MASTER THESIS

Theme: The study of interaction between Calcium and Phosphorus and their effect on the performance of young broilers

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Je dédie ce mémoire de Master

A Nadia
A mes très chers parents

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Abstract

This research was conducted to determine the optimum calcium and phosphorus levels, and the physico-chemical characteristics of different Ca sources (solubility and buffering capacity) as likely factors on the growth performance, Ca and P retention and digestibility, and tibia ash content in broiler chicks from 0 to 14 days.

In order to achieve this main objective, a set of three experiments were designed. **The first experiment** (*in vivo)* was designed to study the interaction between different levels of Ca and non phytic P (NPP) on the productive performances, Ca and P retention and tibia parameters. During this experiment 420 *Ross* broiler male chicks were distributed into 60 battery brooders cages from 0 to 14 days and submitted to 12 treatments (5 replicates/ treatment) with different levels of Ca (0.5, 0.7, 0.9%) and NPP (0.25, 0.31, 0.38, 0.45%) with 1150 U/kg of phytase. The results obtained show a significant interaction between the level of Ca and NPP on ADFI, tibia weight and ash ($P<0.05$). Increasing the level of NPP from 2.5 to 3.8 g/kg increased the ADFI ($P<0.05$) on chickens fed the high Ca diet (9 g/kg) compared to Ca (7 and 5 g/kg). Broilers were able to achieve their maximum growth and bone formation with the calcium level 0.7% Ca and 0.38% NPP level / Kg. The increase on dietary Ca decreased its fractional retention from values close to 74% with diet 5 g Ca/kg to 46% with diet 9 g Ca/kg. The increase on the levels of dietary P steadily increased the fractional retention of Ca from 53% to 61%, and increased the whole-body Ca content (g/kg BW).

The second experiment was an *in vitro* trial that was used to compare the solubility and acid binding capacity of different calcium sources (Ca carbonate, Ca chloride and Lipocal, a fat encapsulated source of tricalcium phosphate) and levels of calcium in the absence and presence of phytic acid at different pH values. The results showed that Ca chloride has the highest Ca solubility and the lowest ABC as compared to the rest of Ca sources.

The third experiment was an *in vivo* trial, in which 300 *Ross* broiler male chicks were distributed into 60 battery brooders cages , and submitted to 12 treatments (5 replicates/ treatment) which differed in the levels of NPP (0.3, 0.35, 0.4, 0.45% NPP) and also on the dietary calcium sources (Ca carbonate, Ca chloride and Lipocal). The

birds performance **(**ADFI, BW and ADG) was not influenced by the Ca sources and NPP level interaction (P>0.05). Dietary source of Ca influenced ADFI (P<0.05), and ADG (P<0.01) from day 0 to 14. The ADG and BW on day 14 was higher in birds fed Lipocal and Ca carbonate than birds fed Ca chloride (P<0.01). Tibia weight was the highest in birds fed Lipocal at 4 g NPP/kg, and Ca carbonate at 3.5 g NPP/kg; and was the lowest for treatments including Ca chloride in the diet and 3.5 and 4 g NPP/kg. On the other hand, birds fed Ca chloride showed the highest Ca ileal digestibility as compared to birds fed Ca carbonate and Lipocal. Calcium ileal digestibility was also progressively increased with higher levels of NPP, being significantly higher in birds fed 4.5 g NPP/Kg than 3g NPP/Kg. Phosphorus ileal digestibility was influenced by the level of NPP, being the highest with the level 4.5g NPP/Kg and the lowest with 3 g NPP/ Kg .

It can be concluded that a dietary level of 3.8 g NPP /Kg in diets containing an overdose of phytase, and a calcium level of 7g Ca/kg are adequate to ensure a good growth and bone formation of broilers from day 0 to day 14. Higher levels of Ca or the use of high-soluble sources of Ca may determined early decreases on feed intake with negative responses on the bird performance and bone mineralization.

Key Words: Calcium, Phosphorus, *in vitro*, Tibia, Solubility

Resumen

Esta investigación se llevó a cabo para determinar el óptimo nivel de calcio y fósforo, así como las características físico-químicas de las diferentes fuentes de Ca (solubilidad y capacidad tampón) como factores que influyen en el crecimiento, la digestibilidad ileal y la retención de Ca y P y el contenido de cenizas de la tibia en pollitos de 0 a 14 días.

Para alcanzar este objetivo principal, diseñamos una serie de tres experimentos. **El primer experimento** (in vivo) fue diseñado para estudiar la interacción entre los diferentes niveles de Ca y P no fítico (NPP) en los rendimientos productivos, la retención de Ca y P, y los parámetros de la tibia. Durante este experimento 420 pollitos Broiler machos *Ross* se distribuyeron en 60 jaulas de batería del Día 0 a 14 de vida. Los animales estuvieron expuestos a 12 tratamientos (5 repeticiones / tratamiento) con diferentes niveles de Ca (0.5, 0.7, 0.9%) y NPP (0,25, 0,31, 0,38, 0,45%) con 1150 U / kg de fitasa en el pienso. Los resultados obtenidos muestran una interacción significativa entre el nivel de Ca y el NPP en ADFI, peso y cenizas de la tibia ($P < 0,05$). El aumento del nivel de la NPP 2,5 a 3,8 g / kg aumentó el ADFI ($P < 0,05$) en los pollos alimentados con la dieta alta en Ca (9 g / kg) en comparación con Ca (7 y 5 g / kg). Los pollos fueron capaces de alcanzar su máximo crecimiento y la formación dehueso con el nivel de calcio 0,7% Ca y 0,38% de nivel / Kg NPP. El aumento de Ca en la dieta disminuyó su retención corporal en valores cercanos al 74% con la dieta de 5 g / kg de Ca a 46% con la dieta de 9 g / kg de Ca. El aumento en los niveles de P dietético aumentó de forma constante la retención corporal de Ca del 53% al 61%, y aumentó el contenido de Ca en todo el cuerpo (g / kg PV).

El segundo experimento fue un ensayo *in vitro,* que se utilizó para comparar la solubilidad y la capacidad de unión de ácido de diferentes fuentes de calcio (Ca carbonato, cloruro de Ca y Lipocal, una fuente encapsulada de grasa de fosfato tricálcico) y niveles de calcio en la ausencia y presencia de ácido fítico a diferentes valores de pH. Los resultados mostraron que el cloruro de Ca tiene la solubilidad de Ca más elevada, y el ABC más bajo en comparación con el resto de las fuentes de Ca.

El tercer experimento fue un ensayo *in vivo*, en el que 300 pollitos Broiler machos *Ross* se distribuyeron en 60 jaulas de batería de 0 a 14 dias, y se sometieron a 12 tratamientos (5 repeticiones / tratamiento) que diferían en los niveles de NPP (0.3, 0.35, 0.4, 0.45% de NPP) y también por las fuentes de calcio en la dieta (Ca carbonato, cloruro de Ca y Lipocal). El rendimiento de las aves (ADFI, BW y ADG) no fue modificado por la fuente de Ca y la interacción con el nivel de NPP (P> 0,05). La fuente de Ca afectó el ADFI (P <0,05), y ADG (P <0,01) desde el día 0 a 14. El ADG y BW en el día 14 fue mayor en las aves alimentadas con Lipocal y Ca carbonato que las aves alimentadas con cloruro de Ca (P <0,01). El Peso de la tibia fue el más alto en los pollos alimentados con Lipocal a 4 g NPP / kg, y carbonato de Ca de 3,5 g NPP / kg, y fue el más bajo para los tratamientos, incluyendo cloruro de Ca en la dieta con el 3,5 y 4 g NPP / kg. En cambio, aves alimentadas cloruro de Ca mostró la mayor digestibilidad ileal de Ca en comparación con las aves alimentadas con carbonato de Ca y Lipocal. La digestibilidad ileal del Calcio también se aumentó progresivamente con mayores niveles de NPP, siendo significativamente mayor en las aves alimentadas con 4,5 g NPP / Kg que de 3 g NPP / Kg. El nivel de NPP también incrementó la digestibilidad ileal del P, alcanzando los valores más elevados con 4,5 g NPP / Kg y el más bajo con 3 g NPP / Kg.

Se puede concluir que un nivel de 3,8 g de NPP / Kg en las dietas que contienen una sobredosis de fitasa, y un nivel de calcio de 7 g Ca / kg son suficientes para garantizar un buen crecimiento y formación ósea de pollos de broiler del día 0 al día 14. Los niveles más altos de Ca o el uso de fuentes de solubilidad alta de Ca pueden producir primeros reducciones en el consumo de alimento con respuestas negativas sobre el rendimiento de las aves y la mineralización ósea.

Palabras clave: Calcio, Fósforo, *in vitro*, Tibia, Solubilidad

Resumé

Cette recherche a été menée pour déterminer l'optimum de calcium et de phosphore, et les caractéristiques physico-chimiques des différentes sources de Ca (solubilité et la capacité tampon) en tant que facteurs influencent les performances zootechniques, la rétention et la digestibilité du Ca et du P, et le contenu en cendre dans le tibia des poussins de chair de 0 à 14 jours.

Afin d'atteindre cet objectif principal, une série de trois expériences a été conçue. **La première expérience** *in vivo* a été établie pour étudier l'interaction entre les différents niveaux de Ca et P non phytique (NPP) sur les performances productives, les paramètres du tibia et la rétention de Ca et P. Au cours de cette expérience 420 poussins Broiler Ross mâle ont été distribués dans 60 cages en batterie, et soumis à 12 traitements (5 répliqués / traitement) avec différents niveaux de Ca (0.5, 0.7, 0.9%) et NPP (0,25, 0,31, 0,38, 0,45%) avec 1150 U / kg de phytase. Les résultats obtenus montrent une interaction significative entre le niveau de Ca et NPP sur la consommation journalière de l'aliment (ADFI), le poids et la teneur en cendres du tibia ($P < 0,05$).

L'augmentation du niveau de NPP de 2,5 à 3,8 g / kg a augmenté l'ADFI ($P < 0,05$) pour les poussins nourris au régime riche en Ca (9 g / kg) par rapport aux autres niveaux de Ca (7 et 5 g / kg). Les poulets de chair ont réussi à atteindre leur maximum de croissance et de formation osseuse avec le niveau de 0,7% de Ca et 0,38% NPP/ Kg. L'augmentation du Ca dans la ration a réduit sa rétention corporel, en effet la fraction de rétention était de valeurs proches de 74% avec un régime alimentaire de 5 g Ca / kg et 46% avec le régime 9 g Ca / kg. L'augmentation des niveaux de P dans l'aliment, a constamment augmenté la rétention de la fraction de Ca de 53% à 61%, et a augmenté la teneur en Ca dans l'ensemble du corps (g / kg de poids vif).

La deuxième expérience a été un essai *in vitro*, qui a été utilisé pour comparer la solubilité et la capacité de liaison aux acides de différentes sources de calcium (carbonate de Ca, le chlorure de Ca et Lipocal qui est une source encapsulée de grasse de phosphate tricalcique) et les niveaux de calcium en absence et en présence de l'acide phytique à différentes valeurs de pH. Les résultats ont montré que le chlorure de Ca a la plus forte solubilité de Ca et le plus bas ABC (capacité de liaison aux acides) par rapport au reste des sources de Ca.

La troisième expérience était un essai *in vivo*, dans laquelle 300 poussins Broiler Ross mâle ont été distribués dans 60 cages en batterie de 0 à 14 jours, et soumis à 12 traitements (5 répliqués/traitement) qui diffèrent par les niveaux de NPP (0.3, 0.35, 0.4, 0.45%) et également par les sources de calcium alimentaire (carbonate de Ca, le chlorure de Ca et Lipocal). Les performances des animaux (ADFI, BW et ADG) n'ont pas été influencées par l'interaction entre les sources de Ca et les niveaux du NPP (P> 0,05). La source de Ca a influencé ADFI (P <0,05), et de l'ADG (P <0,01) du jour 0 à 14. L'ADG et BW à 14 jours était plus élevée chez les poulets nourris avec le Lipocal et le carbonate de Ca que ceux nourris avec le chlorure de Ca (P <0,01). Le poids du tibia était plus élevé chez les animaux nourris avec le Lipocal à 4 g NPP / kg et avec le carbonate de Ca à 3,5 g NPP / kg, et était le plus bas pour les rations contenant le chlorure Ca dans l'alimentation avec 3,5 et 4 g NPP / kg. En revanche, les poussins alimentés avec le chlorure de Ca ont montré la plus forte digestibilité iléale du Ca par rapport aux poussins nourris avec le Ca carbonate et le Lipocal. La digestibilité iléale du Ca a également augmenté progressivement avec des niveaux plus élevés de NPP, étant significativement plus élevée chez les oiseaux nourris 4,5 g NPP / Kg que ceux alimentés avec 3g NPP / Kg. La digestibilité iléale du P a été influencée par le niveau de NPP, étant la plus élevée avec 4.5g NPP / Kg et la plus basse avec 3 g NPP / Kg.
On peut conclure que le niveau alimentaire de 3,8 g NPP / kg dans des rations contenant une surdose de phytase, et un niveau de calcium de 7g Ca / kg sont suffisants pour assurer la bonne croissance et la formation des os de poulets broiler de 0 à 14 jours. Des niveaux élevés de calcium, ou l'utilisation de sources de haute solubilité de Ca peut provoquer des diminutions précoces sur la consommation de l'aliment, avec aussi des réponses négatives sur les performances des poussins et la minéralisation osseuse.

Mots clés: Calcium, Phosphore, *in Vitro*, Tibia, Solubilité

Abbreviations used

µm: microns

ABC: Acid binding capacity

ADFI: Average Daily Feed Intake

ADG: Average Daily Gain

AOAC: Association of Official Analytical Chemists

ATP: Adenosine triphosphate

BW: Body Weight

BWG: Body Weight Gain

Ca: Calcium

$CaCl_2$: Calcium chloride

$CaCO_3$: Calcium carbonate

FCR: Feed Conversion Ratio

meq: milliequivalents

N : Nitrogen

NPP: Non Phytic Phosphorus

P: Phosphorus

PTH: Parathyroid hormone

SBM: Soybean Meal

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Introduction

Calcium is, together with Phosphorus, the main mineral retained in the body of broilers (Brown, 2002). It is a structural component of bones, but also plays a role in many metabolic and functional aspects of the animal physiology. Calcium is considered a Type I nutrient (Emery, 2005) because it accumulates reserves in some tissues and mobilizes them in case of a deficient diet is provided. In the animal industry, Ca requirements in the diet have been measured following criteria to maximize performance and bone mineralization (values range from 0.9 to 1.1 total Ca% for starting chicken (FEDNA, 2008). As Ca is mainly stored in bones, calcium requirements for bone mineralization are usually higher than those established to optimize body weight gain (Driver et al, 2005b).

There are also two statements to take into account. First, Ca animal requirements have been usually measured using limestone as a Ca source in the diets (a low soluble source); and second, Ca is considered a low cost nutrient and low environmental impact compound. Then, little efforts have been done to optimize the use and availability of Ca sources. For instance, calcium requirements are always described on a total Ca basis.
However, calcium has been also related with numerous negative interactions in the digestive tract. It is well described that calcium may form soap precipitates with free saturated fatty acids, decreasing the energy digestibility of the diet and animal growth (Pepper et al., 1955; Edwards et al., 1960). Calcium may also precipitate mineral phosphates and phytic acid; reducing the activity of endogenous and exogenous phytase (Tamim et al., 2004). Thus, a marginal increase on the dietary Ca levels may be associated with a significant decrease on P availability (De kort et al., 2009). Other factors derived from the incorporation of mineral sources in the diet, such as the buffering capacity of mineral sources or the kosmotropic characteristics of ions, have been also related with significant decreases on the protein and P solubility in the gizzard, and may affect N and P digestibility (Tamim and Angel, 2003).

The young chick, by its immature gastrointestinal tract, in contrast to the adult hen, could be more sensitive to the level and properties of the Ca source in the diet. Coon and Manangi (2007) described that broilers increased weight gain with $CaCO_3$ particles sizes between 137 and 388 µm compared to the gains obtained by feeding either smaller (28 µm) or larger particle (1306 µm) size. In contrast, feeding large particle size Ca (3300-4700 µm) to layers and broiler breeders hens compared to small particle size (500-800 µm) resulted in a significant reduction on the P faecal excretion and an improvement in tibia ash. Larger particles with a lower solubility for adult birds may allow calcium to be retained for a longer time period in the gizzard, leading to more availability of a Ca source in the gut at the time of shell formation.

Therefore, different values for broilers requirements in phosphorus and calcium have been proposed by FEDNA, NRC, INRA and many other researchers and institutions. However, these values vary from one to another because of the multiple factors that may influence Ca availability of vegetable and mineral sources (pH, particle size, interaction between Ca and P) as well as the animal criteria adopted to be optimized (bone mineralization, feed efficiency, weight gain, feed efficiency). Moreover, phytase may increase phytate P and Ca availability, reducing P excretion. Inorganic P can be minimized to make poultry production cost effective and environmental friendly.

Consequently, in the present work we propose to study some of the factors, not all simultaneously, that affect the performance of broilers and the Ca and P retention. **The hypothesis will propose that a significant decrease on the level of Ca in the diet and the use of alternative sources with a higher or a lower Ca solubility, as compared to limestone, may improve broiler performance and bone mineralization** by reducing digestive interactions with the rest of the components of the diet (namely P, N and energy). The effects will depend on the average levels of Ca and P in the diet, but also on the main physico-chemical properties of the mineral sources used to fortify these diets. We propose to identify and characterize these interactions in this Master thesis by using a double *in vitro* and *in vivo* approach.

A. Literature Review

1. Importance of Calcium and Phosphorus for chicken broilers

1.1. Calcium and Phosphorus in few words

Calcium is, together with Phosphorus, the main mineral retained in the body of broilers (Table 1). It is a structural component of bones; but also plays a role on blood coagulation, adhesion of molecules, neural transmission, muscle contraction, cellular motility, differentiation and proliferation, hormonal secretions, and apoptosis (Brown, 2002). Therefore, calcium homeostasis becomes an important driving force in the maintenance of bone strength and function of the body. Low blood Ca levels stimulate secretion of PTH and vitamin D synthesis, which in turn activate release of bone minerals stores and mineral absorption.

In the poultry industry, Ca is mainly supplied with inorganic sources to reach Ca requirement level in the diet, which it has been up to now described in a total Ca basis. Calcium fortification of vegetable diets with calcium carbonate and calcium diphosphate or monophosphate, together with a proper supplementation of vitamin D, reduces the risks of Ca deficiency in birds. However, there is still controversy in relation to the proper Ca levels and sources to provide to the animals as well as the digestive factors that may affect Ca absorption (Perry et al., 1991).

Table 1. Calcium and Phosphorus content in the chicken whole body and bones (g/kg)

	Ca	P
Whole body (g/kg)		
[1]Hatching	3.4	3.3
[1]7 weeks	6.8	5.1
[2]Day 0	4.05	3.38
[2]Day 7	3.18	4.33
[2]Day 14	2.8	3.42
Tibia content (g/kg DM)		
[3]Day 35, Male	168	80
[3]Day 35, Female	165	78
[4]Day 2, male	193	83

[1]Larbier & Leclerq, 1992, [2]Olukosi et al., 2008, [3]Venäläinen et al., 2006; [4]Walk et al., 2012

Phosphorus is also a constituent of bones, nucleic acids, high-energy compounds (ie.-ATP), and phospholipids found in membranes. It is also involved in a variety of enzymatic reactions as well as in oxygen transport as a constituent of phosphoglycerate compounds (Drezner, 2002; Applegate and Angel, 2008). It plays a critical role in cellular metabolism as a part of the energy currency of cells, in cellular regulatory mechanisms, and in the bones structure.

As it occurs for Ca, bone is the main storage organ for P, but at a lower proportion of the whole body total P content (Table 1). Through its involvement in many metabolic and structural processes, P is essential for animals to attain their optimum genetic potential in growth and feed efficiency as well as skeletal development. Because of the key role of P in growth and bone development and mineralization, the requirements of the animals for P are the highest during the time the animal is growing (Applegate and Angel, 2004). Therefore, growing broilers usually requires P fortification of vegetable diets with calcium diphosphate or monocalcium phosphate.

1.2. The symptoms of a Ca and P deficiency in poultry

As cited above, calcium and phosphorus are the main minerals in the whole body; share a common storage in the bones structure, and highly affect each other during their absorption and metabolism (we´ll see later on). However, there are major differences between both minerals in relation to the consequences of a dietary deficit. In fact, Ca is considered a Type I nutrient (Table 2), while P is considered a Type II nutrient (nutrients are classified as either Type I or Type II based on the effect a deficiency has on the body).

Deficiency of Type I nutrients results in specific physical signs; such as it is anemia after Fe deficiency or scurvy after vitamin C deficiency (Emery, 2005). An animal respond to a deficiency on Type I nutrients by continuing the growth and consuming body stores with eventual reduction in the bodily functions. Diagnosis is simple by the symptoms, but also via measurement of the concentration of the nutrient itself in the whole body or storage tissues. Examples of other Type I nutrients, in addition to Ca, are Fe, Cu, Se and vitamins.

An animal respond to a deficiency on Type II nutrients by reducing growth and avidly conserving the nutrient to maintain the concentration of the nutrient in the tissues. The

animals reduce excretion to conserve the nutrient, and a reduction of appetite usually accompanies this condition. Individuals with a Type II deficiency are stunted in growth and have no visual signs or differences from ″normal″ individuals. Other examples of Nutrients Type II, in addition to P, are Nitrogen, essential amino acids, K, Na or Zn.

Table 2. Examples of Type I and Type II nutrients

Type I nutrients	Type II nutrients
All vitamins, most trace elements, **Calcium**	Nitrogen, sulphur, essential amino acids Potassium, Sodium, Magnesium, **Phosphorus**, Zinc,Water,Dietary sources of energy (including carbohydrate and fat)

(Emery, 2005)

Bone status is commonly used as an indicator of mineral adequacy in poultry diets. Well over 90% of Ca is found in the bones where it combines with P to form calcium phosphate crystals or hydroxyapatite with the molecular formula Ca_{10} $(PO_4)_6(OH)_2$ (Scott et al., 1982). Other elements including Na, Mg, Fe and Fl may also be incorporated into the hydroxyapatite crystal (Frandson and Spurgeon, 1992).

With this description, it is easy to understand that a deficient Ca diet affects bone mineralization and strength (Reichmann and Connor, 1977), and maybe associated with increased risk of fractures (Blake and Fogelman, 2002). The modern broiler chicken has been selected for rapid growth and increase muscle mass; but may also be associated with poor leg health and lameness due to reduced bone mineralization. Reducing Ca and P in the diet can also cause broken bones and bloody meat during processing of the carcass (Chen and Moran, 1995). In particular, bone breakage during catching and transportation create problems during processing (Gregory and Wilkins, 1992; Julian, 1998; Knowles and Wilkins, 1998). Broken bones, especially fractured clavicle bones, may find their way into the meat, and must be removed at great expense. Hemorrhages in the meat are another major quality defect, which can lead to downgrading of the broiler carcass. This is very significant due to the increased current importance of selling cut up chicken parts, in which the emphasis is no longer only on yield but also on characteristics such as bloody breast meat and broken bones (Gregory and Wilkins, 1990).

Because of the complex interaction among Ca, P, vitamin D, and other calcitropic hormones, it is necessary to judiciously balance the amount of Ca and P added in the poultry diet (Lundy et al., 1992; Rennie et al., 1997, Rath et al., 1999). The interactions of these two minerals are highly complex and are not easy interpreted. In the literature, Létourneau-Montminy et al. (2007, 2009) show the importance of the Ca / P non phytic (NPP) ratios on growth performance and bone mineralization of broilers from 1 to 21 days. Similarly Driver et al. (2005a) and Rama Rao et al. (2006) studied the effects of changes in Ca and P or NPP intake in chickens from 1 to 16 days and from 1 to 42 days, respectively. Their work demonstrates clearly that the Ca/P ratio has a greater impact on the quality and bone strength that the intrinsic level of each mineral.

The literature is also plenty of evidences of the consequences associated to a deficient level of Phosphorus in the diet. Results with moderate deficient levels of available P in the diet (for example by adding variable amounts of mineral sources of P or phytase) usually had greater effects on the rates of nutrient accumulation (growth) rather than on the proportion of nutrients deposited in the carcass (Olukosi et al., 2008). However, lower levels of the dietary available P are also related with pronounced decreases on bone mineralization (Walk et al., 2012a), as it reflects the percentage of ash in the tibia (Figure 1. Phillips et al., 2012)

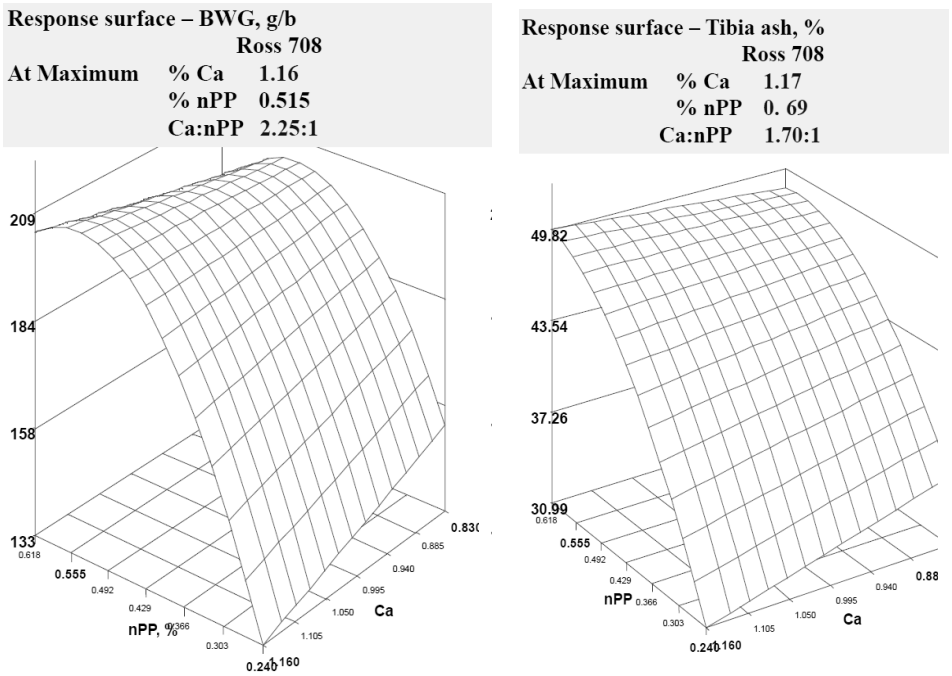


Figure 1. Response surface of the body weight gain (BWG, g/broiler) and tibia ash to different levels of non phytic P (nPP, %) and Ca (%) in the diet (Angel and al., 2008)

2. Phosphorus and Calcium requirements

The Ca and P requirements of domestic animals are usually discussed together as the requirement of each mineral depends on the concentration of the other in the diet. An excessive or deficient level of Ca or P in the diet often leads to a deficiency or excess of the other, which it is due to the interactions between the two minerals on Ca and P availability and endogenous excretion (Al Masri et al., 1995). We´ll see these interactions at more detail in section 6.

2.1. Calcium

As referred above, requirements of Ca have been established based on its effect on performance, but mainly on bone mineralization. They are described on a total Ca basis (Table 3), and no information is published in relation to digestible Ca requirements for broilers. There are different causes that may justify this apparent lack of interest on improving description on a digestible or available Ca basis. Calcium is considered, in contrast to phosphorus, a cheap nutrient, and its implication on environmental contamination is low. Moreover, absorption of Calcium is highly regulated as dependent on the levels of Ca and P in the diet.

It is generally assumed that Ca requirements levels include a wide security margin to supply Ca requirements. However, apart from the fact that inorganic Ca sources replace other ingredients in the diet, an excess on the levels of dietary Ca may interferes with the availability of other minerals, including phosphorus, magnesium, manganese, and zinc (NRC, 1994), as well as may form fatty acid soaps which affect the energy digestibility of diet (Pepper et al., 1955; Edwards et al., 1960). Calcium, provided as $CaCO_3$ may also increase the pH in the proximal segments of the gastrointestinal tract due to its high acid-binding capacity leading to a decrease in P and amino acid digestibility (Selle et al., 2009).

Table 3. Calcium and P requirements for starting Broilers

	FEDNA(2008)	NRC(1994)	INRA(1989)
Age(d)	0-15	0-21	0-15
Calcium (%)	0.95-1.05	1	1-1.1
Phosphorus	-	-	-
Total P (%)	0.65	-	0.67-0.70
Available P (%)	0.45	-	0.42-0.45
Non phytic P (%)	-	0.45	-
Ca /NPP	-	2.2:1	-
Ca /aP	2.11 to 2.33:1	-	2.3 to 2.4:1

Therefore, calcium requirements are affected by the criteria use to maximize or optimize. According to Driver et al (2005b), calcium requirements determined for 0- to 16-d-old chicks suggest that current NRC (1994) recommendations (1.0% Ca) are adequate for maximum bone ash but excessive for all other measured variables. Both, BWG and FCR were optimized at or below 0.625% dietary Ca, which may suggest that a lower total Ca concentration in general is desirable. Furthermore, significant sex differences were observed; males appeared to require more Ca than females to maximize tibia ash but less Ca to optimize weight gain (0.49% ±0.11 vs. 0.62%±0.18 for males and females, respectively). Calcium requirements for FCR were very similar for both males and females (0.63% ±0.40 vs. 0.61% ±0.19), respectively.

On the other hand, Calcium must be soluble in the medium of the gastrointestinal digesta before absorption. Limestone, which it is the main Ca source in the poultry industry, requires an acidic medium to reach a solubility of 80% (Walk et al., 2012c). This implies that Ca can be mostly solubilised in proventriculus and gizzard, but it becomes mostly insoluble in the small intestine. This may suggest that the current total Ca recommendation may be high, as they are associated to an intrinsically low available source. Recent reports suggest that provision of an alternative highly digestible Ca source at lower dietary concentrations may circumvent this problem (Bradbury et al., 2012).

2.2. Phosphorus

As we will see later, phosphorus in plants is present in different organic forms, such as phospholipids and proteins, but mostly as part of the phytic acid molecule. Phytic acid P is variably available to poultry (0 to 50%), which imply that inorganic P must be usually added to the diet in order to meet P requirements in birds (Applegate and Angel,2008). Phosphorus is also an expensive nutrient in the diet (aprox 150-300 $ per ton vs. 15-30 € per ton for Ca), and an environmental concern for the animal industry. Therefore, a higher effort has been done in order to improve and assure a minimum P availability in the diet as well as to avoid an excess of P in the diet and excreta. Historically, there has been a move from the use of total P (tP, NRC, 1950) to inorganic P (iP, NRC, 1954), available P (aP, NRC, 1984), and non phytic P (NPP, NRC, 1994).

Thus, the terms used to describe phosphorus requirements are:

a. - **Total P** (tP) is generally referred to as phosphorus and encompasses any and all forms of phosphorus in the diet. It doesn´t take into account differences on P availability.

b. - **Digestible P** (dP) refers to the P that is truly or apparently absorbed from the diet into the animal (i.e. feed P minus P within the distal ileum).

c. - **Available P** (aP) refers historically to the inorganic and animal based P + 30% of P from plant sources (Motzok et al., 1956).

d. - **Non phytic P** (nPP = Total P – phytate P) is the term used by NRC (1994) to define P requirements. By definition it is not the same that aP (some phytic P is available and some inorganic P not) but values are very similar to aP in corn-soy diets.

d.- **Retained P** refers to the P that stays in the body (i.e., feed P minus excreta P, Applegate and Angel, 2008). It highly depends on the P availability, but also on the levels of Ca and P in the diet (Al Masri ,1995).

Recent research has reported substantial differences in the non-phytate P (nPP) requirement of broilers compared with those published by the NRC (1994). Waldroup et al. (2000) reported that nPP requirement for the starter phase ranges from 0.37 to 0.39%.

Many studies have been realized to determine the need for phosphorous and calcium in broilers at different life stages, these studies are summarized in the following table.

Table 4. Different requirements of Ca and P for starter broilers depend on the used criteria.

Reference	Criteria	Age(wk)	Ca %	P %	nPP %	aP%	Ca:tP	Ca/nPP
Moran and Todd, 1994	Growth/ Bone ash	0-3	1.00	0.68	NS*	0.45	1.47:1	
Chen and Moran, 1995	Growth/ Bone ash	0-3	1.05	0.68	NS	NS	1.54:1	
Rao et al., 1999	Growth	3-30 d	1	NS	0.44	NS		2.27:1
Angel et al., 2000	Tibia/femur ash/strength	0-17 d	0.91		0.45-.037	NS		2.21:1
[1]Driver et al., 2005b	BWG	0-16d	0.48-0.62	0.74	0.45		0.76:1	1.08:1
	Tibia ash	0-16d	0.6-0.72	0.74	0.45		0.94:1	1.34:1
[2]Phillips et al., 2012	BWG	1-10d	1.16		0.515			2.25:1
	Tibia ash	1-10d	1.17		0.69			1.70:1

1. Variable levels of Ca were evaluated with a fixed level of nPP (0.45).

2. Variable levels of Ca and P were factorially analysed following surface analyse

*NS: Not specified

3. Calcium sources and availability

Except for some vegetable ingredients, such as rapeseed meal, vegetable feedstuffs are very low in Ca content (Figure 2) and therefore, provision of adequate dietary Ca supply is almost entirely achieved through the use of animal-based and inorganic feedstuffs whose Ca bioavailability is > 66% (NRC, 2005).

Little is known about Ca availability in vegetable feedstuffs, perhaps a consequence of the fact that most of these feedstuffs contribute so little Ca to the diet (NRC, 2005). It is known that 20–30% of Ca in plant tissues is bound to oxalate, which is relatively unavailable (NRC, 2001). Moreover, the high phytic content of some ingredients (such as rapeseed meal) make Ca low available. Based on Ca digestibility in corn soy diets with no added inorganic Ca and P sources, Tamim and Angel (2003) calculated an availability of Ca in the corn and SBM of the diet of 20 to 33%.

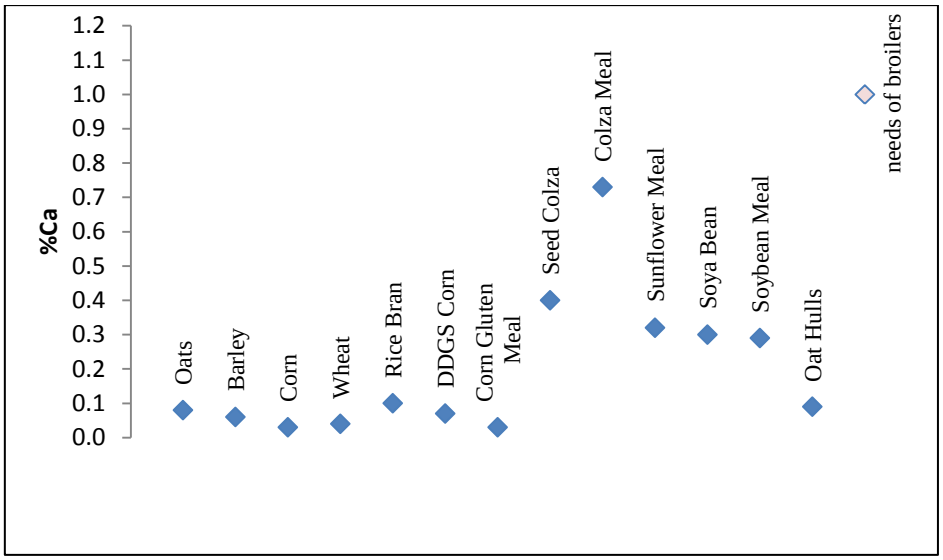


Figure 2.Calcium content in different cereal, vegetable protein and fibrous ingredients as compared to total Ca requirements in broilers (FEDNA, 2008)

Main used inorganic sources for Ca in the poultry industry are limestone as well as dicalcium or monocalcium phosphate (Walk et al., 2012c). The primary source of Ca for diet supplementation is ground limestone (also known chemically as $CaCO_3$), because more than 80% of the Ca in the earth's crust exists as limestone. The bioavailability of Ca from these different sources has been extensively discussed (Shafey, 1993; Walk et al., 2012c). In the diet, it can be present in a fine (e.g. limestone, which it may content a range of particle size) or coarse (e.g. oyster shell) form (Figure 3).

Limestone (large particles)

Limestone (small particles)

Oyster shell

Figure 3. Various sources of dietary calcium

Oyster shell, a common source of Ca in laying bird diets, also has highly (100%) available Ca. Marble dust and aragonite are considered less common sources of Ca for domestic animals (Peeler, 1972). Considerably more research on Ca bioavailability has been reported for poultry than for other animals, underscoring the importance of Ca to these species. However, as reported above, Ca bioavailability is highly dependent on different factors, such as the physical form of the ingredient, the dietary level, as well the animal age.

4. Phosphorus sources and availability

4.1. Vegetable sources of P

The P content in the raw materials used in animal feed presents a wide range of variation. In general, seeds (cereal grains, legumes and oilseeds) have a greater P content than forages. The by-products of processing grains (wheat bran, corn gluten or oilseed meal) are especially rich in P (Rebollar and Mateos, 1999). The raw materials of animal origin including the skeleton are foods with high levels of P (FEDNA, 2011). The level of P varies not only among sources but also within each source. In raw materials of plant origin, P content depends on soil type, cultivar, maturation state, culturing conditions, weather, etc., (Ravindran et al., 1995; Rebollar and Mateos, 1999).

Phytic acid is the major storage form of P in plant seeds. It contains 28.2% of bound P and represents on average 70% of the total P (tP) in feed ingredients commonly used in poultry diets (Figure 4) (Maenz, 2001; Kornegay, 2001; Gregori et al., 2006). It's present in grains and seeds as a mixed salt, phytate, which refers to the phytic acid molecule chelated to mineral cations, proteins, starch, lipids, or both starch and lipids (Figure 4, Ravindran et al., 1999; Selle et al., 2000; Kornegay, 2001; Gregori et al., 2006).

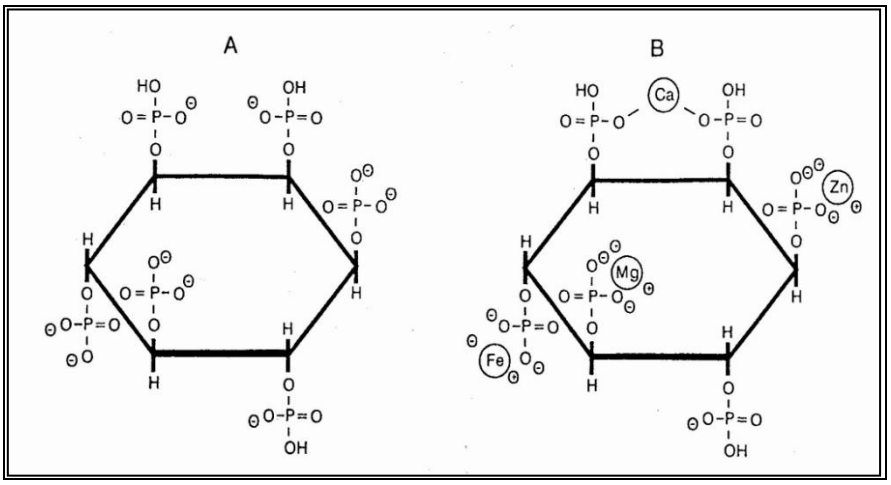


Figure 4. Structure of Phytic Acid (A) and Phytic Acid Chelate with metal cations

Phytic acid is an essential component of all seeds which constitutes a P and mineral reserve for the new plant as they are released during germination (Wodzinski and Ullah, 1995). Its location varies depending on the type of grain. In wheat and rye, as well as part of monocotyledons, phytate (between 80 and 90%) is located in the aleurone layer and in the pericarp; whereas corn and sorghum accumulate in the germ. In legumes, phytate is concentrated in cotyledons, and for oilseeds it is diffusely distributed throughout the seed associated with protein-rich globular bodies (Cosgrove, 1980; Sauveur, 1989).

Cereals contain between 0.2 and 0.3% phytic P; Their by-products (except for maize and sorghum) around 0.5 and 1.0%; and protein meals between 0.3 and 0,9% (Pointillart, 1994).

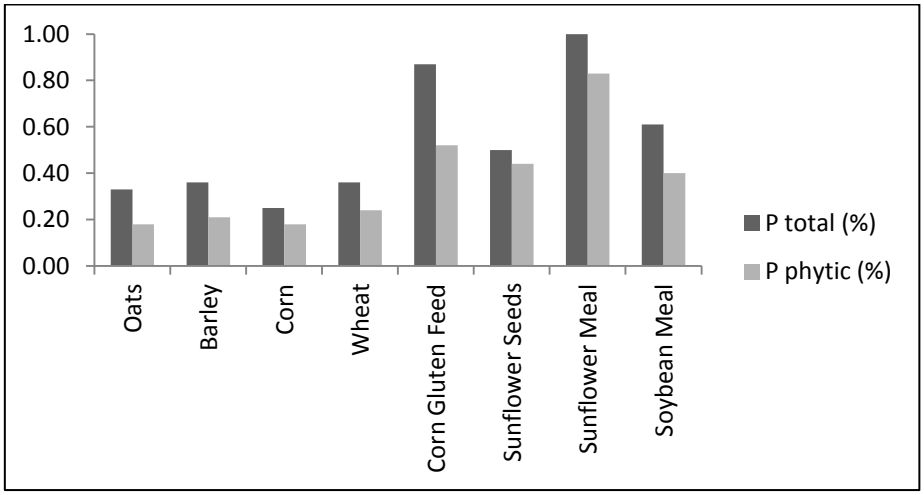


Figure 5. Total and phytic P content (%) in different feedstuff (FEDNA,2008)

From a practical standpoint it is recognized that the availability of inorganic P and non-phytate organic P is similar and nearly 100% (range 80-100%). On the contrary, the phytate P content is low available for poultry (by assigning a value of 0) as the monogastric animals lack the precise enzyme, at least sufficient to break and separate the P-inositol molecule (Kornegay, 1999). Hydrolysis of organic P in the gastrointestinal tract releases PO_4^{3-} , which is the only way that the animal can absorb and utilize P (De Groote, 1990). The aP values described in the feedstuffs evaluation tables are values obtained without any exogenous phytase addition.

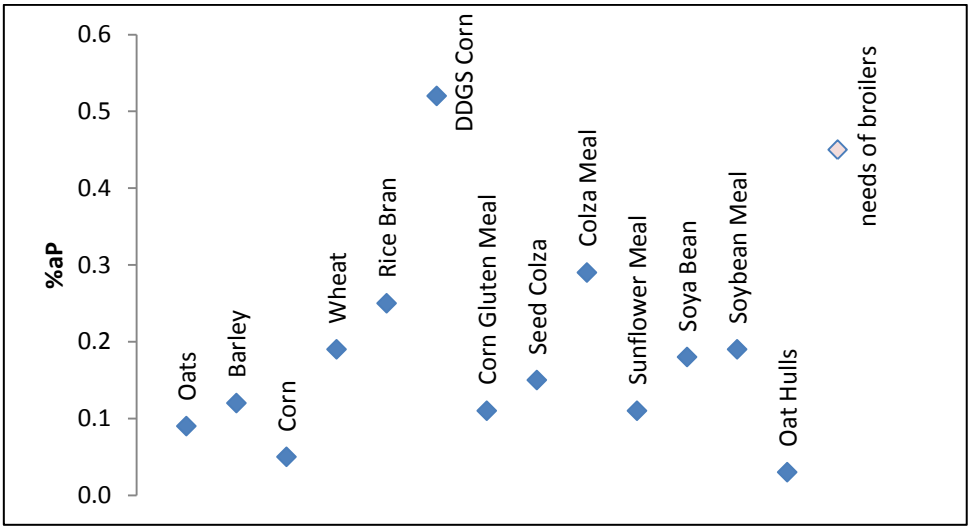


Figure 6. Comparison between available P content of some raw materials and broilers requirements (FEDNA, 2008)

4.2. Phytase

Phytase are phosphatases that catalyze the process of hydrolysis of the phytic acid, making P fully available to monogastric animals (Irving, 1980; Gibson and Ullah, 1990). Phytase is the only recognized enzyme that can initiate the release of phosphate from phytin (IUB, 1979). Phytase hydrolyze only phytate in solution, and with certain optimum conditions of pH and temperature that they are variable according to the type of phytase (Wodzinski and Ullah, 1995).

One unit of phytase is defined as the amount of enzyme required to liberate one µmol of orthophosphate from phytin per minute at pH 5.5 and 37° C (Zyla et al., 1995, AOAC). However, the problem is that a phytase showing the same activity level measured at pH 5.5 can have dramatically different activities at lower pH values (ie, proventriculus and

gizzard). This may explain some of the differences and discrepancies in results obtained with different phytase *in vivo*.

4.2.1 Vegetable phytase

Some feedstuffs contain considerable phytase activity (wheat, wheat bran, rye, barley), whereas others have little or no phytase activity (corn, oats, sorghum, and oilseeds) (Eeckhout, and de Paepe, 1994). No correlation exists between the phytic P content in the grain and his phytase activity (Eeckout and De Paepe, 1994; Rebollar and Mateos, 1999).

Phytase of plant origin is the 6-phytase. This phytase converts the myo-inositol 1, 2, 3,4,5,6 hexakis dihydrogen phosphate starting with the 6-position to yield a first product, the D-myo-inositol pentakis dihydrogen phosphate plus inorganic phosphate (Pi). This reaction is repeated until the terminal products are myoinositol and 6 Pi (Greiner et al, 1993)

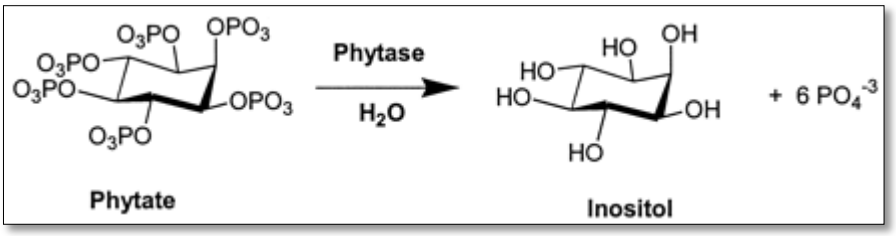


Figure 7. The mode of action of phytase

Phytase activity in grains, such as wheat, has a very high correlation with overall P retention in broilers when diets are fed in mash form (i.e. diets that are not pelleted) (Barrier-Guillot et al., 1996). Within wheat samples, phytase activity can be highly variable (915 to 1581 U/kg; Eeckhout and De Paepe, 1994). Much of this variation can be explained through cultivar differences (Barrier-Guillot et al., 1996; Applegate and Angel, 2004) and possibly through grain storage time and conditions.

It is estimated that phytase contained in plants are at least 10 % less efficient than those of fungus origin (Kornegay et al., 1996). The reason might be the narrow range of pH at which plant phytases are active (Hoppe, 1992). Optimum pH for maximum activity is higher than that found in the stomach of poultry (pH of 2.5-3.5), principal point of action of phytases (Liebert et al., 1993; Rebollar and Mateos, 1999). For example, 6-

phytases from wheat have only one optimum pH at 5.5 (Kies et al., 2001). Because vegetal phytases are active at a pH of 5 and are very sensitive to changes, pH too acidic or too alkaline may inactivate them irreversibly (Pointillart, 1994). Moreover, in certain regions of the gastrointestinal tract, where pH is 5-6, phytic acid can react with other minerals (such as Ca, Fe, Cu or Zn) and precipitate, avoiding the activity of phytase on this precipitate. In areas with lower pH (such as proventriculus and gizzard in poultry), phytin is more soluble, but plant phytase is less active.

Optimal temperature ranges of plant phytases are from 45 to 60°C (Wodzinski and Ullah, 1996; Applegate and Angel, 2004). Plant phytases, however, may be partially or totally inactivated by over-heating or high steam-pelleting temperatures (Ravindran et al., 1995). Phillippy (1999) also demonstrated that wheat phytase lost substantial activity when incubated with pepsin, a proteolytic digestive enzyme. Temperature stability of plant phytases is not good and, therefore, is a primary drawback when diets are pelleted. Producers that feed mash (diets that are not pelleted) diets may find some benefit from plant phytases but must consider the high inherent variability of vegetable phytase.

4.2.2. Microbial phytase

Phosphorus retention by broilers was improved from 50 to 60% by supplementing diets with a fungal phytase (Simons et al., 1990; Kornegay et al., 1996). However, efficacy of phytase supplementation may be dependent on different factors, such as: 1.- the microbial source and form of the enzyme (coated, size of the particle, etc.); 2.- temperature, and optima pH of the enzyme; 3.- the diet mineral concentration (Ca, Fe, Mg, Cu, and Zn), ingredients used or diet manufacturing methodology (pelleted, mash, or liquid); 4.- location of addition of phytase (post pelleting or mixer); 5.- type and level of vitamin D metabolites; 6.- the animal status (ie. disease), and other factors (Ravindran et al., 1995).

The form of microbial origin is 3-phytase. The difference with vegetable phytase is that the microbial phytase starts hydrolysis by position 3 but the terminal products are the same, myo-inositol and 6 Pi. The phytase produced by Aspergillus Niger is the first to be marketed by the company BASF under the trademark Natuphos ®. Its maximum activity occurs at pH 5.5 to 6.0 with a second area of activity at pH 2.5 (Simons et al.,

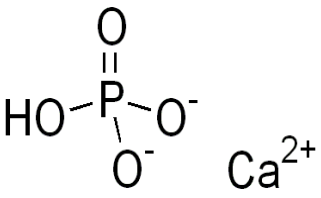
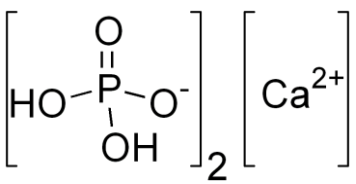
1990), similar to the phytase from *Aspergillus ficuum* that has pH optima at 2.5 and 5.0 (Gibson and Ullah, 1990).

Shirley and Edwards (2003) indicated 94.8% phytate P disappearance could be achieved using 12,000 units of phytase (Natuphos 5000)/kg diet. Coon and Manangi (2004) indicated 99.5% phytate hydrolysis in broilers fed diets supplemented with 5,000 units of phytase (Phyzyme XP) per kg diet. In recent years, the use of higher levels of exogenous phytase, referred to as super dosing, has been promoted as a strategy to release more phytic P and to reduce the antinutritive effects of phytase (Cowieson et al., 2011). As refered above, phytase activity is characterized by measuring the activity at pH 5.5, but may show different activities at lower pH values. There was good evidence that the majority of phytase activity takes place in the stomach and gizzard; which it implies that measurements carried out at perhaps 2.5-3.0 could be more relevant to predict bio-efficacy of phytase activity (Bedford, 2011).

4.3. Mineral sources of phosphorus

Main ingredient in the diet and feedstuff phosphorus availability can be quite variable. The use of phytase is critical for an efficient and sustainable use of the vegetable sources in the animal industry. However, most of the diets need to be fortified with mineral sources of P, such as dicalcium phosphate (anhydrous or hydrated), monocalcium phosphate (table 5), or defluorinated rock phosphate. In commercial feed manufacturing, it is important to note that these commercial products can contain other phosphate forms (Joseph and Scares, 1995).

Table 5. Comparison between Dicalcium phosphate and Monocalcium phosphate

	Dicalcium phosphate	Monocalcium phosphate
Molecular formula	$CaHPO_4$	$CaH_4P_2O_8$
Solubility in water	0.02 g/100 ml	2 g/100 ml
Molecule		

Baker (1989) described the typical commercial products; dicalcium phosphate ($CaHP0_4$) and monocalcium phosphate [$Ca\ (H_2P0_4)_2{}^{-}H_20$] contain mixtures of $CaHP0_4$, $Ca\ (H_2P0_4)_2{}^{-}H_20$, and $CaHP0_4$-$2H_20$. Monocalcium phosphate generally contains 13% $CaHP0_4$, and 61% $Ca(H_2P0_4)^{2-}H_20$, with the remainder small amounts of other phosphates and minerals. Commercial dicalcium phosphate generally contains about 14% $Ca\ (H_2P0_4)_2{}^{-}H_20$, 35% $CaHP0_{4\text{-}2}H_20$, and 26% $CaHP0_4$ (Joseph and Scares, 1995). In general, dicalcium phosphate, which it is less soluble than monocalcium phosphate, is the preferred source in the poultry feeding.

5. Absorption of Calcium and Phosphorus

Factors that affect the absorption of Ca and P from the digestive tract include the dietary concentrations of Ca and P and the Ca:total P ratio, which should normally be within the range of 1:1 to 2:1 for broilers (NRC, 1994, 1998).

The apparent absorption of Ca and P occurs primarily in the duodenum and jejunum in the small intestine of monogastrics (Partridge, 1978; Liu *et al*., 2000).

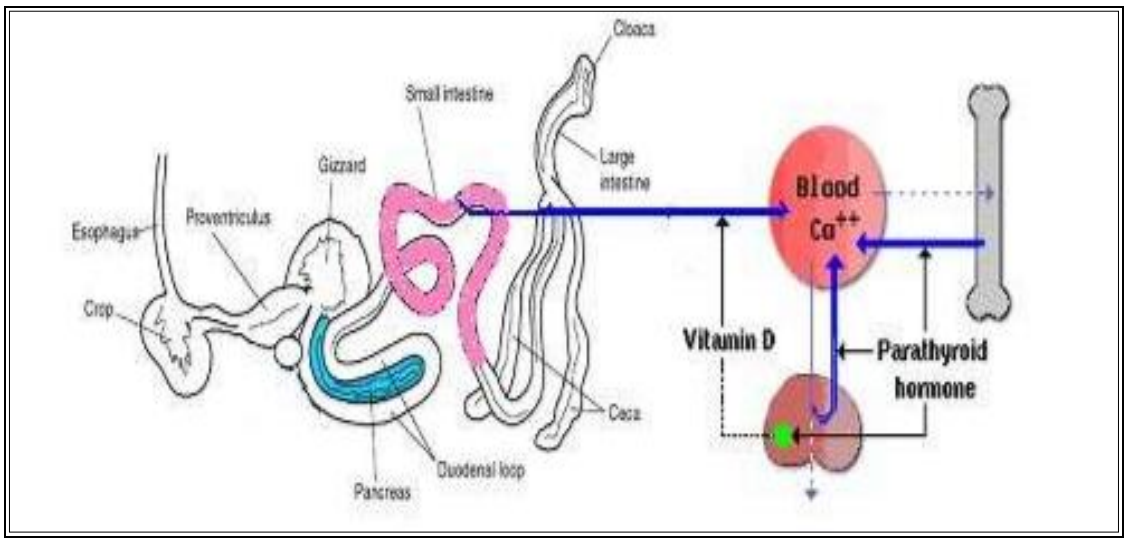


Figure 8.- System of regulation of the absorption of calcium and phosphorus

Vitamin D_3, also named cholecalciferol, is a fat-soluble vitamin that is found almost exclusively in animals and not in plants. Vitamin D_3 does not have functional biological activity until it is converted metabolically to 1, $25(OH)_2D_3$, also named calcitriol, a metabolite that is classified as a secosteroid hormone because of its functional roles in the absorption of Ca and P in the intestine, resorption of Ca^{2+} and P in the kidney and

mobilization or accumulation of Ca^{2+} and P in bone (Lee et al., 1990; Bouillon et al., 1995).

Vitamin D_3 can be obtained either directly from the diet or it can be synthesized from its precursor, 7 dehydroxycholesterol, which is formed in the liver. 7 Dehydroxycholesterol is then transported to the skin, where it is transformed to vitamin D_3 under the influence of ultraviolet light and skin temperature. In the liver, and to a lesser extent in the kidney and intestines, vitamin D_3 is transformed to 25-hydroxyvitamin D_3 (25-OHD_3). In the kidney, and to a lesser extent in other tissues, including intestine, bone and skin, this is converted into 1, 25-$(OH)_2D_3$, which is the hormonal form of the vitamin. The conversion takes place with the help of 1-α-hydroxylase and is homeostatically regulated by plasma Ca^{2+}, the secretion of parathyroid hormone (PTH) and possibly also by plasma P, calcitonin and the secretion of gonad hormones (Soares, 1984).

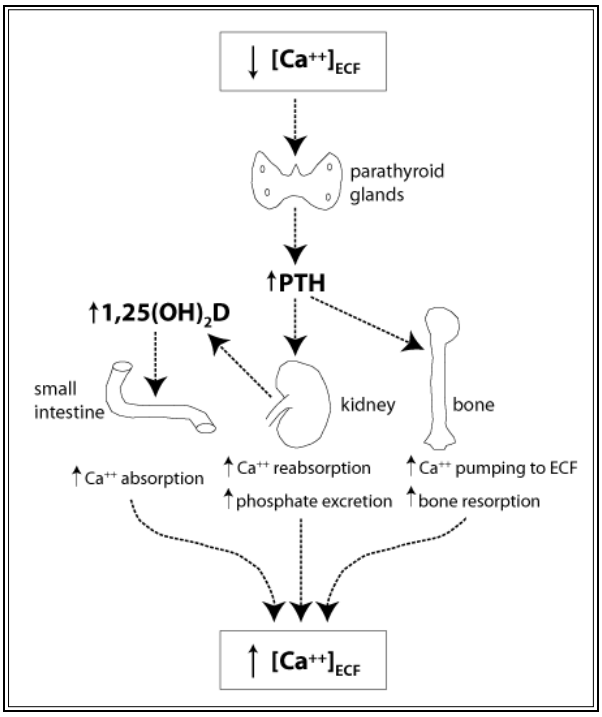


Figure 9. Hormonal regulation of calcium and phosphorus

In situations of sustained low Ca^{2+} concentrations, PTH stimulates the conversion of vitamin D_3 to the steroid hormone 1,25$(OH)_2D_3$, the active form of vitamin D_3, which functions to increase the intestinal absorption of Ca^{2+} and the deposition of Ca^{2+} in bone, both beneficial for increasing the overall body Ca status (Hoenderop *et al*., 2005).

Dietary P is absorbed in the phosphate form from the small intestine. It is necessary that phosphate is in solution at the point of contact with the intestinal mucosa, as any compound forming insoluble complex with the phosphate ion will decrease P absorption.

6. Interactions between Calcium and Phosphorus

Large amounts of calcium (Ca) can cause calcium phytate (Selle et al., 2009) or calcium phosphate precipitation (De kort et al., 2009) within the small intestine, reducing P absorption. Therefore the Ca:P ratio of a feed is an important factor affecting P ileal digestibility (for example from 0.54 to 0.40 % for dietary levels of Ca from 0.45 to 0.9%, (Walk et al., 2012b) .

Al-Masri (1995) describes also a decrease on the availability of feed P from 0.66 to 0.30 as Ca: P ratios were changed from 1:1 to 2.5:1. However, the author refers that increasing Ca concentration (ie, from 0.66 to 1.58%) showed a greater effect on P absorption than on P retention, as the animals tended to reduce the endogenous P excretion trying to conserve the nutrient (ie. Type II nutrient). High dietary Ca has also been implicated in reduced phytase efficacy (Ballam et al., 1984; Tamim and Angel, 2003; Tamim et al., 2004) because it increased gastrointestinal pH (Guinotte et al., 1995) which gives in unfavorable pH in the proventriculus/gizzard.
An increase in gizzard pH significantly reduced Ca solubility in broilers (Guinotte et al., 1995), and a higher pH has been implicated in Ca-phytate interactions in the gastrointestinal tract and interference with macromineral absorption (Simpson and Wise, 1990).

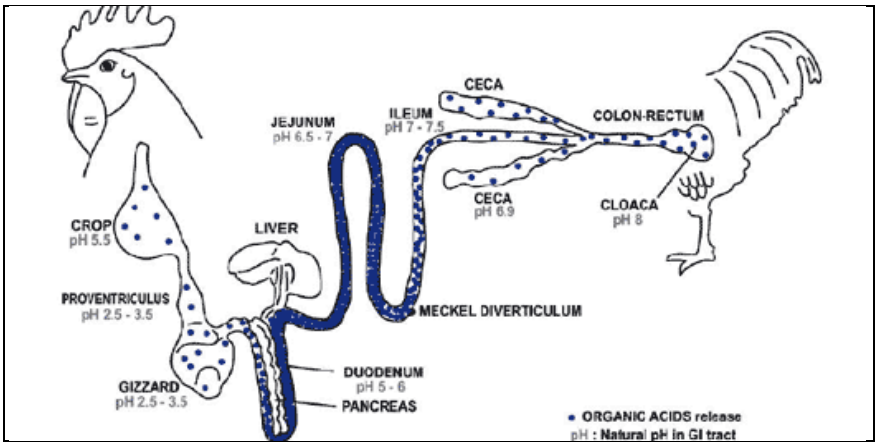


Figure 10. The pH value in the different part of the Broilers digestive tract

The literature describes different evidences about the influence of the digesta pH value on the Ca–phytate precipitation. In *in vitro* conditions, Wise and Gilburt (1981) found that Ca–phytate was soluble below pH 4, but precipitation was observed at pH 5. In contrast, Marini et al. (1985) reported Ca–phytate binding from pH 2.0 to 12.0 and Champagne (1987) also found soluble Ca^{2+}–phytates complexes at pH 2.4 to 5.9.

Limestone, the dominant source of Ca in poultry diets, has a high acid-binding capacity (Lawlor et al., 2005), so high dietary limestone may act as an antacid in the distal portions of the gizzard and ileum. According to Guinotte et al., (1995) increasing dietary limestone increased gizzard pH of immature pullets and increased crop and ileal pH in 12-d-old broilers, Shafey et al. (1991) reported that increasing dietary Ca from 10.7 to 25.3 g kg−1 increased crop pH from 4.89 to 5.32.

7. Effect of calcium particle size

The solubility of Ca in the gastrointestinal tract may have a direct effect on the formation of phytic P- mineral complexes. Research usually neglects to describe the limestone particle size and Ca solubility on the mineral studies in poultry, or those aiming to evaluate exogenous phytase in broilers (Manangi and Coon, 2007). Despite it looks a contradiction, broilers may gain more from feeding phytase by feeding larger particle $CaCO_3$ with lower solubility to minimize the solubility of $CaCO_3$ in the crop and in anterior portion of the gastrointestinal tract. A low solubility form of $CaCO_3$ may allow the phytase enzyme more access to phytic acid P in the gut and provide more available P from phytic acid hydrolysis in the broiler (Manangi and Coon, 2007). The phytic P hydrolysis was reduced 8% in an *in vitro* assay when incubation mixture was pH 2.5 and contained the smallest particle size $CaCO_3$ compared to a mixture with the largest particle size of $CaCO_3$ (Manangi and Coon, 2007).

The $CaCO_3$ with very small particles has a high solubility and may pass through the gastrointestinal tract at a faster rate and decrease maximum retention. The highly soluble Ca from the small particles may also enhance the formation of a mineral-phytic complex that limits the ability of added dietary phytase to hydrolyze phytic acid. These mechanisms may explain that feeding chicks a diet with $CaCO_3$ particle sizes between 137 and 388 μm increased the body weight gain of animals as compared to that obtained by feeding either smaller (28 μm) or larger particle (1306 μm) sizes (Manangi and

Coon, 2007). An increased ash tibia content was also obtained for the chicks fed $CaCO_3$ particle sizes ranging from 137-388 μm as compared to the smallest (28 μm) or largest particle (1306 μm) sizes.

However, recently Walk et al (2012c) have presented the results about the influence of a highly soluble Ca source (from 0.45 to 0.9 % Ca in the diet) on performance and bone mineralization. Their results showed that feeding broiler chicks with a higher soluble source of Ca with phytase allowed for reductions in dietary Ca while maintaining broiler performance and bone ash. Their results again suggest that current recommendation of total Ca for broilers may be overestimated as they have been mostly defined using limestone containing diets, which encourages the interest of moving forward to know better Ca requirements on a digestible basis.

B. Experimental Part

1. Objectives and experimental plan

Considering the hypothesis declared in the previous introduction, the present study will aim:

1. To associate the physic-chemical characteristics of various calcium sources (solubility and buffering capacity) with their biological response in broiler chicks.

2. To determine optimum Ca and P levels, ratio of Ca and P, and Ca source characteristics on growth performance, Ca and P retention, and tibia ash content in broiler chicks from 0 to 14 days.

To achieve these objectives we propose to use a double *in vitro* and *in vivo* approach:

-In the two *in vitro* trials we propose to determine pH values which cause the precipitation of calcium and phytic phosphorus in order to define favorable or unfavorable conditions for the phytase activity and make the most from calcium and phosphorus in the diet. The pH values in the digesta may also vary depending on the level and buffering capacity of the Ca source (carbonate calcium, calcium chloride, monocalcium phosphate, calcium lactate).

-In two *in vivo* trials we propose to evaluate the effect of different dietary levels of calcium (0.9, 0.7, 0.5 % Ca) and non-phytic phosphorus (0.25, 0.316, 0.38, 0.45 % NPP) on the productive performance and bone mineralization. For this trial we will use calcium carbonate and monocalcium phosphate as main sources of Ca and NPP, respectively.

In the second *in vivo* experiment we will try to compare the effect of different sources of calcium (a high soluble source, calcium chloride; a medium soluble source, carbonate calcium; and a low soluble source, Lipocal, encapsulated tricalcium phosphate from Lipofoods) on the performance and bone mineralization of young chicks. The diets will contain one level of calcium (0.55% Ca) and 4 levels of phosphorus (0.30, 0.35, 0.40, 0.45 % NPP).

2. Materials and Methods

2.1. Experiment 1 (*In vivo*)

The experiment was conducted at the experimental farm of the Veterinary Faculty in the Universitat Autònoma de Barcelona.

2.1.1. Birds and management

420 Day-old Ross broiler male chicks were obtained from a local hatchery where they had received vaccinations for Newcastle Disease and Infectious Bronchitis post hatch. The birds were weighed individually and distributed in 60 battery brooders cages (7 chicks per cage) in order to get a similar initial average body weight for each cage. Chicks were individually labeled in order to register individual body weight as well the group body weight along the experimental period. The brooder temperature was maintained at 35-37ºC during the 4 first days, and was progressively reduce to 30ºC on day 14. Continuous monocromatic lighting was provided for the duration of the experiment. Feed and water was offered ad libitum.

2.1.2. Diet

All diets were formulated to meet NRC (1994) recommendations, with the exception of Ca and aP in some treatments. Diets were fed in mash form and contained 0.3% titanium dioxide as an indigestible marker. The phytase activity in the diets was 1150 U/kg (Quantum Blue, AB Vista Feed Ingredients).

Table 6. Calculated and analyzed composition of experimental diets (experiment 1)

Treatment Diet												
Ca (g/kg)	9				7				5			
NPP (g/kg)	**2.5**	**3.1**	**3.8**	**4.5**	**2.5**	**3.1**	**3.8**	**4.5**	**2.5**	**3.1**	**3.8**	**4.5**
Ingredients (%)												
Corn	23.87											
Wheat	25											
Soybean meal 44%	27.15											
Extruded soybean	13.27											
Na phosphate	0.29											
L-Lis	0.29											
DL-Met	0.33											
L-Thr	0.04											
Soy oil	6											
Salt	0.22											
Vit-mineral premix	0.3											
Celite	1.08	0.93	0.77	0.61	1.61	1.45	1.29	1.13	2.13	1.97	1.81	1.65
limestone	1.9	1.76	1.63	1.49	1.38	1.24	1.1	0.97	0.85	0.72	0.58	0.44
Monocalcium phosphate	0.26	0.55	0.85	1.14	0.26	0.55	0.85	1.14	0.26	0.55	0.85	1.14
Calculated Composition (%)												
DM %	88.80											
M.E(Kcal/Kg)	2960											
CP	22											
Lys	1.38											
Met	0.64											
Met+Cys	1.01											
Thr	0.86											
Try	0.27											
Ca	0.9	0.9	0.9	0.9	0.7	0.7	0.7	0.7	0.5	0.5	0.5	0.5
Total P	0.49	0.55	0.62	0.69	0.49	0.55	0.62	0.69	0.49	0.55	0.62	0.69
Available P	0.24	0.31	0.37	0.44	0.24	0.31	0.37	0.44	0.24	0.31	0.37	0.44
PP	0.25											
NPP	0.25	0.31	0.38	0.45	0.25	0.31	0.38	0.45	0.25	0.31	0.38	0.45
Na	0.22											
Cl	0.17											
Analyzed Composition (%)												
DM	89.85											
CP	22.33											
Ca	0.96	0.96	0.96	0.96	0.79	0.79	0.79	0.79	0.62	0.62	0.62	0.62
Total P	0.64	0.68	0.78	0.9	0.66	0.7	0.8	0.84	0.64	0.68	0.76	0.92

2.1.3. Experimental Design

The experimental design was completely randomized with a 3 × 4 factorial arrangement of 3 levels of Ca (0.9, 0.7and 0.5%) and 4 levels of NPP (0.25, 0.31, 0.38, and 0.45%). Each treatment was replicated 5 times.

2.1.4. Traits measured

a. Animal performance

Individual and group Body weight (BW) and feed intakes were measured on Day 1, 7 and 14. From these values average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) from Day 1 to 7 and from Day 7 to 14 were calculated.

b. **Tibia parameters**

On day 14, the 3 birds with the closest body weight to the average cage BW were killed by cervical dislocation. Later, the right tibiotarsus was removed, boiled, and cleaned from adherent tissue. As described by Brenes et al. (2003), the bones were dried at 110°C for 12 h, defatted with ether for 48 h, dried again at 110°C for 12 h, and finally ashed at 550°C for 12 h in a muffle.

c. Gizzard and proventriculus pH

The pH of the gizzard and proventriculus contents were recorded by immersing the electrode of digital pH meter into the center of each part. The pH was measured for 3 birds /cage.

d. Calcium and Phosphorus retention

The rest of chicks were fasted during 2h and killed by cervical dislocation to determine Ca and P content of the whole body. The four whole bodies were grounded together and the percentage of ash in the mince was determined following incineration of samples (8g) for 12 hours at 550 ° C.

Calcium and Phosphorus content were analyzed using an atomic absorption and mass spectrophotometer in ash samples (0.5 g) that were digested in 5 ml nitric acid and 1 ml of hydrogen peroxide using microwave digestion.

2.1.5. Statistical analysis

Data were subjected to analysis of variance (ANOVA) using the General Linear Model (GLM) of SAS software (SAS, 2008), version 9.2.

The statistical model used for the analysis of dependent variables was:

$$Y_{ijk} = \mu + Ca_i + P_j + Ca_i * P_j + e_{ijk}$$

where Y_{ijk} is the individual observation, μ the experimental mean, Ca_i the Ca level effect, P_j the phosphorus level effect, Ca_i*P_j the Ca and P interaction, and e_{ijk} the random error. Body weight, body weight gain, feed intake, feed conversion Ratio (FCR) and bone parameters were analyzed on a cage basis. Treatment means were compared using Tukey´s multiple comparisons.

2.2. Experiment 2 (*In vitro*)

2.2.1. Calcium and Phosphorus Solubility *in vitro*

Soluble Ca and P were measured in different solutions containing Ca carbonate, Ca chloride, and tricalcium phosphate (Lipocal (Lipofoods, Barcelona, Spain)) as sources of Ca, and phytic P as organic source of P. The influence of pH on Ca and P solubility was measured at different pH buffers. We prepared seven phosphate-citrate buffer solutions with pH 2.96, 3.53, 4.18, 4.77, 5.27, 6.01, 6.52 from solutions of 0.2M dibasic sodium phosphate (Na_2HPO_4) and 0.1 M citric acid, according to Pearse (1980). All buffers were prepared and pH adjusted before the addition of the phytic acid.

Table 7. Buffering solutions

pH	Na_2HPO_4(ml) (0.2M)	Citrate(ml) (0.1M)
2.96	10.2	39.8
3.53	16.1	33.9
4.18	20.6	29.4
4.77	24.8	25.2
5.27	27.8	22.2
6.01	32.1	17.9
6.52	36.4	13.6

The level of Ca to be added in each solution was calculated to simulate a similar level to that derived from the consumption of diets containing 0.9% Ca in the diets and 2:1 water: feed consumption (90 mg Ca/ml). For $CaCl_2$, 75 ml of buffer and 90 mg of Ca

shaped $CaCl_2$ (250 mg anhydro $CaCl_2$ / ml) were added in each tube. For the $CaCO_3$ tubes, we added 224.75 mg $CaCO_3$ / ml; and for Lipocal we added 250 mg Lipocal / ml). The citric acid part of the buffering solution was used to maximize the Ca carbonate solubilization.

Solubility of P phytic acid (0.2475g solution 40% phytic acid) was also studied in a batch of tubes for each source of calcium and level of pH. The tubes were vortexed and incubated for 60 min at 37ºC to observe the amount of mineral precipitate, and to obtain supernatant samples that were analyzed for soluble Ca and P content. Calcium and phosphorus were analyzed using Optical emission spectroscopy inductively coupled plasma Perkin-Elmer, model Optima 4300DV.

2.2.2. Buffering capacity of different calcium sources *in vitro*

We chose four sources of calcium to determine buffering capacity: calcium chloride, calcium carbonate, calcium lactate and tricalcium phosphate (Lipocal). A batch of solutions was prepared containing 0.2, 0.4, 0.6, 0.8, 1, 1.2% Ca for each Ca source (Table 8) in 75 ml of distilled water. The buffering capacity for each Ca source against reaching a pH=3 was measured by titration using increasing amounts of a solution of HCl (0.1N), and measuring the pH continuously until reaching pH=3. We made two repetitions for each% Ca tube), registering volume of HCl added until pH=3. ABC (acid-binding capacity) was calculated as the amount of acid in milliequivalents (meq), pH = 3 (ABC-3).

Table 8. Solutions prepared from different sources of calcium with different level of Ca

% Ca	0.2	0.4	0.6	0.8	1.0	1.2
gCa/100ml	0.1	0.2	0.3	0.4	0.5	0.6
$CaCO_3$(g/100ml)	0.192	0.388	0.582	0.777	0.971	1.165
$CaCl_2$(g/100ml)	0.270	0.539	0.809	1.079	1.348	1.618
$C_6H_{10}CaO_6$(g/100ml)	0.454	0.909	1.363	1.818	2.272	2.727
Lipocal (g/100ml)	0.278	0.556	0.833	1.111	1.389	1.667

2.3. Experiment 3 (*in vivo*)

The experiment was conducted at the experimental farm in the Veterinary Faculty of the Universitat Autònoma de Barcelona.

2.3.1. Birds and management

300 Day-old Ross broiler male chicks were obtained from a local hatchery where they had received vaccinations for Newcastle Disease and Infectious Bronchitis post hatch. The birds were weighed individually and distributed in 60 battery brooders cages (5 chicks per cage) in order to get a similar initial average body weight for each cage. Chicks were individually labeled in order to register individual body weight as well the group body weight along the experimental period.

The brooder temperature was maintained at 35-37ºC during the 4 first days, and was progressively reduced to 30ºC on day 14.

Continuous monocromatic lighting was provided for the duration of the experiment. Each part contained one feeder that allows feeding 5 animals at the same time and one nipple drink which gave free access to fresh water.

2.3.2. Diet

All diets were formulated to meet NRC (1994) recommendations, with the exception of Ca and aP. Diets were fed in mash form and contained 0.3% titanium dioxide as an indigestible marker. The phytase used allowed to reach an analyzed activity of 1150 FTU/kg (Quantum Blue, AB Vista Feed Ingredients)

Table 9. Calculated and analyzed composition of experimental diet (experiment 3)

Treatment Diet												
Ca source	Limestone				Ca Chloride				Lipocal			
NPP (g/kg)	**3**	**3.5**	**4**	**4.5**	**3**	**3.5**	**4**	**4.5**	**3**	**3.5**	**4**	**4.5**
Ingredients (%)												
Corn	23.87											
Wheat	25											
Soybean meal 44%	27.15											
Extruded soybean	13.27											
Na phosphate	0	0	0	0	0.48	0.48	0.48	0.48	0	0	0	0
L-Lis	0.29											
DL-Met	0.33											
L-Thr	0.04											
CaCl[2]	0	0	0	0	0.7	0.7	0.7	0.7	0	0		0
Limestone[1]	0.66	0.56	0.46	0.36	0.34	0.23	0.13	0.03	0.17	0.17	0.17	0.17
Lipocal [3]	0	0	0	0	0	0	0	0	0.93	0.74	0.54	0.35
Soy oil	6											
Monocalciumphosphate[4]	0.72	0.94	1.16	1.38	0.32	0.55	0.77	0.99	0	0.37	0.74	1.11
Salt	0.57	0.57	0.57	0.57	0.01	0	0	0	0.57	0.57	0.57	0.57
Vit-mineral premix	0.3											
SUCROSE	1.5	1.38	1.26	1.14	1.6	1.49	1.37	1.25	1.78	1.6	1.43	1.25
Titanium dioxide	0.3											
Calculated Composition (%)												
DM %	88.75											
M.E(Kcal/Kg)	2960											
CP	22											
Lys	1.38											
Met	0.64											
Met+Cys	1.01											
Thr	0.86											
Try	0.27											
Ca	0.55											
Total P	0.54	0.59	0.64	0.69	0.54	0.59	0.64	0.69	0.54	0.59	0.64	0.69
Available P	0.29	0.34	0.39	0.44	0.29	0.34	0.39	0.44	0.29	0.34	0.39	0.44
PP	0.25											
NPP	0.3	0.35	0.4	0.45	0.3	0.35	0.4	0.45	0.3	0.35	0.4	0.45
Analyzed Composition (%)												
DM	89.85											
CP	22.33											
Ca	6	5.7	6.4	6.3	7.6	8.1	8.5	8.1	7.4	7.8	6.8	6.8
Total P	6.7	6.7	7.8	8.3	7.1	7.6	8.9	9.5	7.2	7.5	8.1	8.6

[1]Limestone supplied 38% Ca.[2]Calcium chloride supplied 36% Ca.[3]Lipocal supplied 38% Ca. [4]Monocalcium phosphate supplied 16% Ca.

2.3.3. Experimental Design

The experimental design was completely randomized with a 3 × 4 factorial arrangement of 3 sources of calcium (0.55% Ca from Calcium Carbonate, Calcium chloride and Lipocal) and 4 levels of NPP (0.3, 0.35, 0.4, and 0.45%). Each treatment was replicated 5 times.

2.3.4. Traits measured

a. Animal performances

Individual and group Body weight (BW) and feed intakes were measured on Day 1, 7 and 14. From these values average daily gain (ADG), average daily feed intake (ADFI), and feed conversion ratio (FCR) from D 1 to 7 and from D 7 to 14 were calculated.

b. **Tibia parameters**

On day 14, the 3 birds with the closest body weight to the average cage BW were killed by cervical dislocation. Later, the right tibiotarsus was removed, boiled, and cleaned from adherent tissue. As described by Brenes et al. (2003), the bones were dried at 110°C for 12 h, defatted with ether for 48 h, dried again at 110°C for 12 h, and finally ashed at 550°C for 12 h in a muffle.

c. Gizzard and proventriculus pH

The pH of the gizzard and proventriculus with contents were recorded by immersing the electrode of digital pH meter into the center of each part separately. The pH of the gizzard and proventriculus were measured for 3 birds /cage.

d. ileal digesta

The ileal digesta were collected from three animals in each cage from the Meckel's diverticulum to about 2 cm to the ileo-cecal junction, and stored at -20ºC. Samples were digested in nitric perchloric and fluoridric acids and subsequently analyzed for P, Ca, Ti and Zn by flame atomic absorption spectroscopy. Ileal digestibility of calcium and Phosphorus (%) was calculated as follows:

$$\textbf{Ileal Ca Digestibility} = 1 - ([Ti]_D / [Ca]_D / [Ti]_M / [Ca]_M)$$

$[Ti]_{D:}$ the concentration of Ti in the diet; $[Ca]_D$ the Ca or P content in the diet; $[Ti]_{M:}$ the concentration of Ti in the ileal digesta; $[Ca]_M$ the Ca or P content in the ileum digesta.

2.3.5. Statistical analysis

Data were subjected to analysis of variance (ANOVA) using the General Linear Model (GLM) of SAS software (SAS, 2008), version 9.2.

The statistical model used for the analysis of dependent variables was:

$$Y_{ijk} = \mu + Ca_i + P_j + Ca_i * P_j + e_{ijk}$$

where Y_{ijk} is the individual observation, μ the experimental mean, Ca_i the Calcium Source effect, P_j the phosphorus effect, $Ca_i * P_j$ the Ca source and P interaction, and e_{ijk} the random error. Body weight, body weight gain, feed intake, food conversion Ratio (FCR) and bone parameters on an individual bird basis, whilst ileale digestibility parameters were analyzed on a cage basis. Treatment means were compared using Tukey´s multiple comparisons.

3. Results

3.1. Experiment 1

3.1.1 Influence of dietary Ca, P levels and their interaction on early bird performance, bone mineralization and whole-body mineral retention

The nutrients of the diets are presented in Table 6. It is worth noting that Ca was higher than formulated likely as a consequence of the Ca content of celite (the ingredient used in the trial to pair the diets) and calcium carbonate added as filler of vitamin and mineral premixes. The Ca content in celite was 5.6% and in the vitamin and mineral premix was 13.5 %. The calculated Ca content of the diets when taking into account these values was 6.22, 7.92 and 9.65 g Ca/kg for the three levels of Calcium.

a. Bird performance

Feed intake and growth performance are presented in Table 10. A significant interaction (Ca x P levels) was observed on ADFI from day 7-14 and from day 1-14. Increasing the level of NPP from 2.5 to 3.8 g/kg increased ($P<0.05$) the ADFI on chickens fed the high Ca diet (9 g/kg) but not on birds fed lower levels of dietary Ca (7 and 5 g/kg). A similar pattern was observed for the growth performance from day 7-14 (Probability Ca x P: 0.068). The rest of performance parameters did not show Ca x P level interactions.

Dietary Ca influenced ($P<0.05$) ADFI from the first day (Day 1 to 14), and growth performance from day 7 to 14 (Table 11). Body weight gain from day 7 to 14 was the highest in broilers fed the 7 g Ca diet and lowest for birds fed the 9 g Ca diet. Also, the feed intake was the greatest on birds fed the 7 g Ca diet, showing higher values than birds fed the 9 g Ca diet.

Dietary P influenced ($P<0.05$) growth performance, being higher for birds fed 3.8 than 2.5 g NPP/kg.

b. Gizzard and proventriculus pH

No significant differences ($P>0.05$) were observed on the effects of calcium or phosphorus on the pH of the proventriculus (average value of pH= 2, 91) and gizzard (average value of pH= 2.31).

Table 10. Influence of Ca and NNP levels on feed intake and growth performance of broilers from Day 1 to 14 (Interaction; Experiment 1)

Ca[1](g/kg)	5				7				9				P-value			
NPP[2](g/kg)	**2.5**	**3.1**	**3.8**	**4.5**	**2.5**	**3.1**	**3.8**	**4.5**	**2.5**	**3.1**	**3.8**	**4.5**	***S.E.M***	**Ca**	**P**	**Ca*P**
Initial BW[3] (g)	44.1	44.4	44.6	44.3	44.3	44.3	44.3	44.5	44.4	44.5	44.3	44.6	0.13	0.357	0.325	0.295
Day 7 BW(g)	185.1	187.3	184.8	170.1	179.2	185.3	186.6	188.3	173.1	182.3	187.9	180.0	5.54	0.574	0.264	0.321
Day 14 BW (g)	427.9	435.7	431.5	407.8	428.1	444.1	459.9	440.1	390.8	417.0	445.5	446.5	12.72	0.086	0.050	0.122
ADFI[4]1-7 d (g/d)	21.5	21.0	20.2	19.3	21.4	20.8	22.4	20.8	18.6	19.9	20.6	19.6	0.84	0.023	0.396	0.386
ADFI 7-14 d (g/d)	53.7^{a}	52.1^{ab}	51.2^{ab}	52^{ab}	54.0^{a}	53.9^{a}	55.9^{a}	52.5^{a}	42.0^{b}	49.3^{ab}	54.4^{a}	51.1^{ab}	2.18	0.001	0.200	0.042
ADFI 1-14 d (g/d)	37.6^{a}	36.6^{ab}	35.7^{ab}	35.7^{ab}	37.7^{a}	37.3^{a}	39.2^{a}	36.6^{a}	30.3^{b}	34.6^{ab}	37.5^{a}	35.3^{ab}	1.35	0.005	0.242	0.048
ADG[5] 1-7 d (g/d)	20.2	20.4	20.0	17.9	19.3	20.1	20.4	20.5	18.4	19.7	20.5	19.3	0.79	0.550	0.262	0.311
ADG 7-14 d (g/d)	34.7	35.5	35.3	34.0	35.4	37.0	39.0	36.0	30.9	33.5	36.6	37.9	1.30	0.045	0.025	0.068
ADG 1-14 d (g/d)	27.4	28.0	27.7	25.9	27.4	28.5	29.7	28.3	24.7	26.6	28.7	28.7	0.91	0.086	0.045	0.107
FCR[6] 1-14 d (g/d)	1.38	1.31	1.29	1.39	1.38	1.31	1.32	1.30	1.23	1.31	1.31	1.23	0.060	0.22	0.960	0.634

[1]Calcium[2]NonPhytic Phosphorus [3]Body Weight [4]Average Daily Feed Intake [5]Average Daily Gain [6]Feed Conversion Ratio

[a,b] Means not sharing a common superscript are significantly different at P <0.05.

Table 11. Influence of Ca and NNP levels on feed intake and growth performance of broilers from Day 1 to 14 (Main factors Experiment 1)

	Ca (g/kg)			P-value		NPP (g/kg)				P-value	
	5	**7**	**9**	***S.E.M***	**Ca**	**2.5**	**3.1**	**3.8**	**4.5**	***S.E.M***	**P**
Initial BW (g)	44.3	44.3	44.4	0.06	0.357	44.2	44.3	44.4	44.4	0.07	0.325
Day 7 BW (g)	181.8	184.8	180.8	2.77	0.574	179.1	184.9	186.4	179.4	3.20	0.264
Day 14 BW (g)	425.7	443	424.9	6.36	0.086	415.6^{b}	432.3^{ab}	445.6^{a}	431.4^{ab}	7.34	0.050
ADFI 1-7 d (g/d)	20.5^{ab}	21.3^{a}	19.6^{b}	0.42	0.023	20.5	20.6	21	19.9	0.48	0.396
ADFI 7-14 d (g/d)	52.2^{ab}	54^{a}	49.1^{b}	1.09	0.001	49.9	51.7	53.8	51.8	1.26	0.200
ADFI 1-14 d (g/d)	36.3^{ab}	37.7^{a}	34.4^{b}	0.67	0.005	35.1	36.1	37.4	35.8	0.78	0.242
ADG 1-7 d (g/d)	19.6	20	14.4	0.40	0.550	19.2	20	20.2	19.2	0.46	0.262
ADG 7-14 d (g/d)	34.8^{ab}	36.8^{a}	34.7^{b}	0.65	0.045	33.6^{b}	35.3^{ab}	36.9^{a}	35.9^{ab}	0.75	0.025
ADG 1-14 d (g/d)	27.2	28.4	27.1	0.45	0.086	26.5^{b}	27.7^{ab}	28.7^{a}	27.6^{ab}	0.52	0.045
FCR 1-14 d (g/d)	1.34	1.32	1.27	0.030	0.22	1.33	1.30	1.30	1.30	0.030	0.960

c. Bone mineralization and whole-body mineral retention

Tibia weight, tibia ash weight (%, mg/tibia), whole body ash content, and Ca and P retention are presented in Table 12. Tibia weight and tibia ash content were influenced by a Ca x P level interaction. Tibia weight was the highest in birds fed the 7 and 9 g Ca/kg at 3.1, 3.8 or 4.5 g NPP/kg, being the highest for birds fed the diet 9 g Ca and 3.8 g NPP/kg. The lowest tibia ash percent was observed in birds with the highest unbalance diets, 4.5gP/kg with 5 g Ca/kg diet, and for the level 2.5 g NPP/kg on the 9 g Ca/kg diet.

Bone weight and mineralization as affected by main factors are presented in Table 13. Changing the levels of Ca in the diet promoted significant effects on the tibia weight, tibia ash content, Ca whole body content and Ca retention. Tibia weight was significantly higher for diet 7 and 9 g Ca than diet 5 g Ca. However, tibia ash percent showed significant differences among the three Ca levels, being the highest values for birds fed diet 7 g Ca/kg and the lowest for birds fed the 5 g Ca/kg diet.

The increase on dietary Ca decreased its fractional retention from values close to 74% with diet 5 g Ca/kg to 46% with diet 9 g Ca/kg. Surprisingly, an increase on the levels of dietary Ca from 5 to 7 g Ca/kg decreased the whole-body Ca content (g/kg BW).

Changing the levels of P in the diet promoted significant effects on tibia weight, tibia ash content, Ca whole body content, and Ca and P retention. A tendency was observed on P whole-body content. Tibia weight was significantly higher for diet 3.1, 3.8 and 4.5 g NPP/kg than diet 2.5 g NPP/kg. Tibia ash percent was significantly higher for diet 3.1 and 3.8 g NPP/kg than diet 2.5 g NPP/kg. The increase on dietary P decreased its fractional retention, with higher retention values with the two lowest dietary P diets (2.5 and 3.1 g NPP/kg diet). The increase on the levels of dietary P steadily increased the fractional retention of Ca from 53% to 61%, and increased the whole-body Ca content (g/kg BW), with values in birds fed diets 3.1, 3.8 and 4.5 g NPP/kg higher than birds fed diet 2.5 g NPP/kg.

Table 12. Influence of Ca and NPP levels on tibia weight and ash and whole-body ash of 14-d-old broilers (Interaction; Experiment 1)

Ca(g/kg)	5				7				9				P-value			
NPP(g/kg)	**2.5**	**3.1**	**3.8**	**4.5**	**2.5**	**3.1**	**3.8**	**4.5**	**2.5**	**3.1**	**3.8**	**4.5**	***S.E.M***	**Ca**	**NPP**	**Ca*P**
Tibia																
Tibia weight(g)	0.88^{bc}	0.87^{bc}	0.87^{bc}	0.80^{bc}	0.85^{bc}	0.92^{a}	0.93^{a}	0.94^{a}	0.77^{c}	0.90^{ab}	1^{a}	0.97^{a}	0.027	0.003	<0.001	<0.001
Tibia ash (%)	50.17^{bc}	51.38^{ab}	50.09^{bc}	49.55^{c}	51.44^{ab}	51.97^{a}	51.87^{a}	51.38^{ab}	49.65^{c}	50.83^{abc}	51.86^{a}	51.39^{ab}	0.341	<0.0001	0.0019	0.0007
Tibia ash (mg/tibia)	439^{b}	451^{b}	437^{b}	395^{b}	439^{b}	479^{a}	484^{a}	481^{a}	381^{b}	460^{a}	522^{a}	500^{a}	15.6	0.001	<0.001	<0.001
Whole Body																
Body ash (%)	2.40	2.63	2.55	2.58	2.33	2.36	2.39	2.50	2.30	2.60	2.48	2.43	0.105	0.160	0.161	0.833
Ca/KgPV(g/Kg)	5.36	6.28	6.13	6.12	5.23	5.39	5.40	5.94	5.12	6.13	5.78	5.65	0.246	0.025	0.003	0.394
P/kgPV(g/Kg)	4.27	4.75	4.61	4.68	4.17	4.23	4.26	4.57	3.99	4.56	4.43	4.35	0.189	0.089	0.052	0.772
Ca retention	0.65	0.77	0.76	0.77	0.51	0.52	0.56	0.58	0.43	0.49	0.46	0.48	0.031	<0.001	0.0152	0.543
P retention	0.66	0.65	0.58	0.53	0.65	0.58	0.57	0.51	0.66	0.63	0.55	0.52	0.031	0.507	<0.001	0.939

Table 13. Influence of Ca and NPP levels on tibia weight and ash and whole-body ash of 14-d-old broilers (Main factors Experiment 1)

	Ca (g/kg)			P-value		NPP(g/kg)				P-value	
	5	**7**	**9**	***S.E.M***	**Ca**	**2.5**	**3.1**	**3.8**	**4.5**	***S.E.M***	**P**
Tibia											
Tibia weight(g)	0.85^{b}	0.91^{a}	0.91^{a}	0.013	0.0032	0.83^{b}	0.90^{a}	0.93^{a}	0.90^{a}	0.015	<0.001
Tibia ash (%)	50.30^{c}	51.67^{a}	50.93^{b}	0.172	<0.001	50.42^{b}	51.39^{a}	51.28^{a}	50.77^{ab}	0.199	0.0019
Tibia ash (mg/tibia)	431^{b}	471^{a}	466^{a}	7.3	0.001	420^{b}	463^{a}	481^{a}	459^{a}	8.7	<0.001
Whole Body											
Body ash (%)	2.54	2.39	2.45	0.052	0.160	2.34	2.53	2.47	2.50	0.060	0.161
Ca/Kg PV(g/Kg)	5.97^{a}	5.49^{b}	5.66^{ab}	0.123	0.025	5.23^{b}	5.93^{a}	5.76^{a}	5.90^{a}	0.142	0.003
P/Kg PV(g/Kg)	4.57	4.30	4.33	0.092	0.089	4.14^{b}	4.51^{a}	4.43^{a}	4.53^{a}	0.106	0.052
Ca retention	0.74^{a}	0.54^{b}	0.46^{c}	0.015	<0.001	0.53^{b}	0.59^{a}	0.60^{a}	0.61^{a}	0.020	0.015
P retention	0.60	0.57	0.59	0.015	0.507	0.658^{a}	0.622^{a}	0.564^{b}	0.519^{b}	0.017	<0.001

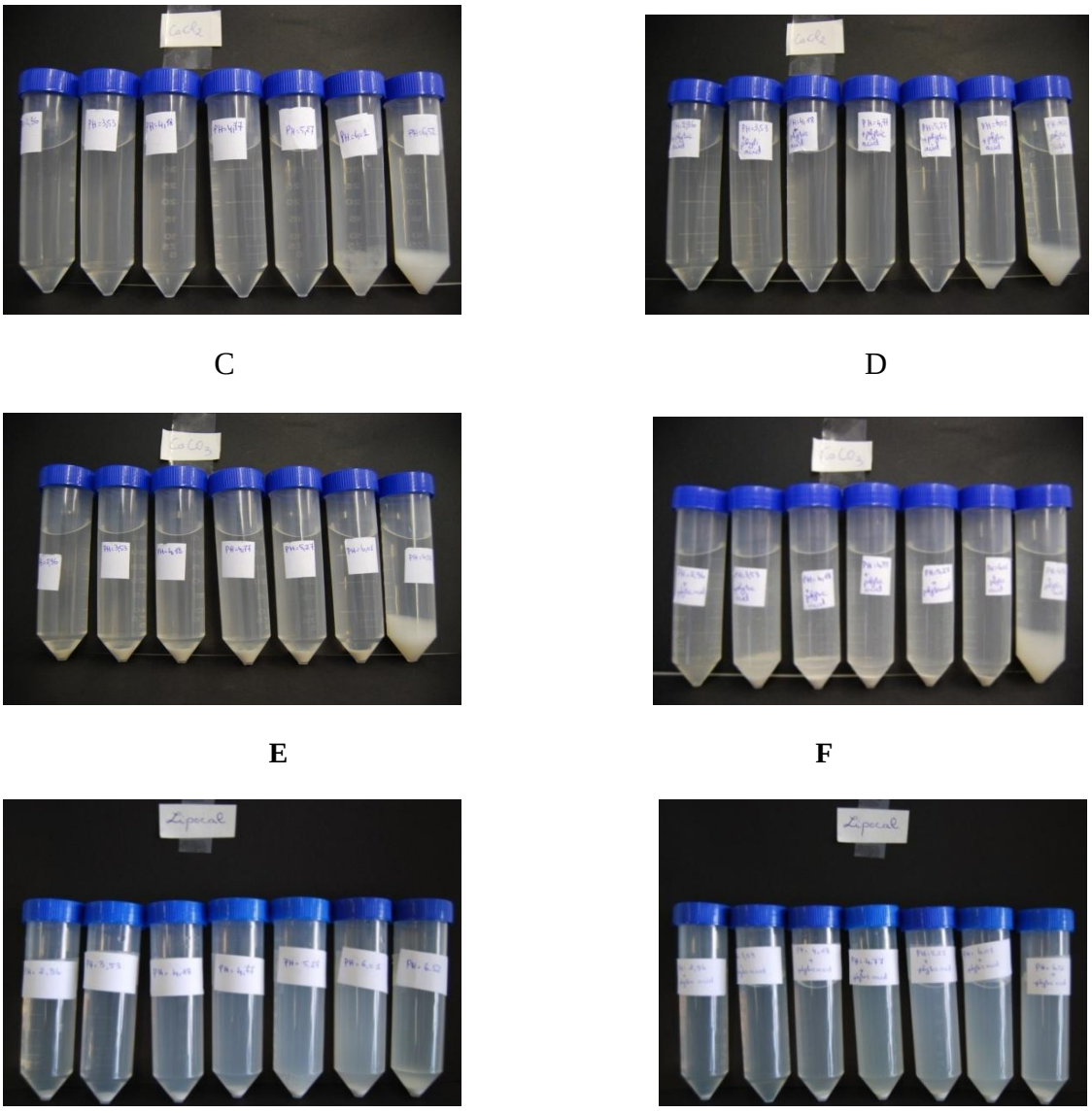
3.2. Experiment 2

3.2. 1.Calcium sources, physico-chemical differences

a. Calcium and Phosphorus Solubility

Figure 11, shows the evolution of mineral precipitate of Ca chloride (without or with phytic acid, A and B, respectively), Ca carbonate (without or with phytic acid, C and D, respectively) and Lipocal (without or with phytic acid, E and F, respectively). The images show that Ca Carbonate and Lipocal precipitated from the lowest pH 2.96 - 3.53 and reached maximum levels at the highest registered pH = 6.52. In contrast, calcium chloride were more soluble and started to show a precipitate at pH = 6.01-6.52.

A B



C D

E F

Figure 11. Ca chloride (A and B) Ca carbonate (C and D) and Lipocal (E and F) solutions at different pH (from left to right, 2.69, 3.53, 4.18, 4.77, 5.27, 6.01, 6.52).

The concentration of Ca in the supernatant of the above solutions is also shown in Table 14 and Figure 12. The values are described as depending on the pH, source of calcium (Ca carbonate, Ca chloride or Lipocal) and the addition of phytic acid. Clear differences among Ca sources were observed on the calcium solubility. Ca carbonate increased solubility of Ca with values below pH=4, reaching practically twice soluble Ca content with a pH of 2.69. On the other hand, Ca chloride showed a higher Ca solubility in the range between 2.69 and 6. An increase on the pH above 6 drastically reduced the levels of soluble Ca from calcium chloride. No relevant differences were observed on the two lines derived from the phytic acid incorporation. Lipocal showed a linear increased in the release of Ca as pH decreased from 6.52 to 2.69, showing lower values of soluble Ca than limestone and Ca chloride at pHs above 5.

Table 14. Concentration of Ca and P (mg/L) in the supernatant of Ca chloride and Ca carbonate solutions at different pH (from left to right, 2.69, 3.53, 4.18, 4.77, 5.27, 6.01, and 6.52) or additional phytic acid.

		$CaCO_3$		$CaCl_2$		Lipocal	
pH	A.phytic*	Ca (mg/l)	P (mg/l)	Ca	P	Ca	P
2,69	(-)	862	1264	781	1279	870	2498
3,53	(-)	472	1959	838	1936	734	2905
4,18	(-)	435	2526	842	2562	601	3668
4,77	(-)	490	3009	869	3063	460	3878
5,27	(-)	534	3362	830	3572	373	4188
6,01	(-)	491	3876	801	3919	184	4489
6,52	(-)	430	3841	332	4053	47	5095
2,69	(+)	977	1674	756	1558	1076	2149
3,53	(+)	609	2426	873	2206	840	2637
4,18	(+)	417	3391	877	2960	732	3182
4,77	(+)	495	3582	834	3361	519	3413
5,27	(+)	526	3951	874	3843	312	3655
6,01	(+)	507	4441	869	4266	152	4180
6,52	(+)	450	4695	452	4465	35	4799

***** (-): absence of phytic acid (+):presence of phytic acid

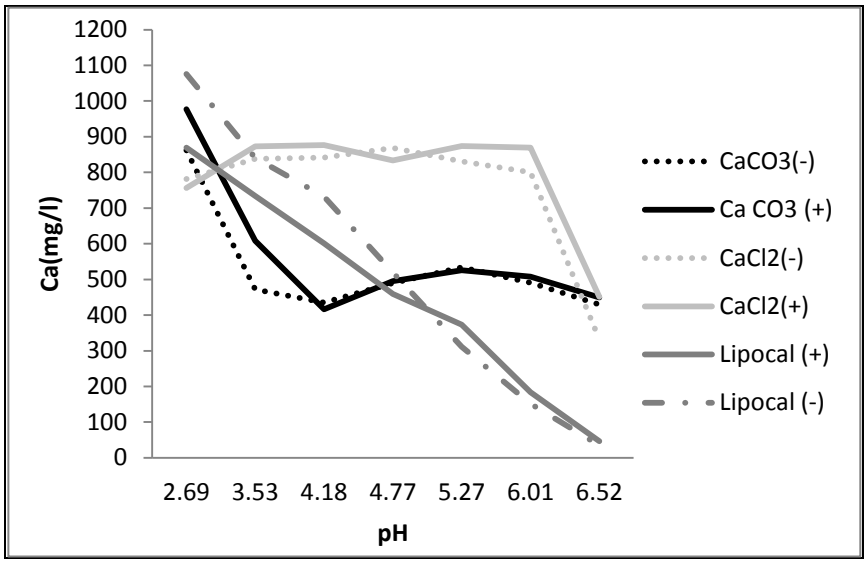


Figure 12. Concentration of Ca (mg/L) in the supernatant of Ca chloride Ca carbonate and Lipocal solutions at different pH (from left to right, 2.69, 3.53, 4.18, 4.77, 5.27, 6.01, 6.52) and with the addition or not ofphytic acid (+ vs -).

The concentration of P in the supernatant of calcium carbonate and calcium chloride solution is also shown in Figure 13. Soluble P was increased with the pH, as it was associated with the higher amount of phosphate contribution to buffer. The increase was practically linear. When comparing Ca sources, we noted that P levels (mg/l) were almost the same for $CaCO_3$ and $CaCl_2$ in the absence of phytic acid ($CaCO_3$ and $CaCl_2$), and slightly higher for Lipocal. As expected, the addition of phytic acid increased the soluble P content.

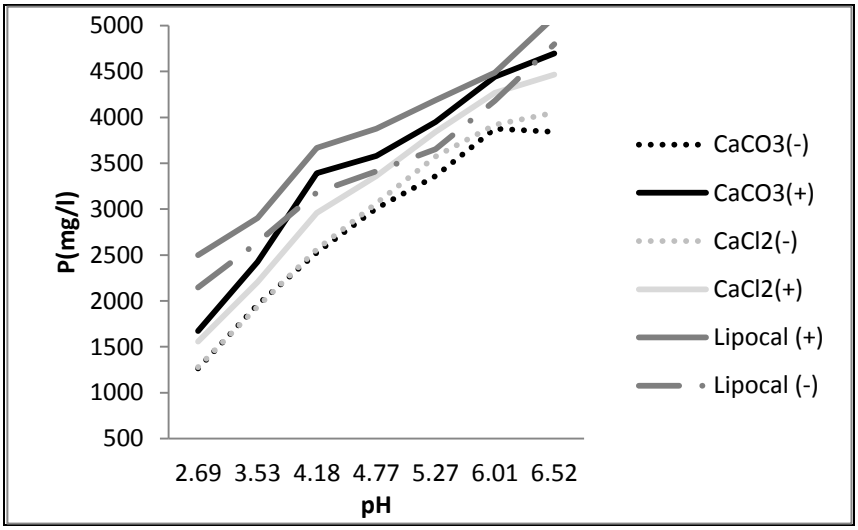


Figure 13. Concentration of P (mg/L) in the supernatant of Ca chloride Ca carbonate and Lipocal solutions at different pH (from left to right, 2.69, 3.53, 4.18, 4.77, 5.27, 6.01, 6.52) and with the addition or not of phytic acid (+ vs -).

b. Buffering capacity of different calcium sources

In Table 15 and Figure 14 is shown the volume of HCl required to reach a pH=3 and the acid-binding capacity in solutions with different Ca sources and concentrations. Two vertical axes were included to show better the large differences among Ca sources: the right one is related to the $CaCl_2$, and the left axe in relation to the $CaCO_3$, Ca lactate and Lipocal. Ca chloride showed the lowest ABC as compared to the rest of Ca sources.

If we compare these four sources, for example at 0.8% Ca, we note that ABC pH=3 are 0.08, 6.29, 8.76 and 10.53 meq respectively for $CaCl_2$, $CaCO_3$, Ca lactate and Lipocal.

Table 15. Hcl volume required to reach pH=3 and acid-binding capacity (ABC) of different concentrations and sources of Calcium

	vol Hcl PH=3(ml)				vol Hcl PH=3(meq)			
%Ca	$Ca\ Cl_2$	$Ca\ CO_3$	Ca lactate	Lipocal	$Ca\ Cl_2$	$Ca\ CO_3$	Ca lactate	Lipocal
0.2	0.64	17.55	20.65	27.25	0.06	1.76	2.07	2.73
0.4	0.7	35.25	49	52.6	0.07	3.53	4.90	5.26
0.6	0.71	52.9	62.4	82.15	0.07	5.29	6.24	8.22
0.8	0.78	62.85	87.55	105.3	0.08	6.29	8.76	10.53
1	0.81	71.35	108.85	128.65	0.08	7.14	10.89	12.87
1.2	0.89	91.45	128.75	157.6	0.09	9.15	12.88	15.76

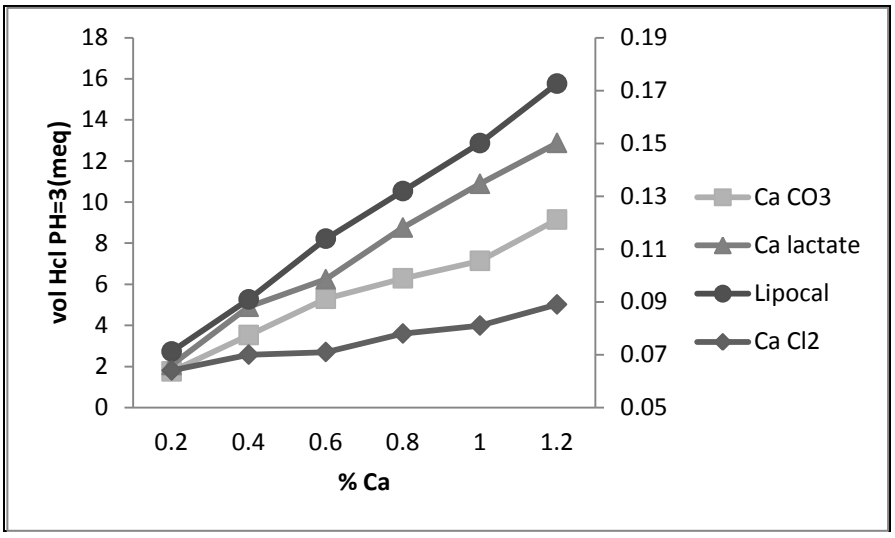


Figure 14. Acid-binding capacity of different concentrations and sources of Calcium

3.3. Experiment 3

3.3.1. Influence of Ca source and P levels on early bird performance and bone mineralization

The nutrients of the diets are presented in Table 9. It is worth noting that Ca was higher than formulated likely as a consequence of Ca carbonate added as filler of vitamin and mineral premixes.

a. Bird performance

Feed intake and growth performance were not influenced by a Ca source x P level interaction (P>0.05, Table 16). Then, average values for main factors (source of calcium and NPP levels) are shown in Table 17. Dietary P did not influence (P>0.05) feed intake or growth performance. Dietary source of Ca influenced ADFI (P<0.05), and growth performance (P<0.01) from Day 7 to 14 and from Day 1 to 14. The feed intake was higher on birds fed the Lipocal diet than birds fed the Ca chloride diet. Birds fed on Calcium Carbonate showed intermediate values. The increase in ADG and BW on day 14 was higher in birds fed Lipocal and Calcium Carbonate than birds fed Ca chloride.

b. Gizzard and proventriculus pH

No significant effects (P>0.05) were observed associated to the source of calcium, phosphorus or their interaction on the pH of proventriculus and gizzard. Average values were 2.08 and 2.15, respectively.

c. Bone mineralization

Tibia weight and tibia ash content (%, mg/tibia) are presented in Table 18. Tibia weight was influenced by a Ca source x P level interaction. Tibia weight was the highest in birds fed Lipocal at 4 g NPP/kg, and Ca carbonate at 3.5 g NPP/kg; and was the lowest for treatments including Ca chloride in the diet and 3.5 and 4 g NPP/kg.

Bone weight and mineralization as affected by main factors are also presented in Table 19. Changing the source of Ca in the diet promoted significant effects on the tibia weight and tibia ash content (% and mg/tibia). Tibia weight was significantly higher for birds fed Lipocal and Ca carbonate than Ca chloride. However, tibia ash percent was significantly higher for Lipocal than for Ca carbonate and Ca chloride.

Table16. Influence of Ca source and P levels on feed intake and growth performance of broilers from Day 1 to 14 (Interaction; Experiment 3)

Source Ca	Calcium Carbonate				Calcium chloride				Lipocal				**P-value**			
NPP(g/kg)	**3**	**3.5**	**4**	**4.5**	**3**	**3.5**	**4**	**4.5**	**3**	**3.5**	**4**	**4.5**	**S.E.M**	**S Ca[1]**	**P**	**S Ca*P**
Initial BW (g)	41.7	42.1	41.81	42	41.9	42.2	42	41.8	42.6	41.6	41.8	42.2	7.26	0.401	0.429	0.448
Day 7 BW(g)	150.5	144.3	155	147.7	138.6	149	135.3	144.3	149.6	150.1	149	151	9.34	0.320	0.993	0.874
Day 14 BW (g)	442.2	436.4	441.2	431.1	413.3	410.1	402.9	416.7	424.5	432.1	460.7	447.5	15.59	0.006	0.849	0.686
ADFI 1-7 d (g/d)	19.1	17.7	18.8	18.8	17.8	19.3	15.9	17.8	18.3	18.8	18.9	18.3	1.14	0.531	0.957	0.648
ADFI 7-14 d (g/d)	48	46.1	49.9	47.9	46.6	44.3	45.2	45.6	47.6	48	51	50.6	1.98	0.012	0.355	0.859
ADFI 1-14 d (g/d)	33.6	31.9	34.3	33.4	32.1	32	31	31.7	33	33.4	35	34.5	1.43	0.045	0.778	0.855
ADG 1-7 d (g/d)	15.5	14.6	16	15.1	13.8	15.2	13.3	14.6	15.3	15.5	15.2	15.5	1.33	0.329	0.991	0.884
ADG 7-14 d (g/d)	41.2	41.7	40.8	40.5	39.2	37.2	38.2	38.9	39.2	40.1	44.5	42.3	1.34	0.001	0.435	1.137
ADG 1-14 d (g/d)	28.4	28.2	28.5	27.8	26.5	26.3	25.8	26.8	27.3	27.8	29.9	28.9	1.11	0.007	0.819	0.692
FCR 1-14d (g/d)	1.20	1.16	1.20	1.22	1.24	1.23	1.25	1.21	1.21	1.21	1.19	1.17	0.050	0.473	0.903	0.614

[1]S Ca : Source Calcium

Table 17. Influence of Ca source and P levels on tibia weight. and tibia and whole-body ash of 14-d-old broilers (Main factors Experiment 3)

	Source			P-value		NPP (g/Kg)				P-value	
	Calcium Carbonate	**Calcium chloride**	**Lipocal**	***S.E.M***	**S Ca**	**3**	**3.5**	**4**	**4.5**	***S.E.M***	**P**
Initial BW (g)	47.5	42	42.1	3.35	0.401	42.1	42.0	49.3	42.0	3.90	0.429
Day 7 BW(g)	149.4	141.9	149.9	4.30	0.321	146.3	147.9	146.5	147.7	5.02	0.993
Day 14 BW (g)	437.7[a]	410.7[b]	441.2[a]	7.19	0.006	426.7	426.2	435.0	431.8	8.38	0.849
ADFI 1-7 d (g/d)	18.6	17.93	18.6	0.52	0.531	18.4	18.7	18.2	18.4	0.61	0.957
ADFI 7-14 d (g/d)	48[ab]	45.46[b]	49.3[a]	0.91	0.012	47.4	46.2	48.7	48.1	1.06	0.355
ADFI 1-14 d (g/d)	33.3[ab]	31.6[b]	33.9[a]	0.66	0.045	32.9	32.4	33.5	33.2	0.77	0.778
ADG 1-7 d (g/d)	15.3	14.2	15.4	0.62	0.329	14.9	15.1	14.9	15.1	0.71	0.992
ADG 7-14 d (g/d)	41[a]	38.4[b]	41.5[a]	0.62	0.001	39.9	39.7	41.2	40.6	0.72	0.435
ADG 1-14 d (g/d)	28.1[a]	26.3[b]	28.4[a]	0.51	0.007	27.4	27.4	28.0	27.8	0.59	0.819
FCR 1-14d (g/d)	1.20	1.23	1.20	0.020	0.47	1.22	1.20	1.21	1.21	0.027	0.903

[a,b] Means not sharing a common superscript are significantly different at P <0.05.

As a consequence, the ash content per tibia was the greatest for birds fed Lipocal, and the lowest for birds fed the Ca chloride diet. Ca carbonate showed intermediate results.

d. Phosphorus and Calcium Ileal digestibility

The calcium and phosphorus ileal digestibility were not influenced by the Ca source x P level interaction ($P>0.05$). Then, average values for main factors (source of Calcium and NPP levels) are shown in Table 19. Calcium ileal digestibility was influenced by both, source of Ca and NPP levels. Birds fed Ca chloride have the highest Ca ileal digestibility as compared to birds fed Ca carbonate and Lipocal. Calcium ileal digestibility was also progressively increased with higher levels of NPP, being significantly higher in birds fed 4.5 g NPP/Kg than 3g NPP/Kg.

P ileal digestibility was influenced by the level of NPP, being the highest with the level 4.5g NPP/Kg and the lowest with 3 g NPP/ Kg .

Table 18. Influence of Ca and P levels on tibia weight and tibia ash of 14-d-old broilers (Interaction Experiment 3)

Source Ca	Calcium Carbonate				Calcium chloride				Lipocal				P-value			
NPP (g/kg)	**3**	**3.5**	**4**	**4.5**	**3**	**3.5**	**4**	**4.5**	**3**	**3.5**	**4**	**4.5**	**S.E.M**	**S Ca**	**P**	**S Ca*P**
Tibia weight(g)	0.78^{ab}	0.83^{a}	0.79^{ab}	0.80^{ab}	0.77^{ab}	0.74^{b}	0.70^{b}	0.78^{ab}	0.77^{ab}	0.78^{ab}	0.89^{a}	0.80^{ab}	0.031	0.011	0.883	0.029
Tibia ash (%)	50.51	50.35	50.65	50.77	50.61	50.46	50.68	51.24	50.96	51.81	51.08	51.27	0.320	0.001	0.381	0.291
Tibia ash (mg/tibia)	398	418	405	407	393	377	359	404	394	406	450	409	16.95	0.011	0.800	0.058

Table 19. Influence of Ca and P levels on tibia weight and tibia ash of 14-d-old broilers (Main factors Experiment 3)

	Source			P-value		NPP (g/Kg)				P-value	
	Calcium Carbonate	**Calcium chloride**	**Lipocal**	***S.E.M***	**S Ca**	**3**	**3.5**	**4**	**4.5**	***S.E.M***	**P**
Tibia weight(g)	0.80^{a}	0.75^{b}	0.81^{a}	0.014	0.011	0.78	0.79	0.79	0.80	0.017	0.883
Tibia ash (%)	50.57^{b}	50.75^{b}	51.29^{a}	0.148	0.002	50.69	50.88	50.80	51.10	0.172	0.382
Tibia ash (mg/tibia)	407^{ab}	383^{b}	415^{a}	7.58	0.011	395	400	404	407	9.11	0.800

Table 20. Influence of Ca and P levels on Ca ileal digestion and P ileal digestion of 14-d-old broilers (Interaction Experiment 3)

Source Ca	Calcium Carbonate				Calcium chloride				Lipocal				P-value			
NPP (g/kg)	**3**	**3.5**	**4**	**4.5**	**3**	**3.5**	**4**	**4.5**	**3**	**3.5**	**4**	**4.5**	**S.E.M**	**S Ca**	**P**	**S Ca*P**
Ca ileal digestibility (%)	64,8	68,8	64,6	70,2	70,4	72,8	74,6	77	62,25	67,4	67,2	70,4	3,11	0.001	0.047	0.948
P ileal digestibility (%)	78,8	81	83	86	78,8	80	84	89,2	72,75	83	79,8	83,2	2,88	0.188	0.001	0.575

Table 21. Influence of Ca and P levels on Ca ileal digestion and P ileal digestion of 14-d-old broilers (Main factors Experiment 3)

	Source			P-value		NPP (g/Kg)				P-value	
	Calcium Carbonate	**Calcium chloride**	**Lipocal**	***S.E.M***	**S Ca**	**3**	**3.5**	**4**	**4.5**	***S.E.M***	**P**
Ca ileal digestibility (%)	$67,1^{b}$	$73,7^{a}$	$66,8^{b}$	1,43	0.001	$65,8^{b}$	$69,7^{ab}$	$68,8^{ab}$	$72,5^{a}$	1,67	0.047
P ileal digestibility (%)	82,2	83,0	79,7	1,33	0.188	$76,8^{b}$	$81,3^{ab}$	$82,3^{ab}$	$86,1^{a}$	1,55	0.001

4. Discussion

4.1. Influence of calcium levels on the broiler performance

The results obtained in Experiment 1 showed that a level of 7g Ca/Kg optimized feed intake, tibia ash and tibia weight for broilers chicken from 1 to 14 days and also gave the best growth compared to lower and higher levels of Ca (see Figure 15). Birds exposed to diets low in Ca (7gCa/kg), and standard NPP (3.8 g P/kg) performed the best, while the high Ca treatment (9 g Ca/kg) induced negative responses, which shows that a lower total calcium concentration is desirable to reach better performance. These results agree with Driver et al., (2005a) who described BWG and FCR optimized at 0.625% Ca in the diet. On other hand, Rao et al., (2006) did not found differences on the body weight gain at Day 14 due to variation in dietary Ca level.

There are different reasons which may explain the negative effects of high levels of calcium on performance. Calcium is known to form insoluble complexes with phytate phosphorus, which may hinder phytase activity (Angel et al., 2002). Calcium may also react with dietary inorganic P to form insoluble calcium orthophosphate (Plumstead et al., 2008), which may also make inorganic P less available for absorption at high dietary intakes. This effect could explain our results that the lowest performance was observed with high Ca diets containing limiting values of NPP (2.5 g nPP/kg). The effect of high dietary calcium on the retention of phosphorus has been reported by Sebastian et al (1996). They also reported that high dietary Ca increase the intestinal pH, reduces the solubility of minerals and limits P availability for absorption. Thus, high concentrations of $CaCO_3$ may increase the pH in the proximal gastrointestinal tract due to its high acid binding capacity (see *in vitro* results of Experiment 2) leading to a decrease in P and amino acid digestibility. However, our results did not show significant differences on the pH in the gizzard and proventriculus.

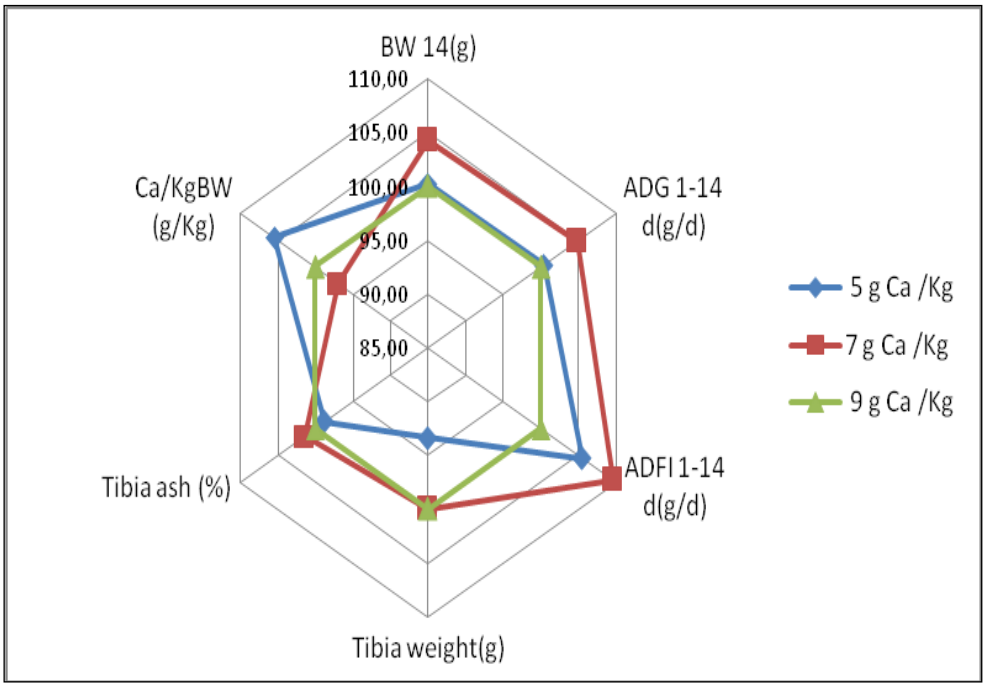


Figure 15.Effect of Calcium on the various parameters (Experiment 1,The results obtained for the level 9g Ca/kg represent 100%, the other results are compared relative this level)

Gacs and Barltrop (1977) showed some aggregations between minerals and dietary polymers in the digesta may contribute to reduce the digestibility coefficients for protein and fat. Calcium is able to form insoluble soaps with free fatty acids and bile acids and there is some evidence that these soaps limit the absorption of fat *in vivo* (Gacs and Barltrop, 1977, Govers, et al., 1996, Shahkalili et al., 2001). These soaps could lower the utilization of energy derived from lipids, particularly saturated fats, in broiler diets.

However, it is relevant that feed intake was early depressed on the high calcium diet during the first week, without affecting the feed conversion rate. This result could suggest that broiler may have detected these high levels of calcium, or they reduced feed intake in order to avoid a larger Ca and P unbalance. Some recent reports suggest that broilers are able to detect calcium in the diet, which could explain specific appetites depending on the current status of the animals (Wilkinson et al., 2012).

The results of tibia weight and bone mineralization (Figure 15) were also influenced by the level of Ca, with the lowest bone weight and mineralization observed for the low

calcium diet. It is interesting to observe the two highest levels of dietary calcium were paired when we considered tibia weight in contrast with body weight which confirms that calcium requirement for bone mineralization may be higher than for performance. This result agree with the result of Onyango et al., (2003) who found that bone mineral content, bone mineral density and percentage of ash increased linearly as the level of dietary Ca increased from 4.5 to 9.1 g/kg. However, we also found a significant interaction between the level of Ca and P on the tibia ash percent, which confirm that high levels of calcium may affect P availability for bone mineralization. The results of this study emphasis the importance of formulating diets that meet or exceed P requirements of broilers, particularly when high Ca diets are used.

Al Masri (1995) saw that the values of dietary Ca and its ratio with P may affect the phosphorus retention; with lower values as higher are the levels of calcium in the diet. However, we did not observe this difference on the P retention with the levels of calcium used in our experiment.

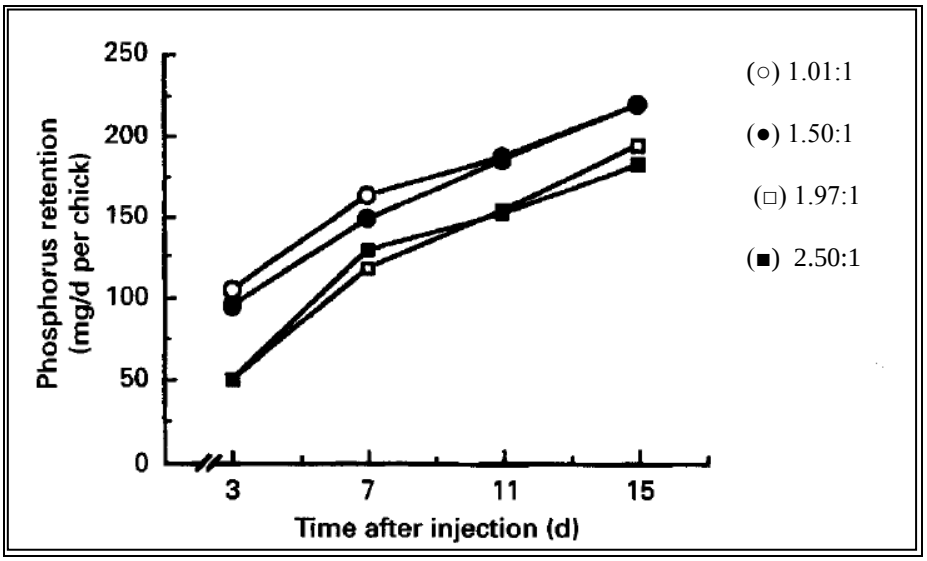


Figure 16.Influence of the calcium:phosphorus ratio of the diet on daily retention of phosphorus (mg P/d per chick) in the whole body of growing chiks from 3 to 15d. (Al Masri, 1995).

Our results on Ca and P retention concur with those of Mitchell and Edwards (1996) and Ziaei, (2008) who reported that reduced mineral content of diets resulted in a higher apparent retention of Ca, leading to a reduction in mineral excretion. Browning et al., (2012) show that reducing dietary Ca/avP concentrations were associated with increased efficiency of Ca retention as compared to high Ca/avP diets which indicates a

physiological response by the chicken to overcome a Ca deficiency by up-regulating its nutrient transfer and deposition infrastructure.

4.2. Influence of NPP levels on the broilers performance

The introduction of NPP phosphorus at a level of 3.8 g / kg maximized the growth of chicks on day 14. This level were significantly different from the lowest level 2.5 g NPP / kg($P < 0.05$) and similar to the productive performance of birds fed the level of 4.5 g NPP / kg, which is the level recommended by NRC 1994 for the age between 0-3 Weeks of age. Moreover, the performance results obtained for these treatments were close to the standard of the breed for this period (473 g BW on D14).

Recent research has reported substantial differences in the non phytic P (NPP) requirement of broilers compared with those published by the NRC (1994). Waldroup et al. (2000) reported that the NPP requirement for the starter phase ranges from 0.37 to 0.39%. The difference between the recommendations of NRC and the needs of the animals can be explained by the fact that the NRC (1994) NPP recommendations for broilers are based on peer-reviewed research published between 1952 and 1983. Modern commercial birds are very different from commercial birds available prior to 1983, due in part to genetic selection, but also management practice have changed (Havenstein et al., 1994), as it has occurred with the addition of phytase to feed. In the present trial we incorporated an overdose of a commercial phytase (Quantum blue, AB vista) to the diets to reach a phytase activity of (1,150 U/kg).

Increasing the levels of NPP in the diet increased the bone mineralization up to 3.8 g NPP / kg, but not further increases were observed with 4.5 g NPP / kg.

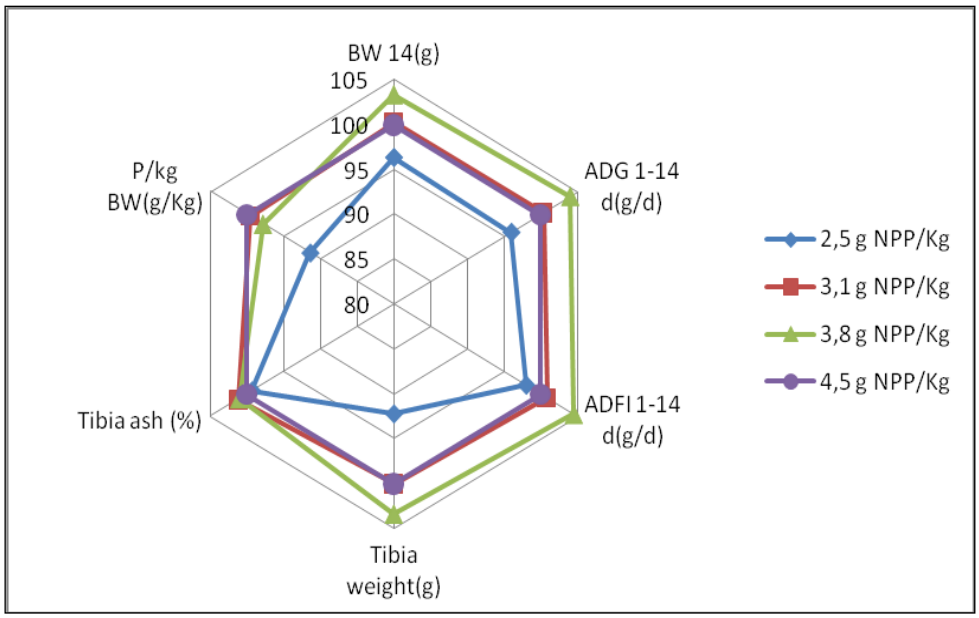


Figure 17. Effect of Phosphorus on the various parameters(experiment 1,The results obtained for the level 4,5 g NNP/kg represent 100%, the other results are compared relative this level)

Ravindran et al., (1995) observed that a bone mineralization criterion is a good sensitive indicator of the P status of the birds. Despite phosphorus is largely contained in all the tissues, bone is the main storage organ for P, containing 85% of the body's total P. Through its involvement in metabolic and structural processes, P is essential for animals to attain their optimum genetic potential in growth and feed efficiency as well as skeletal development (Applegate and Angel, 2008). Increasing the levels of NPP in the diet allowed increases on bone mineralization and on the quantity of Ca and P in the whole body.

However, it is remarkable that increases on the levels of NPP in the diet reduced the fractional retention of phosphorus, which it is a similar response to that observed previously for increasing levels of calcium. The results could reflect a decrease on the P digestibility, (not analyzed in this experiment), but more likely an increase on the endogenous excretion of P in the urine (Al Masri, 1995). When broilers receive P levels that are higher than the physiological threshold for maximum utilization and retention, there is the possibility that the additional P may be eliminated most likely through the kidney (Leske and Coon, 2002). To know this threshold is important to integrators to avoid the wasting of P into the litter.

It is very interesting to see that increasing the levels of NPP in the diet from 2.5 to 3.1 g NPP/kg were associated with significant higher values of Ca retention, likely reflecting how body growth and bone mineralization respond to an improve on Ca/P ratio in the diet. Driver et al. (2006) reported too that the integrity of the tibia was dependent on the Ca and NPP levels fed to broilers in the early stages of growth. However, based on the effect on growth performance during the grower phase of NPP levels fed in the starter phase, Powell et al., (2011) suggested that broilers fed lower levels of NPP in the starter phase are better able to adapt to a lower level of NPP in the grower phase than those fed a higher level of NPP in the starter phase. Our results confirm that broilers respond very early to changes on the NPP levels in the diet in growth performance and bone mineralization. However, the consequences of these changes on later performance and leg quality during the following weeks and the whole growing period deserve further studies.

Therefore, the results reflect that changes on the levels of calcium and phosphorus in the diet may decrease chick growth and also affects bone formation and body retention of calcium and phosphorus, which has been explained by the likely formation of insoluble calcium phosphate and phytic acid complexes due to the high intake of calcium.

It has been also shown in the literature review that the solubility of Ca and P may change depending on the ingredient, phytate, or Ca content of the diet. In addition, conditions in the gastric phase of digestion may have a profound effect on phytase efficacy and digestibility of Ca and P. For example, an increase in the crop and gizzard pH may promote Ca, phytate, and P precipitation and reduce the solubility of Ca and P (Selle et al., 2009, Walk et al., 2012c).

The objectives of the following experiment were to evaluate the solubility of three different sources of Ca *in vitro* (Ca carbonate, Ca chloride and a commercial source of tricalcium phosphate, Lipocal (Lipofoods, Barcelona, Spain), and their likely interaction with the solubility of phosphorus as well as the buffering capacity of the solution. To attain this objective we measured the in *vitro* soluble Ca content at various pH (from pH=2.96, simulating gastric pH in the proventriculus, to pH=6.52, simulating intestinal pH).

4.3. Calcium and Phosphorus Solubility

Precipitation obtained in the case of Ca carbonate and Lipocal at low pH is consistent with Selle *et al*. (2000), who have suggested that most mineral complexes are soluble at low pH's (less than 3.5) with maximum insolubility occurring between 4 and 7. Champagne (1988) has reported that Ca-PP complexes precipitate at pH's between 4 and 6 which is the approximate pH of the intestine where the Ca ions should be absorbed. Previous *in vitro* research in corn-based diets would suggest limestone is approximately 80% soluble in the acidic medium of the gastrointestinal tract but decreased to 77% solubility in neutral conditions of the intestine, suggesting no further dissolution of Ca in the intestinal phase (Walk et al., 2012c).

Our results on soluble Ca and P were coincident with the image of precipitates and we can say that Ca chloride was more soluble than Ca carbonate and Lipocal. Taylor (1965, Manangi and Coon (2008)) has suggested that the primary factor affecting PP utilization is the Ca ion concentration in the small intestine where insoluble Ca-PP-complexes form. Thus, a precipitated PP-mineral complex would not be accessible for hydrolysis or absorption in the intestine. Recent studies suggest that a more digestible calcium source may be effective on performance and reduced risks of leg problems.

According to Manangi and Coon (2008) a smaller particle size and higher Ca solubility had the lowest phytate P hydrolysis indicating the interference due to Ca-phytate complex formation. We observed higher soluble P values in the case of $CaCO_3$ and Lipocal than in the case of $CaCl_2$, likely because $CaCO_3$ and Lipocal are less soluble than $CaCl_2$. Thus, dissolved calcium ions may bind to phytic phosphorus to form a complex, which decrease the concentration of P.

These results could suggest that broilers may gain more from feeding phytase by feeding larger particle $CaCO_3$ with lower solubility to minimize the solubility of $CaCO_3$ in the crop and in anterior portion of the gastrointestinal tract. A lower solubility form of $CaCO_3$ as compared to very finely ground limestone may allow the phytase enzyme more accessibility to the PP in the gut and provide more available P from PP hydrolysis in the broiler (Manangi and Coon, 2007).

4.4. Buffering Capacity of different calcium sources

Lipocal and Ca carbonate showed a high buffering capacity relative to Ca chloride; Jasaitis et al. (1987) found that carbonates and dibasic ortribasic mineral additives had the highest ABC. Bolduan (1988) found that increasing the mineral supplementation of

a diet from 0 to 4% tripled the ABC-4 value. At this respect, mineral sources are major contributors to the buffering capacity of the diet, which can interfere with the ability of the young chick gizzard to reach a low pH. From these results, we planned to do a trial to evaluate if different sources of Ca, differing on their inherent solubility at different pH and acid- binding capacity could allow the use of low calcium diets in broilers feeding.

4.5. Influence of calcium source and its interaction with NPP levels on performance, bone mineralization and ileal digestibility

The calcium source promoted significant differences on feed intake. The introduction of tricalcium phosphate (Lipocal) in the diet promoted higher ADFI in comparison with the more soluble Ca and P sources (ie., Ca chloride + sodium phosphate). This difference directly affected the growth of the animals, being birds fed on Ca carbonate and Lipocal the ones showing the highest body weight gain. Then, it could be suggested that sources of calcium with a lower solubility may allow better performances than the high soluble Ca chloride. This result agrees well with the result obtained by Walk et al., (2012c) who found that broiler chickens fed 0.90% Ca from limestone ate more and were heavier than birds fed 0.90% Ca from a high soluble calcium source (HSC). Due to the soluble nature of the HSC source, the authors suggested that feeding 0.90% Ca from HSC may have reduced broiler performance as a result of a wide Ca:P ratio and an increase in calcium phosphate or calcium phytate precipitation. The authors also described that N digestibility was reduced as Ca from the high soluble source increased, most likely due to the buffering capacity of high inclusion levels of the HSC, which may have reduced pepsin efficacy in the proventriculus and gizzard (pH optimum at 2.8, Bohak, 1969).

A similar response was observed by Coon and Manangi (2008) when they evaluated the effect of different Ca particles sizes (from 28 to 1306 µm) in broilers, The higher weight gains were obtained in chicks fed intermediate $CaCO_3$ particles sizes, between 137 and 388 µ, compared to the gains obtained with the smallest (28 µm) or largest particles sizes. Our study included limestone with an average particle size of 200µm. Both *in vivo* and *in vitro* studies (Coon and Manangi, 2008) indicated that limestone with a high solubility (>70.0%, ie. 28 microns) limited phytate hydrolysis to provide available P for growth and bone ash formation.

Therefore, it could be proposed that the effects of soluble Ca limiting phytate hydrolysis could be counteracted by an overdose of phytase. Walk et al (2012c) described that an overdose of phytase in feed to reach 2,000-2,500 U/kg was able to increase the performance of broilers fed on 0.9% Ca of a high soluble Ca source to values close to those presented by a limestone source without phytase. Similar performance were observed with lower levels of Ca, which suggest that reductions in dietary Ca may be obtained with high soluble sources of Ca while maintaining broiler performance and bone ash.

In the present study, however, all the diets were overdosed with phytase to reach an analyzed phytase activity of 1,150 U/kg. The results also showed that Ca digestibility was higher in Ca chloride than limestone and Lipocal, but no differences were observed in P ileal digestibility or not responses were observed to the NPP supplementation, which make questionable the explanation of a limited P digestibility with the high soluble source of Ca. Moreover, acid-binding capacity was the lowest for Ca chloride which also pose doubts about any negative effect on protein digestibility. On the other hand, the results showed a consistent effect of Ca chloride on feed intake and weight gain, which could suggest that broiler, could have reduced feed consumption after detecting a high soluble Ca and P source in the beak and the crop. However, further research should be performed in order to find a likely explanation to these results.

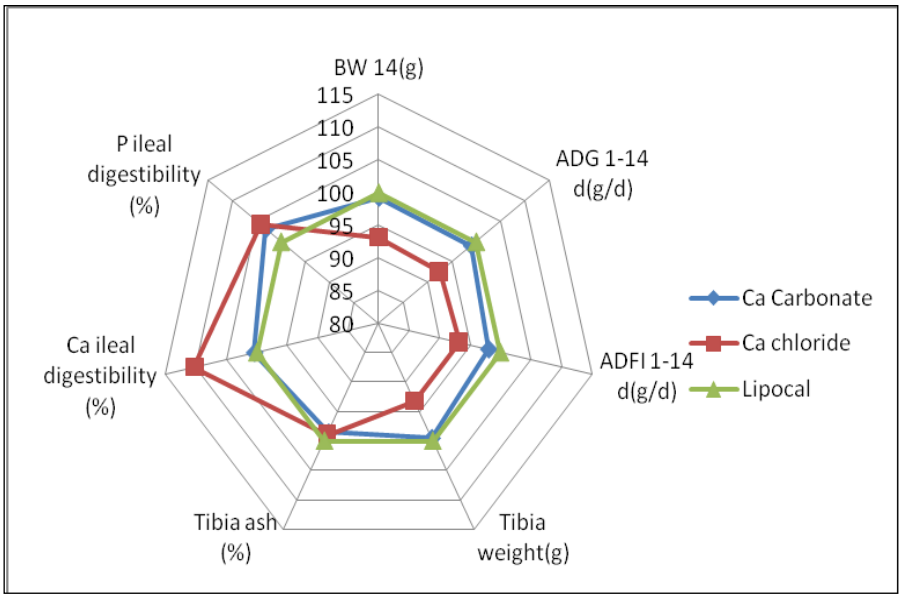


Figure 18. Effect of calcium sources on the various parameters (Experiment 1, the results obtained for the Lipocal represent 100%, the other results for calcium Carbonate and calcium chloride are compared relative to Lipocal).

The tibia weight, percentage of tibia ash and mg ash/ tibia were influenced by changes in the calcium source, with Lipocal and Ca carbonate showing higher results than that obtained in the case of Ca chloride. The results are coherent with differences observed on feed intake and weight gain, but not with the results on Ca and P digestibility. Moreover, it is remarkable the significant interaction observed between the calcium source and level of NPP. Ca chloride at average levels of NPP (3.5-4 g NPP/kg) in the diets caused a lower weight of the tibia relative to Lipocal (0.70 vs 0.89 g) and Ca carbonate (0.83g), respectively. It is difficult to find an explanation to this effect on bone mineralization which it appears to be larger than the effects observed in feed intake and performance. Thus, the results could suggest that bone mineralization is a more complex process which requires providing daily total digestible Ca and P amounts, but also appropriate rates of absorption for each mineral. It appears this rate could be better when derived from less soluble sources. However, this hypothesis deserves further studies.

On the other hand, limestone and Lipocal showed similar responses on performance, bone mineralization and Ca and P digestibility. The responses to NPP were also coherent with our first experiment, with the higher weight gains observed with values of NPP between 3.5 and 4 g NPP/kg.

In the present experiment, we studied also the calcium and phosphorus ileum digestibility as affected by the Ca source and the NPP level. Increasing the level of NPP increased the apparent ileal P digestibility, which it agrees well with the results of Rodehutscord and Dieckmann, (2005). Al Masri (1995) saw that the values of dietary Ca and its ratio with P may affect phosphorus absorption; with lower values on P absorption as higher were the ratios between Ca and P in the diet. However, this result could also reflect that the mineral sources used to increase NPP have a higher P digestibility than vegetable sources, even after including phytase in the diet. At this respect, when we used the level 3 gNPP /kg to compare P digestibility among diets (1.- Ca carbonate diet with a main contain on monocalcium phosphate, 2.- Ca chloride with higher contents of sodium phosphate and monocalcium phosphate , and 3.- the Lipocal diets with a main content of tricalcium phosphate) the values were 78.8, 78.8 and 72.7, respectively. The results showed a higher P digestibility for the monocalcium and sodium phosphate than the tricalcium phosphate (Lipocal), likely reflecting the low solubility of this source at the pH of small intestine (see *in vitro* results, Experiment 2).

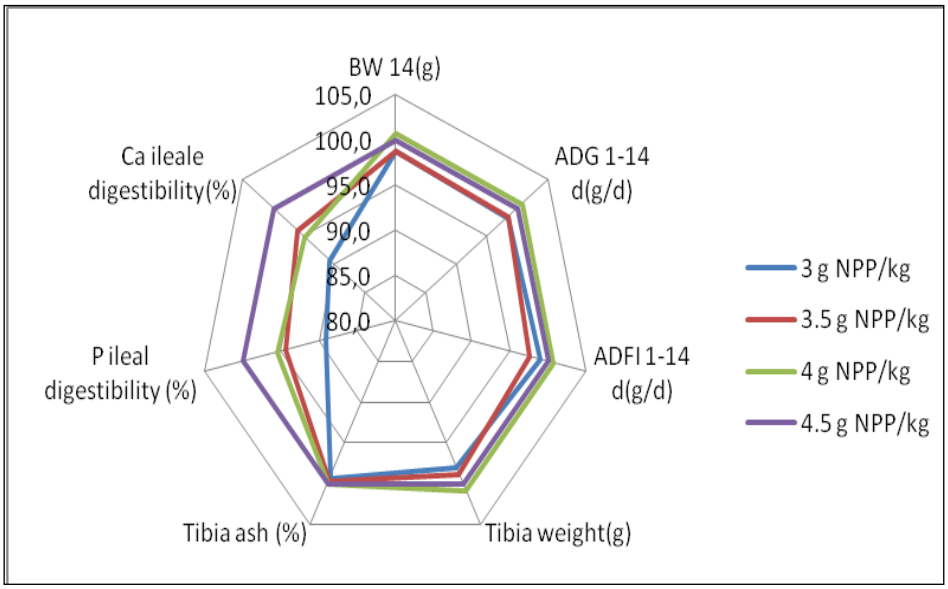


Figure 19. Effect of NPP on the various parameters (Experiment 3 The results obtained for the level 4,5 g NPP/kg represent 100%, the other results are compared relative to this level).

It is also relevant that higher levels of NPP also promoted an increase on calcium digestibility, which it could reflect the changes on the main mineral ingredients used in each diet. Angel (2013) has recently described that true digestibility of Ca for limestone (34.1%) is lower than for monocalcium phosphate (67.9%). The increase on ileum P digestibility with increasing levels of NPP on the Lipocal treatments, also suggest that calcium digestibility is also lower for tricalcium phosphate than monocalcium phosphate. Then a change on the levels of inclusion of mineral ingredients, as those promoted when phytase are included in the diet, are expected to promote also changes on calcium digestibility.

Conclusions

1. Calcium and phosphorus dietary recommendations should be considered together because both macrominerals show clear interactions in the digestive tract as well as on the bird performance and bone formation.

2.- A dietary level of 3.8 g NPP /Kg in the diet, which it was simultaneously overdosed with phytase, was adequate to ensure the best growth and bone formation of broiler from day 0 to day 14 and in diet.

3.- A dietary level of 7.9 g Ca/kg may optimize performance and bone mineralization of chicks during the first two weeks of life; being this value below those currently used by the industry. Higher values of Ca promote significant early decreases on feed intake and body weight gain during the first week of life.

4. - Different Ca and P sources show clear differences on their solubility at a wide pH range, and promote differences on the dietary Ca and P ileum digestibility in the animals, which encourages the interest of using digestible Ca values on feed formulation.

5.- However, Calcium chloride, which it was the calcium source with the highest solubility and ileum digestibility, depressed the intake of low Ca diets and affected bone mineralization of the young chicks. These results may suggest that young birds detect and refuse high levels of soluble Ca in the beak and reinforce the interest of considering rates of Ca and P absorption as important to reach an optimum bone formation.

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