Separate feeding of calcium for poultry

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Foreword

This project was conducted to evaluate the response of bird performance and egg quality to a range of dietary Calcium (Ca) and non-phytate phosphorus (npP) concentrations and ratios, and to investigate the potential to implement a separate Ca source in the form of limestone grit to support egg production in hens offered suboptimal dietary Ca.

This project was funded from industry revenue which is matched by funds provided by the Australian Government.

This report is an addition to Australian Eggs Limited's range of peer reviewed research publications and an output of our R&D program, which aims to support improved efficiency, sustainability, product quality, education and technology transfer in the Australian egg industry.

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Abbreviations

AME	Apparent metabolisable digestivity
ATTD	Apparent total tract digestibility
AID	Apparent ileal digestibility
BD	Becton Dickinson (North Ryde, Sydney)
BW	Body weight
Ca	Calcium
CP	Crude protein
d	Day
DM	Dry matter
FCR	Feed conversion ratio
g	Gram
HSD	Honestly significant difference
IU	International Unit
MJ	Megajoule
npP	Non-phytate phosphorus
NRC	National Research Council
P	Phosphorous
PRF	Poultry Research Foundation (the University of Sydney)
REML	Restricted maximum likelihood
RIRDC	Rural Industries Research and Development Corporation
SEM	Standard error of the mean

Executive Summary

Much research has been conducted on the appropriate concentrations and ratios of calcium and phosphorus requirements for optimum egg production and feed conversion efficiency in laying hens. However, a consensus on the appropriate concentrations and relative proportions of dietary calcium and non-phytate phosphorus (npP) remains elusive. This uncertainty reflects the high requirement for dietary calcium to produce good quality eggshells, a need which is in conflict with maximising digestibility of phosphorus and other nutrients due to the high acid buffering capacity of calcium and complexing with phytate-P. The concept of supplementing the hen with an additional, external source of dietary calcium in the form of a grit has the potential dual purpose of both i) introducing time and space between the ingestion of calcium and the other dietary nutrients, and ii) reducing dietary Ca while maintaining the high total Ca intake required of laying hens. While studies have been conducted in the past to develop our understanding of the role of external Ca consumption, much remains to be done to realise the advantages and disadvantages of this feeding strategy.

In this series of studies, an attempt is made to: i) investigate the presence and extent of an 'appetite' for an extra-dietary source of Ca in laying hens; ii) identify the voluntary intake of dietary Ca and P of laying hens provided with opportunities to adjust macromineral intake; iii) establish the optimum Ca and P concentrations and ratios of dietary Ca for optimum egg production and nutrient digestibility; and iv) investigate the interaction between a source of extra-dietary Ca and dietary phytase on Ca appetite in hens and egg production.

In the first study, ISA Brown laying hens were presented with diets ranging in dietary Ca levels from 10 g/kg to 40 g/kg and supplemented with an adjacent continuous supply of limestone grit. The concentrations of dietary npP were 0.25% across all treatments giving a ratio of 16:1, 12:1, 8:1 and 4:1 Ca:npP for the 10, 20, 30 and 40 g/kg Ca dietary treatments respectively. The objective of the study was to investigate the presence of a Ca-specific appetite in hens when presented with increasingly sub-optimal levels of dietary Ca. The results of this study show evidence for Ca appetite in laying hens. Hens assigned to low dietary Ca diets consumed greater quantities of the limestone grit and had a greater proportion of their total Ca intake coming from the limestone grit. However, the practice of consuming limestone grit was not uniformly expressed and appears to be partly controlled as part of a learned behaviour. The ATTD of various minerals showed that there was no benefit to providing Ca as a separate source, as digestibility of Ca and P was equivalent across dietary treatments. The 10 g/kg dietary Ca group showed the poorest performance in terms of egg quality and egg production despite evidence for increased limestone grit intake. In contrast, the 30 g/kg Ca group were broadly comparable with the 40 g/kg Ca group. The findings of this study suggest that when presented with sub-optimal dietary Ca, a proportion of hens will consume larger quantities of a limestone grit source. However, the capacity for reducing dietary Ca appears to be limited and going below 30 g/kg resulted in compromised bird performance and egg production and quality.

The second study had two objectives. Firstly, to identify the optimum ratios and concentrations of dietary Ca and npP to support bird performance and egg production and quality. This was achieved using a geometric framework design arrangement which facilitated visualisation of response variables to a range of Ca and npP concentrations and ratios using contour plots. Secondly, the capacity for hens to regulate Ca and P intake through voluntary feed intake was assessed when layers were presented with a choice of diets varying in mineral density.

For the first objective, dietary treatments were arranged into three nutrient densities (Ca+npP) of 30, 40 and 50 g/kg and five ratios (Ca:npP) ranging from 16:1 to 6:1 creating a geometric nutrient space. Maximising both dietary Ca and npP resulted in a greater egg mass per day and greater shell weight.

Increasing concentrations of npP resulted in a greater egg Haugh unit at lower Ca levels, however, this effect was not observed at higher Ca levels. Increasing dietary npP intake was also important for egg weight with dietary Ca intake being less important. AME increased in line with increasing npP concentration. However, this was only observed when within dietary total Ca parameters of 37-43 g/kg Ca. Therefore, the results of this study suggest that maximising dietary Ca (40-45 g/kg) stimulates greater egg mass but this must be matched by maximising dietary npP (6-7g/kg) also. This demonstrates that the ratio of Ca:npP is important, with a narrow ratio being preferred. In contrast, shell thickness was maximised in line with increasing dietary Ca regardless of the level of dietary npP, suggesting that the ratio and concentration of npP is less important for this production trait. Haugh unit was affected by dietary treatments also with increasing dietary npP associated with a higher Haugh unit but only when dietary Ca was low. This suggests that the ratio of Ca:npP is important here also with a low Ca:npP improving Haugh unit. Optimum digestibility for Ca and P was observed for a dietary Ca range of between 30-35 g/kg in this study while dietary levels of npP and the ratio of Ca:npP being less important for these digestibility variables. The overall findings of this study suggest that maximising dietary Ca and npP intake is important for egg mass, increasing dietary Ca is important for shell quality, and increasing dietary npP is important for increasing egg weight. The observation that Ca and P digestibility were improved at lower dietary Ca levels was not reflected in improved feed efficiency, suggesting limited capacity to improve overall feed efficiency through improving mineral digestibility.

The third and final study attempted to expand on the observations on limestone grit use by laying hens observed in the initial study. In this study, the tendency for hens to consume an extra-dietary source was investigated in response to suboptimal or optimal levels of dietary Ca (20, 30 and 40 g/kg). Additionally, because dietary phytase can influence the digestion dynamics of Ca and P, and hence may influence Ca-specific appetite, the separate Ca intake of hens provided with or without phytase was also investigated. Similarly to the first study, it was observed that approximately 50% of the hens engaged in limestone grit consumption regardless of what dietary Ca level they were assigned to. The remaining hens consumed negligible amounts of grit per day. However, of the sub-population of hens which did engage in limestone grit consumption, a clear increase in quantities of limestone grit consumption was observed in response to decreasing dietary Ca concentrations. This suggests that there are both behavioural and physiological factors underlying limestone consumption. The results of the study show strong dietary Ca effects across both bird performance, and egg production and quality parameters. Egg mass was greatest in the 30 g/kg dietary Ca birds while the 40 g/kg dietary Ca group had the greatest eggshell weight, eggshell thickness and eggshell breaking force. Egg weight and yolk colour was greatest in the 40 g/kg dietary Ca group. The 20 g/kg Ca group performed the poorest in terms of egg quality. The inclusion of a dietary phytase (3000 FYT/kg phytase) did not influence the consumption of limestone grit or influence any other measured parameters compared with unsupplemented diets. The AME and FCR of birds was not affected by dietary Ca treatments, suggesting that providing a separate source of Ca did not improve nutrient digestibility significantly to influence bird feed efficiency.

The results of this study show that overall, the consumption of limestone grit does not ameliorate the impact of reducing dietary Ca concentrations of bird performance and egg production. This may be due to a portion of the experimental population not engaging in limestone grit consumption. There was no positive benefit observed for free choice limestone grit feeding in nutritionally adequate diets.

Overall Conclusions

The series of studies presented here had the following broad objectives – to:

1. evaluate the response of bird performance and egg quality to a range of dietary Ca and npP concentrations and ratios
2. investigate the potential to implement a separate Ca source in the form of limestone grit to support egg production in hens offered suboptimal dietary Ca.

The overall conclusions of the work are as follows.

This study shows that in a caged facility using individually-housed hens presented with a complete diet and a separate Ca source as limestone grit, that limestone grit consumption is not carried out uniformly. Birds could be categorised as non-grit or grit consumers. Birds that do consume limestone grit respond to decreasing dietary Ca levels by consuming greater quantities of limestone grit. Offering 30 g/kg dietary Ca resulted in comparable performance for many production traits when compared with 40 g/kg Ca when both groups were supplemented with a limestone grit. However, the findings of this study suggest, particularly where limestone grit is not offered, that maximising dietary Ca and keeping the ratio of Ca:npP at 6:1 is the best strategy to maximise bird performance and egg quality, particularly as this pertains to egg mass, egg weight and shell quality.

Maximising dietary Ca with a Ca:non-phytate P ratio of 6:1 is the best strategy to maximise hen performance, egg mass, egg weight and shell quality

The formation of bones and eggshells utilise similar processes in laying hens, both of which rely heavily on the ratio of calcium (Ca) and phosphorus (P), however, there is little clear evidence of what the optimum Ca:P ratio should be to maximise hen health, egg quality and production. Ca is present in many ingredients in feed, but sometimes a separate source of Ca is provided in the diet in the form of limestone to supplement this. This project sought to provide some guidance on the optimum Ca:P ratio, whether a separate source of Ca was beneficial, and what factors might influence the consumption of Ca in hens (Isa Brown were used in this study), for egg quality and production traits. Further work is required to investigate the effect of Ca:P on bone mineral content and bone density in hens.

Key findings

1. Maximising dietary Ca with a Ca:non-phytate P ratio of 6:1 is the best strategy to maximise hen performance, egg mass, egg weight and shell quality.
2. Hens on a low Ca diet consumed greater amounts of separate Ca providing evidence that hens have an appetite for Ca and will seek to include it in their diet. However, only 50% of hens consumed the separate source of Ca (regardless of the level of Ca in the diet) suggesting in part that it may also be a learned behaviour.
3. Hens offered a diet with 3% or 4% Ca plus a separate Ca source had comparable productivity and egg quality.
4. Going below 3% Ca in the diet negatively impacted on hen performance and egg production and quality.
5. Providing a separate source of Ca hens on a low (1%) Ca diet was not sufficient to maintain egg production.
6. Maximising dietary Ca (4.0-4.5%) stimulated greater egg and eggshell mass, but must be matched by maximising dietary non-phytate P (0.6-0.7%) to obtain higher shell thickness and Haugh unit.

7. The inclusion of a dietary phytase did not influence the consumption of limestone or influence any other measured parameters compared with unsupplemented diets. However, this result may have been influenced by the fact that not all hens consumed the limestone.
8. The AME and FCR of birds were not affected by dietary Ca treatments, suggesting that providing a separate source of Ca did not improve nutrient digestibility significantly to influence bird feed efficiency.

Implications

Maximising dietary Ca with a Ca:non-phytate P ratio of 6:1 is the best strategy to maximise hen performance, egg mass, egg weight and shell quality. The practice of calcium consumption may be a learned behaviour in addition to a physiological need and appetite for calcium. Supplementation of the diet with a separate source of Ca will not prevent egg quality deterioration at low Ca levels.

1 Review of the literature – separate feeding of calcium for poultry

1.1 Calcium and phosphorus requirements of laying hens

The utilisation of dietary Ca and P, and the interaction between these two macrominerals in poultry, has undergone sustained research over the past 30 years particularly in the broiler chicken. As a consequence, our understanding of the complex biology of Ca and P nutrition to maximise growth performance and skeletal integrity is advanced (Driver et al. 2005; Selle et al. 2009; Wilkinson et al. 2014). Furthermore, the implications of, and consequently widespread uptake of, phytase in the chicken meat industry is the result of detailed investigation and characterisation with many examples of this work in the literature (Simons et al. 1990; Marounek et al. 2008; Cowieson et al. 2015; and Manobhavan et al. 2016).

While there has been some seminal research work published exploring the importance and utilisation of Ca and P (Keshavarz & Nakajima 1993; Roberts 2004) and phytase (Lim et al. 2003) in egg production, generally there is comparatively less literature available on Ca and P biology in laying hens. Consequently, there is still no general consensus on appropriate concentrations of dietary Ca and P for optimum performance and nutrient digestibility. The lack of agreement on appropriate dietary Ca and P inclusion rates for the laying hen stems from the desire to match the hen's large requirement for Ca, which is in conflict with the complex, inhibitory interactions of these two minerals post-ingestion.

1.1.1 Calcium requirements of the laying hen

Calcium is an essential nutrient for poultry and is typically provided to laying hens at approximately 4.2-4.6% of the total feed volume. Calcium is usually supplied to layers as a calcite grit or flour, i.e. limestone, and also as part of the inorganic P supply, e.g. dicalcium or monocalcium phosphate. Only a minor part of the birds' Ca requirement is met by vegetable ingredients such as wheat, canola or soybean meal, although other ingredients such as meat and bone meal can contribute substantially to this source. Historically, the optimum range of dietary calcium has been well defined. Scott et al. (1971) revised the optimum inclusion rate for dietary calcium upwards to 3.5% but noted a decline in egg production at inclusion rates of 5% driven by a decrease in voluntary feed intake. More recently Safaa et al. (2008) supported this finding, showing the modern layer in late phase requires at least 3.5% dietary Ca to maintain optimum egg quality. Pelicia et al. (2009) show in a study comprising dietary Ca levels ranging from 3.0-4.5% that 4.5% dietary Ca provided the best outcomes for bird performance and egg quality, regardless of npP levels. Overall, the literature suggests that there is little scope to reduce dietary Ca intake without severely compromising important production traits.

1.1.2 Phosphorus requirements of the laying hen

Similar to Ca, cereal grains provide little in the way of dietary P and much of this is in phytate form and hence largely unavailable to the host (Selle et al. 2009). Adequate levels of dietary npP are provided by inclusion of inorganic sources predominantly in the form of dicalcium phosphate, with other bioavailable sources including monosodium phosphate, monocalcium phosphate and defluorinated phosphate. Excellent reviews and studies (albeit generally with a focus on broilers) on the high bioavailability (relative to plant sources) of these P sources are available elsewhere (Bikker et al. 2016; Li et al. 2016).

There is markedly less focus on P requirements in layers outside of research concerning phytase. The interest in P and phytase in hens, like broilers, stems from a traditional understanding of phytate-P being largely unavailable for digestion and absorption in the gastrointestinal tract. For example, Nelson (1976) showed that the phytate-P recovery in laying hen faeces was 92% for corn and 87% for corn/wheat diets. Cereal grains may contribute some inherent phytase activity where mash is offered although this will be markedly reduced where steam pelleting feed is in place (Jongbloed & Kemme 1990; Slominski et al. 2007). Marounek et al. (2008) reported that the greatest level of phytase activity is in the caeca of both young and aged hens where opportunity for P recovery is minimal. However, more recently in mash-fed broilers Morgan et al. (2015) showed that both intestinal and cereal-derived phytase activity was substantial in the gizzard and small intestine, increased with age and resulted in an ileal P digestibility range of between 0.69 and 0.82.

The relatively lesser amount of studies with a strong focus on P also likely reflects the greater importance placed on Ca intake for egg and eggshell optimisation. Despite the apparently poor utilisation of overall P in hens, Keshavarz and Nakajima (1993) reported the capacity to reduce dietary P in aged hens without affecting egg production, however, bone ash was compromised. Some studies suggest the response of egg production generally seems to be less sensitive to changes in dietary P when compared with Ca. Aged hen egg production showed no response to increasing dietary P (4.3-7.5 g/kg), rather the authors concluded that dietary P should be lifted in response to increased dietary Ca (Bar et al. 2002). This finding is supported by Pelicia et al. (2009) who showed no response of bird performance or egg quality to dietary npP ranging from 0.25-0.40%. Most recently, Li, Bryden and Zhang (Report to Australian Eggs Limited 2016; 1UQ101A) concluded that modern laying hens met minimum P requirements at levels of npP of 1.5g/kg. In contrast to this, Usayran and Balnave (1995) report that increasing dietary npP level impeded egg production. Interestingly, these authors also noted an increase in mortality in birds offered low total P diets, suggesting that in that study there was a narrow ideal range for the dietary concentration of this mineral. A lot of research focusing on optimising phosphorus utilisation stems from reducing the environmental impact of phosphorus and acknowledging the finite supply of this resource from conventional sources (Selle et al. 2007).

Adjustment of P may be considered in three aspects: i) reducing dietary P to reduce costs and pollution; ii) increasing P for possible improvements in egg production and bird performance; and iii) adjusting P levels in line with Ca level to maintain an optimum Ca:npP ratio.

1.2 Interactions between Ca and P and consequences for availability

The aforementioned requirements for Ca and P by the laying hen must be considered in tandem due to the tendency for Ca-phytate-P complexes, which impede the digestibility and hence availability of Ca and to a greater extent P. Our understanding of Ca-phytate chemistry is advanced (Waldenstedt 2006; Selle et al. 2009; Wilkinson et al. 2011) and will be reviewed only briefly here. The main storage form of P in cereal grains is as phytate (*myo*-inositol hexaphosphate; IP_6), a polyanionic compound that is largely recalcitrant to endogenous enzyme hydrolysis. The polyanionic properties of phytate result in a propensity for complex-forming with minerals including Ca and also proteins and lipids, and thus may inhibit the availability of these nutrients to the bird also. In the hen, the availability of phytate-P and other nutrients is potentially further hindered by the high levels of dietary Ca required to support optimum egg production and physiological Ca homeostasis. Phytate-P hydrolysis is thwarted as the ratio of Ca:phytate-P increases, as demonstrated *in vitro* by Grynspan and Cheryan (1983). The poor solubility of phytate-P is largely attributed to the high lumen cation concentration of Ca used in poultry diets, which causes the formation of insoluble Ca-phytate complexes (Maenz et al. 1999; Angel et al. 2002). Hurwitz and Bar (1965) showed this relationship rather elegantly using a radioactive tracer to demonstrate reduced phosphorus absorption in the small intestine of laying hens in response to increasing dietary Ca levels. In this study, the ratio of Ca to total P was 5.65:1 and 3:1

for the high and low dietary Ca treatments respectively.

Therefore the requirements of either Ca or P must be considered in tandem rather than separately. For this reason, a ratio of dietary Ca:non-phytate phosphorus (npP) is a useful metric to describe the relationship of the two minerals.

1.3 Exogenous phytase

The inclusion of exogenous phytase to hydrolyse phytate-P is now a frequent feature of monogastric diets, particularly for broilers (Selle et al. 2009). The addition of exogenous phytase to poultry diets improves dietary P retention, along with performance parameters when added to diets with low P concentrations (Ravindran et al. 2000; Tamim & Angel 2003; Tamim et al. 2004). These improvements are due to phytase liberating P from phytate-P (Selle et al. 2011; Walk et al. 2012a). The ability for almost complete phytate hydrolysis has been demonstrated in broilers using dietary phytase. Manangi and Coon (2008) showed in broilers that dietary phytase up to 5000 FTU/kg diet resulted in complete phytate destruction although maximum P retention was achieved at 1000 FTU/kg diet. While the response to phytase has shown clear benefits in broilers, there are less data available for layers. Marounek et al. (2008) report some phytase activity in adult hens, which increases as they age, however, it is mostly confined to the caecum where opportunities for absorption are lost. The available studies suggest promising responses of mineral digestibility to phytase inclusion (Silversides & Hruby 2009; Liu et al. 2007) with improvements in production traits reported in multiple studies (Francesch et al. 2005; Lim et al. 2003; Liu et al. 2007).

Other studies have reported less promising results on the benefits of phytase in laying hens. Usayran and Balnave (1995) reported that the addition of a dietary phytase (500 IU/g diet) had an adverse impact on egg production and egg quality. Similarly, in a report prepared for Australian Eggs Limited (Li, Zhang & Bryden 2016; 1UQ101A), the researchers showed that laying hens were not P limiting at 1.5 g npP per kg of diet, and that addition of phytase did not improve bird performance and egg production. Importantly in the context of this literature review, in addition to liberating P from phytate, phytase may also increase Ca digestibility. Jalal and Scheideler (2001) showed that, although dependent on source, phytase could markedly increase Ca digestibility in hens 45 and 60 weeks old. This suggests that phytase may contribute to Ca homeostasis while the potential role of phytase in influencing a Ca-specific appetite is unknown.

1.3.1 Response surface methodology to assist identifying optimal dietary Ca and P levels

Due to the aforementioned interactions between dietary Ca and P, it is often necessary to resort to an experimental design comprised of varying factors, i.e. different levels of dietary Ca and P within a series of diets. This experimental design approach is in an attempt to capture both the response to absolute levels of dietary Ca and P and also the ratio between the two minerals. These factorial designs are very useful, but it becomes difficult to interpret and facilitate selection of the optimum Ca and P levels to choose. Response surface methodology is an approach that greatly improves this data visualisation process, and permits easier interpretation of complex experimental designs that incorporate various levels of Ca and P, and possibly phytase as well. For example, as can be viewed in Figure 1 (adapted from Roush et al. 1986), the optimum eggshell percentage was achieved with a dietary inclusion rate of 4.73% dietary calcium and 0.48% non-phytate phosphorus.

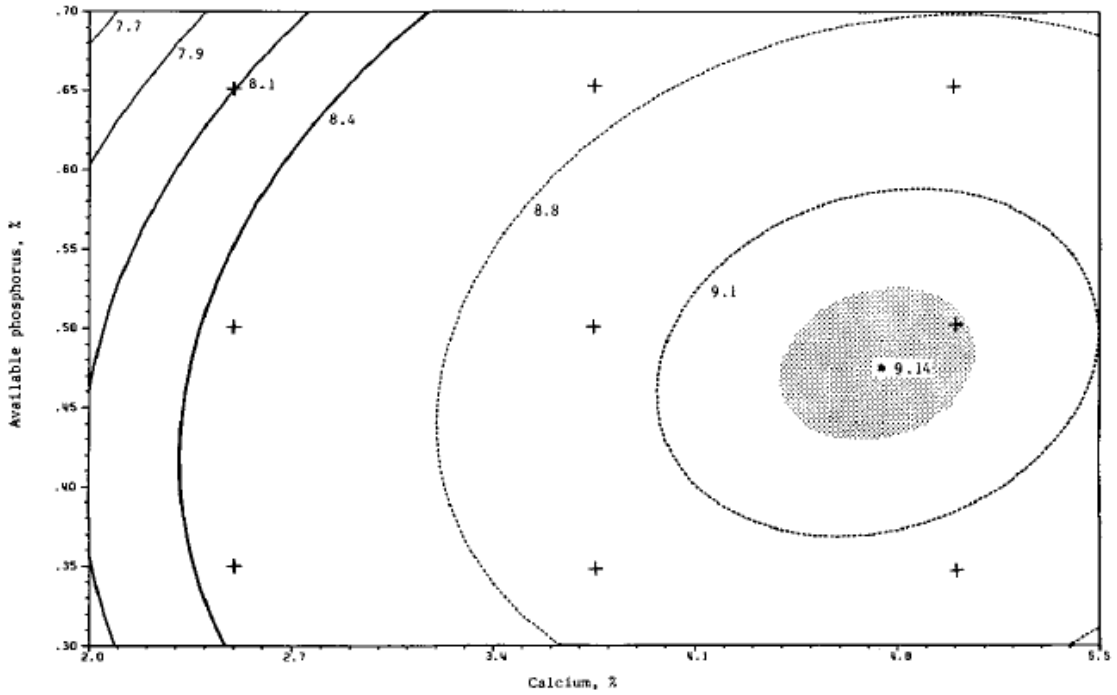

Figure 1.1 Two dimensional response surfaces for eggshell percentage with varying levels of Ca and npP

The experimental design points are defined as crosses.
The shaded oval area represents a 95% confidence area for the optimal calcium**,** and available phosphorus levels are the predicted optimal yield (indicated by *).
Adapted from Roush et al. (1986).

1.4 Choice feeding concept

Conventionally it is believed that the predominantly plant-derived phytate-P complex is not significantly hydrolysed by poultry due to the absence of endogenous phytase activity in the avian gastrointestinal tract (Nelson 1976). However, it has been shown that when dietary Ca is kept to a minimum, phytate-P utilisation is significantly improved (Taylor 1965). Tammin et al. (2004) at the University of Maryland have shown that the addition of 0.5% Ca to a broiler diet without phytase, reduces the birds' endogenous capacity to hydrolyse phytate-P from around 70% to 30%. Furthermore, Marounek et al. (2008) demonstrate endogenous phytase activities, albeit predominantly in the caeca, in laying hens throughout the gastrointestinal tract, a finding that was observed to be heightened in older birds (47 weeks of age). More recently, Morgan et al. (2015) reported considerable pre-caecal phytate hydrolysis in mash-fed broilers due to a combination of intestinal and plant-derived phytase. This observation suggests that poultry do have some capacity to hydrolyse phytate-P and the calcium-led reduction in the solubility of phytate-P reflects the chelation and precipitation of the two as a Ca-'phytin' salt. In addition to complex forming with phytate-P, dietary Ca also has a significant acid binding capacity (Lawlor et al. 2005) and may prevent the young chick, pullet or adult bird from satisfactorily acidifying the diet in the gastric gut, compromising protein digestibility (Grynspan & Cheryan 1989). It is important to note that the improved digestibility of phytate-P and other nutrients in poultry diets is thought to be the result of the physical separation of Ca and phytate, and not simply that of feeding reduced amounts of Ca. Thus, though Ca is necessary for optimal performance and bone strength, Ca addition (notably as limestone) is contrary to the concept of maximising nutrient digestibility and minimal feed conversion ratio (FCR).

Some early development of regulating the intake of calcium and 'metering' calcium digestion and absorption in the gastrointestinal tract has an origin in influencing the particle size of Ca within diet. For example, Scott et al. (1971) showed that replacing limestone with oyster shell improved eggshell strength and numerically improved other production parameters. The concept of 'choice feeding' in layers is equally not recent (Cowan et al. 1978), while a specific appetite for calcium was reported in hens by Taher at al. (1984). However, while current egg production systems rely on a homogenous uniform feed to achieve targeted nutrient intake, choice feeding and physical separation of calcium have recently regained interest, particularly in broilers (Wilkinson et al. 2011).

Therefore, layer hens have been found to regulate nutrient intake through choice feeding and to possess a Ca-specific appetite. Presumably this originates from the history of modern fowl, as historically they would not have consumed a nutritionally complete diet in one bolus (as modern layer hens do) but would rather have foraged, consuming seeds, roots, insects and soils/silicates to attain nutritional targets. Importantly, the intake of phytate-P (in seeds) would have been quite separate in temporal-spatial terms from the intake of calcium (as soils, clays and silicates). This may explain why poultry are able to digest phytate-P in a low Ca environment, as most of the P requirement of poultry may actually be met by the consumption of phytate-P. The poor (<20%) retention of phytate-P by modern layers may be an artefact of the concerted provision of Ca as limestone or dicalcium phosphate with the vegetable component of the diet.

Wood-Gush and Kare (1966) were perhaps the first to demonstrate that Ca-deficient chickens preferred and would seek out feed containing high levels of Ca compared to Ca-deficient feed when given free choice in food selection. This specific appetite for Ca, was later confirmed by Hughes and Wood-Gush (1971) and Joshua and Mueller (1979), both validating that, when given the choice to self-regulate their nutrient intake, poultry were able to meet their nutritional Ca requirements. The modern laying hen has a greatly amplified capacity to produce egg mass and so is phenotypically quite distinct from these earlier birds. It follows that the nutrient requirements and appetite for nutrients including Ca may also be amplified in order to satisfy egg synthesis requirements. There is a scant recent literature available on choice feeding in laying hens as this pertains to Ca. Furthermore, the return of free range systems provides opportunities for birds to engage in more foraging behaviour including Ca sources than was previously facilitated under caged production systems.

2 The effect of graded concentrations of dietary calcium offered with a separate free choice calcium source on laying hen performance and egg quality

2.1 Experiment 1 – key findings

This chapter is largely based on a paper written by Emma Bradbury for her PhD thesis. In part the work has been revised in keeping with the context of the entire project.

Hypotheses tested

The hypothesis of this study was that laying hens provided with suboptimal levels of dietary calcium will stimulate a calcium-specific appetite and encourage consumption of limestone grit.

The objective of the study was to evaluate whether consumption of a low calcium diet supplemented with limestone grit would maintain egg production and quality, and improve nutrient digestibility relative to a high calcium diet.

Key findings in relation to separate source calcium intake, hen productivity and egg quality

Reducing dietary Ca concentrations from formulated 40-10 g/kg in a complete ration resulted in increased consumption of a separate source of Ca. However, providing a separate source of Ca to birds offered 40-10 g/kg dietary Ca was not sufficient to maintain egg production or improve nutrient digestibility. Consumption of the separate Ca source was not observed to be a uniform practice regardless of dietary Ca level. Birds offered 30 g/kg dietary Ca and a separate Ca source had comparable productivity and egg quality with birds offered 40 g/kg dietary Ca and a separate Ca source.

Implications

In this study, consumption of a separate calcium source was practised by some but not all birds, regardless of the dietary Ca level. This suggests the practice of calcium consumption may be a learned behaviour in addition to a physiological need and appetite for calcium. In the presence of a separate calcium source, increasing dietary Ca from 30 to 40 g/kg had no impact on hen productivity or egg quality.

2.2 The effect of graded concentrations of dietary calcium offered with a separate free-choice calcium source on laying hen performance and egg quality

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Abstract

Modern laying hen diets typically contain at least 40 g/kg calcium (Ca) to sustain optimum biological processes, skeletal integrity and eggshell production. The high level of dietary Ca may impede the optimum digestion of phosphorus (P) and other minerals. Previous studies have demonstrated the ability of poultry to self-select their own diets in order to meet their nutritional requirements. Poultry are known to possess a specific appetite for Ca and will seek feedstuffs with a high Ca concentration when the diet is deficient. To confirm that contemporary layers maintain a Ca-specific appetite and assess the effect of separate Ca feeding on egg production, animal performance and mineral retention, a total of 80 Isa Brown laying hens were allocated to one of four dietary treatments each with 20 replicates consisting of an individual bird. Dietary treatments were formulated to one of four levels of total dietary Ca (10, 20, 30 and 40 g/kg) in conjunction with *ad libitum* access to a separate Ca source. Birds that were fed high dietary Ca ate significantly less separate Ca than those on low Ca diets. Birds that received the diet with 10 g/kg total dietary Ca laid significantly fewer eggs than birds receiving 40 g/kg total dietary Ca. Dry eggshell weight and eggshell thickness were significantly lower in birds receiving 10 g/kg total dietary Ca than birds that received 30 g/kg total dietary Ca. The results of the study confirm that Ca-specific appetite is present in laying hens, however, it may not be uniformly expressed.

2.3 Introduction

Calcium is an essential nutrient that is involved in many biological processes including bone development, nerve conduction and eggshell formation (Wilkinson et al. 2011; De Vries et al. 2010). Due to the high Ca requirements of layers for egg production, Ca makes up approximately 4% of the total feed volume. Insufficient Ca is provided from the cereal grains present in the diet, therefore the diet is supplemented with Ca to meet the bird's requirements. Dietary Ca is provided as either a calcium carbonate grit or flour, such as limestone, and meat and bone meal where permitted (Selle et al. 2009) with a small proportion provided by the inorganic phosphorus (P) source. The addition of limestone can result in a reduction in the digestibility of key nutrients in the diet, in particular phytate-P (Selle et al. 2009).

Commercial poultry diets are typically corn- and/or wheat-soy based and contain relatively high concentrations of phytate-P, which has limited availability for poultry (Cowieson et al. 2004; Selle et al. 2009). Phytate carries a strong negative charge and, due to the high inclusion levels of Ca in the diet, the Ca^{2+} is the dominant cation that chelates with phytate forming insoluble Ca-phytate complexes (Selle et al. 2009). The formation of Ca-phytate complexes reduces the bioavailability of both Ca and P, negatively affecting skeletal health and egg quality. Decreasing the amount of Ca in layer diets would improve growth performance and P digestibility, however, this can lead to a reduction in eggshell quality and skeletal integrity and increase osteomalacia (Whitehead 2004; Wilkinson et al. 2011). Physical and temporal separation of Ca from phytate may be necessary to

improve phytate-P digestibility and other nutrients whilst maintaining egg production and skeletal health.

Richter and Eckert (1937) showed that parathyroidectomised rats had an increased intake of Ca lactate solution and actively sought out Ca-rich feedstuff. Wood-Gush and Kare (1966) showed Ca-deficient chickens preferred a high Ca food compared to a Ca deficient food when given free choice, demonstrating that laying hens possessed a specific appetite for Ca. Hughes and Wood-Gush (1971) and Joshua and Mueller (1979) further confirmed this finding. This specific appetite is thought to originate from the ancestral Red Jungle Fowl, whereby the bird would have foraged diverse nutrient sources, consuming seeds, roots, insects and soil/silicates to satisfy nutritional requirements.

Commercially, laying hens are fed a single mixed diet that is formulated to meet all of the nutritional requirements of the bird to support optimum performance. A single mixed diet enables birds to only exercise their appetite for energy (Henuk & Dingle 2002). This can result in some laying hens having to rely on their medullary bone reserves, leading to a decrease in egg production, egg quality and osteoporosis (Webster 2004). The ability to exploit the Ca-specific appetite of laying hens may facilitate the Ca component to be reduced in the diet and allow the birds to consume Ca to their individual requirements and circadian rhythm (Taylor et al. 2013). Allowing birds to self-regulate their Ca intake may also aid and increase the digestion of P and phytate-P. This study investigated whether the Ca-specific appetite of layers may be used to feed Ca separately to improve nutrient digestibility, and its effect on bird performance, egg production and egg quality.

2.4 Materials and methods

2.4.1 Animals and housing

All birds were housed at the Poultry Research Foundation at The University of Sydney, Camden Campus. All experimental procedures conducted had approval from The University of Sydney Animal Ethics Committee and were in accordance with the Australian Code for the care and use of animals for scientific purposes (National Health and Medical Research Council 2004).

A total of 80 Isa Brown laying hens approximately 42 weeks of age were randomly allocated to one of four dietary treatments in a randomised blocked design. Each dietary treatment was replicated 20 times, with one replicate classified as a single bird and cage. Birds were provided with feed, water and the separate Ca source (limestone grit; 1.4 mm AB Grit, Omya, Australia) *ad libitum* throughout the study. Birds were housed in individual cages (23 cm W x 45 cm D x 45 cm H) in an environmentally controlled caged production system shed through the use of evaporative cooling pads and fans, and were exposed to a lighting regime of 16 hr:8 hr (light:dark). To ensure birds were habituated to their environment, birds were placed into the experimental cages two weeks prior to the commencement of the study.

2.4.2 Dietary treatments and experimental procedures

During the habituation period, birds were fed a wheat-soy diet, which provided 11.72 MJ/kg AME, 150 g/kg CP, 40 g/kg Ca and 2.5 g/kg npP. The compositions of the experimental diets are shown in Table 2.1. Experimental mash diets based on wheat-soy were formulated to meet or exceed the nutrient requirements of Isa Brown laying hens, with the exception of dietary Ca (NRC 1994). Experimental diets were formulated to contain one of four levels of dietary Ca: 10, 20, 30 or 40 g/kg. Birds also had access to a separate Ca source, approximately 95% CaCO3 with an average particle size of 1.4 mm (AB Grit, Omya, Australia). Experimental diets were fed for six weeks.

At the commencement and end of the study all birds were individually weighed, and a blood sample collected to measure plasma Ca and P concentrations. Approximately 3 ml of blood was collected from the brachial vein of each bird. Samples were refrigerated overnight before being spun to separate the plasma. Plasma samples were then analysed for Ca and P levels. Feed intake, separate Ca source intake and egg production were recorded daily.

Egg weight, albumen height, dry eggshell weight, eggshell thickness and Haugh units were measured three times per week following the procedures as outlined by Roberts (2004). At the time of egg collection, any egg abnormalities were recorded on the collection sheet. Egg quality parameters were analysed using treatment averages. On days 28-30 of the study, a 48 hr total collection procedure was undertaken for three out of the six replicate groups whereby the feed input and faecal output were measured from individual birds to determine energy, nitrogen and mineral retention. Collection trays were placed under each of the cages of individual birds. Excreta samples were dried in an oven at 100°C until a consistent weight was achieved.

Table 2.1 Ingredient and nutrient specifications (as fed g/kg) of the experimental diets fed to Isa Brown laying hens

	Calcium concentration (g/kg) of the mixed diet			
	10	**20**	**30**	**40**
Composition				
Wheat	562.7	573.3	584.0	594.6
Canola meal	50.0	50	50.0	50.0
Soybean meal	67.3	83.9	100.6	117.2
Wheat bran	250.0	192.9	135.8	78.7
Vegetable oil	39.4	42.9	46.5	50.0
Salt	1.3	1.3	1.4	1.4
Sodium bicarbonate	2.1	2.1	2.0	2.0
DL-Methionine	0.7	0.7	0.8	0.8
Lysine HCL	0.8	0.6	0.4	0.2
Limestone	19.7	45.8	72.0	98.1
Dicalcium phosphate	3.8	4.2	4.7	5.1
Vitamin/Mineral Premix[a]	2.0	2.0	2.0	2.0
Calculated values (g/kg)				
Apparent Metabolisable energy (MJ/kg)	11.72	11.72	11.72	11.72
Crude protein (N x 6.25)	150.0	150.0	150.0	150.0
Calcium	10.0	20.0	30.0	40.0
Available phosphorus	2.5	2.5	2.5	2.5
Methionine+ Cystine	6.2	6.2	6.2	6.2
Lysine	6.9	6.9	6.9	6.9
Sodium	1.5	1.5	1.5	1.5
Chlorine	2.0	2.0	2.0	2.0
DEB meq/kg	182	182	182	182
Analysed values (g/kg)				
GE (MJ/kg)	16.81	16.64	16.11	16.04
Crude protein (N x 6.25)	148.1	150.0	156.7	155.5
Calcium	9.3	15.8	27.8	32.0
Phosphorus	4.7	4.5	4.5	4.0

DEB = dietary electrolyte balance.
GE = gross energy.

[a] Provided the following nutrients per kilogram of diet: vitamin A, 10 000 IU; vitamin D, 2500 IU; vitamin E, 25 mg; vitamin K, 2.5mg; thiamine, 2.5 mg; riboflavin, 5.0mg; pyridoxine, 3.5 mg; vitamin B12, 0.015 mg; niacin, 0.0mg; pantothenic acid, 9mg; folic acid, 1.0mg; biotin, 0.10mg; Fe, 60.0mg; Zn, 60.0mg; Mn, 50.0mg; Cu, 5.0 mg; I, 1.0mg;Co, 0.4mg;Mo,0.5mg;Se, 0.2mg; apo-ester, 2.9mg; canthaxanthin, 3.1 mg; ethoxyquin, 25.0mg. Amino acid values are calculated on a standardised ileal digestible basis.

2.4.3 Chemical analysis

Gross energy of the diets was determined using an adiabatic bomb calorimeter (Parr Instrument Company, Moline, Il, USA) and standardised with benzoic acid. Nitrogen content of diets was determined using a FP-428 nitrogen analyser (LECO Corporation, St. Joseph, MI, USA) as described by Sweeny (1989). Samples were wet acid digested using nitric acid and hydrogen peroxide (Peters et al. 2003) prior to the determination of mineral concentration by inductively Coupled Plasma-Optical Emission Spectroscopy using a PerkinElmer OPTIMA 7300 (PerkinElmer Inc, Waltham, MA, USA).

The calculations for the retention values for the specific minerals were calculated as (intake−output)/intake where *intake* represents total gross energy, DM, N and mineral quantity consumed, and *output* is the total gross energy, DM, N and mineral excretion over the 48 hr collection period.

2.4.4 Blood sample analysis

After collection into Vacutainers (SST II Advance BD Vacutainer, BD North Ryde, Australia) blood samples were refrigerated overnight. The tubes were then centrifuged at 1455 x g for 10 minutes at 4°C in a Beckman Coulter Allegra® X-15R. The serum was then transferred into serum tubes for analysis of serum Ca and P using a colourimetric method of analysis with a Konelab 20 XTi (thermo Electron). A direct UV method without reduction was applied in assessing P concentration. The procedure used to assess P concentration was based on the method first describes by Daly and Ertinghausen (1971). Calcium concentration was analysed using the Ca reagent, Arsenazo III.

2.4.5 Statistical analysis

Bird performance and egg production data were analysed using REML analysis of linear mixed models in GenStat (14th Edition, VSN International). The model of analysis included day of measurement and dietary treatment, and their interaction was fitted as fixed effects. Differences were considered significant when $P < 0.05$. If significance was determined, a Tukey's HSD was performed to differentiate between dietary treatments.

2.5 Results

2.5.1 Bird performance

Feed intake increased across dietary treatments each week over the experimental period (Figure 2.1). Birds that were fed diets containing 10 g/kg dietary Ca consumed less feed than all other dietary treatments ($P < 0.05$, Table 2.2) over the six-week experimental period. Birds that were fed diets containing 30 g/kg dietary Ca had the highest feed intake and consumed more feed than birds that were fed diets containing 40 g/kg dietary Ca ($P < 0.05$). Separate Ca intake decreased with increasing dietary Ca concentration ($P < 0.05$). Birds that were fed diets containing 20 g/kg dietary Ca had the highest intake of the separate Ca source. Birds fed 40 g/kg dietary Ca consumed less of the separate Ca than all other treatment groups ($P < 0.05$). The amount of separate Ca consumed as a proportion of total Ca intake of the birds decreased with increasing dietary Ca concentration ($P < 0.001$). Bird body weight (BW) was not influenced by the dietary treatments, however, the birds were all lighter at the conclusion of the study. Birds that were fed diets containing 10 g/kg dietary Ca had the highest BW loss, with an average of 172 g per bird over the experimental period ($P = 0.08$). Feed conversion ratio (FCR, calculated per unit egg mass) was highest for birds fed diets containing 10 g/kg ($P < 0.05$)

compared to all other dietary treatments. Birds that were fed diets containing 30 g/kg dietary Ca had the lowest FCR over the experimental period (P = 0.002).

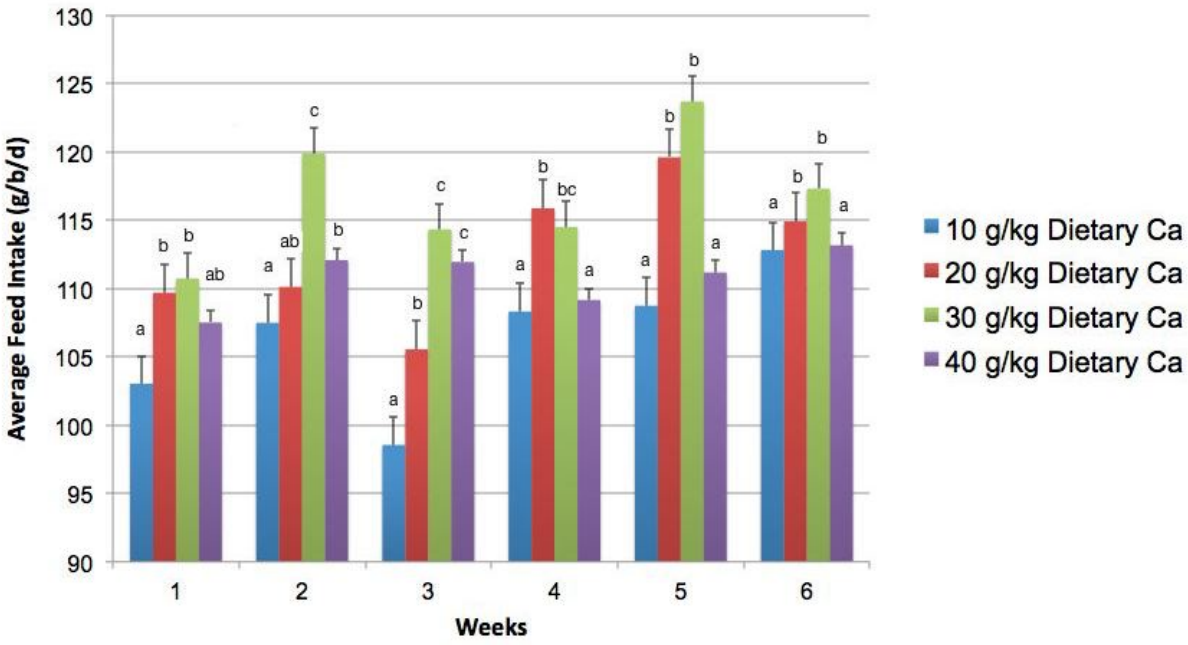

Figure 2.1 Weekly feed intake (g/b/d) for mixed diet fed to Isa Brown laying hens over the six-week experimental period
Values with different letters (a-c) are significantly different.

Table 2.2 The effect of dietary calcium (Ca) concentration on the intake of a separate Ca source, total Ca intake and Isa Brown laying hen performance over the six-week experimental period

	Ca concentration (g/kg) of mixed ration				SEM[A]	*P-Value*
	10	**20**	**30**	**40**		
Average mixed diet intake (g/b/d)	106.5[a]	112.6[b]	116.8[c]	110.9[b]	1.1	*<0.001*
Separate Ca source intake (g/b/d)	46.9[b]	58.7[b]	50.26[b]	28.2[a]	3.4	*0.005*
Total feed intake [B]	153.4[b]	171.4[c]	167.1[bc]	139.1[ab]	3.6	*0.001*
Total Ca intake (g/b/d)[C]	18.9[bc]	24.6[c]	22.6[c]	15.2[ab]	1.2	*0.02*
Ca intake as a proportion of total feed intake (%)	12.1	14.2	13.4	10.9	0.5	*0.062*
Separate Ca intake as a proportion of total Ca intake (%)	93.8[a]	90.4[b]	83.9[c]	70.3[d]	0.6	*<0.001*
FCR[D]	2.36[a]	2.01[b]	1.97[b]	1.84[b]	0.05	*0.002*
Starting body weight (g)	2325	2294	2349	2213	26.1	*0.278*
End body weight (g)	2153	2184	2257	2143	27.4	*0.465*
Body weight loss (g)	172	110	92	70	14.6	*0.08*

[A] Pooled standard error of mean of separate Ca feeding treatments.
[B] Combined mixed diet and separate Ca source intake.
[C] Combined Ca from mixed diet and Ca concentration of separate Ca source.
[D] Feed conversion ratio (FCR) calculated per unit egg mass.
Within rows, values with different letters are statistically different, $P < 0.05$.

The retention values for minerals are presented in Table 2.3. No effect of dietary treatments was observed for Ca retention (P = 0.095) or P retention (P = 0.970). Birds that were fed diets containing 10 g/kg and 20 g/kg dietary Ca had lower retention of Na compared to birds fed diets containing 30 g/kg and 40 g/kg dietary Ca (P < 0.05). Birds that were fed 30 g/kg dietary Ca were observed to have a lower Na retention than birds fed diets containing 40 g/kg dietary Ca (P < 0.05). Birds fed diets containing 40 g/kg dietary Ca had the highest retention of Na. Birds fed 10 g/kg dietary Ca had the lowest retention of Cu compared to all other dietary treatments (P < 0.05), while birds fed diets containing 40 g/kg dietary Ca had the highest retention of Cu. The retention of Zn increased with increasing dietary Ca concentration (P = 0.006). Birds receiving diets containing 10 g/kg Ca had the lowest retention of Zn compared to the other dietary treatments (P < 0.05). Birds that were fed diets containing 40 g/kg dietary Ca had the highest retention of Zn. There was no dietary effect on the retention of Mg, K, Fe, Mn or Sr (P > 0.05).

Blood plasma analysis (data not shown) showed no difference in blood plasma Ca and P between dietary treatments at the commencement of the trial (P = 0.355 and P = 0.996, respectively). Blood plasma analysis from the end of the trial showed no difference in plasma P concentrations between dietary treatments (P = 0.125). At the end of the trial, birds that were fed diets containing 10 g/kg dietary Ca had a lower Ca blood plasma concentration than birds that were fed diets containing 30 g/kg and 40 g/kg dietary Ca (P < 0.01).

Table 2.3 Mineral retention (coefficient) for Isa Brown laying hens offered a separate source of Ca

	Ca concentration (g/kg) of mixed diet				SEM[A]	*P-Value*
	10	**20**	**30**	**40**		
Calcium	0.535	0.473	0.278	0.548	0.031	*0.095*
Phosphorus	0.354	0.367	0.412	0.395	0.020	*0.970*
Magnesium	0.193	0.178	0.162	0.236	0.123	*0.979*
Sodium	0.349^{a}	0.444^{a}	0.629^{b}	0.746^{c}	0.060	*<0.001*
Potassium	0.274	0.292	0.370	0.334	0.020	*0.392*
Iron	-0.099	-0.033	-0.036	0.147	0.053	*0.316*
Copper	0.161^{a}	0.266^{b}	0.373^{b}	0.386^{b}	0.021	*<0.001*
Manganese	0.075	0.175	0.048	0.163	0.041	*0.700*
Zinc	-0.489^{a}	-0.032^{b}	0.027^{b}	0.089^{b}	0.040	*0.006*
Strontium	0.476	0.542	0.418	0.564	0.073	*0.844*

[A] Pooled standard error of mean of separate Ca feeding treatments.
Within rows, values with different letters are statistically different, P < 0.05.

2.5.2 Egg production and quality

Egg production increased with increasing dietary Ca, and birds that were fed 10 g/kg dietary Ca laid significantly less eggs than birds fed other dietary treatments (P < 0.001, Table 2.4). Eggshells from birds fed 10 g/kg dietary Ca were significantly thinner than those from birds fed diets containing 30 and 40 g/kg dietary Ca. Dry eggshell weight was lowest in birds fed 10 g/kg dietary Ca when compared to the other dietary treatments (P < 0.05). No effect of dietary treatment was observed on egg weight, albumen height or Haugh unit (P > 0.05). Hen day production (HDP, Table 2.5) increased with increasing dietary Ca (P < 0.001). Birds that were fed 10g/kg dietary Ca had a significantly lower HDP than birds fed other dietary treatments (P < 0.05). Increasing dietary Ca from 20 g/kg to 40 g/kg significantly increased HDP (P < 0.05).

The total number of egg abnormalities was highest in birds that were fed 10 g/kg dietary Ca (P = 0.029, Table 2.5). Birds that were fed 40 g/kg dietary Ca had significantly less abnormalities when compared

to birds fed 10 g/kg dietary Ca. Birds that fed 10 g/kg dietary Ca had the highest number of cracked eggs when compared to the other dietary treatment groups. Dirty eggshells were highest in birds that were fed 30 g/kg dietary Ca, while birds that were fed 20 g/kg dietary Ca had the least incidence of dirty eggshells (P = 0.025). No significant difference was found for the number of soft shell eggs and broken eggs between dietary treatment groups (P > 0.05).

Table 2.4 Egg production of Isa Brown laying hens, taken as dietary treatment averages over the six-week experimental period

	Ca concentration (g/kg) of mixed ration				SEM	*P*-Value
	10	20	30	40		
Average egg production per bird (n)	30[a]	38[b]	40[b]	41[b]	0.8	*<0.001*
Hen day production (%)[A]	72.9[a]	89.6[b]	94.2[bc]	96.8[c]	3.8	*<0.001*
Egg weight (g)	61	63	63	62	0.75	*0.838*
Egg mass (g)	45[a]	56[b]	59[bc]	60[c]	1.25	*0.001*
Albumen height	7.67	7.35	6.85	7.25	0.13	*0.187*
Dry eggshell weight (g)	5.4[a]	5.8[ab]	6.3[b]	6.1[b]	0.09	*0.004*
Dry eggshell thickness (mm)	0.35[a]	0.37[b]	0.40[b]	0.39[b]	0.01	*<0.001*
Haugh unit[B]	86	84	80	84	1.0	*0.115*

[A] Calculated as total daily eggs/number of live birds.

[B] Haugh unit over 72 is desirable as the highest quality grade. Haugh unit calculated using the formula: HU = 100*log (h – 1.7$w^{0.37}$ + 7.6), where h = observed height of albumen in millimetres, w = weight of the egg in grams.

Within rows data with different superscripts are statistically different, P < 0.05.

Table 2.5 Egg abnormalities for egg produced by Isa Brown laying hens fed dietary treatments over the six-week experimental period

	Ca concentration (g/kg) of mixed ration				SEM	*P*-Value
	10	20	30	40		
Total eggs laid (n)	582[a]	753[b]	752[b]	813[b]	0.8	*<0.001*
Total abnormalities (n)[A]	102[a]	74[ab]	81[ab]	42[b]	0.4	*0.029*
Percentage abnormalities[B]	17.5[a]	9.8[b]	10.8[b]	5.1[b]	1.4	*<0.001*
Cracked eggs (n)	47[a]	23[b]	13[b]	3[b]	0.2	*<0.001*
Dirty eggs (n)	12[b]	3[a]	27[b]	11[b]	0.1	*0.025*
Soft shell eggs (n)	9	4	2	1	0.1	*0.182*
Broken eggs (n)	6	4	2	1	0.1	*0.614*

[A] Defined as the number of eggs with eggshell quality problems.

[B] Defined as the total number of abnormalities divided by the total number of eggs laid.

Within rows data with different superscripts are statistically different, P < 0.05.

2.6 Discussion

Calcium is a critical mineral for egg formation in laying hens but may interfere with nutrient digestibility due to a high buffering capacity (Lawlor et al. 2005) and interaction with phytate-P (Grynspan et al. 1983). The hypothesis of this study was that providing a separate Ca source in the form of grit to layers may allow optimum Ca intake to be achieved while also improving retention of other minerals. Poultry have been shown to possess a specific appetite for Ca (Wood-Gush & Kare 1966; Hughes & Wood-Gush 1971; Joshua & Mueller 1979) and the results of this study are in general agreement with this. With increasing dietary Ca birds consumed less of the separate Ca source. This is consistent with the findings of Griminger and Lutz (1964) who showed birds consumed less supplemental Ca when fed high Ca diets (30 g/kg dietary Ca), while birds had a high intake of supplemental Ca when fed low Ca diets (10 g/kg dietary Ca). These results are also consistent with the findings of Taher et al. (1984) who noted that birds in their study responded to the weekly decrease in dietary Ca by increasing their Ca intake from the free choice supplement. In this study, birds that were fed 10 g/kg dietary Ca consumed the least amount of feed, which is consistent with the findings of Summers et al. (1976) who reported that low level Ca (15 g/kg) resulted in a significant reduction in production and feed intake. Taylor (1970) reported that hens increased feed intake on egg formation days and attributed this to the need for Ca. No such trend was found in this study. The results of the present study show birds fed 30 g/kg dietary Ca consumed the most feed although this could not be attributed to egg forming days.

The results from the present study also show that some birds when fed low Ca diets did not adapt to the separate Ca feeding, particularly those fed 10 g/kg dietary Ca, and it is possible that 10 g/kg dietary Ca was too low in the context of this study. Roland et al. (1973) showed that birds fed Ca deficient diets (5 g/kg dietary Ca) had significantly reduced feed intake within 24 hours of being placed on the Ca deficient diet. The drop in feed consumption was also reflected in a decrease in body weight. This is consistent with the findings of the present study for birds fed 10 g/kg dietary Ca. One bird in the 10 g/kg dietary Ca treatment group was observed to consume its own eggshell each day to obtain the required Ca. Similarly, Salim (1981) observed that some individual hens refused to eat a separate Ca supplement even when the dietary Ca was as low as 5 g/kg. These hens were found to completely stop egg production. A similar result was observed in this study; although no hen completely stopped laying, there was a significant reduction in egg production for birds fed 10 g/kg and 20 g/kg dietary Ca. Salim (1981) attributed this stop in production to a sudden switch from total reliance on dietary Ca to a free choice supplement.

The mechanisms that control the Ca-specific appetite remain uncertain. Animals are thought to respond to excess, deficits or imbalances of nutrients (Villalba et al. 2006; Forbes 2007). However, there is debate as to whether the Ca appetite in poultry is innate or a learned behaviour. Hughes and Wood-Gush (1971) concluded that the Ca-specific appetite in broilers is a learned preference, reinforced by effects post-ingestion. The authors of that study found broilers were able to distinguish between deficient and supplement diets through visual and gustatory cues. Joshua and Mueller (1979) observed in one study that only two out of eight broilers were able to adapt to a separate Ca feeding regime, when fed Ca deficient diets when housed in individual cages. Due to the ability for laying hens to quickly adapt to separate Ca feeding, Hughes (1979) proposed that the Ca-specific appetite in layers is predominantly an innate behaviour in conjunction with some ‘fine tuning’ learning. Holcombe et al. (1975) observed that laying hens did not respond to gustatory cues for Ca supplemented food. In the present study, birds did not receive any visual cues between feeders and the separate Ca feeder, supporting the theory of being an innate behaviour. However, this does not explain why some birds did not adapt to the separate Ca feeding regime. Overall, 25 birds across the dietary treatments were unable to adapt to the separate Ca feeding regime. Birds that were fed 30 g/kg dietary Ca were able to maintain egg production whilst separate Ca feeding, with only three birds in the treatment group

unable to adapt, compared to eight birds in the 10 g/kg dietary Ca treatment. Further investigation is required into why some of the birds were unable to adapt to a separate Ca feeding regime.

A potential benefit of providing a separate source of Ca is placing distance between Ca and other nutrients in the lumen, and thus altering the buffering and chelating impact of Ca on nutrient digestion (Lawlor et al. 2005; Grynspan & Cheryan. 1983). Further, Ca particle size plays a role in Ca solubility in the gut, and therefore the subsequent retention of this mineral (Anwar et al. 2016; Anwar et al. 2017). The absorption and secretion of Ca by the different segments of the intestinal tract in laying hens is dependent on the stage of eggshell formation (Waddington et al. 1989). There was no effect of Ca inclusion rate on the retention of Ca in this study although birds offered the 30 g/kg Ca treatment tended to have the lowest Ca retention. The highest plasma Ca level was found in birds offered diets containing 40g/kg. As plasma Ca is greatest during egg production (Winget & Smith 1958) this observation coincides with the higher egg output and egg production rate of this treatment group. While the contribution of Ca from the separate Ca source was lowest for the 40g/kg Ca group, it was still substantial at 70% of total Ca intake, and therefore it may be assumed contributed meaningfully to the egg production performance of this group. Interestingly, the retention of copper, sodium and zinc was greatest for the birds offered 40g/kg Ca, which is in conflict with the hypothesis of greater digestibility at lower dietary Ca.

However, as this group had the lowest overall total Ca intake, this may have had a bearing on the availability and retention of other minerals. In possible support of this observation, the level of egg normalities as a proportion of total eggs produced was improved with increasing dietary Ca levels.

Calcium makes up approximately 40% of the eggshell therefore shell deposition and quality are directly related to the Ca level in the diet (Keshavarz 1996). The results of this study show that increasing dietary Ca increased egg production and eggshell quality. These findings are in agreement with Abdallah et al. (1993) and Miller and Sunde (1975) who both reported increased egg production with increasing dietary Ca (from 15 g/kg to 45 g/kg dietary Ca).

Of the literature available on separate Ca feeding in layers, very little has focused on eggshell quality. Taher et al. (1984) showed that hens that received the Ca supplement produced significantly more eggs than the control (35 g/kg dietary Ca), and reported a trend for eggshell strength to improve, a finding that is consistent with the results of the present study. In contrast, Salim (1981) reported that oyster shell supplement fed ad lib did not cause any improvement in the egg production rate in young laying hens.

Egg specific gravity is closely related to shell thickness and CaCO3 deposition. This correlation between the two measures can be used to compare the data available in the literature (reported as egg specific gravity) to the results of the present study (reported as eggshell thickness). Eggshell thickness was expected to increase with increasing dietary Ca, which was observed to some extent in the results. Birds fed the highest dietary Ca level (40 g/kg) did not have the thickest eggshell, rather it was birds fed 30 g/kg dietary Ca. This finding is inconsistent with Lim et al. (2003) who reported that eggshell thickness was higher in eggs from birds fed 40 g/kg dietary Ca than those fed 30 g/kg dietary Ca. The differences observed between that study and the present one are the higher intake of the separate Ca source of the birds offered the 30g/kg Ca diet.

The present study does show a general increase in eggshell thickness with increasing dietary Ca, which is consistent with the findings of Gordon and Roland (1998) who reported that increasing dietary Ca resulted in increasing egg specific gravity. This observation is further supported by other examples from the literature (Abdallah et al. 1993; Keshavarz & Nakajima 1993). Birds that received 10 g/kg dietary Ca had the thinnest eggshells and the highest number of eggshell abnormalities, which is

consistent with Lim et al. (2003) who found low dietary Ca levels was associated with reduced eggshell quality. Current commercial standards recommend a dietary Ca inclusion of 4.5% at the end of the laying cycle. According to the Isa Brown Commercial Management Guide laying hens at the same age as those used in the study should be consuming 4.1-4.3 g of Ca per day. This is considerably lower than what the birds were seen to consume in the present study, which ranged from 15-25 g of total Ca per day. The results of the study could indicate that laying hens may have a higher Ca requirement than what is thought, and are able to self-regulate their own intake when presented with separate Ca feeding.

Conclusion

It can be concluded that the Ca-specific appetite is present in a contemporary layer hen, however, this may not be universally expressed. The findings of this study suggest that some but not all birds were able to increase intake of a separate Ca source in order to compensate for lower dietary Ca from a mixed ration. Overall, birds that were fed 30 g/kg dietary Ca had optimum performance with separate Ca feeding, and were able to maintain egg production and quality measures. Further work is required to explore the optimum time, ontogenetically, to introduce a separate Ca source and to consider groups of birds where birds with reduced ability to self-regulate may learn from the early adopters. Further work on separate Ca feeding should also investigate the effect on bone mineral content and bone density, and Ca deposition in the eggshell. Further evaluation of the application of separate Ca feeding in commercial laying hen systems needs to be undertaken to verify the validity of separate Ca feeding, not only for maintaining bird production and performance but also to determine if it is economically viable. In the future it may be possible to spatially separate Ca from the basal diet and exploit the Ca-specific appetite of layer hens to specifically improve the digestibility of phytate-P and amino acids. This would enhance the profitability and sustainability of the egg industry.

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3 An investigation into the interaction between dietary calcium and phosphorus on egg production and quality of laying hens using the geometric framework

3.1 Experiment 2 – key findings

- Hens were offered 15 diets comprised of 3 nutrient densities (total Ca and npP) and 5 ratios of dietary Ca:npP to create a geometric nutrient space.
- Hens had higher egg mass and shell weight when offered higher levels of dietary Ca and npP.
- Haugh unit and AME were maximised when dietary Ca was around 4% and dietary npP was maximised, suggesting a low dietary Ca:npP ratio is important for albumen quality and nutrient digestibility.
- Calcium and phosphorus digestibility were maximised at a dietary Ca of 3.0-3.5% and largely unaffected by dietary npP levels.
- When offered a choice of dietary Ca and npP concentrations hens show a capacity to converge on a common Ca and npP intake target.

3.2 An investigation into the interaction between dietary calcium and phosphorus on egg production and quality of laying hens using the geometric framework

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Abstract

An optimum inclusion rate of dietary calcium (Ca) and non-phytate phosphorus (npP) for laying hen diets is not clear due to complex interactions post-ingestion between these two essential minerals and involvement with other digestive processes. Identifying appropriate inclusion rates and ratios for Ca and npP is complicated further depending on the outcome variable of interest – for example, optimum feed utilisation may necessitate a lower dietary total Ca level when compared with that required for optimum eggshell quality. The effect of total Ca and npP levels on laying hen performance was investigated using 270 mid-lay ISA Brown birds in diets arranged in a geometric design. Birds were offered wheat-soybean based diets that differed only in total Ca and npP concentrations. Diets were clustered into low, medium and high total Ca+npP densities (30, 40 and 50 g/kg respectively) and at each density, five total Ca:npP ratios were formulated to generate a geometric nutrient space. To identify whether hens possess an ability to regulate Ca intake and therefore gather evidence of a Ca-specific appetite in hens, an adjacent group of 54 mid-lay ISA Brown hens was offered a choice of

diets selected for diverse dietary Ca and npP concentrations and ratios (three nutrient densities of low, medium and high values of total Ca plus npP (30, 40 and 50 g/kg respectively) and, within each density, two ratios of dietary Ca:npP (15.7:1 and 6.1:1). Egg production and quality, and feed utilisation were assessed over an eight-week period and surface mapped using the Thin Plate Spline procedure of the fields package in R. Increasing total Ca concentrations and npP concentrations led to greater egg mass per day ($P < 0.05$) and greater shell weight ($P < 0.05$). There was an interaction between Ca and npP found for Haugh unit ($P < 0.05$) and AME ($P < 0.01$). Increasing concentrations of npP resulted in a high egg Haugh unit at lower Ca levels, however, this effect was not observed at higher Ca levels. AME increased in line with increasing npP concentration, however, this was only observed when within dietary total Ca parameters of 3.7-4.3% Ca. When offered a choice of two diets varying in dietary Ca and npP concentrations and ratios, all hens consumed a comparable amount of total dietary Ca and npP.

In conclusion, the results of this study suggest that egg mass and shell weight can be increased through higher levels of dietary Ca and npP. However, achieving optimum Haugh units and AME were more specific with high levels of Ca suppressing egg quality parameters and feed utilisation. Hens show a capacity to converge on a common Ca and npP intake target when offered a choice of dietary Ca and npP concentrations.

3.3 Introduction

The laying hen has a substantial requirement for dietary calcium (Ca) and phosphorus (P) to support optimum egg production and egg quality. In particular, a high intake of Ca ranging from 4.0 to 4.5% of the diet is considered important as the laying cycle progresses from early to late lay to achieve optimum egg number and eggshell quality. While cereal grains provide some dietary Ca, the majority is delivered in the form of limestone and Ca phosphates. Meat and bone meal when used are also an important source of Ca. Dietary P which is available to the bird predominantly in the form of non-phytate phosphorus (npP) is recommended to account for between 0.44 and 0.38% of the diet as the bird's progress from early to late lay (ISA Brown Commercial Management Guide). Observing these macromineral guidelines results in a total Ca:npP ratio ranging from 9.1:1 to 11.7:1 approximately for a typical commercial hybrid.

However, while egg production may be supported by high levels of dietary Ca, other biological processes particularly nutrient digestibility that underpin important production traits may be impacted. Therefore, the amount of Ca required for optimum egg production can have negative consequences for other processes such as P and other mineral and nutrient availability (Plumstead et al. 2008). The relatively high acid buffering capacity of Ca-rich feed ingredients such as limestone (Lawlor et al. 2005) may impact the hydrolysis of nutrients as they pass through the gastrointestinal tract. Grynspan and Cheryan (1989) showed that the solubility of soy protein isolate was diminished with the addition of Ca at pH above 6.5. Furthermore, the tendency for Ca to form complexes with phytate can directly impact phytate solubility and reduce digestibility of P (Grynspan & Cheryan 1983; Selle et al. 2009). The importance of dietary P is undisputed and has given rise to much research on the application of phytase (Jalal et al. 2001) to improve P availability in poultry (Selle et al. 2009). Interestingly, several early studies report a limit on the upper level of dietary P required, demonstrating an inverse relationship between egg quality and dietary P level (Ousterhout 1980; Miles et al. 1983).

Therefore, while there appears to be much information in the literature reporting on Ca and P nutrition, optimum inclusion rates and appropriate ratios for total Ca and npP are not clearly defined and may differ depending on the production traits of interest.

Resolving this complex relationship between Ca and npP to gain a greater understanding of appropriate dosage is complicated further by the limitations of factorial design experiments that are commonly used, but quickly become difficult to interpret when more than two factors are employed. In this study, a geometric framework approach is employed to generate a geometric nutrient space fashioned on two axes – total Ca, and npP which enables more accessible interpretation of the response to varying Ca and npP levels when data are presented as contour plots (Wilkinson et al. 2014; Cowieson 2014).

In Chapter 2 of this report, a capacity for birds to increase calcium intake from a separate calcium source as dietary Ca levels diminished was observed. The ability of the hen to regulate calcium intake in response to physiological need for egg formation and maintenance may be an important tool to instruct on optimum Ca intake to achieve optimum productivity and egg quality.

The objectives of this study were hence two-fold:

1. to identify optimum dietary Ca and npP densities and ratios for important production traits in laying hens
2. to evaluate the total Ca intake of hens offered a choice of diets containing high and low densities of Ca and npP and high and low ratios of Ca:npP.

It was hypothesised that increasing dietary Ca will maximise egg production but may impede feed efficiency variables particularly as the ratio of Ca:npP increases.

3.4 Materials and methods

3.4.1 Experimental design and animal management

All birds were housed at the Poultry Research Foundation at The University of Sydney, Camden Campus. All experimental procedures conducted had approval from The University of Sydney Animal Ethics Committee and were in accordance with the Australian Code for the care and use of animals for scientific purposes (National Health and Medical Research Council 2013). A total of 270 mid-lay Isa Brown laying hens were randomly allocated to individually housed cages and fed for eight weeks on 15 dietary treatment groups arranged in a Geometric Framework design. Each treatment group consisted of 18 birds (six replicates with three birds per replicate). Each bird was housed separately in cages measuring 23 x 45 x 45 cm, with three adjacent cages forming the replicate unit located evenly throughout the experimental laying house. Birds were offered experimental diets based on wheat-soybean meal that differed in total Ca and npP concentrations for two weeks prior to commencement of data collection. Diets were clustered into low, medium and high total Ca plus npP densities (30, 40 and 50 g/kg respectively) and within each density, five total Ca:npP ratios (15.7:1, 11.5:1, 9:1, 7.3:1, 6.14:1) were formulated to generate a geometric nutrient space. Aside from differing Ca and npP concentrations, diets were formulated to ensure nutrient specifications satisfied the recommended requirements of ISA Brown laying hens. Feed and water were supplied *ad libitum.* The photoperiod regimen was 16 hours of light and eight hours of dark. In a smaller study run concurrently, 54 mid-lay ISA Brown hens were randomly allocated to individually housed cages and fed for eight weeks on a choice of two diets, which were selected from the initial set of 15 dietary treatments to a choice of dietary Ca:npP (high versus low) and a choice of high and low concentrations of both dietary Ca and npP.

Egg quality and feed conversion efficiency were assessed once weekly and on a weekly basis respectively, over an eight-week period. On day 28, a 48 hr total collection procedure was undertaken for the six replicate groups from the main study whereby the feed input and faecal output were measured to determine energy, nitrogen and mineral retention. Collection trays were placed under

each of the cages of individual birds. Excreta samples were dried in an oven at 100°C until a consistent weight was achieved.

3.4.2 Chemical analysis

To estimate apparent metabolisable energy (AME), gross energy of feed and faecal output was determined using a Parr 1281 adiabatic bomb calorimeter (Parr Instrument Company, Moline, IL, USA), which was standardised with benzoic acid. The nitrogen concentration of samples was determined by the Dumas method using an FP-428 nitrogen analyser (LECO Corp., St Joseph, MI, USA) as described by Sweeney (1989). The mineral composition of the feed and faeces ash was determined by inductively coupled plasma optical emission spectrometry (ICP) using a PerkinElmer OPTIMA 7300 (PerkinElmer Inc., Waltham, MA, USA) following digestion with nitric acid and hydrogen peroxide beforehand.

Throughout the eight-week trial, all eggs were collected and weighed for each replicate group. In order to determine egg quality, eggs were collected one day per week, weighed, and their egg yolks and eggshells weighed. Egg quality assessment was as reported earlier in Chapter 2.

The calculations for apparent metabolisable energy (AME), apparent coefficients for dry matter (ACDM) and nitrogen (ACNR), and the retention values for the specific minerals, were calculated as (intake−output)/intake where *intake* represents total gross energy, DM, N and mineral quantity consumed, and *output* is the total gross energy, DM, N and mineral excretion over the 48 hr collection period.

3.4.3 Statistical analysis

Data were surface mapped using the Thin Plate Spline procedure of the fields package (R Development Core Team 2011). Treatments were represented as dots overlaid on the contour plots. Interrogation of the data was performed using the PROC MIXED procedure of SAS (Littell et al. 1996). The fixed effects were total Ca and npP density, the ratio of total Ca to npP, and the interaction between the two. For the smaller choice study, experimental data were analysed as a one-way ANOVA of dietary treatments using the IBM® SPSS® Statistics 20 program (IBM Corporation, Somers, NY USA). Treatment differences were considered significant at $P < 0.05$.

Table 3.1 Ingredient and nutrient specifications (as fed, g/kg) of the experimental diets offered to Isa Brown laying hens

Treatment	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Ca (%)	4.70	4.60	4.50	4.40	4.30	3.76	3.68	3.60	3.52	3.44	2.82	2.76	2.70	2.64	2.58
avP (%)	0.30	0.40	0.50	0.60	0.70	0.24	0.32	0.40	0.48	0.56	0.18	0.24	0.30	0.36	0.42
Ca+avP	5.00	5.00	5.00	5.00	5.00	4.00	4.00	4.00	4.00	4.00	3.00	3.00	3.00	3.00	3.00
Ca:avP	15.7	11.5	9.0	7.3	6.1	15.7	11.5	9.0	7.3	6.1	15.7	11.5	9.0	7.3	6.1
Ingredient (g/kg)															
Wheat	300	300	300	300	300	300	300	300	300	300	300	300	300	300	300
Soybean 46%	262	262	261	261	261	247	247	247	246	246	317	318	232	232	232
Sorghum 9.2%	199	200	202	203	204	258	259	260	261	262	232	232	319	319	320
Limestone	121	115	108	102	96	97	92	87	82	76	73	69	65	61	57
Canola meal 38%	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Sunflower oil	44.9	44.5	44.1	43.7	43.4	28.0	27.7	27.4	27.1	26.8	11.1	10.9	10.7	10.4	10.2
Dicalcium phosphate	13.1	18.8	24.5	30.2	35.9	9.5	14.1	18.7	23.2	27.8	6.0	9.4	12.8	16.3	19.7
Sodium bicarbonate	3.1	3.1	3.1	3.1	3.1	3.4	3.4	3.4	3.4	3.4	3.7	3.7	3.7	3.7	3.7
Salt	2.1	2.1	2.1	2.1	2.1	1.8	1.8	1.8	1.8	1.8	1.6	1.6	1.6	1.6	1.6
Layer premix	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
DL-Methionine	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9
Porzyme 9310[1]	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
L lysine HCl	0.2	0.2	0.2	0.2	0.2	0.5	0.5	0.5	0.5	0.5	0.8	0.8	0.8	0.8	0.8
Threonine	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

[1] endo-1, 4-beta-xylanase.

Table 3.2 Calculated and analysed nutrient and energy specifications (as fed) of the experimental diets offered to Isa Brown laying hens

Treatment	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Calculated (g/kg)															
Dry matter	908	908	908	908	908	903	903	903	903	902	898	898	898	897	897
Fat	17.5	17.5	17.6	17.6	17.6	19.1	19.1	19.1	19.1	19.2	20.6	20.6	20.6	20.7	20.7
AMEn kcal/kg	2750	2750	2750	2750	2750	2750	2750	2750	2750	2750	2750	2750	2750	2750	2750
Calcium	47	46	45	44	43	37.6	36.8	36	35.2	34.4	28.2	27.6	27	26.4	25.8
npP	3.0	4.0	5.0	6.0	7.0	2.4	3.2	4.0	4.8	5.6	1.8	2.4	3.0	3.6	4.2
Sodium	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
Chloride	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Analysed (g/kg)															
DM (g/kg)	90.5	90.5	90.2	90.0	89.8	89.4	90.2	89.8	89.7	89.5	89.5	90.2	89.4	89.6	56.8
Ca	57.5	52.3	40.8	43.9	47.1	43.0	46.9	36.2	37.8	38.6	33.8	29.3	22.0	23.2	16.8
P	7.1	7.5	7.4	8.5	10.1	5.5	6.8	7.2	8.2	9.2	5.9	6.2	6.0	6.6	6.5
GE (MJ/kg)	15.6	15.6	15.6	15.5	15.9	15.8	15.4	15.7	15.6	15.5	15.9	15.6	15.9	15.8	16.2

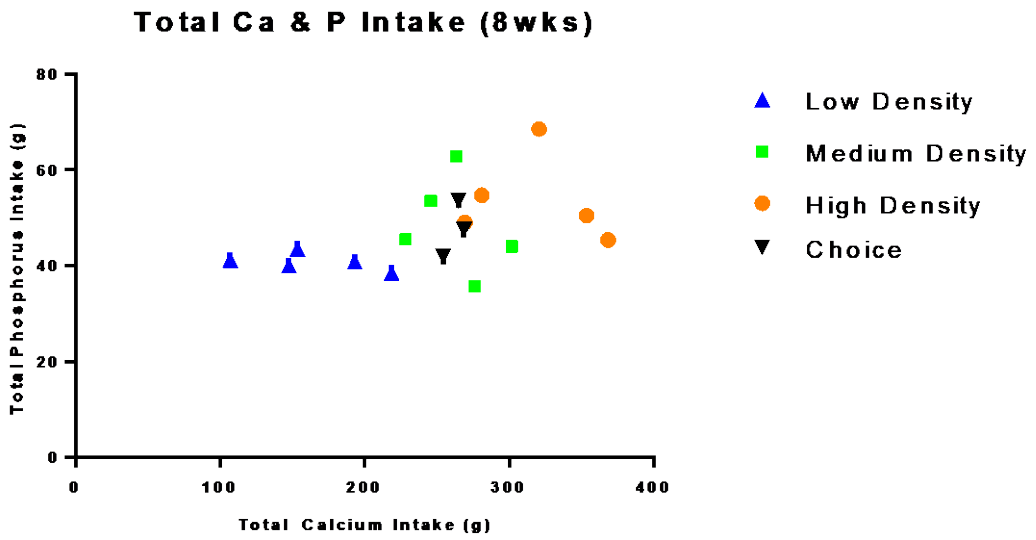

Figure 3.1 The cumulative intake of hens offered single diets differing in macromineral density (total Ca+npP), and of birds offered a choice of diets comprising high and low Ca densities, high and low npP densities, and high low ratio of Ca:npP

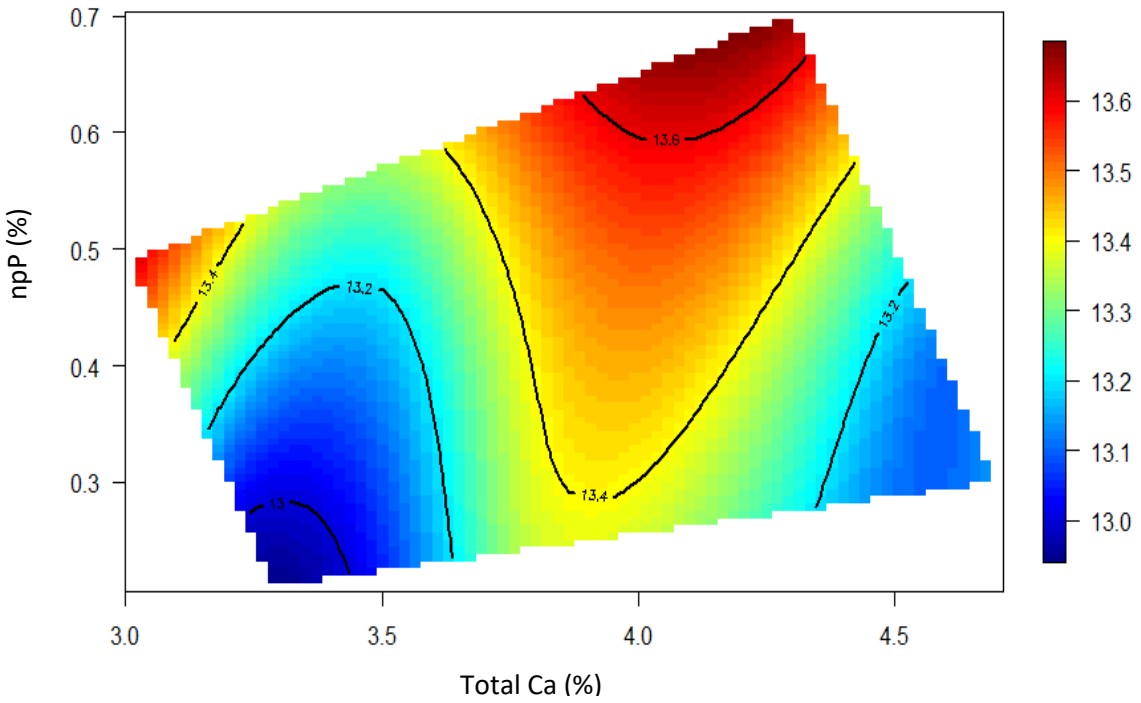

Figure 3.2 The effect of dietary total Ca and npP on the AME of single diets fed to laying hens

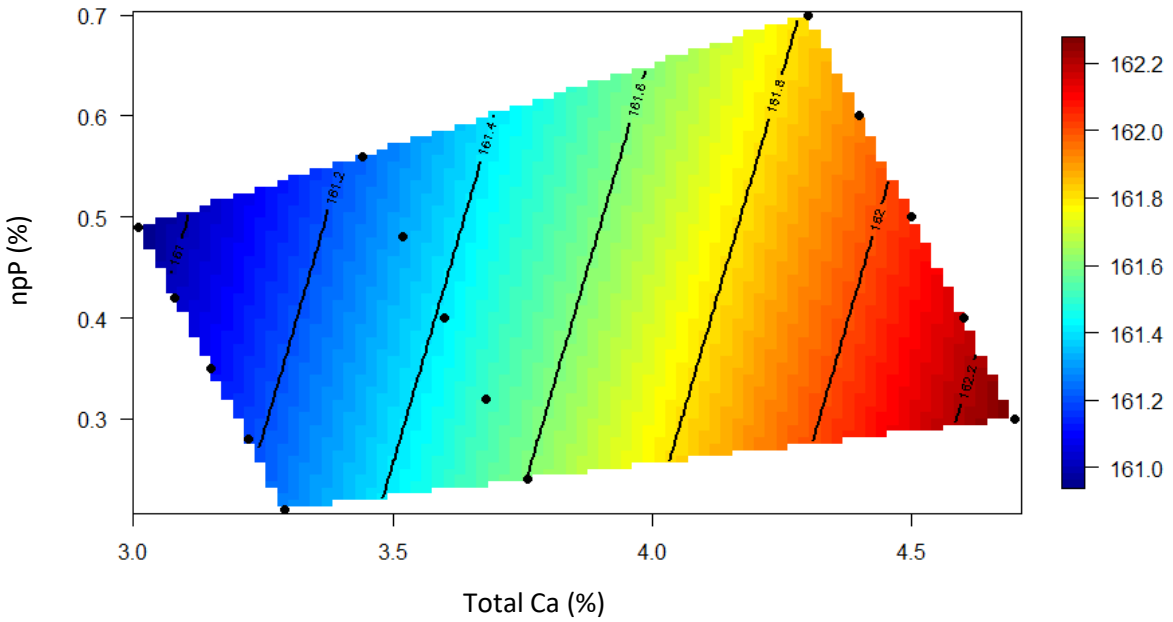

Figure 3.3 The effect of dietary total Ca and npP on total egg production of laying hens over eight weeks (eggs produced per three hens)

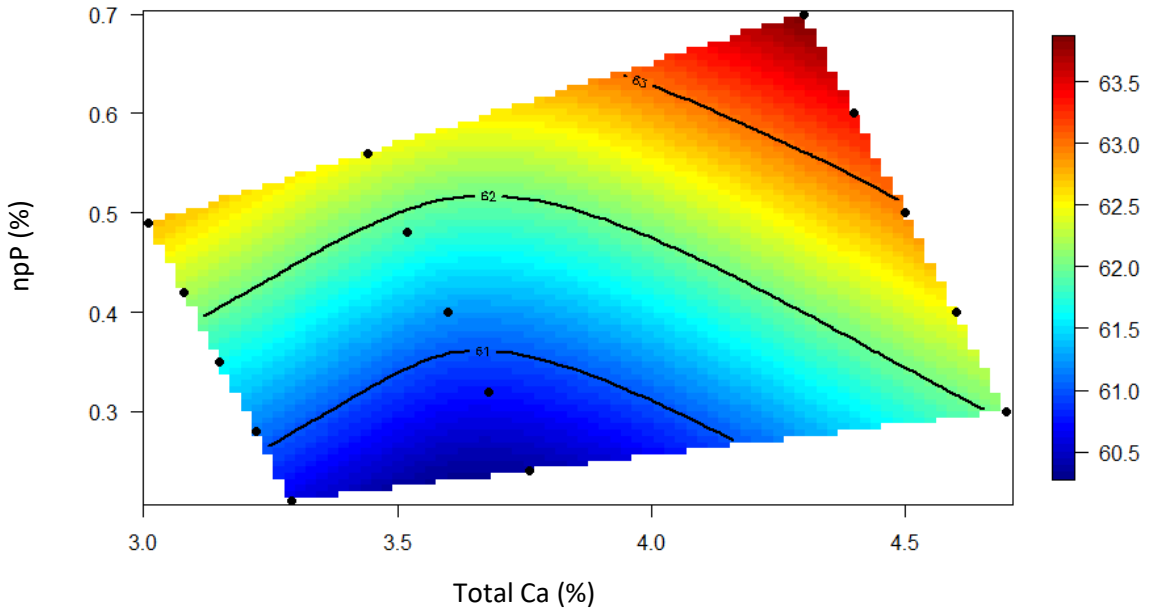

Figure 3.4 The effect of dietary total Ca and npP on egg mass (g) per day of laying hens

3.5 Results and discussion

3.5.1 Intake and performance

While maximising the intake of dietary Ca particularly, and to a lesser extent npP, is recognised as an important target for optimum egg production, high levels of Ca can impede other important production traits by interfering with nutrient and energy availability (Selle et al. 2009). In this study a geometric framework approach was taken to facilitate investigation of the response of egg production and quality, and AME and nutrient digestibility to a range of dietary Ca and npP densities and ratios. Additionally, the capacity for hens to select from diets containing diverse quantities and ratios of Ca and npP was also investigated.

The effect of dietary Ca and npP concentrations on performance measurements of Isa Brown laying hens are shown in Table 3.3. The overall performance of Isa Brown laying hens from over the eight-week period during the mid-lay production cycle was acceptable in this study in comparison to the Isa Brown Commercial Performance Objectives, as there was an average finishing weight of 2078 g, daily feed intake of 123.5 g/bird and FCR of 1.97. Dietary treatment did not influence daily feed intake, finishing body weight or average body weight gain. The transition of diet Ca+npP densities from 3.5% to 5.0% Ca and npP increased body weight gain percentage by 1.54 percentage units (5.95 vs. 7.49; $P < 0.05$). There were significant Ca+npP density and Ca:npP ratio interactions in Ca retention, P retention, dry matter retention and AME ($P < 0.05$). The effect of dietary treatment on Ca retention appears to mimic P retention where the highest retention of Ca and P is achieved in the high Ca+npP density, with Ca:npP ratios of 15:7 and 6:1 respectively. The lowest Ca and P retention of 1.02 g and 0.120 g respectively was obtained in the low Ca+npP density diet with a Ca:npP ratio of 6:1. The elevation of Ca:npP ratios increased DM retention ($P < 0.01$) in both the high (0.695 vs. 0.720) and low density diet (0.698 vs. 0.705) but had a negligible effect across the medium density diet, where a consistently high DM retention was achieved. Similarly, the effect of Ca and P on AME was dependant on the Ca+npP density ($P < 0.01$). The optimal AME of 13.8 MJ/kg DM was achieved in diet 5 (high diet mineral density and Ca:npP ratio of 6:1).

The effect of dietary treatment and choice feeding on Ca and npP intake is shown in Figure 3.1. Birds offered high Ca+P density diets obtained the highest overall Ca and npP intake while the medium Ca+P density diet followed with the second highest and the low Ca+P density diets resulted in the lowest intake. Somewhat unexpectedly, high and medium Ca+P density diets did not appear to drive the consumption of Ca and npP as both diets were quite Ca and P adequate. The most interesting outcomes were that of the low density and choice feeding treatments. Birds offered low density diets had a constant npP intake of 40g while calcium intake ranged from 100-200g. This result suggests that birds on a low Ca+npP diet will eat to meet a npP requirement, regardless of Ca intake. However, when birds are given a choice, the reverse effect was observed where calcium intake was consistent at 275 g, while intake of npP varied between 40-60 g, suggesting that birds ate until they meet a certain calcium requirement.

The response surface of the effect of dietary Ca and npP concentrations on AME is shown in Figure 3.2 and suggests the optimal concentration of dietary Ca resides between 3.8-4.2%, whereby diverging from this range will impede AME. The most favourable concentration of npP appears to be more lenient but is dependent on Ca concentrations being held within its optimal range.

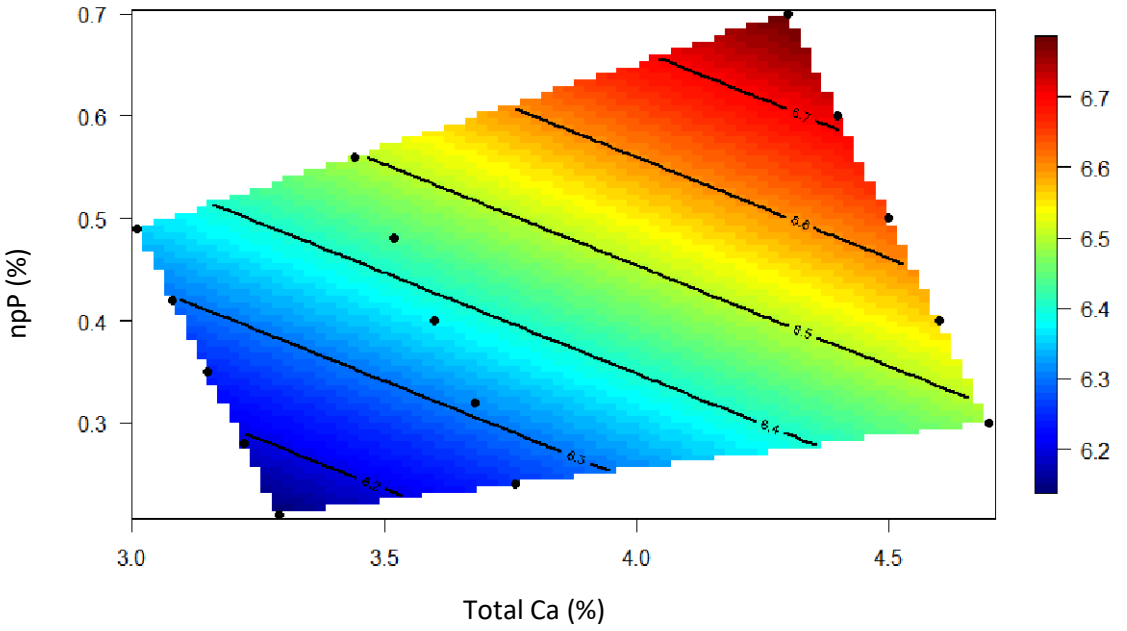

Figure 3.5 The effect of dietary total Ca and npP on eggshell weight (g) of laying hens

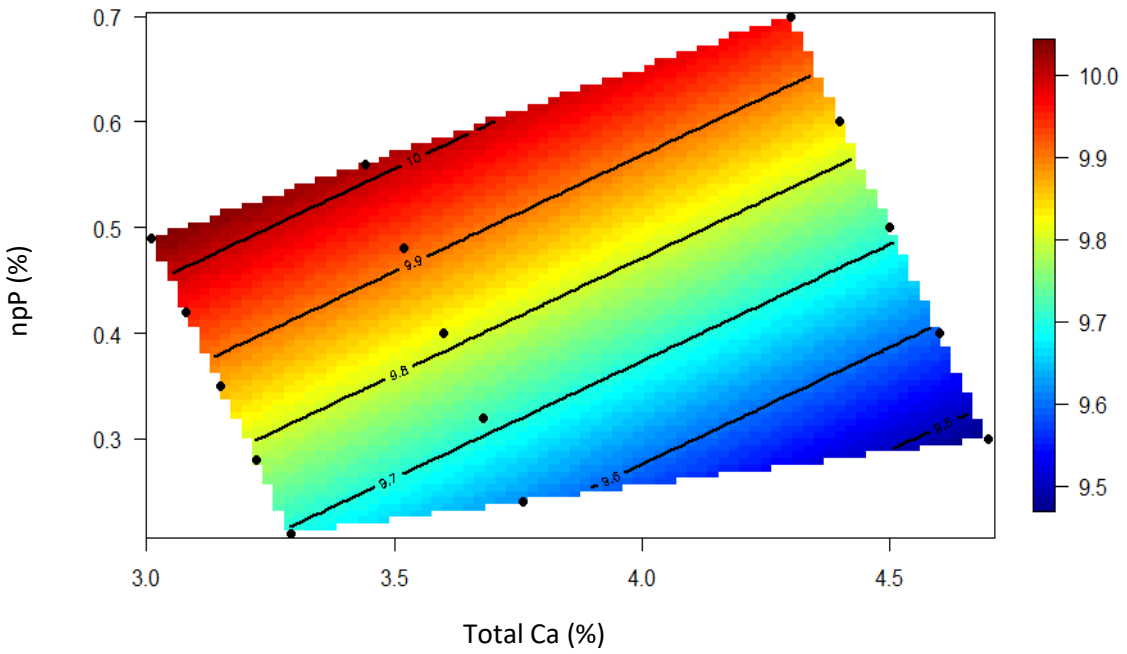

Figure 3.6 The effect of dietary total Ca and npP on albumen height (mm) of laying hens

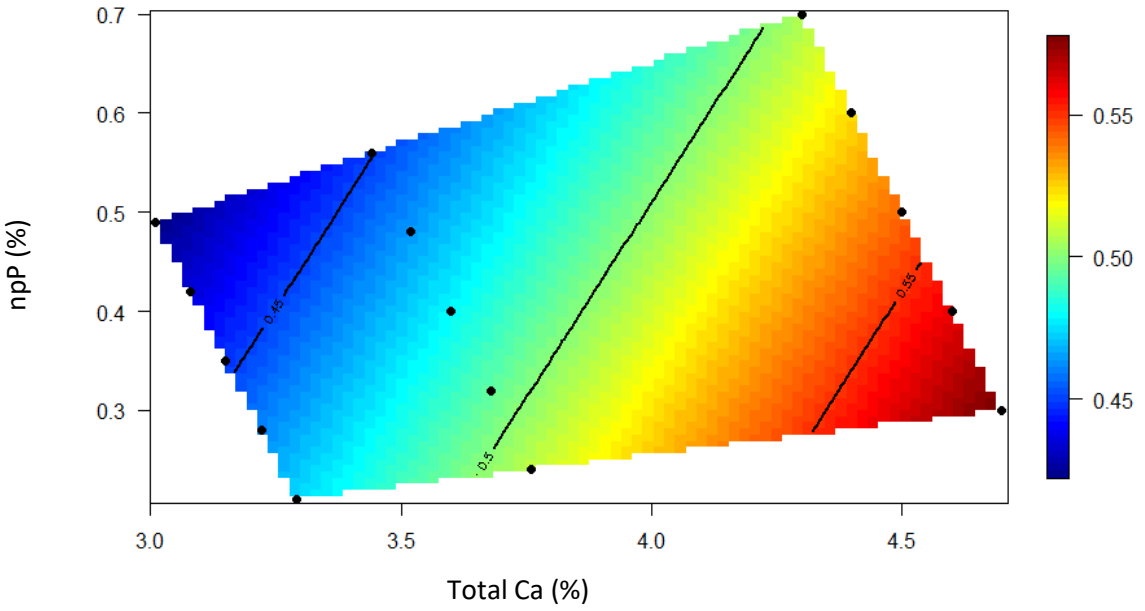

Figure 3.7 The effect of dietary total Ca and npP on shell thickness of laying hens

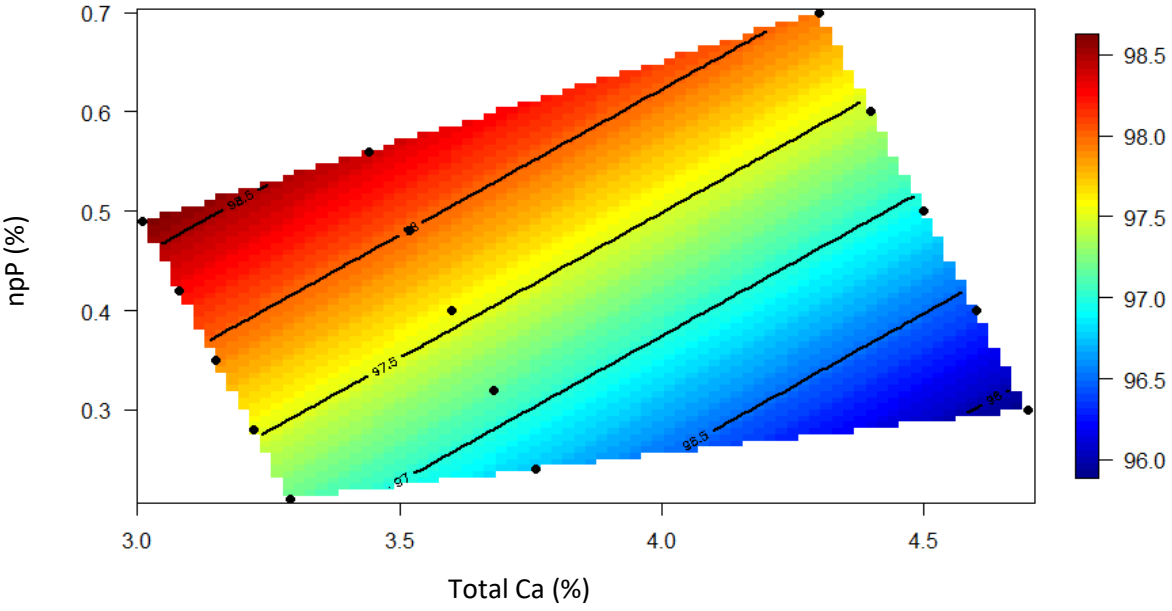

Figure 3.8 The effect of dietary total Ca and npP on Haugh units of Isa Brown laying hens

3.5.2 Egg quality and mineral digestibility

The effect of dietary Ca and npP on egg production is presented in Table 3.4 and in Figures 3.3 to 3.8. There was no significant treatment interaction effect on egg production measurements, however, the main effects of Ca:npP and Ca+npP density did influence albumen height ($P < 0.05$) and dry shell weight ($P < 0.05$), respectively. Ca:npP ratio of 11:5 produced the lowest albumen height (0.9413 mm) while dry shell weight increased with elevating diet density from 3.5% to 5.0% (6.237 vs. 6.645 g).

The response surface showing the effect of dietary Ca and npP on total egg production is shown in Figure 3.3, where there is a distinct linear relationship between Ca and egg production although this

was not significant and likewise may not be relevant unless there is a greater decrease in dietary Ca not captured by the parameters of this study.

The response of average egg mass to dietary Ca andnpP % ($P < 0.05$) is presented in Figure 3.4. There was a quadratic relationship between average egg weight and dietary Ca. However, dietary npP concentrations had a greater and linear impact on average egg weight than Ca, whereby average egg weight increased with dietary npP levels. Laying hens offered diets containing high densities of total Ca (4.0-4.5%) and npP (0.6-0.7%) had the greatest average egg weight.

The response of eggshell weight to Ca and npP concentration ($P < 0.05$) is presented in Figure 3.5. Birds offered diets containing high densities of total Ca (4.0-4.5%) and npP (0.65-0.7%) had the greatest eggshell weight. In this study the response for eggshell weight appears to be more sensitive to changes in dietary npP rather than total Ca.

The responses of albumen height to Ca and npP concentrations are presented in Figure 3.6. Birds offered diets containing 0.5% npP and 3.0-3.5% Ca had the highest albumen height in contrast with birds offered 0.3% npP and 4.5% Ca resulting in the lowest albumen height.

The effect of diet on albumen height was similarly reflected in the Haugh units of eggs. There was a strong trend ($P = 0.051$) towards an interaction between total Ca+npP densities and the ratio of total Ca to npP on egg Haugh units (Figure 3.8). Birds offered diets containing 0.5% npP and 3% total Ca had the highest Haugh units, in contrast with birds offered 0.3% npP and 4.5% Ca resulting in the lowest Haugh units.

The effect of Ca and npP on shell thickness, shown in Figure 3.7, appears to mirror the effect shown in Figure 3.3. Again, Ca is the primary driver of responses to shell thickness.

Dietary concentrations of calcium appear indicative of egg qualities related to shell thickness and total egg production where the uppermost level of Ca obtained superior quality outcomes. However, concentrations of npP appeared to be the primary driver of albumen height and Haugh units where npP% above 0.4 produced higher albumen height and greater Haugh units. Both Ca and npP concentrations were required at maximum levels to achieve optimal egg mass and eggshell weight.

Mineral digestibility

The effect of dietary Ca+npP density and Ca:npP on ATTD digestibility of eight minerals is presented in Table 3.5. There was a significant dietary Ca+npP density and Ca:npP interaction effect for all mineral digestibilities in which the highest combined mineral digestibilities were observed in the medium Ca+npP density. The digestibility of Zn was negative across all dietary treatments. Pearson correlations were obtained and revealed that all eight minerals were positively correlated with each other ($r = 0.215$-0.839; $P = 0.46$-< 0.001).

The response surface of dietary Ca and npP (%) and their effect on the digestibility of eight minerals are shown in Figures 3.9-3.10 and Figures 6.1-6.6, which are presented in Appendix 1. It appears that the concentration of npP does not affect the digestibility of the eight minerals assessed, while the concentration of Ca will impede mineral digestibility after a certain point. The dietary Ca range of 2.5 to ~4.0% is optimal for the digestibility of Ca, K, Mn, Na, Sr, Mg and P, however, the digestibility of Zn is the lowest at this Ca range and will be heightened at dietary Ca concentrations past 4.0%. Importantly, increasing dietary Ca does not result in an increase in Ca digestibility and the same outcome was seen for dietary P and P digestibility.

Acknowledgements

The authors are grateful to Australian Eggs Limited for its financial support of this study. We would like to acknowledge the support of the technical team at the Poultry Research Foundation (PRF) at the University of Sydney, led by Joy Gill.

The references cited in this paper are provided at Chapter 5, References.

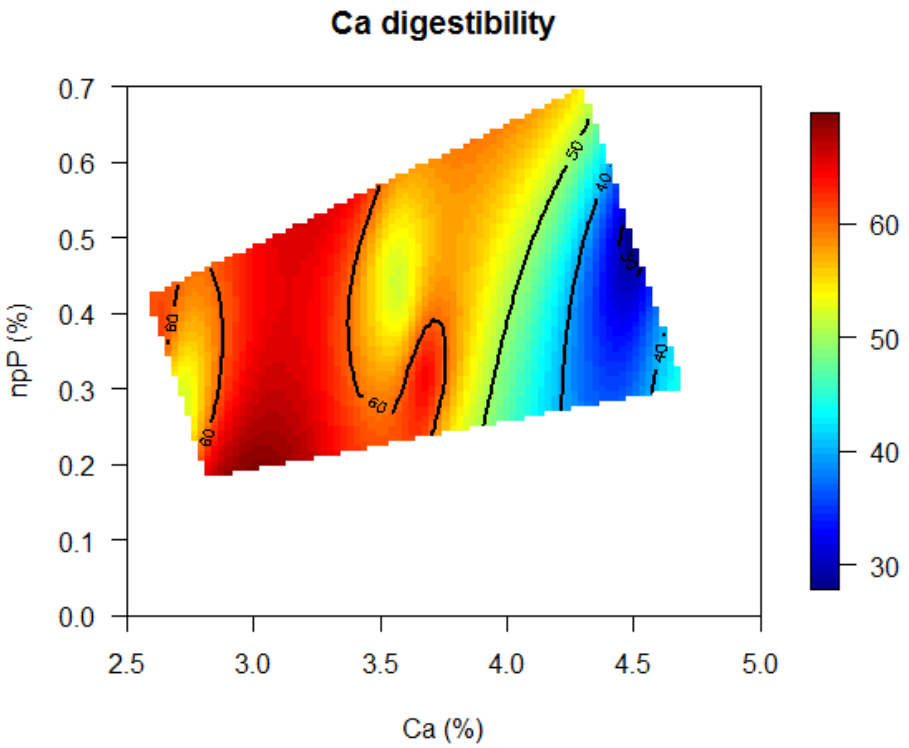

Figure 3.9 The effect of dietary total Ca and npP on Ca digestibility of laying hens

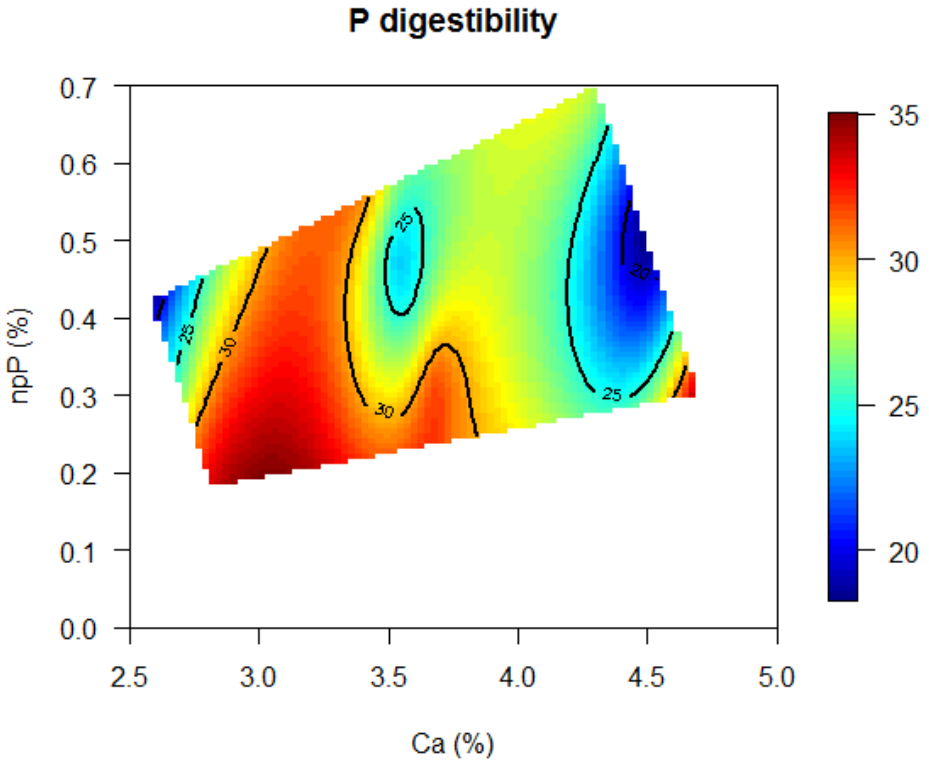

Figure 3.10 The effect of dietary total Ca and npP on P digestibility of laying hens

Table 3.3 Interaction of dietary Ca and npP concentration (%) on performance measurements of Isa Brown laying hens

Treatment	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**s.e.m.**	***P*-value**		
Ca (%)	4.70	4.60	4.50	4.40	4.30	3.76	3.68	3.60	3.52	3.44	2.82	2.76	2.70	2.64	2.58				
npP (%)	0.30	0.40	0.50	0.60	0.70	0.24	0.32	0.40	0.48	0.56	0.18	0.24	0.30	0.36	0.42				
Ratio Ca:npP	15.7	11.5	9.0	7.3	6.1	15.7	11.5	9.0	7.3	6.1	15.7	11.5	9.0	7.3	6.1		Diet density (Ca+P)	Ca:nPP	Diet density x Ca:npP
Ca Intake (g)	6.1	5.9	4.6	4.8	5.3	4.7	5.3	3.9	4.1	4.5	4.0	3.4	2.5	2.6	1.9				
P Intake (g)	0.76	0.83	0.83	0.92	1.13	0.61	0.77	0.77	0.90	1.08	0.70	0.71	0.68	0.74	0.75				
Ca retention (g)	2.77abc	2.00bcdef	1.28fg	1.95cdef	2.89ab	2.62abcd	3.44a	2.03bcdef	2.20bcde	2.84abc	2.62abcd	1.82defg	1.33efg	1.61efg	1.02g	0.323	0.001	0.001	0.001
P retention (g)	0.276abc	0.154cd	0.113d	0.212bcd	0.330ab	0.178cd	0.275abc	0.187cd	0.180cd	0.358a	0.234bcd	0.233bcd	0.159cd	0.195cd	0.120d	0.036	0.118	0.003	0.001
AME (kcal/kg)	13.4abc	12.7de	13.3abc	13.4abc	13.8a	13.6ab	13.2bcd	13.2bcd	13.1bcde	13.3abc	13.0cde	12.6e	13.5abc	13.3abc	13.8a	0.093	0.775	0.001	0.005
Dry matter retention	0.695de	0.668e	0.697de	0.700cd	0.720abcd	0.72abcd	0.733ab	0.714abcd	0.703bcd	0.730abc	0.698de	0.697de	0.734a	0.732ab	0.705abcd	0.006	0.001	0.118	0.002
Average daily feed intake	120	127	124	121	123	122	121	119	123	129	125	124	127	125	123	1.78	0.598	0.894	0.509
FCR[3]	1.96	2.09	1.99	1.97	2.05	2.04	2.04	2.04	2.00	2.11	2.08	2.16	2.16	2.00	2.02	0.028	0.233	0.831	0.314
Finishing body weight	2056	2121	1994	2063	2120	1992	2047	2056	2066	2134	2071	2134	2116	2123	2074	25.9	0.344	0.387	0.653
% Body Weight[4]	7.13ab	7.05ab	7.51ab	8.20a	7.59a	5.21ab	5.77ab	7.02ab	7.66a	7.27ab	4.53b	7.16ab	5.45ab	6.70ab	6.29ab	0.481	0.042	0.151	0.803
Average daily gain	2.72	2.78	2.69	3.05	2.93	1.92	2.16	2.59	2.85	2.79	1.76	2.84	2.09	2.54	2.42	0.199	0.101	0.230	0.832

[1] Mean values from six treatment replicates.
[2] Pooled s.e.m.
[3] Feed Conversion Ratio (FCR) calculated per unit egg mass.
[4] Means of main effect: Diet density (Ca+P) of 3.5 is 5.95b, 4.0 is 6.59ab and 5.0 is 7.43a.
abcdefg – Means of parameters with a significant interaction effect within rows not sharing a common suffix are significantly different at the 5% level of probability.

Table 3.4 Interaction of dietary Ca and npP concentration (%) on egg production measurements of Isa Brown laying hens[1]

Treatment	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**s.e.m.[2]**		***P*-value**	
Ca (g/kg)	4.70	4.60	4.50	4.40	4.30	3.76	3.68	3.60	3.52	3.44	2.82	2.76	2.70	2.64	2.58				
npP (g/kg)	0.30	0.40	0.50	0.60	0.70	0.24	0.32	0.40	0.48	0.56	0.18	0.24	0.30	0.36	0.42				
Ratio Ca:npP	15.7	11.5	9.0	7.3	6.1	15.7	11.5	9.0	7.3	6.1	15.7	11.5	9.0	7.3	6.1		Diet density	Ca:nPP	Diet density x Ca:npP
Total egg production	163.7	161.8	166.5	163.3	154.5	160.7	162.7	155.5	164.7	164.8	162.8	154.8	160.2	164.3	162.5	2.23	0.925	0.736	0.363
Egg weight (g)	63.2	63.4	62.9	63.2	65.1	62.3	61.1	62.7	62.6	62.4	61.9	62.4	62.0	63.9	62.8	0.489	0.072	0.488	0.757
Albumen height (mm)[3]	9.28	9.53	9.64	10.01	10.09	9.96	9.44	10.39	9.77	9.62	10.05	9.27	9.68	9.98	10.3	0.137	0.561	0.033	0.056
Dry shell weight (g)[4]	6.5	6.56	6.50	6.47	7.15	6.36	6.25	6.35	6.43	6.49	6.12	6.29	6.09	6.81	5.90	0.126	0.022	0.562	0.122
Shell thickness	0.48	0.83	0.47	0.46	0.48	0.47	0.49	0.46	0.47	0.47	0.45	0.46	0.44	0.47	0.44	0.049	0.274	0.333	0.516
Haugh units	95	96	97	98	98	98	96	100	98	97	99	95	97	98	100	0.597	0.304	0.053	0.051
Egg mass / day (g)	61	61	62	61	60	60	59	58	61	61	60	57	59	63	61	0.413	0.311	0.238	0.219
Hen day production %	97	96	99	97	92	96	97	93	98	98	97	92	95	98	97	0.8	0.373	0.461	0.255

[1] Mean values from six treatment replicates.
[2] Pooled s.e.m.
[3] Means of main effect: Ca:P of 6.14 is 10.02a, 7.33 is 9.92a, 9.00 is 9.90a, 11.50 is 9.41b and 15.70 is 9.79ab.
[4] Means of main effect: Diet density (Ca+P) of 3.5 is 6.24b, 4.0 is 6.38b and 5.0 is 6.65a.

Table 3.5 Interaction of dietary Ca and npP concentration (g/kg) on mineral digestibility (%) of laying hens[1]

Treatment	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Ca (g/kg)	47	46	45	44	43	37.6	36.8	36	35	34	28	27.6	27	26	25.8
npP (g/kg)	3	4	5	6	7	2.4	3.2	4	4.8	5.6	1.8	2.4	3.0	3.6	4.2
Ca:npP	15.7	11.5	9.0	7.3	6.1	15.7	11.5	9.0	7.3	6.1	15.7	11.5	9.0	7.3	6.1
Apparent ileal digestibility (units?)															
Ca Dig	44.9cde	38.1ef	27.6f	40.9bde	54.3abc	55.6abc	65.0ab	52.8bcd	53.4abcd	63.0ab	66.0a	54.1abc	52.2bcd	61.9ab	59.8ab
P Dig	36.3a	23.5cde	16.1e	22.5cde	28.6abcd	29.3abcd	35.5ab	24.0bcde	20.2de	32.8abc	33.2abc	32.7abc	23.6cde	26.4abcde	15.7e
Mg Dig	26.7ab	14.6def	11.9ef	19.6abcdef	25.3abc	21.3abcd	26.6ab	20.3abcde	16.9cdef	27.3a	20.4abcde	17.9bcdef	18.0bcdef	18.4bcdef	11.5f
Na Dig	71.8a	41.0fg	40.9g	55.9bcd	44.7defg	52.0bcdefg	63.3ab	59.5abc	49.4cdefg	53.7bcdef	50.7cdefg	43.1efg	55.2bcde	57.2bcd	44.7defg
K Dig	16.4ab	-0.5d	8.0bcd	7.5abcd	16.8a	12.2abcd	14.9abc	6.9cd	9.1abcd	13.0abcd	5.8d	8.1bcd	13.6abcd	16.7abc	11.6abcd
Mn Dig	6.9abcd	-3.0cdef	-10.2efg	1.3bcdef	11.0abc	7.6abcd	21.3a	4.2bcde	-7.6def	14.6ab	10.9abc	-2.2cdef	-6.2def	-14.8fg	-29.4g
Zn Dig	-45.0abc	-68.2bcde	-65.7bcde	-57.1abcd	-52.7abcd	-80.6bcde	-10.2a	-94.2de	-105.5e	-38.0ab	-65.1bcde	-61.4bcde	-86.9cde	-47.6bcde	-101.7de
Sr Dig	38.3bcd	28.8de	19.8e	31.0cde	42.8abc	46.5ab	54.5a	39.5bcd	39.7bcd	48.9ab	48.7ab	41.5bcd	38.9bcd	47.4ab	32.6bcd

P-value

Apparent ileal digestibility (units?)	SEM[2]	Diet density	Ca:nPP	Diet density x Ca:npP	LSD
Ca Dig	3.7	0.001	0.001	0.015	12.909
P Dig	3.6	0.408	0.001	0.005	11.829
Mg Dig	2.8	0.016	0.072	0.001	9.180
Na Dig	3.1	0.015	0.001	0.001	12.673
K Dig	0.3	0.696	0.210	0.009	8.638
Mn Dig	4.5	0.001	0.001	0.001	16.427
Zn Dig	15.3	0.316	0.090	0.002	48.496
Sr Dig	3.8	0.001	0.005	0.001	12.539

[1] Mean values from six treatment replicates.

[2] Pooled s.e.m.

abcdefg – Means of parameters with a significant interaction effect within rows not sharing a common suffix are significantly different at the 5% level of probability.

4 The effect of a separate calcium source and inclusion of phytase on egg production and quality of hens offered diets differing in calcium concentration

4.1 Experiment 3 – key findings

- Early lay ISA Brown hens were offered a choice of diets ranging in total Ca concentrations from 20-40 g/kg with a fixed npP content of 0.25% and the inclusion or not of dietary phytase. All hens had access to a separate source of limestone grit (3.4 ± 1 mm).
- Hens exhibited a behavioural response to the external source of dietary Ca, with approximately 50% of birds engaging in limestone grit consumption regardless of dietary Ca concentration.
- Of the hens that did limestone consume grit, a clear increase in consumption was observed with decreasing dietary Ca concentration in the mixed feed.
- Decreasing dietary Ca concentrations had a negative impact on a range of measurements related to egg quality and particularly shell quality.
- Dietary phytase had no impact on limestone grit consumption.

4.2 The effect of a separate calcium source and inclusion of phytase on egg production and quality of hens offered diets differing in calcium concentration

Y. Akter, A.J. Cowieson, P. Hughes, D. Caporale, S. Bucker, S. Wilkinson, and C.J. O'Shea

Abstract

Separate Ca sources may improve nutrient digestibility in laying hens without compromising total Ca intake. Limestone grit consumption may be stimulated or inhibited by factors such as dietary Ca level and the presence or absence of dietary phytase. This study investigated the response of egg production to providing a separate Ca source in the form of limestone grit to hens offered diets differing in Ca level supplemented with or without phytase. A 3 x 2 factorial arrangement of treatments in a completely randomised design experiment comprising three dietary Ca levels (20, 30 and 40 g/kg) and the inclusion or not of dietary phytase (3500 FTU/kg diet). The study investigated the effect of dietary treatment on egg performance of early lay ISA Brown hens. All cages were offered a separate limestone grit (3.4 ± 1 mm). Feed intake, egg production and quality were assessed over a six-week period.

The results of this study showed approximately 50% of the hens consumed limestone grit regardless of dietary Ca level or the inclusion of phytase, suggesting grit consumption to be a learnt behaviour. However, of the birds that did engage in limestone grit consumption, a clear increase in grit consumption was observed in response to a reduction in dietary Ca. Overall, separate Ca intake as a proportion of total Ca intake increased with decreasing dietary Ca levels ($P < 0.001$). Egg mass was greatest in birds receiving 3% dietary Ca compared to other treatments ($P < 0.01$). Egg quality

parameters were broadly influenced by dietary Ca level. The birds offered 4% dietary Ca had significantly increased egg weight, yolk weight, higher (darker) yolk colour score, and eggshell quality compared with the 2% dietary Ca group, while the 3% dietary Ca group had intermediate results. There was a clear effect of dietary Ca group on egg breaking force, with the 4% dietary Ca group having a greater egg breaking strength compared with the 2% dietary Ca group ($P < 0.01$). There was no effect of dietary phytase inclusion on limestone grit consumption in laying hens.

The results of this study confirm the findings of study 1, showing that limestone grit consumption was only partially adopted regardless of dietary Ca level or phytase inclusion. However, of the hens that did consume grit (>2 g / day), a clear increase in grit consumption was observed in response to decreasing dietary Ca. In summary, limestone grit consumption was not universally adopted in laying hens regardless of dietary Ca levels or phytase inclusion, and egg quality – particularly shell quality – was reduced in groups receiving suboptimal dietary Ca levels.

4.3 Introduction

Commercial laying hens have a substantial need for dietary Ca and non-phytate phosphorus (npP) to achieve optimum egg production and maintain physiological Ca and P homeostasis. An inadequate supply of these minerals can impact both the quantity and quality of eggs produced, and the longevity of the bird in the flock due to diminished bone mineral reserves leading to a compromised skeleton (Wilkinson et al. 2009; Whitehead 2004). Conversely, providing too much Ca and P or an inappropriate ratio of Ca to P can impede egg production, egg quality and hen performance. The conventional approach to achieving adequate macromineral intake is to provide a homogenous feed supplemented with ingredients rich in highly digestible Ca and P to satisfy production needs. For the ISA Brown hybrid, the recommended Ca intake ranges from 3.9 to 4.6 g/d and for npP from 0.44 to 0.38 g/d over the laying cycle (17 to 50+ weeks; ISA Brown breed standard). This means that for Ca at least, inclusion rates of ~4.2% across a production cycle are standard in commercial formulations.

Dietary Ca (and to a lesser extent P) play a contradictory role in the availability of other nutrients due to a high acid buffering capacity (Lawlor et al. 2005), which may impede the initial hydrolysis of proteins and later bind to proteins in the small intestine at luminal pH above 6.5 (Grynspan & Cheryan 1989). Of the common ingredients used to provide a source of Ca, limestone exhibits the greatest acid buffering capacity (Lawlor et al. 2005). Furthermore, Selle et al. (2009) report on the tendency for Ca to bind to phytate-P *in vivo*, forming complexes with implications for the digestibility of both nutrients. *In vitro* studies show that the solubility of phytate-P diminishes markedly as the ratio of Ca:phytate-P increases (Grynspan & Cheryan 1983). Other nutrients including lipids and microminerals may also be a target for Ca complexes as reviewed by Wilkinson et al. (2011).

That the critical need for dietary Ca and P to meet production needs is in disaccord with optimising feed utilisation is a challenge to achieving the dual targets of maximising egg production and feed economy. The concept of a nutrient-specific appetite in poultry has recently undergone review by Wilkinson et al. (2011) and shows potential to concomitantly provide adequate Ca to support production needs whilst also minimising the effect of Ca on nutrient digestibility. Thus, a specific appetite for Ca may be exploited by providing a separate Ca-rich source that can be selected and consumed in response to a physiological stimulation of appetite for Ca.

The inclusion of phytate-degrading phytase enzymes in poultry diets is pervasive and demonstrates great capacity to improve the availability of nutrients, principal among these being phosphorus and calcium. However, how the use of phytase may influence an appetite for Ca and consequently Ca intake from alternate sources are unknown. The objective of this study was to investigate for the presence of a Ca-specific appetite in laying hens offered diets containing diminishing levels of dietary Ca, and to evaluate the effect of phytase on Ca-specific appetite.

4.4 Materials and methods

4.4.1 Experimental design and dietary treatments

The study was conducted at Poultry Research Foundation, the University of Sydney, Camden Campus, and all experimental procedures conducted had approval from the University of Sydney Animal Ethics Committee, and were in accordance with the Australian Code for the care and use of animals for scientific purposes (National Health and Medical Research Council 2013).

A total of 144 Isa Brown (18 weeks old) were randomly selected and raised individually in battery (25 × 50 × 50 cm) cages (with a separate feeder and water nipple) for six weeks to facilitate individual weekly feed intake and daily egg production. Each hen was assigned a unique individual identification number. Each treatment group consisted of 24 birds (six replicates with four birds per replicate). Birds were offered *ad libitum* water and access to experimental diets comprised primarily of wheat and soybean meal that differed in total Ca with or without phytase. Each cage was equipped with a separate calcium grit feeder. Dietary treatments in this experiment consisted of:

1. 20 g/kg Ca;
2. 20 g/kg Ca with 3500 unit phytase/kg of diet;
3. 30 g/kg Ca;
4. 30 g/kg Ca with 3500 unit phytase/kg of diet;
5. 40 g/kg Ca; and
6. 40 g/kg Ca with 3500 unit phytase/kg of diet (Ronozyme HiPHos, DSM Nutritional Products).

Phytase was added on top of formulated nutrient specifications. Aside from differing Ca, and with and without phytase, all experimental diets were formulated to ensure nutrient specifications satisfied the recommended requirements of ISA Brown laying hens. The composition of the experimental diets fed to the birds during the experimental period is shown in Tables 4.1 and 4.2. The photoperiod regimen was 16 hours of light and eight hours of dark. The limestone used had an average particle size of 3.37 ± 1.02 mm, and an analysed Ca content of 384 g/kg and a Mg content of 10 g/kg.

Table 4.1 Ingredient composition of experimental diets (as is basis)

	Treatments					
Calcium (g/kg)	40	40	30	30	20	20
Phytase Added (±) / kg	0	3500	0	3500	0	3500
Phytase Activity U / kg	106	3588	99	3436	137	4061
Ingredients (g/kg)						
Wheat	207.9	207.9	260.7	260.7	313.5	313.5
Soybean Meal -48%	265.6	265.6	254.9	254.9	244.2	244.2
Soybean oil	38.9	38.9	24.2	24.2	9.5	9.5
Sorghum 9.2%	350	350	350	350	350	350
Limestone	101.1	101.1	74.0	74.0	46.9	46.9
Dical. Phos.	6.9	6.9	6.5	6.5	6.1	6.1
Salt	2.4	2.4	2.3	2.3	2.3	2.3
Sodium bicarbonate	2.5	2.5	2.5	2.5	2.5	2.5
L-Lysine HCl	0.8	0.8	1.0	1.0	1.1	1.1
DL-Methionine	2.2	2.2	2.1	2.1	2.1	2.1
L-Tryptophan	0.3	0.3	0.3	0.3	0.3	0.3
Threonine	0.3	0.3	0.3	0.3	0.4	0.4
Layer premix[a]	1	1	1	1	1	1
Celite	20	20	20	20	20	20
TOTAL	1000	1000	1000	1000	1000	1000

[a] Provided the following nutrients per kilogram of diet:
vitamin A, 10 000 IU; vitamin D, 2500 IU; vitamin E, 25 mg; vitamin K, 2.5mg; thiamine, 2.5 mg;
riboflavin, 5.0mg; pyridoxine, 3.5 mg; vitamin B12, 0.015 mg; niacin, 30.0mg;
pantothenic acid, 9mg; folic acid, 1.0mg; biotin, 0.10mg; Fe, 60.0mg; Zn, 60.0mg;
Mn, 50.0mg; Cu, 5.0 mg; I, 1.0mg;Co, 0.4mg;Mo,0.5mg;
Se, 0.2mg; apo-ester, 2.9mg; canthaxanthin, 3.1 mg; ethoxyquin, 25.0mg.

Table 4.2 Nutrient specifications of the experimental diets (as is basis)

		Treatments		
Calcium g/kg	Units	40	30	20
Phytase ±		±	±	±
Calculated				
AME	MJ/kg	11.5	11.5	11.5
Crude protein (N × 6.25)	g/kg	183	183	184
Crude fibre	%	2.1	2.1	2.2
Ca	g/kg	40.0	30.0	20.0
P	g/kg	4.8	4.8	4.8
npP	g/kg	2.5	2.5	2.5
Ca:npP	ratio	16.0	12.0	8.0
Cl	g/kg	2.0	2.0	2.0
Na	g/kg	1.7	1.7	1.7
SID ARG	g/kg	10.4	10.3	10.2
SID GLY	g/kg	5.0	5.1	5.1
SID SER	g/kg	8.0	8.0	8.0
SID GLY & SER	g/kg	11.6	11.6	11.7
SID HIS	g/kg	4.1	4.1	4.1
SID ILE	g/kg	7.1	7.1	7.0
SID LEU	g/kg	13.8	13.8	13.8
SID LYS	g/kg	8.5	8.5	8.5
SID MET	g/kg	4.4	4.4	4.4
SID CYS	g/kg	2.6	2.6	2.7
SID TSAA	g/kg	7.0	7.0	7.1
SID PHE	g/kg	8.3	8.3	8.3
SID TYR	g/kg	5.6	5.6	5.6
SID THR	g/kg	6	6	6
SID TRP	g/kg	1.9	1.9	1.9
SID VAL	g/kg	7.7	7.7	7.7

SID – Standard ileal digestible content.
npP – non-phytate phosphorus.

4.4.2 Performance assessment of experimental hens

Egg quality and feed conversion efficiency were assessed weekly over a six-week period. The body weights of all experimental birds were taken at the beginning and the end of the experimental period. Body weight change (g) was calculated. The feed conversion ratio (FCR) was calculated from daily egg production and weekly feed intake over six weeks to verify the feed efficiency of each group.

4.4.3 Quality assessment of experimental eggs

For quality assessment, each egg was weighed, the height and width were measured using a digital caliper. and broken on a flat glass slide for measuring the height of the thickest part of the albumen and yolk. Albumen height was measured using an albumen height gauge TSS (Technical Services and Supplies). Albumen and yolk width were measured using a digital caliper. Yolk height was measured by a tripod micrometer. Albumen and yolk were separated and weighed. Yolk colour was measured using a DSM Yolk Colour Fan. Eggshell thickness was measured using a digital caliper. The ratio of albumen and yolk was calculated by dividing the mean albumen weight by the yolk weight. Haugh unit (HU) values were calculated using the formula (Altuntas & Sekeroglu 2008):

$100 \times \log (h - 1.7 \times w^{0.37} + 7.6)$ where h = albumen height (mm), w = egg weight (g)

4.4.4 Chemical analysis

To estimate apparent metabolisable energy (AME), gross energy of feed and faecal output were determined using a Parr 1281 adiabatic bomb calorimeter (Parr Instrument Company, Moline, IL, USA), which was standardised with benzoic acid. The nitrogen concentration of samples was determined by the Dumas method using an FP-428 nitrogen analyser (LECO Corp., St Joseph, MI, USA) as described by Sweeney (1989). The mineral composition of the feed and faeces ash was determined by inductively coupled plasma optical emission spectrometry (ICP) using a PerkinElmer OPTIMA 7300 (PerkinElmer Inc., Waltham, MA, USA) following digestion with nitric acid and hydrogen peroxide.

4.4.5 Statistical analysis

Experimental data were analysed as a 2 x 3 factorial arrangement of treatments using the GLM procedure (SAS Inst. Inc., Cary, NC). The statistical model investigated the main effects of phytase inclusion, calcium level and the associated two-way interactions. A trio of individually housed hens served as the experimental unit. All data in the tables are presented as least squares means ± the standard error of the mean (LSM ± sem).

4.5 Results and discussion

The objective of this study was to firstly confirm the observation of the 1st experiment that decreasing dietary Ca intake resulted in an increase in consumption of a separate limestone grit. Approximately 38% of birds in this study did not consume limestone grit regardless of dietary Ca level and inclusion or not of phytase (Figure.4.1). This observation supports the findings of study 1, which suggest that appetite for Ca is not uniformly expressed across modern layers even when dietary Ca is limited. Egg breaking force decreased with decreasing dietary Ca level regardless of providing a separate limestone source. Secondly, the role of phytase addition in influencing a Ca-specific appetite in hens when provided with a separate limestone grit was investigated. Hens consuming diets containing background or added phytase (150 versus 3500 IU/kg diet) did not show different levels of a separate limestone grit consumption.

4.5.1 Production traits

The effect of Ca level and phytase of birds on performance and egg production of birds offered a separate Ca source are presented in Table 4.3.

Overall, there was no effect of decreasing dietary Ca on the total intake level of the separate limestone grit. This confirms that the birds did not uniformly choose to consume limestone from a separate feeder in sufficient quantities to reach or approximate their Ca requirement as had been reported by Wilkinson et al. (2014). According to Wilkinson et al. (2014), although the Ca-specific appetite has been demonstrated, not all birds respond to Ca deficiency equally, which is consistent with the results of this study. Birds need to perceive a Ca deficiency as well as associate the organoleptic properties of the separate source limestone with Ca. Hence, it would appear that learned behaviour is an important factor for this mechanism (Wood-Gush 1966; Taher et al. 1984). Despite the observation that birds did not consume extra limestone grit in response to decreasing dietary Ca levels, limestone grit intake as a proportion of total Ca intake was greater as dietary Ca levels decreased. Therefore, limestone grit Ca accounted for 21%, 69% and 83% of the total Ca intake for birds assigned to the 4%, 3% and 2% dietary Ca treatments respectively.

Decreasing dietary Ca levels and inclusion of phytase had no effect on egg production rate, feed intake, and FCR. Body weight (Table 4.3) was not affected by dietary treatments although there was a difference in percentage BW change between treatment groups over the course of the study. Birds offered 20g/kg had the lowest change in BW over the course of the experiment and were significantly lower ($P = 0.014$) than birds offered 40g/kg.

Neither the dietary Ca level or phytase affected laying performance indices ($P > 0.05$). This finding suggests that when offered a separate Ca source such as limestone grit, that 30 (g/kg) Ca in the diet is sufficient to supply hens' requirements for egg production and to maintain good laying performance. Pelicia et al. (2009) reported that increasing dietary levels of Ca (3.0-4.5%) did not influence laying performance. Similarly, Cufadar et al. (2011) reported no differences in egg production, feed intake and FCR when hens' diets were supplemented with 3.0%, 3.6% or 4.2% Ca. Similar findings as to Ca levels were obtained by Clunies et al. (1992), who worked with 3.5% and 4.5% dietary Ca. In our study, birds offered diets containing 30 g/kg dietary Ca had increased egg mass when compared with birds offered 20 g/kg ($P = 0.010$). Saafa et al. (2008) reported that an increase in dietary Ca levels to 40 (g/kg) improved egg mass. Araujo et al. (2005) worked with 3.5 to 4.2% Ca levels and noticed that EM increased with increasing dietary Ca levels.

Dietary phytase was included at a 'superdosing' rate to evaluate the response of a separate limestone grit intake to differences in total Ca:npP ratio. Calcium sensing in the lumen influences secretion of several hormones related to Ca homeostasis including parathyroid hormone and calcitonin, which may subsequently have implications for voluntary calcium intake (Tordoff 2001). Phytase has been shown to increase Ca digestibility through liberating phytate-bound Ca and thus may influence voluntary intake of Ca. The dietary phytase activity was 150 IU and 3500 IU per kg of diet for the unsupplemented and phytase supplemented diets respectively. However, there was no effect of phytase supplementation on limestone grit intake or other response variables related to bird performance and egg quality in this study. This suggests that separate consumption of a limestone source is not substantially influenced by phytate degradation and putative Ca and P availability. While Chapter 3 of this report suggests that there are consequences for reducing npP levels for egg quality and important interactions between npP and Ca level, in this study hens may not have been P limiting even as dietary Ca:npP increased. This observation is broadly in agreement with an investigation carried out at the University of Queensland (Li et al. 2016; 1UQ101) which found hens were not limiting at 1.5 g npP per kg of diet and no benefit was found to providing a source of phytase.

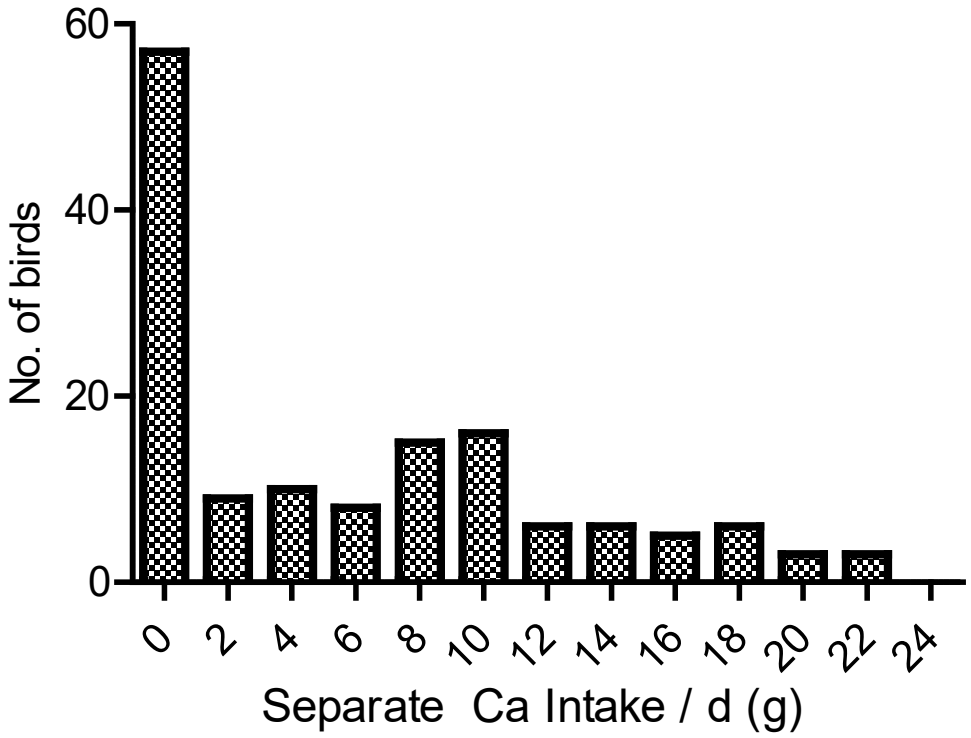

Figure 4.1 Separate Ca intake of the experimental hens

Approximately 38% of hens did not consume a separate limestone grit source over the six-week experimental period.

Table 4.3 Egg production of Isa Brown laying hens, taken as dietary treatment averages over the six-week experimental period

	Ca concentration (g/kg) of mixed ration						SEM [A]	*P*-Value		
Calcium %	**40**	**40**	**30**	**30**	**20**	**20**		**Ca**	**Phy**	**Ca × Phy**
Phytase (±)	-	+	-	+	-	+				
Hen day egg production(%)	91.0	95.3	95.9	95.8	90.7	91.8	2	0.060	0.259	0.487
Average plant-based diet intake (g/b/d)	115	115	115	118	113	112	2	0.322	0.704	0.709
Calcium Intake (from diet)	5.3	5.8	3.8	3.9	2.9	3.6	0.085	0.001	0.001	0.001
Limestone grit intake (g/b/d)	6.4	3.3	6.6	6.5	6.5	6.8	0.526	1.44		
Total feed intake (g/b/d)[B]	121	118	122	125	120	119	3	0.354	0.908	0.607
Total Ca intake (g/b/d)[C]	7.8	7.1	6.5	6.5	5.5	6.3	0.528	0.015	0.936	0.433
Separate Ca intake as a proportion of total Ca intake (%)	27	15	71	67	91	75	13.5	0.001	0.335	0.914
FCR without limestone[D]	2.25	2.01	1.99	2.03	2.23	2.21	0.096	0.099	0.338	0.323
FCR with limestone[D]	2.38	2.07	2.10	2.12	2.37	2.33	0.098	0.078	0.203	0.198
AME	13.2	13.5	13.7	13.7	13.5	13.6	0.36	0.572	0.609	0.923
Egg weight (g)	57	60	60	61	57	56	1.4	0.001	0.721	0.526
Egg mass (g/b/d)	52	57	58	59	52	51	1.8	0.013	0.315	0.421
Starting body weight (g)	1890	1857	1857	1893	1854	1862	27	0.871	0.791	0.459
Final body weight (g)	1952	1930	1924	1936	1859	1881	31	0.052	0.873	0.762
Body weight change (%)	+3.29	+3.96	+3.69	+2.24	+0.37	+1.16	1.0	0.014	0.998	0.460

[A] Pooled standard error of mean of Separate Ca feeding treatments.
[B] Combined mixed diet and separate Ca source intake.
[C] Combined Ca from mixed diet and Ca concentration of separate Ca source.
[D] Feed Conversion Ratio (FCR) calculated as per unit egg mass.

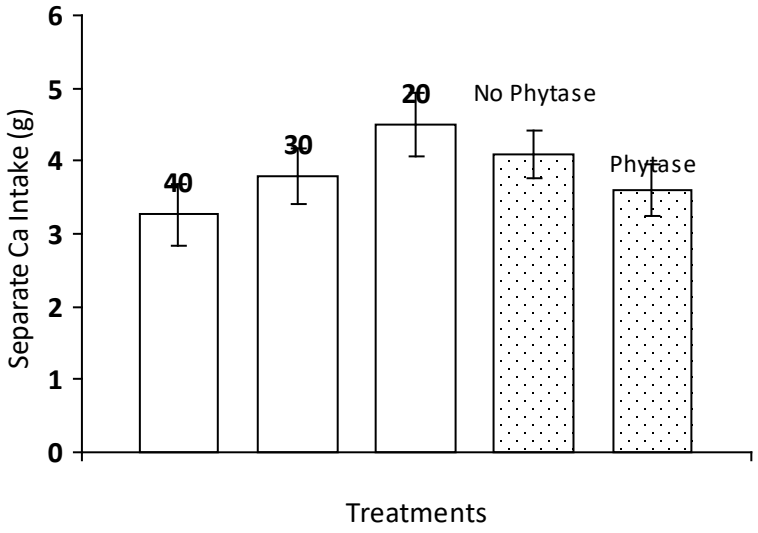

Figure 4.2 Limestone intake of birds offered varying levels of dietary Ca and inclusion or not of phytase

Birds that did not habituate to grit consumption have been excluded from this graph.

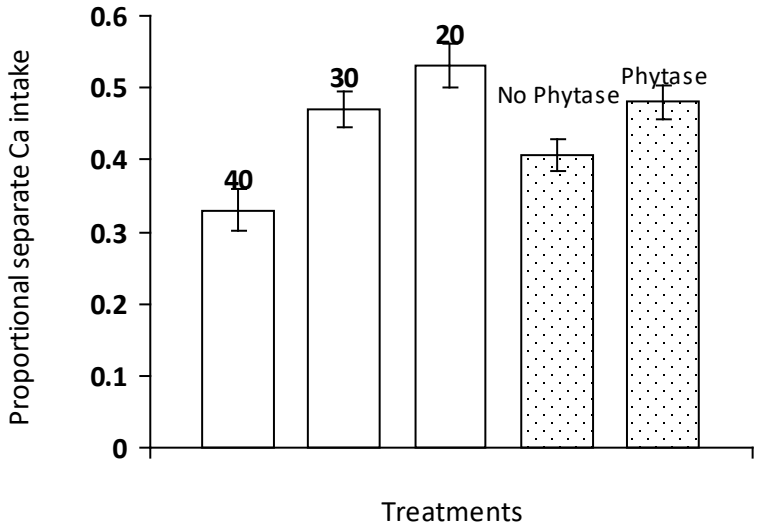

Figure 4.3 Separate Ca intake as a proportion of total Ca consumption of birds offered varying levels of dietary Ca and inclusion or not of phytase

Birds that did not habituate to grit consumption have been excluded from this graph.

4.5.2 Egg quality

Egg quality was broadly improved with increasing dietary Ca level, particularly in regard to eggshell quality. There was no effect of phytase inclusion on egg quality in this study. The 4% Ca had increased egg weight compared with the 2% Ca group (P = 0.003) while the egg weight for the 30 g/kg Ca group was intermediate. Clunies et al. (1992) reported that the shell weight of eggs increased by increasing the Ca level (from 3.5 to 4.5%) in the diet. This finding suggests that at lower levels of dietary Ca, consumption of the limestone grit was not sufficient to prevent a decrease in egg weight.

No significant effects ($p > 0.05$) of dietary Ca and phytase levels, nor of the interaction between Ca and phytase levels were observed on Haugh unit (Table 4.4).

The present study shows that the yolk colour score of eggs was increased with increasing dietary Ca levels. Albano Jr et al. (2000) reported that 2% and 6% dietary Ca reduced yolk colour intensity as compared to 3%, 4%, and 5% Ca. This finding reveals that there is an optimal Ca level, between 3% and 5%, for yolk colour intensity in commercial layer production. Hurwitz (1987) indicated that Ca regulates some important biological processes, such as the transference of cell information, hormone biosynthesis and release, and cell replication and differentiation. The higher total Ca levels resulting from higher dietary Ca levels may have significant influence on metabolic processes of birds that resulted in the greater yolk colour score observed in this study.

Eggshell weight, as well as eggshell weight percentage, was affected by the Ca levels such that birds consuming 30 and 40 g/kg dietary Ca had increased eggshell percentage as compared to 20 g/kg, indicating that positive responses were obtained as dietary Ca levels increased. The increase in eggshell percentage as dietary Ca level increased, observed in the present experiment, may have resulted from the possible increase in eggshell Ca content. This outcome is in agreement with the results of Casartellli et al. (2005), but not with those of Oliveira (2001) or Lichovnicova (2007). Keshavarz and Nakajima (1993) have demonstrated a positive correlation between the dietary Ca level and its retention into the body and eggshell quality. Similarly, Attech and Lesson (1983) reported that the quality of eggshell improves when the amount of Ca in the diet increases. The results of this study confirm that the supplementation of 30 g/kg Ca in layer diet improves egg quality traits and therefore provides more benefits to egg producers. Consumption of limestone grit did not result in equivalent eggshell weight or percentage between dietary Ca levels.

There were significant effects ($P < 0.05$) of Ca levels on eggshell thickness and breaking strength, as shown in Table 4.4. Rodrigues et al. (2005) found that 3.5% dietary Ca increased eggshell thickness as compared to 2.0% Ca, whereas Rodrigues (1995) did not observe any effect of Ca levels on eggshell thickness. Similar to these findings, other reports revealed improved eggshell quality at higher concentrations of Ca in diet (Roland et al. 1985; Hartel 1989; Lim et al. 2003). Some reports also indicated that by increasing the amount of Ca in the diet, shell thickness and egg breaking strength increases (Hill et al. 1998; NRC 1994; Scott et al. 1999). In the present study, significant differences in egg breaking strength were detected in birds fed different levels of Ca. This outcome is supported by Jiang et al. (2013) who noted that layers on a diet with 2.6% Ca had a lower egg breaking strength than those on a diet with 3.7% or 4.4% Ca, while Cufadar et al. (2011) did not find any significant effects of dietary Ca levels on eggshell breaking strength and eggshell thickness. The present study showed a linear effect in the egg breaking strength in terms of eggshell thickness and percentage of shell weight (Figure 4.4 and 4.5), which indicated that egg breaking strength and thickness as well as egg breaking strength and thickness linearly increased as dietary Ca intake increased. Importantly, provision of a separate limestone grit source did not preserve egg thickness or egg breaking force as dietary Ca levels decreased.

The levels of phytase and the interaction between dietary Ca concentration and phytase supplementation did not influence ($P > 0.05$) the eggshell quality parameters (shell weight, shell thickness and egg breaking strength). Gordon and Roland (1997) and Punna and Roland (1999) reported significant improvement in shell quality in layers fed phytase supplemented diets. In this study, there was little evidence to suggest phytase impacted egg production and egg quality, and therefore dietary P may not have been limiting for production needs. This study was conducted on early lay hens and a response to phytase may be more evident in latter stages of egg production when FCR worsens and egg quality deteriorates.

Table 4.4 Egg production of Isa Brown laying hens, taken as dietary treatment averages over the six-week experimental period

	Ca concentration (g/kg) of mixed ration						SEM	*P*-Value		
Calcium g/kg	**40**	**40**	**30**	**30**	**20**	**20**		**Ca**	**Phy**	**Ca × Phy**
Phytase (±)	-	+	-	+	-	+				
Average egg production per bird (n)										
Egg weight (g)	63.9	64.7	62.4	64.0	61.3	58.9	1.174	0.003	0.981	0.195
Egg height (mm)	56.9	57.1	56.4	56.4	56.7	56.1	0.511	0.432	0.786	0.752
Egg width (mm)	44.2	44.8	44.0	44.5	43.5	42.7	0.358	0.001	0.701	0.092
Shape index	77.8	78.6	78.0	79.0	76.8	76.1	0.959	0.078	0.662	0.613
Yolk height (mm)	16.9	17.3	17.1	16.3	17.1	18.4	0.629	0.260	0.568	0.265
Yolk width (mm)	40.0	39.6	38.6	39.5	39.3	38.9	0.316	0.027	0.924	0.069
Yolk weight (g)	15.4	15.4	14.9	15.3	14.9	14.3	0.269	0.010	0.784	0.165
Yolk colour	11.8	11.7	11.2	11.6	10.9	11.1	0.180	0.002	0.279	0.476
Yolk index	42.3	43.7	44.4	41.4	43.6	47.4	1.663	0.228	0.598	0.130
Albumen height (mm)	9.8	10.4	9.7	9.6	9.8	9.6	0.399	0.449	0.861	0.496
Albumen width (mm)	68.9	67.2	68.7	70.1	67.5	64.7	1.226			0.217
Albumen index	14.3	15.5	14.2	13.8	14.7	14.8	0.778	0.450	0.643	0.527
Albumen weight (g)	37.9	39.2	37.6	38.7	37.3	35.3	0.967	0.053	0.883	0.168
Dry eggshell weight (g)	6.6	6.8	6.5	6.3	5.8	5.7	0.174	0.001	0.874	0.614
Dry eggshell weight %	10.4	10.5	10.4	9.9	9.4	9.7	0.255	0.006	0.866	0.220
Dry eggshell thickness (mm)	0.40	0.41	0.39	0.38	0.34	0.34	0.006	0.001	0.892	0.737
Haugh unit	97.6	100.1	98.0	97.4	97.4	96.4	1.788	0.555	0.842	0.570
Egg breaking strength (g)	4696	4829	4471	4499	4158	4157	174	0.009	0.699	0.917

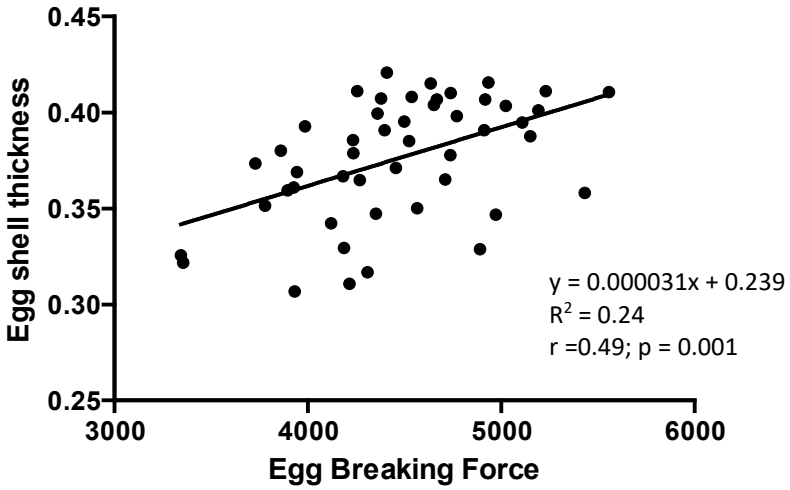

Figure 4.4 Association between average eggshell thickness (mm) and egg breaking force (g)

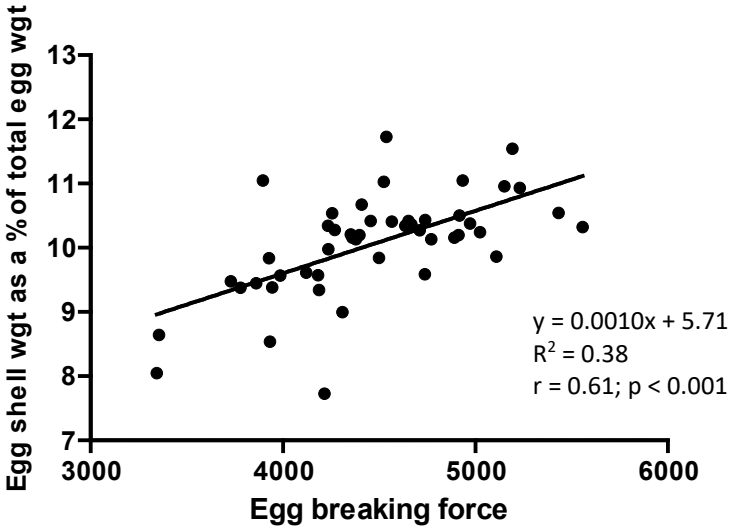

Figure 4.5 Association between eggshell weight as a % of total egg weight and egg breaking force (g)

Table 4.5 Pearson (r) correlation coefficients for associations between various intake measurements and egg quality and bird performance indices in ISA Brown hens

	Egg quality measurements							Bird productivity		
	Egg weight	**Eggshell thickness (mm)**	**Eggshell breaking force**	**Haugh unit**	**Albumen: Yolk**	**Yolk colour**	**Egg Mass**	**Feed conversion ratio (including grit consumption)**	**Egg Production (%)**	**Final Body weight**
Average daily feed intake (g)	0.52***	0.38 **	0.14 NS	0.14 NS	-0.18 NS	0.42**	0.45 **	0.23 NS	0.39 **	0.65***
Total Calcium intake (dietary and grit sources)	0.37**	0.30*	0.25†	-0.34*	-0.27†	0.34*	0.36*	0.17 NS	0.38**	0.33*
Grit Ca intake	0.11 NS	0.05 NS	0.11 NS	-0.44**	-0.24 NS	0.05 NS	0.14 NS	0.19 NS	0.24 NS	0.07 NS
Dietary Ca intake	0.32*	0.71***	0.35*	0.16 NS	-0.07 NS	0.56***	0.25†	0.09 NS	0.21 NS	0.49***

† $P < 0.1$.
Asterisks indicate significant correlation coefficients.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4.5.3 Conclusion

In summary, the results of this study show that limestone consumption was not uniformly adopted in hens offered diets differing in Ca levels, with or without phytase. Consequently, reducing dietary Ca resulted in compromised egg quality particularly as this relates to eggshell quality. Limestone grit consumption was not practised by all hens regardless of dietary Ca level or phytase inclusion. Of the birds that did consume limestone grit over the course of the study, a clear increase in limestone grit was observed in response to decreasing dietary Ca levels. Hens offered diets containing 30 g/kg Ca had similar performance and egg quality to those offered 40 g/kg Ca when provided with a separate limestone source.

4.6 Acknowledgements

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6 Appendix 1

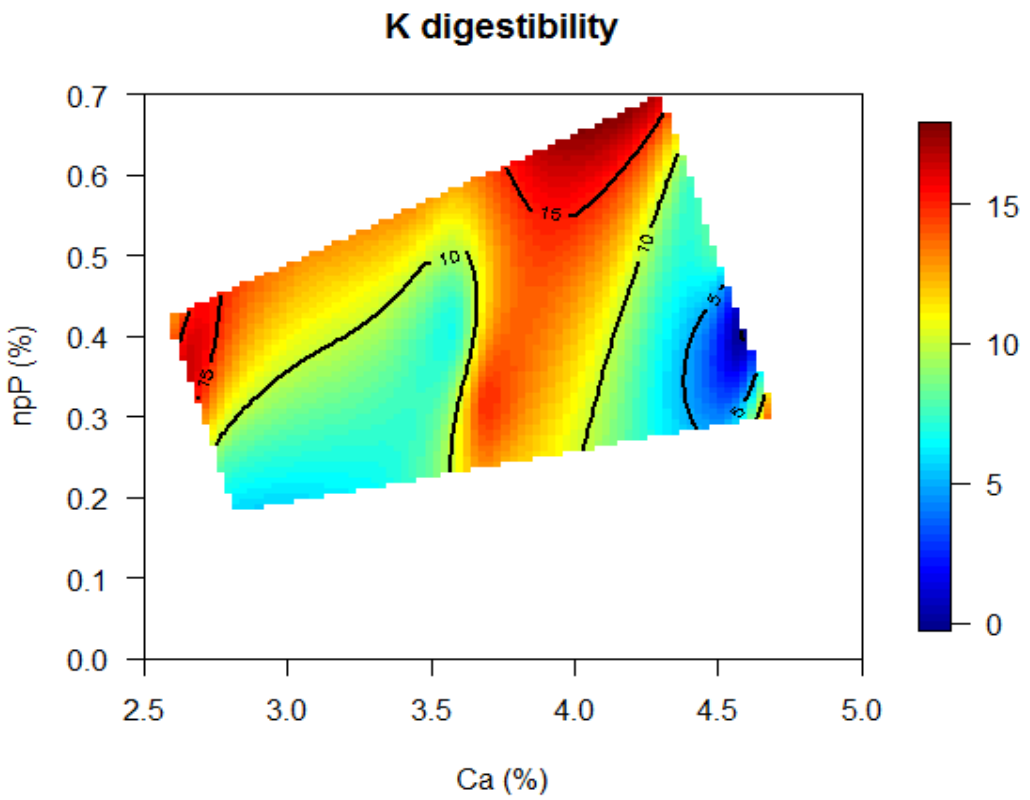

Figure 6.1 The effect of dietary total Ca and npP on K digestibility of laying hens

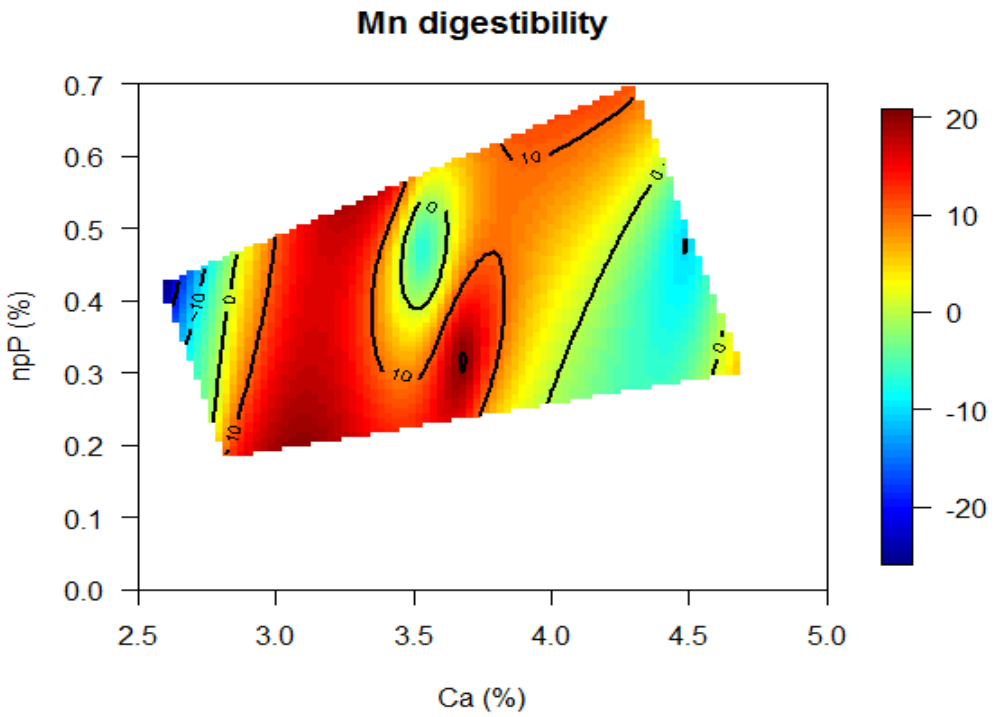

Figure 6.2 The effect of dietary total Ca and npP on Mn digestibility of laying hens

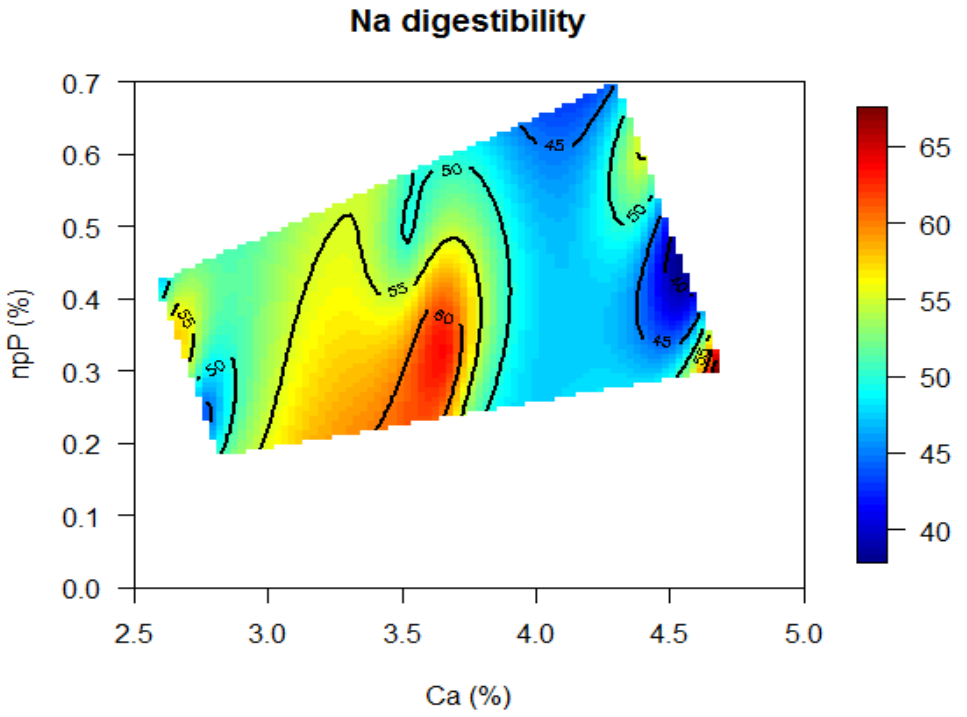

Figure 6.3 The effect of dietary total Ca and npP on Na digestibility of laying hens

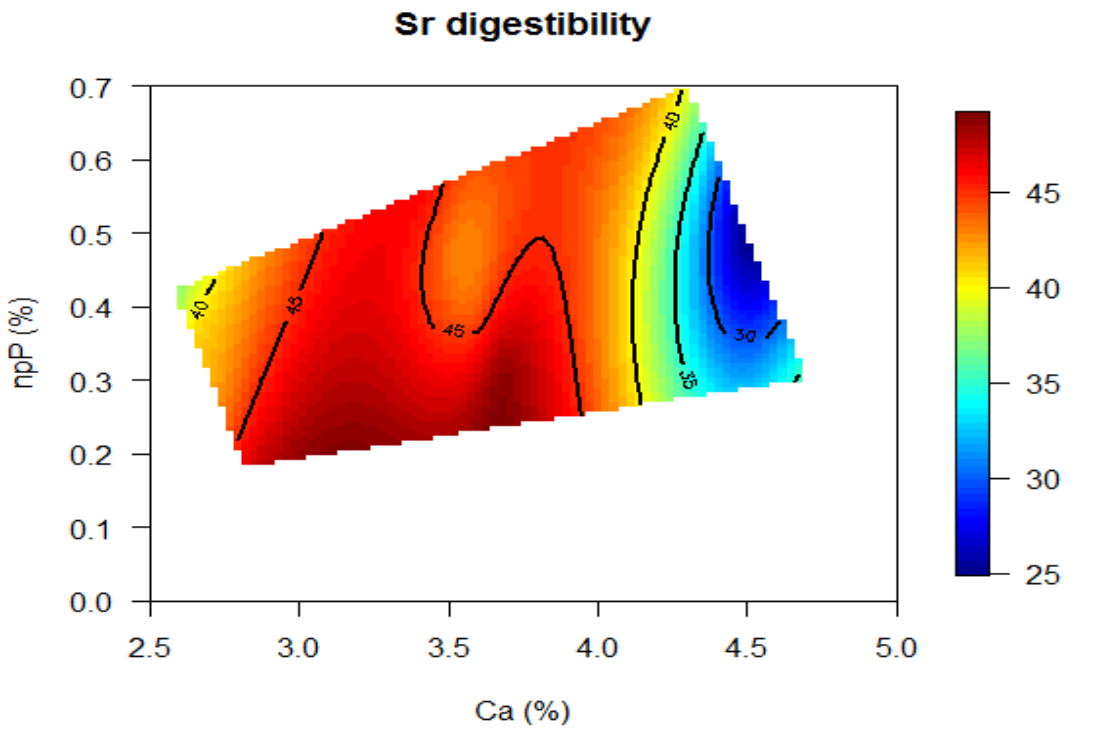

Figure 6.4 The effect of dietary total Ca and npP on Sr digestibility of laying hens

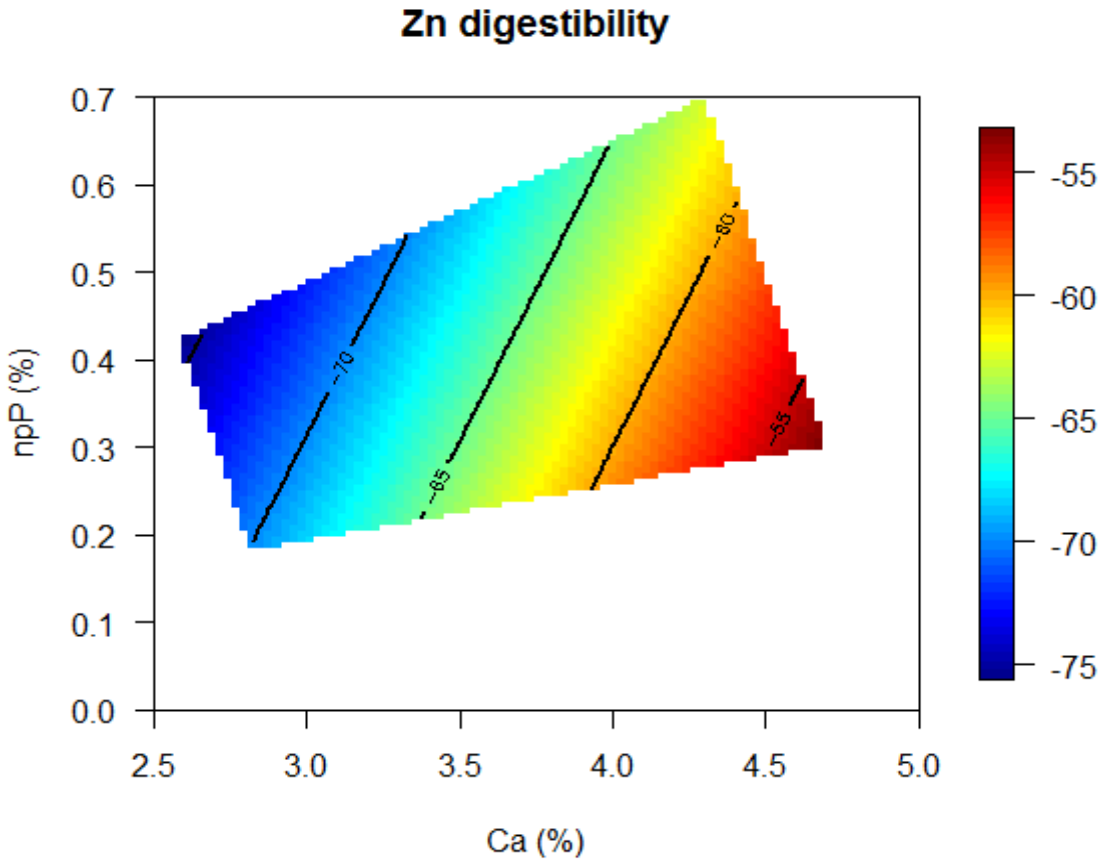

Figure 6.5 The effect of dietary total Ca and npP on Zn digestibility of laying hens

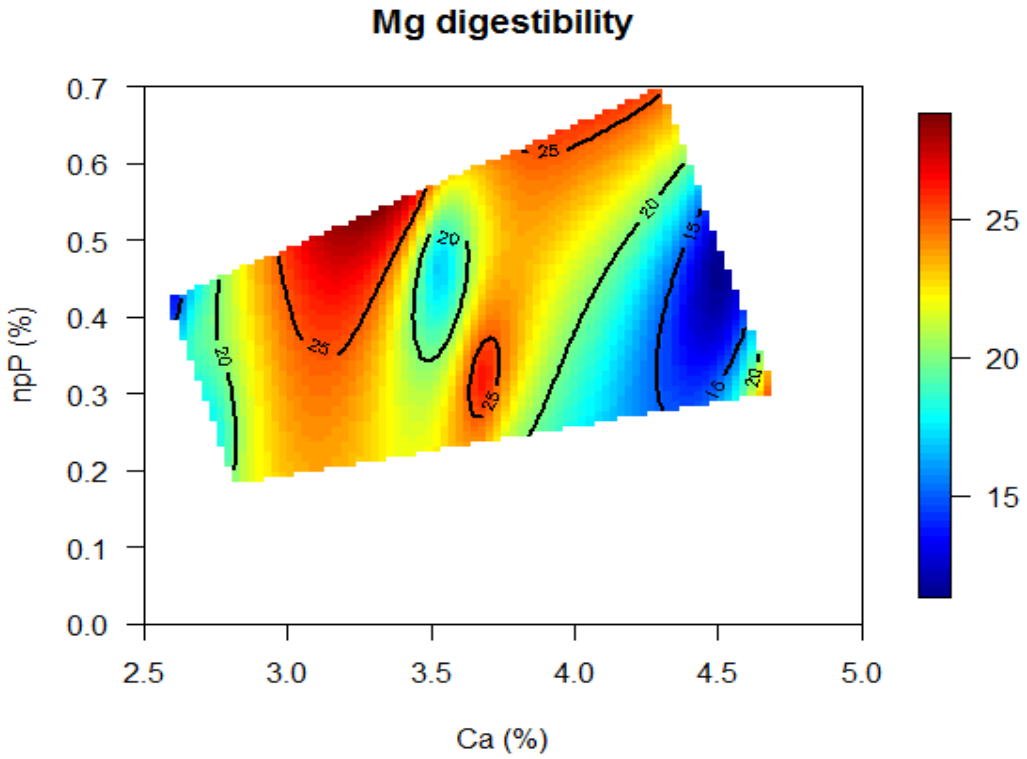

Figure 6.6 The effect of dietary total Ca and npP on Mg digestibility of laying hens

7 Plain English Summary

Project Title	Separate feeding of calcium for poultry
Australian Eggs Limited Project No.	1US112
Researchers Involved	C.J. O'Shea, S. Liu, S. Wilkinson, Y. Akter, H.H. Truong, Y. Bao, E. Bradbury, and A. Cowieson
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Objectives	Evaluate the appetite for a separate Ca source in laying hens and effect on bird performance and egg quality. Identify optimum dietary Ca and non-phytate phosphorus levels in laying hens for bird performance and egg quality.
Background	High levels of dietary Ca are required for maximum egg production and quality, but conversely high Ca buffers the pH of the digestive tract and impairs maximum nutrient absorption. Providing a separate source of dietary Ca may allow for greater digestion and retention of nutrients from a balanced ration.
Research	Three studies were conducted: 1. Ca-specific appetite in hens using limestone grit. 2. Optimum Ca and non-phytate P to maximise bird performance and egg quality. 3. Investigating the influence of decreasing dietary Ca and phytase inclusion on a limestone grit consumption in hens.
Outcomes	The results of this study were as follows: 1. Limestone grit consumption is not uniformly adopted in laying hens regardless of dietary Ca level. Providing a source of limestone grit does not prevent against poorer egg quality as dietary Ca levels decrease. 2. A range of dietary Ca and non-phytate P levels were evaluated. Maximising dietary Ca resulted in improved egg quality but it was important to increase dietary non-phytate P also to ensure the ratio remains narrow. 3. Separate limestone grit consumption was not influenced by superdosing phytase in laying hens.
Implications	Limestone grit consumption is not uniformly practiced in laying hens and will not prevent egg quality deterioration as dietary Ca levels decrease.
Key Words	Calcium phosphorus phytate appetite egg hen
Publications	Study 1: Forms part of Dr Emma Bradbury's PhD thesis Study 1: Submitted to Journal of Applied Poultry Science Study 2: Presented in part at APSS 2016

Study 2: In preparation for submission to Animal
Study 3: In preparation for submission to British Poultry Science