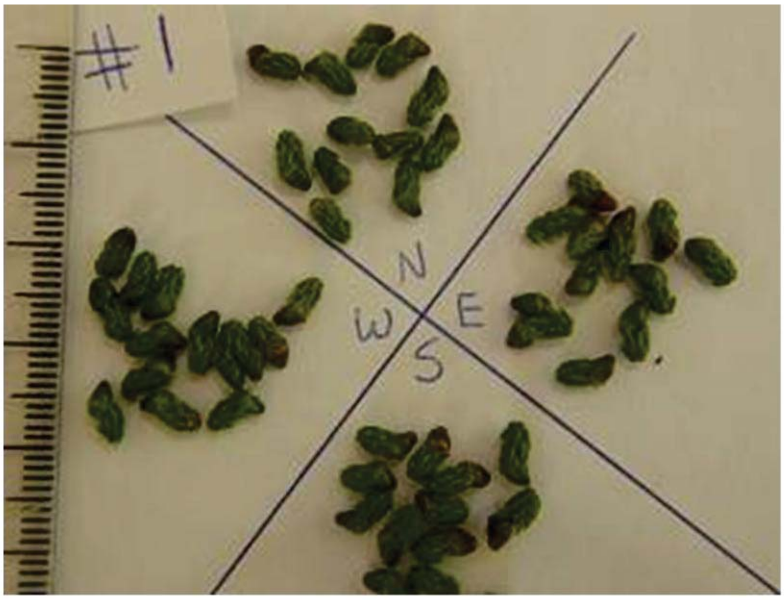


Figure 8. An example of the rimu fruit collected from the four aspects (branches facing north, east, south, west) around Tree 1, collected to examine within-tree variation.

Each of the main three-monthly samples (taken from a total of 17-21 trees), was pooled to give one sample for each time-point, with the same mass of fruit from each tree represented in each pooled sample. Samples taken from the subset of six trees on 9 Jan 08, 21 Jan 08 and 6 Feb 08 were split into two, with part of the sample kept separate (one sample per tree) and the rest of the sample pooled to give one sample per time point as for the 3-monthly samples. Samples collected in Feb-08 from the four orientations around each of six trees were also kept separate, with four samples per tree. All the samples were frozen, freeze-dried and ground before analysis.

2.3 Chemical analyses

2.3.1. Gross nutrient analyses

The full range of gross nutrient analyses were carried out on the seven composite samples i.e. the four 3-monthly samples (May-07, Aug-07, Nov-07, Feb-08) and the three extra samples (9 Jan 08, 21 Jan 08 and 6 Feb 08). These ground samples were analysed for dry matter (DM, the measure of water content, used to adjust the proportions of all nutrients to a common basis), crude protein, lipid, amino acids (a total of 17 individual amino acids), fatty acids (a total of 10 fatty acids), minerals, ash, energy, fibres and sugars. The aspect samples were analysed only for dry matter, ash, crude fat and crude protein. Gross energy was calculated as (crude protein*17)+(crude fat*37)+(carbohydrates*17).

Dry matter was determined by oven drying for 16 hours at 105°C while ash was determined by heating the sample to 550°C for 16 h. Crude protein was determined by multiplying nitrogen by 6.25 with nitrogen determined by the Kjeldahl technique. Lipid (crude fat) was analysed using petroleum ether extraction (AOAC, 2000). Cellulose, hemicellulose and lignin were calculated from neutral detergent fibre, acid detergent fibre and acid detergent lignin analyses conducted using methodology described by Van Soest (1973) and Van Soest (1991). Amino acids were determined by hydrochloric acid hydrolysis followed by high performance liquid chromatography separation with cysteine and methionine oxidation before hydrolysis as described by Hendriks *et al.,* (2000). Fatty acids were analysed using gas chromatography, with mineral determination carried out by plasma emission spectrophotometer. Simple sugars were analysed by a combination of gas chromatography and spectrophotometry according to the method of Englyst (1994).

2.3.2. Phenolic contents and antioxidant activity

Polyphenolic and antioxidant activity analyses were carried out on the seven composite samples and the aspect samples collected in Feb 08, using the methods described by Molan *et al.,* (2009). The freeze-dried fruit samples were extracted in either distilled water or ethanol, and analysed at three concentrations, 5 mg/ml, 2.5 mg/ml and 1.25 mg/ml. All samples were analysed in duplicate and each assay was repeated twice.

Total polyphenolic content (TPC) was quantified by Folin-Ciocalteu test. In brief, an aliquot of 12.5 µl of extract was mixed with 250 µl of 2% sodium carbonate solution in 96-well microplates and allowed to react for 5 minutes at room temperature. Then 12.5 µl of Folin-Ciocalteu phenol reagent (50 %) was added and left to sit at room temperature for a further 30 minutes. Absorbance was then read at 650 nm using a plate reader. Calibration was carried out using an aqueous gallic acid solution (100-1000 µg/ml). TPC was expressed as mg gallic acid equivalents per gram of dry powder.

The ability of the extracts to reduce ferric ions was determined using the FRAP (reducing ferric ion antioxidant potential) assay. An aliquot of 8.5 µl of extract was added to 275 µl of diluted FRAP reagent on a 96-well microplate, and incubated at 37°C for 30 minutes. Absorbance was read at 395 nm using a plate reader. The FRAP reagent was prepared by mixing 10 parts of 300 mmol/l acetate buffer (pH 3.6), 1 part of 10 mmol/l TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mmol/l hydrochloric acid and 1 part of 20 mmol/l ferric chloride. The standard curve was prepared using 200-2000 µmol/l of $Fe_2SO_4.7H_20$. The antioxidant capacity of the extracts are based on the ability to reduce ferric ions, and expressed as mmol $Fe_2SO_4.7H_20$ per litre of aqueous extract.

The radical scavenging activity of the crude extracts were measured using the DPPH (1,1-diphenyl-2-picrylhydrazyl hydrate) assay. DPPH is a stable nitrogen-centred free radical, with a colour change of violet to yellow when reduced by the addition of H or electron donation. This action can be carried out by antioxidants (radical scavengers). An aliquot of 25 µl of extract was allowed to react with 250 µl of 0.2 mM DPPH in 95 % ethanol in a 96-well microplate. This was incubated at 37°C for 30 minutes and then the absorbance was read at 550 nm using a plate reader. Scavenging activity of the extract was compared to ascorbic acid (0.1 – 1.0 mM) as a positive control. Antiradical activity was calculated as the percentage of DPPH decolourisation relative to a negative control:

$$\text{Antiradical activity (\%)} = \frac{(\text{absorbance of control} - \text{absorbance of sample})}{\text{absorbance of control}} \text{ x } 100$$

Chelating activity was measured using the ferrous-ion chelating (CHA) assay. Diluted Fe_2SO_4 (100 µl of 2 mM solution) was mixed with 100 µl of extract and then 100 µl diluted ferrozine (5 mM solution) was added. This was allowed to equilibrate for 10 minutes then absorbance was read at 560 nm using a plate reader. The ability of the extract to chelate ferrous ions was calculated relative to the negative control:

$$\text{Chelating activity (\%)} = \frac{(\text{absorbance of control} - \text{absorbance of sample})}{\text{absorbance of control}} \text{ x } 100$$

2.3.3. Flavonoid identification

Flavonoid analyses were carried out on the seven composite samples (May-07, Aug-07, Nov-07, 9-Jan-08, 21-Jan-08, 6-Feb-08 and Feb-08) and on five samples (Nov-07, 9-Jan-08, 21-Jan-08, 6-Feb-08 and Feb-08) from three individual trees (tree # 1, 4, 10). A sample of ripe rimu fruit collected in 2002 was also analysed for comparison.

The flavonoids (anthocyanins, flavans, flavanones, flavones, flavonols and isoflavonoids) present in the crude extracts and freeze-dried fruits were identified by HPLC (high-performance liquid chromatography) and LC-MS (liquid chromatography-mass spectrometry) methods. The samples were prepared using the modified method of Reynertson *et al.,* (2008).

In brief, 50 mg of freeze-dried sample was measured, 1 ml of MeOH-formic acid (9:1) was added, and the sample sonicated for one hour. The sample was spun in a micro-centrifuge at 12000 rpm for 2 minutes, the supernatant was pipetted off and 1 ml of MeOH added to the pellet. Extracts were sonicated, centrifuged and the marc (solids) extracted twice more. Extracts were combined and reduced from 3 ml to approximately 0.5 ml by evaporation under nitrogen (approximately an hour). HPLC/GC/MS analyses were then carried out on the samples as follows. The extracts were analysed using a Thermo Surveyor HPLC system equipped with a LTQ linear ion-trap Mass Spectrometer operating in positive electrospray mode, scanning from 150-1500 m/z.Aliquots (5µL) were injected onto a Phenomenex Luna C18 column (150 x 2.0mm, 5µm particle size) with a column flow rate of 0.2ml/min and an oven temperature of 25^{o}C. The mobile phase solvents were A) water containing 0.1% formic acid and B) acetonitrile containing 0.1% formic acid. The initial gradient was 5% B held constant for 6 minutes, then increased to 10% B at 11 minutes, 17% B at 26 minutes, 23% B at 31 minutes, 30% B at 41 minutes, 50% B at 45 minutes and then held for a further 7 minutes at 50 % B, then increased to 97% B at 55 minutes and held for a further 4 minutes before being reduced back to 5% B at 62 minutes and held at that concentration till 70 minutes.

The method of concentration of the sample before the HPLC analyses were carried out results in the samples being at slightly different dilutions making it impossible to compare absolute peaks between samples. The area under the curve was calculated for the main peaks, to enable comparison between ratios.

2.3.4. Oestrogenic Activity

The seven composite samples (May-07, Aug-07, Nov-07, 9 Jan 08, 21 Jan 08, 6 Feb 08 and Feb-08) and samples from to individual trees (trees 1 and 18) were tested for oestrogenic activity. A YES/hER system, utilising a yeast strain with the human oestrogen receptor alpha inserted into the genome, was used to test for oestrogenic activity in plant extracts (Mortensen, 2009). The strain was also transfected with a reporter plasmid containing an oestrogen response element joined to a *LacZ* reporter gene. Samples of rimu fruit were extracted using a beta-glucoside assisted water technique. The media contained CPRG, a yellow compound which is converted to red by B-galactoside (product of the *LacZ* gene) and the optical density was measured at

540 nm. These analyses were carried out by Kelly Mortensen as part of an honours project (Mortensen, 2009).

2.4. Statistical analyses

As this is a descriptive study with limited sample sizes there were a restricted number of statistical analyses that could be carried out. The gross nutrient composition and the polyphenolic/antioxidant results from the aspect samples were subject to analysis of variance with tree and aspect as fixed effects. The effect of time on polyphenolic and antioxidant results was tested using repeated measures analyses. Correlations were carried out between the phenolic contents and antioxidant activity assays.

All of the analyses were carried out using the statistical package SAS (SAS, 1999).

3. Results

3.1 Gross nutritional composition

The gross nutrient composition of the seven composite rimu fruit samples is presented in *Table 2*, from the four aspects around six individual trees in *Figure 9*, the amount of crude protein and fat in *Figure 10* and the total amount of nutrients (sum of crude protein, crude fat, crude fibre, ash, sugars) from each sample in *Figure 11*. There was a highly significant difference in percentage dry matter between the six individual trees ($P<0.001$) and a trend towards significance for aspect ($P=0.08$). Tree 18 had a significantly lower level of dry matter (32.0%) than the other five trees (ranging from 33.8 - 34.6%). For both ash and crude fat, there was a difference between trees (ash $P<0.001$, crude fat $P<0.05$), but no difference within trees (i.e. aspect). There was a trend for crude protein to differ between trees ($P=0.09$) but no difference within trees.

Estimates of variability were made from the results for six individual trees (and four aspects) collected in mid-Feb-08, with standard errors of 0.52% for dry matter, ash 0.12%, crude protein 0.2% and fat 0.22% (see *Figure 10*). Crude protein was lowest in Aug-08 (5.5%), and increased up to 7.0% over the season. There were no obvious changes in individual amino acids. The levels of crude fat dropped from 2.3% to 1,6% in late January, up to 2.7% in the mid-February sample. Total fatty acids increased by 76% over the season (from 9.93 to 17.48 mg/g DM). This increase mainly occurred between 6-Feb-08 and mid-February. Sugar content was 3.5 to 4% for May-07 to November-07, then decreased in the 21 January sample (down to 1%) then increased again in the 6 February sample (up to 2.7%) and the mid-February sample (up to 4.1%). Dry matter content decreased over the season, from around 40% in the May and August samples to 36% in the February sample. Crude fibres increased over time, with total crude fibre increasing from around 37% to 42%, and NDF and ADF both increasing 12 - 15% from the May sample to the February sample. Calcium did not change over the year (ranging from 1.1 to 1.3% DM), whereas phosphorus increased by 27% over the season, which resulted in a 23% lower Ca:P ratio.

The total amount of nutrients (sum of crude protein, crude fat, crude fibre, ash, sugars) in each sample remained around 54% of the total dry matter from Aug-07 to Nov-07 then increased up to 61% in the Feb-08 sample.

Table 2. Gross nutrient content of rimu fruit.

Sample	**May-07**	**Aug-07**	**Nov-07**	9 Jan	21 Jan	6 Feb	**Feb-08**
Fresh sample (%):							
Dry Matter	39.7	40.1	35.7	32.5	33.7	35.1	35.6
Dry matter basis (%):							
Ash	5.4	5.4	6.1	6.2	6.0	6.0	5.5
Crude protein	6.0	5.5	6.7	6.6	6.6	7.0	6.6
Amino acids	5.9	5.5	6.0	6.2	6.6	5.7	6.5
Crude fat	2.3	2.4	2.2	1.8	1.6	2.0	2.7
Fatty acids	1.0	1.1	1.1	1.0	1.0	1.3	1.7
Fatty acids:crude fat	0.44	0.44	0.48	0.60	0.63	0.67	0.65
Sugars	3.5	4.9	4.6	5.5	1.0	2.7	4.1
Crude fibre	36.6	37.0	34.3	35.5	39.0	40.6	42.0
NDF	48.7	48.2	47.6	48.6	53.4	53.3	54.6
ADF	39.5	39.4	40.2	41.6	44.8	44.4	45.5
Ca (mg/g)	11.6	11.5	12.2	12.7	12.1	12.7	11.2
P (mg/g)	1.1	1.1	1.3	1.4	1.3	1.4	1.4
Ca:P	10.1	10.4	9.4	8.8	9.2	9.3	8.2
CP+CF+CFibre+ ash+sugars[a]	53.7	55.3	54.0	55.6	54.1	58.3	60.9

[a] crude protein + crude fat+ fibre + ash + sugars

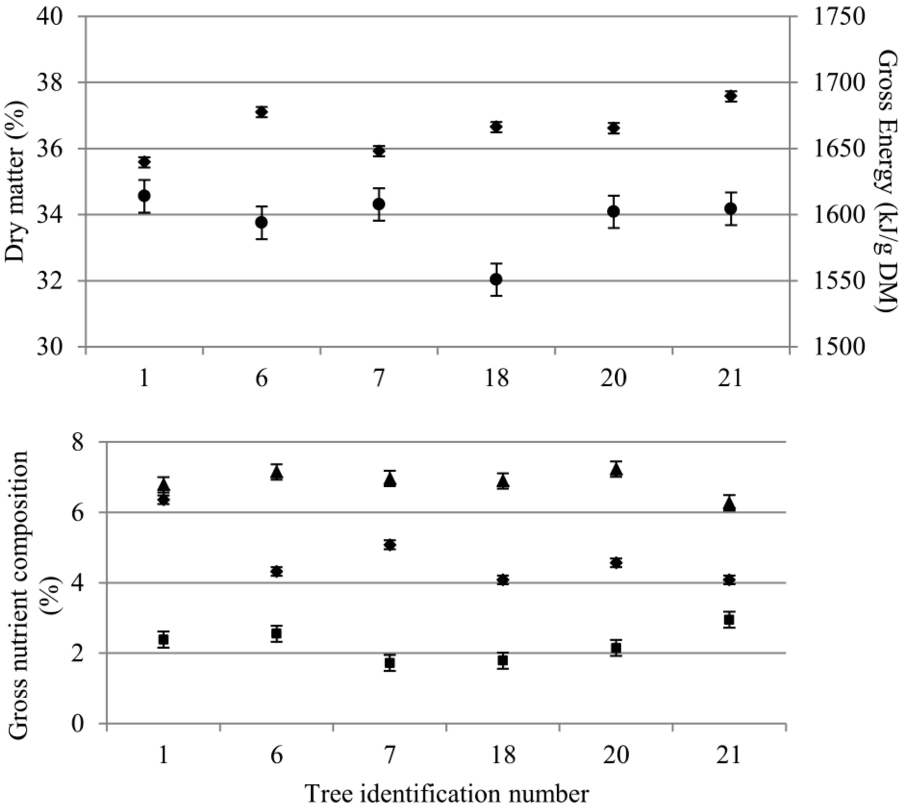


Figure 9. Dry matter (circles) and gross energy (diamonds) (top graph), crude protein (triangles), crude fat (squares) and ash (circles) in samples of fruit from six individual trees. Symbols are LSmean ± standard error.

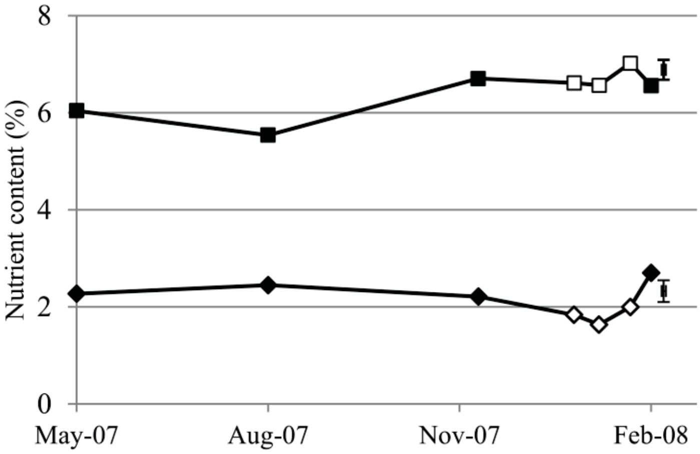


Figure 10. Amount of crude protein (squares) and crude fat (triangles) in rimu fruit over the year. Solid symbols are samples taken at 3-month intervals (composite of 17-21 trees), hollow symbols are more frequently sampled (composite of six trees). Standard errors are shown for the six individual trees sampled in mid-Feb-08.

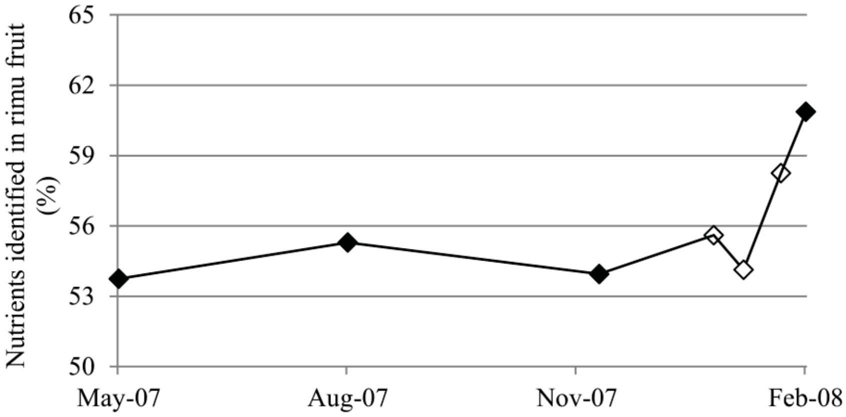


Figure 11. Total amount of gross nutrients (crude protein, crude fat, crude fibre, ash, sugars) in rimu fruit over the year. Solid symbols are samples taken at 3-month intervals (composite of 17-21 trees), hollow symbols are more frequently sampled (composite of six trees).

Figure 12 presents the amount of crude protein, crude lipid and calcium eaten if 100 g of dry matter was eaten by a kakapo, based on figure 10 in Raubenheimer & Simpson (2006).

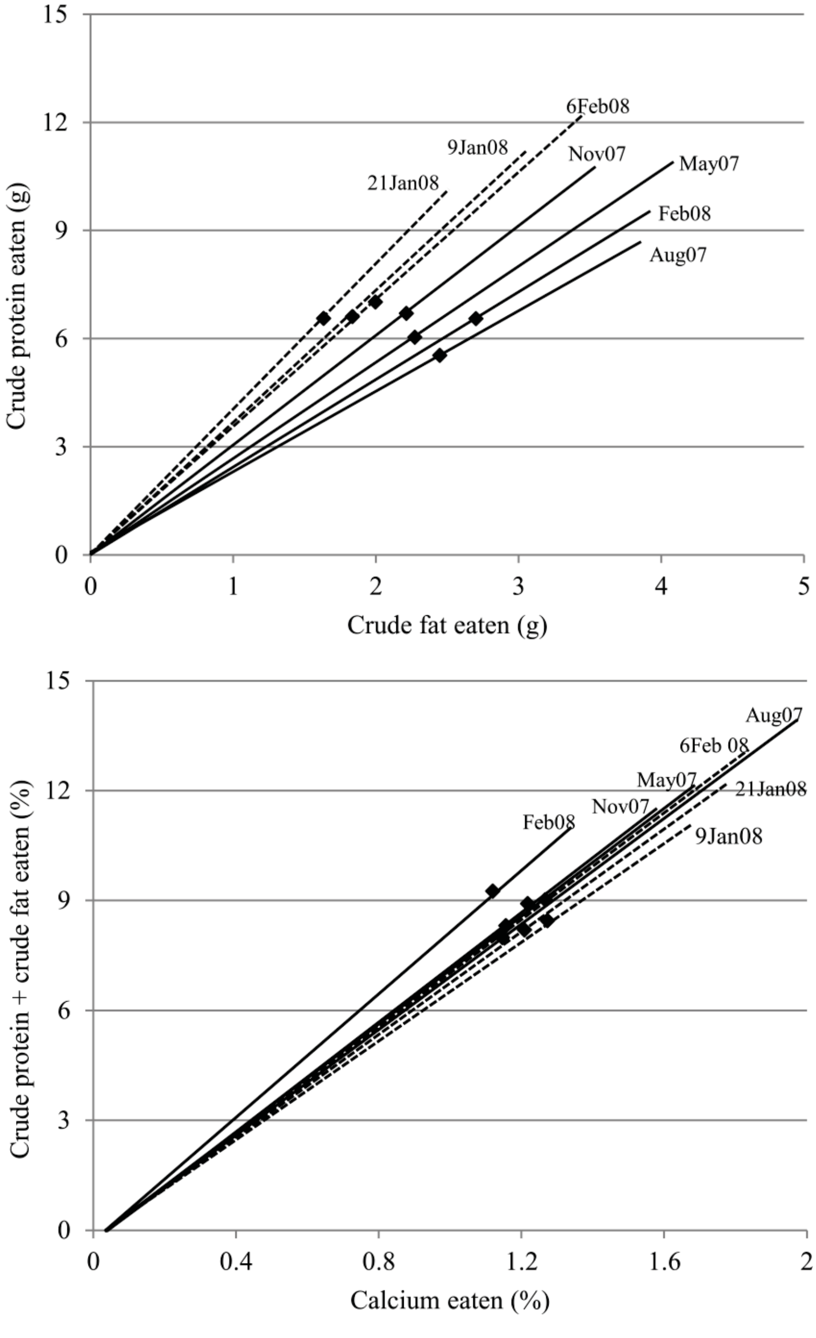


Figure 12. The amount of crude protein vs. crude fat (upper graph) and crude protein + crude lipid vs. calcium (lower graph) consumed per 100g of rimu fruit eaten. Solid lines are samples taken at 3-month intervals (composite of 17-21 trees), dashed lines are more frequently sampled (composite of six trees). Labels denote when samples were collected.

The amino acid content of the seven composite rimu fruit samples are presented in *Table 3*, fatty acids in *Table 4*, with the omega-3 and omega-6 content and the ratio of linoleic (18:2) : linolenic acid (18:3) in *Figure 13* and the amounts of the four main fatty acids in rimu fruit (linolenic, palmitic, oleic and linoleic) (*Figure 14,* upper graph) also expressed as the percent of the total fatty acids (*Figure 14,* lower graph).

The total amount of fatty acids as a proportion of crude fat increased by 47% over the season, from 0.44 to 0.65 (*Table 4*). This change occurred between the November and early January samples. However, although the amounts of some individual fatty acids increased others decreased over the season. Individual fatty acids which showed the largest increase over the season were palmitic (2.4 to 3.1%), stearic (0.2 to 0.35), oleic (0.7 to 2.1), linoleic (1.8 to 2.9), C 18:3 -cis9,12,15 linolenic (3.3 to 7.7), C18:1 -cis11 vaccenic (0.07 to 0.19). The fatty acids showing the largest decrease were lauric (0.10 to 0.07), capric (0.05 to 0.01), myristic (0.13 to 0.09), caprylic (0.05 to 0.03), arachidic (0.20 to 0.17). Expressed as the percentage of total fatty acids, linolenic was the most common (30.7 to 44.0 % of total fatty acids), then in order of decreasing abundance: palmitic (18 to 27%), linoleic 16.8 to 23.5%, and oleic (4.5 to 11.7%) (lower graph, *Figure 14*). Actual levels of these fatty acids in rimu fruit seemed to increase over the January-February period (upper graph, *Figure 14*). Of the four principal fatty acids in rimu fruit, linolenic and oleic both increased over the December-February period, and palmitic and linoleic both decreased as a percentage of total fatty acids.

The amount of omega-3 fatty acids in rimu fruit increased over the season to more than double in the February sample compared with the May sample. Omega-6 fatty acids did not change over the year, therefore the omega3:omega6 fatty acid ratio increased with season (lower panel, *Figure 14*). The ratio of linoleic:linolenic acid increased slowly from August to the 9 January sample, then dropped rapidly in the February sample (top panel, *Figure 14*).

Table 3. Amino acid content of rimu fruit.

Sample	**May-07**	**Aug-07**	**Nov-07**	9 Jan	21 Jan	6 Feb	**Feb-08**
Dry matter basis (%):							
Aspartic acid	0.624	0.584	0.664	0.661	0.708	0.624	0.733
Threonine	0.284	0.266	0.276	0.291	0.305	0.262	0.300
Serine	0.358	0.316	0.351	0.372	0.392	0.345	0.395
Glutamic acid	0.715	0.670	0.718	0.812	0.831	0.738	0.850
Proline	0.347	0.334	0.360	0.377	0.404	0.357	0.420
Glycine	0.378	0.348	0.368	0.375	0.395	0.340	0.387
Alanine	0.395	0.373	0.394	0.417	0.444	0.389	0.436
Valine	0.340	0.315	0.323	0.349	0.373	0.326	0.368
Methionine	0.084	0.074	0.088	0.083	0.087	0.068	0.084
Isoleucine	0.268	0.251	0.263	0.288	0.303	0.258	0.294
Leucine	0.547	0.518	0.587	0.582	0.605	0.518	0.587
Tyrosine	0.242	0.216	0.236	0.250	0.256	0.201	0.233
Phenylalanine	0.311	0.300	0.301	0.328	0.338	0.291	0.325
Histidine	0.187	0.168	0.192	0.191	0.196	0.173	0.202
Lysine	0.515	0.468	0.499	0.510	0.546	0.467	0.537
Arginine	0.345	0.330	0.346	0.350	0.385	0.329	0.387
Sum	5.939	5.532	5.966	6.236	6.570	5.685	6.537

Essential amino acids in bold.

Table 4. Fatty acid content of rimu fruit.

Sample	**May-07**	**Aug-07**	**Nov-07**	9 Jan	21 Jan	6 Feb	**Feb-08**
Dry matter basis (mg/g):							
C6:0 Caproic	0.067	0.072	0.032	0.045	0.054	0.049	0.022
C 8:0 Caprylic	0.049	0.047	0.040	0.029	0.030	0.033	0.031
C 10:0 Capric	0.047	0.038	0.021	0.019	0.013	0.015	0.010
C 12:0 Lauric	0.096	0.111	0.070	0.084	0.078	0.069	0.069
C 14:0 Myristic	0.129	0.146	0.117	0.107	0.091	0.086	0.088
C 16:0 Palmitic	2.368	2.290	2.537	2.924	2.802	2.974	3.114
C 16:1-cis9 Palmitoleic	0.046	0.039	0.039	0.065	0.065	0.068	0.081
C17:0 Margaric	0.094	0.105	0.077	0.062	0.054	0.066	0.068
C17:1 - cis10 Heptadecenoic	0.073	0.128	0.054	0.032	0.030	0.028	0.025
C 18:0 Stearic	0.212	0.215	0.226	0.244	0.218	0.283	0.353
C18:1 -trans11 Vaccenic	0.006	0.006	0.004	0.007	0.009	0.011	0.008
C 18:1-cis9 Oleic	0.691	0.638	0.477	0.784	0.655	1.380	2.051
C18:1 -cis11 Vaccenic	0.068	0.056	0.063	0.076	0.144	0.162	0.187
C18:2 -cis9,12 Linoleic	1.799	1.828	2.305	2.600	2.111	2.448	2.935
C 20:0 Arachidic	0.203	0.221	0.130	0.116	0.127	0.160	0.166
C18:3n6 -cis6,9,12 γ-linolenic	0.013	0.016	0.017	0.020	0.020	0.033	0.054
C20:1 -cis11 Eicosenoic	0.025	0.024	0.012	0.017	0.019	0.032	0.039
C 18:3 -cis9,12,15 linolenic	3.277	4.001	3.787	3.399	3.325	4.959	7.682
C21:0 Heneicosanoic	0.019	0.017	0.014	0.018	0.018	0.019	0.022
C20:2 -cis11,14 Eicosadienoic	0.016	0.014	0.012	0.007	0.006	0.010	0.017
C 22:0 Behenic	0.368	0.457	0.251	0.194	0.166	0.192	0.170
C 22:1-cis13 Erucic	0.020	0.020	0.011	0.019	0.019	0.008	0.010
C20:3n3-cis11,14,17 Eicosatrienoic	0.018	0.044	0.023	ND	0.008	0.031	0.057
C 20:4n6-cis5,8,11,14 Arachidonic	0.051	0.048	0.045	0.040	0.045	0.053	0.046
C22:2 -cis13,16 Docosadienoic	0.036	0.032	0.023	0.012	0.009	0.015	0.013
C 24:0 Lignoceric	0.129	0.121	0.138	0.159	0.155	0.165	0.162
Sum of fatty acids (mg/g)	**9.93**	**10.74**	**10.53**	**11.08**	**10.27**	**13.35**	**17.48**

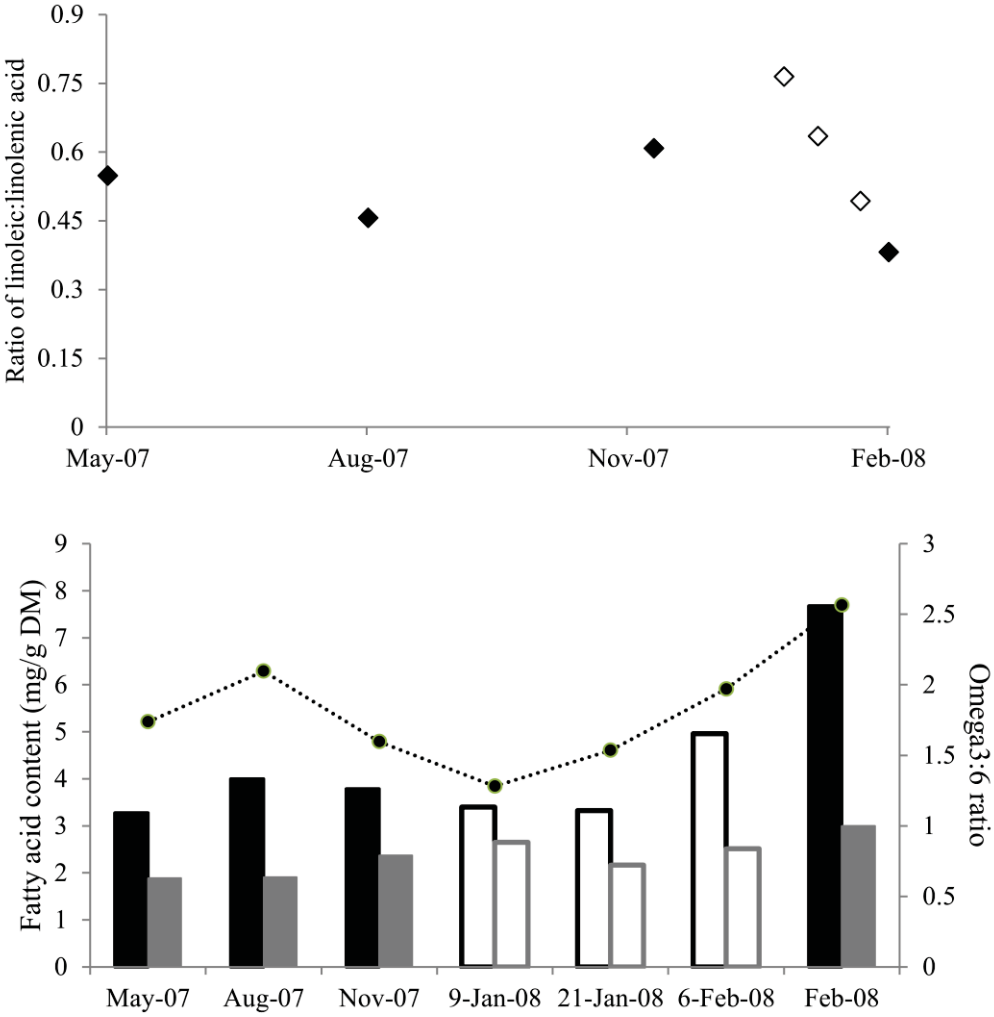
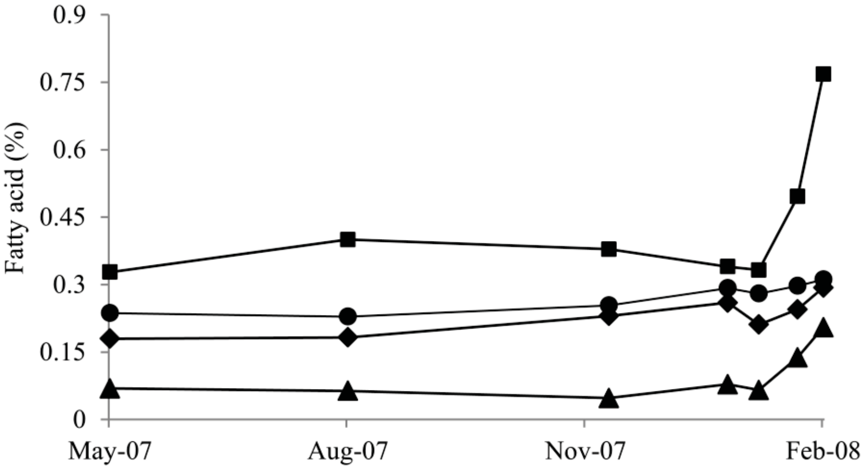


Figure 13. The ratio of linoleic (18:2):linolenic (18:3) fatty acids (upper graph) and the omega-3 (black bars) and omega-6 (grey bars) fatty acid content of rimu fruit (lower graph). The omega3:6 ratio is the black dotted line. Solid symbols are samples taken at 3-month intervals (composite of 17-21 trees), hollow symbols are more frequently sampled (composite of six trees).



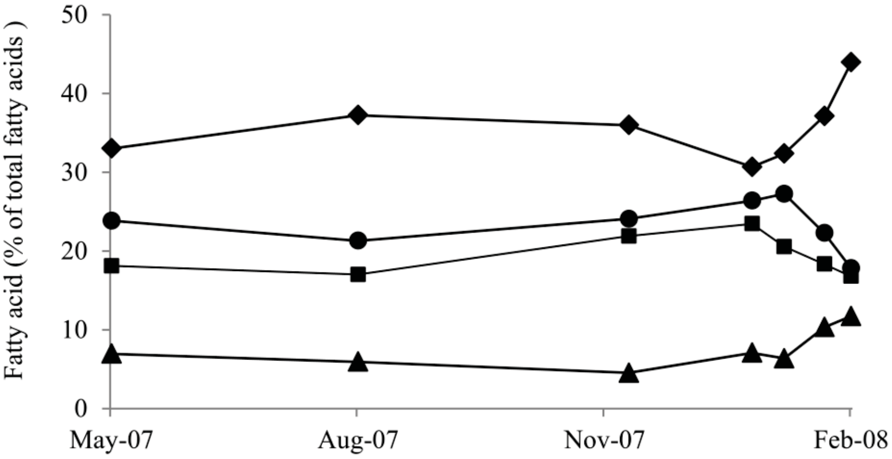


Figure 14. Levels of the four main fatty acids found in rimu fruit (upper graph) and the percent of each fatty acid as a proportion of the total fatty acids (lower graph). Symbols are linolenic (diamonds), palmitic (circles), oleic (triangles) and linoleic (squares). Solid symbols are samples taken at 3-month intervals (composite of 17-21 trees), hollow symbols are more frequently sampled (composite of six trees).

3.2. Phenolic contents and antioxidant activity

The correlations between phenolic contents and antioxidant activities are presented in *Table 5*, with a summary of phenolic content and antioxidant activity for the 5 mg/ml dilution in *Table 6*.

Table 5. Correlation coefficients for relationships between assays.

	FRAP	DPPH	TPC
CHA	0.33^{**}	0.40^{***}	0.43^{***}
FRAP		0.52^{***}	0.81^{***}
DPPH			0.60^{***}

** P<0.01, *** P<0.001

Table 6. Total polyphenolic content (TPC) and antioxidant activities of rimu samples from seven composite samples and the aspect samples collected in February 2008.

	Water		Ethanol	
	Range	Mean ± se	Range	Mean ± se
TPC (µg gae/g)	107 – 154	142 ± 7	109 – 152	137 ± 7
DPPH (% inhibition)	45 – 66	54 ± 4	53 – 81	68 ± 4
FRAP (µmol/l)	1541 – 2220	1933 ± 100	1986 – 2856	2486 ± 121
CHA (% inhibition)	9 – 22	16 ± 2	19 – 30	25 ± 2

3.2.1. Total polyphenolic content

The total polyphenolic content (TPC) of seven composite rimu fruit samples is shown in *Figure 15*, and from the aspect samples in *Figure 16*. There was no difference between water and ethanol extract for TPC (P>0.10), with TPC changing over time (P<0.001). There was a significant effect of concentration (P<0.001), tree (P<0.001) and aspect (P<0.05) for TPC.

The levels of TPC in the pooled samples were similar over most of the year (*Figure 16*), with a decrease in TPC in both the 21-Jan-08 and 6-Feb-08 samples, and an increase in Feb-08 as the fruit ripened.

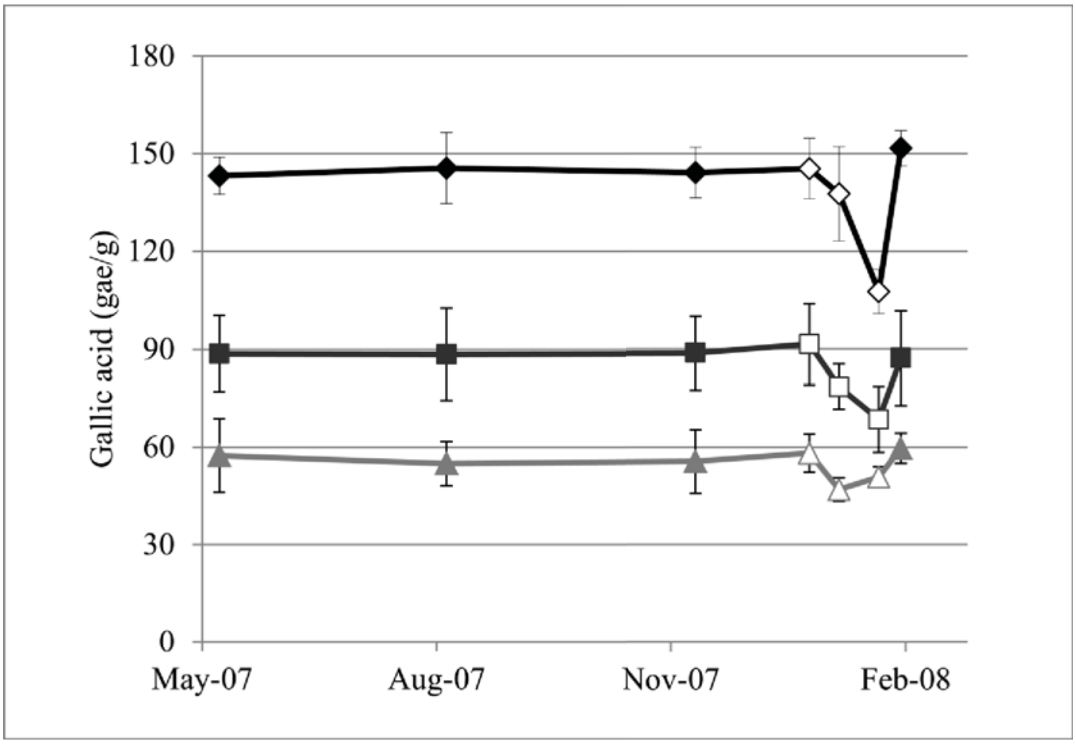


Figure 15. Average total polyphenolic content (gallic acid equivalents) of the three dilutions of extracts (black 5 mg/ml red; 2.5 mg/ml, blue 1.25 mg/ml) of composite rimu fruit samples taken over one year. Solid symbols are samples taken at 3-month intervals (composite of 17-21 trees), hollow symbols are more frequently sampled (composite of six trees).

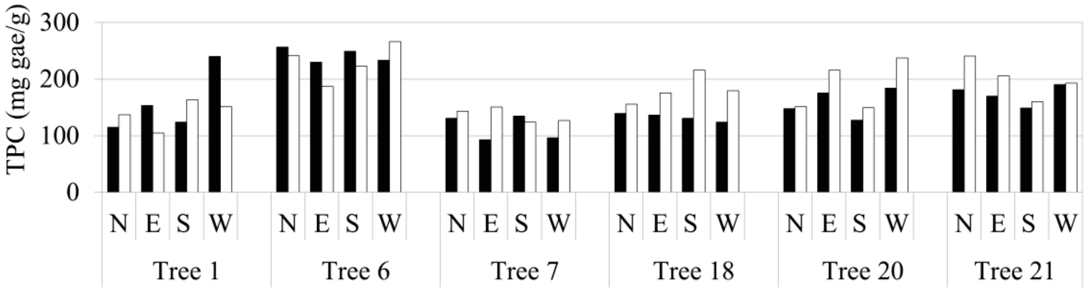


Figure 16. Total polyphenolic content (gallic acid equivalents) (water extracted (black bars) or ethanol extracted (white bars)) of rimu fruit extract from six individual trees, four aspects per tree, collected in Feb 2008. Only the 5 mg/ml dilution is shown here, as similar trends were observed for other dilutions.

3.2.2. DPPH

The 1,1,-diphenyl-2-picrylhydrazide free radical scavenging ability (DPPH) of seven composite rimu fruit samples is shown in *Figure 17* and from the aspect samples around 6 trees in *Figure 18*. There was no difference between water and ethanol extract for DPPH, with DPPH decreasing over time ($P<0.001$), dropping from 75% to 40% by the final sample (Feb-08), with the most dramatic drop between 9-Jan-08 and 21-Jan-08. There was a significant effect of concentration ($P<0.001$), tree ($P<0.001$) and aspect ($P<0.05$) for DPPH.

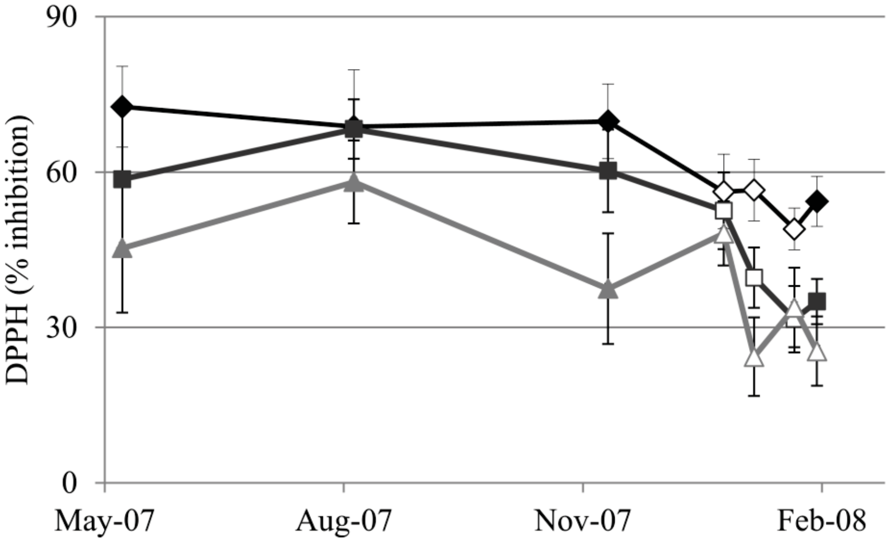


Figure 17. DPPH content of the three dilutions of extracts (black 5 mg/ml red; 2.5 mg/ml, blue 1.25 mg/ml) of composite rimu fruit samples taken over one year. Solid symbols are samples taken at 3-month intervals (composite of 17-21 trees), hollow symbols are more frequently sampled (composite of six trees). Bars are standard errors.

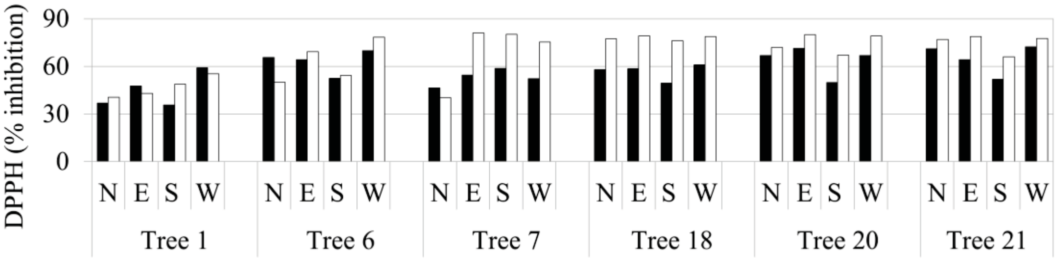


Figure 18. DPPH content (water extracted (black bars) or ethanol extracted (white bars)) of rimu fruit from six individual trees, four aspects per tree, collected in Feb 2008. Only the 5 mg/ml dilution is shown here, as similar trends were observed for other dilutions.

3.2.3. Ferric reducing/antioxidant power

The ferric reducing/antioxidant power (FRAP, µmol/l) of seven composite rimu fruit samples is shown in *Figure 19* and from the four aspects around six trees in *Figure 20*. There was no difference between water and ethanol extract for FRAP

(P>0.10), with FRAP changing over time (P<0.001). There was a highly significant effect of concentration (P<0.001), tree (P<0.001) and aspect (P<0.001) for FRAP. As with TPC, FRAP levels decreased over the year, declining in both 21-Jan-08 and 6-Feb-08 samples.

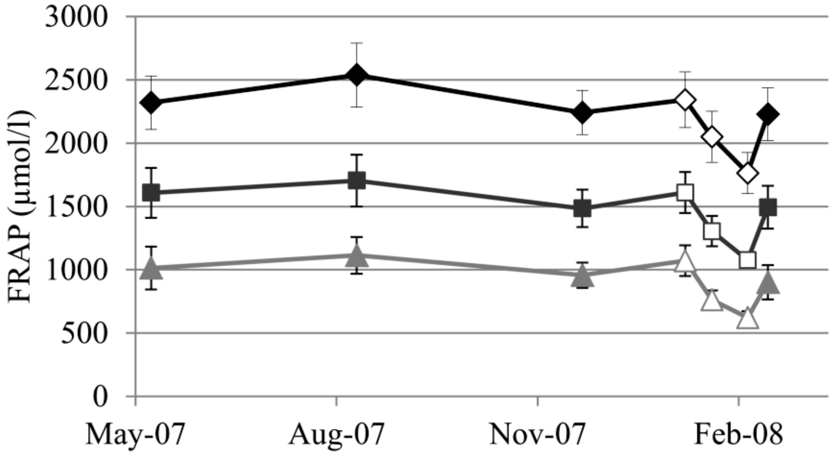


Figure 19. FRAP content of the three dilutions of extracts (black diamonds 5 mg/ml, dark grey squares 2.5 mg/ml, light grey triangles 1.25 mg/ml) of composite rimu fruit samples taken over one year. Solid symbols are samples taken at 3-month intervals (composite of 17-21 trees), hollow symbols are more frequently sampled (composite of six trees). Bars are standard errors.

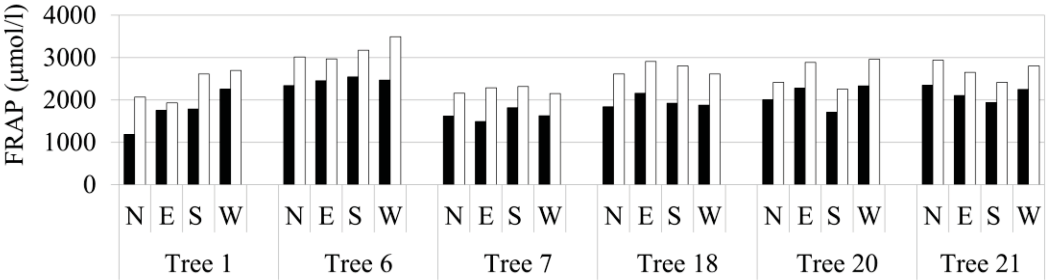


Figure 20. FRAP (water extracted (black bars) or ethanol extracted (white bars)) of rimu fruit from six individual trees, four aspects per tree, collected in Feb 2008. Only the 5 mg/ml dilution is shown here, as similar trends were observed for other dilutions.

3.2.4. Chelating Activity (CHA)

The chelating activity (CHA) of seven composite rimu fruit samples is shown in *Figure 21* and from the four aspects around six trees in *Figure 22*. There were significant differences between water and ethanol extract for most of the seven samples for CHA. CHA changed over time (P<0.01). There was a significant effect of concentration (P<0.001) on CHA for both solvents, a significant effect of tree for ethanol extract only (P<0.001) but no difference between the aspect around the tree (P>0.10) for either solvent for CHA. In the water extract, the levels of CHA increased over the year, to a maximum (double the initial inhibition) in the sample from 6-Feb-08. The level then decreased by the mid-Feb-08 sample.

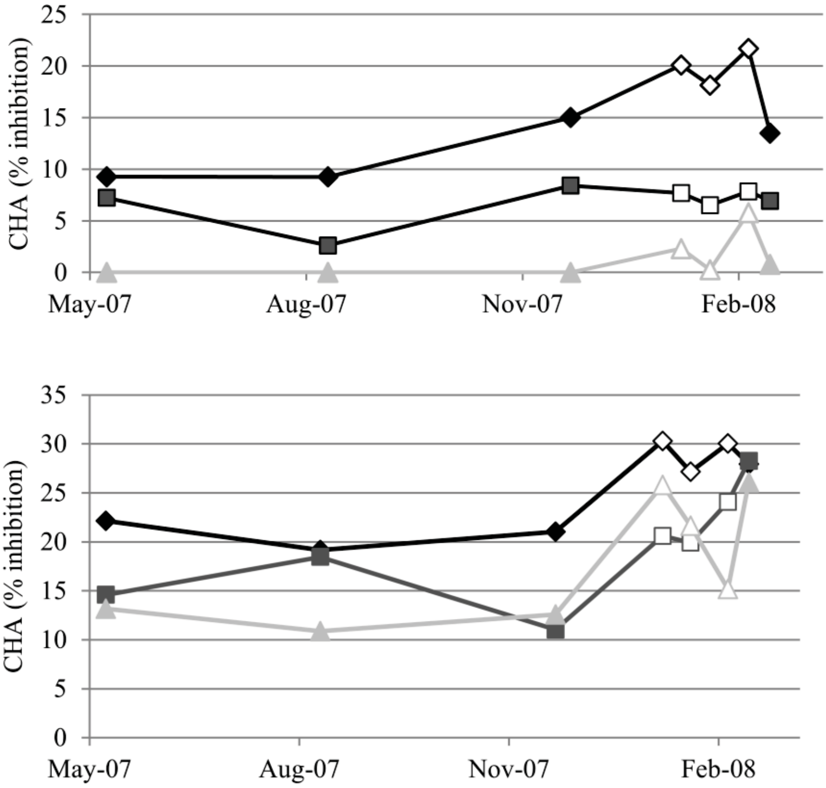


Figure 21. CHA content (water extracted (top graph) and ethanol extracted (bottom graph)) of the three dilutions of extracts (5 mg/ml black diamonds; 2.5 mg/ml dark grey squares, light grey triangles 1.25 mg/ml) from composite rimu fruit samples taken over one year. Solid symbols are samples taken at 3-month intervals (composite of 17-21 trees), hollow symbols are more frequently sampled (composite of six trees).

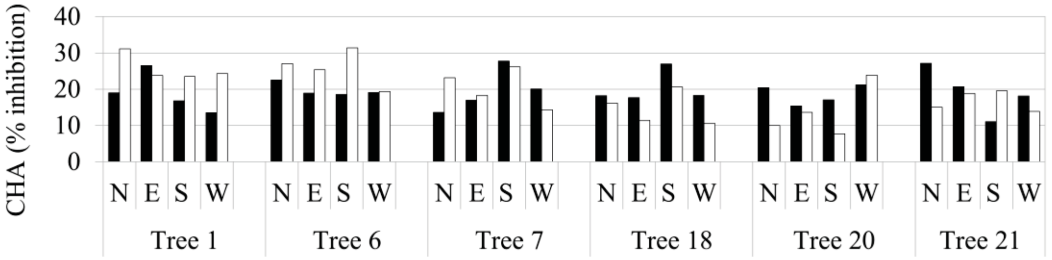


Figure 22. CHA content (water extracted (black bars) or ethanol extracted (white bars) of rimu fruit from six individual trees, four aspects per tree, collected in Feb 2008. Only the 5 mg/ml dilution is shown here as similar trends were observed for other dilutions.

3.3 Flavonoid identification

HPLC/GC/MS chromatograms of the extracts from the seven composite rimu fruit samples are shown in *Figure 23*, with chromatograms from the five time-points for each of the three individual trees (#1, #4 and #10) in *Figures 24-26*, respectively. An example of the areas under the curve is presented in Figure 27, with the areas under the curve, expressed as a percentage of the total area for each time point, for the seven composite rimu fruit samples in *Figure 28* and the three individual trees (#1,#4 and #10) in *Figure 29*.

The amount of compound 48.95 increased the most over the season, comprising 5.4 % of the total area (a measure of the amount present in the sample) in May-07, increasing up to 32.5 % of total area on 6 Feb 08, then dropping slightly. The amounts of a number of compounds present decreased over the season, notably the three peaks with the shortest retention times (12.4, 17, 18). The compound with a retention time of 12.4 was the most prevalent compound in rimu fruit over the May-07 to Nov-07 period. The amount in the sample then dropped markedly in Jan-Feb and increased again in the last sample. The peak 20.1 was present in the first three samples, and then was not detected in the last four samples.

As in most of the analyses reported previously, individual trees showed markedly different compositions. Note that the results from the three individual trees focus on

Nov-07 to Feb-08 rather than the whole season. Tree 1 showed a similar profile to the composite samples. Each of the individual trees showed a similar increase over the season in the peak 48.95, although not as dramatically as in the seven composite samples. As in the composite samples, the individual trees also showed decreasing amounts of the peaks 12.4, 17 and 18 over the summer. In the Nov-07 sample, two trees had the highest level of 12.4, and one had 52.1 as the most abundant. Over the summer period, tree 1 had 48.95 as the most abundant compound, tree 7 had 52.1 then 48.95 and tree 10 was mostly 12.4.

A few select of polyphenols have been identified from these profiles, using the time eluted from the column. Preliminary identification shows the rimu fruit contains catechins, epicatechins and two flavonoid glucosides found by Markham (Markham *et al.*, 1989) in rimu foliage (3-methyl-myricetin-3-O-oxloside and quercetin 3-O-glucoside), No ferruginol was found in our rimu fruit samples.

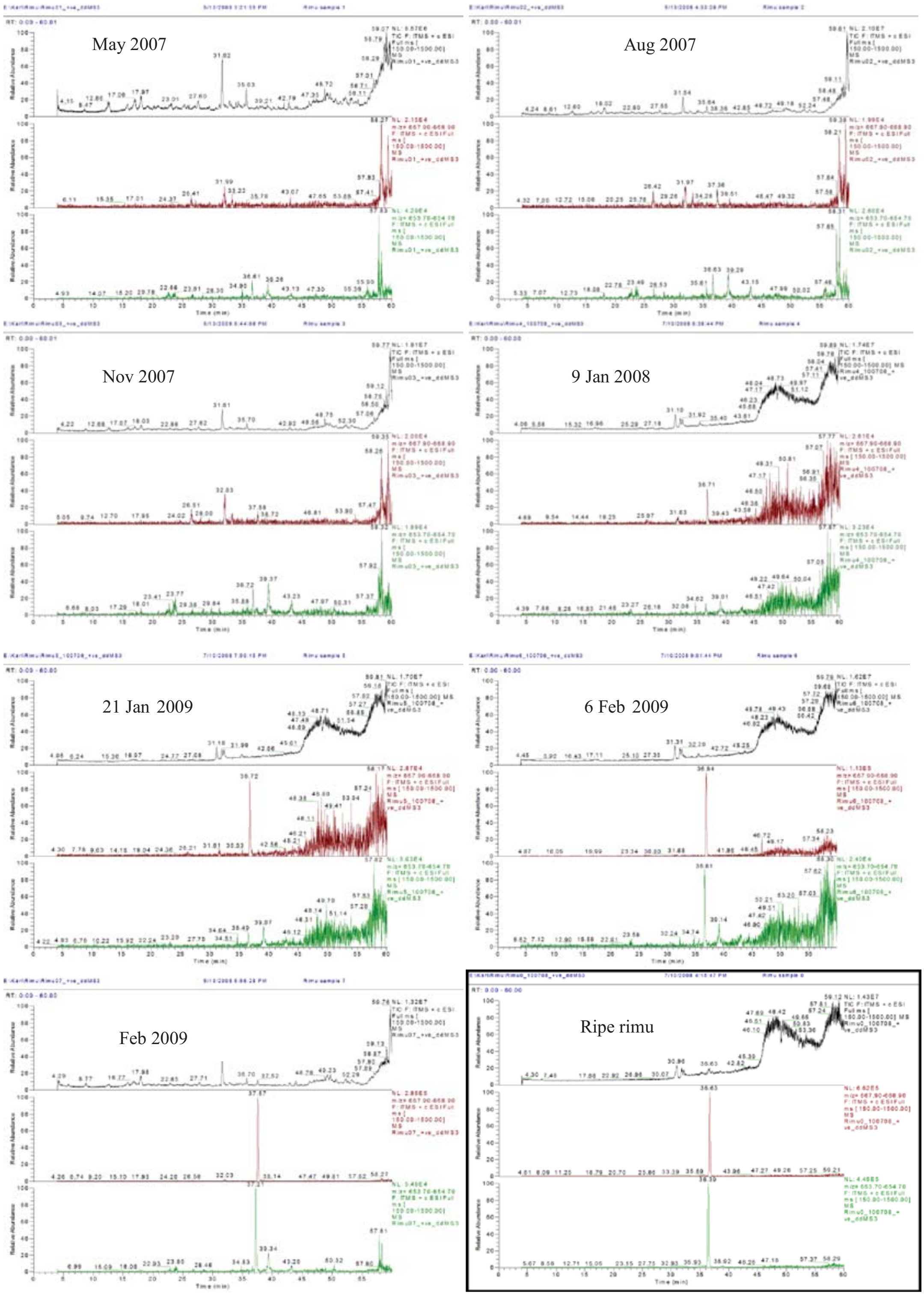


Figure 23. HPLC/GC/MS profiles of composite rimu fruit samples collected over one year. The bottom right profile (outlined in black) is the ripe rimu sample from 2002.

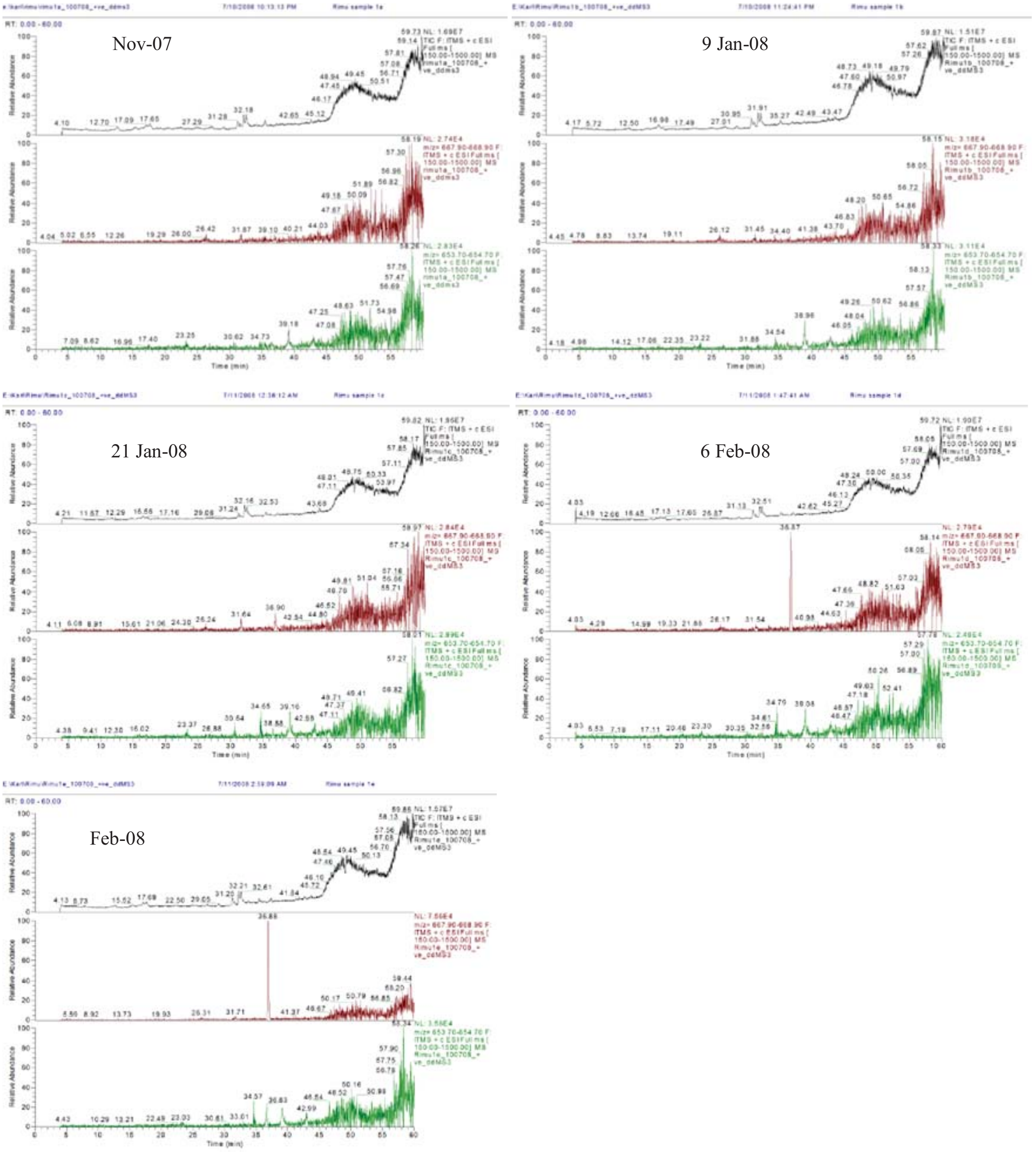


Figure 24. HPLC/GC/MS profiles of rimu fruit from Tree 1 collected between November 2007 and February 2008.

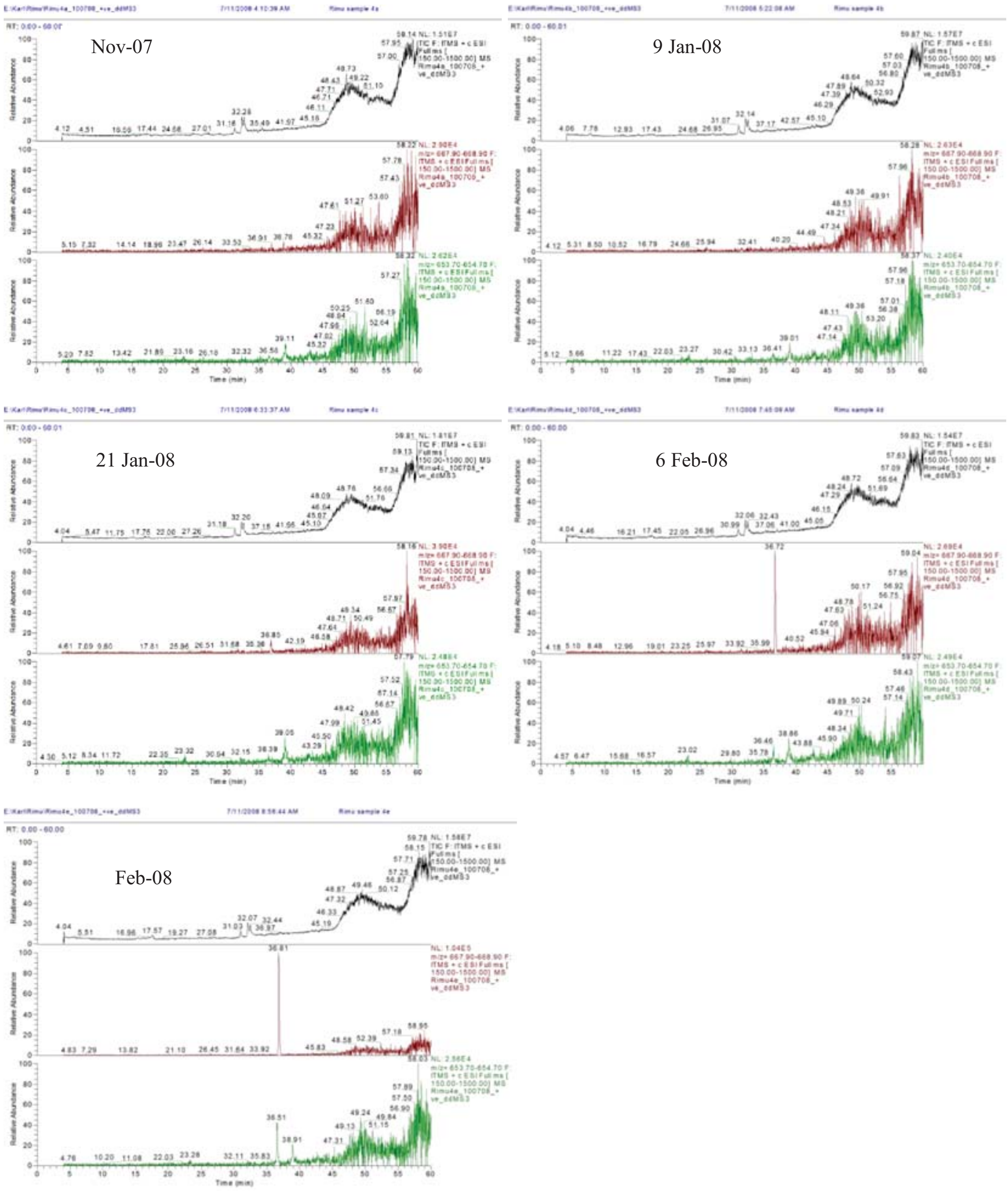


Figure 25. HPLC/GC/MS profiles of rimu fruit from Tree 4 collected between November 2007 and February 2008.

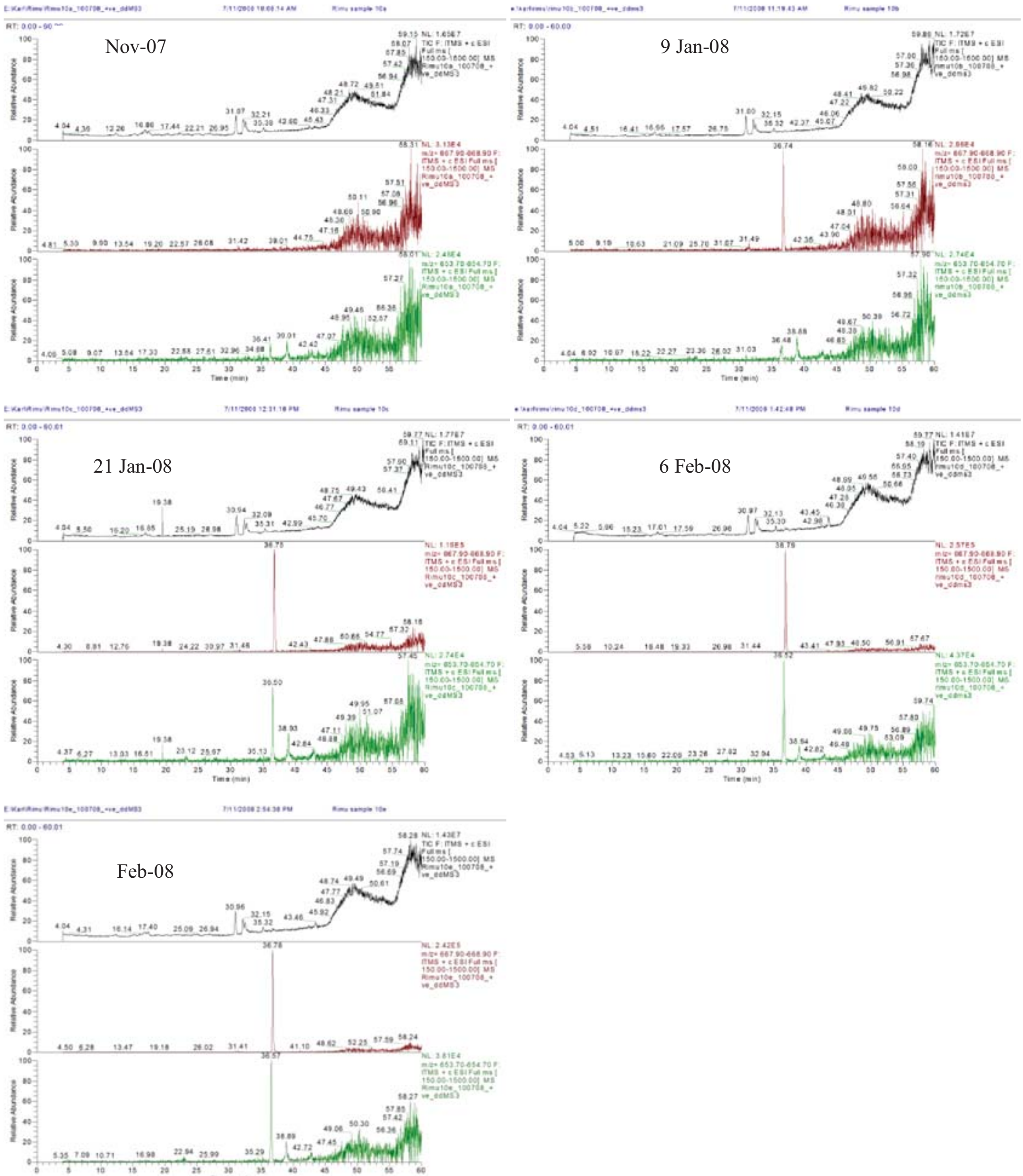


Figure 26. HPLC/GC/MS profiles of rimu fruit from Tree 10 collected between November 2007 and February 2008.

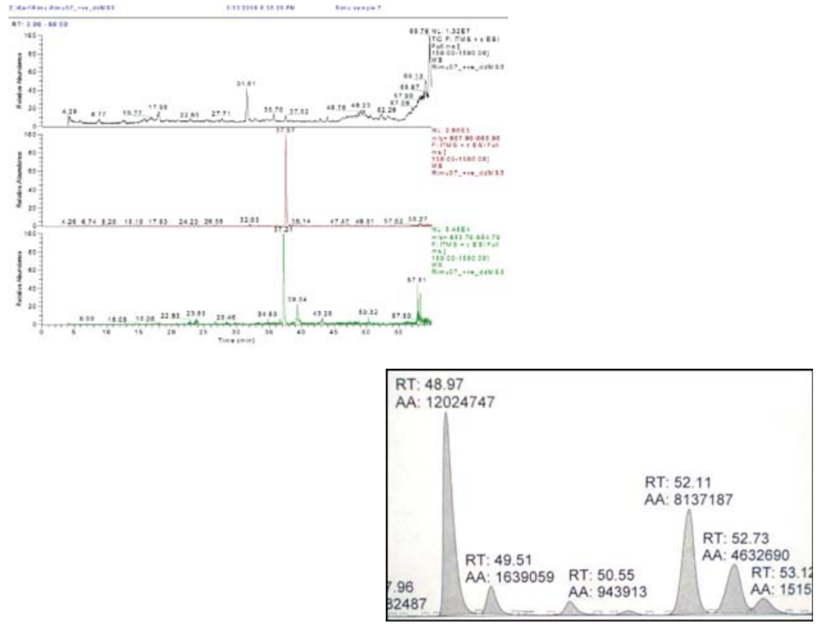


Figure 27. Example of areas under the curve for the Feb-08 composite rimu fruit sample. RT is retention time on the column, AA is the area under the curve.

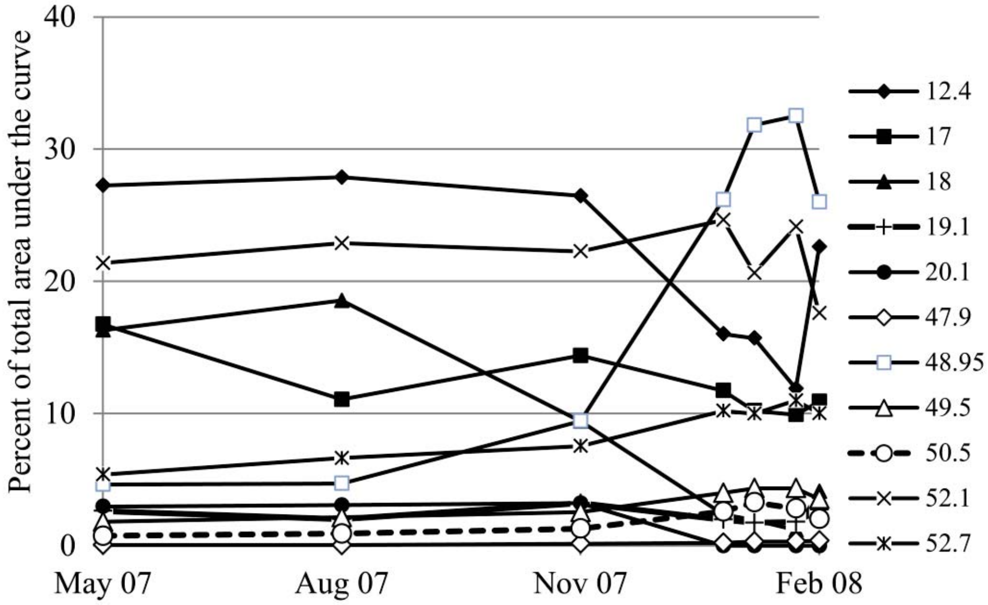


Figure 28. Areas under the curve expressed as a percentage of total area for each time point for composite rimu fruit samples taken over one year. Different symbols represent the different HPLC peaks, with the retention time on the column as identification.

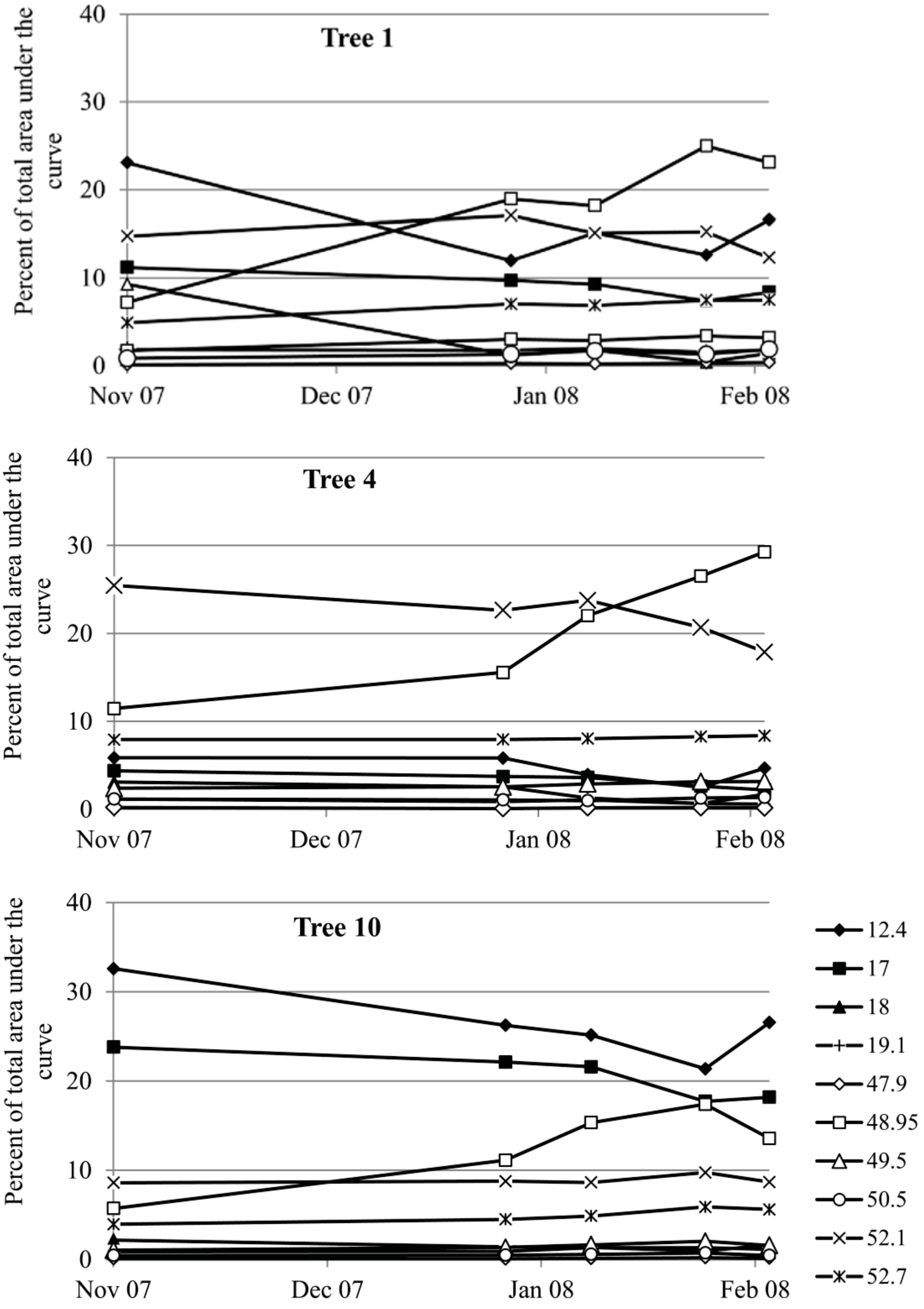


Figure 29. Areas under the curve expressed as a percentage of total area for each time point for three individual trees from Nov-07 to Feb-08. Different symbols represent the different HPLC peaks, with the retention time on the column as identification.

3.4 Oestrogenic Activity

None of the rimu fruit samples tested exhibited any oestrogenic activity, although this may be due to limitations with the system used (Mortensen, 2009). Several of the rimu fruit extracts inhibited growth, indicating that they may contain antimicrobials.

4. Discussion

The aim of this work was to collect rimu fruit from a range of trees over a one year period and to chemically analyse these samples with the objective of elucidating possible changes in these compounds (increases or decreases) which could potentially be triggering breeding in kakapo.

There was no within-tree variation in dry matter, crude protein, crude fat or ash, indicating that samples from different positions within the same tree had similar gross nutrient compositions. There were significant differences between trees in dry matter, crude fat and ash, and a trend for crude protein.

As there was only one sample per 3-month period (a pooled sample representing between 17 and 21 individual trees), no statistical comparisons of nutrient levels could be made over time. The percentage changes discussed here are indicative, with no measure of variation about the values available. Consequently there is no way of determining whether these percentages are significant. Samples from the six individual trees (and four aspects) were used to calculate standard errors for crude protein, crude fat, dry matter and ash. These can be used as an indication of variation about the mean, and highlight possible significant differences. Crude protein and crude fat showed differences of 1.5% and 0.7%, respectively over the season. With estimated standard errors of 0.2%, and 0.22%, these differences are likely to be significant. Individual amino acids and total fatty acid content increased by 76% over the season (predominantly between the last two samples). Sugar content fluctuated over the season, and was highest early January. Dry matter content also fluctuated, and was lowest also in early January. All of the fibres increased over time, and were highest in the February 2008 samples. Calcium did not change over the year, whereas phosphorus increased by 27% over the season, which resulted in a 23% lower Ca:P ratio.

The winter (Aug-08 sample) rimu fruit sample had the lowest amount of protein, with levels changing over the season by 27%. Seasonal differences in the amount of protein have been found in plants, specifically algae. Both Australian marine microalgae (Renaud & Luong-Van, 2006) and marine macroalgae (*Gelidium* *latifolium*) (Rubenstein & Wikelski, 2003, Rico, 1996 #169) had higher levels of nitrogen and protein in winter, whereas fat content in Australian marine microalgae showed no obvious differences. For example, the average protein in rhodophytes changed by 11% between summer and winter (Rubenstein & Wikelski, 2003).

The amount of crude protein, crude fat, crude fibre and ash in four kakapo food plants (*Aciphylla crenulata, Chionochloa conspicta, Phormium cooki, Schoenus pauciflorus*) collected in Fiordland by Grey (reviewed in Butler, 2006) were similar to those in rimu fruit. Crude protein ranged from 5.5 to 9.2%, crude fat 1.5 to 2.4%, crude fibre 31.2 to 46.9%, ash 4.2 to 8.9%). In rimu fruit, crude protein ranged from 5.5 to 7.0%, crude fat 1.0 to 2.7%, crude fibre 34.3 to 42.0%, ash 5.4 to 6.2%. The gross nutrient values found in rimu fruit were higher than those found in kahikatea fruit by (Dijkgraaf, 2002), however, because only the receptacle of the kahikatea fruit was analysed these data are not directly comparable. Sugar content was similar, but all other components (crude protein, crude fat, crude fibre, ash) were much higher (4- to 12-fold) in rimu fruit than in kahikatea fruit and calcium was 69-fold higher (12.0 vs. 0.172 mg/g).

The amount of crude protein in rimu fruit ranged from 55 – 66 g crude protein per kg dry matter. This agrees with James *et al.,* (1991), who calculated the breeding diet of kakapo on Stewart Island to contain 59 g crude protein per kg dry matter, which they concluded was barely adequate for survival or chick production. As kakapo are known to raise chicks solely on rimu fruit, it would certainly appear to be adequate.

The protein levels in rimu fruit were low compared with many other plant parts eaten by birds. Similar levels of protein were found in heather (*Calluna vulgaris*), the main food item eaten by red grouse (Moss & Parkinson, 1972), with 7% protein and 25% fibre. Voluntary intake of heather by red grouse was very high relative to body weight compared to poultry, therefore Moss *et al.,* (1972) concluded that the intake of energy was adequate. The digestibility of protein was higher in spring (42 - 48%) compared to autumn (24 - 31%) and heather had a higher level of protein in spring (average 6.9%) than in autumn (6.1% protein) (Moss & Parkinson, 1972). Heather in spring had more digestible protein and cellulose, similar levels of fat and lignin, and less soluble carbohydrates than in autumn. Plant-eating bats eat fruit rich in soluble carbohydrates and low in crude protein (Ruby *et al.*, 2000). Therefore to get enough protein it is suggested that these bats overeat fruit and 'dump' carbohydrates to meet their protein requirements, or alternatively eat leaves (which are higher in protein and calcium) so they do not need to overeat (Ruby *et al.*, 2000).

Animals cannot synthesize the essential amino acids (arginine, isoleucine, leucine, lysine, methionine, phenylalanine, valine, tryptophan, threonine) (Koustos *et al.*, 2001) and so need to get these from their diet. Glycine, histidine and proline are 'almost essential'. There was no change in levels of amino acids in rimu fruit over the season (Table 3). Growing meat chickens require a minimum of 18% protein (NRC, 1994). With respect to amino acids, their highest requirements are for arginine (1%), phenylalanine (1.04%), glycine + serine (0.97%), leucine (0.93%) and lysine (0.85%) (NRC, 1994). The levels of these amino acids in the rimu fruit are much lower than the dietary levels recommended for growing meat-type chickens. This type of chicken was chosen for comparison as the growth rates of meat-type chickens (increasing 50-55-fold in 6 weeks) are similar to the growth rates of kakapo chicks (males 54-fold, females 44-fold increase in body weight over the first 6 weeks). The lower levels of amino acids in rimu fruit, which are known to be the sole food provided to kakapo chicks, agree with the conclusion by Koustos *et al.,* (2001), that Psittacine birds require lower energy, protein and calcium than poultry during all stages of their life cycle. For example, a diet containing 6.8% crude protein (including 0.33% sulphur amino acids, 0.15% lycine) maintained weight in budgerigars (Koustos *et al.*, 2001). Kakapo have a very low metabolic rate and daily energy expenditure (Bryant, 2006) which allows them to survive on a low-energy diet. Any excess energy consumed in the diet is usually expelled by an increase in metabolic rate, but in kakapo it is converted into fat (Bryant, 2006).

As seeds are produced, amino acids are synthesised and deposited as stored proteins. Germination is the reverse of this process, with protein mobilised to fuel the growth of the new plant, making nutrients more available to seed predators. For some species, it has been demonstrated that it is the quality of the seed rather than the quantity that is important in triggering breeding For example, house mice cease to breed when only mature spilt grain is available, but quickly recommence breeding when given food with high levels of protein (White, 2002). During mouse plagues in

Australia, breeding is sustained at high levels due to the abundant ripening grain seeds followed by germinating seed (White, 2002). This confirms the conclusion by Bomford (1987) that the key to breeding in mice is the flush of ripening seed, not the mature/ripe seed. Protein levels in ripe seed were sufficient to maintain mice but not sustain breeding (White, 2002). Ripening and germinating seeds contain high levels of soluble amino acids which are necessary for successful breeding. For example, lycine levels in cereal grains are higher earlier in development (Allen & Hume, 1997). This did not appear to be the case in rimu fruit. Ripening and germination are the only periods in a seed's development when sufficient nitrogen is converted to an easily digestible form that can provide the nutrition required by growing young (White, 2002). This hypothesis has support from work on birds (Allen & Hume, 1997). For example, the zebra finch is able to breed on green grass seeds. This soft ripening seed contained more of the essential amino acids that are limiting for breeding than mature seed (Allen & Hume, 1997).

It is obvious from these studies that it is neither the amount of seed present, nor the amount of nitrogen present in the seeds that affects reproduction; instead it is the amount of nitrogen that is available as one or more essential of the amino acids. The levels of protein in ripe rimu fruit changed over the season and, combined with the possibility that the fruit provided more nutrients as it ripened (as seen in ripening grain seeds White, 2002), this could possibly trigger breeding in kakapo.

The most abundant amino acid in rimu fruit was glutamic acid (average 0.62 mg/100 g), followed by aspartic acid (0.66 mg/100 g) then either arginine (0.35%) or leucine (0.56%). The lowest was methionine (0.084%) then histidine (0.187 mg/100 g) or tyrosine (0.233 g/100 g). The ranking of amino acids in rimu fruit remained similar over the season, i.e. glutamic acid was most abundant, followed by aspartic acid and methionine the lowest. The ranking of amino acids found in rimu fruit were similar to those found in kiwi egg yolks, in which glutamic acid was also the most abundant, followed by aspartic acid and arginine with methionine the least abundant (Body & Reid, 1978). However, the absolute levels of these amino acids were up to three times higher in kiwi eggs than in rimu fruit. Young nestling tits are provided with a relatively high number of spiders by their parents compared to older nestlings. Spiders are protein rich, with high levels of sulphur amino acids, including cysteine, methionine, and taurine (Ramsay & Houston, 2003). Ramsay *et al.,* (2003) argued that the tendency for parents to feed younger chicks more spiders could be because taurine may be an essential nutrient during this critical stage of chick development. Note that dietary amino acids are hydrolysed to free amino acids before being incorporated into the eggs of birds (White, 1991). Taurine is conjugated with bile acids during the formation of bile, and can by synthesised from methionine which can be synthesised from cysteine. When taurine is present in the diet, cysteine can be redirected into feather growth. If the maternal diet is deficient in cysteine, then it is possible that the bird can detect this, and delay the onset of breeding. Methionine in rimu ranged from 0.068 to 0.087%, with no obvious changes over the season. Neither taurine nor cysteine was measured in rimu fruit in this study. Methionine in ripe rimu in 2002 was 0.121%, cysteine was 0.143% (Cottam *et al.*, 2006).

The total fatty acid content (as a proportion of crude fat) increased by 47% over the season, with this change mainly occurring between the November and early January samples. The amounts of some individual fatty acids increased (palmitic stearic, oleic, linoleic, linolenic, vaccenic) while others decreased over the season (lauric, capric, myristic, caprylic, arachidic). The most abundant fatty acids were the same as in the ripe fruit sample collected in 2002, with linolenic acid making up around 40% of the total (Cottam *et al.*, 2006). The amount of omega-3 fatty acids in rimu fruit increased over the season, more than double in the February sample compared with the May sample. Omega-6 fatty acids did not change over the year, therefore the omega3:omega6 fatty acid ratio increased with season. The ratio of linoleic:linolenic acid peaked in the 9-Jan-08 sample then dropped rapidly.

The only fatty acid with a dietary requirement in poultry is linoleic (NRC, 1994), and all rimu fruit samples tested contained more than the required 1%. Most animals contain triacylglycerols (fatty acids) as their main lipid, storing excess energy in this form in adipose tissue for later use (Body, 1988). Polyunsaturated C20 and C22 fatty acids are essential for embryonic development, and bird sperm is rich in C20, C22 and sometimes C24 (Coniglio, 1994). There is little research into the effects of fatty acids on reproduction in birds, but there is evidence of the effects in other vertebrate species. Fatty acids in the diet are deposited in adipose tissue (Body, 1988). Nutritional deficiencies in polyunsaturated fatty acids can alter lipid and fatty acid composition of the testis leading to reduced fertility or infertility (Coniglio, 1994). Diets deficient in n-3 fatty acids have a negative effect on spawning in fish. Omega-6 polyunsaturated fatty acids are apparently not limiting resource for reproduction in the edible dormouse (Lebl *et al.*, 2010).

The fatty acid composition of dietary items is reflected in the ratio of individual fatty acid classes in egg yolks (Body & Powlesland, 1990) and also in the fatty acid profiles in the adipose tissues of animals (Fietz *et al.*, 2005). The fatty acid content of kiwi adipose tissue (Body, 1988) was similar to that found in kakapo eggs (Body & Powlesland, 1990) and kiwi eggs (Body & Reid, 1978). These pools were highest in oleic acid (35 – 52% of total fatty acids) followed by palmitic (15 – 24%) and linolenic (9 – 10%). The fatty acid compostion of the eggs laid by captive kiwi reflected that of their diet, with the exception of C18:0 (stearic). The amino acid and fatty acid levels in kiwi egg yolks were similar to those found in the domestic chicken, so the maternal diet of kiwi (as reflected in the egg yolk) was thought to be sufficient to produce viable embryos/chicks (Body & Reid, 1978).

Differences in the levels of fatty acids in the maternal diet of wild alligators were linked to differences in fatty acid composition of eggs and their associated hatching rates (Noble *et al.*, 1993). Egg yolks from wild alligators had higher C20, C22 and lower C18:1 levels than those from captive animals. The captive animals had a greatly reduced hatching rate (50% compared with 94% in the wild) which was attributed to the differences in the maternal diet (and consequently in egg yolk fatty acid profiles between captive and wild animals).

The fatty acid content of the diet of edible dormice was reflected in their white adipose tissue (Fietz *et al.*, 2005). The proportions of oleic and linoleic acid in the white adipose tissue differed between mast and non-mast years, In a mast year linoleic acid was 43% of total fatty acids, and oleic was 42%. In the non-mast year linoleic comprised only 28% of total fatty acids, with 54% oleic, and 2-3% linolenic (Fietz *et al.*, 2005). It is suggested that beechnuts were less available in non-mast years and oleic acid was synthesised to compensate for the dietary deficiencies. Fietz *et al.,* (2005) concluded that the polyunsaturated fatty acid content in the white adipose tissue of edible dormice did not explain their reproductive status, as there was no differences between reproducing and non-reproducing animals

The natural diet of wild tuatara (*Sphenodon punctatus*) has a different fatty acid profile to that of captive tuatara (Cartland-Shaw *et al.*, 1998). Plasma levels of fatty acids in wild tuatara reflected that of fairy prions (*Pachyptila turtur*), the main prey of tuatara in the wild. In this study the natural diet of wild tuatara was comprised of adult and fledgling fairy prions, tree weta (*Hemideina crassidens*) and two types of darkling beetle (*Mimopeus* spp. and *Artystoma* spp.). In contrast, the captive diet consisted of commercially available insects: locusts (*Locusta* spp.), mealworm larvae (*Tenebrio molitor*), waxmoth larvae (*Galleria mellionella*) and huhu grubs (*Prionopus reticularis*). The natural diet was rich in C20, C22, C18:3, C20:5, C22:5 and C22:6. In contrast, the diet of the captive tuatara contained no C20 or C22, and their plasma was higher in C18:1 (oleic) and C16:0 (palmitic) than wild tuatara. The association between diet and plasma has been shown in experimental studies on other vertebrate species, including tuatara. Differences in long-chain fatty acid levels between wild and captive tuatara have the potential to affect growth, reproduction and the ability to survive (Cartland-Shaw *et al.*, 1998).

Infertility in birds has been linked with low dietary quantities of essential fatty acids, including linolenic acid (C18:2), reflected in the composition in the yolk, which could seriously affect the survival of chicks (Body & Powlesland, 1990). Body *et al.,* (1990) found that the cones and fruit of pink pine (*Halocarpus biformis*), a relative of rimu, were higher in oleic acid (C18:1) and C20:0, C22:0 and C24:0 fatty acids than the foliage. They concluded that if sufficient foliage is consumed then satisfactory levels of essential fatty acids should be available. The fatty acid profiles of pink pine were fairly similar to rimu fruit, both were highest in palmitic (C16:0) (26.9 vs. 17.8-27.3% of total fat), linoleic (C18:2) (17.1% vs. 17.0 to 23.5%), higher in oleic (C18:1) (17.7% vs. 4.5-11.7%) and much lower in linolenic (18:3) (8.9% vs. 30.7 to 44.0%). Linolenic is a precursor to C22:6 (Body & Powlesland, 1990), which was not found in the rimu fruit. It is interesting to note that erucic acid (C22:1), considered by Body & Powlesland (1990) to reduce the breeding success of hens, is present at low levels in rimu fruit.

The immune-modulating features of poly- and mono-unsaturated fatty acids may depend on environmental factors (Parmentier *et al.*, 2002). Dietary additions of different fatty acid sources (sunflower, safflower or linseed oil) resulted in different effects (both stimulatory and inhibitory) on antibodies and inflammatory or immune responses in chickens (Parmentier *et al.*, 2002). These authors concluded that it was unclear if these different responses were due to the different oil sources, differences in the amount of linoleic acid (n-6), in the amount of other fatty acids (e.g. oleic acid), or dietary oil ratios. Dietary oleic acid has been shown to have variable effects on immune responses (Parmentier *et al.*, 2002). The oleic acid content in rimu fruit rose from 0.7 to 2.1 mg/g over the season, although these levels were much lower than in ripe fruit (21.8 mg/g) (Cottam *et al.*, 2006). As dietary fatty acid ratios and oleic acid are known to affect reproduction in other animals, these could be involved in triggering reproduction in kakapo. It has been shown in rats that the regulation of the oestrus cycle depends on oestrogen levels plus the availability and types of fatty acids available (Collins *et al.*, 2003).

When food is available *ad libitum* and all nutrients are balanced, birds generally eat the amount of food required to satisfy their energy requirements (Sales & Janssens, 2003). Although the nutritional content of food is considered a potential trigger for reproduction in birds, most studies have looked only at macronutrients, ignoring individual fatty acids or amino acids. The amounts of the sulphur amino acids cysteine and methionine levels in eggs are much higher than those in plant material, and it is possible that birds could build up protein muscle reserves containing amino acids poorly represented in the diet, for use in egg production (Houston *et al.*, 1995a).

In the Florida scrub jay, the combination of protein and fat in the pre-breeding diet advanced the laying date, increased clutch and egg size and altered egg composition (Reynolds *et al.*, 2003). A number of authors have found that birds specifically select certain plant material just prior to breeding, which may trigger breeding in some way. Moss (1972; 1975) found that gallinaceous birds increasingly selected nitrogen- and phosphorus- rich food items from winter to spring until after egg laying. This was also found in female capercaillie which tend to switch from their winter food plants to nitrogen and phosphorus-rich foods (catkins, flower buds, berries, stems, tips from various species of plants) in the early spring following snow melt (Pulliainen &

Tunkkari, 1991). This switch was not seen to the same extent in males. Ruffed grouse also select dietary items high in protein and potassium (Doerr *et al.*, 1974).

A number of authors have investigated the theory that the ingestion of buds and other plant material might provide a chemical signal to trigger reproduction in birds. Although most of these authors concluded that this is unlikely, there is a clear well-documented example of a mammalian breeding cycle that is synchronised with a single variable plant food. Selas *et al.,* (2002) tested a number of theories concerning the possible nutritional or chemical mechanisms for the cyclical relationship between vole reproduction and bilberry masting. They concluded that masting provides a good source of nutrients until the plants chemical defence is re-established the following year, therefore improved food quality after the mast was the most probable explanation.

Squirrels may follow a resource tracking strategy, tracking an alternative energy source correlated with future seed crops (Boutin *et al.*, 2006). The candidates for this trigger include reproductive structures of food plants (e.g. buds, flowers, pollen cones) which are consumed in advance of the forthcoming seed crop. The fat dormouse feed on floral buds in spring/summer, and it is possible that these contain specific compounds which trigger reproduction well before the seed is available (Pilastro *et al.*, 2003). This has also been reported for the meadow vole (Berger *et al.*, 1981). There is evidence to suggest that increased testis length in Australian Zebra finches may be caused by a chemical trigger contained in the green grass shoots which appear soon after rain (Vleck & Priedkalns, 1985). It is also possible that grass shoots stimulate reproduction in the finches because it is nest building material.

Corsican blue tits (*Cyanistes caeruleus*) have a breeding schedule tightly linked to the phenology of oak trees (Bourgault *et al.*, 2006). Oak buds are a large part of the diet of blue tits in spring (> 50% of total gizzard content in 63% of tits, and > 75% of total gizzard content in over half of these) compared with other times of the year (< 5% total gizzard content in 9% of birds) (Betts, 1955). The percentage of piñon seeds in the stomach of piñon jays increased from 20% at breeding time, to 70% at fledging, and increased to 100% a couple of months later (Ligon, 1978). The kakapo also show a similar trend, with the proportion of podocarp fruits in the diet of female kakapo increasing from 20% in December (breeding time), 80% in January, and 100% in both February and March (fledging time) (Wilson *et al.*, 2006). These studies suggest that the abundance of reproductive structures could be at least as important as a reproductive cue as chemical substances in the plants and bud tissue.

Poor neonatal nutrition could lead to impaired reproductive performance in adulthood. Zebra finches with poor neonatal nutrition during the critical 2-week period of nestling development took longer to initiate egg laying and had a slower egg laying rate when they reached adulthood (Blount *et al.*, 2006). Reducing food intake led to reproductive inhibition in white-footed mice without an accompanying weight loss or body condition loss (Merson & Kirkpatrick, 1981).

The calcium:phosphorus ratio of rimu fruit decreased over the season as a result of , phosphorus increasing by 27%, from 10:1 to 8.2:1. Calcium levels in rimu fruit did not change much over the season.

Calcium limitation of avian reproduction may be a widespread phenomenon which affects egg properties and chick development (Tilgar *et al.*, 2004). In habitats of limited calcium availability birds may obtain less protein and other nutrients required for successful reproduction, resulting in smaller eggs and clutch sizes (Tilgar *et al.*, 2002). There is evidence that calcium is a common limiting factor for reproduction in a variety of vertebrates (Barclay, 1994), and strong indications that calcium might be interacting with relatively high levels of macronutrients in supplementary food (Raubenheimer & Simpson, 2006). Limited calcium causes bats to reduce their litter size to usually raising single offspring (Barclay, 1994). The usually multi-annual intervals between breeding in kakapo could reflect the periodic availability of key nutrients required for breeding, including calcium, in the environment.

Given that endogenous calcium stores in the bone medulla are insufficient to meet egg-laying demands birds must actively seek calcium-rich foods during egg production (Wilkin *et al.*, 2009). Insects are a poor source of calcium for birds (Barclay, 1994; Bernard & Davison, 1996) and do not provide enough calcium for egg production. For example, the calcium of insects ranged from 1.62 – 3.40 mg/g (Barclay, 1994) compared to 11.2 – 12.7 mg/g in rimu fruit). Breeding and clutch size in birds is thought to be limited by calcium, with environmental calcium playing a proximate role in determining clutch size (Patten, 2007; Wilkin *et al.*, 2009). In general, calcium availability tends to increase from the equator to the poles, with an associated increased clutch size with latitude (Patten, 2007). Environmental conditions, such as acid rain, increases soil acidity and associated leaching of calcium, leading to lowered uptake of calcium by plants (Ramsay & Houston, 1999). A reduction in calcium availability may manifest itself in smaller clutch sizes but also thinner eggshells or fewer fledglings.

Bernard & Davison (1996) suggested that calcium availability may play a significant role in controlling the timing of reproduction in insectivorous bats (long-fingered bats, *Miniopterus schreibersii*), and that the timing of parturition is fixed by energy and calcium constraints. Bone calcium is in constant flux and changes in bone calcium reflect the extent to which dietary calcium is meeting requirements. They concluded it was likely that calcium availability has played a major role in evolution of timing of birth and lactation. The timing of birth and lactation in these bats is likely to be determined by calcium availability, as the late pregnancy and lactation periods are times of high calcium demand. These bats copulate before winter but implantation was delayed during winter so that young are born in summer when calcium is relatively abundant.

Raubenheimer & Simpson (2006) argued that calcium could be a key nutrient limiting reproduction in kakapo. The kakapo skeleton is large and dense so raising chicks would place a considerable calcium demand on females who do this without male assistance. Female kakapo consume the shells of hatched eggs, presumably to recycle the calcium these contain. This hypothesis could explain why kakapo breeding on Codfish Island is dependent on an abundance of calcium-rich rimu fruit.

The amount of calcium and phosphorus, and their respective bioavailability, is important in animal diets. For example, the growth diet of green iguanas requires adequate calcium but not excessive phosphorus (Frye, 1997). If they are fed a diet with too much phosphorus relative to calcium, the iguana’s body tries to re-establish the normal ratio by recovering calcium from blood, or from existing calcified bone. Excess calcium has been shown to decrease growth and feed intake in growing chickens (Shafey, 1993). It is thought that excess calcium reduces the soluble fractions of other minerals, leading to decreased availability of these other minerals needed for growth. Both these mechanisms have implications for reproduction and egg shell development in kakapo.

Wild figs from Belize, Indonesia and Uganda have been shown to contain over three times as much calcium (0.33 to 1.91% DM basis) compared to other wild fruits (0.10 to 0.53%) and their calcium:phosphorus ratio is 3.7 times higher (approximate ratios for each region 4, 10, 11) than other fruits (2, 3, 3.5) (O'Brien *et al.*, 1998). The average calcium and the ratio of calcium to phosphorus found in figs was comparable with the levels measured in rimu fruit in the present study. O'Brien *et al.,* (1998) concluded that the amount of calcium in the figs was high enough to promote egg shell deposition and bone growth in birds. In chickens, a calcium:phosphorus ratio of 1.4:1 to 4:1 is suggested, assuming an adequate level of vitamin D (Shafey, 1993). Vitamin D is produced on exposure to sunlight, and because kakapo are nocturnal they presumably get their vitamin D from another source (although there are reports of kakapo 'sunning' themselves (R. J. Moorhouse, pers. comm.)). Mineralisation of eggshells requires mobilisation of calcium from bone, therefore increased calcium in the diet is usually needed to maintain bone density (Koustos *et al.*, 2001). The calcium content of vegetation is usually high but is often in a form that is not readily available to animals (Koustos *et al.*, 2001). It is possible that calcium could be a limiting factor in kakapo reproduction, and that the increase in the amount of phosphorus in the rimu fruit over the season could be a potential trigger.

Four common methods were used to determine polyphenolic content and antioxidant activities. TPC (total polyphenolic content) gives the amount of phenolic compound present in an extract, DPPH determines the primary antioxidant activity (i.e. the free radical scavenging activities), FRAP measures the ability of the plant extracts to reduce ferric ions and CHA measures the ability of the plant extract to sequester free metal ions. With the exception of CHA which showed no differences within trees, all other measures of phenolic content and antioxidants showed significant changes over time, and a significant difference between individual trees and aspect within each tree. Both TPC and FRAP showed a relatively constant level over the May 2007 to 9

January 2008 samples, then a drop in the next two samples, and an increase back to original levels for the last sample.

A number of plant chemical defences against herbivory, such as phenols and fibres, could be involved in triggering reproduction, either directly or indirectly. TPC levels were different between individual trees, and also between different aspects within the same tree, however no one aspect consistently contained more TPC than another. The levels of TPC in this study (137 - 142 gae/g) were much higher than that found by Reynardson (2008) in fourteen edible Myrtaceae fruits (average 2.57-44.1 gae/g, max. 101), and similar to those found in *Quercus coccifera* L. and *Juniperus phoenicea* L. fruit extracts by Hayouni (2007) (45-201 gae/g dm). Liu *et al.,* (2008) found total phenolics in emblica fruit (*Phyllanthus emblica* L.) varied with growing conditions. Water extract of Moldavian balm (*Dracocephalum moldavica* L.) had a TPC of 102 mg gae/g DM, whereas methanol extract had a content of 488 mg gae/g DM (Dastmalchi *et al.*, 2007). This contrasts with our results in which water and ethanol gave very similar TPC levels (142.2 ± 6.8 vs. 136.5 ± 6.5 μg gae/g).

The antioxidant activity in rimu fruit, as measured by DPPH, decreased over time and differed between individual trees and between aspects of the same trees. Again no aspect consistently had more antioxidant activity than others. The observed decrease in both TPC and DPPH inhibition in rimu fruit over the ripening period has also been found in other plant species. Polyphenolic content and antioxidant activity of various plant parts are known to be affected by both the stage of maturation (ripening) and growing conditions (rainfall, temperature). For example, the antioxidant activity in plant leaves differed between location and season in *Moringa oleifera* (Iqbal & Bhanger, 2006). Significant seasonal differences in total polyphenolic content were found with TPC highest in the coldest month, and lowest in the warmest month and TPC was lowest in newly opened leaves, increasing gradually as the leaves matured (Iqbal & Bhanger, 2006).

Blueberry (*Vaccinium corymbosum* L.) fruit showed a decrease in antioxidant activity and TPC content with ripening (Castrejón *et al.*, 2008), with TPC decreasing from 60 to 33 mg gae/g DM as the fruit ripened. TPC content of various fruits: including guava (*Psidium guajava*) and banana (*Musa sapientum*) (Lim *et al.*, 2007) and hot peppers (*Capsicum annuum* L. var. *acuminatum* L.)(Conforti *et al.*, 2007) also decreased with ripening. TPC in hot peppers decreased from 76 to 43 mg/g, with antioxidant activity increasing as ripening occurred. The highest antioxidant activity was found in green bitter gourds, with a correlation of 0.95 between TPC and FRAP (Kubola & Siriamornpun, 2008). Radical scavenging activity (DPPH %) decreased from 90% in green fruit to 60% in ripe gourds, with FRAP decreasing from 43.8 to 9.4 μmol FeSO4/g DM, and TPC decreasing from 324 ± 1.6 to 224 ± 0.9 mg gae/g DM. All of the gourd fractions tested were rich in phenolics and had strong antioxidant and radical scavenging activities (Kubola & Siriamornpun, 2008). Ripening stage affected the phenolic content and antioxidant activity of wild mushrooms (Barros *et al.*, 2007). The highest antioxidant content and the lowest antioxidant activity were found in the ripest sample. Phenol content of the mushrooms decreased from 5.52 mg/g in the immature mushroom to 3.09 mg/g in the mature mushroom.

Wong *et al.,* (2006) found a strong correlation between DPPH and FRAP, which implied that compounds in the extracts were capable of scavenging DPPH free radicals and reducing ferric ions. A ‘satisfactory’ correlation between TPC and both DPPH (r=0.57) and FRAP (r=0.55) suggested that polyphenols in the extracts were partly responsible for the antioxidant activity (Wong *et al.*, 2006). Our results show similar correlation coefficients of 0.60 and 0.52, respectively. Kubola & Siriamornpun (2008) found a much higher correlation in gourds, with TPC vs. DPPH r=0.71 and a similar correlation for TPC vs. FRAP (r=0.55). Wong *et al.,* (2006) and Kubola & Siriamornpun (2008) both found a very strong correlation between DPPH and FRAP (r=0.95), meaning that compounds which are able to reduce DPPH radicals were also able to reduce ferric ions. Our correlation coefficient was 0.52. This could be due to our CHA activity of 15.6 ± 2.2% for the water extract (range 9.3 – 21.7%) and 25.0 ± 2.0% range (19.2 – 30.3%) for the ethanol extract, compared with 39 - 95% chelation found by Wong *et al.,* (2006). Manian *et al.,* (2008) found that the amount of DPPH activity appeared to be dependent on the total phenolic content of the extracts of tea (*Camellia sinensis* L.).

Oxidation is essential to all living organisms for the production of energy to fuel biological processes. This endogenous oxidative metabolism results in the production of reactive oxygen species (oxygen- and nitrogen-centre free radicals, 'reactive oxygen species', ROS) as by-products. A free radical is capable of containing one or more paired electrons, which can accept electrons from other atoms, converting them into secondary free radicals, leading to biological damage (Costantini, 2008). Overproduction of free radicals can be caused by an imbalance in the body's antioxidant defence system and can also be caused by various environmental chemicals/pollutants. These free radicals can react with biomolecules causing oxidative damage to DNA, lipids and proteins, resulting in failure of cellular function, cellular injury and death. Humans and other organisms possess innate antioxidant defences to protect against oxidative damage (i.e. stress responses allow an organism to maintain body homeostasis), but if the response is inadequate then it may have adverse effects health and biological fitness (Costantini, 2008).

Antioxidants are substances which delay the oxidation process, inhibiting the polymerization chain initiated by free radicals and other subsequent oxidising reactions (Céspedes *et al.*, 2008). Antioxidants are either enzymatic or non-enzymatic, and have a number of functions, including binding metal ions to avoid generation of ROS and scavenging reactive species in the body (Costantini, 2008). Primary antioxidants scavenge radicals to inhibit chain initiation whereas secondary antioxidants suppress the formation of radicals and protect against oxidative damage (Lim *et al.*, 2007). Exogenous antioxidants are constantly required to maintain an adequate level of antioxidants to balance reactive oxygen species in the body. Breeding affects circulating levels of antioxidants in both males and females (Costantini, 2008). These exogenous antioxidant compounds are found in many plants and are consumed as part of the diet of many organisms, including humans and birds. It is possible that food quality or availability may alter the body's response to oxidative stress (Costantini, 2008). Uric acid has antioxidant properties, and since the level of uric acid excreted is affected by the amount of protein in the diet of birds, this could alter the body's oxidative status (Costantini, 2008).

There are few studies on the effects of oxidative stress in non-human animals, and it is not clear to what extent life history and oxidative stress is linked (Costantini, 2008). It is thought that oxidative stress (resulting from an imbalance of pro-oxidants and anti-oxidants) may affect reproduction, longevity and immune responses.

Oxidative damage can also result in secondary damage to biomolecules, such as changing ion levels. A decline in essential dietary metal cofactors such as selenium, magnesium or zinc may also result in oxidative stress (Costantini, 2008). The fact that birds are generally more long-lived than mammals may be because they are subject to lower oxidative stress (Costantini, 2008).

There is a link between antioxidant protection and reproduction in birds, with oxidative stress underlying the cost of reproduction (Costantini, 2008). Lipids are targets for oxidative damage, saturated fatty acids are more susceptible to oxidative damage than unsaturated, and polyunsaturated n-3 fatty acids are more susceptible to oxidative damage than n-6 fatty acids (Costantini, 2008). Bird phospholipids contain more n-6 than n-3 fatty acids. It is possible that changing dietary fatty acid profiles could alter membrane composition, leading to increased (or reduced) susceptibility to oxidative damage (Costantini, 2008). When a bird enters the reproductive state, nutrients and antioxidants are reallocated from maintenance to reproduction, possibly leading to lowered future fecundity or survival.

Flavonoids/polyphenols possess antioxidant properties, and interfere with the activity of enzymes involved in free radical formation (Wong *et al.*, 2006). For example, in fruits the most abundant antioxidants are polyphenols and most of these are flavonoids (Lim *et al.*, 2007). These dietary phenolic antioxidants can counteract the effects of the free-radicals, and have a role in prevention of many oxidative and inflammatory diseases (Reynardson *et al.*, 2008). Generally a high level of polyphenols is associated with a high antioxidant activity. For example, polyphenols are responsible for 76-84% of the antioxidant activity of fruit extracts of *Quercus coccifera* L. and *Juniperus phoenicea* L. (Hayouni *et al.*, 2007).

The ethanol extracted samples showed some odd results for CHA. For example, unlike all the other analyses, the most dilute samples (1.25 mg/ml) did not always show the lowest CHA. In the water extract of rimu fruit the levels of CHA increased over the year, to a maximum (double the initial inhibition) in the sample from 6-Feb-08. The level then decreased by the mid-Feb-08 sample. Chelating agents may serve as secondary antioxidants as these reduce the redox potential and stabilise the oxidised form of metal ions (Manian *et al.*, 2008). Low to moderate ferrous ion chelating effects (7.4 to 22.3 mg/g) in fractions of tea extracts could be beneficial to protect against oxidative damage (Manian *et al.*, 2008). The increase in CHA activity as the rimu fruit ripens may protect against oxidative damage with an associated effect on reproduction in kakapo.

The HPLC/GC/MS profiles of composite rimu fruit samples show differences between the compounds over time, and differences between trees. As mentioned previously, it is not possible to compare the profiles between samples because the amount of sample used in each HPLC run varied. The amounts of compounds in sample were estimated using areas under the curve which were used for comparison between samples and trees.

The compound with retention time of 48.95 minutes changed dramatically over time, with the amount of this peak increasing over 6-fold over the season. The compounds with shorter retention times (12.4, 17, 18) decreased over the season. Compound 18 was identified as a catechin and the compound at 48.95 could not be identified. Kakapo start eating rimu fruit in late October/early November, so some of the unknown compounds that changed in abundance over the season (especially the compound with the 48.95 retention time) may deserve more investigations as a possible trigger.

A chemical compound in plant material has been found to trigger reproduction in the montane meadow vole. This compound was subsequently isolated and identified as 6-methoxybenzoxazolinone, a nonestrogenic compound. This compound could be an ultimate cue because it indicates that a high quality food supply, mature grasses, is available to support reproduction. Since kakapo have reproduced where no rimu is present, such as on Little Barrier Island (Hauturu) it is unlikely that a single chemical compound unique to rimu fruit is their only trigger for reproduction. It is thought that breeding on Little Barrier could have been triggered by the mast seeding of kauri (*Agathis australis*).

A number of possible non-nutrient chemical cues, such as tannins, may be involved in the regulation of consumption of the plant material, and could trigger reproduction. For many animals, tannins are repellents with a bitter taste that form insoluble complexes with protein and inhibit protein absorption (Liukkonen-Anttila *et al.*, 2001). Eating plant material containing tannins could therefore reduce the amount of protein (amino acids) available for reproduction. Phytoestrogens, oestrogenic compounds found in plants, have been reported to have adverse effects on reproductive function in ungulate ruminants and birds, and can have both agonistic and antagonistic effects under different conditions. It is possible that insects that have fed on leaves containing tannins have reduced nutritive quality, or at least palatability, to birds.

Partridges (*Perdix perdix*) were fed a high protein diet containing 6% tannin developed a longer small intestine (Liukkonen-Anttila *et al.*, 2001). This may indicate more effective nutrient absorption and increased gastro-intestinal detoxification. After release into the wild, partridges not previously exposed to tannins had relatively high mortality, thought to be due to their inability to detoxify plant secondary compounds.

There are a number of deterrents such as phenols and fibres in food eaten by birds, which could be potential triggers for reproduction, or for fine-tuning the timing of nesting. Phenolic substances (tannins) in date fruit were high in the unripe stage, and declined as the fruit ripened (Biglari *et al.*, 2008). Jensen (1999) demonstrated a link between the periodic production of lethal toxins by food plants and the death of voles. Microtine rodents deplete preferred food plants during the breeding season forcing them to switch to plants they usually avoid because they contain deterrents/toxins. These plants produce deterrents and digestibility reducers, such as tannins but also lethal toxins. Consequently as the voles run out of palatable food they consume more lethal toxins causing an increase in mortality (Jensen & Doncaster, 1999).

Birds are thought to be less affected by secondary plant metabolites than other animals (Bairlein, 1996). The Hoatzin (*Opisthocomus hoazin*), a species of bird found in the Amazon and South America, is an obligate folivore like the kakapo, and is the only bird known to have a well developed foregut fermentation system (Grajal, 1995). This fermentation system selectively retains solid food particles in the foregut, resulting in increased absorption of water and digestible soluble nutrients, and possibly detoxifies secondary plant compounds. Kakapo have a similar digestive morphology and strategy as the Hoatzin (Klasing, 1998), except that there is no evidence that they have foregut fermentation (G. P. Elliot, pers comm.). It seems unlikely as kakapo ingest little fibre. The strategy of digestion of plant fibre is also employed by species such as the ostrich, emu and takahe (Grajal, 1995).

The terpenes found in trees of the Podocarpaceae are dominated by totarol derivatives (phenolic diterpenes) and structures related to ferruginol, but only shoots/branchlets have been analysed in previous studies (Cox *et al.*, 2007). No ferruginol was found the rimu fruit samples. Podocarpic acid is confined to the Podocarpaceae family. Monoterpenoids have been shown to trigger the onset of reproduction in the desert locust (Ellis *et al.*, 1965). A diet of aging Brassica leaves delayed egg-laying by several weeks compared with those on a diet of green leaves, attributed to gibberellins (plant hormones), eugenol and other monoterpinoids (Ellis *et al.*, 1965). The flavonoid glucoside biochemistry of New Zealand *Dacrydium* species was first reported by Markham, preliminary studies were reported in Markham (Markham *et al.*, 1987) with a full investigation into *Dacrydium* species reported in Markham (1989). The main aim of these studies was to support the classification of the genera within the *Podocarpus* genus. The major components of rimu leaves/foliage was found to be flavonol mono- and di-glycosides (including quercetins and kaemferols), and apoglycones/biflavonoids (Markham *et al.*, 1989). The different genera of the Podocarpaceae (*Halocarpus, Lepidothamnus, Lagarostrobus* and *Dacrydium*), have very different flavonol glycoside profiles. Rimu is characterised by a predominance of flavolnol-3-o-rhamnoglucosides.

The isolation and identification of polyphenols/flavonoids is a complex process, and beyond the scope of this study. They could be an important component of the rimu fruit affecting kakapo reproduction, as they have been shown to affect reproduction in a number of species. There are many potentially toxic secondary plant metabolites, and it is not known how these affect normal metabolism of birds.

Fidler *et al.,* (2008) hypothesised that low levels of dietary phytochemicals may affect reproduction in the kakapo. In brief, in non-mast years low levels of phytochemicals result in the ovarian follicles failing to develop, whereas in mast years the increased exposure to low levels of phytochemicals results in egg production. Preliminary results from an earlier study (Fidler *et al.*, 2000) did not find oestrogenic activity in green rimu fruit, although this was possibly due to the use of a human oestrogen receptor (Fidler *et al.*, 2008). Analysis of a range of rimu fruit from our study did not show any evidence of oestrogenic activity either, although this was also using a human oestrogen receptor alpha inserted into a yeast genome (Mortensen, 2009). No podocarpic acid (slightly oestrogenic) or isoflavones (e.g. diedzein, genistein) was found in any of the rimu fruit samples in our study.

A summary of the possible chemical triggers affecting kakapo reproduction is presented in *Table 7*. All of the compounds I found in rimu fruit either remained constant or increased/decreased, either of which could trigger breeding. Based on my results in addition to the literature review, the most likely candidates are crude protein, crude fat and fatty acids, calcium and polyphenolics/antioxidants.

Table 7. Summary of possible chemical triggers.

Possible chemical Trigger	Change over the season	Possibly involved in triggering breeding in kakapo?	Unlikely to trigger breeding in kakapo
Crude protein	increased	yes	
Individual amino acids	no	yes	
Crude fat	increased	yes	
Individual fatty acids	increased	yes	
Phosphorus	increased	yes	
Calcium	no	yes	
Total polyphenolic content	decreased	yes	
Antioxidant activity	decreased	yes	
Phytoestrogens	none found	unlikely	yes
Flavenoids	some increased	yes	

There are inherent difficulties carrying out nutritional research on critically endangered birds, especially those with unusual life-history and dietary characteristics. The type of research which can be undertaken with suchbirds is very limited, so commonly used techniques (such as digestibility trials) are not possible. Attempts at radio-tracking of kakapo observe foraging behaviour proved ineffective, although offering branches of rimu fruit to tame but free-living kakapo allowed us to confirm which parts of the fruit they were eating, assuming that this is what they would eat in a natural situation. We can analyse the diet of the birds, what we think they are eating, but not the bioavailability of the nutrients which they extract from these items. We can also theoretically estimate the extraction rates of some of the nutrients from analysis of nutrient content.

The feeding strategy of plant-visiting bats is to chew leaves and fruits to extract the readily-digestible cell contents and juices and expel the fibre (Ruby *et al.*, 2000). Kakapo do the same with the exception of rimu fruit which is consumed whole. The extracted fractions from food plants eaten by the short-nosed fruit bat (*Cynopterus sphinx*) were analysed and compared with uneaten plant material, giving extraction rates for organic nutrients ranging from 50 - 64% and for macrominerals from 0 – 36%. Specific extraction estimates were 15 - 36% for calcium, 33 – 50% for phosphorus, 55 - 64% for protein and 40 – 50% for fat (Ruby *et al.*, 2000). Similar calculations of estimated extraction rates of lipid and protein for kakapo gave extraction rates of 27% for protein and 58% for lipid (Klasing, 2007). This method of food processing enables the animal to process a greater amount of food per unit of time (Ruby *et al.*, 2000), but the differences in extraction rates of birds and bats complicate the estimation of digestibility from the nutrient content of plant material.

5. Summary and Conclusions

The initial predictive cue to initiate reproduction in birds, including the kakapo, is photoperiod (Cockrem, 2006). In most birds, supplementary cues (such as food cues) are then required to actually initiate reproduction. A number of species display anticipatory reproduction, the ability to anticipate future food supplies and breed so that abundant food is available when the offspring need it. The animal needs to track environmental cues to provide information on when successful reproduction is most likely. Some appear to do this by specifically feeding on certain plant parts, often the reproductive structures of food plants (e.g. buds, flowers, pollen cones) which are present in advance of the forthcoming seed crop. In the kakapo, breeding is known to be dependent on infrequent mast fruit crops of a number of native plants, namely rimu on Codfish Island. Kakapo start eating the unripe rimu fruit in late October/early November, and if >10% of branch tips are bearing fruit breeding takes place from January to July. Successful breeding depends on there being sufficient ripe fruit to raise nestlings. There is always some rimu fruit every year but it is only when its abundance reaches a certain level that breeding occurs.

There are many theories about what triggers breeding in species like the kakapo, however most authors concede that it is likely to be a number of inter-related cues. Many authors have investigated the association between the ingestion of buds and other plant material, and most have found that these food sources do trigger reproduction, but the identity of the actual component which triggers reproduction within these structures remains unclear. Kakapo have bred in response to a number of plant species, so if there is a chemical trigger it can not be specific to a single plant species. A number of compounds have been proposed as potential triggers for reproduction, including protein (and individual amino acids), fat (and fatty acids), fibres, polyphenolic compounds and antioxidants.

A number of the compounds found in rimu fruit are known to affect reproduction in other species and therefore could be potential triggers for reproduction in kakapo. Some of these increase (or decrease) in concentration in rimu fruit over time, or may trigger breeding when a sufficient quantity has been ingested In non-mast years insufficient fruit is available to trigger reproduction and there is no breeding. Crude protein and crude fat levels increased in rimu fruit over the season, with some individual fatty acids increasing up to 70%. Changes in protein levels have been shown to trigger breeding in a number of other species. Although the level of protein found in rimu fruit are low compared to that in foods eaten by other birds, an increase in protein levels through increased rimu fruit consumption in October could be the trigger for breeding in kakapo. Changes in the solubility of amino acids within the fruit may also make limiting amino acids more available for reproduction.

The total polyphenolic content of rimu fruit changed over time, decreasing around the time kakapo breed. This was associated with a decrease in antioxidant levels. This is found in the fruit of many species of plant, with polyphenolic levels and antioxidant levels decreasing as fruit ripens and so could be a general proximate cue for species that depend on fruit to breed. Such changes may allow kakapo to track the ripening of fruit crops so that they can time nesting to achieve the greatest synchrony between the nestling period and the availability of ripe fruit.

One alternative theory is that low levels of dietary phytochemicals may affect reproduction in the kakapo (Fidler *et al.*, 2008). This theory was not supported by evidence from the current study, as no phytochemicals or oestrogenic activity were found in rimu fruit.

The theory which has the largest body of supporting evidence is that the trigger for reproduction in many species is the size, or abundance, of the developing fruit crop. Instead of responding to a chemical trigger in plant foods birds and animals can somehow assess the size of developing seed crops and are triggered to breed when this appears sufficiently abundant to raise young. The trigger is more likely a combination of these two theories, both the abundance of the fruit crop and some compound/s (nutritional or chemical) present in the fruit crop. The triggering of breeding by abundant food sources, or the precursors of these, has been reported in a number of birds, including the piñon jay (Ligon, 1978), great tit (Nager & van Noordwijk, 1995), red crossbill (Hahn, 1998) and neotropical spotted antbirds (Hau *et al.*, 2000; O'Brien & Hau, 2005). The trigger for breeding in these birds could be ingestion of a threshold level of a trigger substance or substances, or, as in the antbird, it could be entirely visual. The combined abundance/specific compound theory is consistent with kakapo biology because kakapo only breed if the abundance of rimu fruit exceeds a certain threshold. It is also consistent with the fact that kakapo have been triggered to breed by plant foods other than podocarp fruit; for example, pine seed on Maud Island and possibly kauri seed on Little Barrier (R. J. Moorhouse, pers. comm.). This makes a single, rimu-specific chemical trigger unlikely, although the trigger could be some aspect of the profiles of nutritional compounds common to the foods which trigger breeding (e.g. high calcium).

Another contender is dietary fibre. Dietary fibre is known to bind to calcium and decrease its availability (Ruby *et al.*, 2000), so an increase in fibre over the season may affect calcium availability in rimu fruit. Calcium limitation is thought to be a widespread phenomenon that affects egg properties and chick development in many species. There is evidence that calcium is a common limiting factor for reproduction in a variety of vertebrates (Barclay, 1994), and there are strong indications that calcium might be interacting with relatively high levels of macronutrients in supplementary food provided to kakapo (Raubenheimer & Simpson, 2006). Supporting evidence that calcium could be a limiting factor for reproduction in kakapo includes the fact that kakapo fledglings have large, dense skeletons, mothers alone provision the chicks, the nestling period is long, and small clutch size (Raubenheimer & Simpson, 2006). Female kakapo also consume egg shells after chicks have hatched thereby recycling the calcium these contain. Kakapo are entirely vegetarian so it is possible that rimu fruit provides the only readily available and sufficiently abundant source of calcium for growing chicks.

Amounts of most compounds measured in this study differed between individual trees, with some of the phenolic content and activity measures even differing within trees. Previous studies have shown that individuals of some species actively select vegetation particularly rich in certain nutrients. Although we do not know which, if any, of the trees sampled were actually used by kakapo, these marked nutritional differences between (and within) trees could be important.

In summary, kakapo (and many other birds) are able to anticipate an abundance of ripe fruit of sufficient nutrition to raise their young, and time nesting to ensure that this food supply is available during the nestling period when chicks are dependent on their mother for food. Rimu fruit is on the tree for a year before it ripens. This gives kakapo a long period in which to detect that sufficient ripe fruit will be available to raise young but they still need to be able to anticipate the timing of ripening so that there will be sufficient ripe fruit available for nestlings. It is possible that the physiological changes necessary for reproduction are triggered by the abundance of the unripe fruit crop but that nesting itself is triggered by changes in the green, undeveloped fruit associated with ripening.

Candidates for the trigger include crude protein, crude fat, individual fatty acids, phosphorus, calcium, total polyphenolic content and antioxidant levels and some flavenoids. With the exception of calcium, most of these components increased over time. As calcium did not change over the season, it is possible that maybe even naïve kakapo could potentially identify rimu as a suitable food for chicks long before it was ripe. The changes in the other compounds could be a trigger that allows kakapo to predict when the fruit is likely to ripen time nesting appropriately.

Key points:

- Kakapo on Codfish Island are clearly triggered to breed by some property of the abundance of green rimu fruit. As they consume this fruit, it is possible that chemical changes within it allow them to synchronise nesting with the ripening of the fruit crop.

- The nutritional profile of rimu fruit did not change markedly over the early part of the season, which may allow kakapo to identify it as a potential food for nestlings long in advance of ripening

- Since kakapo have bred where there is no rimu any putative trigger for breeding can not be specific to rimu

- Potential candidates for triggers for nesting include increased levels of crude protein, increasing crude fat levels, individual fatty acid levels and ratios, increasing phosphorus, crude fibres (changing digestibility of other nutrients), phenols and polyphenols/flavonoid compounds, or a combination of these.

- All trees are not equal, a number of compounds measured differed between trees, and sometimes within trees.

The 2007-2008 season was a relatively minor mast year, with only 13% of tips upturned in contrast with 39% of tips upturned in the bumper year of 2001-2002. It did result in five females nesting and 6 chicks fledging (R. J. Moorhouse, pers. comm.), compared with 24 nests (15 successful) and 24 chicks fledging in the 2001-2002 season (Elliott *et al.*, 2006).

Further investigation needs to be carried out to identify individual trees that the birds are using when they first switch to eating rimu fruit, and to collect and analyse some of this fruit, to confirm if the results from this study are comparable.

My results show that there are major differences in nutritional composition between and even within the individual trees which could have implications for kakapo management. A number of changes in fruit composition over time were also detected, some of which could be involved in the timing of nesting. It is likely that reproduction is triggered by a two-stage, rather than a single trigger and that this is unlikely to be specific to rimu.

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