PEARL MILLET AND GHRELIN IN POULTRY

By

JOSHUA DEAN HAMBURG

(under the direction of Adam J. Davis)

ABSTRACT

Poultry production allows the efficient production of animal protein in the form of meat and eggs. In developing countries where affordable animal protein sources for human consumption are deficient, the consumer price of poultry products and the expansion of the commercial poultry industry are negatively impacted by the utilization of costly imported feed ingredients to make poultry diets. Identifying and utilizing locally grown feed ingredients would be beneficial in these locations. Pearl millet is a drought resistant plant that produces a nutritious grain. Its cultivation in present-day Mali spans thousands of years. It is still widely cultivated in this country where poultry production is limited. The nutrient composition of different varieties of pearl millet grown in Mali was assessed and then experiments were completed which indicated that whole pearl millet grain grown in this region is a suitable replacement for corn in broiler and laying hen diets.

INDEX WORDS: pearl millet, broilers, laying hens

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DEDICATION

In loving memory of my grandfather Dean Bural Hamburg. Thank you for all of your advice and encouragement, I miss you every day.

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TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS……………………………………………………………………………..v

CHAPTER

1 MILLET………………………………………………………………………………………1

General overview……………………………………………………………………1

Pearl millet……………………………………………………………………………1

Nutrient composition ……………………………………………………………3

Pearl millet grain in poultry production……………………………………4

Laying hens ……………………………………………………………………………5

Broilers…………………………………………………………………………………6

Whole pearl millet in poultry diets……………………………………………7

Pearl millet as an alternative grain in poultry diets………………………8

Summary………………………………………………………………………………9

2 GHRELIN AND THE AVIAN OVARY……………………………………………10

The avian ovary……………………………………………………………………10

Avian follicular tissues and follicular maturation………………………11

Feed restriction and ovarian function………………………………………11

Ghrelin overview……………………………………………………………………14

Ghrelin synthesis……………………………………………………………………15

Ghrelin secretion and tissue distribution……………………………………16

Ghrelin receptor……………………………………………………………………..17

Physiological effects of ghrelin……………………………………………………18

Regulation of ghrelin secretion……………………………………………………18

Ghrelin and reproduction…………………………………………………………19

Avian ghrelin……………………………………………………………………...22

Avian GOAT………………………………………………………………………23

Avian ghrelin receptor……………………………………………………………23

Avian ghrelin synthesis and functions…………………………………………24

Summary…………………………………………………………………………...25

3 STATEMENT OF PURPOSE……………………………………………………28

4 MATERIALS AND METHODS…………………………………………………30

Pearl millet experiments…………………………………………………………30

Experiment 1: Whole pearl millet in laying hen diets …………………………31

Experiment 2: Whole pearl millet in broilers ……………………………………33

Experiment 3: Ground pearl millet in broilers……………………………………39

Ghrelin experiments………………………………………………………………39

Experiment 4: Ghrelin expression in the hen ovary……………………………39

Experiment 5: Ghrelin in fed and fasted broiler breeder hens…………40

RNA extraction……………………………………………………………………42

Real time RT-PCR…………………………………………………………………42

Statistics……………………………………………………………………………45

5 RESULTS AND CONCLUSIONS…..46

Experiment 1…..46

Experiment 2…..52

Experiment 3…..57

Experiment 4…..66

Experiment 5…..66

6 DISCUSSION…..76

Pearl millet…..76

Protein and energy content of Malian pearl millet…..76

Malian pearl millet in poultry production…..77

Whole pearl millet…..80

Ghrelin…..81

7 REFERENCES…..85

CHAPTER 1

MILLET

General overview

Millets are a collection of divergent small seeded grasses that are grouped together based on their agronomic characteristics rather than close genetic relatedness. Thus, millet grains vary widely in color, size and shape. Millets are known for their short growing season, drought resistance and tolerance of high temperatures. They are important crops in Southeast Asia, particularly India and in Sub-Sahara Africa in countries such as Mali, Niger and Nigeria. Millet grain is an important human food staple in developing countries and the grass serves as important forage for livestock. In the United States, millet is often planted in southern areas of the country such as Texas, where it serves as pasture forage for livestock. In addition, proso millet is grown for feeding wild and domesticated birds.

Pearl millet

Although cultivated around the world, millet likely evolved in western Africa and many varieties of wild and cultivated millet are still found there. Worldwide, pearl millet (Pennisetum *glaucum*) is the most widely grown millet and accounts for approximately 50% of millet production. Pearl millet domestication can be traced back to the Lower Tilemsi Valley in northeastern Mali to the period of 2500 to 2600 BC (Manning *et al.*, 2011). From this location domesticated pearl millet spread eastward across the Sahel

region and reached India by sea contact within less than 100 years of its domestication (Manning *et al.*, 2011). Its rapid spread was likely by mobile pastoralist societies that valued pearl millets drought resistance and suitability as a pasture forage.

Today pearl millet is still a favored crop in the semiarid, low soil fertility regions of Southeast Asia (especially India) and in the Sahel and tropical savanna regions south of the Sahara Desert in Africa. Pearl millet is a tall, fast growing, deep-rooted forage and cereal grain that is utilized for both human and livestock consumption (Baltensperger, 2002) and can be grown successfully under environmental conditions where corn and wheat fail to survive. Pearl millet grain out-yields other cereal grains under poor environmental conditions such as: drought, heat stress, infertile soil quality, and limited growing season (Freeman and Bocan, 1973). There are several genetic strains of pearl millet which means the color of millet grain can vary from white, pale yellow, brown, grey, slate blue or purple and the kernel shape can be classified as obovate, hexagonal, lanceolate, globular and elliptical. Pearl millet grains are typically about 2 to 4 mm long (Lee *et al*., 2004). In the Sahel region of Africa, pearl millet grain can account up to 60% of total cereal food consumption. It is often eaten as porridge, used as a flour to make flat bread or processed into a beverage component.

The agronomic characteristics that allow pearl millet to grow in the poor soils of semi-arid Africa and southeast Asia makes it suitable to be grown in the coastal plain region of the southeast United States, where soils tend to be acidic, arid, and have poor fertility. Pearl millet also matures quickly under proper environmental conditions (maturing in 75-80 days) allowing for double-cropping in the long summers of the southeast as well as rotational cropping systems. Pearl millet can be planted as early as

May 1st, when temperatures are ideal; around 70°F. and as late at August 10th (Lee *et al.*, 2004). However, widespread use and cultivation of pearl millet has been limited in the U.S. because of its susceptibility to rust disease. This fungal disease can cause significant losses of yield and grain weight (Wilson *et al.*, 1995). More recently the development of rust resistant pearl millet varieties has renewed interest in cultivating pearl millet in the southeastern United States (Davis *et al.*, 2003; Lee *et al.*, 2004). These rust resistant varieties have reliably produced 70 or more bushels/acre with proper management and environmental conditions (Lee *et al.*, 2004).

Nutrient composition

The nutrient composition of pearl millet is similar to or better than corn. Although variable, the crude protein level of pearl millet typically ranges from 10-16% (Burton *et al.,* 1972; Sullivan *et al.*, 1990; Adeola and Rogler, 1994; Amato and Forrester, 1995; Davis *et al.*, 2003; Singh *et al.*, 2005; Vasan *et al.*, 2008a, b). Protein levels vary from harvest to harvest and are different depending on the variety of pearl millet and the agronomic conditions, particularly the nitrogen content in the soil from which it was harvested. The recommended amount of nitrogen fertilizer is 80 to 100lbs per acre in shallow soils and up to 140lbs per acre in deep soil (Lee *et al.,* 2004).

On a weight-to-weight comparison, millet has up to 60% more protein, 40% more lysine and methionine, and up to 30% more threonine than corn (Singh and Perez-Maldonado, 2003). Other research also indicates that pearl millet has higher lysine concentrations than corn (Adeola and Rogler, 1994; Amato and Forrester, 1995; Davis *et al.,* 2003; Singh, 2004; Vasan *et al.*, 2008a, b). Singh *et al.*, (2005) reported that newly

developed pearl millet varieties in Australia had about 50% more lysine, methionine, threonine, and tryptophan than sorghum and that the digestibility of the essential amino acids determined in poultry was superior (cystine, lysine, and threonine) or equivalent to sorghum. The apparent digestibilities of the essential amino acids of pearl millet are similar to corn (Vasan *et al.*, 2008a, b).

Pearl millet is rich in oil, typically having a fat content above 5%, slightly higher than corn (3.8%) and other common cereal grains (Rooney, 1978; Hill and Hanna 1990; Sullivan, *et al.*, 1990; Adeola and Rogler, 1994; Hidalgo, *et al.*, 2004). The nitrogen corrected true metabolizable energy (TME_n) of corn is 3,350 kcal/kg compared to 3,300 to 3450 kcal/kg for pearl millet (Adeola, *et al.,* 1994; Collins, *et al.*, 1997; Davis, *et al.*, 2003; Hidalgo, *et al.*, 2004). Pearl millet has a high proportion of linolenic acid (LNA; $C_{18:3n\text{-}3}$), about 4% of its fatty acid content, giving it a much higher content of n-3 fatty acids than many other cereal grains (Rooney, 1978). Pearl millet has a lower content of omega-6 fatty acids, specifically linoleic acid (LA; $C_{18:2n\text{-}6}$).

Pearl millet grain in poultry production

The nutritional profile of millet would allow it to replace corn as an energy source, as well as a portion of the soybean meal as a protein source in poultry diets. Thus, a growing body of research has been conducted to determine the feasibility of including pearl millet in both laying hen and broiler diets.

Laying hens

Collins *et al.* (1995) reported that replacing 100% of the dietary corn (68% of the formulated diet) with millet caused no reduction in egg production for a 6-week trial period. Similar results were reported by Purushothaman and Thirumalai (1997) and Collins *et al.* (1997). In contrast, Mehri *et al.* (2010) conducted a 12-week experiment and found that 100% replacement of corn (58% of the formulated diet) with millet decreased egg production ($P<0.05$). However, replacing 25, 50, or 75% of the corn content of the laying diet with millet did not affect egg production. The authors attributed the decrease in egg production when corn was completely replaced by millet in the diet to the high fiber content of the local Iranian variety of millet which had a crude fiber content of 64 g/kg while the fiber content of the corn was 22 g/kg. Similarly, Rama Rao *et al.* (2000) suggested that the higher crude fiber level of their millet (44.4 g/kg versus corn 19.3 g/kg) might have increased passage rate, therefore reduced nutrient utilization in hens.

Egg weights and egg quality are typically unaffected by replacing dietary corn with millet, even when the dietary corn is completely replaced by millet (Collins *et al.*, 1997; Amini and Ruiz-Feria, 2007). However, yolk pigmentation is reduced when dietary corn is replaced by millet (Collins *et al.*, 1997; Amini and Ruiz-Feria, 2007). Kumar *et al.* (1991) reported an increase in egg size when dietary corn was completely replaced by millet. As with egg production, Mehri *et al.* (2010) reported replacing the corn content of a laying diet with 25, 50 or 75% millet did not affect egg weight, but a 100 replacement of dietary corn with millet reduced egg weight. Similar reductions in egg weight were reported by Purushothaman and Thirumalai (1997) in laying hens and by Rama Rao *et al.* (2000).

In corn omega-3 fatty acids only comprise 0.9% of the total fatty acids while in pearl millet 4% of the fatty acids are n-3 fatty acids (Rooney, 1978). The benefit of having high n-3 fatty acids in feed ingredients for hens is that more of these fatty acids can then be deposited into eggs. Through marketing strategies, omega-3 enriched eggs are sold for higher prices to humans for consumption. There are potential benefits of elevated n-3 fatty acid intake from the eggs, which include: increased immune function, prevention of cardiovascular disease, diabetes, some cancers and a significant role in neonatal growth (Kinsella *et al.,* 1990; Simopoulos, 2000). Traditionally omega-3 enriched eggs have been produced by incorporating flaxseed and menhaden fish oil but both of these ingredients lead to the production of eggs with off-flavors and that are more susceptible to oxidative rancidity (Scheideler *et al.,* 1994). The utilization of pearl millet as a replacement for corn in laying hen diets allows the levels of flaxseed or menhaden fish oil to be reduced while yielding omega-3 enriched eggs that are of better consumer quality.

Broilers

Total replacement of corn with pearl millet improved body weight and feed conversion in male Ross 508 broilers during a 42-day grow-out experiment conducted in cages (Baurhoo *et al.*, 2011). Additionally, digesta viscosity, jejunum villus, height, width and surface area were equal between broilers fed the control corn containing diet or diets in which the corn was replaced by millet (Baurhoo *et al.*, 2011). In a 42 day floor pen study, male and female Ross broilers were fed diets containing 0, 25 or 50% pearl millet and live performance and carcass yields were equivalent or better for the broilers fed the pearl millet diets relative to the control (0% millet) corn and soybean meal diet (Davis, *et*

al., 2003). Similarly, pearl millet was reported to be equivalent or superior to corn as a grain source for broiler rations in prior research (Sharma *et al.*, 1979; Smith *et al.*, 1989; Collins *et al.*, 1994; Amato and Forrester, 1995).

Whole pearl millet in poultry diets

Often with the introduction of new cultivars of pearl millet there has been limited supply of the grain for poultry research and for incorporation into commercial diets. Additionally, many commercial poultry feed mills lack the post-grinding storage capacity for minor grains such as pearl millet. Therefore, the majority of research that has been conducted was to determine the feasibility of incorporating whole millet into poultry diets. Incorporation of up to 10% whole pearl millet in diets fed throughout the entire broiler production period of 42 days did not adversely affect broiler performance, carcass yields, or pellet quality (Hidalgo *et al.*, 2004). Disappearance of whole millet determined by the absence of millet seed in the excreta was greater than 95% for broilers that were fed diets containing 5, 10, 15 or 20% whole millet from 1 to 15 days of age (Hidalgo *et al.*, 2004) or in laying hens fed for 7 days diets containing 5, 10, 20, 30 or 40%whole millet (Garcia and Dale, 2006). Laying hens fed a diet containing 10% whole millet for 4-weeks had no effect on feed consumption or egg production (Garcia and Dale, 2006).

Laying hens fed diets containing 15% whole millet versus 15% ground millet had significantly higher starch digestibility of the diet than the hens fed the diet with ground millet. Previous studies have also indicated that broilers fed diets containing whole wheat or barley had increased dietary starch digestibility (Hetland *et al.*, 2002; Plavnik *et al.*, 2002; Svihus *et al.*, 2004). The increased starch digestibility may be due to increased

enzyme accessibility to starch granules, which may result from enhanced gizzard function in birds fed whole grains. Hidalgo *et al.*, (2004) reported that broilers fed diets containing 10, 15 or 20% whole millet had increased relative gizzard weights to body size than birds fed diet with no whole millet. Similar increases in gizzard weight have been seen in studies using whole grains, such as wheat, triticale, and barley as reported by Kiiskinen (1996), Svihus *et al.* (1997) and Jones and Taylor (2001).

Pearl millet as an alternative grain in poultry diets

The positive performance results obtained in poultry when using millet in poultry research along with its favorable agronomic characteristics has stimulated interest in developing pearl millet as an alternative feed grain for poultry diets. With the increase in corn prices due to ethanol production, pearl millet becomes increasingly more attractive as a feed ingredient since its nutritive value is typically at least as good as, if not better than corn. Alternative feed ingredients are often locally produced crops or crop by-products , which also can make them more attractive as it is more economical to use a product that is harvested locally to save on shipping and handling costs. Thus, millet, which could be easily grown in the southern U. S., where poultry production is concentrated, has tremendous potential as a crop. In the Sahel Region of Africa where millet production is abundant and corn does not thrive, millet has been used as a human food staple for centuries. Poultry production in this region of Africa has not developed greatly in part because commercial producers use imported corn and soybean meal to feed their birds, which results in very elevated and inflated consumer prices. However, the use of locally grown pearl millet in poultry diets, as a corn replacement, could greatly reduce

poultry production costs, stimulate commercial development, and offer an affordable alternative protein source.

Summary

Pearl millet is the leading millet variety produced in the world. As a native of the western and southern edges of the Sahara desert, it grows well under conditions characterized by sporadic irregular rainfall, high temperatures and poor soil quality. Pearl millet is a fast growing crop that produces a grain that has energy and protein values that are variable based on variety and growing conditions, but that are typically similar to or better than the values for corn. Research indicates that pearl millet can easily replace a portion or all of the corn in poultry diets without negatively effecting growth and egg production.

CHAPTER 2

GHRELIN AND THE AVIAN OVARY

The avian ovary

The functional left ovary of the sexually mature laying hen typically contains a visually evident hierarchy of follicles based on size and time until ovulation. In the laying hen, there are commonly four to six large yellow yolk-filled follicles, referred to as hierarchical follicles that range from approximately 12 to 40 mm in diameter. There are several additional follicles, called small yellow follicles (SYF), measuring 5 to 12 mm in diameter. In addition to the SYF, there are a large number of white follicles that are less than 5 mm in diameter. The SYF and white follicles are referred to as pre-hierarchical follicles.

The large yellow hierarchical follicles are named according to size and expected time of ovulation. The largest follicle, designated F1, will ovulate within 24 hours. The next largest follicle is called the F2 follicle and will ovulate approximately 24-26 hours after the ovulation of the F1 follicle. The remaining large yolk filled hierarchical follicles are named accordingly (F3-Fn). With the ovulation of each F1 follicle, the next follicle in position advances one position forward in the naming hierarchy while a new follicle is recruited into the hierarchy from the pool of SYF. Meanwhile, some of the larger white follicles begin the uptake of yellow yolk and advance to the pool of SYF. The vast majority of small yellow and white follicles will never advance into the hierarchy (Gilbert *et al.,* 1983) and instead will undergo atresia by apoptosis (Johnson *et al.*, 1996).

Avian follicular tissues and follicular maturation

Each preovulatory follicle consists of distinct tissue layers that surround the yolk-filled oocyte. In each hierarchical follicle, the yolk-filled oocyte is surrounded by its plasma membrane, then the inner perivitelline layer, followed by the granulosa cell layer, the basement membrane, and theca tissue layers. The theca tissue is highly vascularized, in contrast to the avascular granulosa cell layer, and facilitates the transfer of yolk precursors from plasma to the developing follicles in the ovary (Etches and Cheng, 1981).

In general terms, follicular maturation can be described by the accumulation of yolk and the development of endocrine capabilities within the follicular tissues. The theca cells of the small yellow and white follicles are steroidogenically competent and are the primary source of plasma estrogen (Lee and Bahr, 1989; Senior and Furr, 1975). However, the granulosa cells of these small follicles are steroidogenically incompetent because they lack P450 side chain cleavage (P450 SCC) enzyme activity which catalyses the initial step in the metabolic pathway that converts cholesterol to steroid hormones (Li and Johnson, 1993). Once selected into the hierarchy, the granulosa cells become increasingly steroidogenically competent and luteinizing hormone (LH) sensitive (Li and Johnson, 1993; Johnson and Bridgham, 2001). This LH sensitivity promotes progesterone production (Calvo and Bahr, 1983; Robinson *et al.*, 1988; Davis *et al.,* 1999; Davis *et al.*, 2001; Johnson *et al.*, 2004) which allows the F1 follicle to ovulate.

Feed restriction and ovarian function

Through genetic selection, better nutrition and better bird management today's broilers reach a market weight of about 2.5 kilograms in 5 to 6 weeks after hatching. To

support this rapid growth rate, broilers have been bred to possess voracious appetites. These appetites and rapid growth rates are problematic for optimal reproductive performance in the genetically similar parent stocks of broilers. Optimum reproductive efficiency in broilers is dependent in large part on attaining and maintaining an ideal body weight to support reproduction, consuming a nutritionally adequate diet, and being photostimulated. Although the ideal body weight for reproduction is a little less than market size, the optimum sensitivity to photostimulation, for reproduction. in broilers does not occur until about 20 weeks of age.

To prevent broiler breeder pullets from growing too quickly and becoming too large and fat by the photosensitivity-based sexual maturity that occurs at 20 weeks of age, their dietary intake is restricted. Typically, feed allocations are 60-80 percent less during the rearing period and 25-50 percent less during the laying period than what breeder hens would consume *ad libitum* (Renema and Robinson, 2004). Feed restriction of broiler breeder hens is a successful management tool in increasing the reproductive efficiency of these birds. Feed restricting broiler breeder hens delays sexual maturation (Robbins *et al.*, 1986; Yu *et al.*, 1992a; Heck *et al.,* 2004; Bruggeman *et al.*, 2005; Hocking and Robertson, 2005; Onagbesan *et al.*, 2006) and decreases mortality (Robbins *et al.*, 1986; Katanbaf *et al.*, 1989; Heck *et al.*, 2004; Bruggeman *et al.*, 2005). Additionally, feed restriction during the rearing and the laying period reduces the development of an abnormally high number of large follicles on the ovary of broiler breeder hens (Hocking, 1987; Hocking *et al.,* 1989; Heck *et al.,* 2004; Hocking and Robertson, 2005). More importantly, broiler breeder hens that have been feed-restricted produce more eggs (Yu *et al.*, 1992a; Heck *et al.,* 2004; Bruggeman *et al.*, 2005; Onagbesan *et al.*, 2006) because they lay longer sequences

(Robinson *et al.,* 1991), persist in lay longer (Fattori *et al.*, 1991), lay fewer abnormal eggs and have fewer multiple ovulations in a single day (Fattori *et al.*, 1991; Yu *et al.,* 1992a; Heck *et al.,* 2004) compared to full-fed broiler breeder hens. Overweight broiler breeders also have compromised fertility due to reduced locomotion and their physical difficulty in successfully copulating (Duff and Hocking, 1986). Fertility is reduced in overweight hens even when artificial insemination is used (Brake and McDaniel, 1981), this may be due to the fact that the excess body fat may actually make the insemination more difficult, possibly block the sperm storage tubules or inhibit sperm movement (Hocking, 1987).

The implementation of feed restriction in the United States typically results in broiler breeder pullets being fed once every other day during rearing and once a day sometime between photostimulation and when total egg production reaches about 5% for the flock. This feed is quickly consumed by the birds resulting in significant fasting periods for the birds between feedings. Morris and Nalbandov (1961) suggested that the lack of gonadotropin secretion from the pituitary was responsible for the loss of egg production in fasted birds. Subsequently, Scanes *et al.* (1976) reported that plasma LH concentrations were significantly depressed in 6 week old male chicks fasted for 12 hours compared to control-fed cockerels. In addition, fasted laying hens have lower plasma concentrations of LH after 48 hours of fasting and lower estradiol and progesterone concentrations after 24 hours of fasting compared to *ad libitum* fed control hens (Tanabe *et al.*, 1981).

Research supports the idea that the fasting periods created as a result of current poultry industry feed restriction practices depress total egg production in broiler breeder hens. Egg production through peak lay was reported to be improved by reducing the fasting duration between meals in broiler breeder hens by feeding them twice a day rather

than once a day (Spradley, 2007). In addition, reducing the fasting period by feeding once a day instead of every other day during the period after photostimulation for reproduction until the flock reached 8% egg production significantly enhanced total egg production by 19 eggs per hen (Gibson, 2006).

The research of Gibson (2006) and Spradley (2007) reinforces the hypothesis that nutritional status and caloric intake are both intricately connected to reproductive function in birds. Furthermore, despite the success of feed restriction in broiler breeder hens, these hens still produce annually over 100 eggs less than their Leghorn laying hen counterparts, and follicular maturation and ovulation are still plagued by an unacceptable incidence of atresia of large yellow follicles and internal ovulations. The complex hormonal interactions defining how caloric intake affects reproduction have yet to be clarified, but a key component of this interaction may be the hormone ghrelin.

Ghrelin overview

Ghrelin is a 28-amino acid peptide hormone involved in the regulation of energy homeostasis and growth hormone (GH) secretion. In 1999, Kojima *et al.*, isolated ghrelin from rat gastric tissue and identified it as the endogenous ligand for the growth hormone secretagogue receptor (GHSR). Since its discovery, ghrelin has been found to be a potent orexigenic compound that centrally and peripherally effects metabolism. In addition to modulating a variety of metabolic processes, ghrelin has been found to influence reproduction, both centrally and peripherally, in mammalian species (Van der Lely *et al.* 2004). There are two identified isoforms of GHSR, 1a and 1b, however only GHSR1a activation results in GH secretion in the pituitary (Howard *et al.*, 1996). Ghrelin also has

two identified forms; des-acyl ghrelin (DAG) and acylated ghrelin (ghrelin), both of which are found in circulation. Only acylated ghrelin binds to the ghrelin receptor.

Ghrelin synthesis

In humans, the ghrelin gene (GHRL) encodes for a 117-residue peptide, preproghrelin, which undergoes proteolysis of a signal peptide comprised of 23 amino acids to generate a 94 amino acid peptide known as proghrelin (amino acids 24-117). While this immature peptide form of ghrelin is still within the leaflets of the endoplasmic reticulum, acylation by ghrelin O-acyltransferase (GOAT) may occur (Yang *et al.*, 2008b). Thus, both the des-acyl proghrelin and acylated proghrelin may be transported from the endoplasmic reticulum to the golgi body for further processing (Kojima and Kangawa, 2010). Once within the golgi body, prohormone convertases (PC1/3) cleaves proghrelin after amino acid 51 (Zhu *et al.*, 2006). The result is the 28 amino acid ghrelin (amino acids 24-51) and a 66 amino acid C-terminal propeptide named C-ghrelin (amino acids 52-117) (Hosoda *et al.*, 2003).

The acylated form of ghrelin has a characteristic N-octanoic acid functional group on Ser3 (Thr3 in frogs), which is added to DAG by GOAT. In 2008, two separate groups identified GOAT as the membrane bound O-acyltransferase (MBOAT4) responsible for modifying ghrelin. Currently, ghrelin is the only known peptide hormone in which a fatty acid modification is crucial for biological function, as only the acylated ghrelin binds to and activates GHSR1 (Kojima *et al.* 1999; Gutierrez *et al.*, 2008; Yang *et al.*, 2008a).

The acylated form of ghrelin was originally believed to be the only biologically active form, however, recent studies indicate that DAG is also involved in cell signaling

pathways independent of the GHSR system (Inoue *et al.*, 2010). It has now become generally accepted that DAG is not a biologically inert hormone (Baldanzi *et al.*, 2002; Murata *et al.*, 2002; Gauna *et al.*, 2005; Toshinai *et al.*, 2006; Barazzoni *et al.*, 2007; Zhang *et al.*, 2008), but the mechanisms by which it causes its effects are unknown. DAG is involved in a wide range of regulatory pathways including insulin homeostasis, orexigenesis, cell proliferation, and adipogenesis (Cassoni *et al.*, 2004; Muccioli *et al.*, 2004; Thompson *et al.*, 2004; Asakawa *et al.*, 2005; Granata *et al.*, 2006; Sato *et al.*, 2006; Granata *et al.*, 2007).

Ghrelin secretion and tissue distribution

In mammals, approximately two thirds of plasma total ghrelin (DAG + ghrelin) is produced in the stomach by cells within the gastric fundus mucosa while the remaining source of plasma total ghrelin is the small intestine (Date *et al.*, 2000; Ariyasu *et al.*, 2001; Gualillo *et al.*, 2003). Other tissues that express ghrelin mRNA include the pancreas, heart, adipose tissue, adrenals, thyroid, pituitary, hypothalamus, placenta, ovary, and testes (Gnanapavan *et al.*, 2002; Volante *et al.*, 2002; Gronberg *et al.*, 2008). Of the total plasma ghrelin content, the concentration of DAG is approximately 50 times higher than that of ghrelin (Hosoda *et al.*, 2000; Murakami *et al.*, 2002).

Initially, many researchers measured plasma total ghrelin levels based on the assumption that DAG was inactive and that increases in total ghrelin reflected increases in ghrelin. While the ratio between total ghrelin and ghrelin secretion have been reported to remain constant under a variety of conditions (Ariyasu *et al.*, 2002; Murikami *et al.*, 2002), the scientifically preferred method for determining plasma ghrelin concentration is to

measure it directly. Furthermore, since the N-octanoic acid modification of ghrelin is relatively unstable, collected plasma samples should be acidified immediately to prevent hydrolysis of ghrelin to DAG (Hosoda *et al.*, 2004). Thus, research reports dealing with plasma ghrelin concentrations should be reviewed carefully to determine if total ghrelin or acylated ghrelin was being measured and if the samples were processed properly to prevent degradation of acylated ghrelin.

Ghrelin receptor

Discovery of GHSR preceded the successful isolation of its natural ligand, ghrelin (Howard *et al.*, 1996). Because binding of synthetic substrates to the G-coupled protein receptor stimulated the release of growth hormone (GH), the receptor was reasonably named Growth Hormone Secretagogue Receptor. Currently, there are two identified isoforms, GHSR1a and GHSR1b; however, only GHSR1a is activated by ghrelin and can initiate downstream signal transduction of secondary messengers to stimulate GH secretion. The protein structure of GHSR1a is predicted to consist of an extracellular N-terminal domain, 7 transmembrane domains, and an intracellular C-terminal domain (Howard *et al.*, 1996). GHSR1b is composed of 289 amino acids and lacks the first 77 amino acids encoded by the beginning of the second exon. It is predicted that this truncated form of GHSR has only the first 5 of the 7 transmembrane domains. Though the latter GHSR form cannot bind ghrelin, it may serve to down regulate availability of GHSR1a for binding. The highest expression levels of GHSR1a mRNA are found in somatotrophs, cells responsible for GH secretion within the pituitary, and in the hypothalamus in the arcuate nucleus, an area crucial for neuroendocrine regulation of appetite stimulation

(Guan *et al.*, 1997; Kojima and Kangawa, 2005). However, GHSR1a mRNA is expressed at lower levels in a variety of other tissues such as heart, lung, liver, pancreas, intestine, adipocytes, thyroid, spleen, adrenal, ovarian and testicular tissue (Guan *et al.*, 1997; Kojima *et al.*, 2001; Gnanapavan *et al.*, 2002; Barreiro *et al.*, 2003; Gaytan *et al.*, 2003). GHSR1b mRNA has been detected within the same tissues in which GSHR1a has been identified (Gnanapavan *et al.*, 2002), but the mRNA expression of GHSR1b tends to be less than the expression of GHSR1a (Korbonits *et al.*, 2001; Gauna *et al.*, 2005).

Physiological effects of ghrelin

Ghrelin was originally discovered as the natural ligand for a growth hormone secretagogue receptor within the pituitary. Therefore, it is not surprising that ghrelin causes the potent release of growth hormone in somatotrophs both in vitro and in vivo in a dose dependant manner (Kojima *et al.*, 1999; Peino *et al.*, 2000; Hataya *et al.* 2001). However, the most striking physiological effect of ghrelin, independent of growth hormone releasing activity, is the stimulation of appetite and feeding behavior. Infusion of ghrelin, either intracerebroventricularly or peripherally, into mice or rats stimulates feeding behavior and if the injections are given long enough the animals will gain weight (Tschop *et al.*, 2000; Wren *et al.*, 2000; Kamegai *et al.*, 2001).

Regulation of ghrelin secretion

Plasma ghrelin concentrations and mRNA expression within the stomach directly reflect energy balance within mammals. Rats that have been fasted for 24 or 48 hours, and consequently have a negative energy balance, have higher levels of ghrelin mRNA

expression in their stomachs, as well as increased plasma levels of total ghrelin, when compared to rats that have been fed (Toshinai *et al.*, 2001). Six hours after refeeding the fasted rats, there was a decrease in stomach ghrelin mRNA expression and plasma levels of total ghrelin (Toshinai *et al.*, 2001). Mechanical distention of the stomach with non-nutritive substances, such as water, will not suppress plasma ghrelin levels in mice or rats (Tschop *et al.*, 2000; Williams *et al.*, 2003), but providing total parenteral nutrition will reduce plasma ghrelin levels (Qader *et al.*, 2005).

Ghrelin and reproduction

Hyperghrelinemia delays puberty in male rats, but has no effect on puberty onset in females (Fernandez-Fernandez *et al.*, 2005a; Martini *et al.*, 2006). Prepubertal males receiving subcutaneous injections of ghrelin have lower levels of plasma LH than normal controls, as well as decreased plasma testosterone levels. However, females undergoing the same treatment had no changes in plasma LH, FSH, or estradiol concentrations (Fernandez-Fernandez *et al.*, 2005a). These results suggest that male reproduction may be more sensitive to the influence of ghrelin than female reproduction.

In female and male rats, female monkeys, ewes, and human males, intracerebro-ventricular or peripheral ghrelin injections will decrease the pulse frequency of LH release from the pituitary and plasma LH levels, regardless of whether or not the animal is gonadally intact (Furata *et al.*, 2001; Tena-Sempere *et al.*, 2002; Fernandez-Fernandez *et al.*, 2004; Vulliemoz *et al.*, 2004; Iqbal *et al.*, 2006; Kluge *et al.*, 2007). The effect on LH secretion appears to be mediated by gonadotropin releasing hormone (GnRH), since GnRH secretion by hypothalamic fragments from ovariectomized female rats is inhibited by

ghrelin, as is pituitary cell responsiveness to GnRH, as measured by LH production (Fernandez-Fernandez *et al.*, 2005b). Interestingly, ghrelin will stimulate LH and FSH secretion in a dose dependant manner when it is added to isolated pituitary cells collected from ovariectomized female rats (Fernandez-Fernandez *et al.,* 2004; Fernandez-Fernandez *et al.,* 2005b; Lebrethon *et al.,* 2006). The biological basis for this unique effect in vitro of ghrelin on gonadotropin secretion is not known, but it has not affected the overall conclusion that ghrelin inhibits LH production in vivo and provides a mechanism that when there is caloric insufficiency the further energy demands of reproduction are prevented (Barreiro and Tena-Sempere, 2004; Tena-Sempere, 2005).

Peripheral injection of ghrelin into human males lowers plasma testosterone levels (Kluge *et al.,* 2007). This is likely due to ghrelin inhibiting LH production, since LH is the primary stimulus for leydig cells to produce testosterone (Moyle and Ramachandran, 1973). However, ghrelin produced by leydig cells, possibly in response to LH stimulation (Barreiro, *et al.,* 2002) may have a role in fine-tuning testosterone production by the leydig cells (Barreiro and Tena-Sempere, 2004). Neither ghrelin or GHSR1a (mRNA. or protein) is expressed in leydig cells before they are steriodogenically competent (Barreiro *et al.,* 2002; Barreiro *et al.,* 2003). Once the testes are capable of producing steroids, rat testicular tissue incubated with ghrelin produces less testosterone than untreated control tissue (Tena-Sempere *et al.,* 2002). This direct effect of ghrelin on testosterone production is associated with a decrease in mRNA expression of steriodogenic enzymes, such as steriodogenic acute regulatory protein (StAR) and cholesterol side chain cleavage enzyme required for testosterone synthesis (Tena-Sempere *et al.,* 2002) and would diminish the stimulatory effect of LH on testosterone production. Ghrelin also decreases proliferative

activity of immature rat leydig cells differentiating into adult leydig cells (Barreiro *et al.,* 2004).

Although there is evidence of ovarian production of ghrelin and for the presence of GHSR1a in most ovarian tissue, the role of ghrelin in follicular development has not been widely researched and remains unclear. Ghrelin and GHSR1a (mRNA and protein) are expressed in mature human and rat ovaries (Caminos *et al.,* 2003; Gaytan *et al.,* 2003). Ghrelin mRNA expression within the rat ovary is cyclic in nature, with the lowest expression found during proestrus and highest expression level occurring during the luteal phase (Caminos *et al.,* 2003). In humans, ghrelin protein is present in interstitial cells and granulosa cells, but not detectable in oocytes or theca cells (Gaytan *et al.,* 2003). In addition, ghrelin protein is not detectable in newly formed or regressing corpus luteum, but is detected in the young and mature corpus luteum (Gaytan *et al.,* 2003).

Ghrelin mRNA and protein are expressed by rodent endometrial and placental cells (Gualillo *et al.,* 2001a) and are secreted by endometrial cells into uterine fluid (Kawamura *et al.,* 2003). The injection of exogenous ghrelin during early pregnancy significantly decreased litter size in rats, most likely due to an inhibition in the development of preimplantation embryos (Fernandez-Fernandez *et al.,* 2005a). However, placental ghrelin production may be crucial to some aspects of fetal development. Ghrelin mRNA and protein expression in human and rat placentas sharply peaks during the last half of gestation (Gualillo *et al.,* 2001a). Furthermore, exogenous ghrelin given to pregnant rats late in gestation increases fetal body weight and immunization against ghrelin will decrease fetal body weight (Hayashida *et al.,* 2002; Nakahara *et al.,* 2006). Thus, during implantation and early pregnancy, ghrelin may serve to link nutrient status with the

demands of pregnancy by preventing pregnancy or limiting litter size in females with energy insufficiency. However, during the later stages of pregnancy ghrelin from placental production may actually promote fetal growth due to the stimulatory effect of growth hormone secretion.

Avian ghrelin

Avian ghrelin was cloned in 2002 by Kaiya *et al.* Avian preproghrelin is composed of 116 amino acids and shares very little amino acid sequence homology with mammalian preproghrelin except in the core DAG sequence (Yuan *et al.,* 2007). Similar to the processing of human ghrelin, the first 23 amino acids of chicken preproghrelin are cleaved to yield proghrelin. Although the chicken proghrelin sequence has no amino acid deletions in the amino acid DAG core sequence that follows the signal sequence, it is processed differently than human ghrelin. N-terminal sequencing of isolated chicken ghrelin reveals that it consists of only 26 amino acids (Kaiya *et al.,* 2002). When DAG is cleaved from proghrelin the amino acid residues in position 27 and 28 are left with the proghrelin portion of the protein (Kaiya *et al.,* 2002; Yuan *et al.,* 2007). The same as mammalian species, it is the third amino acid residue, a serine, which is acylated (Kaiya *et al.,* 2002).

The tissue distribution pattern of avian ghrelin mRNA and protein expression is similar to that of mammalian species as well. The highest expression levels are found in the proventriculus, the glandular portion of the avian stomach, followed by the small intestines (Kaiya *et al.,* 2002; Wada *et al.,* 2003; Richards *et al.,* 2005). Avian ghrelin mRNA is also expressed in the pancreas, adipose tissue, lung, spleen, and brain, but at levels lower than what is detected in the digestive tract (Kaiya *et al.,* 2002; Wada *et al.,*

2003; Richards *et al.,* 2005; Kaiya *et al.,* 2007). It is assumed that the proventriculus is the main source of circulating ghrelin in avian species (Richards *et al.,* 2005; Kaiya *et al.,* 2007).

Avian GOAT

Avian GOAT was cloned and the mRNA expression of GOAT is detected in the proventriculus of broilers and broiler breeders, but is not detected in most tissues (Dimova, 2012). However, GOAT mRNA is detected in granulosa cells, but not theca cells of preovulatory follicles, and its expression decreases with follicular maturity. Fasting broiler breeder hens increases GOAT mRNA expression in the proventriculus, but not in granulosa cells isolated from preovulatory follicles of any size.

Avian ghrelin receptor

Avian GHSR was characterized in 2003 by two separate research groups (Geelissen *et al.*, 2003; Tanaka *et al.*, 2003). Both groups reported that chickens have a GHSR gene structure analogous to that seen in humans with the avian GHSR gene being composed of two exons. The GHSR1a mRNA sequence in chickens codes for a protein of 347 amino acids. Alternate splicing of the GHSR transcript yields two other forms of the chicken ghrelin receptor, GHSR1aV and GHSRtv (Tanaka *et al.*, 2003; Sirotkin *et al.*, 2006). GHSR1aV lacks the first 16 amino acids coded by exon 2 and is predicted to lack transmembrane region 6 (Tanaka *et al.*, 2003) and has its C-terminal region located on the extracellular side of the cell membrane. The GHSR1tv transcript forms from a premature splicing from exon 1, retention of a 126bp fragment from intron 1, and premature

initiation of exon 2, all of which results in a shift in the open reading frame of the message that results in a new stop codon at amino acid 221. It is unclear if both of these truncated forms of the chicken GHSR receptor are even translated from their altered mRNA sequences (Sirotkin *et al.*, 2006).

GHSR1a mRNA expression in avian species is very high in the hypothalamus and pituitary. Lower levels of expression are found in the proventriculus, duodenum, adrenals, ovary, testes, liver, muscles, heart, and skin (Geelissen *et al.*, 2003; Richards *et al.*, 2006). The mRNA for the ghrelin receptor has been detected in the theca and granulosa cells from hierarchical and nonhierarchical follicles and the mRNA expression of the ghrelin receptor is down regulated by FSH and LH in cultured granulosa cells (Freeman, 2008).

Avian ghrelin synthesis and functions

In 2006, Richards, *et al*. reported that broilers fasted for 48 hours had increased ghrelin mRNA levels in the proventriculus, but that plasma concentrations of total ghrelin remained unchanged. In addition, ghrelin mRNA expression remained high in the proventriculus 12 hours after the birds had been re-fed. Rodents ,on the other hand, have an increase in both ghrelin mRNA levels in the stomach and plasma levels of total ghrelin when they are fasted and both levels decrease within 6 hours after re-feeding (Toshinai *et al.*, 2001). Research reports in quail and male Leghorn chicks, in which acylated ghrelin was specifically measured, indicate that plasma ghrelin levels increased and subsequently decreased upon re-feeding (Shousha *et al.*, 2005; Kaiya *et al.*, 2007). However, it was again noted by Kaiya *et al.* (2007) that the level of ghrelin mRNA in the proventriculus remained high 24 hours after the Leghorn chicks had been re-fed. Plasma acylated ghrelin levels

also increase in broiler breeder hens that are fasted (Freeman, 2008). Similar to mammalian species, ghrelin has been found to be a potent in vivo and in vitro stimulator of growth hormone release in Leghorn chickens (Ahmed and Harvey, 2002; Baudet and Harvey, 2003). However, the effects of ghrelin upon feeding behavior are not as clearly defined in avian species as they are in rodents and humans. Since the discovery of ghrelin in avian species, there has been only one report of peripherally injected ghrelin stimulating feeding behavior in birds and that occurred in adult quail (Shousha *et al.*, 2005). Intracerebro-ventricular injection of ghrelin into broiler chicks or peripheral injection of ghrelin into Leghorn chicks inhibited (Furuse *et al.*, 2001; Saito *et al.*, 2002; Saito *et al.*, 2005) or had no effect on feed intake (Kaiya *et al.*, 2007).

Very little research has been performed on ghrelin and reproduction in avian species. In 2005, Yoshimura *et al.* reported that ghrelin mRNA and protein was expressed in the mucosal epithelium layers in the infundibulum and magnum of mature quail, yet there was no ghrelin expression found in the oviducts of immature quail. Sirotkin *et al.* (2006) found ghrelin and GHSR mRNA to be present in follicular wall fragments of developing hierarchal follicles, but Freeman (2008) was unable to replicate these results or detect ghrelin in theca or granulosa tissue from preovulatory follicles. However, Dimova (2012) reported that GOAT mRNA was detectable in granulosa tissue from preovulatory follicles.

Summary

Ghrelin plays a key role in signaling a negative energy balance and stimulating appetite in mammalian species. In addition, ghrelin has a negative impact on reproduction

by decreasing LH secretion in both males and females when caloric intake is not sufficient or during fasting. In avian species, ghrelin is also prominently produced by the glandular stomach and implicated in regulation of energy homeostasis. The broiler breeder hen provides a unique opportunity to explore the role of ghrelin in ovarian development. Restricting caloric intake is essential in broiler breeder hens to prevent excessive weight gain that is detrimental to reproductive efficiency. At the same time, management practices in implementing this caloric restriction may negatively impact reproduction. The negative impact of fasting on reproduction could be mediated in part by ghrelin and the histological structure of the avian preovulatory follicles, as well as the arrangement of the preovulatory follicles in a size hierarchy relative to ovulation make it an ideal model to determine the role of ghrelin in ovarian development.

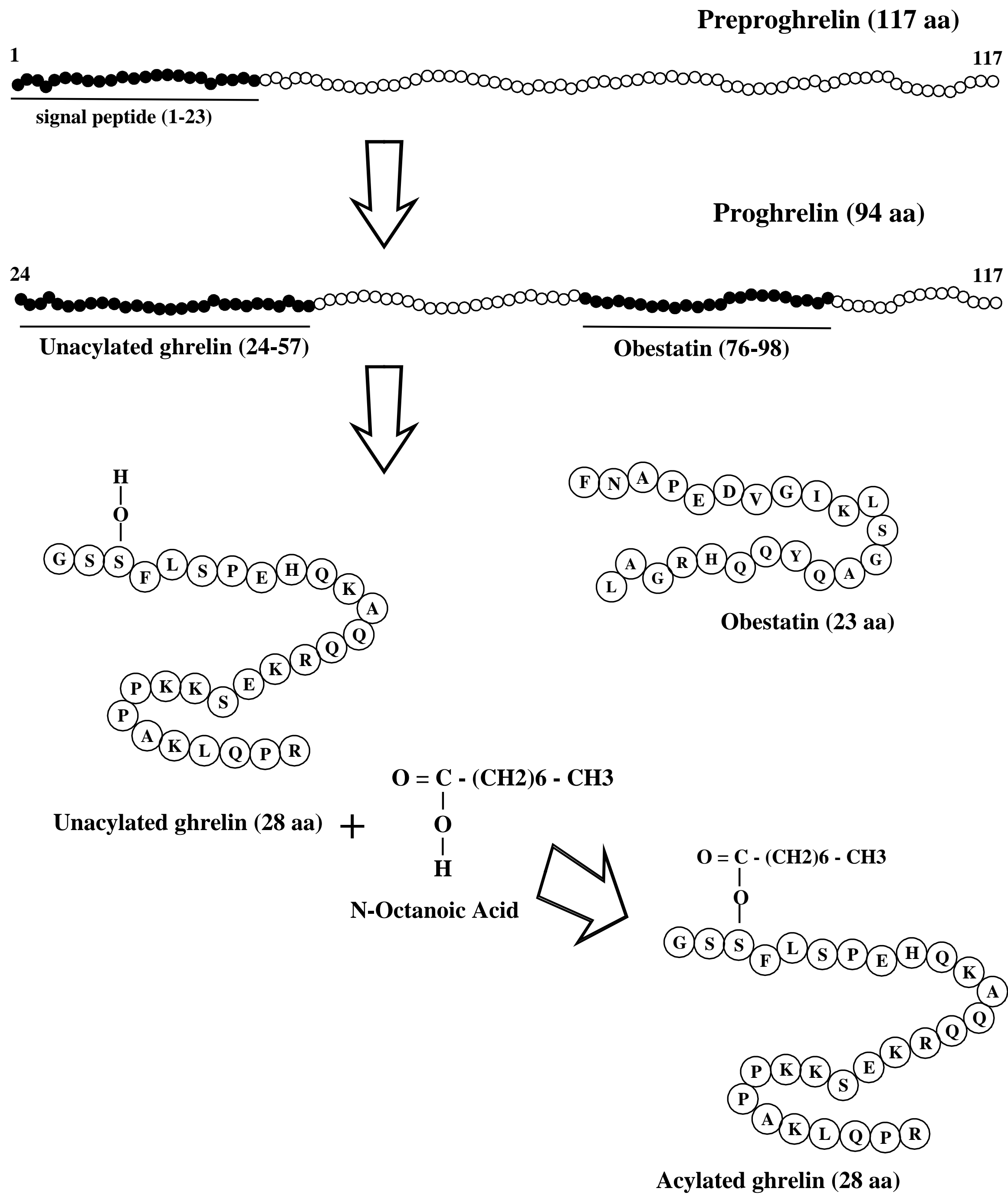

Figure 1.1. Post-translational processing of human preproghrelin into unacylated ghrelin (UAG), acylated ghrelin, and obestatin.

CHAPTER 3

STATEMENT OF PURPOSE

Pearl millet was domesticated in Mali over 4,500 years ago and Mali is still one of the top 5 countries in the world for pearl millet production. Pearl millet is well suited to growing in the arid environmental conditions of Mali. Pearl millet produced in other parts of the world typically yields grain that is comparable to, or slightly better than, corn with regards to nutritional characteristics. Pearl millet grain has been successfully used as a corn replacement in poultry diets without compromising growth or feed efficiency in broilers or egg production and feed efficiency in laying hens. Commercial poultry production in Mali is not widespread and utilizes imported corn and soybean meal as dietary ingredients that make poultry production expensive and consumer prices for poultry products high. Despite being a leading producer of pearl millet, the grain from pearl millet is not utilized in poultry production in Mali. This is based on following the lead of commercial poultry production in developed countries such as the U. S. where corn and soybean meal are used in commercial poultry production. Ghrelin is a hormone that regulates the effects of nutrient status on reproductive function in mammalian species. Broiler breeders have to be severely feed restricted not only during rearing, but also during production, to prevent them from over eating and becoming too large and reproductively unfit with poor livability. Previous research indicates the presence of ghrelin receptor mRNA in the developing follicles of the hen ovary. For ghrelin to bind to its receptor it needs to be acylated and this is accomplished by the enzyme GOAT, which is

expressed in the developing follicles of the hen ovary. Further characterization of the ghrelin system in the hen ovary, in particular the broiler breeder hen ovary, by detecting ghrelin itself could be important for determining why follicular development is abnormal in these hens relative to laying hens. Therefore, the goals of the present research are 1) to characterize the nutrient composition of pearl millet produced in Mali, 2) to determine if whole pearl millet produced in Mali could be incorporated in its whole unground form into laying hen and broiler diets without compromising performance, 3) to characterize the mRNA expression of ghrelin in developing hen follicles and 4) to determine whether fasting changes ghrelin mRNA expression in the developing follicles of the ovary of the broiler breeder hen.

CHAPTER 4

MATERIALS AND METHODS

Pearl millet experiments

Seven different widely available varieties of pearl millet, grown in different regions of Mali, were obtained. In addition, an eighth sample was obtained, which was a commercial blend of the most widely available varieties of millet in Mali. The obtained millet samples were ground and then proximate composition (AOAC, 2006), amino acid content (AOAC, 2006), digestible amino acid content and nitrogen corrected true metabolizable energy (TME_N) (Sibbald, 1976; Dale and Fuller, 1984) were determined for the different pearl millet samples. For the digestible amino acid content determinations of each pearl millet sample, 8 cecectomized White Leghorn roosters (60 weeks of age) underwent a feed withdrawal of 30 hours to clear the digestive tract, followed by each rooster being fed 35 grams of the given pearl millet variety, using the precision fed rooster assay describe by Sibbald (1976). An additional 8 un-fed roosters were used as endogenous controls. All of the roosters were placed in individual wire cages suspended over an aluminum pan. Feces were collected for 48 hours after feeding for each individual rooster. Fecal samples were dried and analyzed for amino acid content (AOAC, 2006) at Ajinomoto Heartland Laboratories, Chicago, Illinois.

The TME_N procedure mirrored the digestible amino acid procedure except non-cecectomized roosters were utilized and the dried feces was analyzed at The University of

Georgia Ag. Services, Athen, GA. as previously described (Sibbald, 1976; Dale and Fuller, 1984) for the determination of TME_N.

Experiment 1: Whole pearl millet in laying hen diets

Based on nutrient analyses and its continual and widespread availability, the commercial blend of pearl millet from Mali was chosen for the live bird research. A few thousand kilograms of this pearl millet was purchased and imported from Mali via Senegal. In the first experiment, whole pearl millet was tested as a feed ingredient in laying hen diets. The decision to use whole pearl millet was based on the potential savings for Malian poultry farmers from not having to grind pearl millet, if it was proven that whole pearl millet could replace a large portion of the corn in a typical laying hen diet.

For this experiment, 300 individually caged, 33 week old, Hy-line W-36 White Leghorn laying hens, were selected from a larger farm stock flock, in which egg production and hen body weight were monitored for 3 weeks, prior to the start of the experiment. Each cage had a sloped floor for egg displacement from the interior of the cage. The height of the cage was 41cm in the rear and 46 cm in the front. The width of the cage was 25 cm and the depth was 41 cm. Each cage was equipped with a nipple drinker and access to a galvanized trough feeder. The birds were housed in an environmentally controlled building, had free access to a standard laying hen diet and were maintained on daily lighting schedule of 16 hours light and 8 hours dark.

At the end of the 3-week period, the 300 birds with the best egg production and body weights were selected and divided into 4 dietary treatments; each treatment consisted of 3 blocks of 25 individually caged hens. The hens were distributed such that

Table 4.1. Ingredient composition of layer diets (Experiment 1)[1].

Ingredient	Diet 1	Diet 2	Diet 3	Diet 4
	---------------------(%)	-------------------		
Corn	56.357	45.122	33.795	22.381
Whole pearl millet	0.000	14.193	28.387	42.580
Soybean meal	27.754	26.036	24.372	22.719
Limestone	9.785	9.794	9.802	9.810
Dicalcium phosphate	1.360	1.343	1.325	1.307
Salt	0.239	0.107	0.043	0.185
S-carbonate	0.088	0.181	0.220	0.098
L-Lysine, 78.8%	0.016	0.060	0.102	0.144
DL-Methionine, 99.0%	0.249	0.255	0.260	0.266
L-Threonine, 98.0%	0.023	0.033	0.043	0.053
L-Tryptophan, 98.0%	0.008	0.001	0.000	0.000
Choline chloride, 60%	0.020	0.059	0.097	0.136
Soybean oil	3.780	2.497	1.232	0.000
Phytase[2]	0.020	0.020	0.020	0.020
Trace mineral mix[3]	0.075	0.075	0.075	0.075
Vitamin mix[4]	0.227	0.227	0.227	0.227
<u>Calculated analyses</u>				
CP, %	17.36	17.32	17.30	17.27
ME, kcal/kg	2856	2856	2856	2856
Calcium, %	4.20	4.20	4.20	4.20
Available phosphorus, %	0.480	0.480	0.480	0.480
Digestible lysine, %	0.850	0.850	0.850	0.850
Digestible threonine, %	0.595	0.595	0.595	0.595
Digestible methionine, %	0.488	0.491	0.494	0.497

[1]Pelleted diets were fed for 16 weeks (hen age at start 36 weeks).
[2]Quantum 2500 XT (AB Vista, NC)
[3]Trace mineral premix provides the following in milligrams per kilogram of diet: manganese, 176; zinc, 176; iron, 64; copper, 8.8; iodine, 2.8; selenium, 0.5.
[4]Vitamin premix provides the following per kilogram of diet: vitamin A, 7,000 IU; vitamin D_3, 2,500 IU; vitamin E, 19 IU; vitamin K, 1.3 mg; vitamin B_1, 1.6 mg; vitamin B_2, 6.3 mg; vitamin B_6, 2.4 mg; vitamin B_{12}, 0.01 mg; niacin, 40 mg; pantothenic acid, 11 mg; folic acid, 0.7 mg; biotin, 0.08 mg.

the 12 blocks of hens did not differ in egg production or body weight profile at the start of the experiment, when the birds were 36 weeks of age.

The hens in dietary treatment 1 were fed a standard corn/soybean meal laying hen diet (Table 4.1) while the hens in dietary treatments 2, 3 or 4 were fed a diet in which 25, 50 or 75 percent of the corn in dietary treatment 1 was replaced with whole pearl millet, respectively (Table 4.1). The hens were fed these diets for 16 weeks. The diets were formulated on digestible amino acid basis.

During the 16-week experimental period, all hens were individually weighed every 4 weeks and total feed consumption per block of 25 hens was also determined every 4 weeks. Egg production was recorded daily for each bird and hen-housed and hen-day egg production was calculated weekly from daily egg counts. Every other week, the eggs from two days worth of production were weighed for each replicate block of hens. Specific gravities were determined by using the saline flotation method (Phillips and Williams, 1944) on 2 days of egg production from each replicate block starting at week 4 of the experiment (hen age 40 weeks) and continuing every two weeks thereafter until the conclusion of the experiment when the birds were 52 weeks of age. Finally, egg yolk color was assessed during the last week of the experiment on eggs collected from 2 days worth of production using a Minolta colorimeter to measure L* (lightness), a* (redness), and b* (yellowness).

Experiment 2: Whole pearl millet in broilers

This experiment was conducted to determine the performance of broilers fed diets containing whole pearl millet. The dietary treatments for this experiment were similar to

Table 4.2. Ingredient composition of the broiler starter diets (Experiments 2 and 3)[1].

Ingredient	Diet 1	Diet 2	Diet 3	Diet 4
	(%)			
Corn	51.738	40.718	29.624	18.453
Pearl millet[2]	0.000	14.193	28.387	42.580
Soybean meal	40.481	38.627	36.784	34.953
Limestone	1.294	1.302	1.310	1.318
Dicalcium phosphate	1.192	1.176	1.159	1.143
Salt	0.277	0.152	0.019	0.046
S-carbonate	0.236	0.324	0.416	0.384
L-Lysine, 78.8%	0.113	0.160	0.208	0.255
DL-Methionine, 99.0%	0.347	0.354	0.361	0.368
L-Threonine, 98.0%	0.045	0.057	0.069	0.081
Choline chloride, 60%	0.020	0.020	0.056	0.095
Soybean oil	3.935	2.596	1.283	0.000
Phytase[3]	0.020	0.020	0.020	0.020
Trace mineral mix[4]	0.075	0.075	0.075	0.075
Vitamin mix[5]	0.227	0.227	0.227	0.227
Calculated analyses				
CP, %	23.17	23.09	23.00	22.92
ME, kcal/kg	3031	3031	3031	3031
Calcium, %	0.950	0.950	0.950	0.950
Available phosphorus, %	0.475	0.475	0.475	0.475
Digestible lysine, %	1.250	1.250	1.250	1.250
Digestible threonine, %	0.813	0.813	0.813	0.812
Digestible methionine, %	0.651	0.655	0.658	0.662

[1]Starter diet fed from 0 to 14 days of age (crumble diet).
[2]Whole pearl millet was used in Experiment 2 while ground pearl millet was used in experiment 3.
[3]Quantum 2500 XT (AB Vista, NC)
[4]Trace mineral premix provides the following in milligrams per kilogram of diet: manganese, 176; zinc, 176; iron, 64; copper, 8.8; iodine, 2.8; selenium, 0.5.
[5]Vitamin premix provides the following per kilogram of diet: vitamin A, 7,000 IU; vitamin D_3, 2,500 IU; vitamin E, 19 IU; vitamin K, 1.3 mg; vitamin B_1, 1.6 mg; vitamin B_2, 6.3 mg; vitamin B_6, 2.4 mg; vitamin B_{12}, 0.01 mg; niacin, 40 mg; pantothenic acid, 11 mg; folic acid, 0.7 mg; biotin, 0.08 mg.

Table 4.3. Ingredient composition of broiler grower diets (Experiments 2and 3)[1].

Ingredient	Diet 1	Diet 2	Diet 3	Diet 4
	(%)			
Corn	55.509	44.423	33.323	22.165
Pearl millet[2]	0.000	14.193	28.387	42.580
Soybean meal	36.619	34.775	32.933	31.101
Limestone	1.264	1.272	1.280	1.289
Dicalcium phosphate	1.092	1.075	1.059	1.043
Salt	0.266	0.135	0.002	0.000
S-carbonate	0.232	0.324	0.417	0.407
L-Lysine, 78.8%	0.104	0.152	0.200	0.247
DL-Methionine, 99.0%	0.315	0.322	0.329	0.336
L-Threonine, 98.0%	0.046	0.058	0.070	0.082
Choline chloride, 60%	0.000	0.032	0.072	0.111
Soybean oil	4.232	2.917	1.606	0.319
Phytase[3]	0.020	0.020	0.020	0.020
Trace mineral mix[4]	0.075	0.075	0.075	0.075
Vitamin mix[5]	0.227	0.227	0.227	0.227
Calculated analyses				
CP, %	21.60	21.52	21.44	21.35
ME, kcal/kg	3085	3085	3085	3085
Calcium, %	0.900	0.900	0.900	0.900
Available phosphorus, %	0.450	0.450	0.450	0.450
Digestible lysine, %	1.150	1.150	1.150	1.150
Digestible threonine, %	0.759	0.759	0.759	0.759
Digestible methionine, %	0.603	0.606	0.610	0.613

[1]Grower diet fed from 14 to 28 days of age (pelleted diet).
[2]Whole pearl millet was used in Experiment 2 while ground pearl millet was used in experiment 3.
[3]Quantum 2500 XT (AB Vista, NC)
[4]Trace mineral premix provides the following in milligrams per kilogram of diet: manganese, 176; zinc, 176; iron, 64; copper, 8.8; iodine, 2.8; selenium, 0.5.
[5]Vitamin premix provides the following per kilogram of diet: vitamin A, 7,000 IU; vitamin D_3, 2,500 IU; vitamin E, 19 IU; vitamin K, 1.3 mg; vitamin B_1, 1.6 mg; vitamin B_2, 6.3 mg; vitamin B_6, 2.4 mg; vitamin B_{12}, 0.01 mg; niacin, 40 mg; pantothenic acid, 11 mg; folic acid, 0.7 mg; biotin, 0.08 mg.

Table 4.4. Ingredient composition of broiler finisher diets (Experiments 2and 3)[1].

Ingredient	Diet 1	Diet 2	Diet 3	Diet 4
	---------------------(%)------------------------			
Corn	64.694	53.705	42.595	31.410
Pearl millet[2]	0.000	14.193	28.387	42.580
Soybean meal	27.854	25.999	24.155	22.327
Limestone	1.228	1.236	1.244	1.252
Dicalcium phosphate	1.033	1.016	0.999	0.984
Salt	0.240	0.110	0.000	0.061
S-carbonate	0.231	0.322	0.397	0.339
L-Lysine, 78.8%	0.118	0.166	0.214	0.261
DL-Methionine, 99.0%	0.242	0.249	0.256	0.263
L-Threonine, 98.0%	0.045	0.057	0.069	0.081
Choline chloride, 60%	0.020	0.043	0.083	0.122
Soybean oil	3.972	2.584	1.278	0.000
Phytase[3]	0.020	0.020	0.020	0.020
Trace mineral mix[4]	0.075	0.075	0.075	0.075
Vitamin mix[5]	0.227	0.227	0.227	0.227
<u>Calculated analysis</u>				
CP, %	18.13	18.05	17.96	17.88
ME, kcal/kg	3152	3152	3152	3152
Calcium, %	0.850	0.850	0.850	0.850
Available phosphorus, %	0.425	0.425	0.425	0.425
Lysine, %	0.950	0.950	0.950	0.950
Threonine, %	0.637	0.636	0.636	0.636
Methionine, %	0.494	0.497	0.501	0.505

[1]Finisher diet fed from 28 to 42 days of age (pelleted diet).
[2]Whole pearl millet was used in Experiment 2 while ground pearl millet was used in experiment 3.
[3]Quantum 2500 XT (AB Vista, NC)
[4]Trace mineral premix provides the following in milligrams per kilogram of diet: manganese, 176; zinc, 176; iron, 64; copper, 8.8; iodine, 2.8; selenium, 0.5.
[5]Vitamin premix provides the following per kilogram of diet: vitamin A, 7,000 IU; vitamin D_3, 2,500 IU; vitamin E, 19 IU; vitamin K, 1.3 mg; vitamin B_1, 1.6 mg; vitamin B_2, 6.3 mg; vitamin B_6, 2.4 mg; vitamin B_{12}, 0.01 mg; niacin, 40 mg; pantothenic acid, 11 mg; folic acid, 0.7 mg; biotin, 0.08 mg.

those of the laying hen experiment in that the levels of whole pearl millet incorporated into the broiler diets (Tables 4.2, 4.3, 4.4) were the same as those in the laying hen diets. Thus, the starter, grower and finisher diets had 0% whole pearl millet for dietary treatment 1, 14.193% whole pearl millet for dietary treatment 2, 28.387% whole pearl millet for dietary treatment 3, and 42.580% whole pear millet for dietary treatment 4. The starter diets were fed from days 1 to 14 of age, the grower diets were fed from days 14-28 of age and the grower diets were fed from 28 to 42 days of age. The starter diets were in crumble form while the grower and finisher diets were in pellet form.

This experiment was conducted in a facility with two identical, but separate, rooms. Each room was equipped with 24 (3.05 m by 1.22 m) floor pens. All pens were provided with 7 nipple drinkers, originating from a common water line and one pan feeder (0.09 m^2). The stocking density calculations accounted for the area occupied by the pan feeders. Prior to chick placement, new pine shavings were placed in the pens. A continuous lighting program was implemented with a light intensity of 20 lux for 24 hours (0 to 4 days), 20 lux for 20 hours (5 to 7days), 10 lux for 16 hours (8 to 14 days), and 2 lux for 16 hours (15 to 35 days) and 2 lux for 23 hours (35 to 42 days). Light intensity was verified by placing a Light ProbeMeter™ (model 403125, Extech Instruments Corp. Waltham, MA) into the pens at bird height. Management protocols were followed according to the Cobb brooding and broiler management guidelines (Cobb-Vantress, 2008a and 2008b).

For each room, two computerized controller regulated gas-fired furnaces, an exterior evaporative cooling system, present on both sides of the room for cool air intake, four 45.7 cm ceiling circulation fans, and two 91.4 cm exhaust fans and one 61cm exhaust fan, located on the ends of the rooms for air evacuation. The ambient temperature was set to

34°C on day 1 and decreased by 0.28°C until 24°C was reached and maintained. No significant differences in temperature and humidity were noted throughout the study, between the two rooms.

Prior to placing chicks, the 32 pens were assigned to one of the 4 dietary treatments in a random block design [8 replicates per treatment (7 replicates in one room 1 replicate in the other room)]. A total of 840 day of hatch Cobb 500 male broiler chicks, originating from the same breeder flock, were obtained from a primary breeder hatchery. The chicks were sorted and those with extreme weights were discarded before the remaining birds were assigned to the 32 pens (21 birds per pen).

Feed and water were provided *ad libitum* throughout the duration of the experiment. Diets were formulated on a digestible amino acid basis. All diets were formulated to meet or exceed NRC (1994) requirements. All animal procedures were approved by the University of Georgia Animal Care and Use Committee, Athens, GA.

For each room, humidity, temperature, water consumption, and pen mortality were recorded twice daily. Birds and feed were weighed on days 0, 14, 28 and 42 to determine body weight (BW), feed intake (FI), body weight gain (BWG), and feed conversion (FC). On day 42, the mean bird weight for each pen was determined and 8 birds per pen, within 300 g above/below the mean weight of their pen, were selected for processing. Individual weights for the selected birds were recorded and each bird was leg banded prior to placement in a coop for an overnight feed withdrawal. On day 43, birds were weighed again to determine their fasted live weight and processed at the University of Georgia's Pilot Processing Plant as previously described (Hidalgo *et al.*, 2004). During evisceration the gizzard was removed. Once removed, fat tissue was removed from the outside of the

gizzard and then the gizzard was cut open to remove any contents and the koilin membrane, before the gizzard was weighed. Subsequently, eviscerated hot carcass weights were recorded for each bird prior to static chilling in an ice bath for 4 hours. After a 4-hour chill, chilled carcasses were drained prior to cut up and deboning. Weights were recorded for: drained chilled carcass, pectoralis major, pectoralis minor, wings, and leg quarters of each bird. Percent yield calculations were based on the fasted, live weight of the bird.

Experiment 3: Ground pearl millet in broilers

This experiment followed a very similar protocol as experiment 2 except that the diets contained ground millet rather than whole millet. In addition, dietary treatment 3 (28.387% millet) was not included in this experiment, nor were gizzard weights recorded. There were 10 replicates (5 replicate pens per room) per dietary treatment.

Ghrelin experiments

Experiment 4: Ghrelin expression in the hen ovary

To determine if ghrelin is expressed in the granulosa and theca tissue of chicken, preovulatory follicles and initial characterization was completed using Hy-line W-36 laying hens. Three 50 to 55 week old hens were killed by cervical dislocation 2 to 4 hours prior to ovulation and the ovary was collected from each hen. The theca and granulosa layers from each of the F1, F2, F3, and F4 follicles were manually separated from one another (Huang and Nalbandov, 1979) while the theca and granulosa layers from the large small yellow follicles (SYF1, 8 to 12 mm in diameter) small yellow follicles (SYF2, 5 to 8 mm in

diameter), large white follicles (LWF1, 2 to 5 mm in diameter) and small large white follicles (LWF2, less than 2 mm in diameter) were separated enzymatically (Davis *et al.,* 2000a). The individual theca and granulosa tissue for each follicle size obtained from one hen were combined with corresponding samples from the other two hens for RNA extraction and subsequent RT-PCR detection of ghrelin mRNA. This procedure was repeated two more times and a total of 3 sets (n = 3) of samples for each follicle size were obtained. The theca layers collected from each individual F1 through F4 follicle and from the individual pools of SYF and LWF were placed in 3 mL of guanidinium isothiocyanate solution and homogenized for 30 seconds with a PowerGen 700 tissue disrupter (Fisher Scientific, Pittsburg, PA). The single cell layer of granulosa tissue from each hierarchical follicle and granulosa cells isolated from the individual pools of SYF and LWF was placed in 3 mL of guanidinium isothiocyanate solution and vortexed for 20 seconds. The tissue solutions were stored at -80ºC for subsequent RNA extraction.

Experiment 5: Ghrelin in fed and fasted broiler breeder hens

This experiment was completed to determine if fasting influenced the mRNA expression of ghrelin in the hierarchical or prehierarchical follicles of broiler breeder hens. The Cobb 500 broiler breeder hens utilized for this experiment were between 45 and 55 weeks of age. The birds were reared as previously described (Spradley *et al.*, 2008) using a skip a day feed restriction program. At 21 weeks of age the pullets were placed in individual cages and were photostimulated to initiate reproduction with a lighting program that provided 14L:8D (lights on at 06:30 hours) per day. The hens were given free access to water and were fed a standard broiler breeder layer diet each morning at 8am. The daily

amount of feed provided to the hens was determined using the guidelines set forth by the primary breeder (Cobb-Vantress, 2005a and Cobb-Vantress 2005b) based on the weekly body weight measurements and egg production rates of the hens. Eggs were collected twice daily and individual hen egg production was recorded. All animal procedures were approved by the University of Georgia Animal Care and Use Committee, Athens, GA.

Four hens in mid-laying sequence were divided into two treatment groups. The hens in one treatment group continued to receive their daily allotment of feed while the hens in the other treatment group did not receive food. After 72 hours of fasting, all 4 hens were killed and the ovary was collected from each hen. The theca and granulosa layers from each of the F1, F2, F3 and F4 follicles were collected as described in experiment 4. The theca and granulosa layers from the SYF (5 to 10 mm in diameter) and LWF follicles (2 to 5 mm in diameter) were separated enzymatically (Davis *et al.,* 2000a). The individual theca and granulosa samples for each follicle size from one hen of each treatment were combined with the corresponding samples from the other hen of that treatment for RNA extraction. This collection procedure was repeated 2 more times to give 3 total replications for each treatment (n = 3).

Approximately 300 mg of proventriculus was also collected from the 6 individual fed and 6 individual fasted birds. Immediately after collection, each tissue was placed in 3 mL of guanidinium isothiocyanate solution and homogenized for 30 seconds with a PowerGen 700 tissue disrupter (Fisher Scientific, Pittsburg, PA). Individual homogenized tissue solutions were frozen and stored at -80ºC for future RNA extraction.

RNA Extraction

Total RNA was extracted from tissue samples using the guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). RNA samples were stored at -80ºC.

Real time RT-PCR

Extracted RNA samples were DNase treated using TURBO-DNA-free kit (Ambion, Austin, TX) to remove any potential genomic DNA contamination. TaqMan minor groove-binding probes and primers (Table 4.5) for detecting ghrelin (GenBank accession #AY303688) and the endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH, GenBank accession #M11213) were designed using Primer Express software (Version 4.0, Applied Biosystems). Each probe was labeled at the 5' end with FAM (6-carboxyfluorescein) as the reporter dye and at the 3' end with TAMRA (6-carboxy-N,N,N',N'-tetramethylrhodamine) as the quencher dye. Primer and probe sets were validated for real-time PCR by determining the optimal amplification efficiency and primer/probe concentration as described by the manufacturer (Applied Biosystems, Foster City, CA).

Reverse-transcription cDNA synthesis reactions were performed using the TaqMan Reverse Transcription Kit (Applied Biosystems) following the manufacturers protocol. For two-step real-time PCR, 200 ng of cDNA was used for each sample for the ghrelin and GAPDH amplification. The reactions were performed in a 25ul volume of reaction buffer containing 1x TaqMan Universal PCR Master Mix (Applied Biosystems) and 900nM of either, ghrelin or GAPDH primer pairs and 25nM of the appropriate probe. The reactions

were completed in an ABI 7500 thermocycler (Applied Biosystems). The thermocycler conditions were 10 minutes at 95°C and 40 cycles each 15 seconds at 95°C and 1 minute at 60°C. The reactions for each sample were performed in duplicate for ghrelin and GAPDH assays.

Table 4.5 Real-time RT-PCR primer and probe designs for ghrelin and GAPDH

Gene	Primer	Oligonucleotide Primer and Probe Sequence	Product Size (Base Pairs)
Ghrelin	Forward	5'-GGATACAAGAAAACCAACAGCAAGA-3'	71
	Reverse	5'-CTGTTTCATCTGTATCCCAAAAGCT -3'	
	Probe	5'-TACATCGCCGAGGCAC-3'	
GAPDH	Forward	5'-TTGGCATTGTGGAGGGTCTT-3'	87
	Reverse	5'-GGGCCATCCACCGTCTTC-3'	
	Probe	5'-TGACCACTGTCCATGCCAT-3'	

The Ct (the cycle number at which the fluorescence exceeds the threshold level) was determined for each reaction using the Sequence Detection Software (version 1.2.2, Applied Biosystems) and quantification was completed using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Briefly, the ghrelin samples Ct's were determined and then normalized to the GAPDH Ct from the same sample (GAPDH Ct subtracted from the ghrelin Ct to yield the ΔCt). After all the ΔCt values were obtained for an experimental replicate, the ΔCt values for each individual ghrelin sample were compared to the sample within the replicate that had the highest mRNA expression for ghrelin using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Thus, all data for ghrelin is expressed as the fold-difference relative to sample with the highest expression.

Statistics

Data from each experiment was subjected to ANOVA according to the General Linear Model (GLM) with replicate and dietary treatment as factors in experiments 1, 2 and 3 and replicate and tissue as factors in experiment 4 and replicate, feeding state and tissue as factors in experiment 5. Tukey's multiple-comparison procedure (Neter *et al.,* 1990) was used to detect significant differences among individual dietary treatments, tissues and follicle sizes. Differences were considered significant when $P < 0.05$. All statistical procedures were completed with the Minitab statistical software package (Release13, State College, PA).

CHAPTER 5

RESULTS AND CONCLUSIONS

The protein content of the pearl millet varieties from Mali ranged from about 8.5 to 10.5%, while the nitrogen-corrected true metabolizable energy values ranged from about 3,400 to 3,750 kcal/kg on as-is basis (Table 5.1). The pearl millet variety Koutiala 1 had among the best energy and protein content of the tested varieties, also had some of the best essential amino acid concentrations (Table 5.2) and digestibility coefficients for these essential amino acids (Table 5.3) relative to the other tested varieties. The mixed industrial sample, which is a commercial blend of millet varieties grown in Mali, also had very good available energy and amino acid contents relative to the individual varieties tested.

Experiment 1

Laying hens fed diets in which the corn content of a standard laying hen diet had been reduced by 25, 50 or 75% and replaced with the mixed industrial pearl millet in whole form, that had been imported from Mali, performed equivalently to one another. There were no differences in body weight gain (Table 5.4) or weekly hen day egg production (Table 5.5) between the hens fed the 4 different dietary treatments from 36 to 52 weeks of age. The total hen housed egg production (mean ± SEM) was 100 ± 1, 100 ± 0.5, 98 ± 1 and 98 ± 0.5 eggs, during the 120 day experimental period for hens fed diets containing 0, 14, 28 and 43% whole pearl millet, respectively. Total hen day egg

Table 5.1. Nutrient characteristics of commercially available pearl millet varieties in Mali[1].

Variety	GE[3] (kcal/kg)	TME_N (kcal/kg)	Protein (%)	Fat (%)	Fiber (%)	Ash (%)	Moisture (%)
Segou	4,045	3,395	8.99	4.10	2.50	5.79	7.22
Koutiala 1	4,266	3,698	10.47	4.09	2.09	1.58	7.19
Koutiala 2	4,164	3,572	8.70	5.27	2.30	3.12	7.45
Bla	4,221	3,601	9.55	4.74	1.87	2.34	7.51
Mopti Sanio	4,292	3,638	10.20	5.31	1.59	1.70	7.60
Mopti Suna	4,132	3,465	10.47	3.60	2.17	3.82	7.46
Bankass	4,229	3,738	9.18	5.08	2.36	1.41	7.41
Mixed industrial[2]	4,259	3,630	10.11	4.42	2.29	1.68	7.08

[1]Values are reported on an as is basis. Abbreviations: GE is gross energy, TME_N is nitrogen corrected true metabolizable energy.
[2]The mixed industrial pearl millet is a commercial blend of different millet varieties from across the country.
[3]Abbreviations: GE equals gross energy, TME_N equals nitrogen corrected true metabolizable energy.

Table 5.2. Amino acid content of commercially available pearl millet varieties in Mali[1].

	Variety							
	Segou	Koutiala 1	Koutiala 2	Bla	Mopti Sanio	Mopti Suna	Bankass	Mixed industrial
	(%)							
Dry matter	93.20	93.22	93.05	92.94	92.91	92.37	92.66	92.87
Alanine	0.64	0.76	0.66	0.68	0.72	0.75	0.68	0.74
Arginine	0.40	0.44	0.40	0.41	0.43	0.43	0.38	0.43
Aspartic acid	0.70	0.81	0.71	0.74	0.77	0.79	0.73	0.79
Cysteine	0.18	0.18	0.18	0.18	0.18	0.19	0.17	0.17
Glutamic acid	1.57	1.90	1.62	1.72	1.86	1.91	1.58	1.87
Glycine	0.30	0.32	0.31	0.30	0.31	0.31	0.31	0.30
Histidine	0.19	0.21	0.19	0.20	0.21	0.20	0.20	0.20
Isoleucine	0.30	0.46	0.39	0.41	0.43	0.45	0.36	0.45
Leucine	0.71	1.00	0.81	0.88	0.93	0.97	0.84	0.97
Lysine	0.25	0.30	0.29	0.36	0.33	0.28	0.28	0.29
Methionine	0.17	0.20	0.20	0.20	0.21	0.20	0.22	0.19
Phenylalanine	0.41	0.52	0.45	0.46	0.49	0.50	0.46	0.50
Proline	0.52	0.60	0.54	0.53	0.56	0.58	0.52	0.57
Serine	0.41	0.48	0.42	0.43	0.45	0.46	0.43	0.46
Threonine	0.33	0.37	0.34	0.34	0.35	0.37	0.35	0.36
Tryptophan	0.14	0.17	0.14	0.14	0.16	0.16	0.14	0.16
Valine	0.47	0.57	0.49	0.51	0.54	0.55	0.48	0.55

[1]Values are reported on an as is basis.

Table 5.3. Amino acid digestibility coefficient of commercially available pearl millet varieties in Mali[1].

Amino acid	Variety							
	Segou	Koutiala 1	Koutiala 2	Bla	Mopti Sanio	Mopti Suna	Bankass	Mixed industrial
	----------%							----------
Alanine	87.70	92.51	90.58	88.88	89.52	90.83	92.87	89.99
Arginine	88.23	92.48	91.21	88.45	89.07	89.12	92.17	89.20
Aspartic acid	85.14	90.27	88.34	86.05	86.63	88.11	90.47	85.71
Cysteine	85.05	91.21	92.53	89.08	87.04	86.71	94.04	93.10
Glutamic acid	91.78	94.87	93.82	92.04	92.84	93.33	94.69	92.71
Glycine	62.07	66.36	65.27	50.47	57.80	63.40	68.53	45.26
Histidine	88.48	94.87	91.44	89.38	92.95	89.89	96.49	91.13
Isoleucine	82.80	91.60	89.64	87.31	88.23	89.78	90.85	90.11
Leucine	88.63	94.40	92.57	91.30	91.88	93.29	94.04	92.27
Lysine	74.89	84.24	80.96	88.73	82.88	78.30	86.99	79.53
Methionine	86.45	90.61	90.08	87.47	88.81	89.26	91.39	86.84
Phenylalanine	86.53	92.45	89.98	88.40	89.39	90.10	93.06	89.50
Proline	90.27	94.71	95.37	91.40	91.64	93.64	95.81	93.55
Serine	86.30	92.23	91.65	86.89	88.42	88.68	92.30	89.52
Threonine	81.46	87.93	85.95	82.30	83.50	84.97	90.18	82.68
Tryptophan	88.77	94.86	92.70	90.81	91.90	92.99	95.83	92.18
Valine	86.49	91.84	90.20	87.93	88.63	90.20	91.12	88.99

[1]Values are reported on an as is basis.

Table 5.4. Body weight of Leghorn hens fed one of four diets containing varying amounts of whole pearl millet from 36 to 52 weeks of age (Experiment1)[1].

	Dietary treatment			
Body Weight	1 (0% millet)	2 (14% millet)	3 (28% millet)	4 (43% millet)
(week)	--(g)---			
36	1650 ± 76	1649 ± 76	1650 ± 76	1650 ± 76
40	1756 ± 90	1736 ± 80	1722 ± 82	1708 ± 83
44	1810 ± 95	1803 ± 77	1784 ± 90	1791 ± 90
48	1810 ± 106	1790 ± 81	1797 ± 85	1811 ± 93
52	1836 ± 111	1841 ± 97	1857 ± 95	1867 ± 92

[1]Values are means ± SEM, n = 3 replicate groups of 25 individual caged laying hens for each dietary treatment.

Table 5.5. Weekly hen day egg production of leghorn hens fed one of four diets containing varying amounts of whole pearl millet from 36 to 52 weeks of age (Experiment 1)[1].

	Dietary treatment			
Age	1 (0% millet)	2 (14% millet)	3 (28% millet)	4 (43% millet)
(week)	(%)			
37	95.2 ± 1.33	93.0 ± 1.49	91.6 ± 1.56	94.7 ± 1.33
38	92.2 ± 0.38	90.9 ± 0.66	89.1 ± 2.31	90.7 ± 0.50
39	93.9 ± 0.19	95.2 ± 1.66	92.0 ± 0.87	92.8 ± 1.16
40	89.9 ± 0.76	90.3 ± 2.01	92.0 ± 2.70	91.0 ± 0.19
41	93.1 ± 0.66	91.8 ± 0.69	89.5 ± 1.33	91.8 ± 1.16
42	91.6 ± 0.38	90.7 ± 0.38	90.1 ± 0.83	90.9 ± 1.44
43	91.6 ± 0.69	94.5 ± 1.06	91.4 ± 0.87	93.0 ± 0.19
44	89.1 ± 0.00	86.3 ± 0.87	88.3 ± 1.77	87.8 ± 1.63
45	87.4 ± 0.66	90.7 ± 1.90	87.8 ± 1.82	88.2 ± 0.76
46	90.3 ± 2.31	88.0 ± 0.87	88.1 ± 1.17	87.8 ± 0.69
47	90.1 ± 0.95	91.0 ± 0.19	88.2 ± 0.67	90.1 ± 0.50
48	89.3 ± 1.06	86.3 ± 0.87	85.5 ± 0.70	85.7 ± 1.51
49	84.8 ± 2.02	85.1 ± 1.87	80.5 ± 1.83	79.0 ± 1.44
50	79.6 ± 2.12	84.8 ± 2.29	80.9 ± 2.00	77.5 ± 4.14
51	83.8 ± 3.30	85.2 ± 2.36	85.5 ± 2.20	83.6 ± 2.43
52	88.6 ± 0.57	87.1 ± 0.53	86.5 ± 1.28	85.9 ± 1.57

[1]Values are means ± SEM, n = 3 replicate groups of 25 individual caged laying hens for each dietary treatment.

production for the experimental period was 100 ± 1, 100 ± 0.5, 100 ± 0.5 and 99 ± 1 eggs for hens fed diets containing 0, 14, 28 and 43% whole pearl millet, respectively. Egg weight (Table 5.6) and the specific gravity (Table 5.7) of the eggs produced by the hens did not vary among the dietary treatments. The yellowness of the egg yolks from eggs produced during the last week of the experiment was 79.3 ± 0.5, 78.6 ± 0.1, 77.1 ± 0.3 and 75.6 ± 0.5 for hens fed diets containing 0, 14, 28 and 43% whole pearl millet, respectively. The degree of yellowness of egg yolks was significantly ($P < 0.05$) less in the eggs produced from hens fed the two dietary treatments containing the most pearl millet compared to the eggs produced by the hens fed the corn and soybean meal control diet. In addition, egg yolk yellowness was also less in eggs produced from hens fed the highest level of dietary pearl millet relative those fed the lowest level of pearl millet.

Daily feed consumption (mean ± SEM) was 103.00 ± 1.90, 104.11 ± 2.22, 105.56 ± 3.56 and 107.11 ± 1.64 grams per bird for hens fed diets containing 0, 14, 28 and 43% whole pearl millet, respectively. There were no significant differences in feed intake among the 4 dietary treatments.

Experiment 2

During the starter period (1 to14 days of age), broilers fed diets containing 28 and 43% whole millet gained less body weight and had a lower feed efficiency than those fed diets containing 0 or 14% whole millet (Table 5.8). During the grower period, as in the starter period, the inclusion of 14% whole millet in broiler diets did not affect bird performance relative to the performance of birds fed the control diet, containing no whole pearl millet (Table 5.9). Broilers fed a diet containing 43% whole pearl millet during the

Table 5.6. The weight of eggs produced by Leghorn hens fed different dietary levels of whole pearl millet from 36 through 52 weeks of age (Experiment1).[1]

	Dietary treatment			
Age	1 (0% millet)	2 (14% millet)	3 (28% millet)	4 (43% millet)
(wk)	(g)			
36	59.54 ± 0.15	60.14 ± 0.20	59.51 ± 0.34	59.42 ± 0.67
38	61.44 ± 0.49	61.59 ± 0.68	61.11 ± 0.69	60.51 ± 0.49
40	62.25 ± 0.57	62.11 ± 0.66	61.02 ± 1.03	60.81 ± 0.89
42	62.97 ± 0.47	62.91 ± 0.56	61.66 ± 0.95	61.70 ± 0.91
44	62.68 ± 0.73	63.56 ± 0.48	61.67 ± 0.89	62.14 ± 0.88
46	63.49 ± 0.27	63.65 ± 0.24	63.05 ± 0.90	62.94 ± 0.87
48	64.36 ± 0.63	64.15 ± 0.12	64.06 ± 0.72	63.55 ± 0.78
50	64.16 ± 0.25	64.97 ± 0.63	63.99 ± 1.11	63.66 ± 0.60
52	65.14 ± 0.49	64.86 ± 0.78	64.15 ± 0.85	65.06 ± 0.78

[1]Values are means ± SEM, n = 3 replicate groups of 25 individual caged laying hens for each dietary treatment. Egg weights were determined for all eggs collected over a 2-day period at a given week of age.

Table 5.7. Specific gravities of eggs produced by Leghorn hens fed different dietary levels of whole pearl millet from 36 through 52 weeks of age (Experiment1).[1]

	Dietary treatment			
Age (wk)	1 (0% millet)	2 (14% millet)	3 (28% millet)	4 (43% millet)
46	1.081 ± 0.0003	1.080 ± 0.0013	1.083 ± 0.0008	1.083 ± 0.0008
48	1.079 ± 0.0003	1.078 ± 0.0006	1.080 ± 0.0010	1.081 ± 0.0003
50	1.082 ± 0.0001	1.088 ± 0.0069	1.082 ± 0.0008	1.082 ± 0.0008
52	1.080 ± 0.0003	1.079 ± 0.0007	1.080 ± 0.0010	1.079 ± 0.0007
54	1.080 ± 0.0003	1.079 ± 0.0009	1.081 ± 0.0008	1.081 ± 0.0006
56	1.077 ± 0.0003	1.078 ± 0.0006	1.076 ± 0.0011	1.077 ± 0.0004

[1]Values are means ± SEM, n = 3 replicate groups of 25 individual caged laying hens for each dietary treatment. Specific gravities were determined for all eggs collected over a 2 day period at a given week of age.

Table 5.8. Performance of male broilers from 0 to 14 days of age fed diets containing varying levels of whole pearl millet (Experiment 2)[1].

Dietary treatment	Body weight	Body weight gain	Feed to gain
	--------------- (g)	--------------	
1 (0% millet)	469 ± 3[a]	425 ± 3[a]	1.191 ± 0.007[a]
2 (14% millet)	471 ± 4[a]	427 ± 4[a]	1.203 ± 0.006[a]
3 (28% millet)	436 ± 5[b]	393 ± 5[b]	1.243 ± 0.012[b]
4 (43% millet)	442 ± 5[b]	399 ± 5[b]	1.256 ± 0.007[b]

[a-b]Means within each column with different superscripts are statistically different, $P < 0.05$.
[1]Values are means ± SEM, n = 8 replicate pens of 20 birds.

Table 5.9. Performance of male broilers from 14 to 28 days of age fed diets containing varying levels of whole pearl millet (Experiment 2)[1].

Dietary treatment	Body weight	Body weight gain	Feed to gain
	--------------------(g)------------------		
1 (0% millet)	1,657 ± 11[a]	1,192 ± 8[a]	1.356 ± 0.008[a]
2 (14% millet)	1,655 ± 10[a]	1,185 ± 8[a]	1.367 ± 0.008[a]
3 (28% millet)	1.590 ± 17[b]	1,154 ± 14[ab]	1.399 ± 0.007[b]
4 (43% millet)	1,576 ± 13[b]	1,134 ± 10[b]	1.432 ± 0.006[c]

[a-c]Means within each column with different superscripts are statistically different, $P < 0.05$.
[1]Values are means ± SEM, n = 8 replicate pens of 20 birds.

finisher phase (28 to 42 days of age) gained less body weight and were less efficient in converting ingested feed to body weight than those fed the control diet (Table 5.10). For the overall experimental period, only the broilers fed the diet containing 43% millet had lower body weight and body weight gains relative to the broilers fed the control diet (Table 5.11.).

Gizzard weight as a percent of fasted live body weight was greater in the broilers fed diets containing 28 and 43% whole pearl millet than those fed a diet containing no whole pearl millet (Table5.12). As a percent of fasted live body weight, the yields of wings, leg quarters, and total white meat did not differ between the broilers fed the four different dietary treatments. (Table 5.13).

Experiment 3

During the starter period, broilers fed diets containing 43% ground pearl millet gained less body weight and were less efficient in converting ingested feed to body weight gain than broilers fed a diet containing no ground pearl millet (Table5.14). In the grower period, the broilers fed the diet containing 14% ground millet were more efficient in converting ingested feed to body weight gain and weighed more at the end of this period than the broilers fed a diet containing no ground millet (5.15). During the finisher period, (Table 5.16) and in the overall period from 1 to 42 days of age (Table 5.17), there were no differences in body weight or body weight gain between the broilers fed the different dietary treatments, but the broilers fed the diet containing 43% millet had poorer feed conversions during these time periods relative to the control broilers.

Table 5.10 Performance of male broilers from 28 to 42 days of age fed diets containing varying levels of whole pearl millet (Experiment 2)[1].

Dietary treatment	Body weight	Body weight gain	Feed to gain
	--------------(g)--------------		
1 (0% millet)	3,131 ± 17[a]	1,490 ± 17	1.823 ± 0.012[a]
2 (14% millet)	3,150 ± 13[a]	1,496 ± 13	1.798 ± 0.010[a]
3 (28% millet)	3,038 ± 39[a]	1,474 ± 39	1.868 ± 0.012[ab]
4 (43% millet)	2,992 ± 37[b]	1,416 ± 27	1.903 ± 0.015[b]

[a-b]Means within each column with different superscripts are statistically different, $P < 0.05$.
[1]Values are means ± SEM, n = 8 replicate pens of 20 birds.

Table 5.11. Performance of male broilers from 0 to 42 days of age fed diets containing varying levels of whole pearl millet (Experiment 2)[1].

Dietary treatment	Body weight	Body weight gain	Feed to gain
	-------------(g)------------		
1 (0% millet)	3,131 ± 17[a]	3,088 ± 17[a]	1.553 ± 0.007[a]
2 (14% millet)	3,150 ± 13[a]	3,106 ± 13[a]	1.557 ± 0.015[a]
3 (28% millet)	3,038 ± 39[ab]	2,995 ± 39[ab]	1.611 ± 0.010[b]
4 (43% millet)	2,992 ± 37[b]	2,949 ± 37[b]	1.631 ± 0.006[b]

[a-b]Means within each column with different superscripts are statistically different, $P < 0.05$.
[1]Values are means ± SEM, n = 8 replicate pens of 20 birds.

Table 5.12. Processing yields from 42-day-old broilers that had been fed diets containing varying levels of whole pearl millet (Experiment 2)[1].

Dietary treatments	Chilled carcass		Hot carcass		Gizzard	
	(g)	(%)[2]	(g)	(%)[2]	(g)	(%)[2]
1 (0% millet)	2,211 ± 32[a]	74 ± 0.4	2,195 ± 31	74 ± 0.2	31 ± 1.0[a]	1.1 ± 0.04[a]
2 (14% millet)	2,237 ± 10[a]	75 ± 0.2	2,229 ± 10	74 ± 0.3	34 ± 0.8[bc]	1.1 ± 0.02[ac]
3 (28% millet)	2,164 ± 28[ab]	75 ± 0.4	2,152 ± 24	74 ± 0.6	35 ± 0.4[bc]	1.2 ± 0.01[bc]
4 (43% millet)	2,116 ± 19[b]	75 ± 0.7	2,105 ± 19	74 ± 0.8	33 ± 0.6[ac]	1.2 ± 0.02[bc]

[a-c]Means within each column with different superscripts are statistically different, $P < 0.05$.
[1]Values are means ± SEM, n = 8 replicate pens with 8 birds per pen processed.
[2]As a percent of live fasted weight.

Table 5.13. Processing yields from 42 day old broilers that had been diets containing varying levels of whole pearl millet (Experiment 2)[1].

Dietary Treatment	Pectoralis major		Pectoralis minor		Total white meat[2]		Wings		Leg quarters	
	G	%[3]	g	%[3]	g	%[3]	g	%[3]	g	%[3]
1 (0% millet)	562 ± 13	18.9 ± 0.3	106 ± 1[ab]	3.6 ± 0.03	667 ± 14[b]	22.5 ± 0.3	243 ± 5	8.0 ± 0.1	712 ± 10[a]	24.0 ± 0.1
2 (14% millet)	583 ± 5	19.5 ± 0.2	108 ± 1[a]	3.6 ± 0.06	695 ± 6[a]	23.2 ± 0.3	247 ± 3	8.3 ± 0.1	721 ± 5[a]	24.0 ± 0.1
3 (28% millet)	548 ± 12	18.9 ± 0.2	101 ± 2[b]	3.5 ± 0.07	650 ± 12[b]	22.4 ± 0.2	237 ± 5	8.2 ± 0.1	699 ± 8[ab]	24.1 ± 0.2
4 (43% millet)	549 ± 14	19.3 ± 0.3	103 ± 1[ab]	3.6± 0.03	650 ± 14[b]	22.9 ± 0.3	235 ± 3	8.3 ± 0.1	674 ± 7[b]	23.8 ± 0.1

[a-c]Means within each column with different superscripts are statistically different, $P < 0.05$.
[1]Values are means ± SEM, n = 8 replicate pens with 8 birds per pen processed.
[2]*Pectoralis major* plus *Pectoralis minor.*
[3]As a percent of live fasted weight.

Table 5.14. Performance of male broilers from 1 to 14 days of age fed diets containing varying levels of ground pearl millet (Experiment 3)[1].

Dietary treatments	Body weight	Body weight gain	Feed to gain
	---------------(g)---------------		
1 (0% millet)	475± 5[a]	429 ± 5[a]	1.188 ± 0.010[a]
2 (14% millet)	463 ±6[ab]	418 ± 6[ab]	1.203 ± 0.006[a]
4 (43% millet)	455 ± 2[b]	410 ± 3[b]	1.247 ± 0.004[b]

[a-b]Means within each column with different superscripts are statistically different, $P < 0.05$.
[1]Values are means ± SEM, n = 10 replicate pens of 20 birds.

Table 5.15. Performance of male broilers from 14 to 28 days of age fed diets containing varying levels of ground pearl millet (Experiment 3)[1].

Dietary treatments	Body weight	Body weight gain	Feed to gain
	---------------(g)---------------		
1 (0% millet)	1,713 ± 16	1,238 ±14[a]	1.418 ± 0.001[a]
2 (14% millet)	1,737 ± 16	1,275 ± 11[b]	1.384 ± 0.004[b]
4 (43% millet)	1,691 ± 9	1,237 ± 8[a]	1.445 ± 0.006[c]

[a-c]Means within each column with different superscripts are statistically different, P <0.05.
[1]Values are means ± SEM, n = 10 replicate pens of 20 birds.

Table 5.16. Performance of male broilers from 28 to 42 days of age fed diets containing varying levels of ground pearl millet (Experiment 3)[1].

Dietary treatment	Body weight	Body weight gain	Feed to gain
	----------------(g)----------------		
1 (0% millet)	3,247 ± 22	1,533 ± 18	1.806 ± 0.014[a]
2 (14% millet)	3,239 ± 28	1,507 ± 20	1.867 ± 0.014[b]
4 (43% millet)	3,183 ± 16	1,495 ± 10	1.936 ± 0.015[c]

[a-c]Means within each column with different superscripts are statistically different, $P < 0.05$.
[1]Values are means ± SEM, n = 10 replicate pens of 20 birds.

Table 5.17. Performance of male broilers from 0 to 42 days of age fed diets containing varying levels of ground pearl millet (Experiment 3)[1].

Dietary treatment	Body weight	Body weight gain	Feed to gain
	----------------(g)---------------		
1 (0% millet)	3,247 ± 22	3,201 ± 22	1.573 ± 0.000[a]
2 (14% millet)	3,239 ± 28	3,193 ± 28	1.580 ± 0.010[a]
4 (43% millet)	3,183 ± 16	3,137 ± 16	1.653 ± 0.010[b]

[a-c]Means within each column with different superscripts are statistically different, $P < 0.05$.
[1]Values are means ± SEM, n = 10 replicate pens of 20 birds.

To the contrary, the percent carcass yield relative to live fasted weight was greater in the broilers fed a diet containing 43% ground millet than those fed a diet containing no millet (Table 5.18). In addition, as a percent of live fasted weight, total white meat yield and pectoralis major were higher and wing yield was less, by weight and percent, for broilers fed a diet containing 43% ground pearl millet relative to those consuming a diet containing no pearl millet (Table 5.19).

Experiment 4

Ghrelin mRNA was detected in both the granulosa and theca cells of the preovulatory follicles of the laying hen ovary (Figures 5.1 and 5.2). Overall, the mRNA expression of ghrelin was greater in granulosa tissue than in theca tissue (Figure 5.1). The mRNA expression of ghrelin was higher in granulosa cells obtained from 8 to 12mm diameter small yellow follicles (SYG1) than from granulosa cells isolated from any of the hierarchical follicles (Figure 5.2).

Experiment 5

There were no differences in the mRNA expression of ghrelin in the ovarian preovulatory follicular tissue (Figure 5.3) or proventriculus (Figure 5.4) tissue between fed and fasted broiler breeder hens. Since there were no difference in the expression of ghrelin mRNA in the preovulatory follicle tissue with feeding state, the 3 fed and 3 fasted replicates were combined for further analyses of ghrelin mRNA expression in the broiler breeder hen ovary. Overall, ghrelin mRNA expression is greater in granulosa tissue than theca tissue (Figure 5.5).

However, for any given follicle size there is not a difference in ghrelin expression between the theca and granulosa cells and there were no differences in ghrelin mRNA expression between different sized preovulatory follicles (Figure 5.6).

Table 5.18. Processing yields from 42 day old broilers that had been fed diets containing varying levels of ground pearl millet (Experiment 3)[1].

Dietary treatments	Chilled carcass		Hot carcass	
	(g)	(%)[2]	(g)	(%)[2]
1 (0% millet)	2290 ± 26	75 ± 0.2[a]	2273 ± 26	74 ± 0.2[a]
2 (14% millet)	2279 ± 18	75 ± 0.2[ab]	2261 ± 20	75 ± 0.2[ab]
4 (43% millet)	2285 ± 20	76 ± 0.2[b]	2261 ± 19	75 ± 0.2[b]

[a-b]Means within each column with different superscripts are statistically different, $P < 0.05$.
[1]Values are means ± SEM, n = 10 replicate pens with 8 birds per pen processed.
[2]As a percent of live fasted weight.

Table 5.19. Processing yields from 42 day old broilers that had been fed diets containing varying levels of ground pearl millet (Experiment 3)[1].

Dietary treatments	Pectoralis major		Pectoralis minor		Total white meat[2]		Wings		Leg quarters	
	(g)	(%)[3]	(g)	(%)[3]	(g)	(%)[3]	(g)	(%)[3]	(g)	(%)[3]
1 (0% millet)	596 ± 13	19.4 ± 0.2[a]	117 ± 2	3.8 ± 0.04	713 ± 14	23.3 ± 0.2[a]	246 ± 2[a]	8.0 ± 0.05[a]	708 ± 6	23.1 ± 0.2
2 (14% millet)	601 ± 9	19.8 ± 0.2[ab]	116 ± 2	3.8 ± 0.03	717 ± 10	23.7 ± 0.2[ab]	243 ± 3[ab]	8.0 ± 0.05[ab]	704 ± 5	23.2 ± 0.2
4 (43% millet)	616 ± 9	20.4 ± 0.3[b]	115 ± 1	3.8 ± 0.03	732 ± 10	24.2 ± 0.2[b]	237 ± 2[b]	7.9 ± 0.05[b]	698 ± 7	23.2 ± 0.1

[a-b]Means within each column with different superscripts are statistically different, $P < 0.05$.
[1]Values are means ± SEM, n = 10 replicate pens with 8 birds processed.
[2]*Pectoralis major* plus *Pectoralis minor.*
[3]As a percent of live fasted weight.

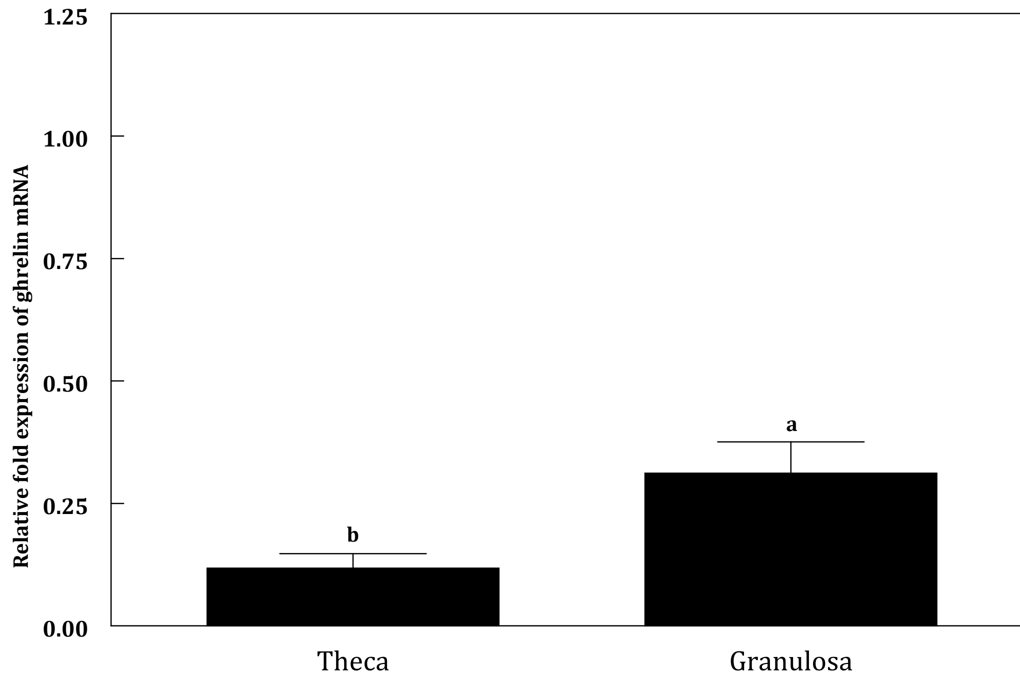

Figure 5.1. The overall relative fold expression of ghrelin mRNA in theca and granulosa tissue collected from the four largest hierarchical (F1 through F4) follicles the large small yellow follicles (8 to 12 mm in diameter) small yellow follicles (5 to 8 mm in diameter), large white follicles (2 to 5 mm in diameter) and small large white follicles (less than 2 mm in diameter) from 50 to 55 week old Leghorn (Experiment 4). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 48 [6 samples (3 theca and 3 granulosa) from each of the 8 follicle sizes].

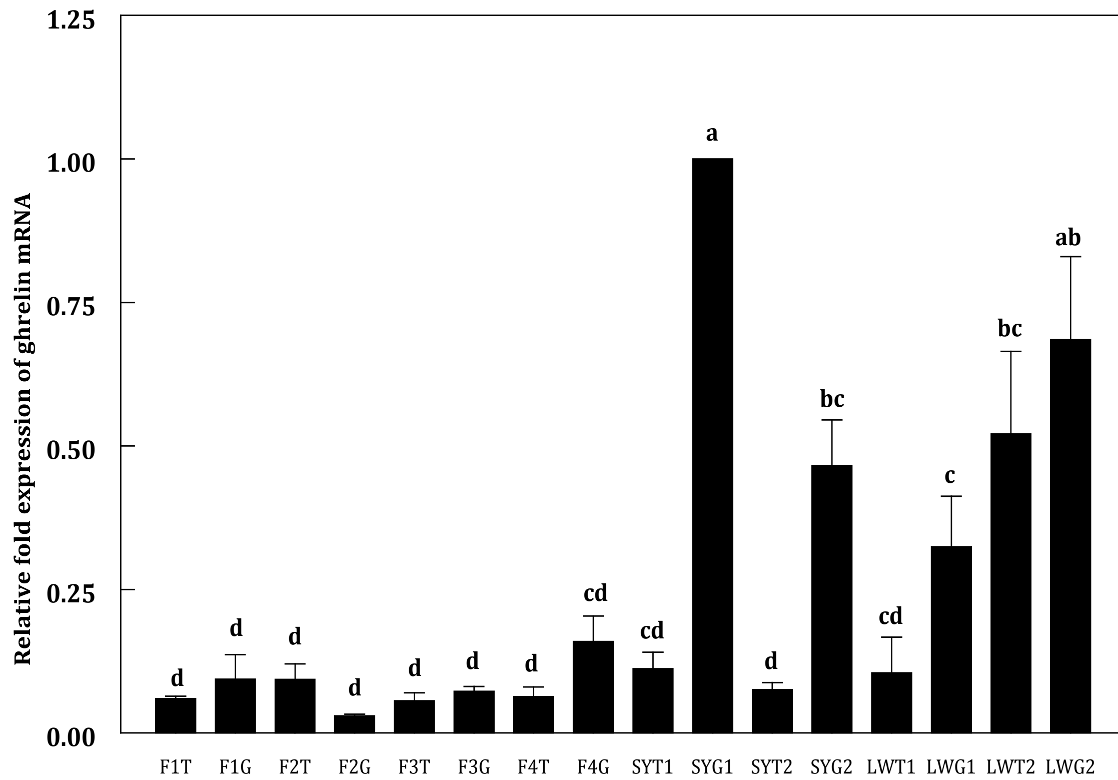

Figure 5.2. The relative fold expression of ghrelin in theca (T) and granulosa (G) tissue collected from the four largest hierarchical preovulatory follicles (F1 through F4), large small yellow follicles (SY1, 8 to 12 mm in diameter), small yellow follicles (SY2, 5 to 8 mm in diameter), large white follicles (LW1, 2 to 5 mm in diameter) and small large white follicles (LW2, less than 2 mm in diameter) from 50 to 55week old Leghorn hens (Experiment 4). The data is expressed as mean fold differences ± SEM, n = 3. [a-d]Means with different letters differ, $P < 0.05$.

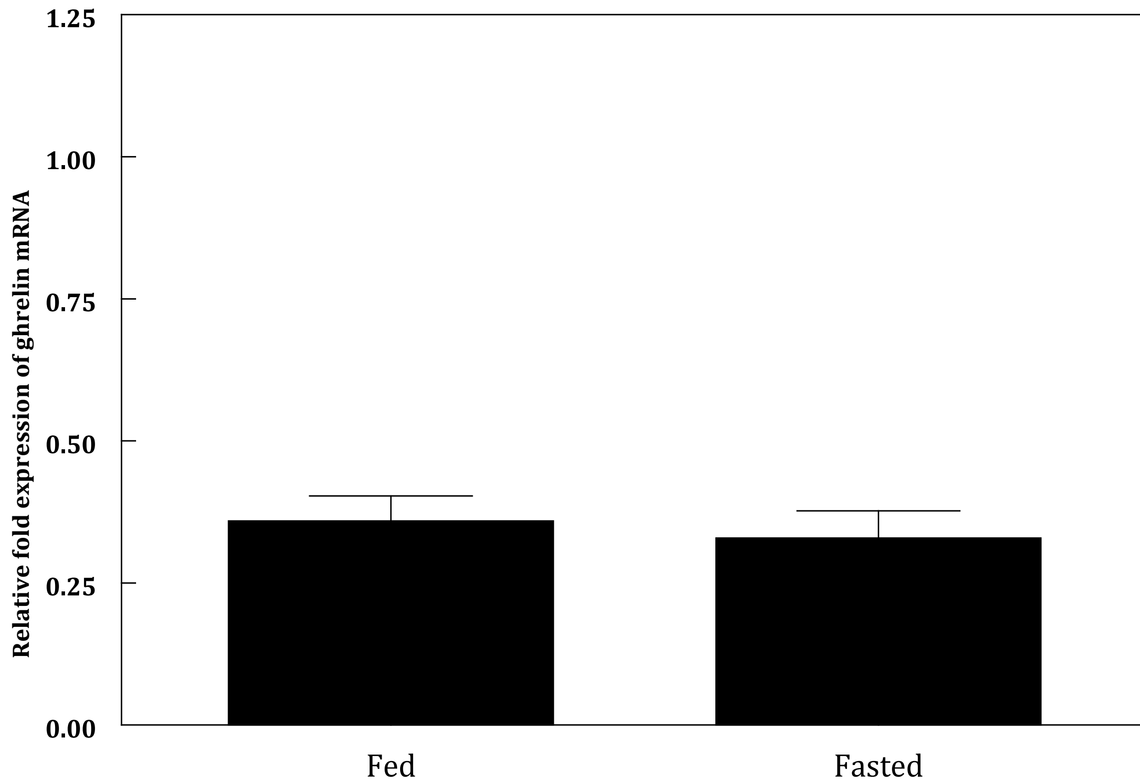

Figure 5.3. The overall relative fold expression of ghrelin mRNA in theca and granulosa tissue collected from the four largest hierarchical (F1 through F4) follicles and the small yellow and large white follicles from 45 to 55 week old broiler breeder hens fed daily or fasted for 72 hours (Experiment 5). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 36 [6 samples (3 theca and 3 granulosa) from each of the 6 follicle sizes].

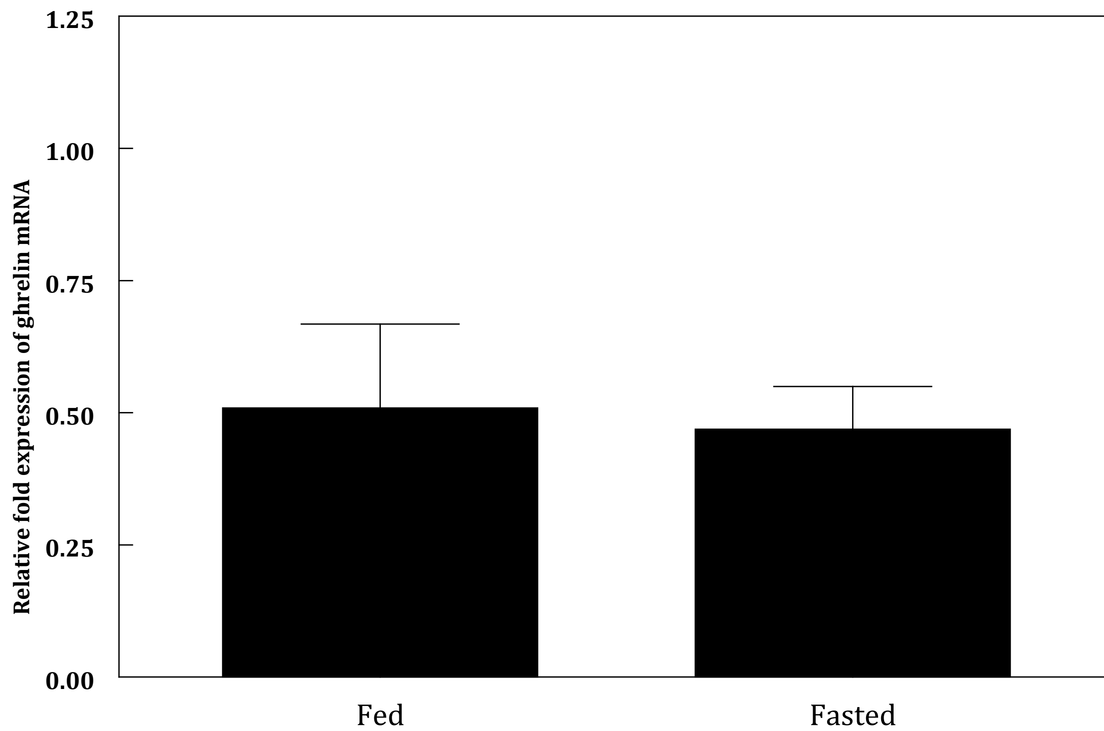

Figure 5.4. The overall relative fold expression of ghrelin mRNA in proventriculus collected from 45 to 55week old broiler breeder hens fed daily or fasted for 72 hours (Experiment 5). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 6.

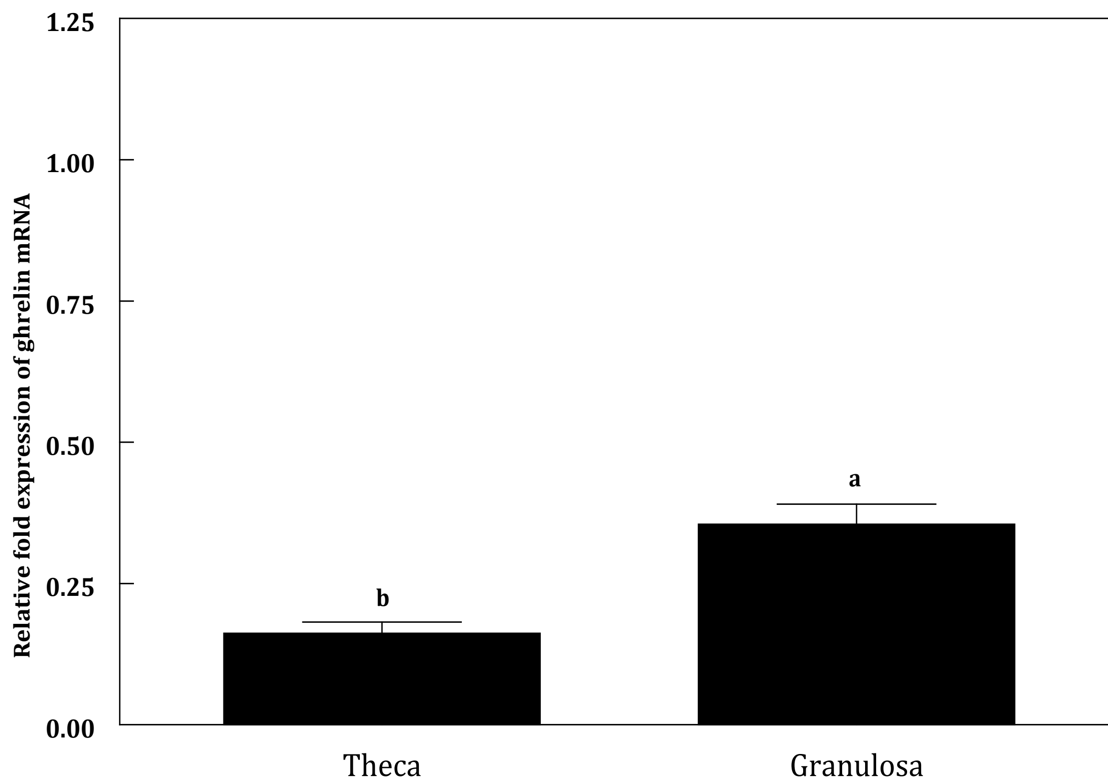

Figure 5.5. The relative fold expression of ghrelin mRNA in theca or granulosa tissue collected from the four largest hierarchical (F1 through F4) follicles and the small yellow and large white follicles from 45 to 55 week old broiler breeder hens (Experiment 5). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 36 [6 samples (3 theca and 3 granulosa) from each of the 6 follicle sizes]. [a-b]Means with different letters differ, $P < 0.05$.

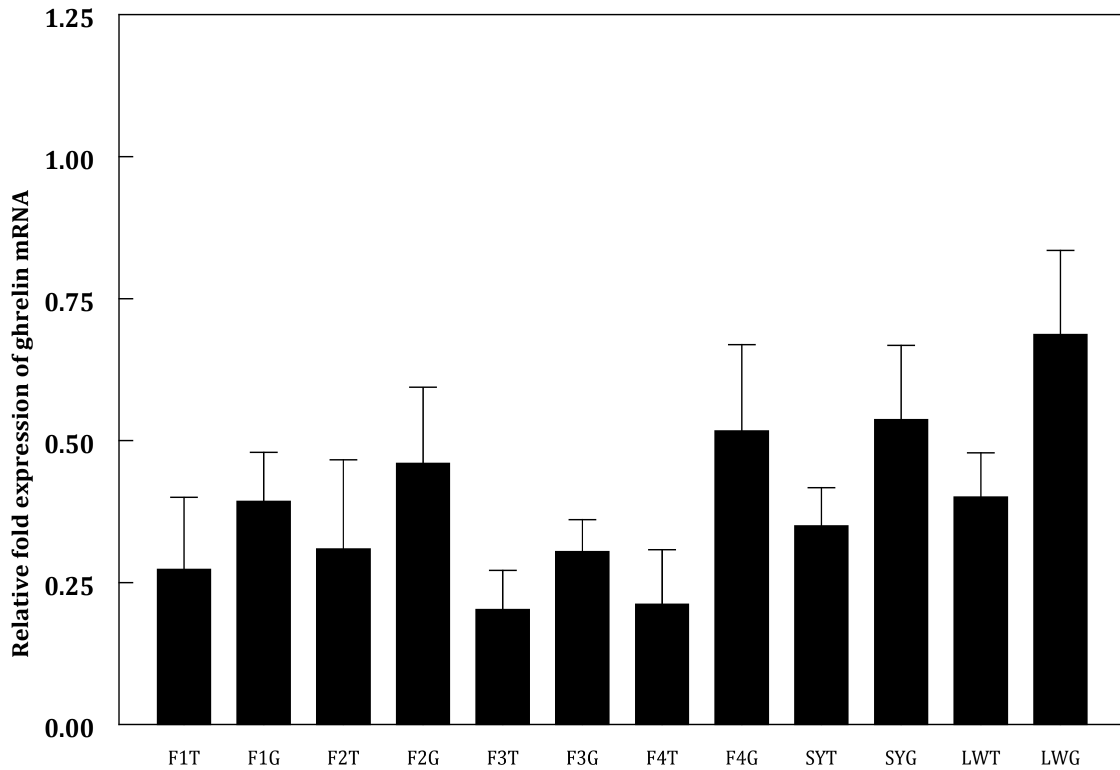

Figure 5.6. The relative fold expression of ghrelin mRNA in theca (T) and granulosa (G) tissue collected from the four largest hierarchical (F1 through F4) follicles and the small yellow (SY) and large white (LW) follicles from 45 to 55 week old broiler breeder hens (Experiment 5). The mRNA expression data were normalized with glyceraldehyde-3-phosphate dehydrogenase and expressed as the mean fold difference ($2^{-\Delta\Delta CT}$) ± SEM, n = 6.

CHAPTER 6

DISCUSSION

Pearl millet

The overall goal of the pearl millet research was to test whether Malian grown pearl millet would be an effective feed ingredient for poultry production. The current research indicates that pearl millet grown in Mali could successfully replace corn in laying hen and broiler diets. Furthermore, it indicates that performance of laying hens and broilers would likely be unaffected even if the millet was incorporated into poultry diets in whole form.

Protein and energy content of Malian pearl millet

Genetic selection of pearl millet has been focused on improving its disease resistance, yield and nutrient composition. Research from an animal feeding perspective has been focused on testing these developed varieties, which have been produced and grown almost exclusively in developed countries. Although variable, the crude protein level of pearl millet typically ranges from 10-16% (Burton *et al.*, 1972; Sullivan *et al.*, 1990; Adeola and Rogler, 1994; Amato and Forrester, 1995; Davis *et al.*, 2003; Singh *et al.*, 2005; Vasan *et al.*, 2008a, b). Protein levels vary from harvest to harvest and are different depending on the variety of pearl millet and also agronomic differences, particularly the nitrogen content in the soil from which it was harvested. Thus, given the arid conditions and poorer soil fertility in Mali, it is not surprising that the protein concentration of the most widely grown varieties of pearl millet were less or on the lower end of the

concentration range seen with the previously researched highly selected varieties of pearl millet. However, all of the Malian pearl millet varieties tested had protein concentrations, which were higher than corn and had similar apparent digestible amino acid coefficients as corn.

The energy content of the tested Malian pearl millet varieties was also similar to or slightly higher than corn and also similar to previously reported levels in the highly genetically selected pearl millet varieties grown in developed countries. The nitrogen corrected true metabolizable energy (TME_N of corn is typically about 3,350 kcal/kg compared to 3,300 to 3,450 kcal/kg for pearl millet (Adeola *et al.,* 1994; Collins *et al.*, 1997; Davis *et al.,* 2003; Hidalgo *et al.*, 2004) and the TME_N values for the tested Malian varieties of pearl millet were all above 3,395 to as high as 3,738 kcal/kg on an as is basis.

Malian pearl millet in poultry production

In the present research, lowering the corn content of a control laying hen diet by 25, 50 or 75% by replacing it with whole millet imported from Mali, did not significantly affect egg production in white leghorn hens during the 4-month experiment. Similarly, Collins *et al.* (1995) reported that replacing 100% of the dietary corn (68% of the formulated diet) with ground pearl millet caused no reduction in egg production for a 6-week trial period. Similar results were also reported by Purushothaman and Thirumalai (1997) and Collins *et al.* (1997). In contrast, Mehri *et al.* (2010) conducted a 12-week experiment and found that 100% replacement of corn (58% of the formulated diet) with whole millet decreased egg production ($P<0.05$). However, replacing the corn content of the laying diet with 25, 50 or 75% millet did not affect egg production.

Egg weights and egg quality are typically unaffected by replacing dietary corn with millet even when the dietary corn content is completely replaced by pearl millet (Collins *et al.*, 1997; Amini and Ruiz-Feria, 2007). Egg weight was not significantly affected by dietary millet inclusion in the current research. However, yellow yolk pigmentation was reduced in the eggs produced by the hens fed diets containing 28 and 43% whole millet. This result is in line with previous research in which the yellowness of egg yolks was reduced when dietary corn was replaced by pearl millet (Collins *et al.*, 1997; Amini and Ruiz-Feria, 2007). Thus, birds fed pearl millet based diets need additional dietary sources of xanthophylls to obtain the degree of yellow pigmentation associated with feeding corn-based diets, for consumer preference.

Although daily feed intake was not significantly different among the laying hens fed 0, 14, 28 or 43% whole millet, there was a clear trend that feed consumption increased as the dietary levels of whole millet in the diet increased. The significance of this became more apparent when broilers were fed the same levels of dietary whole pearl millet and feed conversion ratios in these broilers increased as the level of dietary millet increased. However, the degree to which body weight and body weight gain were affected by increasing levels of dietary millet became less as the birds got older. Taken together, the results suggested that the birds might be compensating for a nutrient deficiency in the millet diets by eating more of the diet, rather than the idea that the whole millet seeds were not completely digested or that the energy cost of digesting the whole millet was more than anticipated.

Gizzard weight relative to body weight increased for the broilers fed the diets containing 28 and 43% whole millet in the current research and this agreed with previous

research that our laboratory had conducted with a U.S. variety of pearl millet in which broilers fed diets from 1 to 15 days of age containing 10, 15 or 20% whole millet had increased relative gizzard weights to body size relative to birds fed diet with no whole millet (Hidalgo *et al.*, 2004). The anticipated increase in gizzard size in the current research was actually expected to improve nutrient digestibility as previous research had indicated that laying hens fed diets containing 15% whole millet versus 15% ground millet had significantly higher starch digestibility of the diet than the hens fed the diet with ground millet (Garcia and Dale, 2006). Previous studies had also indicated that broilers fed diets containing whole wheat or barley had increased dietary starch digestibility (Hetland *et al.*, 2002; Plavnik *et al.*, 2002; Svihus *et al.*, 2004). The increased starch digestibility is likely due to increased enzyme accessibility to starch granules, which may result from enhanced gizzard function in birds fed whole grains.

Given the likely enhanced nutrient digestibility stimulated by feeding whole pearl millet, our focus was not on the poor feed conversion ratios. Instead, we focused on a possible amino acid deficiency. Although a representative sample of the pearl millet utilized for the current research had been procured and analyzed for protein and digestible amino acid content (mixed industrial variety, tables 5.1-5.3) prior to importation, another sample of the imported millet remaining after completion of experiments 1 and 2 was taken and analyzed. The protein content of the second analyzed sample was only 8.85% compared to the original 10.11%. More importantly, while the digestible lysine, methionine, and other essential amino acid contents between the two samples remained essentially the same, the levels of digestible threonine and tryptophan decreased by 26 and 22%, respectively, which was enough to cause marginal deficiencies in the diets containing

28 and especially 43% pearl millet. This marginal deficiency would be especially apparent for rapidly growing broilers that could not compensate by eating more of the less nutrient diet.

Experiment 3 was conducted with the remaining pearl millet utilizing diets that were equivalent in composition as to those used in Experiment 2 with whole pearl millet. However, in this experiment, the millet was ground before being incorporated into the diets. The results from Experiment 3 support the idea that the decrease in body weight gain and efficiency seen in experiment 2 at the 43% dietary millet inclusion level were not the result of feeding whole millet, but likely due to marginal amino acid deficiencies., because the diet containing 43% ground millet also resulted in broilers that were less efficient in converting feed to body weight gain. However, the results from Experiment 3 also indicated that there could be some benefit to feeding ground pearl millet as it resulted in improved carcass yields relative to the control diet.

Whole pearl millet

The utilization of whole pearl millet at a dietary level of 43% in laying hens and broilers in the current research far exceeds the levels of whole millet utilized in previous research. Hidalgo *et al.*, (2004) reported the incorporation of up to 10% whole pearl millet in diets given throughout an entire broiler production period of 42 days did not adversely affect broiler performance, carcass yields or pellet quality. Similarly, Garcia and Dale (2006) reported that laying hens fed a diet containing 10% whole millet for 4 weeks had no adverse effects on feed consumption or egg production. The energy savings from not having to grind pearl millet for inclusion in poultry diets would be significant and the

ability to feed millet in whole form eliminates the need for extra storage capacity for ground millet.

In the Sahel Region of Africa where millet production is abundant and corn does not thrive, millet has traditionally been used as a human food staple. Poultry production in this region of Africa has not developed greatly in part because commercial producers use imported corn and soybean meal to feed their birds which results in very elevated consumer prices. However, the use of locally grown pearl millet in poultry diets as a corn replacement could greatly reduce poultry production costs and stimulate commercial development. The current research indicates that the widely grown millet varieties in Mali are comparable or slightly better than corn in nutrient composition and would make an ideal substitute for corn in poultry diets. In addition, the results from the current research also indicates that pearl millet could be utilized in whole form when feeding broilers and laying hens without compromising bird performance and the use of whole millet would further reduce poultry production costs.

Ghrelin

Previously, our laboratory reported that the mRNA for ghrelin could not be detected in theca and granulosa samples from broiler breeder hens (Freeman, 2008). In this previous research using reverse transcriptase PCR followed by detection of product with ethidium bromide staining, ghrelin production was confirmed in the proventriculus of the broiler breeder hen. In the current research using a much more sensitive detection system with real time reverse transcriptase PCR, ghrelin mRNA was detected in theca and granulosa tissue from preovulatory follicles of all maturity stages in both the laying and

broiler breeder hen. In the laying hen, ghrelin mRNA expression was greater in granulosa cells relative to theca cells and ghrelin expression was higher in granulosa cells from the largest small yellow follicles than in granulosa cells isolated from any the hierarchical follicles. While the overall expression of ghrelin mRNA was also greater in the granulosa cells than theca cells of the broiler breeder hen ovary, there was not any differences in ghrelin mRNA expression based on follicular size (maturity). This difference in the expression of ghrelin relative to follicular development between laying and broiler breeder hens needs to be further investigated in future research as does a direct determination of ghrelin mRNA expression levels between the two types of hens.

In research from our laboratory, Dimova (2012) reported on the cDNA cloning of GOAT in chickens and the tissue distribution of GOAT mRNA in broilers. GOAT is the enzyme that acylates ghrelin, which allows it to bind to its receptor and it plays a critical role in ghrelin biology. Expression of GOAT mRNA was detected in the proventriculus, which is the primary site of endocrine ghrelin production, but most tested tissues had no detectable GOAT mRNA expression. However, GOAT mRNA was detected in the granulosa cells, but not the theca cells of hierarchical and prehierarchical preovulatory follicles (Dimova, 2012). GOAT mRNA expression decreased with follicular maturity and was not detected in granulosa cells from the F1 follicle (Dimova, 2012). Thus, it would appear that is the granulosa cells of the small yellow and large white follicles that that are the primary site for ovarian acylated ghrelin production.

Freeman (2008) reported that plasma acylated ghrelin levels increased when broiler breeders were fasted as plasma acylated ghrelin levels, 72 hours after the last feeding, were about double of the levels found 6 hours after the last feeding. Interestingly,

in the current research, ghrelin mRNA was not increased in broiler breeder hens fasted for 72 hours. However, in these same tissue samples GOAT mRNA expression was significantly elevated after the broiler breeder hens had been fasted for 72 hours (Dimova, 2012). Thus, in broiler breeder hens it would appear that GOAT may be the key regulator of the production of acylated ghrelin.

The ghrelin receptor is also found in the theca and granulosa cells of prehierarchical and hierarchical follicles of broiler breeder hens (Freeman, 2008). Furthermore, in broiler breeder hens fasted for 72 hours the level of the mRNA for the ghrelin receptor is up-regulated in theca tissue, while it remains unchanged in the granulosa tissue (Freeman, 2008). The increase in GOAT mRNA levels in the proventriculus at 72 hours of fasting and the associated increase in plasma acylated ghrelin combined with the fasting induced up-regulation of theca ghrelin receptor mRNA at 72 hours is important. The theca cells are vascularized while the granulosa cells are avascular. Thus, ghrelin could be responsible for communicating caloric insufficiency to the developing follicles through the theca cells, which could trigger the initiation of atresia. These prehierarchical follicles are the most sensitive to undergoing atresia with estimates that 90% of the follicles in these stages of development undergo atresia (Gilbert *et al.*, 1983) by apoptosis (Johnson *et al.*, 1996). Although the mRNA expression of GOAT and ghrelin in the granulosa cells of the preovulatory follicles of broiler breeder hens was not affected by fasting, the very fact that granulosa cells are one of the few tissues to even express GOAT and ghrelin mRNA is an interesting indication that ghrelin is an important potential regulator of follicular development.

In summary, the current research indicates that ghrelin mRNA is present in the developing follicles of the broiler breeder hen ovary. This result combined with our previous results indicates that the mRNA for all of the components of the ghrelin system (ghrelin, GOAT, and ghrelin receptor) is expressed within the theca or granulosa cells of the preovulatory follicles of the broiler breeder hen ovary. Therefore, ghrelin could be a key regulator of follicular development during both the fed and fasted state and provide a link between nutritional status and reproductive capability. However, further research is needed to develop this hypothesis.

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Analysis of variance was performed using the general linear model procedure of Minitab (Release 8.2, State College, PA). Tukey's multiple-comparison procedure was used to detect significant differences.