University of New England

Metabolism of energy and implementation of net energy system in laying hens

Submitted by:

Shahram Barzegar Nafari

B.Sc. in Animal Science (University of Tehran, Iran)

M.Sc. in Poultry Nutrition (Technical University of Esfahan, Iran)

A thesis submitted for the degree of Doctor of Philosophy

January 2019

Certification of dissertation

I certify that the substance of this thesis has not already been submitted for any degree and is not currently being submitted for any other degree qualification.

I certify that any help received in preparing this thesis, and all sources used, have been acknowledged in this thesis.

Shahram Barzegar Nafari

January 2019

Acknowledgments

I would like to express my special appreciation and thanks to my supervisors Prof. Robert Swick and Associate Prof. Shubiao Wu. You have been tremendous mentors for me. The door to offices was always open whenever I ran into a trouble spot or had a question about my research, findings or writing. They consistently allowed this paper to be my own work but steered me in the right direction whenever they thought I needed it. I have to also appreciate Prof. Mingan Choct, my co-supervisor, for his brilliant comments and suggestions, academic guidance and all his support for this project. I would like also to appreciate Dr. Jean Noblet for his priceless directions and guidance. I am gratefully indebted to his very valuable comments on this study.

I would also like to thank the experts and classmates at UNE who were involved and contributed in this research project: Drs, Sarbast Kheravii, Matt Hilliar, Chuck Keerqin, Sami Samiullah, Mehdi Toghyani, Natalie Morgan, Zafar Iqbal, Manisha Kolakshyapati, Guangda Xue and Nikki Zhang. Without their passionate participation and input, the current research could not have been successfully conducted. I also acknowledge Shuyu Song, Andrew Cohen-Barnhouse, Grahame Chaffey, Mark Porter, Michael Raue, Jenny Wittig, Leanne Lisle, and John Pesor for their friendship and technical support.

Finally, I must express my very profound gratitude to my wife, Sepideh and to my son, Sorena, for providing me with unfailing support and continuous encouragements throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you.

A special thanks to my mother and father for all of the sacrifices that you've made on my behalf. Your support for me was what sustained me thus far.

Preface

This thesis has been written and edited according to the standard format of the University of New England. I have made every effort to minimize the repetition of materials between chapters. However, some overlap remains, particularly in the methodology sections.

Abstract

Dietary energy is an expensive component of poultry diet formulations and may be as high as 60% of diets costs in certain situations. Different energy evaluation systems have been used for poultry diets formulations. The apparent metabolizable energy system (AME) is widely accepted and has been applied in the industry for 50 years in most world areas. The system compares the total gross energy of the diet to that present in excreta to determine what is retained. Development of energetic measurement techniques such as open and closed circuit calorimetry have enabled researchers to measure the wasted heat energy to determine the true available amount of energy for different body functions as net energy (NE). While the NE system has been proposed as a more accurate system for expressing feed energy and birds energy requirements compared to the AME system some difficulties remain in the poultry area with respect to the effect of age, environmental conditions and lack of data. Net energy based feeding systems are in successful use for pig and cattle feed formulation. For laying hens most nutritionists use the same AME values used for broilers. While this may be adequate, the use of broiler NE values for laying hens would not likely be acceptable as broilers are growing at a much higher rate than layers. This thesis examined the application of the NE system in laying hens. Chapter 1 provides general information about energy metabolism in body with more focus on the objectives of this study experiments. Chapter 2 is the literature review that provides the scientific background for the comparison of different feed energy evaluation systems and their limitations in practice. Included is a discussion on the metabolism of energy in chickens, partitioning of energy for different metabolic activities (maintenance, growth, and production) and dietary energy utilization for various body functions. The effect of the dietary profile, age, genotype, physiological status and environment on the energy metabolism, specifically on the net energy of the diets are considered and discussed.

Chapter 3 studies the application of the bioassay method for measuring the AME, AMEn (AME adjusted for zero nitrogen retention) and AMEs (AME adjusted to 50% nitrogen retention) values of common dietary ingredients in layers feed as specific ingredients AME values are rarely available for laying hens. The bioassay evaluation used the reference diet substitution method and compared the data with the regression estimation method. The results confirmed that the *in vivo* measured AME values of ingredients using laying hens were close to those calculated from proximate composition using the European prediction equation and tabulated values based on adult cockerels. The results showed a good agreement between the reference

diet substitution and regression methods to estimate ingredients AME content. In conclusion, the AMEn values are not representative of production conditions, in particular for the high-protein ingredients. In addition, AME values as obtained from the difference method should be interpreted with caution as it is affected by the CP content of the test diet. AMEs would then be the most representative of productive conditions.

Chapter 4 evaluates the energy efficiency and net energy prediction of feed in laying hens. Using closed-circuit calorimetry chambers by feeding different diets with various nutrient contents to the laying hens in different ages in the production phase enabled the measurement of gas exchange, heat production, AME and NE of diets. Then AME and NE equations were generated based on diets and applied to or ingredients. The equations were further validated in calorimetry chambers. It was confirmed that the NE of diets can be predicted from AME or AMEn, crude protein and ether extract levels in laying hen diets.

Chapter 5 describes two production experiments that were conducted to investigate the influence of different energy ratios (NE/ AMEn) by increasing dietary ether extract (EE) levels on birds performance and egg quality parameters. This chapter examined the effect of formulating diets based on the NE system compared to the default system (AME) and is intended to provide recommendations for nutritionists serving the layer industry. The results indicate that higher NE/AMEn diets with added EE improved hen performance and egg quality with higher albumen and Haugh units and darker yolk color score.

Chapter 6 examines the energy metabolism at the molecular level. The effect of dietary NE/AME levels on messenger RNA (mRNA) expression of genes involved in energy metabolism and lipogenesis in laying hens was examined. Feeding laying hens diets with different NE/AME and levels of EE over time increased mRNA expression of peroxisome proliferator-activated receptor gamma (PPARG) a gene involved in fatty acid storage and glucose metabolism, in jejunal mitochondria. The different dietary treatments did not alter the mRNA expression of genes involved in cellular energy metabolism, oxidative phosphorylation or fatty acid synthesis. Furthermore, mitochondrial content per cell remained unchanged as a result of changes in dietary NE/AME ratio.

This studies conducted in this thesis have provided the data necessary for nutritionists to begin implementation of an NE based formulation system for layer feed. An NE database of ingredients has been provided along with equations that can be applied to ingredients not present in the database so the NE value can be generated. The system gives higher NE values

to ingredients with higher EE levels and lower NE values to ingredients with high protein levels relative to the AME system. This should give nutritionists operating in the layer industry to formulate diets more efficiently than before with improved performance and lower dietary costs. Further study is warranted to further confirm the benefits of the NE system with the existing AMEn system for layers

Table of contents

List of figures

List of tables

List of abbreviations

AA	amino acid
ACACA	acetyl-CoA carboxylase alpha
ADF	acid detergent fiber
AHP	activity heat production
AME	apparent metabolizable energy system
AMEn	AME adjusted for zero nitrogen retention
AMEs	AME adjusted to 50% nitrogen retention
AMP	adenosine monophosphate
AMPK	adenosine monophosphate-activated protein kinase
ANOVA	analysis of variance
ATP	adenosine triphosphate
ATP15W	ATP synthase subunit alpha
avANTP	avian adenine nucleotide translocator
BMR	basal metabolic rate
BW	body weight
cDNA	complementary DNA
COXIII	cytochrome c oxidase subunit III
CP	crude protein
DM	dry matter
DNA	deoxyribonucleic acid
EE	ether extract
ETC	electron transport chain
FCR	feed conversion ratio
FHP	fasting heat production
FI	feed intake
GE	gross energy
HDP	hen day production
HI	heat increment
HU	Haugh unit
MEI	metabolizable energy intake
MEm	maintenance energy requirement
mRNA	messenger RNA
N	nitrogen
ND2	NADH dehydrogenase subunit 2
NDF	neutral detergent fiber

NE	net energy
NEm	net energy requirements for maintenance
NEp	net energy requirements for production
NSP	non-starch polysaccharide
PCR	polymerase chain reaction
PPARG	peroxisome proliferator-activated receptor gamma
PRKAG2D	protein kinase AMP-activated non-catalytic subunit gamma 2
RE	retained energy
RNA	ribonucleic acid
RQ	respiratory quotient
SBM	soybean meal
SDHA	succinate dehydrogenase complex flavoprotein subunit A
TDN	total digestible nutrients
THP	total heat production
TME	true metabolizable energy
UQCRFS1	ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1

List of publications

Manuscripts published and/or submitted for publication

Hiep, TD., PW. Hunt, N. Sharma, R A. Swick, S. **Barzegar**, B. Hine, J. McNally, and Isabelle Ruhnke. 2018. Analysis of antibody levels in egg yolk for detection of exposure to *Ascaridia galli* parasites in commercial laying hens. *Poultry Science*, https://doi.org/10.3382/ps/pey383.

Barzegar, S., S.-B. Wu, M. Choct, and R. Swick. 2019. Energy metabolism and factors affecting net energy evaluation in chicken feed. *Poultry Science* (To be submitted).

Barzegar, S., S.-B. Wu, J. Noblet, and R. Swick. 2019. Metabolizable energy of corn, soybean meal and wheat for laying hens. *Poultry Science* (Submitted).

Barzegar, S., S.-B. Wu, J. Noblet, M. Choct, and R. Swick. 2019. Energy efficiency and net energy prediction of feed in laying hens. *Poultry Science* (To be submitted).

Barzegar, S., S.-B. Wu, M. Choct, and R. Swick. 2019. Implementation of net energy evaluating system in laying hens: Validation by performance and egg quality. *Poultry Science* (To be submitted).

Barzegar, S., R. Swick, S. Kheravii, and S.-B. Wu. 2019. Peroxisome proliferator-activated receptor gamma (PPARG) upregulation in laying hens fed different NE/AME diets. *Poultry Science* (To be submitted).

Conference proceedings

Barzegar, S., S.-B. Wu, and R. Swick. 2017. Metabolizable energy of ingredients in peak layers. *Proceedings of the 28th Australian Poultry Science Symposium, Sydney, Australia.*

Barzegar, S., S.-B. Wu, and R. Swick. 2017. Validation of predicted net energy ingredient values for laying hens. *Proceeding of Recent Advances in Animal Nutrition, Armidale, Australia.*

Barzegar, S., S.-B. Wu, and R. Swick. 2018. Performance and egg quality of layers fed diets with low and high net energy: metabolizable energy ratio. *Proceedings of the 29th Australian Poultry Science Symposium, Sydney, Australia.*

Barzegar, S., S.-B. Wu, and R. Swick. 2018. Implementation of net energy formulation system for laying hen feed. *The Poultry Information Exchange (PIX) Conference, Queensland, Australia.*

Barzegar, S., S.-B. Wu, and R. Swick. 2018. Implementation of net energy evaluating system in layer hens: Validation by performance and egg quality. *Proceedings of 2018 Poultry Science Association Annual Meeting. Texas, USA. Poult. Sci 97(E-suppl. 1): 47*

Chapter 1. General introduction

Metabolism refers to the assimilation of absorbed nutrients for use within the body. Energy metabolism is integrated with other metabolic processes such as feed and nutrient uptake, digestion, absorption and excretion of waste compounds. These processes result in producing energy for fundamental body functions such as thermoregulation and maintenance or for body weight gain and production purposes. Evaluating feedstuffs by different energy systems enable nutritionist to feed animals according to nutrient requirements and so they perform according to their genetic potential.

The AMEn system used in poultry has widespread use globally as it is a simple and reliable method to measure the absorbed energy of ingredients. However, it does not explain energy partitioning in the body for different functions and activities. The NE takes AMEn a step further and incorporates the energy loss as heat increment (HI). The relationship is described as NE = AME - HI which was introduced by Armsby and Fries (1915). Heat increment of the feed is truly waste energy and can be regarded as a "direct tax" on the feed energy. Considerable effort has been devoted to establishing the relationship between gas exchange and heat production. The equation developed by Brouwer (1965) has enabled researchers to calculate total heat production in respiratory chambers using CO_2 production and O_2 consumption data in indirect calorimetry. To calculate heat increment or wasted heat, the heat required to keep the body at optimum thermoneutral temperatures must be subtracted from total heat production. A more accurate measurement of the amount of energy available for productive purposes is given by the NE system (Noblet et al., 2010a). De Groote (1974) compared NE and ME systems for feedstuff energy evaluation in broilers and reported improved feed efficiency (feed/gain) using the NE system compared to the AME system. Pirgozliev and Rose (1999) evaluated 40 different feedstuffs with a wide range of AME contents and NE contents using predicted NE (Fraps, 1946). They reported that NE values gave an improved evaluation of utilizable energy for feedstuffs compared to the AME system, as the latter overestimated the net energy values for production (NEp) in high protein animal by-products feedstuffs compared to cereals, cereal by-products and high-protein vegetable feeds in broilers. The NE/AME (the efficiency of AME for NE) of protein was reported to be lower than EE in broilers as 50 vs 85% (Wu et al., 2019) and 68 vs 85% (Carré et al., 2014) and pigs (60 vs 90%) (Noblet et al., 2010a); therefore, all these studies justify the NE system application for poultry feed evaluation. Prediction of diets NE content from their digestible nutrient contents or from dietary compositions and AME

content was performed in growing pigs (Noblet et al., 1994). Using close-circuit calorimetry chambers recently enabled researchers to predict dietary NE from their AME and nutrient contents in broilers diets which were applicable to estimate the NE value of ingredients (Wu et al., 2019). Attempts have been made to assess the net energy and energy efficiency in layer feed (Farrell, 1975; Reid et al., 1978; Sakomura et al., 2005a). However, further studies and application of the data in practice have been scarce, probably due to, at least partially, the methodology and variations of the measurements.

The major parts of this thesis were:

- Bioassay measurement of the AME, AMEn, and AMEs values of common ingredients in layers at production,
- Measuring the gas exchange and heat production of different feeds with various dietary nutrients in indirect calorimetry method to propose the NE prediction equation to generate an NE database for layers industry,
- Validating of the NE prediction equation to estimate the dietary NE in calorimetry chambers,
- Validation of the NE system in small and big scale production experiments to verify the effect of NE system formulation on the birds performance, egg quality parameters and economic criteria in short- and long-term period,
- Investigate the effect of dietary NE/AME ratio on the expression of genes involved in energy metabolism at the mitochondrial level in the cell.

Chapter 2. Energy metabolism and factors affecting net energy evaluation

2.1. Energy evaluation systems in poultry

Nutrients such as proteins, carbohydrates, and fats when assimilated yield energy which is vital to body function. The main system of ingredient energy evaluation that has been used for decades is AME where available energy is calculated as gross energy ingested minus energy excreted in the faeces and urine. The final value is often corrected to zero nitrogen retention to allow its use in both growing and adult birds (Bourdillon et al., 1990a). The TME system was proposed by researchers to include endogenous energy losses in the calculation (McNab and Fisher, 1981). This was a rapid test taking around 24 hours with small amounts of feed or single ingredients given to fasted adult birds. In most areas, the AME system is deemed as the most practical energy evaluation system in poultry. Large amounts of AME data are available for various feedstuffs as well as predicted energy values based on simple chemical components. AME values of feed can be measured using birds at various ages, including adult birds.

It has been well-documented that adult birds utilize the energy of feedstuffs to a greater degree with less variation than growing broilers (Garnsworthy et al., 2000; Svihus and Gullord, 2002; Cozannet et al., 2010a). As different bird breeds (meat or egg producers) and ages differ physiologically in their digestion and absorption of nutrients, different energy values may be obtained from ingredients (Begin, 1967; Pym and Farrell, 1977; Lopez and Leeson, 2005; Cozannet et al., 2010b). In addition, AME values depend on the composition and form of the test diet (Nitsan et al., 1997; Noblet et al., 2010b). For instance, AME values obtained for high-fat ingredients are often underestimated and those for high-protein ingredients are typically overestimated in the AME system (De Groote, 1974; Carré et al., 2014).

AME values reported are most often corrected to either zero nitrogen retained in the body for AMEn or AMEs (retention of N equal to 50% of nitrogen intake). The argument is to make AME values more consistent across various bird types (Mollah et al., 1983; Hätel, 1986; Bourdillon et al., 1990b; Farrell et al., 1997) and different ages (Lopez and Leeson, 2008a).

Although the AME system is simple to use and is the current default system for energy measurement in poultry, it is by no means an accurate or indeed a comprehensive system that accounts for energy partitioning in the body for maintenance, production (meat, egg) and heat

increment (HI). Such a system does exist, which is the NE system (Noblet et al., 2010b). This system considers energy for maintenance, as well as HI, being the energy wasted as heat. De Groote (1974) compared NE and AME systems for feedstuff energy evaluation in broilers and reported better feed efficiency when using the NE system. Thus, it appears to indicate that taking heat loss into feed energy evaluation could prove to be economically advantageous. Pirgozliev and Rose (1999) evaluated 40 different feedstuffs with a wide range of AME contents and NE contents using predicted NE (Fraps, 1946). They reported that NE values gave an improved evaluation of utilizable energy for feedstuffs compared to the AME system, as the latter overestimated the net energy values for production (NEp) in high protein feedstuffs of animal origin compared to cereals, cereal by-products, and high-protein vegetable ingredients. Therefore, they proposed NE to be more predictable from a chemical analysis of feed, provided that digestibility coefficients for protein, fat, and carbohydrate are known for the feedstuff. Despite this, there have been criticisms against the NE system as a useable method. The first and foremost issue is the tedious nature of the NE system and the difficult in measuring, let alone tabulating, HI values for individual ingredients. Its accuracy also depends on highly experienced operators and flawless equipment. For instance, De Lange and Birkett (2005) lamented that NE was unable to estimate the energy requirements for maintenance (NEm) and production (NEp) because of inaccuracy in methodology for HP calculations in indirect calorimetry; further, the NEp of different body tissue stores cannot be precisely explained by the NE system.

Emmans (1994) proposed an alternative evaluation system called the effective energy model. In this system, the effective energy of a feed or feedstuff is estimated from AME, digestible crude protein, digestible fat and faecal organic matter. This system is similar to NE, and heat production can be calculated as the difference between ME and effective energy intake. However, the application of effective energy values is debatable. The Emmans Model assumed that the higher faecal organic matter (undigested organic matters) increase the heat increment of feeding resulting in decreased NE of diets. According to the effective energy model, the high-fiber content diets should be able to decrease the NE of diet as dietary fiber is not a well-digested nutrient in poultry. However, later researches confirmed that dietary fiber content had no significant effect on HP, HI and NE/ME in broilers (Noblet et al., 2010b; Carré et al., 2014).

Moreover, both the effective energy and AME systems involve corrections which may underestimate energy values of feedstuffs; for example, AMEn of ingredients with high crude protein level is underestimated due to the correction to zero N retention.

2.2. Energy partitioning in the body - maintenance requirements

The AME value is obtained by subtracting urinary and faecal energy losses, usually determined as excreta energy as poultry species void their urine and faeces together, from the total or the gross energy (Figure 2.1). The NE value can be calculated by deducting heat loss or heat increment from the AME value. The NE value represents the energy available for maintenance, growth, and production. Further removal of maintenance energy results in NEp. Total heat production is made up of heat produced for maintenance or fasting heat production (FHP) and HI. The latter includes the thermic effect of diet and heat production associated with the activity (AHP) as a normal level of animal physical activity (van Milgen et al., 1997; Noblet et al., 2010b) .

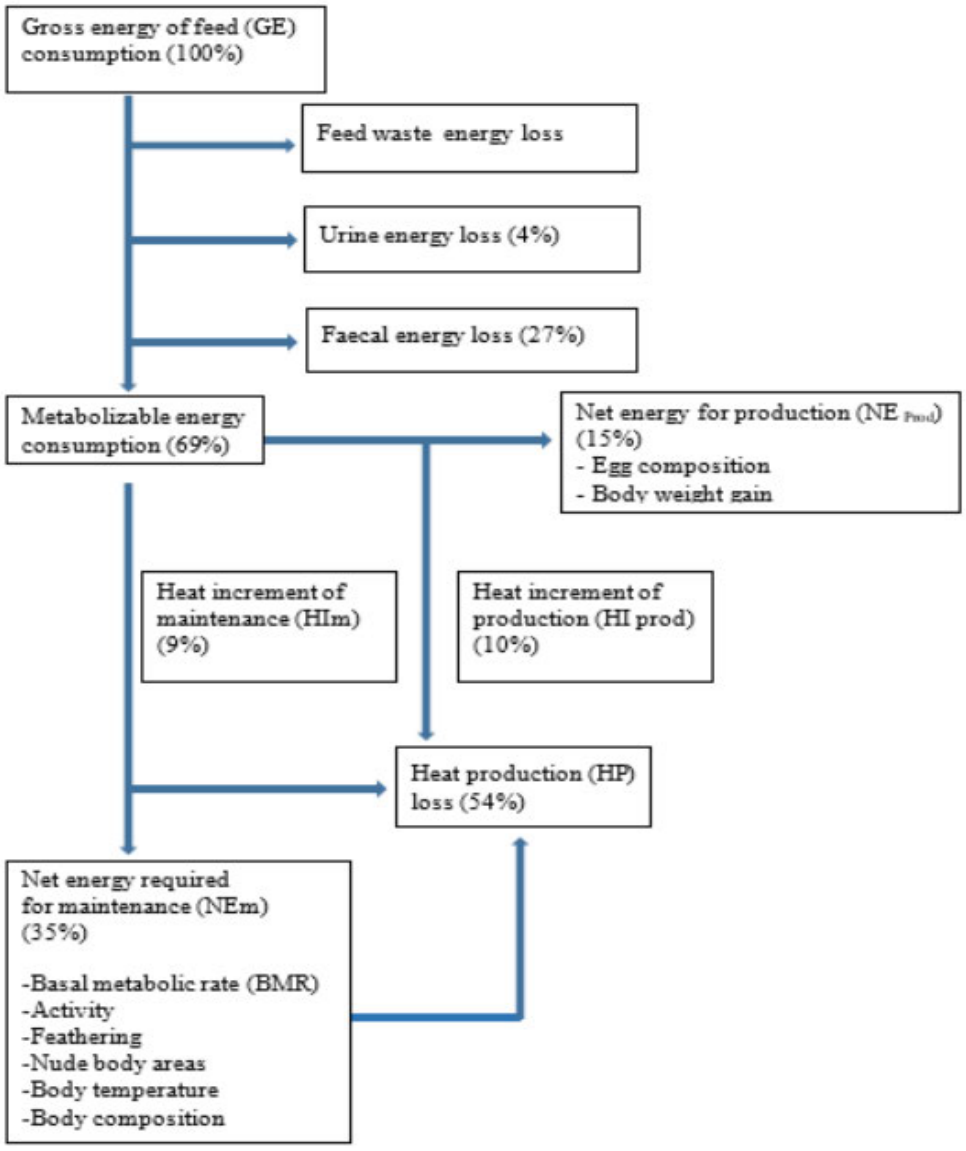

Figure 2-1 Gross energy partitions in laying hens (Luiting, 1990)

2.3. Total heat production

Total heat production accounts for approximately 50% of ME intake in broilers (van Milgen et al., 2001) with reported values of 54% for layers (Luiting, 1990). THP can be measured by either calorimetric methods or by the comparative slaughter method. Calorimetry methods, either direct or indirect, measure THP with which retained energy (RE) can be calculated by ME intake subtracting THP (McDonald et al., 2011).

Direct calorimetry measures the heat produced by the animal for 24 hours or more, assuming that the amount of dissipated heat is equal to the quantity produced. The indirect calorimetry method is commonly used for measuring heat production using respiration chambers. Oxidation of carbohydrates, fats, and proteins during metabolism leads to the production of metabolites and heat energy. The average heat of combustion of protein when completely oxidized is 22.2 kJ/g. The oxidization of glucose and tripalmitin as a typical carbohydrate and fat source produces the following values of energy, respectively:

$$C_6H_{12}O_6 + 6O_2 \rightleftharpoons 6CO_2 + 6H_2O + 2820\ kJ$$

$$C_3H_5(COO\text{-}C_{15}H_{31})_3 + 72.5O_2 \rightleftharpoons 51CO_2 + 49H_2O + 3202\ kJ$$

The modified Brouwer equation (given the loss of CH_4 and N being ignorable in poultry) can be used to predict heat production from the gaseous exchange in calorimetry (Brouwer, 1965; McLean, 1972) as THP (kcal) = (3.866 ×liter of O_2 consumed) + (1.200 ×liter of CO_2 expired)

Other techniques, such as the comparative slaughter technique, calculate THP from measured RE in body tissues. The comparative slaughter method was introduced by Fraps (1946). In this approach, the birds are divided into two groups, and the first group is slaughtered at the beginning of the experiment and their body energy content is measured by bomb calorimetry. The second group is slaughtered at the end and their body energy content is measured. The difference between the initial and final body energy content is used to calculate the retained energy (McDonald et al., 2011). This method is time-consuming as it involves serial slaughter and measurement of birds. In addition, the sampled birds at the beginning of the measurement must be representative of the birds used for the final measurement.

2.4. Respiratory quotient (RQ)

The respiratory quotient (RQ) is the ratio between the volume of carbon dioxide produced by an animal and the volume of oxygen it used. RQ changes with diet composition, metabolic rate, physiological status and feed intake (van Ouverkerk and Pedersen, 1994). RQ for oxidization of carbohydrates, protein, and fat is 1.0, 0.74, and 0.70, respectively, in uricotelic species (McLean et al., 1987; Walsberg and Wolf, 1995). Utilization of protein as a source of energy involves more complex metabolic pathways and a higher metabolic rate. Dietary composition in terms of carbohydrates, protein, and fat affects the RQ of growing broilers with a tendency for lower values with diets both higher in fat and protein but higher values when carbohydrates are supplied as the main ingredients of the diets (MacLeod, 1990). The RQ was higher in broilers fed low-protein diets, as this group mostly retained energy as fat with more efficient fatty acids synthesis (lipogenesis) compared to low-fat counterparts fed isoenergetic diets (Swennen et al., 2004). Furthermore, the utilization of body resources for providing energy affects RQ such as in the state of controlled feeding or fasting, fat or protein is used as energy and thus lower RQ is expected. On the other hand, lipogenesis, the conversion of carbohydrates to fats in some pathways, results in increased RQ. Conversely, the conversion of fat to carbohydrate decreased RQ to values less than 0.70 in humans (Blaxter, 1989). When birds synthesize fat as a reserve, RQ values become higher than 1 (King, 1957; Blaxter, 1989). During periods of starvation, body oxidation patterns change such that more fat than protein is catabolized (Chwalibog et al., 2004). Fasted laying hens showed lower RQ in the last day of a 3-day calorimetry measurement, implying that the birds were oxidizing more body fat reserves to meet energy requirements as starvation continued (Ning et al., 2014).

Recent findings confirmed that RQ can be affected by both dietary nutrients (as an energy source) and lipogenesis. RQ tends to be as low as 0.70 if dietary fat is used for energy, however, due to de novo lipogenesis, RQ values can be much higher than 1 as, in this pathway, no O_2 is consumed and only CO_2 released (Rivera-Torres et al., 2010). Researchers showed that de novo lipogenesis accounted for 60% of total lipid retention and increased as birds grew older. RQ is influenced by the level of feed intake. Increased level of feed intake resulted in higher THP and RQ in pigs (Noblet et al., 1994).

Since the body changes its metabolic patterns for energy utilization at maturity, RQ values can be affected by bird age. Choct (2004) showed RQ values greater than 1 with less variability in growing broilers compared to 56 week-old layers fed the same ingredients. Chepete et al.

(2004) found an average RQ of 0.94 and 0.91 for Hy-Line W-36 pullets and layers, respectively. Broiler breeder hens showed the lowest RQ values at 43 weeks of age compared to the beginning of egg production (Caldas et al., 2018). Those researchers assumed that at 43 weeks of age, birds oxidize fat or protein to meet the energy requirements compared to the beginning of production which is mostly dependant on carbohydrates. Broiler breeders use glucose for egg lipogenesis at the beginning of production but mostly utilize dietary fat for egg lipid synthesis at the end of production (Salas et al., 2016).

The mode of CO_2 excretion from the body affects gas measurement in calorimetry chambers and thus RQ values in laying hens. Walsberg and Wolf (1995) reported RQ values of less than 0.71 during fasting may be due to incomplete oxidation of fat and non-pulmonary loss of CO_2 through non-respiratory sinks for CO_2 or excretion as bicarbonate ions. Therefore, CO_2 excretion varies in layers at the different level of egg production.

2.5. Factors affecting heat production

It has been well-documented that animal THP varies owing to different factors. Energy intake is positively correlated to heat production (Chudy et al., 2003; Ning et al., 2013; Ning et al., 2014) as more feed intake increases metabolic rate and thus releases more heat. Feeding diets with different nutrient composition change THP; dietary protein and amino acids contents are more important than other dietary nutrients. MacLeod (1997) found that increased lysine intake enhanced protein retention in the body and this resulted in higher heat production in broilers while providing diets with imbalanced or excess amino acids levels had no effect on heat production.

Utilization of ME in different body tissues alters heat production with different anabolic pathways. Protein synthesis, excreting the nitrogen compounds from body and protein turnover requires more energy compared to anabolism of other tissue components (Latshaw and Moritz, 2009). Synthesis of each gram protein requires 380% more oxygen compared to the synthesis of each gram fat (Teeter et al., 1996). In the same way, broiler breeder hens produced more heat at the end of the production period as they retain dietary energy in muscles and catabolize fat to meet energy requirements (Caldas et al., 2018).

Enzyme application, particularly carbohydrases, improves nutrient digestibility and energy metabolism as this hydrolyzes polysaccharides to release encapsulated starch and protein in feedstuffs. Therefore, it reduces THP and increases NE of diets (Choct et al., 2010; Nian et

al., 2011). Enzymes reduce the weight of gastrointestinal tract which accounts for more than 50% of HI and maintenance energy requirement (MEm) (Nian et al., 2011). Barekatain et al. (2014) reported enzyme supplementation (xylanase, protease, amylase, and glucanase) increased NE, NE intake and RE, but did not change THP in broilers.

Photoperiod affects metabolic rate, and accordingly, THP will change based on the diurnal patterns and physical activity. Physical activity accounts for 20-25% of total heat production in laying hens (MacLeod et al., 1982; Boshouwers and Nicaise, 1985). Physical activity accounts for about 10% of ME intake in different species such as growing broilers, pigs, and calves (Noblet et al., 2010b). The THP was significantly decreased in the dark period compared to the light period as physical activity was higher when lights were on (Chepete et al., 2004). The same research showed higher values of THP for modern pullets and layers compared to those reported some years ago, reflecting genetic improvements. Lighting period and intensity changed heat production and physical activity in laying hens (Li et al., 1992; Ning et al., 2014). This is an important point for poultry production, as different lighting schedules might affect THP and NE of layers fed the same diets. THP decreases after oviposition, as the hen loses some weight with less energy required for keeping the egg warm within the same temperature range of the body tissues (Pesti et al., 1990).

Diseases such as necrotic enteritis affects metabolism, energy balance and nutrient digestion. Broilers challenged with necrotic enteritis showed lowered feed intake and lower energy intake, RQ and NE. Infected birds had decreased body temperature and THP probably as a consequence of hypothyroidism (M'Sadeq et al., 2015).

2.6. Fasting heat production

Basal metabolic rate (BMR) is heat produced in fasted animals estimated by measuring FHP and adjusting to zero activity (Noblet et al., 2015a). Also as an alternative, FHP and MEm can be extrapolated from regression estimation of different ME intakes on different THP measurements (Birkett and de Lange, 2001; Noblet et al., 2010b; Ning et al., 2013). Fasting heat production should be measured at the thermoneutral zone or the environmental temperature where the animal produces a constant minimal level of heat loss to maintain body temperature in homoeothermic animals (Arieli et al., 1980). Increased levels of ambient temperature from thermoneutral decreased THP and MEm (Vohra et al., 1975; Chudy et al., 2003). FHP accounts for 80% of MEm variations; therefore, the factors affecting FHP might

affect MEm as well (Vohra et al., 1975). Likewise, the main source of variation in THP is due to variation in MEm and is affected by physical activity, feathering, FHP, body composition and temperature (Luiting, 1990).

The FHP is assumed to be an allometric function of body weight. The fasting metabolic rate was defined as FHP = a × BW^b, where ***a*** is some constant number of kcal, BW is body weight (kg), and ***b*** is the power that correlates bodyweight to surface area (Vohra et al., 1975). Researchers applied regression estimations to find the correct power to express metabolic BW (Close et al., 1973; Bikker, 1994; Noblet et al., 1994). The power of 0.75 for metabolic body weight was originally stated by Kleiber (1947) for a wide range of animals, although Noblet et al. (2015a) recommended 0.70 for growing broilers. Lopez and Leeson (2005) reported that applying the power of 0.75 underestimated MEm estimations of smaller and younger broilers, and 0.60 is more accurate for these birds. Since MEm accounts for 42-44% (large portion) of ME intake (Lopez and Leeson, 2005), an accurate estimation of MEm is necessary for correct calculations for production requirements.

The ME intake alters FHP and the efficiency of ME intake for MEm. Higher ME intake showed higher FHP compared to lower ME intake; in addition, starvation decreased THP and FHP during a three-day calorimetry measurement, and the more elongated the starvation the less the contribution of feed to heat production (Ning et al., 2013). The efficiency of ME intake for MEm was higher (0.67-0.80%) when feed intake met maintenance requirements compared to lower values (0.57-0.69) when consumption was higher than maintenance requirements (Sakomura, 2004). MacLeod (1990) reported dietary composition had no effect on MEm and FHP in growing broilers.

As birds age their maintenance requirement changes (Sakomura, 2004). The composition of body weight gain in different ages affects MEm (Sakomura et al., 2005b). Growing birds require higher MEm than adult birds. Mature birds tend to deposit energy mostly as fat resulting in lower MEm. However, growing birds use dietary energy for protein synthesis. Protein synthesis requires complicated metabolic pathways and embraces higher energy cost for the body (Blaxter, 1989).

Different bird types varied in FHP and MEm requirements. The MEm of broilers was reported to be 594-618 kJ/$BW^{0.75}$/day (Liu et al., 2017); however, values for laying hen strains were 469 -502 kJ/$BW^{0.75}$/day (Jadhao et al., 1999; Sakomura, 2004). FHP values obtained in broilers

were 386 - 404 kJ/$BW^{0.75}$/day (Liu et al., 2017) and higher than values for layers at 370 - 395 kJ/$BW^{0.75}$/day (Farrell, 1975; Wu et al., 2016).

FHP and MEm vary by gender. MacLeod et al. (1979) found 30% lower MEm in cockerels compared to hens fed the same diet, indicating cockerels to have a lower metabolic rate. O'Neill and Jackson (1974) reported higher FHP (404 - 464) (kJ/kg, $BW^{0.75}$/d) for hens compared to lower values in cockerels (223-349) (kJ/kg, $BW^{0.75}$/d).

MEm is not a constant value and varies with ambient temperature. MEm of different bird strains increased with decreasing environmental temperature (Sakomura, 2004). MEm of broilers was negatively correlated by the quadratic effect of ambient temperature (Sakomura et al., 2005b). Birds have to produce heat when housed below the thermoneutral zone limits and, conversely, for the temperatures above that zone, they need to dissipate heat in order to maintain body temperature (Leeson and Summers, 1997). The environmental effect of energy requirements can be discussed from endocrinology point of view. Thyroid hormones play a pivotal role in body temperature homeostasis. Both ambient temperature and feed intake affect thyroid hormone production. For example, fasted hens after 4.5 hours had lower triiodothyronine (T_3) (May, 1978) and less THP (Klandorf et al., 1981). Increased ambient temperatures beyond thermoneutral decreased T_3 level in plasma and heat production as the latter is predominantly controlled by T_3 hormone and not by thyroxine (T_4) in laying hens (Klandorf et al., 1981).

MEm requirements vary with body feather cover, for similar reasons. Layers housed at a thermoneutral zone with no feather coverage required 38% more MEm than their peers kept on the same environmental conditions with 100% feather coverage (Peguri and Coon, 1993). Accordingly, birds with poor plumage conditions were found to be more resistant to heat stress than birds with normal feather coverage as the former was able to dissipate heat more easily (Balnave, 2004).

AHP and MEm requirements change by different housing conditions. AHP accounts for 20-25% of THP variations or 8-10% of MEm requirements (van Milgen et al., 2001). Broiler breeder hens reared on the ground produced more THP, lost more energy as AHP and required 20% higher MEm compared to those kept in the cages (Sakomura, 2004).

Lighting program changed AHP and THP of birds as activity level and THP decreased during the dark period (Ning et al., 2014). Birds are also more active under a continuous lighting program. Ohtani and Leeson (2000) observed that THP of broilers reared under an intermittent

lighting program was higher than of those under continuous lighting which might be because of higher activity rate of birds with continuous light.

The diurnal pattern of FHP and THP will change as the birds experience different metabolic status during the day. Damme et al. (1987) found an increased metabolic rate in hens just before oviposition, which resulted in the increased level of FHP and THP followed by a sharp fall to the resting level after oviposition.

2.7. Heat increment and net energy

The proportion of gross energy lost via excreta approximately 30%, that is to say, about 70% of gross energy of a common diet fed to poultry is metabolized. From AME to NE, the amount of energy lost as heat is approximately 75% for most common ingredients fed to poultry. This means that 25% of ME is lost as heat during the digestive and metabolic utilization of energy. Indeed, the extensive work by Wu et al. (2019) reports a thermic effect of feed accounting for 26% of ME intake, although others have reported values of 20-23% in broilers (Swennen et al., 2004).

Diet composition was reported to have no effect on NE/AME or HI in broilers (Noblet et al., 2003; Noblet et al., 2010b). Using diets with different nutrient composition resulted in low variation in NE/AME and did not affect HI (Farrell, 1976; Carré et al., 2014; Carre and Juin, 2015). Low variation of NE/AME might be attributed to the low digestibility of fiber in poultry (Carré et al., 2014). Conversely, a well-digested dietary fiber fraction was mentioned as an important source of NE/AME variation in pigs (Noblet et al., 1994). Dietary amino acids had no effect on NE/AME as diets containing high amino acid concentrations showed the same NE/AME compared to those with a low amino acid concentrations (Carré et al., 2013). Diet composition also can alter the expression of genes which are involved in metabolism of enery (lipogenesis) in mitochondria. Dietary fats are important modulators of PPARG (Peroxisome proliferator-activated receptor gamma) and this may relate to the regulation of energy balance (Cecil et al., 2006). Kliewer et al. (1997) suggested that PPAR α and γ are physiological sensors for lipid homeostasis which can be triggered by dietary fatty acids.

The efficiency of the use of different nutrients as sources of energy varies. For example, when protein is used as an energy source, NE/AME is lower than when fat or carbohydrates are used as energy sources (Blaxter, 1989). An increase in HI with elevated protein levels might be due to two main reasons. Firstly, the catabolism of protein leads to nitrogenous wastes that require

energy to be excreted. Secondly, dietary protein stimulates protein turnover in the body and this needs the energy to fuel corresponding metabolic pathways (Musharaf and Latshaw, 1999). For the same reason, under high ambient temperatures, lowering dietary protein and increasing fat inclusion in feed has been adopted as a strategy for reducing heat stress (Lin et al., 2006). Noblet et al. (2010b) reported utilization of NE/AME between 65-85% for protein and fat in poultry. NE/AME ratios were 84, 78, and 68%, respectively, when broiler chickens used fat, carbohydrates or protein as a source of energy (Carré et al., 2002).

Feed and ME intake also alter NE/AME efficiency and HI. Liu et al. (2017) reported higher levels of feed intake in broilers lowered NE/AME. They stated that while feed intake increased, the proportion of ME used for HI increased and resulted in less NE and a lower NE/AME ratio. In addition, laying hens with access to *ad libitum* feed showed higher HI and lower NE/ME than the feed-restricted hens (MacLeod et al., 1979),

The utilization of AME for NE depends on the purpose for which the energy is retained. Utilization of AME for fat retention is more efficient compared to that for protein retention (Farrell, 1975; Noblet et al., 1999). Utilization of AME for NEp produces more heat than that for NEm. Blaxter (1989) showed that different energy sources as protein, carbohydrates or fat used for maintenance showed 20% higher efficiency; similarly, HI was less when the nutrients were used for maintenance than for growth and production. Feeding animals to the level of maintenance produces heat that is used for basal heat requirements of body (thermostasis) and spares dietary energy to be used for FHP; however, feeding above maintenance requirements, produces heat that is surplus to basal requirements and hence it is wasted, leading to decreased energy efficiency (Musharaf and Latshaw, 1999).

2.8. Energy partitioning in the body: growth and production

Partitioning of ME as MEm and retained energy in body (either fat or protein) is based on $MEI = MEm + (1/K_f \times RE_f) + (1/K_p \times RE_p)$ (Kielanowski, 1965), where MEm is ME for maintenance as a function of body weight, and K_f and K_p efficiencies of utilization of ME for fat and protein retention, respectively. The values for K_f (86%) and K_p (66%) have been estimated using statistical models with different feed restriction levels applied in broilers (Boekholt et al., 1994).

Utilization of energy for protein deposition (NEp/ME) is assumed to be lower than for fat deposition. Energy utilization above maintenance requirements was reported to be 51% for protein and 78% for fat retention in broilers (Petersen, 1970) and 51% for protein and 96% for fat retention in different layer strains (Farrell, 1975). More recently, it was estimated that the efficiency of retained energy was 86% for egg protein and 100% for egg fat in layers using the comparative slaughter technique (Jadhao et al., 1999). The modified efficiency values were reported as 66% for protein accretion and 86% for fat accretion in broilers (Lopez and Leeson, 2005); however, genetic selection is changing the pattern of retained energy over time to meet the goal of producing more lean meat in broilers. Consequently, the recently reported values for the efficiency of total energy retention of 51 and 49% for protein and fat retention, respectively, in broilers compared to values of 62 and 38% in layers reflect different genetics (Lopez and Leeson, 2008b).

Examining retained energy as a balance of ME intake and heat production in the body, the amount retained as NE per unit of product (egg) and body weight gain in layers can be ascertained. The efficiency of ME for deposition in the egg was lower than for growth (62 vs 65%) in layer hens, whilst those correspondent values were 64 and 47% in broiler breeder hens (Rabello, 2001; Sakomura, 2004). The egg energy content remained constant while ME intake increased in hens (Chudy et al., 2003).

Energy retention in the body depends on the bird energy balance. Layers do not retain energy as fat unless they are in positive energy balance; nonetheless, they can retain energy as protein and eggs or body tissues regardless of body energy balance status (Farrell, 1975). When energy is provided only to meet MEm requirements, energy is totally retained as protein without any fat deposits in broilers (Boekholt et al., 1994). Also in layers the efficiency of ME for egg production is higher when body tissues are used for egg production compared to utilizing a dietary energy source for egg production (Blaxter, 1989).

Bird age affects body composition and energy retention. Broilers are leaner at a young age and thus the proportion of energy intake deposited as protein is more efficient than fat deposition. Protein retention accounted for 23-30% of body weight gain during the first few weeks of age in turkeys, although fat was deposited to a greater extent than protein in older turkeys (Rivera-Torres et al., 2010). The same pattern of age and energy retention was observed during the laying period of hens. Caldas et al. (2018) observed a negative correlation between protein and energy retention in broiler breeder hens. Lean body mass decreased from peak production until

50 weeks of age with the opposite trend for fat mass, which soared after 50 weeks of age. At the beginning of the production period, birds sacrificed muscle protein to maintain egg production, but increased their retained energy as abdominal fat when they get older and towards the end of production.

Diet composition can change energy and protein metabolism in birds. Broilers fed low-protein diets consumed more energy from isoenergetic diets with an increased level of THP and more retained energy as fat compared to their counterparts fed low-fat diets (Swennen et al., 2004). Low-protein diets are formulated with a higher level of fat and the extra caloric effect of fat might be a reason for better-retained energy as fat. Extra caloric effect of fats is the synergistic function of fat supplementation on the enhancement of digestibility and energy utilization of other nutrients (non-lipid portion) in the diet. It is perhaps also for the fact that the utilization of fat produces less heat and hence more NE, accentuating the amount of energy available for production. Furthermore, low-protein fed broilers showed a higher propensity for energy retention as protein in this research.

Different bird strains have different body composition that may affect growth and energy utilization. During the first six weeks of life, layer chickens showed a sharp increase in the conversion of energy into protein deposition compared to broilers. Broilers undergo some physiological changes like feather replacement, which requires protein and influences body retention pattern during the first weeks of their life (Lopez and Leeson, 2005). Different broiler lines showed different responses to energy retention. Lean broiler lines showed higher retention for protein compared to fat retention; contrarily, the fat-line counterparts degraded higher amount of dietary amino acids resulted in higher uric acid excretion and lower potential for protein retention. Both genetic lines showed the same ME intake, THP, HI and MEm (Geraert et al., 1988; Geraert et al., 1990).

2.9. Comparison of energy evaluation systems for energy partitioning

Different feed energy systems can be compared if the measurements performed are under standardized conditions, i.e. with the same genotype, sex, age, housing, and environmental temperatures. Furthermore, energy partitioning should be expressed on metabolic body size as opposed to body weight. The exponent used to convert body weight to body size should be constant as the use of different exponents to compare results may result in different MEm and

energy partitions values. Estimation of MEm when body weight raised to the exponent 0.60 is more accurate (in particular for younger and smaller birds) with less residual variance compared to the estimations using 0.75 (Lopez and Leeson, 2008b).

Initially, Fraps (1946) used 62 individual feedstuffs to determine their productive energy by comparing the comparative slaughter technique in growing chickens. Results showed that NEp from high protein feedstuffs (e.g. animal sources) was lower than those originating from high starch ingredients (e.g. cereal sources); consequently, the ratio of protein or starch content of a feedstuff changed the utilization of ME. Furthermore, the NEp took into account the total amount of fat and protein retention without any differentiation. This method for the calculation of NEp is arduous and time-consuming and changes with individual circumstances. The advantages of utilizing indirect calorimetry include rapid determination of heat production and shorter restricted feeding times; thus, basal metabolism is less affected. Indirect calorimetry reduces the errors associated with carcass analysis and shortens the total experimental period (Farrell, 1974). Comparing different methods of NE measurements under the same situations, the reported values of energy partitions were different. The THP variation (based on metabolic body weight) was 1% when measured by either indirect calorimetry or by the comparative slaughter method (Farrell, 1972). The THP measured by indirect calorimetry was maximum 3% higher ((kcal/bird/day) than that measured by the comparative slaughter method with 10-week old cockerels (Fuller et al., 1983). The ME intake, THP, RE, NE, NE/ME measured by comparative slaughter were the same as measured by indirect calorimetry in broilers (Liu et al., 2017). However, Barekatain et al. (2014) reported that applying the comparative slaughter method resulted in less THP and higher NE, NE/ME and RE compared to indirect calorimetry method. Different equipment used for THP measurements in indirect calorimetry experiments for animals. McLean (1972) found that open circuit calorimetry was useful in measuring THP of ruminants with an accuracy of ± 2%. Open-circuit chambers were not as accurate as closed-circuit chambers, although open-circuit chambers diminished the individual variation in respiratory measurements for layers (Hilliar et al., 2016).

2.10. Conclusion

Currently, the ME system is considered as the default method for feedstuff energy evaluation. However, it lacks the ability to determine available energy for different body functions (partitioning) and can change with different nutrient composition, bird age, and genotype. The NE system gives more accurate energy values compared to ME, although its application is

complicated in practice. The main component of NE calculation in respiratory measurements is heat production on which is dependent the other parameters such as dietary nutrient levels, age, type (broiler or layer), body composition, physiological status, and environmental conditions. The energy partitioning for different metabolic purposes as growth and production in different body tissues (fat or protein) can be defined by the NE system. Using the NE system with more accurate estimation of the energy value and NE/ME of dietary nutrients and ingredients provides new ground for poultry scientists to predict performance objectives of chickens more efficiently. Further research is required to confirm the NE system application for different sources of feedstuffs and its advantages compared to other energy assessment systems. This will enable nutritionists to formulate more effective diets with lower costs.

Chapter 3. Metabolizable energy of corn, soybean meal and wheat for laying hens

3.1. Introduction

Little data is available on ingredient AME and AMEn assay values using commercial laying hens in peak production. Most nutritionists formulating layer feed use energy data obtained from adult cockerels or growing broilers (Janssen, 1989; Bourdillon et al., 1990a). The EU prediction equations are often used in conjunction with NIRS proximate estimates or wet chemistry proximate analysis to predict AMEn of ingredients for formulation (Janssen, 1989). While the prediction and NIRS based methods have the advantage of being cost effective and fast, they may not accurately predict AME and AMEn values for laying hens without accurate bioassay data available. The *in vivo* methods are either based on apparent or true metabolizable energy with or without adjustments made to zero nitrogen retention (Hill and Anderson, 1958) or to a standardized nitrogen retention coefficient (Cozannet et al., 2010a).

The AME assay uses either adult birds or, more frequently, growing broiler chickens with an adaptation feeding period followed by a three to four-day measurement period (Bourdillon et al., 1990a). In the reference diet substitution method, a portion of the reference or basal diet is replaced with test ingredient (usually 30 to 40% for grains and lower for other ingredients) (Bourdillon et al., 1990a). In these methods, the reference and test diets are generally supplemented with minerals and vitamins to ensure the diets are similar and balanced. Failure to take this into account in the calculation may cause erroneous results. Assuming the GE, AME, AMEn, AMEs, and NE of a complete diet consist of energy additively contributed by individual ingredients, the energy value of those ingredients can also be calculated by linear regression equations. This method has been applied in pigs by Noblet et al. (1993) for a set of 13 ingredients and also in broilers by Lopez and Leeson (2008a) for calculating the AME and AMEn values of corn and SBM.

Although the AME classical total collection bioassay using young broilers has become a preferred method for formulating growing broiler feed (Farrell, 1999), the application of those values or values obtained from adult cockerels to laying hens or other bird species is debatable. Poultries from different species, breeds and ages have various abilities to digest and metabolize feed components (Ravindran et al., 2004; Adeola et al., 2018). For instance, the AMEn of wheat

dried distillers grains with solubles (DDGS) showed the highest value in adult cockerels followed by adult layers and growing broilers. The ability of laying hens to digest energy as AMEn was 97% and 93% for diets and DDGS, respectively, compared to adult cockerels (Cozannet et al., 2010a). The structure and function of the gastrointestinal tract likely affect the energy utilization of various classes of birds. The longer intestine and slower passage rate may decrease microbial fermentation and reduce energy utilization in broilers compared to Leghorn layers (Shires et al., 1987). Other work found layers utilize more AMEn from corn, SBM, and wheat bran compared to broiler strains (Pishnamazi et al., 2005). Thus, the potential of the broiler to utilize dietary energy is less than layers (Lopez and Leeson, 2005). The objective of this study was to measure the metabolizable energy of 3 major ingredients (corn, wheat and soybean meal) by the reference diet substitution and regression methods in laying hens.

3.2. Material and methods

3.2.1. Birds and diets

The study was approved by the Animal Ethics Committee of the University of New England (UNE) and designed to follow the Australian code of practice for the care and use of animals for scientific purposes (NHMRC, 2013). Forty eight Hy-Line Brown hens, 42 weeks of age, laying at 93% hen day production (HDP) were housed two birds per cage. The sloping floor cages were 55 cm by 50 cm, by 50 cm average height and were in an open-sided shed at the University of New England, Australia with 16 h light per day in the spring season; the average temperatures were between 22-24 C. Cages were fitted with individual feeders, nipple drinkers, and 75 × 70 cm dropping collection trays.

Flint corn (Australian origin), SBM (Argentina origin), and hard red wheat (Australian origin) were sourced from the local market. Nutrient composition (%) of the three ingredients is given in Table 3-1. The ingredients and nutrient composition of reference and test diets (fed as mash) are shown in Table 3-2. The reference diet was based on corn, SBM, canola oil and added amino acids (ref diet) and the three experimental diets contained 30% of each test ingredient (as is) and a constant level (65.7%) of ref diet (as is). Supplementary vitamins, minerals (including calcium) and other non-energy ingredients were adjusted to have equivalent inclusion rates across all diets. Each of the 4 diets was fed to six replicate cages for a 7-day adaptation period followed by a 3-day experimental period with feed intake measured and total excreta collected and measured. Birds had *ad libitum* access to water and experimental diets.

Feed spillage was measured from the under cage collection tray and deducted from feed intake. Feathers and down were removed from the collected excreta.

Table 3-1 Composition of ingredients and diets (%, as is) [1].

Nutrients	Ingredients			Reference diet	Test diets		
	Corn	Soybean meal	Wheat		Corn	Soybean meal	Wheat
Dry Matter [2]	88.0	89.9	89.6	89.8	89.5	90.1	90.0
GE, kcal/kg [3]	3920	4189	3918	3641	3601	3634	3602
Crude protein	8.6	47.5	11.2	17.6	14.2	25.7	14.8
Crude fiber	1.7	3.3	2.3	1.8	1.7	2.2	1.9
Ether extract	2.7	1.7	1.4	3.6	3.3	2.9	3.0
Ash	1.5	6.5	1.5	14.3	13.8	15.2	13.7
ADF	3.4	5.1	2.6	3.4	3.2	3.7	3.0
NDF	8.1	8.4	9.8	7.0	7.0	7.1	7.6
Starch	48.9	0.1	63.9	29.6	34.1	19.4	38.6
NSP total	5.7	12	8.1	6.6	6.1	7.9	6.8
NSP soluble	0.3	0.8	1.3	0.4	0.4	0.5	0.3
NSP insoluble	5.4	11.2	6.8	6.2	5.7	7.4	4.1
Calcium	0.03	0.26	0.05	4.1	3.7	5.1	2.7
Avail Phosphorus	0.08	0.23	0.12	0.4	0.4	0.4	0.4
Lysine	0.30	2.90	0.40	0.93	0.73	1.43	0.74
Methionine	0.16	0.60	0.16	0.52	0.48	0.60	0.48
Threonine	0.31	1.90	0.33	0.75	0.62	1.08	0.63
Arginine	0.39	3.40	0.51	1.04	0.80	1.62	0.84
Valine	0.41	2.20	0.50	0.82	0.66	1.21	0.69
Isoleucine	0.32	2.10	0.40	0.68	0.54	1.01	0.57

[1] Measured values.
[2] Measured at time of laboratory analysis.
[3] Abbreviations: GE = gross energy, ADF = acid detergent fiber; NDF = neutral detergent fiber; NSP = non-starch polysaccharide.

Table 3-2 Ingredients composition (%) of the reference and test diets (as is).

Item	Reference diet	Test diets			Ingredient DM (%) [1]
		Corn	SBM	Wheat	
Ingredient, %					
Corn	60.5	39.8	39.8	39.8	89.4
Soybean meal	25.8	16.9	16.9	16.9	90.2
Corn	0.0	30.0	0.0	0.0	89.4
Soybean meal	0.0	0.0	30.0	0.0	90.2
Wheat	0.0	0.0	0.0	30.0	90.5
Canola oil g	1.3	0.8	0.8	0.8	100.0
Limestone	9.7	9.7	9.6	9.6	99.5
Dicalcium phosphate	1.6	1.6	1.6	1.6	98.0
Salt	0.3	0.3	0.3	0.3	99.6
Na bicarbonate	0.2	0.2	0.2	0.2	100.0
Vitamin, mineral premix [2]	0.2	0.2	0.2	0.2	99.0
Choline Cl 60%	0.08	0.08	0.08	0.08	75.0
L-lysine HCl	0.08	0.08	0.08	0.08	99.5
D,L-methionine	0.27	0.27	0.27	0.27	99.5
L-threonine	0.09	0.09	0.09	0.09	99.5
CP (DM)	19.7	15.9	28.4	16.9	
Energy yielding ingredients (%) [3]	86.9	57.4	57.3	57.2	
a (%, DM)[4]	100.0	66.0	65.9	65.8	
b (%, DM)[5]	0.0	29.5	29.7	29.8	

[1] DM measured at time of mixing
[2] Provided per kilogram of diet: vitamin A, 12,000 IU; vitamin D, 3,500 IU; vitamin E, 40 mg; vitamin K, 2 mg; nicotinic acid B3, 50 mg; pantothenic acid B5, 11 mg; folic acid, 1.5 mg; riboflavin B2, 6 mg; vitamin B12, 0.02 mg; biotin, 0.1 mg; pyridoxine B6, 5 mg; thiamine B1, 2 mg; Cu, 8 mg; Co, 0.3; Mo, 1 mg; I, 1 mg; Se, 0.3 mg; Fe, 60 mg; Zn, 60 mg; Mn, 80 mg; Endox (antioxidant), 25 mg.
[3] The total amount of energy-yielding ingredients including grains, SBM, canola oil and amino acids in the reference and test diets (%).
[4] The contributions of energy-yielding ingredients from the reference diet in the test diet.
[5] Substitution level of each ingredient in the test diet (DM basis).

3.3. Measurements and analysis

Feed intake, egg production, and egg weight were measured for each cage. The total excreta voided daily were pooled from each cage and weighed. Multiple subsamples were collected

and homogenized from the total of each cage at the end of the collection period. A 30 g representative sample of excreta was weighed and freeze-dried to a constant weight for gross energy and N analysis. Samples of feed and freeze-dried excreta were finely ground to ensure homogeneity. Approximately 2 g of diet and 3 g of freeze-dried excreta samples were dried in crucibles in a drying oven at 105 °C for 16 hr to determine DM. Excreta DM was calculated by correction for the loss of moisture during both freeze- and oven-drying. The gross energy content of feed and excreta was determined in triplicate 0.5 g samples using an adiabatic bomb calorimeter (IKA Werke, C7000, GMBH, and Co., Staufen, Germany) with benzoic acid as the calibration standard. Ingredient samples were analyzed for GE, CP, EE, crude fiber (CF), ash, neutral detergent fiber (NDF) and acid detergent fiber (ADF) (AOAC, 2016), starch (Megazyme Total Starch Kit, Megazyme International Ireland Ltd., Bray, Ireland), free sugars (mono- and disaccharides) (Annison et al., 1996), total non-starch polysaccharides (NSP), soluble NSP and insoluble NSP (Englyst and Hudson, 1987b, a; Theander and Westerlund, 1993). Nitrogen was measured using a LECO model FP-2000 N analyzer in triplicate on 0.15-g samples of ingredients, diets, and excreta. LECO was calibrated by pure reference EDTA. Birds were fed ad libitum with reference and test diets. Feed intake (g, DM), hen day egg production (%), egg weight (g), egg mass (g) and FCR were measured daily and cumulated over a period of three days.

3.3.1. Calculations

Egg mass was calculated as the product of average egg weight and hen day production. FCR was calculated as the ratio of feed intake to egg mass. Nitrogen intake and nitrogen retained in the body were measured for all the birds fed reference and test diets as g/b/d.

Dietary AME (kcal/kg of DM) values were calculated according to the following equations:

AME (kcal/kg DM of diet) = [(FI × GEf) - (E × GEe)] / FI

AMEn (kcal/kg DM of diet) = [AME - [8.22 × (Ni - Ne)]] / FI

AMEs (kcal/kg DM of diet) = AMEn + 8.22 × Ni% × 10 × 50%

Where GEf is the gross energy of feed (kcal/g DM) and GEe is the gross energy of excreta (kcal/g DM); FI = feed intake (g DM/day/hen); E = excreta output (g DM/day/hen) and 8.22 kcal/g of N as nitrogen correction factor for each gram of N retained in the body and in eggs (Hill and Anderson, 1958); Ni is nitrogen intake from the diet (g/day) and Ne is the nitrogen

output from the excreta (g/day). AMEs is the AME corrected for a retention of N equal to 50% of nitrogen intake; this ratio corresponds to what occurs under practical conditions in laying hens (Cozannet et al., 2010a; Barzegar et al., 2017).

Calculation of test ingredient AME, AMEn, or AMEs according to the reference diet substitution method is as follows:

AME_{ingr} (kcal/kg) = (AME_{test} - AME_{ref} × a%) / b%

Where AME_{ingr} is the AME value of the test ingredient, AME_{test} is the measured AME value of test diet less the AME contributed by the supplemental L-lysine HCl, D,L- methionine and L-threonine and AME_{ref} is the measured AME value of the reference diet less the AME contributed by the supplemented D,L-methionine, L-lysine HCL, and L-threonine; a% is the inclusion level of energy-yielding ingredients from the reference diet in the test diet and b% is the substitution level of the ingredient in the test diet. All the energy-yielding ingredients (including AA's), minerals and vitamins inclusion rates in reference and test diets were converted to DM basis in order to express the a and b values on a DM basis (Table 3-2).

The AME values contributed by supplemental amino acids (D,L-methionine, L-lysine HCL, and L-threonine) added in reference and test diet was considered as GE of these AAs as it has been assumed the digestibility of supplemented AAs is close to 100% (Karakas et al., 2001).

The AMEn contribution of supplemented AAs was calculated from AME by:

$AMEn_{AA}$ (kcal/kg) = AME_{AA} – 8.22 × N% × 10

$AMEn_{AA}$ is the AMEn of the supplemental AA; N% is the percentage of N in the AA which is converted to g/kg by multiplying 10.

The AMEs contribution of supplemented AAs was calculated from AMEn by:

$AMEs_{AA}$ ((kcal/kg) = $AMEn_{AA}$ + 8.22 × N% × 10 × 50%

$AMEs_{AA}$ is the AMEs of the supplemental AA; 50% is the % of N intake that is retained.

3.3.2. Statistical Analysis

Energy values were distributed normally and thus subjected to a one-way ANOVA analysis with a randomized design of treatments using the PROC GLM of SAS (2010) and least squares means option at $P < 0.05$.

PROC REG (SAS, 2010) was used for regressing the determined GE, AME, AMEn and AMEs values (kcal/kg DM) of the reference diet and individual test diet replicates less corresponding supplemental AA contributions on the inclusion rate of reference diet in addition to each ingredient as corn, SBM, and wheat (DM basis) as estimators in all experimental diets.

3.4. Results and discussion

Feed intake and performance measured in the three days of bioassay measurement were not affected ($P > 0.05$) by feeding diets containing different energy values and protein contents (Table 3-3). Feed intake was similar to breeder performance recommendations (Hy-Line, 2016).

The N retention values were 1.28, 1.14, 1.25 and 1.36 (g/b/d) ($P>0.05$) for those birds fed the reference diet and the test diets including additional corn, wheat, and SBM. Therefore, feeding diets with various CP content had no effect on N retention (g/b/d) ($P > 0.05$); the lowest CP and amino acids (0.74% total lysine, for instance) levels in the diets of this study were then sufficient to meet the protein requirements of laying hens and to maintain their production.

Table 3-3 Effect of diet composition on laying hens performance, N balance and diets energy values.

Items	Reference diet	Test diets			RSD	P value
		Corn	SBM	wheat		
Laying performance						
FI, g [1]	90.7	95.2	97.0	96.5	5.2	0.19
HDP, %	55.6	61.1	83.3	56.7	24.1	0.20
Egg weight, g	59.9	59.7	61.1	60.4	5.3	0.97
Egg mass, g	33.2	37.0	51.3	34.6	15.0	0.17
FCR	1.47	1.99	1.03	1.57	0.97	0.42
N balance (g/b/d)						
Intake	2.86 ^{b}	2.41 ^{c}	4.41 ^{a}	2.60 ^{c}	0.16	< 0.001
Excreta	1.58 ^{b}	1.27 ^{c}	3.05 ^{a}	1.35 ^{bc}	0.15	< 0.001
Retained [2]	1.28	1.14	1.36	1.25	0.15	0.14
Energy values, kcal/kg DM [2]						
AME	3195 ^{a}	3228 ^{a}	2885 ^{b}	3166 ^{a}	45	< 0.001
AMEs	3211 ^{a}	3236 ^{a}	2959 ^{b}	3176 ^{a}	39	< 0.001
AMEn	3083 ^{ab}	3134 ^{a}	2774 ^{c}	3067 ^{b}	39	< 0.001

[1] Abbreviations: FI, feed intake (g DM/bird/d); DM, dry matter; HDP, average hen day production (%); Egg weight, average egg weight (g); Egg mass as average egg weight average HDP (g of egg/bird/d); FCR, feed conversion ratio (g of feed DM/g of egg); AME, apparent metabolizable energy; AMEs, AME corrected with nitrogen retention equal to 50% of nitrogen intake; AMEn, AME corrected with zero N retention.
[2] Total N retained calculated as N intake - N in excreta (g/b/d).
[3] By reference diet substitution method. Data are means of 6 replicates of 2 hens per dietary treatment during 3 experimental days. ($P < 0.05$) by one-way ANOVA.
[abc] Means within rows with different superscripts are different ($P < 0.05$).

The AME, AMEs and AMEn values of reference diet, test diets and test ingredients measured by different methods are given in Tables 3-3 and 3-4. All the AMEs values were greater than the AMEn (kcal/kg DM), either for diets or ingredients. The difference was not consistent; with the highest difference of 7% for soybean meal diet followed by 4 and 3% for wheat and corn diets. This was the highest for soybean meal (high-protein ingredient) compared to cereals (12 *vs* 2%). The highest variation of AMEs and AMEn was observed for soybean meal diet (185 kcal/kg DM) in this experiment. That difference was 140 kcal/kg, DM (0.6 MJ/kg, DM) for different categories of birds fed different diets (Cozannet et al., 2010a). AME values were also higher than AMEn (kcal/kg, DM) for both diets and ingredients. The highest difference observed as 4% for soybean meal diet compared to 3% for both corn and wheat diets. The AME of ingredients were 5 and 2% higher than correspondent AMEn values for soybean meal and cereals. Lopez and Leeson (2008a) reported that difference of AME and AMEn by 7-12% and 3-5% for soybean meal and corn in broilers. The AMEs and AME values of low-protein diets and ingredients (cereals) were close to each other. However, the AMEs was higher than the AME for soybean meal diet (+3%) and soybean meal ingredient (+8%) because of an excessive N catabolism due to higher N supply in the soybean meal diet.

Table 3-4 GE, AME, AMEs, and AMEn (kcal/kg DM) values of corn, wheat and soybean meal in laying hens; comparison with literature values.

Method	GE [1]	AME	AMEs	AMEn	EU table [2]	INRA table [3]
					AMEn	AMEn
Bioassay method [4]						
Reference diet	3971	3195	3211	3083		
Test ingredients:						
Corn	4455	3791	3784	3722	3725	3662
SBM	4659	2621	2835	2496	2579	2614
Wheat	4373	3565	3562	3479	3494	3429
Regression equation [5]						
Reference diet	3971	3195	3211	3083		
Test ingredients:						
Corn	4437	3791	3784	3722		
SBM	4539	2621	2835	2496		
Wheat	4418	3565	3562	3479		
RSD	0	45	39	39		

[1] Abbreviations: GE, gross energy; AME, apparent metabolizable energy; AMEs, AME corrected with 50% nitrogen intake; AMEn, AME corrected with zero N retention.
[2] European table of energy values for poultry feedstuffs using our ingredients proximate values as estimators (Janssen, 1989).
[3] Based on the AMEn values of INRA table for adult cockerels (Sauvant et al., 2004).
[4] By reference diet substitution method (n=18).
[5] Estimated by regressing the determined GE, AME, AMEn and AMEs values (kcal/kg DM) of diets on the inclusion rates of reference diet and corn, SBM and wheat in the diets (n=24).

To conclude, the AMEn values are not representative of production conditions, in particular for the high-protein ingredients. In addition, AME values as obtained from the difference method should be interpreted with caution as it is affected by the CP content of the test diet. AMEs would then be the most representative of productive conditions.

Estimation of AME values of ingredients by regressing their inclusion rate in the reference and test diets against the measured AME value of the diets is a statistical approach to validate the AME, AMEs, and AMEn values measured using the reference diet substitution method. Values obtained for ingredient AME, AMEs, and AMEn from the reference diet substitution method were the same as those obtained by diet-ingredient-inclusion-regression (Table 3-4). Using

linear regression to extrapolate the AME and AMEn of ingredients by their inclusion rate in diets was examined by others (Applegate, 2005).

The ingredient AMEn (kcal/kg DM) values derived from the reference diet substitution method were compared to those predicted by applying measured proximate values to equations from the EU table of energy values for poultry feedstuffs (Janssen, 1989) and INRA table (Sauvant et al., 2004). A summary is given in Table 3-4. In all methods, the highest values of AMEn were obtained for corn followed by wheat and SBM, respectively. The AMEn values obtained by reference diet substitution were within 15 kcal/kg to those obtained by using proximate values applied to EU prediction tables (Janssen, 1989) for corn and wheat but were 84 kcal/kg lower for SBM.

The mineral supplement inclusion rate in the diet affects energy utilization in animals. Providing limestone as a source of calcium for egg production leads to endogenous energy loss and decreases the AMEn content of ingredients in laying hens compared to roosters (Cozannet et al., 2010a) and in pigs (Noblet and van Milgen, 2013). Provision of limestone in layer diets in the current study might explain differences between AMEn values of corn, SBM, and wheat grains compared to diets formulated for adult cockerels in EU tables (Table 3-4). The ability of laying hens to metabolize energy has been reported to be lower than cockerels and more than broilers. Slinger et al. (1964) showed higher metabolizable energy values in layer chickens compared to broiler chickens. Bourdillon et al. (1990b) reported higher AMEn values for different diets in adult cockerels compared to growing broilers. Although laying hens showed intermediate AMEn values for different diets and one grain compared to highest and lowest correspondent values by cockerels and broiler (Cozannet et al., 2010a), the AME values of feedstuffs using adult cockerels can be easily used for laying hens (Farrell, 1999). The data achieved in the current study confirm these observations.

Utilization of GE for different AME values are shown in Table 3-4. The AME/GE was 85, 82 and 56% for corn, wheat and soybean meal, respectively. The lower metabolizability of the energy of soybean meal is related to its high CP content and subsequent excessive N oxidation and excretion and, more importantly, to the presence of poorly digested non-starch polysaccharides (Dale, 2000). Utilization of GE for AME, AMEs, and AMEn were almost similar for cereals (83%, on average); corresponding values for soybean meal are 56, 61, and 54% of GE, the 2 lowest values (AMEn and AME) representing underestimated ratios and not being representative of production conditions.

The results in the current study showed agreement between the reference diet substitution and regression methods to estimate ingredients ME content. The regression method has the advantage over the diet substitution method in that a series of diets can be formulated with varying levels of ingredients yet remain balanced in nutrient content with no deficiencies or excesses. These diets can then be assayed for AME, AMEn or AMEs. Ingredient energy values can then be calculated by regressing inclusion level to diet energy values. Further research to assess metabolizable energy of ingredients using regression across different classes of chickens and poultry species will enable nutritionists to more accurately formulate feeds.

Acknowledgments

Authors acknowledge Poultry CRC and the Australian Egg Corporation Limited for supporting this study.

Chapter 4. Energy efficiency and net energy prediction of feed in laying hens

4.1. Introduction

Feed represents 65-75% of the total layer production cost and energy represents at least 60% of total diet cost; hence, the accurate estimate of the real available amount of feedstuffs energy is a necessity in order to meet birds performance objectives and nutrients requirements. The pig and dairy industries have the ability to formulate feed using net energy (NE) system. However, the poultry industry uses true ME (TME) or apparent ME (AME) usually corrected to zero-nitrogen retention (TMEn, AMEn) to formulate feed and as such does not distinguish maintenance from production or does not consider heat increment losses that would depend on nutrients. Proposals for prediction of NE in broilers have been recently done (Wu et al., 2019) but not yet in layers.

Each feed energy system deals with the energy utilization in the body and the calculation method for energy values. The ME is the obtainable portion of feedstuff gross energy present after deducting the excreta energy. Heat increment (HI) is the heat produced in fed animals in excess of their fasting metabolism and net energy is equal to ME minus HI. Net energy can be used for maintenance, body weight gain and egg production (Farrell, 1974). Total heat production (HP) can be measured by calorimetry gas exchange or comparative slaughter technique. The HI can be calculated by subtracting fasting HP from total HP. The simple relationship of NE, ME and HI as NE = ME - HI was primarily introduced by (Armsby and Fries, 1915). They found HI component not affected by feed intake level but dependent on the feed or ingredient specifications.

Taking the NE as the most available portion of feed energy for animals, using the NE system could be expected to overcome limitations of the current ME system. De Groote (1974) examined the relative efficiency of energy utilization for different nutrients and found energy utilization of 100%, 113% and 78% for carbohydrate, fat, and protein, respectively, in growing chicks. Pirgozliev and Rose (1999) compared the NE and AME concentration of 62 different feedstuffs and found the AME system overestimated the NE of high protein animal-based feed compared to cereal grains, cereal by-products, and underestimated high protein vegetable-based feedstuffs. It was also reported by Carré et al. (2014) that NE/AMEn ratios for CP, lipids,

and starch were 76, 86, and 81% in broilers; similarly, Wu et al. (2019) obtained NE/AMEn ratios of 73% for soybean meal (SBM), 88% for soy oil and 81% for corn in growing broilers. These results indicate that the ME system would underestimate the energy value of dietary fat and overestimates the energy value of high-protein ingredients.

NE prediction equations for poultry have been proposed by several researchers (Schiemann, 1972; De Groote, 1974; Carré et al., 2002; Noblet et al., 2003; Swick et al., 2013). The objective of this study was to measure NE values of 16 diets in laying hens and to determine whether NE of diets and ingredients in laying hens could be predicted from their chemical composition. For validation, regression equations obtained from the 16 diets were applied to two further diets differing NE/AME ratio. The AME and NE of the 4 major ingredients were calculated by regression with the AME and NE values of 16 diets against the inclusion rates of the ingredients.

4.2. Material and methods

4.2.1. Birds and experimental design

The study was approved by the Animal Ethics Committee of the University of New England (UNE) and designed to follow the Australian code of practice for the care and use of animals for scientific purposes (NHMRC, 2013). Hy-Line Brown pullets were sourced from the Glenwarrie Partnership, Tamworth, NSW, Australia and reared following Hy-line Brown recommendations (Hy-Line, 2016) in a curtain sided shed. They received 16 hours of light and 8 hours darkness during their production period. A completely randomized design was used to evaluate 16 different diets in 16 calorimetry chambers (one diet per chamber) with 3 birds per chamber. The same birds were kept together in chambers and shed cages between each run. Birds were assigned to diets randomly. The birds were 51-62 weeks of age for the first four runs and a different batch of birds, 29-37 weeks of age, in the last four runs. Birds were moved from the shed to chambers and fed test diets for a 4-day in-chamber adaptation period with chamber lids open and running air pumps in a climate-controlled room. Then, the 3-day respiratory measurements were performed to measure the heat production in respiratory chambers. Birds were fed a standard commercial diet when housed in the shed. Feed and water were provided *ad libitum* all the time.

4.2.2. Diets and experiments

Sixteen diets were formulated to have the same AMEn of 2775 kcal/kg (as is basis) using the values for AMEn of corn, wheat, and SBM determined in a previous experiment with laying hens (Barzegar et al., 2017). This was done in an attempt to minimize the effect of dietary AMEn on feed intake. Half of the diets were based on corn and the other half based on wheat (Table 4-1). All wheat-based diets contained xylanase Econase XT 25 (AB Vista, Marlborough, UK). None of the diets contained phytase. Some diets contained small amounts of alpha cellulose and celite (fine silica) as fillers such that diets could be formulated with various levels of fat and protein at the same calculated AMEn level. Diets ranged in protein content from 13% to 24% (/DM) using single crystalline amino acids added as necessary to ensure required digestible amino acids in order to meet the Hy-Line recommendations (Hy-Line, 2016) (Table 4-2). Diets were formulated such that levels of fat, protein, and starch had minimal correlations to each other (Table 4-3) to improve the probability of developing robust net energy prediction equations.

Table 4-1 Ingredients composition of diets (g/kg; as-is basis).

Diet	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Ingredients																
Corn	661	574	516	395	547	548	648	520	0	0	0	0	0	0	0	0
Wheat	0	0	0	0	0	0	0	0	625	522	730	529	598	480	489	604
Soybean meal	211	111	338	373	307	268	158	256	202	339	62	164	179	278	310	258
Canola oil	1	52	24	60	20	30	17	43	18	20	7	60	32	53	41	13
Alpha cellulose	0	38	0	30	3	13	16	26	0	0	32	44	16	24	10	0
Celite	0	81	0	20	0	17	27	30	30	0	26	71	47	43	28	5
Others [1]	122	125	120	121	121	122	124	122	121	119	122	122	121	120	120	120
Supplemented amino acids [2]	4.4	19.5	1.5	1.5	1.8	2.5	11.3	2.9	4.0	1.1	19.6	10.1	6.7	1.9	1.5	1.7

[1] Others provided as (as-is, g/kg): 100 limestone, 16 dicalcium phosphate, 2.4 salt, 2.0 Na bicarbonate, 1.0 UNE vitamin & mineral premix, 0.6 choline 60%. Xylanase XT 25 added in diets 9 - 16 at 0.08 g/kg. UNE layer premix supplied per tonne: 10.0 MIU Vit A, 3.0 MIU Vit D, 20.0 g Vit E, 3.0 g Vit K, 35.0 g nicotinic acid, 12 g pantothenic acid, 1 g folic acid, 6 g riboflavin, 0.02 g cyanocobalamin, 0.10 g biotin, 5.0 g pyridoxine, 2.0 g thiamine, 8.0 g copper, 0.20 g cobalt, 0.50 g molybdenum, 1.0 g iodine, 0.30 g selenium, 60.0 g iron, 60.0 g zinc, 90.0 g manganese, 20.0 g Oxicap E2 (antioxidant).

[2] Supplemental amino acids (as-is, g/kg): 2.3 D,L-methionine for all diets; 1.2 L-lysine HCL, 78.4% for diets 1, 2, 7, 9, and 11-13; 0.7 L-threonine, 99% for diets 1-2 and 6-9 and 11-13; 0.1 L-tryptophan for diets 2, 7 and 11; 0.5 L-isoleucine for diets 2, 7, and 11- 13; 0.4 L-arginine for diets 2, 7, 11 and 12; 0.5 L-valine for diets 2, 7, and 11-13.

Table 4-2 Nutrient composition of experimental diets (g/kg DM basis; unless noted).

Diet	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Nutrients assayed																
DM% [1]	91.3	92.6	91.6	92.0	91.8	91.7	91.6	91.8	92.0	91.8	92.0	93.0	92.4	92.9	92.4	92.2
CP	185	132	229	226	212	198	154	190	189	244	144	156	171	200	224	215
EE	31	63	57	82	52	55	33	58	35	42	32	66	42	63	51	30
Crude fiber	82	88	59	41	55	67	75	44	69	66	63	74	43	68	66	46
Ash	156	214	165	171	197	166	189	177	204	182	163	208	186	201	169	190
NSP total	66	83	73	98	71	76	69	85	81	89	101	108	87	104	98	88
NSP soluble	6	4	6	6	6	6	5	5	15	13	15	12	13	13	13	14
NSP insoluble	60	79	67	92	65	70	64	80	66	76	86	96	74	91	85	74
NDF	76	85	81	102	82	96	78	93	92	98	116	122	118	97	93	97
ADF	39	57	47	61	43	53	56	56	38	44	47	57	49	39	38	31
Starch	429	395	338	277	321	378	431	360	372	308	460	351	399	302	338	359
Sugars	34	21	43	44	41	38	29	35	39	47	28	29	33	40	42	43
Nutrients calculated, as-is [2]																
Calcium	42	42	42	42	42	42	42	42	42	42	42	42	42	42	42	42
Phosphorus, available	4.0	4.1	4.0	4.1	4.1	4.1	4.1	4.1	4.1	4.0	4.1	4.1	4.1	4.1	4.1	4.1

[1] DM measured at the feed distribution time.
[2] Total digestible amino acids calculated (g/kg as is): 4.5 methionine, 8.8 lysine, 6.8 methionine + cysteine

Table 4-3 Correlations between nutrient compositions and energy values of the 16 diets used for the prediction of net energy values [1].

		ash	CP	EE	Starch	Free sugars	CF	ADF	NDF	NSP	GE	AME
CP	R	-0.378**										
EE	R	0.194*	0.119									
Starch	R	-0.166	-0.738**	-0.642**								
Free sugars	R	-0.330**	0.981**	0.057	-0.728**							
CF	R	0.323**	-0.579**	0.595**	0.023	-0.577**						
ADF	R	0.321**	-0.599**	0.562**	0.053	-0.593**	0.999**					
NDF	R	0.325**	-0.511**	0.178	0.126	-0.440**	0.800**	0.820**				
NSP	R	0.230*	-0.065	0.396**	-0.320**	-0.010	0.678**	0.684**	0.870**			
GE	R	-0.509**	0.661**	0.620**	-0.641**	0.604**	0.025	-0.006	-0.240**	0.138		
AME	R	-0.453**	0.067	0.355**	-0.028	0.019	0.234*	0.221*	-0.006	0.069	0.597**	
NE	R	-0.106	-0.166	0.341**	0.030	-0.197*	0.358**	0.349**	0.140	0.124	0.302**	0.749**

[1] *Abbreviations*: ADF, acid detergent fiber; CF, crude fiber; CP, crude protein; EE, ether extract or fat; GE, gross energy; AME, apparent metabolizable energy; NDF, neutral detergent fiber; NE, net energy; NSP, non-starch polysaccharides.

* Pearson correlation significance level ($P < 0.05$).

** Pearson correlation significance level ($P < 0.01$)

4.2.3. Calorimetry chambers measurements

Closed-circuit calorimetric chamber design, gas exchange, and heat production measurements have been previously described by Swick et al. (2013) and Wu et al. (2019) in broilers. Temperature and humidity of each chamber were monitored with electronic sensors including display and memory capabilities. A 28 L/min diaphragm air pump circulated chamber air through a plastic CO_2 trap containing 2 L of a 320 g/kg KOH solution with bubbler assembly to maximize CO_2 absorption followed by a moisture trap containing approximately 3 kg of dried silica gel before being returned to the chamber. A barometric sensor was connected to a solenoid valve to backfill the chamber with medical grade oxygen as CO_2 was absorbed. The O_2 consumption was determined gravimetrically by weighing cylinders before and after each day run. The recovery of CO_2 from KOH was performed according to the method described by Annison and White (1961) based on a barium chloride ($BaCl_2$) precipitation technique. The changes of O_2 and CO_2 in the calorimetric chambers were measured before the close and opening of the chambers every day during the run using a FoxBox Respirometry System instrument (Sable Systems, Las Vegas, NV, USA). The total consumption of O_2 and expiration of CO_2 were calculated by taking into account the residual proportion of gases in the chambers. The daily gas exchanges were measured for 3 consecutive days. However, it was suspended for about 2 hours each day for replenishing feed, water, KOH and silica gel and collection of excreta. Feed intake (FI) was measured and total excreta collected daily in each calorimetry chamber. Excreta collections were cumulated and weighed for 3 days. All variables were adjusted to a total of 72 hrs for calculation of heat production. Performance measurements over 3 days included initial and final body weight of each laying hen, the daily feed intake (FI) per chamber and the eggs mass and number per chamber.

4.2.4. Analyses of diets and excreta

Feed and excreta were thoroughly homogenized with subsamples taken for analysis. Feed samples were analyzed on an as-is basis and results were expressed on a DM basis. Approximately 2 g of diet and 3 g of freeze-dried excreta samples were dried in crucibles in a forced air oven at 105 °C to constant weight to determine DM. Excreta were freeze-dried for gross energy and N analysis. Wet excreta DM was calculated by correction for the loss of moisture during both freeze- and oven-drying. Gross energy was analyzed using an adiabatic bomb calorimeter (IKA® Werke, C7000, GMBH and CO., Staufen, Germany). Feed samples were analysed for CP, EE, crude fiber, ash, neutral detergent fiber (NDF) and acid detergent

fiber (ADF) (AOAC, 2016), starch (Megazyme Total Starch Kit, Megazyme International Ireland Ltd., Bray, Ireland), free sugars (mono- and disaccharides) (Annison et al., 1996), total non-starch polysaccharides (NSP), soluble NSP and insoluble NSP (Englyst and Hudson, 1987b; Theander and Westerlund, 1993).

4.2.5. Calculations

The AME was determined by the total collection method of Bourdillon et al. (1990a). The AME values were converted to AMEn (AME for zero N retention in body and eggs) and AMEs (AME corrected for a retention of N equal to 50% of nitrogen intake) (Cozannet et al., 2010a) values using a GE of 8.22 kcal per gram of N as the correction factor (Hill and Anderson, 1958). The total heat production (THP) corresponded to the O_2 consumed and the amount of CO_2 produced from birds according to the modified Brouwer equation: total heat (kcal) = 3.866 × liters of oxygen consumed + 1.200 × liters of CO_2 expired (Brouwer, 1965; McLean, 1972). The respiratory quotient (RQ) of each 3-day run was calculated as the ratio of liters of CO_2 expired to liters of O_2 consumed. Heat increment (HI) was calculated by subtracting fasting heat production (FHP) from THP. An FHP value of 88 kcal/kg $BW^{0.75}$ (370 kJ/kg $BW^{0.75}$) per bird per day was used. This corresponds to the asymptotic HP (at zero activity) after a 24 h fasting period as reported for laying hens by (Wu et al., 2016). The net energy content was calculated as AME intake minus HI (per bird per day) divided by feed consumed on a DM basis. The N balance data were expressed per gram per bird per day.

$$TNR = Nint - Nexc \quad \text{Equation 1}$$

where TNR is total nitrogen retained, Nint is N intake, and Nexc is N in excreta (g/bird/day).

$$NRegg = 1.936 \times \text{Egg mass} \quad \text{Equation 2}$$

where NRegg is total nitrogen retained in egg (g/bird/day), 1.936 is N% in egg (Miranda et al., 2015).

$$NRbody = TNR - NRegg \quad \text{Equation 3}$$

where NRbody is total nitrogen retained in the body (g/bird/day).

$$RE = MEI - HP \quad \text{Equation 4}$$

where RE is total retained energy, MEI is ME intake, and HP is total heat production (kcal/kg/d).

$$REprot = TNR \times 6.25 \times 5.7 \quad \text{Equation 5}$$

where REprot is retained energy as protein, 6.25 is the protein equivalent of 1 gram nitrogen, and 5.7 is the energy equivalent of 1 gram protein (kcal/kg/d).

$$REfat = RE - REprot \quad \text{Equation 6}$$

where REfat is retained energy as fat (kcal/kg/d).

$$REegg = -19.7 + 1.81 \times \text{egg weight (Sibbald, 1979)} \quad \text{Equation 7}$$

where REegg is retained energy in egg (kcal/d).

$$REegg\ prot = NRegg \times 6.25 \times 5.7 \quad \text{Equation 8}$$

where REegg prot is retained energy in the egg as protein (kcal/d).

$$REegg\ fat = REegg - REegg\ prot \quad \text{Equation 9}$$

where REegg fat is retained energy in the egg as fat (kcal/d).

$$REbody = RE - REegg \quad \text{Equation 10}$$

where REbody is retained energy in the body (kcal/kg $BW^{0.75}$/d).

$$REbody\ prot = NRbody \times 6.25 \times 5.7 \quad \text{Equation 11}$$

where REbody prot is retained energy as protein in the body (kcal/kg/d).

$$REbody\ fat = REbody - REbody\ prot \quad \text{Equation 12}$$

where REbody fat is retained energy in the body as fat (kcal/d).

Energy balance data as AME intake, HP, retained energy (RE) and its partition between protein and fat and between body and egg production were further expressed per bird per kg $BW^{0.75}$ per day. Energy values of diets were expressed per kg DM, and energy utilization data were expressed as a percent. The performance data were expressed per bird per day, with FI and FCR reported on a DM-basis. Egg mass calculated as the product of percent hen day production (HDP) and average egg size (g/b/d) and FCR as FI (g) divided by egg mass (g).

4.2.6. Statistical analyses

All the performance, nitrogen balance, energy balance, energy values, and utilization data were analyzed using PROC GLM and Tukey's multiple-range test to separate means when appropriate (SAS, 2010). The model included the effects of diet (n=16) and run (n=8). As explained above, the so-called run effect includes the effect of a batch of birds *per se* but also their age, the environmental conditions, etc. that differed between successive runs. Multiple linear regression equations were calculated for estimating ME for maintenance (MEm), FHP and mean utilization of ME for NE, on one hand, and efficiencies of ME for egg and body energy or protein and fat gains, on the other hand. The stepwise procedure of PROC REG was applied with or without intercept to calculate significance of chemical components to predict energy values and energy efficiencies according to linear effects. Measured dietary chemical components were ash, CP, EE, starch, free sugars, CF, ADF, NDF, NSP. AME, AMEn and AMEs were included in the model for predicting NE. The significant components were then used to generate prediction equations using PROC REG without the stepwise procedure.

4.3. Results

Thirteen observations were removed from the 128 total measurements taken because of low feed intake (4), low HDP (3) or technical issues with chambers (6). The calorimetry measurements were performed at different times and each measurement (3d) was regarded as a run. Body weight, FI, HDP, FCR, N intake, N excreted, total N retained, N retained in egg, N retained in body, AME intake, HP, HI, and RE were all affected by run ($P < 0.05$) while retained energy in egg as protein was not affected by run ($P > 0.05$) (Table 4-4). Variance due to run includes bird age, bird individuality, and environmental conditions during the pre-measurement period.

4.3.1. Metabolic utilization of energy in laying hens

The average AME intake and HP of the 115 groups of 3 hens used in the study were 157 and 130 kcal/kg $BW^{0.75}$/d, respectively (Table 4-5). The amount of energy retained or exported to eggs averaged 54 kcal/kg $BW^{0.75}$/d, meaning that on average the laying hens in the present study had to mobilize 27 kcal/kg $BW^{0.75}$/d energy from body reserves. Thus, AME intake was insufficient to meet the energy requirements for both maintenance and egg production. In fact, for all of the 16 treatment groups of laying hens, the mean body energy balance was negative, which means that dietary NE measurements were obtained under a metabolic situation where

the energy required for maintenance and egg production was met by both feed and body reserves. With regard to N utilization, the data presented in Table 4-4 indicate that N retained on average was 1.22 g/b/d of which 1.19 g/b/d was exported to egg with a subsequent close to zero N deposition in the body (0.03 g/b/d, on average). Unlike energy, protein and amino acids supplies were sufficient to meet the requirements of laying hens for egg production and maintenance. This also means that the mobilization of body energy corresponds to fat exclusively. In the present study, this mobilization averaged 28 kcal/kg $BW^{0.75}$ or 48 kcal/b/d being equivalent to about 5 g/b/d of fat.

Retained energy was related to ME intake ($r^2 = 0.89$) as depicted in Figure 4-1 and model 1 in Table 4-6. It was then possible to estimate parameters for energy utilization in laying hens by regression modeling. Different models were calculated in order to estimate the MEm (ME requirements for maintenance) and the energy cost of energy gain in egg or as protein and fat (Table 4-6). Model 1 indicates that MEm (for RE = 0) and FHP (for MEint = 0) equal 120 and 90 kcal/kg $BW^{0.75}$/d, respectively and the efficiency of ME for energy gain is 75%. Model 2 describes the total recovered energy from ME intake into both protein and fat for body and egg combined. This model illustrates that the energy cost of depositing 1 kcal of AME intake as protein (in both egg and body) was 1.98 kcal. Also, the energy cost for retaining 1 kcal of energy intake as fat (in both egg and body) was 0.98 kcal. Therefore, the efficiencies of energy gain as protein and as fat from AME were approximately 50 and 100%. Model 3 examined how energy balance in the body is dependent on ME intake and energy exported to the egg. This model showed that deposition of 1 kcal of egg energy required 1.28 kcal of body energy (i.e. 78% efficiency).

4.3.2. The effect of diet composition on performance and energy utilization

Dietary treatment did not change FI, HDP, egg mass and FCR ($P > 0.05$) as shown in Table 4-4. Total N retained, N retained in egg and N retained in the body were not affected by dietary protein level ($P > 0.05$) as shown in Table 4-4. Body N retention was on average 0.1 g/b/d while that retained in the egg was much higher at 1.2 g/b/d. The lysine consumed by the birds averaged 909 mg/b/d with a range between 777 and 1108 mg/b/d.

Diet composition did not alter the AME intake, HP and HI (kcal/kg $BW^{0.75}$/d; $P > 0.05$) as shown in Table 4-5. However, birds fed the highest AME levels (diets 12 and 14) showed the highest total RE and the lowest mobilization of body energy. The energy gain as protein in

eggs and protein in the body were not affected by diet composition ($P > 0.05$). The RQ values ranged from 0.914 to 1.009 with the lowest values observed in birds fed high-fat diets (diets 4 and 14) and the highest values in birds fed the high starch diets (diets 11 and 1) (Table 4-5). The measured AMEn values among diets were different ranging from 2694 to 2928 kcal/kg DM as shown in Table 4-5 ($P < 0.05$). This was unexpected as diets were formulated to have a constant AMEn of 2750 kcal/kg (as is basis). The average energy metabolizability (AME/GE) was 77% (range: 74-79%) with the highest ratio in birds fed diet 11 that contained the highest level of starch with low CP. The AMEs averaged 2937 kcal/kg DM (ranging from 2832 to 3052 kcal/kg DM) and were similar to the average AME values of 2935 kcal/kg DM (ranging from 2808 to 3066 kcal/kg DM). AMEn values were lower than AMEs or AME and averaged 2815 kcal/kg DM.

The average efficiency of AME for NE was 74% and ranged from 70 to 76%. The NE/AME was lowest in birds consuming diet 16 with an EE of 29 g/kg DM (the lowest) and CP of 215 g/kg DM. NE values ranged from 1969 to 2299 kcal/kg DM in connection with combined differences in ME content and efficiency of ME for NE. The lowest NE/AME value (70%) was observed in birds fed diet 16 that had the lowest AME content (2808 kcal/kg, DM) and lowest NE of 1969 kcal/kg DM, while the highest NE/AME value (76%) was observed in birds fed diet 12 that had an AME content of 2906 kcal/kg DM. Birds fed diet 16 had also had the lowest NE of 1969 kcal/kg DM while birds fed diet 8 had the highest NE of 2299 kcal/kg DM in connection with an AME value of 3047 kcal/kg DM and an efficiency of AME for NE of 75.5%

Table 4-4 Effect of diet composition on performance and N balance in layers.

Diet	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	SEM	P-value (Diet) [1]
Performance [2]																		
BW, g	2074	2056	2035	2063	2022	2002	1992	2052	2030	1999	2017	2030	2047	2036	1983	2042	11	0.645
Daily feed intake, g DM	86	90	87	92	91	84	89	91	92	88	92	97	93	98	89	92	1	0.208
Hen day production, %	100	94	93	97	95	92	92	106	90	97	98	99	102	95	95	97	1	0.482
Egg mass, g/b/d [3]	61	59	62	62	61	62	59	62	62	61	60	61	61	62	62	63	1	0.103
FCR (g/g)	1.41	1.73	1.52	1.54	1.57	1.50	1.64	1.38	1.69	1.51	1.56	1.64	1.49	1.65	1.52	1.56	0.03	0.206
Dig Lys intake mg/b/d	877	846	1097	1108	973	941	839	816	811	1103	817	777	811	841	1015	866	16	< 0.001
Nitrogen balance (g/bird/day)																		
Intake	2.59	1.96	3.06	3.41	3.11	2.70	2.29	2.77	2.78	3.487	2.22	2.51	2.55	3.16	3.25	3.22	0.05	< 0.001
Excreta	1.31	0.88	1.98	2.25	1.95	1.49	1.08	1.45	1.55	2.173	1.06	1.26	1.34	1.88	1.97	1.92	0.04	< 0.001
Retained:																		
Total [4]	1.28	1.07	1.08	1.16	1.16	1.21	1.21	1.32	1.24	1.31	1.16	1.25	1.22	1.28	1.28	1.30	0.02	0.340
Egg [5]	1.19	1.13	1.19	1.20	1.19	1.20	1.15	1.20	1.19	1.18	1.17	1.18	1.18	1.21	1.19	1.22	0.01	0.103
Body [6]	0.09	-0.06	-0.11	-0.04	-0.03	0.01	0.06	0.12	0.05	0.13	-0.01	0.07	0.04	0.07	0.09	0.08	0.02	0.461

[1] From the analysis of variance with diet and run effects; run effect was significant (P < 0.001) for all the performance parameters and N balance components; SEM as the standard error of the mean.

[2] Each value represents the mean of 8 replicates (runs) for each treatment (diet) (n=115) during 3-days respiratory measurements (3 layers per calorimetry chambers).

[3] Egg mass HDP × average egg size (g/bird/day). FCR (g/g) calculated as feed intake (g) divided by egg mass (g)

[4] Total N retained (g/bird/day) calculated as N intake - N in excreta.

[5] Retained N in egg (g/bird/day) calculated as 1.936 (N% in the egg) × egg mass (Miranda et al., 2015).

[6] Retained N in the body (g/bird/day) calculated as total N retained - retained N in the egg.

Table 4-5 Effect of diet composition on energy balance, energy values and energy utilization in layers.

Diet	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	SEM	P-value (Diet) [1]
Energy balance (kcal/kg $BW^{0.75}$/d) [2]																		
AME intake	146	153	151	162	151	154	156	161	156	151	161	166	156	167	161	152	2	0.221
HP [3]	128	126	129	131	132	128	128	128	130	131	131	128	130	131	131	133	1	0.668
HI	40	37	41	43	43	39	40	40	41	43	43	39	41	42	42	45	1	0.668
RE [4]																		
Total	17	27	22	31	19	26	28	33	27	19	30	39	26	36	30	19	2	0.011
As protein	26	22	22	24	24	26	26	27	26	28	25	26	25	27	27	27	1	0.286
As fat	-9	5	0	7	-5	0	2	6	1	-8	5	12	1	10	2	-8	1	< 0.001
RE_{egg}																		
Total	53	50	54	54	54	55	52	54	54	54	53	53	53	55	55	55	1	0.111
As protein	24	23	25	25	25	25	24	25	25	25	24	25	25	25	25	25	1	0.143
As fat	28	27	29	29	29	30	28	29	29	29	28	29	29	29	30	30	1	0.096
RE_{body}																		
Total	-36	-23	-32	-23	-34	-29	-24	-21	-27	-35	-23	-15	-27	-18	-25	-37	2	0.008
As protein	2	-1	-2	-1	-1	0	1	2	1	3	0	1	1	2	2	2	1	0.465
As fat	-37	-22	-29	-22	-34	-30	-26	-23	-28	-38	-23	-16	-28	-20	-27	-38	1	< 0.001
RQ	0.987	0.948	0.954	0.914	0.958	0.950	0.984	0.947	0.985	0.953	1.009	0.958	0.963	0.945	0.947	0.978	0.003	< 0.001

Diet	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	SEM	P-value (Diet) [1]
Energy values, kcal/kg DM																		
AME	2918	2909	2971	3040	2830	3066	2928	3047	2899	2870	2956	2906	2887	2901	3023	2808	8	< 0.001
AMEn	2780	2799	2839	2901	2698	2925	2818	2928	2787	2746	2854	2787	2780	2794	2904	2694	9	< 0.001
AMEs	2894	2880	2988	3045	2835	3052	2913	3050	2909	2901	2942	2885	2890	2923	3045	2832	9	< 0.001
NE	2118	2201	2170	2239	2027	2287	2189	2299	2134	2053	2168	2220	2118	2173	2220	1969	13	< 0.001
Energy utilization, %																		
AME/GE	77.4	78.8	74.7	73.8	74.3	77.1	78.4	77.3	77.8	74.3	79.2	77.1	77.9	74.7	75.8	75.7	0.1	< 0.001
NE/AME	72.6	75.6	73.1	73.6	71.6	74.6	74.8	75.5	73.6	71.5	73.3	76.3	73.3	74.9	73.5	70.1	0.1	0.0145

[1,2] From the analysis of variance with diet and run effects; run effect was significant ($P < 0.05$) for all the parameters in table 4-4, except RE egg as protein ($P > 0.05$). Each value represents the mean of 8 replicates (runs) for each treatment (diet) (n=115) during 3-days respiratory measurements (3 layers per calorimetry chambers).

[3] Abbreviations: GE, gross energy; AME, apparent metabolizable energy as [(FI × GEf) - (E × GEe)] / FI (kcal/kg DM of diet); AMEn, AME corrected for zero N retention as = [AME - [8.22× (Ni - Ne)]] / FI (kcal/kg DM of diet); AMEs, AME corrected for a N retention equal to 50% of nitrogen intake = AMEn + 8.22 × Ni% × 10 × 50% (kcal/kg DM of diet), where GEf and GEe are the gross energy of feed and excreta (kcal/g DM); FI = feed intake (g DM/d/hen); E = excreta output (g DM/day/hen); 8.22 as nitrogen correction factor (kcal/g); HI, heat increment as HP - FHP (FHP = 88 kcal/kg $BW^{0.75}$/d); HP, heat production (kcal) as 3.866 × O_2 consumed (L) + 1.200 × CO_2 expired (L) (Brouwer, 1965); Respiratory quotient (RQ; CO_2/O_2); Net energy (NE) values expressed based on DM of the feed (total collection method); NE (kcal/d) calculated as fasting heat production + RE.

[4] Total retained energy (RE) calculated as ME intake - HP; RE as protein (kcal) calculated as total retained N × 6.25 × 5.7; RE as fat calculated as total RE - RE as protein; Total retained energy in egg (RE egg; kcal) calculated as -19.7 + 1.81 × egg weight (Sibbald, 1979); RE egg as protein (kcal) calculated as retained N in egg × 6.25 × 5.7; Protein content of an egg assumed as 12.1% (Miranda et al., 2015); RE egg as fat calculated as total RE egg - RE egg as protein; RE body calculated as total RE - RE egg; RE body as protein calculated as retained N in body × 6.25 × 5.7; RE body as fat calculated as total RE body - RE body as protein.

Table 4-6 Energy utilization prediction equations in laying hens (kcal/kg $BW^{0.75}$/d) [1,2].

Equation no	Equation	r^2	RSD
1	RE = -90 + 0.75 × ME intake	0.89	5.3
2	ME intake = 105 + 1.98 × RE_{prot} + 0.98 × RE_{fat}	0.92	5.8
3	RE_{body} = -75 + 0.75 × ME intake -1.28 RE_{egg}	0.90	5.3

[1] Multiple linear regression with means of the 115 measurements on 16 diets.
[2] Abbreviations: RE, total retained energy; RE_{body} retained energy in the body; RE_{egg} retained energy in egg; RE_{prot}, retained energy as protein; RE_{fat}, retained energy as fat.

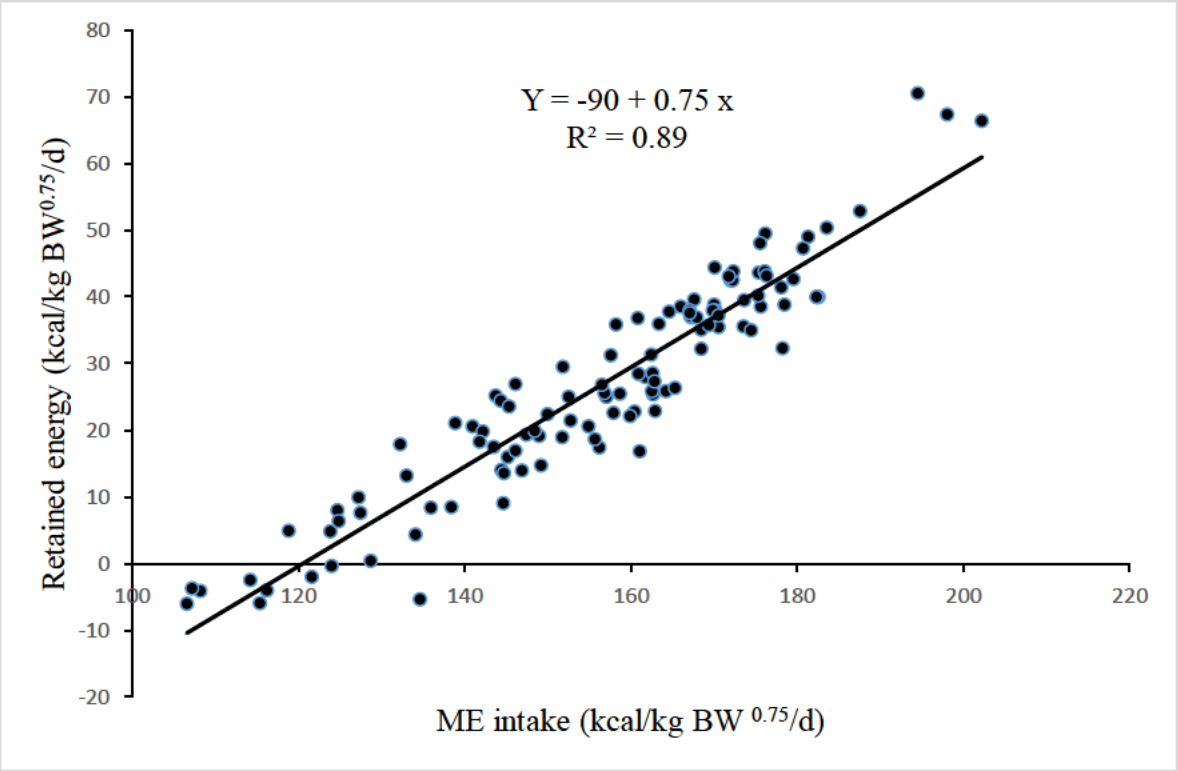

Figure 4-1 Relationship between energy retained and ME intake in laying hens (n=115; kcal/kg $BW^{0.75}$/day).

4.3.3. Prediction of GE, AME, NE, and energy efficiency from the dietary chemical composition

Linear regression was conducted to evaluate the contributions of energy-yielding nutrients to GE, AME, AMEs, AMEn, and NE as shown in Table 4-7. For that purpose, the organic matter was partitioned between CP, EE, starch, and residue (Res). The Res component contained fiber and sugar fractions including soluble and insoluble NSP. The ratios between the AME and GE coefficients for each nutrient provided an estimate of the energy metabolizability of each nutrient while the ratios of nutrient coefficients between the NE equation and AME equation provide an estimate of the efficiency of ME for NE of each nutrient. The ratios for

metabolizability of energy were 60, 78, 95, and 65% for CP, EE, starch, and Res, respectively. The ratios for metabolizability of CP increased from AME/GE (60 %) to AMEs/GE (65%) and decreased for AMEn/GE (52%). Using the same method, the efficiencies of AME for NE were 49, 104, 78 and 70% for CP, EE, starch, and Res, respectively. Thus, the NE content of 1 g of starch was equivalent to 1.84 g of CP and 0.37 g of EE.

Multiple stepwise regression was used to predict the AME, AMEn and AMEs values and metabolizability of energy from diet composition (Table 4-8). The results showed that AME and AMEn can be estimated by dietary nutrients and were positively related to EE and starch and negatively related to ash. The AMEs values were predicted by EE and ash. Energy efficiency ratios of AME/GE, AMEs/GE, and AMEn/GE were positively related to starch and negatively related to CP.

The stepwise multiple linear regression was used to estimate diet NE, NE/AME, NE/AMEn and NE/AMEs from dietary nutrients as shown in Table 4-9. The equation shows NE to be positively related to AME (0.781) and EE (16.4) while CP was negatively related (-11.0) when AME was used as a predictor. Using AMEn as a predictor, NE is also positively related to AMEn (0.796) and EE (18.5) but negatively related to CP (-8.80). Similarly using AMEs as a predictor, NE is positively related to AMEs (0.798) and EE (18.4) and negatively related to CP (-14.2). The intercept was not significant in the equations to predict NE from AME, AMEs or AMEn ($P > 0.05$). Also using multiple liner stepwise regression, the equations to predict the ratio of NE/AME were generated with a significant intercept in each case ($P < 0.001$). The equation shows the NE/AME ratio was positively related to EE (0.66) and negatively related to CP (-0.33) with an intercept of 76.7%; NE/AMEn ratio was positively related to EE (0.72) and negatively related to CP (-0.29) with an intercept of 78.9%; and NE/AMEs ratio was positively related to EE (0.71) and negatively related to CP (-0.45) with an intercept of 78.8%.

Table 4-7 Contribution of energy-yielding nutrients (% of DM) to GE, ME and NE contents of layers diets.

Equation	Energy	Equation [1]				RSD
n°	(kcal/kg, DM)	CP [2]	EE	Starch	Res1	
4	GE	52.7	94.7	39.2	44.1	36
5	AME	31.8	74.2	37.1	28.7	67
6	AMEs	34.2	67.1	35.2	31.6	80
7	AMEn	27.3	66.8	35.2	32.2	80
8	NE	15.7	77.1	28.9	20.2	124

[1] Linear regression without intercept (n = 115 measurements on 16 diets).
[2] The abbreviations: GE, gross energy; AME, apparent metabolizable energy (as measured); AMEn, AME corrected for zero N retention; AMEs, AME corrected for N retention equal to 50% of N intake; CP, crude protein; Res1 for diet organic matter minus CP, EE and starch; EE, ether extract or fat; NE, net energy; RSD, residual standard deviation.

Table 4-8 Prediction of AME, AMEn, AMEs (kcal/kg, DM) and metabolizability (%) in layers from diet composition (% of DM).

Equation	Energy	Equation[1]					RSD
no		intercept	CP [2]	EE	Starch	Ash	
9	AME	3033	-	39.6	5.6	-27.1	67
10	AMEn	2820	-	37.8	6.3	-23.1	79
11	AMEs	3328	-	27.5	-	-28.8	79
12	AME/GE	74.3	-0.25	-	0.19	-	1.5
13	AMEn/GE	72.6	-0.30	-	0.18	-	1.8
14	AMEs/GE	72.3	-0.13	-	0.19	-	1.8

[1] Stepwise multiple linear regression (n = 115 measurements on 16 diets).
[2] Abbreviations: AME, apparent metabolizable energy; AMEn, AME corrected for zero N retention; MEs, AME adjusted corrected for 50% N retention; CP, crude protein; EE, ether extract or fat; GE, gross energy; NE, net energy; RSD, residual standard deviation.

Table 4-9 Prediction of NE and HI feed (kcal/kg DM) and energy efficiencies (%) in layers from diet composition (CP, EE, and starch as % of DM) and ME content (kcal/kg DM).

Equation no	Energy	Equation [1]							RSD
		Intercept	AME [2]	AMEn	AMEs	CP	EE	Starch	
15	NE	-	0.781	-	-	-11.0	16.4	-	93
16		-	-	0.796	-	-8.8	18.5	-	98
17		-	-	-	0.798	-14.2	18.4	-	98
18	HI feed	212	-	-	-	16.4	-	6.8	88
19	NE/AME	76.7	-	-	-	-0.3	0.7	-	3.2
20	NE/AMEn	78.9	-	-	-	-0.3	0.7	-	3.5
21	NE/AMEs	78.8	-	-	-	-0.5	0.7	-	3.4

[1] Stepwise multiple linear regression (n = 115 measurements on 16 diets); if the intercept is not significantly different from zero, it is then fixed to zero.
[2] Abbreviations: AME, apparent metabolizable energy; AMEn, AME corrected for N retention; AMEs, AME corrected for 50% N retention; CP, crude protein; DM, dry matter; EE, ether extract or fat; NE, net energy; RSD, residual standard deviation.

4.4. Discussion

In the current study, digestible lysine was sufficient for maintenance and egg production since the body N balance was close to zero for all diets. This averaged 909 mg/b/d and ranged from 777 to 1108 mg/b/d across treatments as compared to the Hy-Line recommended a value of 780 mg/b/d (Hy-Line, 2016). Recently, Pastore et al. (2018) reported a minimum requirement of standardized ileal digestible lysine for layers in peak production of 813 mg/b/d. With regard to protein intake, it ranged from 11.8 to 21.3 g/b/d, the 17.4 g/b/d average value being quite close to the 17.0 g/b/d Hy-Line (2016) recommendation. Although CP intake of several of the diets in the current study was below this Hy-Line recommendation, these diets were sufficient in digestible lysine and other essential amino acids for getting a zero N balance in the body and maintaining egg production as they were formulated with inclusions of essential amino acids in lieu of intact protein. This situation was achieved despite an insufficient energy supply and a subsequent mobilization of body fat. As in the study of Roberts et al. (2007), our study indicates that a sufficient supply of essential amino acids despite a shortage of energy is able to maintain egg production in laying hens, at least on a short-term basis. This also suggests that depot fat can be easily mobilized to supplement dietary energy to maintain egg production when laying hens are in negative energy balance (Waring and Brown, 1967).

In the current study, an FHP value of 88 kcal/kg $BW^{0.75}$/d in layers was applied; this value originates from previous research using Hy-Line Brown laying hens at 27 weeks of age in open-circuit respiratory calorimetric chamber system (Wu et al., 2016). This is close to the estimated FHP obtained by regressing RE with ME intake (90 kcal/kg $BW^{0.75}$/d; Table 4-6) in the present study. The values are also consistent with those reported in White Leghorn layers by Farrell (1975) (94 kcal/kg $BW^{0.75}$/d) but higher than those reported by (Reid et al., 1978) (69 kcal/kg $BW^{0.75}$/d). Differences in the genetics of birds, in methodological approaches and in housing conditions may explain this variability. Nonetheless, this suggests that the FHP used in the current study for calculating the NE value of diets was rather accurate and representative. Apart from FHP, another indicator of energy requirement for maintenance is represented by the ME requirement for maintenance (MEm). The estimated MEm for the current experiment (120 kcal/kg $BW^{0.75}$/d, model 1) is comparable to the 120 kcal/kg $BW^{0.75}$/d value reported for Rhode Island Red laying hens (Jadhao et al., 1999) but slightly higher than the reported value of 112 kcal/kg $BW^{0.75}$/d in caged layers at 22°C (Sakomura, 2004) or the value that can be calculated from the other models applied to our data (105 and 100 kcal/kg $BW^{0.75}$/d according to models 2 and 3, respectively; Table 4-6). Again, differences in methodologies, genetics, and environment may explain these variations.

In the present study, the utilization of dietary AME intake for fat deposition was more efficient compared to being deposited as protein (100 vs 50%). This corroborates with other research (Spratt et al., 1990) indicating fat deposition to be more energetically efficient compared to protein deposition. However, the 100% efficiency for fat is not fully relevant, probably in connection with the structure and characteristics of the data set including a depot of fat in eggs from both feed energy and body fat energy. A 90% efficiency of ME for fat energy gain (in eggs) would be more relevant. In the specific case of laying hens being on a zero N balance and zero or negative fat balance in their body, the efficiencies obtained above for protein and fat correspond to efficiencies of deposition of fat and energy in the egg. Assuming then that 54 and 46% of egg energy are retained as fat and as protein, respectively, the overall efficiency of ME for egg energy gain would be about 72% (i.e. $(90 \times 54 + 50 \times 46)/100$)). In our study, the energy retained in the egg averaged 54 kcal/kg $BW^{0.75}$ /d. The corresponding ME requirement would then be about 75 kcal/kg $BW^{0.75}$/d. This should be added to the ME requirement for maintenance (105 kcal/kg $BW^{0.75}$/d in order to calculate the total ME requirement of the laying hens in this study (about 180 kcal/kg $BW^{0.75}$/d). For a 2 kg laying hen, that is equivalent to 303 kcal ME per day or 110 g of a conventional feed containing 2750 kcal ME per kg (as is).

According to a rather comparable model of energy utilization in laying hens and using Hy-Line inputs for bird weight, egg production, age, and egg mass, the energy requirements were calculated as 305 and 288 kcal/b/d at 28 and 64 weeks (Sakomura, 2004). The average AME intake in the current study ranged from 252 to 284 kcal/b/d (average 266 kcal/b/d) across the dietary treatments. Lower AME intake of layers in the current study was likely due to reduced feed intake caused by the change in diet and respiration chamber environment, even though there was a 4-d adaptation period.

As expected, the present study showed that fat contributed highest to gross energy, followed by CP and starch as shown in Table 4-7. These results are consistent with reported values of energy-yielding nutrients in food or feed (WHO, 1985; Sauvant et al., 2004; Carré et al., 2013; Wu et al., 2019), confirming also the quality of laboratory measurements and proximate analysis in the current study. Higher AME/GE ratios were observed in diets with high starch and low CP indicating starch to be efficiently absorbed and used as an energy source. The AME/GE ratio for EE, starch, and CP in the current study (78, 95 and 60%) are in agreement with those reported in broilers by Wu et al. (2019) using similar methods and facilities (113, 93 and 57%); however, the ratio was higher, and even higher than 100%, in broilers. Logically, the highest AME/GE ratios in the present study were then observed in diets with high starch ($r= 0.705$, $P < 0.001$) and low CP ($r= - 0.691$, $P < 0.001$). The positive coefficient values of starch and a negative coefficient of CP were also confirmed in the AME/GE prediction equation in our current study (Table 4-8). The EE and starch as predominating dietary nutrients for layers AME prediction equation in the current experiment were confirmed in broilers (Wu et al., 2019). As in the study of Wu et al. (2019) in broilers, this suggests that EE and starch are more digestible than other energy containing components such as non-starch polysaccharides and other fibrous components in layers and broilers. It should be noted that the corresponding ratio calculated for CP is quite difficult to interpret since it includes both undigested and endogenous protein energy and urinary energy, this latter component is highly dependent on the protein supply as compared to protein gain.

The NE/AME of nutrients in the current study was 104, 78, and 49% for EE, starch, and CP, respectively. In broilers, the NE/AME efficiency ratios were 85, 79 and 50% for EE, starch, and CP, respectively using a similar estimation method (Wu et al., 2019). The efficiency of AME for NE from digestible EE, starch, and CP was reported to be 84, 78, and 68% in broilers (Carré et al., 2002). In growing pigs, the efficiency of AME for NE was found to be 90, 82, and 60% for digestible EE, starch, and CP (Noblet et al., 1994). Logically, NE/AME is then

positively affected by dietary fat and negatively by dietary CP in all these studies. Similarly, as for Wu et al. (2019) or Noblet et al. (1994), NE can be predicted from ME (or DE) content and fat and CP contents. In addition, based on the recent prediction equations reported in broilers (Wu et al., 2019) and the equation developed for layers in the current study, it can be concluded that layers are more responsive than broilers to levels of dietary EE and CP levels for estimating NE. Thus formulation on an NE basis may have greater benefit in layers than broilers.

The energy values of ingredients used in the 16 diets were estimated by multiple linear regression according to their inclusion rate (Table 4-10). The AME/GE was 82, 81, 62 and 79% for corn, wheat, soybean meal, and canola oil, respectively in our current study. Low GE efficiency for AME of canola oil might be because of a low inclusion rate and thus the achieved efficiency may not be as accurate as expected. The efficiencies of GE for AME in the current study are similar to those reported previously for corn (85%), wheat (82%) and soybean meal (56%) (Barzegar, 2017) or those that can be calculated from feeding tables (Rostagno et al., 2011). According to the same calculation method, the NE/AME ratio was 75, 74, 62, and 92% for the corn, wheat, SBM and canola oil used in the current study. These efficiency values show that evaluation of ingredients by an NE system compared to an AME system gives higher productive energy values for high EE ingredients and lower energy values for high-protein ingredients. This also confirms the NE prediction equation obtained in our study (Table 4-9) that indicates a positive effect of EE and a negative effect of CP in addition to the ME concentration.

Two diets used in another study were used as a validation trial of the calculated NE prediction equations proposed from our study. The two diets were rather extreme in terms of theoretical NE/ME ratio, one being low in CP and high in EE and the other one, high in CP and low in EE. Eight replicates per diet in a single run with the 16 calorimetry chambers were conducted with 3 laying hens per chamber; the same ingredients as in the main experiment were used (Table 4-11). The trial procedure and measurements of ME, heat production (HP), HI and NE followed the same protocol as described in the main study. The results indicate that the partition of ME intake between HP, HI and retention of energy in this validation trial is comparable to the values measured in the main experiment performed for calculating the prediction equations (Table 4-12). The NE/AME values differed significantly between the diets, being markedly higher in the one with low CP and high EE. In connection with differences in AME content and in efficiencies of AME for NE, the difference in NE content of the 2 diets was accentuated,

which justifies the use of an NE system for laying hens, especially if the diets have rather an unconventional composition. Finally, the application of equation 15 (Table 4-9) to the measured AME values generates calculated NE values of the 2 diets that are quite close to the measured ones and with exactly the same hierarchy. Also, the comparison between measured *vs* predicted NE/AME ratio showed slight differences but similar rankings.

Table 4-10 Estimated energy values (kcal/kg DM) of layer feed ingredients.

Nutrients	Corn	Wheat	Soybean meal	Canola oil	RSD
Composition, % DM (measured)					
Protein	9.6	12.4	52.7	0	
Fat	3.1	1.7	2.0	99.9	
GE, kcal/kg DM	4455	4373	4659	9446 [1]	
Energy values, kcal/kg DM [2]					
GE	4922	4782	4173	14666	77
AME	4024	3885	2566	11608	87
AMEs	3955	3854	2734	11255	96
AMEn	3869	3753	2391	11323	95
NE	3008	2854	1579	10640	130
NE predicted [3]	3089	2925	1455	10712	
Energy efficiency, %					
AME/GE	81.8	81.2	61.5	79.1	
NE/AME %	74.7	73.5	61.5	91.7	
NE/AMEs %	76.1	74.0	57.8	94.5	
NE/AMEn %	77.7	76.0	66.0	94.0	

[1] Based on tabulated values (Rostagno et al., 2011).

[2] Estimated by multiple regression (zero intercept) of GE, AME, AMEs, AMEn and NE values of diets (n=16; kcal/kg DM) on the inclusion rates (DM/DM) of ingredients as corn, wheat, soybean meal and canola oil in the main experiment (kcal/kg, DM) (n=115 observations).

[3] NE predicted based on equation 15 (Table 4-9) with estimated AME of each ingredient based on their inclusion rate in 16 diets.

Table 4-11 Ingredients and nutrients compositions of diets of the validation experiment (as-is).

Diet	High NE/AMEn	Low NE/AMEn
Ingredient, g/kg		
Corn	300	300
Wheat	327	298
Soybean meal	118	273
Canola oil	45	7
Others [1]	192	120
Supplemented amino acids [2]	17	2
Nutrients assayed, g/kg		
DM %	89	88
CP	138	194
EE	66	31
Starch	281	263
Nutrients calculated, g/kg		
Calcium	42	42
Phosphorus, available	4.0	4.0

[1] Others provided as (the average as-is, g/kg): 100 limestone, 16 dicalcium phosphate, 2.2 salt, 2.0 Na bicarbonate, 1.0 UNE vitamin & mineral premix, 0.6 choline 60%. Alpha cellulose and Celite added in High NE/AMEn diet both at 35 gr/kg. UNE layer premix supplied per tonne: 10.0 MIU Vit A, 3.0 MIU Vit D, 20.0 g Vit E, 3.0 g Vit K, 35.0 g nicotinic acid, 12 g pantothenic acid, 1 g folic acid, 6 g riboflavin, 0.02 g cyanocobalamin, 0.10 g biotin, 5.0 g pyridoxine, 2.0 g thiamine, 8.0 g copper, 0.20 g cobalt, 0.50 g molybdenum, 1.0 g iodine, 0.30 g selenium, 60.0 g iron, 60.0 g zinc, 90.0 g manganese, 20.0 g Oxicap E2 (antioxidant).

[2] Supplemental amino acids (In high NE/AMEn and low NE/AMEn diet as-is, g/kg): 3.4 D,L-methionine ; 4.1 L-lysine HCL, ; 2.1 L-threonine, 99% ; 0.3 L-tryptophan; 2 L-isoleucine; 3 L-arginine; 2 L-valine. Low NE/AMEn diet added with only 2 g/kg D,L-methionine.

Table 4-12 Effect of diet composition on performance and energy utilization in layers; comparison of measured and calculated energy values 1.

Diet	High NE/AMEn	Low NE/AMEn	SEM	P-value
Energy balance, kcal/kg $BW^{0.75}$/d				
AME intake	174	163	3	0.044
HP [2]	132	137	1	0.040
HI	43	49	1	0.040
RE [3]				
Total	42	26	3	0.002
As protein	25	28	1	0.010
As fat	18	-2	3	< 0.001
RQ [4]	0.986	0.982	0.005	0.754
Energy values (kcal/kg DM; measured) [5]				
AME	3121	2946	24	< 0.001
AMEn	3019	2829	25	< 0.001
AMEs	3120	2974	20	< 0.001
NE	2346	2070	40	< 0.001
Predicted NE [6]	2389	2117		
NE/AME % (measured energy values)	75.2	70.3	0.1	0.002
NE/AME % (predicted energy values) [6]	76.5	71.8		

[1] Two diets, eight replicates per diet in a single run with 16 calorimetry chambers containing 3 laying hens in each; the same ingredients as in the main experiment were used. The birds in this experiment were the same young birds used in the main experiment at 40 weeks of age and average BW= 2036 g. The trial procedure and measurements of ME, heat production (HP) and dietary nutrients followed the same protocol as have been described in the main experiment.

[2] HP, heat production; Heat production (HP) (kcal) = 3.866 × O_2 consumed (L) + 1.200 × CO_2 expired (L) (Brouwer, 1965) equation.

[3, 4] Total retained energy (RE) calculated as ME intake - HP as (kcal/kg $BW^{0.75}$/d); RE as protein calculated as total retained N × 6.25 × 5.7 as (kcal/kg $BW^{0.75}$/d); RE as fat calculated as total RE - RE as protein (kcal/kg $BW^{0.75}$/d). RQ; Respiratory quotient.

[5] Based on measured values in respiration chambers (n=8 per dietary treatment).

[6] NE predicted based on equation 15 (Table 4-9) with measured AME.

4.5. Conclusion

In conclusion, the current study measured energy partition of diets in layer chickens allowing prediction equations to be developed for AME, NE, and corresponding efficiencies. By using NE prediction equations, the NE content of feedstuffs and compound feeds can be estimated according to their levels of AME, EE, and CP. This gives an opportunity for layer nutritionists to formulate feeds based on NE rather than AME. A first validation trial and the NE evaluation of a few major ingredients confirm the "quality" of the prediction equations proposed in this study. However, additional validation NE trials and also commercial validations of the NE-based formulation system must be performed to assess their applicability to the industry.

Acknowledgment

Authors acknowledge Poultry CRC and the Australian Egg Corporation Limited for supporting this study. Also, we acknowledge Shuyu Song and Nikki Yue Zhang for their technical support.

Chapter 5. Implementation of net energy evaluating system in laying hens: Validation by performance and egg quality

5.1. Introduction

The AME system has been applied for many years as a feed energy evaluation system for poultry. This system does not provide a clear picture of energy metabolism for different body purposes such as maintenance, growth or production. Thus, adjustment of AME values to attain the real available energy for different body functions may offer improved production performances and further cost savings in formulation and production. The NE system takes into account energy lost as heat and fasting heat production to measure the available energy for production. As such, its use may offer economic benefits in feed formulation and production.

Formulation of diets based on an NE system was reported in pigs (Sorensen et al., 1962; Just, 1982; Noblet et al., 1994). The application of NE system as the best estimate of the true energy value of feed was confirmed in different studies in pigs (Pérez and Sauvant, 2004; Noblet et al., 2010b). The use of an NE system has been proposed for broilers by different researchers (Fraps, 1946; De Groote, 1974; Emmans, 1994; Swick et al., 2013; Wu et al., 2019). Comparing the NE and AME system for estimating the energy value of ingredients, the calculated NE/AME efficiencies for dietary nutrients as lipid, starch and protein were 84, 78 and 68% in broilers (Carré et al., 2002). Experimental data of Noblet et al. (2010a) also confirmed that the NE/ME ratio varies with the chemical composition of diets and nutrients (fat>starch>protein=fibre); therefore, the NE systems which take into account the final stage of energy utilization should be better in predicting the performance of monogastric animals. De Groote (1974) performance experiments in broilers confirmed that the prediction of FCR was slightly more precise with NE (r^2 = 0.765) than with ME (r^2 = 0.725). However limited data exist for the implementation of an NE formulation system in layers (Chudy et al., 2003; Sakomura, 2004; Sakomura et al., 2005a). Net energy prediction equations for laying hens have been recently developed (Barzgar et al, 2018); however, this system yet to be assessed in production experiments. The current study was performed to validate the formulation of feed on an NE basis. The experiments have examined diets varying in NE/AMEn ratios on laying hens performance, egg quality parameters and energy partitioning (calorimetry measurements).

5.2. Material and methods

5.2.1. Birds and diets

The experiments were approved by the Animal Ethics Committee of the University of New England (UNE) and animals handled by following the Australian code of practice for the care and use of animals for scientific purposes (NHMRC, 2013).

Exp 1. Sixty two Hy-Line Brown hens laying at 95% hen day production (HDP), 44 weeks of age were used for an 11-week experimental period. Birds were sourced from the Glenwarrie Partnership, Tamworth, NSW, Australia. The hens were housed one bird per cage in an open-sided shed at the University of New England, Australia with 16 h light per day in the winter season. Cages were fitted with individual feeders and nipple drinkers. A completely randomized design was employed with two dietary treatments and 31 replicates per treatment. Ingredient composition of experimental diets is shown in Table 5-1 and nutrient composition in Table 5-2. Diets were formulated with corn, wheat, wheat bran, soybean meal, cold-pressed canola meal, meat, and bone meal and canola oil sourced from the local market and analyzed for nutrient content by NIRS (Evonik Amino NIR). Xylanase (Econase XT 25) and phytase (Axtra TPT 10000) were added at 0.05 g/kg in both diets in Exp 1 per manufactures recommendations using nutrient matrix for phytase. Two diets with high and low NE/AMEn ratio were formulated based on Hy-Line Brown nutrient specifications to meet digestible amino acids requirements. The birds were fed experimental diets for a 1 w adaptation period before production data were collected. Ingredient AMEn and NE values were calculated based on developed prediction equation in layers using AMEn, crude protein (CP) and ether extract (EE) content of ingredients (Barzegar et al., 2017, 2018).

In Exp 2, six hundred Hy-Line Brown pullets obtained from Glenwarrie Farm in Tamworth at 16 weeks of age were housed in the same cage facility as in Exp 1 and maintained with 16 h light per day in winter and spring seasons. The birds were fed their respective diets for an adaptation period beginning at 21 weeks of age and data collection began at 22 w of age when birds were at 78% hen day production (HDP). Mortality was 0.2% (1 bird from fatty liver syndrome) for the total of experimental period which was considered normal. A completely randomized design was used with three dietary treatments, 10 replicates per treatment and each replicate containing 10 cages of 2 birds per cage. Diets were formulated with wheat, barley, wheat bran, soybean meal, cold-pressed canola meal, and canola oil. The ingredients were analyzed for nutrient content by NIRS (Evonik Amino NIR) prior to formulation. Xylanase

(Axtra XB) and phytase (Axtra TPT 10,000) were used at 0.08 and 0.10 g/kg respectively in all diets. Nutrient matrix values were used for phytase in accordance with the manufacturer's recommendation. Treatment 1 (T1) was a normal commercial control diet formulated to have AMEn and NE levels of 3026 and 2324 (kcal/kg, DM). Treatment 2 (T2) was formulated to have the same NE (2315 kcal/kg, DM) but lower AMEn compared to T1. Treatment 3 (T3) was formulated to have the same AMEn but higher NE (2397 kcal/kg, DM) as T1. The NE/AMEn was ranked T3 > T2 > T1. All diets were formulated according to the minimum digestible amino acid specifications of Hy-Line Brown as shown in Table 5-1 (Hy-Line, 2016). Pigment (Jabiru red and yellow) added at 0.04 and 0.03 g/kg to all diets of both experiments 1 and 2. Birds were fed *ad libitum* with free access to water.

In Exp 3 (indirect calorimetric measurement) one hundred extra birds were sourced from Glenwarrie Partnership, Tamworth, NSW, Australia in addition to the same batch of the birds in Exp 2 and housed in the same shed. A total of forty five from those applied two times randomly at the age of 35 and 44 weeks in calorimetry chambers to measure AME, heat production (HP), NE and NE/AME of different diets. These birds fed the rest diet which contained the same ratio of all the 3 experimental diets when they were in poultry shed.

Table 5-1 Ingredients composition of diets (g/kg; as is basis).

	Experiment 1		Experiment 2		
Diet	**1**	**2**	**1**	**2**	**3**
Ingredient					
Corn	150	150	0	0	0
Wheat	446	221	616	434	419
Barley	0	0	100	116	114
Wheat bran	50	240	20	120	120
Soybean meal	131	137	100	54	59
Canola meal-cold pressed	100	100	50	150	150
Meat and bone meal	11	0	0	0	0
Canola oil	4.5	40.6	3.3	19.2	31.8
Limestone	98.7	101.1	95.1	94.4	94.4
Dicalcium phosphate	1.8	3.8	2.0	0.6	0.7
Salt	1.2	1.4	2.0	1.8	1.8
Others [1]	3.6	3.6	3.6	3.8	3.8
Supplemented amino acids [2]	2.4	2.1	7.8	6.2	6.1

[1] Others provided as (as-is, g/kg): 2.0 Na bicarbonate, 1.0 UNE vitamin & mineral premix, 0.6 (average) choline 60%. Xylanase (Econase XT 25) added in experiment 1 diets at 0.05 g/kg. Xylanase (Axtra XB) added to experiment 2 diets at 0.08 g/kg. Phytase (Axtra TPT 10000) added 0.05 g/kg and 0.10 g/kg for diets in experiment 1 and 2, respectively. Pigment (Jabiru red and yellow) added at 0.04 and 0.03 g/kg to all diets. UNE layer premix supplied per tonne: 10.0 MIU Vit A, 3.0 MIU Vit D, 20.0 g Vit E, 3.0 g Vit K, 35.0 g nicotinic acid, 12 g pantothenic acid, 1 g folic acid, 6 g riboflavin, 0.02 g cyanocobalamin, 0.10 g biotin, 5.0 g pyridoxine, 2.0 g thiamine, 8.0 g copper, 0.20 g cobalt, 0.50 g molybdenum, 1.0 g iodine, 0.30 g selenium, 60.0 g iron, 60.0 g zinc, 90.0 g manganese, 20.0 g Oxicap E2 (antioxidant).
[2] Supplemental amino acids (as-is, g/kg) in experiment 1 and 2, respectively : 1.4 and 1.7 D,L-methionine; 0.7 and 2.4 L-lysine HCL, 78.4%; 0.2 and 1.1 L-threonine, 99%; 1.0 L-isoleucine (only for experiment 2); 0.6 L-valine (only for experiment 2).

Table 5-2 Nutrient composition of experimental diets (g/kg, DM).

	Experiment 1		Experiment 2		
Diet	**1**	**2**	**1**	**2**	**3**
Nutrients assayed					
DM %	92	91	90	90	90
CP	202	203	187	185	181
EE	42	81	25	61	73
Crude fiber	34	44	33	61	48
Total amino acids [1]	70	58	53	57	51
Nutrients calculated					
AMEn (kcal/kg, DM)	3011	3023	3026	2949	3026
NE (kcal/kg, DM)	2288	2374	2324	2315	2397
NE/AMEn	0.760	0.785	0.768	0.785	0.792
Total digestible amino acids [2]	55	56	50	50	51
Calcium	46	46	42	42	42
Phosphorus, available	4.0	3.8	3.5	3.5	3.5

[1] Total amino acids assayed (g/kg, as is): 4.6 methionine, 10.2 lysine, 3.5 cysteine, 10.1 arginine, 2.1 tryptophan, 7.5 isoleucine, 6.9 threonine, and 8.5 valine.

[2] Total digestible amino acids (g/kg, as is): 4.0 methionine, 7.9 lysine, 6.6 methionine + cysteine, 8.4 arginine, 1.7 tryptophan, 6.0 isoleucine, 5.4 threonine, and 6.8 valine.

5.2.2. Measurements and analysis

Body weight was measured by weighing all hens at the beginning and at the end of both experiments. Feed intake, egg production, and egg weight were measured for each cage. Egg collected daily and total number and weight recorded. Feed intake measured weekly. Approximately 2 g of diet samples were dried in crucibles in a drying oven at 105 °C for 16 hr to determine DM. Samples of ingredients and feeds were finely ground to ensure homogeneity. Ingredient samples were analyzed for CP, EE, crude fiber (CF), ash, neutral detergent fiber (NDF) and acid detergent fiber (ADF) (AOAC, 2016). Fatty acid profile of all diets measured using the AOAC Official Method 996.06 (Davi, 2000) (Table 5-4). CP was measured as nitrogen using a LECO model FP-2000 N analyzer in triplicate on 0.15-g samples of ingredients and diets. LECO was calibrated by pure reference EDTA.

5.2.3. Eggshell and egg internal quality measurements

External and internal egg quality was measured on freshly-laid eggs collected on two consecutive days determined 5 and 10 times fortnightly for Exp 1 and 2, respectively. Egg external and internal quality measurements were performed following reported procedures (Samiullah et al., 2016) using TSS (Technical Services and Supplies, Dunnington, York, UK) equipment. Shell color reflectivity (%) was measured using a hand-held Konica Minolta spectrophotometer (CM-2600d Ramsey, NJ) calibrated with a white reference tile. The top (wide) part of each egg was measured. As this is a reflectance measurement, lower values indicate darker shell color. Shell breaking strength (N) and shell deformation (μm) were measured by quasistatic compression using TSS QC-SPA (50 N load cell) equipment. Individual eggs were placed horizontally in the egg holder before being compressed by the shell breaking strength machine to record the maximum compression force to break the egg and expressed as Newton. The egg shell was washed and dried overnight. Egg shell thickness (including inner and outer shell membranes) was measured at three different points around the equator of each egg using a custom-built micrometer, based on a Mitutoyo Dial Comparator Gauge Model 2109–10 (Kawasaki, Japan). An average of three thickness measurements of an egg was taken. The dried egg shell weight was determined using a digital Quintix 513-1S

balance (Sartorius Lab Instruments GmbH & Co. KG Goettingen, Germany). Yolk, albumen and shell percentage calculated as correspondent parameters values ratio to the egg weight (%).

For egg internal quality, Haugh unit and yolk color were measured. The egg was cracked carefully and the eggshell separated thoroughly. Albumen height was measured using a digital micrometer measuring one centimeter apart from yolk perimeter. Haugh unit was calculated using the formula with the records of albumen height and egg weight: $HU = 100 \log10 (H - 1.7 W^{0.37} + 7.56)$, where HU = Haugh unit, H = height of the albumen (mm) and W = egg weight (g). The yolk was separated from the albumen by rolling them down to the yolk color reader as a yolk score. Before the yolk weight was determined, the chalazae and any adhering albumen were removed and then the yolk weight measured by a digital scale. Haugh unit and yolk color were measured using the TSS QCEQCM equipment. The yolk color scoring system used in the TSS QCE-QCM is based on the 1 to 15 scale of the DSM (previously Roche) yolk color fan scoring system (DSM, 2008).

5.2.4. Indirect calorimetric measurements

The birds for Exp 3 were moved to calorimetry chambers and fed experimental diets for 4 days in closed-circuit calorimetry chambers with open lid followed by a 3-day heat production measurements. Closed-circuit calorimetric chambers design, gas exchanges, and heat production measurements followed the same procedures as described by Swick et al. (2013) and Wu et al. (2019) in broilers.

5.2.5. Calculations

Egg mass was calculated as the product of average egg weight and hen day production. FCR was calculated as the ratio of feed intake to egg mass. Albumen weight was calculated by subtracting the weight of yolk and shell from the whole egg weight. Shell, albumen and yolk percentage was calculated as their percentage of the egg weight. The AME of the diets was determined by the total collection method as previously described by Bourdillon et al. (1990a). The AME values were converted to AMEn (AME for zero N retention in body and eggs) values using a GE of 8.22 kcal per gram of N as the correction factor (Hill and Anderson, 1958). Heat production (kcal) calculated as $3.866 \times O_2$ consumed (L) + $1.200 \times CO_2$ expired (L) (Brouwer, 1965). The respiratory quotient (RQ) of each 3-day run was calculated as the ratio of liters of CO_2 expired to liters of O_2 consumed. Heat increment (HI) was calculated by subtracting fasting heat production (FHP) from total heat production (THP). A value of 88 kcal/kg $BW^{0.75}$

(370 kJ/kg $BW^{0.75}$) per bird per day was used as FHP (Wu et al., 2016). The net energy was calculated as AME intake minus HI (per bird per day) divided by feed consumed on a DM basis. Total retained energy (RE) calculated as ME intake minus HP. Total retained energy in egg (kcal) was calculated as -19.7 + 1.81 × egg weight (Sibbald, 1979). RE body calculated as total RE minus RE egg. Energy balance data as AME intake, HP, retained energy (RE) and its partition between body and egg production were expressed as kcal per kg $BW^{0.75}$ per bird per day. Energy values of diets were expressed per kg DM, and energy utilization data were expressed as percentage (%). Total N retained was calculated as N intake minus N in excreta. Nitrogen balance values were expressed as g/bird/day.

5.2.6. Statistical Analysis

All the performance, egg quality parameters, energy balance, energy values, energy utilization, and nitrogen balance data were distributed normally and thus subjected to a one-way ANOVA analysis using PROC GLM and Tukey's multiple-range test (SAS, 2010) to separate means ($P < 0.05$) when appropriate. The model included the effects of diet and age in Exp 1 and 2 for egg quality parameters and in Exp 3 for calorimetry measurements.

5.3. Results

5.3.1. Hen performance

Initial body weight measured at the beginning of both Exp 1 and 2 was the same ($P > 0.05$) (Table 5-3). Dietary treatments had no effect on body weight change of laying hens in Exp 1 and 2 ($P > 0.05$). Although feed intake (g/hen/day, as is) remained unchanged by feeding different diets in Exp 1 and 2 ($P > 0.05$), dietary treatments containing higher NE/AMEn improved FCR in both experiments ($P < 0.01$). Diets had no effect on HDP % in both Exp 1 and 2 ($P > 0.05$). The higher NE/AMEn diet increased the egg weight in both experiments ($P < 0.01$). The higher NE/AMEn resulted in numerically higher egg mass values in Exp 1 ($P > 0.05$) and significantly elevated the egg mass in Exp 2 ($P < 0.05$).

5.3.2. Egg quality parameters

The age effect was significant for all the egg quality parameters in experiment 1 and 2 ($P < 0.01$), although it had no effect on albumen weight in experiment 1 ($P > 0.05$) (Table 5-3). The weight of those eggs used for egg measurements was increased by the increased level of fat inclusions in diets ($P < 0.001$). Darker shell color (lower shell color reflectivity measured

value) was observed by feeding low-fat content diet in experiment 1 ($P < 0.01$). Dietary compositions were unable to change breaking strength, deformation, and shell thickness in both experiments ($P > 0.05$). Feeding the diets with higher NE/AMEn in both experiments resulted in higher albumen and less yolk and shell when expressed as a percentage of the egg weight ($P < 0.001$ or 0.05). Higher NE/AMEn diets improved Haugh unit ($P < 0.001$) and yolk color of eggs ($P < 0.001$) in Exp 1 and Haugh unit ($P < 0.05$) and yolk color of eggs ($P < 0.001$) in Exp 2.

5.3.3. Energy partitions of diets from indirect calorimetry measurements in Exp 3

Increased level of dietary NE/AMEn increased the AME intake ($P < 0.05$) with the same HP and HI ($P > 0.05$) (Table 5-5). Feeding the birds with the same NE/AMEn (diet 1 and 2) was unable to change total RE, RE as fat and RE in body (kcal/kg $BW^{0.75}$/d) but feeding diet 3 with higher NE/AMEn increased total RE ($P < 0.05$), RE as fat ($P < 0.05$) and RE in body ($P < 0.01$). Diets with higher NE/AMEn increased the AME ($P < 0.001$), AMEn ($P < 0.001$), and NE ($P < 0.01$) values of diets. The AME/GE changed by feeding different treatments ($P < 0.05$) but NE/AMEn was same for all diets ($P > 0.05$). Different diets NE/AMEn contents had no effect on nitrogen balance ($P > 0.05$).

Table 5-3 Performance and egg quality of laying hens feed different diets.

Diet	Experiment 1		SEM	P value (diet)	Experiment 2			SEM	P value(diet)
	1	2			1	2	3		
Performance parameters [1]									
Initial BW (g/hen) [2]	2123	2084	24	0.417	1935	1912	1940	5	0.078
Final BW (g/hen)	2159	2121	28	0.513	2270 ^{ab}	2211 ^{c}	2253 ^{bc}	8	0.012
BWT change (%)	1.5	1.8	0.3	0.751	17.4	15.7	16.1	0.3	0.081
Feed intake (g/hen/d as is)	123.1	120.5	1.3	0.321	118.5 ^{ab}	117.0 ^{bc}	115.7 ^{c}	0.3	0.001
HDP, %	95.9	94.9	0.5	0.323	95.8	95.6	95.5	0.2	0.827
Egg weight, g	60.5 ^{b}	63.4 ^{a}	0.5	0.002	59.4 ^{bc}	59.3 ^{c}	60.3 ^{a}	0.1	0.004
Egg mass, g/d	58.0	60.1	0.5	0.0504	56.9 ^{bc}	56.7 ^{c}	57.6 ^{ab}	0.1	0.030
FCR, (g/g)	2.124 ^{a}	2.007 ^{b}	0.021	0.004	2.082 ^{ab}	2.065 ^{b}	2.010 ^{c}	0.008	< 0.001
Egg quality parameters [3]									
External									
Egg weight, g	60.2 ^{b}	63.4 ^{a}	0.2	< 0.001	60.0 ^{bc}	59.8 ^{c}	60.8 ^{a}	0.1	< 0.001
Shell colour reflectivity (%)	18.3 ^{b}	19.0 ^{a}	0.1	0.003	18.2	18.0	18.2	0.1	0.460
Breaking strength, N	41.5	42.0	0.2	0.337	47.2	46.9	47.0	0.2	0.820
Deformation, (µm)	255	257	1	0.261	290	285	289	1	0.052
Shell thickness (mm)	0.418	0.419	0.001	0.355	0.410	0.412	0.409	0.001	0.180
Yolk Weight, g	16.1	16.3	0.1	0.102	14.7 ^{a}	14.4 ^{bc}	14.3 ^{c}	0.1	< 0.001
Yolk %	26.7 ^{a}	25.7 ^{b}	0.1	< 0.001	24.5 ^{a}	24.0 ^{b}	23.5 ^{c}	0.1	< 0.001
Albumen weight, g	38.2 ^{b}	41.0 ^{a}	0.1	< 0.001	39.5 ^{c}	39.7 ^{bc}	40.7 ^{a}	0.1	< 0.001
Albumen %	63.5 ^{b}	64.7 ^{a}	0.1	< 0.001	65.9 ^{c}	66.3 ^{b}	66.9 ^{a}	0.1	< 0.001
Yolk/Albumen %	42.2 ^{a}	39.8 ^{b}	0.2	< 0.001	37.2 ^{a}	36.3 ^{b}	35.2 ^{c}	0.1	< 0.001
Shell weight, g	5.9 ^{b}	6.1 ^{a}	0.1	< 0.001	5.8	5.8	5.8	0.1	0.297
Shell %	9.74 ^{a}	9.61 ^{b}	0.02	0.003	9.63 ^{bc}	9.68 ^{ab}	9.57 ^{c}	0.02	0.037
Internal									
Haugh unit	90.2 ^{b}	92.9 ^{a}	0.3	< 0.001	98.4 ^{b}	97.5 ^{c}	98.5 ^{ab}	0.2	0.012
Yolk colour score	11.4 ^{b}	11.7 ^{a}	0.1	< 0.001	10.8 ^{c}	10.9 ^{bc}	11.0 ^{ab}	0.1	< 0.001

[1] Data are means of 31 hens per 2 dietary treatment in experiment 1 and 200 hens of 10 replicates per 3 dietary treatments in experiment 2. ($P < 0.05$) by one-way ANOVA.
[2] Abbreviations: Initial BW, average body weight at the beginning of experiment (g/hen); Final BW, average body weight at the end of the experiment (g/hen); BWT change, body weight change as difference of initial and final body weight divided by initial body weight) (%); HDP, average hen day production (%); Egg weight, average egg weight (g) for

total experimental period; Egg mass as average egg weight × average HDP (g of egg/bird/day); FCR (g/g), feed conversion ratio as total feed intake (g/hen/day, as is) divided by total egg mass (g); Yolk, albumen and shell percentage calculated as correspondent parameters values ratio to the egg weight (%).

[3] From the analysis of variance with diet and age effects; the age effect was significant for all the egg quality parameters in experiment 1 and 2 ($P < 0.05$). The age had no effect on albumen weight in experiment 1 ($P > 0.05$); SEM as the standard error of the mean.

[.abc] Means within rows with different superscripts are different at different P values.

Table 5-4 Fatty acid profile (expressed as g/kg of diet, as is).

Diet	Experiment 1		Experiment 2		
	1	2	1	2	3
Myristic (14:0)	0.07	0.07	0.03	0.06	0.06
C15:0	0.03	0.04	0.02	0.03	0.04
Palmitic (16:0)	3.85	5.81	2.79	4.73	5.47
Palmitoleic (9c-16:1)	0.15	0.18	0.05	0.15	0.17
Margaric (17:0)	0.06	0.07	0.03	0.06	0.06
Stearic (18:0)	0.94	1.43	0.45	1.05	1.25
Oleic (9c-18:1)	15.39	34.73	6.95	24.68	30.10
Linoleic (18:2 n6)	12.39	21.13	9.31	17.21	19.99
Linolenic (18:3 n3)	2.23	4.79	1.32	4.04	4.80
Arachidic (20:0)	0.17	0.36	0.07	0.27	0.33
Behenoic (22:0)	0.11	0.21	0.07	0.18	0.21
Erucic [22:1 n9]	0.01	0.02	0.01	0.02	0.04
Lignoceric (24:0)	0.08	0.13	0.03	0.11	0.12

Table 5-5 Effect of diet composition on energy balance, energy values, energy utilization and N balance in layers in experiment 3.

Diet	1	2	3	SEM	P-value (Diet) [1]
Energy balance, kcal/kg $BW^{0.75}$/day [2]					
AME intake [3]	169 [c]	172 [bc]	186 [ab]	3	0.040
HP	133	134	135	1	0.795
HI	45	45	46	1	0.795
RE					
Total	36 [c]	38 [bc]	51 [a]	2	0.012
As protein	25	25	24	1	0.913
As fat	10 [c]	14 [bc]	27 [a]	3	0.021
RE_{egg}	53	54	52	1	0.215
RE_{body}	-17 [c]	-15 [bc]	-1 [a]	2	0.009
RQ	1.037 [a]	0.986 [bc]	0.982 [c]	0.006	< 0.001
Energy values (kcal/kg DM)					
AME	2968 [c]	2992 [bc]	3129 [a]	17	< 0.001
AMEn	2856 [c]	2894 [bc]	3035 [a]	18	< 0.001
NE	2182 [c]	2211 [bc]	2352 [a]	23	0.003
Energy utilization					
AME/GE	0.759 [a]	0.718 [c]	0.732 [bc]	0.004	< 0.001
NE/AMEn	0.764	0.764	0.775	0.005	0.759
NE/AME	0.735	0.739	0.752	0.005	0.444
Nitrogen balance (g/b/day)					
Intake	2.97	3.03	2.88	0.06	0.634
Excreta	1.72	1.82	1.68	0.04	0.279
Retained	1.25	1.21	1.20	0.05	0.918

[1] From the analysis of variance with diet and age effects; age effect was significant for RE egg and RQ ($P < 0.05$).
[2] Each value represents the mean of 2 replicates (runs) for each treatment (diet) (n=10) during 3-days respiratory measurements (3 layers per calorimetry chambers).
[3] Abbreviations: GE, gross energy; AME, apparent metabolizable energy as [(FI× GEf) - (E × GEe)] / FI (kcal/kg DM of diet); AMEn, AME corrected for zero N retention as = [AME - [8.22× (Ni - Ne)]] / FI (kcal/kg DM of diet); where GEf and GEe are the gross energy of feed and excreta (kcal/g DM); FI = feed intake (g DM/d/hen); E = excreta output (g DM/day/hen); 8.22 as nitrogen correction factor (kcal/g); HI, heat increment as HP - FHP (kcal/kg $BW^{0.75}$/d); HP, heat production (kcal) as 3.866 × O_2 consumed (L) + 1.200 × CO_2 expired (L) (Brouwer, 1965); Respiratory quotient (RQ); Net energy (NE) values expressed based on DM of the feed (total collection method); NE calculated as fasting heat production + RE. Total retained energy (RE) calculated as ME intake - HP; RE as protein calculated as total retained N × 6.25 × 5.7; RE as fat calculated as total RE - RE as protein; Total retained energy in egg (RE egg; kcal) calculated as -19.7 + 1.81 × egg weight (Sibbald, 1979); RE body calculated as total RE - RE egg. Total N retained calculated as N intake - N in excreta (g/b/d). Retained N in egg calculated as 1.936 (N% in the egg) × egg mass (Miranda et al., 2015).
[abc] Means within rows with different superscripts are different at different P values.

5.4. Discussion

The different dietary composition had no effect on body weight change in current experiments. This is consistent with the view that layer body weight is hardly changed by dietary compositions (Leeson and Summers, 2009). Hens are able to adjust their feed intake according to the dietary energy concentration as an increased level of dietary energy results in lower feed intake. In the current study, feed intake was similar to the breeder performance recommendations (Hy-Line, 2016) and was higher in Exp 1 as it was carried out in all winter season compared to Exp 2 which was conducted in winter and spring. Regardless of the effect of temperature and seasonal effects in open shed, the birds received a diet with higher dietary energy consumed less feed in both experiments 1 and 2. Lower feed intake and higher egg production have also been reported with an increased level of dietary energy and fat contents in different studies (Sell et al., 1987; Pérez-Bonilla et al., 2012).

The egg weight is affected by the body weight of hens and dietary nutrients. The hens with higher initial body weight expected to lay heavier eggs at the beginning of production (Summers and Leeson, 1983). Although the average initial body weight of hens in both experiments 1 and 2 was 9% higher than recommended values by the breeder company (Hy-Line, 2016), the egg weight was lower than those performance standards in breeder manual (Figure 5-1). The extraordinary size of eggs is not favorable for the egg industry as it increases the chances for broken eggs. Diets with higher NE/AMEn increased the egg weight at the favorable size range in the current study. The increased level of dietary NE/AMEn is associated with higher inclusion of fat with unsaturated and saturated fatty acids. Linoleic acid availability is a necessity for lipoprotein synthesis for developing ova (March and Macmillan, 1990). The

birds fed diets with higher linoleic acid content (Table 5-4) laid bigger eggs with higher egg mass and albumen weigh compared to their counterparts that fed with less linoleic acid in both experiments 1 and 2. The minimum required linoleic acid for brown layers was reported to be 11.5 g/kg of diet to maintain their production (Grobas et al., 1999). When the linoleic acid provided more than the marginal requirement of laying hens, the extra amount of dietary fat and linoleic acids results in further increase in egg weight (Pérez-Bonilla et al., 2012). Smith and Pourezza (1989) confirmed that the higher linoleic acid content of diet from 10.0-15.5 g/kg resulted in bigger egg size, higher albumen weight, and better egg mass.

The fat inclusion improved the FCR in this study as diets containing higher fat showed better weekly FCR compared to other treatments (Figure 5-2). Fat application in diets may increase the nutrients transition time in gastro intestinal tract which enhanced their digestibility and thus FCR (Mateos and Sell, 1980, 1981). Grobas et al. (1999,) observed that increased level of saturated fatty acid content in diets was an important factor for improved FCR and egg production in laying hens when the linoleic acid content maintained at constant level.

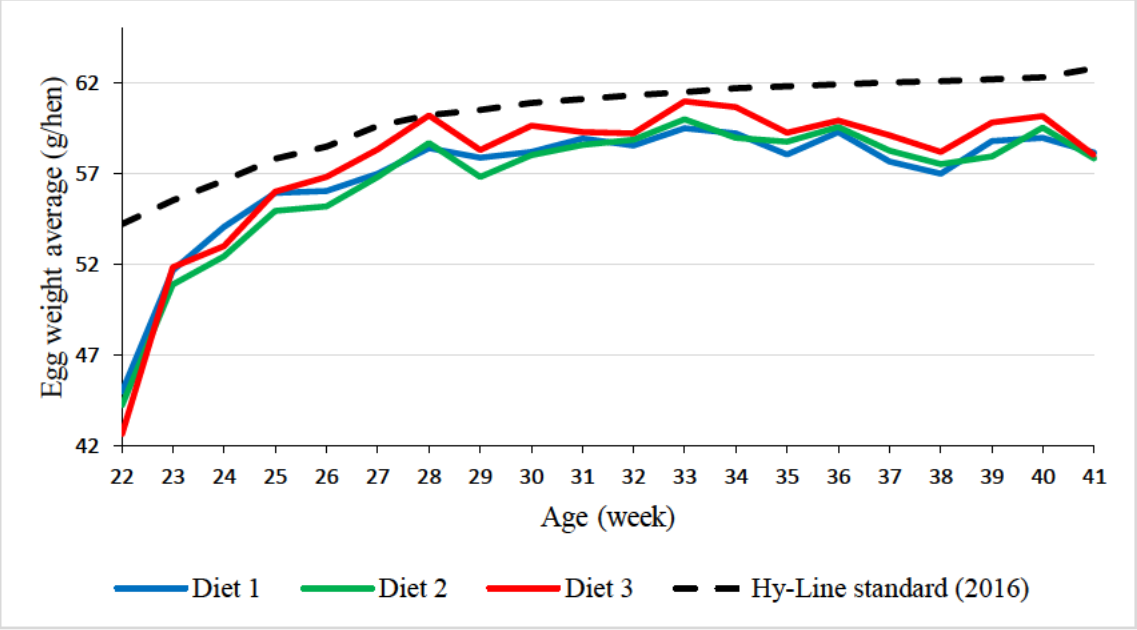

Figure 5-1 Different dietary treatments effect on weekly egg weight variations in experiment 2.

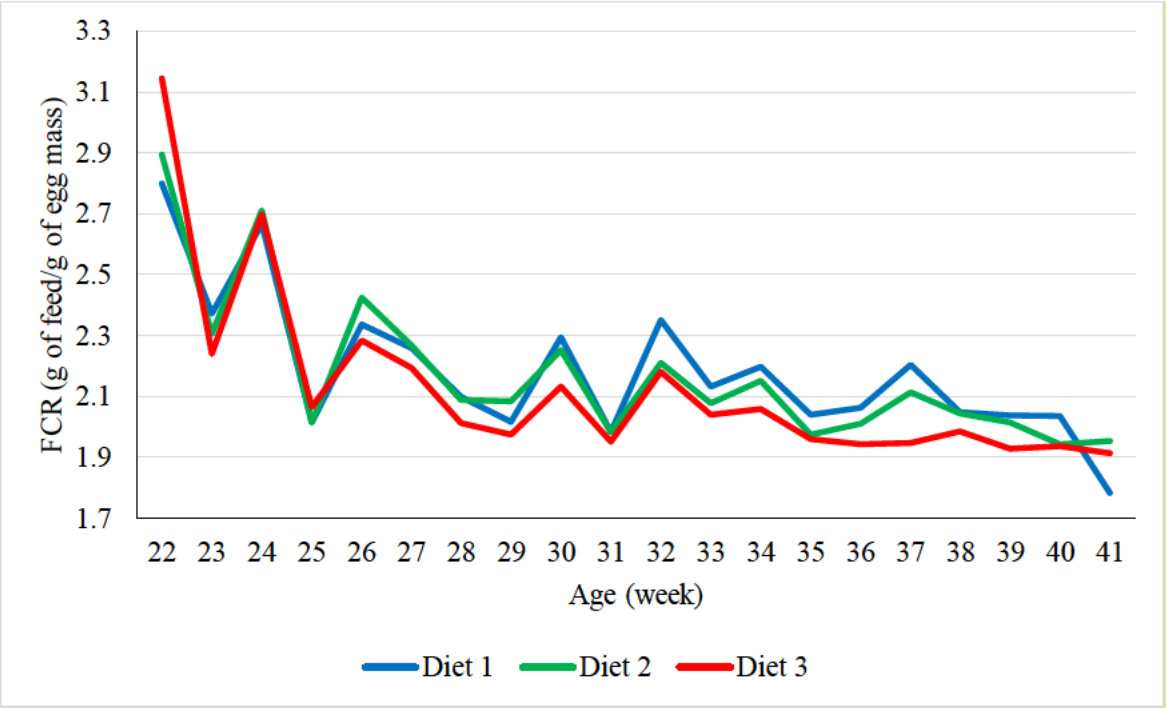

Figure 5-2 Different dietary treatments effect on weekly FCR variations in experiment 2.

Egg shell brown color is important for retailers and consumers. However, there is no correlation between egg shell color and other external or internal egg quality parameters (Yang et al., 2009). The birds fed diet 2 in Exp 1 of the present study produced bigger eggs with the lighter shell color as they deposited the brown eggshell pigments of on the bigger shell surface. When laying hens laid bigger eggs (get older) then the main factor for eggshell color brownness was egg size (Odabaşi et al., 2007).

Higher NE/AMEn diets with an increased level of fat decreased yolk/Albumen ratio in the present study. The yolk/albumen ratio is an age-dependent parameter, i.e., higher in younger birds compared to older birds also observed in the current study with younger (Exp 2) and older (Exp 1) birds. Whitehead (1995) observed that following the fat inclusion in diets of young layers below the 30 weeks of age the egg weight increase is mostly because of both increases in yolk and albumen weight compared to older layers with which egg weight increase was attributed to only higher albumen weight. This researcher assumed that dietary fat inclusion with certain unsaturated fatty acids enhances the production of estrogen hormone which resulted in more albumen secretion.

Shell % decreased in parallel with egg weight increase in the current study. Egg weight increase is not proportional to egg shell weight which resulted in lower shell % (Roberts, 2004). In

addition, more dietary fat inclusion and fatty acids resulted in saponification of calcium, less Ca deposition and less egg shell % in layers (Atteh and Leeson, 1983, 1984; Pérez-Bonilla et al., 2012)

The most important internal quality parameter is measured the albumen viscosity by albumen height and Haugh unit. Laying hens feed efficiency can be a determining factor for the Haugh unit. In both Exp 1 and 2 the higher Haugh unit was observed in layers with better feed efficiency. Akter et al. (2018) observed that high feed efficient laying hens showed the increased systemic level of antioxidant enzyme which enables them to produce eggs with better albumen quality.

The preferred yolk color for consumers varies in different parts of the world but in Australia, the favored value is about 11 on the Roche scale (Roberts, 2004). The birds in both Exp 1 and 2 had the same amount of supplemental pigments in their diets, so all the yolk color difference might be because of the fat effect in diets. The higher NE/AMEn diet with more fat inclusion diets showed better yolk color in the current study. Yolk color is mainly determined by xanthophyll, the pigments responsible for yolk color, are known as fat-soluble nutrients. Higher fat inclusion provides a better situation for these pigments absorption in hens guts (Lázaro et al., 2003; Pirgozliev et al., 2010).

The efficiency of diet AME for NE (NE/AME) is high when heat increment is low. Higher NE/AMEn diets with the beneficial effect of certain nutrients or ingredients resulted in increased RE, NE, NE/AME and enhanced laying performance in the present study. Formulating diets with different sources of protein by-products (soybean meal and canola meal) to meet layers protein requirements might affect the NE/AME efficiency of diets. Canola meal is known for lower AME compared to SBM and SBM higher protein and lower fiber justifies this difference in AME value in chickens diets (Khajali and Slominski, 2012). Broilers fed diets formulated based on SBM showed improved performance and nutrients digestibility with higher RE, NE, and NE/AME compared to their counterparts which fed diets formulated with expeller canola meal (Toghyani et al., 2017).

In the Exp 3, the increased dietary NE/AMEn is almost attributed to the added dietary fat which is known for sparing the protein and amino acids towards the production purposes and nutrient digestibility improvement. Diets with higher NE/AMEn showed higher total retained energy and RE as fat. This might be because of the extra caloric effect which has been reported to be responsible for improved energy utilization of other dietary nutrients in laying hens (Mateos

and Sell, 1980). The retained energy as protein was the same for all the birds in our study. Regardless of energy balance status, the layers prioritize to retain dietary energy to meet protein demands for egg production or body maintenance (Farrell, 1975). Birds fed the diets with lower NE/AMEn retained less RE in the body (or higher body energy mobilization). On the other hand, the AME/GE decreased in parallel with an increased level of fat in diets. Wiseman et al. (1986) observed that the higher inclusion rate of fat decreased the calculated AME of fat and also dietary energy as the broiler response to added dietary fat was curvilinear. . The main difference in dietary composition of this study attributes to a various amount of fat levels in diets which were unable to affect HP, HI and NE/AME in the current study. Formulating diets with different levels of both fat and CP (as contributory factors to HI) significantly changed the HI of diets in broilers (Wu et al., 2019). In addition, a major factor of differentiation between NE and ME in other animal species comes from fiber digestion, while poultry is not able to digest a significant amount of fiber (Carré et al., 1995). Many researchers observed that feeding diets with different dietary compositions were not able to change HI and NE/AME in poultry (Noblet et al., 2010b; Carré et al., 2014).

5.5. Conclusion

To conclude, the current study demonstrates that higher NE/AMEn diets improved the egg mass and FCR of hens at different production stages. Higher NE/AMEn diets also enhanced egg quality external and internal parameters. This can be attributed to the increased level of dietary NE/AME which validates NE-based diet formulation advantages compared to ME system for laying hens. Calorimetry measurements confirmed that diets with higher NE/AMEn utilized more dietary energy and nutrients for egg production resulting in improved performance of laying hens. The efficiency of dietary AME for NE will be varied by diets nutrients composition in particular fat, CP, dietary ingredients, ingredients source, feed processing and feed form for energy partitioning measurements. These are important parameters which should be considered by layers nutritionists to attain more NE/AME at the time of diet formulation.

Acknowledgment

Authors acknowledge Poultry CRC and the Australian Egg Corporation Limited for supporting this study.

Chapter 6. Peroxisome proliferator-activated receptor gamma (PPARG) upregulation in laying hens fed different NE/AME diets

6.1. Introduction

In animals, energy provided by feed is used for absorption, digestion, and metabolism of nutrients. Overall, energy balance is defined by two main components as energy intake and energy expenditure. In endothermic animals, the constituents of energy expenditures are basal metabolism, physical activity, and body thermoregulation. Body thermoregulation refers to changes in heat dissipation in response to environmental temperature, nutritional status, and disease (Puigserver and Spiegelman, 2003). PPARG plays a regulatory role in fatty acid storage and glucose metabolism by mediating the expression of fat-specific genes in adipocyte differentiation and function in mammals (Tontonoz et al., 1995) and chicken (Wang et al., 2008). Any changes to adenosine diphosphate/ adenosine triphosphate (ADP/ATP) ratio in mitochondria will be sensed resulting in activation of the PPARG coactivators (Puigserver and Spiegelman, 2003). Adenosine monophosphate-activated protein kinase (AMPK) is an enzyme that plays a leading role in cellular energy homeostasis and appetite regulation. AMPK is activated (and its associated subunit, PRAKG2) when there is an increased level of AMP/ATP resulting in higher glucose uptake, increased glycolysis, greater fatty acid oxidation and stimulates mitochondrial biogenesis to compensate for low levels of cellular energy (Hardie et al., 2006). Dietary nutrients are oxidized during oxidative phosphorylation and ATP is released as an active form of energy via the electron transport chain (ETC) inside mitochondria. ETC consists of five multi subunit enzyme complexes (I, II, III, IV, and V) and two electron carriers. These complexes transfer electrons from electron donors to electron acceptors via redox reactions, and couples this electron transfer with the transfer of protons across inner mitochondrial membrane (Lehninger et al., 1993). Adenine nucleotide translocator (ANT), also known as the ADP/ATP translocase, exchanges free ATP with free ADP across the inner mitochondrial membrane. Accordingly, ANT is the most abundant protein in the inner mitochondrial membrane (Li et al., 1989). Both ACACA and AMPK are the encoding genes which are involved in energy metabolism and fatty acid synthesis. Energy stores in the form of triacylglycerols are depleted during hydrolysis to glycerol and fatty acids. Fatty acids are beta-oxidized with resulting acetyl CoA transported to mitochondria. Acetyl-CoA is fuel for Krebs

cycle for ATP production. Acetyl-CoA carboxylase converts acetyl-CoA to malonyl-Co A (an essential substrate for fatty acid biogenesis) (Berg et al., 2002). AMPK also inhibits the activity of acetyl-CoA carboxylase resulting in preventing the fatty acid biosynthesis (Richards and Proszkowiec-Weglarz, 2007).

Sato et al. (2004) reported PPARG as a pivotal gene for energy partitioning as fat deposition and egg production in laying hens. High-fat diets increased the PPAR γ expression in adipose tissue of normal mice and induced PPAR γ^2 mRNA expression in obese mice livers; however, fasting downregulated PPAR γ (Vidal-Puig et al., 1996). It has been also reported that the body energy demands increase AMPK activity in all eukaryotic cells (Hardie et al., 2003), while Lei and Lixian (2012) showed that fasting increased the hypothalamic activity of AMPK in broilers. On the other hand, the higher energy provision by diet decreases the AMPK function. Increased level of dietary α-lipoic acid decreases hypothalamic AMPK activity, and in turn results in less feed intake and body weight in broilers (El-Senousey et al., 2013). The function of inner mitochondrial membrane ETC enzymes depends on the fluidity of mitochondrial membrane in particular complex V (Robblee and Clandinin, 1984). Feeding high-fat content diets increased mitochondrial respiration and complex V activity, and decreased complex III and IV activities in rats (Aoun et al., 2012). On the other hand, high-protein diets resulted in less body fat retention and also decreased oxidative phosphorylation and less ATP synthesis in heart and liver of chicken (Toyomizu et al., 1992). Uncoupling the oxidative phosphorylation is a compensatory mechanism for endotherms to produce heat immediately after exposing to highly decreased ambient temperature and fatty acids (natural uncouplers) possibly as uncouplers of oxidative phosphorylation in mitochondria (Skulachev, 1991). Similarly, the avANT (uncoupling protein) as a key gene controls the heat production for endothermy in chicken (Walter and Seebacher, 2009). The avian mitochondrial DNA encodes 22 tRNA, 2 rRNA, and 13 respiratory chain proteins (Desjardins and Morais, 1990). Peroxisome proliferating factor peroxisome proliferator-activated receptor γ coactivator-1 α (PGC1-α), the master regulator of mitochondrial biogenesis (Nisoli et al., 2003), and PPAR-γ stimulate mitochondrial biogenesis to meet the cellular energy production as flared up by AMPK (Bottje and Kong, 2013). The role of PPAR-γ as a key regulator for mitochondrial biogenesis in response to exercise, temperature, diet, and genetics is well-documented in mammals (Hudson et al., 2017).

The jejunum is the main site for absorption and digestion of main dietary nutrients such as fat, protein, and starch in chickens (Svihus, 2014). The jejunum is particularly defined as a most important site for lipid and fatty acids absorption in poultry (Krogdahl, 1985). Also, digestive enzymes activity was reported higher in this part of the intestine as the opening of pancreatic ducts discharges near the anterior jejunum (Denbow, 2015). Much of the digestion of the feed and all of the absorption of the nutrients takes place in the small intestine; hence, jejunum mitochondrial function is important for the observation of dietary effect on energy expenditure and nutrient utilization.

The objective of the study was to find any potential link between dietary NE/AME effects and correspondent dissipated heat (heat increment) on the genes involved in cellular energy homeostasis in layers mitochondria as the primary sites for nutrients digestion, energy metabolism, and ATP production.

6.2. Material and methods

6.2.1. Birds and diets

The study was approved by the Animal Ethics Committee of the University of New England (UNE) and designed to follow the Australian code of practice for the care and use of animals for scientific purposes (NHMRC, 2013).

Six hundred hens Hy-Line Brown pullets obtained from Glenwarrie Farm in Tamworth were housed at 16 w of age in the caged shed facility at the University of New England in Australia (Barzegar et al., 2018). The experiment conducted from 22 - 42 weeks of age when the hen day production (HDP) was 78% from start and up to 96% at peak lay. A completely randomized statistical design performed with three diets (see below) as treatments, 10 replicates each; each replicate composed of 10 cages housing 2 birds each.

The main ingredients used for making diets were wheat, barley, wheat bran, soybean meal, and cold-pressed canola meal (Table 6-1). Canola oil was also used to provide energy. The ingredients were analyzed for nutrient content by NIRS (Evonik Amino NIR) prior to formulation. Diets were formulated according to the minimum digestible amino acid specifications of Hy-Line Brown (Hy-Line, 2016) (Table 6-2). Diet 1 contained 187 and 25 g/kg (DM) CP and EE, diet 2 contained 185 and 61 g/kg (DM) CP and EE, and diet 3 contained 181 and 73 (DM) CP and EE. Diets were formulated with phytase (Axtra TPT 10,000) and

xylanase (Axtra XB) with the dosages at 0.08 and 0.10 g/kg respectively in all diets. Birds were fed *ad libitum* with free access to water.

Table 6-1 Ingredients composition of diets (g/kg; as-is basis).

Treatment	Low NE/AME	Medium NE/AME	High NE/AME
Ingredient			
Wheat	616	434	419
Barley	100	116	114
Wheat bran	20	120	120
Soybean meal	100	54	59
Canola meal-cold pressed	50	150	150
Canola oil	3.3	19.2	31.8
Limestone	95.1	94.4	94.4
Dicalcium phosphate	2.0	0.6	0.7
Salt	2.0	1.8	1.8
Others [1]	3.6	3.8	3.8
Supplemented amino acids [2]	7.8	6.2	6.1

[1] Others provided as (as-is, g/kg): 2.0 Na bicarbonate, 1.0 UNE vitamin & mineral premix, 0.6 (average) choline 60%. Xylanase (Axtra XB) added to experimental diets at 0.08 g/kg. Phytase (Axtra TPT 10000) 0.10 g/kg. Pigment (Jabiru red and yellow) added at 0.04 and 0.03 g/kg to all diets. UNE layer premix supplied per tonne: 10.0 MIU Vit A, 3.0 MIU Vit D, 20.0 g Vit E, 3.0 g Vit K, 35.0 g nicotinic acid, 12 g pantothenic acid, 1 g folic acid, 6 g riboflavin, 0.02 g cyanocobalamin, 0.10 g biotin, 5.0 g pyridoxine, 2.0 g thiamine, 8.0 g copper, 0.20 g cobalt, 0.50 g molybdenum, 1.0 g iodine, 0.30 g selenium, 60.0 g iron, 60.0 g zinc, 90.0 g manganese, 20.0 g Oxicap E2 (antioxidant).

[2] Supplemental amino acids (as-is, g/kg): 1.4 and 1.7 D,L-methionine; 0.7 and 2.4 L-lysine HCL, 78.4%; 0.2 and 1.1 L-threonine, 99%; 1.0 L-isoleucine; 0.6 L-valine.

Table 6-2 Nutrient composition of experimental diets (g/kg, DM).

Treatment	Low NE/AME	Medium NE/AME	High NE/AME
Nutrients assayed			
DM %	90.3	89.9	90.0
CP	187	185	181
EE	25	61	73
Crude fiber	33	61	48
Total amino acids [1]	53	57	51
Nutrients calculated			
Calcium	42	42	42
Phosphorus, available	3.5	3.5	3.5
Energy values(measured)			
AME (kcal/kg, DM)	2968	2992	3129
NE (kcal/kg, DM)	2182	2211	2352
NE/AME	0.735	0.739	0.752

[1] Total amino acids assayed (g/kg, as is): 4.6 methionine, 10.2 lysine, 3.5 cysteine, 10.1 arginine, 2.1 tryptophan, 7.5 isoleucine, 6.9 threonine, and 8.5 valine.

6.2.2. Performance, fat pad, and energy of the feed

Body weight (BW) was measured by weighing all hens at the beginning and at the end of the experiment. Two birds from each replicate (20 hens per dietary treatment) were selected randomly, weighed and killed for abdominal fat pad measurements and tissue sampling at the end of the experimental period (42 weeks of age). Abdominal fat pad (g) were excised and weighed and reported as an average for two birds per replicate. The ratio of fat pad to BW (%) was calculated accordingly. The proximal part of jejunum was excised and immediately frozen in liquid N_2 and then stored at -80 °C until required. AME, heat production (HP) and NE of diets were measured in indirect calorimetry according to a previous study (Barzegar et al., 2018). AME intake was calculated as dietary AME (kcal/kg diet, DM) multiplied by feed intake (g, DM) and expressed as kcal/$BW^{0.75}$/d.

6.2.3. DNA and RNA extraction

Total DNA was extracted from approximately 65 mg of proximal jejunum tissue using ISOLATE II Genomic DNA Kit (Bioline, Sydney, Australia) as per manufacture's protocol. The quantity and purity of total DNA were determined using NanoDrop ND-8000 (Thermo

Fisher Scientific, Waltham, USA). The extracted DNA was stored at -20°C until required for downstream applications.

Total RNA was extracted from approximately 90 mg of proximal jejunum tissues at week 42 using TRIsure^{TM} (Bioline, Sydney, Australia) following the manufacturer's instructions. The total RNA was further purified using ISOLATE II RNA Mini Kit (Bioline, Sydney, Australia) as per the manufacturer's instructions. For each RNA sample, NanoDrop ND-8000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA) was employed to analyze the purity and quantity of the RNA. RNA integrity was evaluated with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Waldbronn, Germany) using RNA 6000 Nano kit as per the manufacturer's protocol. The RNA Integrity Number (RIN) values of the samples ranged from 7.7 to 9.7 in this study were considered high in quality.

6.2.4. cDNA synthesis

Approximately 1 μg RNA was reversely transcribed into cDNA using the SensiFAST cDNA Synthesis Kit following manufacturer's instructions. The cDNA was diluted 10 times with nuclease-free water and stored at -20 ˚C for further analysis.

6.2.5. Primer sources

The NCBI primer tool (https://www.ncbi.nlm.nih.gov) was employed to design the primers for target genes in this study. The primers for the reference genes and mitochondrial quantification were sourced from previously published studies in chickens (Yin et al., 2011; Kuchipudi et al., 2012; Samiullah et al., 2017). Table 6-3 shows the primers that were used in the current study. The specificity for each pair of primers was evaluated with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Germany) using Agilent DNA 1000 Kit (Agilent Technologies, Inc., Germany) following the manufacturer's protocol. The amplification efficiency of each pair was also evaluated and the specific primer pairs with high efficiency were used in the current study.

Table 6-3 Sequences of primers used for quantitative real-time PCR.

Gene	Gene full name	Primer sequence (5'-3')	Ta	size (bp)	Accession No.	Reference
PPARG	Peroxisome proliferator-activated receptor gamma	F- TGGTTGACACAGAAATGCCGT R- CCATTTTGATTGCACTTTGGC	60	234	NM_001001460.1	This study
PRKAG2	Protein kinase AMP-activated non-catalytic subunit gamma 2	F- ACGCTGGAATTACAAACCTGC R- ACTTGGTTGTGGTCTTGGTGG	60	73	NM_001278143.1	This study
ND2	NADH dehydrogenase subunit 2	F-AGGCTCCTCCCTAATCACTGC R-CCCATTCAGCCTCCGATTAG	60	147	JQ970529.1	This study
SDHA	Succinate dehydrogenase complex flavoprotein subunit A	F-ATACGGGAAGGAAGGGGTTG R-TGCTGGGGTGGTAAATGGTG	60	74	NM_001277398.1	This study
UQCRFS1	ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1	F- CATCAGCCTCAACGCACCT R- ATCACATCTTCACGACGGTAGG	61	90	NM_001005843.1	This study
COX III	Cytochrome c oxidase subunit III	F-AGTCACCGTTACATGGGCTCA R-AGAGTTAGTGCGTGGATGGCTT	60	72	KC847880.1	This study
ATP5A1W	ATP synthase subunit alpha	F-GGCAATGAAACAGGTGGCAG R-GGGCTCCAGCTTGTCTAAGTGA	60	232	XM_429118.5	This study
avANT	ATP/ADP antiporter	F-GTCAGGACGCAAAGGAGCTG R-AGCACGAGCACGAAAGCAC	60	147	AB088686.1	This study

Gene	Gene full name	Primer sequence (5'-3')	Ta	size (bp)	Accession No.	Reference
ACACA	Acetyl-CoA carboxylase alpha	F-AGACAAGGCTGCCCGTGAG	60	181	NM_205505.1	This study
		R-GAAATTCCCTCTTCTGTGCCA				
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F:GAAGCTTACTGGAATGGCTTTCC R: CGGCAGGTCAGGTCAACAA -	60	66	NM_204305.1	(Kuchipudi et al., 2012)
ND4 [1]	NADH dehydrogenase subunit 4	F- CGCAGGCTCCATACTACTCG R- TTAGGGCACCTCATAGGGCT	60	137	NC_001323.1	(Samiullah et al., 2017)
GAPDH [2]	Glyceraldehyde-3-phosphate dehydrogenase	F- GGTCACCAAGAAGGTGGAGA	63	137	NC_006088.3	(Samiullah et al., 2017)
		R- GACAGTGCCCTTGAAGTGTC				
HMBS	Hydroxymethylbilane synthase	F- GGCTGGGAGAATCGCATAGG	60	131	XM_417846.2	(Yin et al., 2011)
		R- TCCTGCAGGGCAGATACCAT				

[1] Gene was used to amplify a fragment of mtDNA.
[2] Gene was used to amplify a fragment of gDNA.

6.2.6. Quantitative PCR

Quantitative PCR was performed in a Rotorgene 6000 real-time PCR machine (Corbett Research, Sydney, Australia) using an SYBR Green kit SensiFAST™ SYBR® No-ROX (Bioline, Sydney, Australia). The amplification cycle (Cq) value for each gene was imported into qBase+ version 3.0 (Biogazelle, Zwijnbeke, Belgium) software and analyzed against two optimized reference genes (GAPDH and HMBS) in this study. The optimized reference genes were used to normalize the target genes in the jejunum. Then, the relative quantification of the target genes that obtained by arithmetic means method in qBase+ was exported to SAS (2010) for further analysis.

6.2.7. Mitochondria quantification

Mitochondria were enumerated according to the method described by (Samiullah et al., 2017). Briefly, qPCR was performed to enumerate mitochondrial DNA counts using the SensiFAST™ SYBR® No-ROX Kit (Bioline, Eveleigh, Australia). Quantitative PCR reaction was performed in a total volume of 20 µL with a Rotor-Gene 6000 thermocycler (Corbett Research, Sydney, Australia). The reaction consisted of 10 µL 2× SensiFAST™ SYBR® No-ROX mix, 400 nM each of the primers, 6.4 µL RNase-free water and 2 µL of 10^{-2} diluted DNA. Serial dilutions of linearised plasmid DNA (TOPO® TA Cloning® Kit for sequencing, ThermoFisher Scientific, Australia) inserted with ND4 and GAPDH amplicons were used to construct a standard curve. The cloned plasmid DNA amplification cycle (Cq) values were then used to quantify the mtDNA and gDNA. The equation (count of mtDNA)/(count of gDNA/2) was used to calculate the counts of mtDNA per cell.

6.2.8. Statistical Analysis

All the data of performance parameters, mRNA gene expression and mitochondrial counts were distributed normally and thus subjected to a one-way ANOVA analysis using PROC GLM and Tukey's multiple-range test (SAS, 2010) to separate means ($P < 0.05$) when appropriate.

6.3. Results

6.3.1. Laying hens performance and energy metabolism

Dietary treatment did not affect BW (g), abdominal fat (g), and abdominal fat pad/BWT (%) ($P > 0.05$) (Table 6-4). Birds fed a diet containing high NE/AME increased the AME intake compared to those consuming low NE/AME diets (kcal/$BW^{0.75}$/d) ($P < 0.05$).

Table 6-4 The effect of different treatments on performance parameters [1, 2].

Treatment	Low NE/AME	Medium NE/AME	High NE/AME	SEM	P value
Performance parameters					
BWT (g)	2181	2179	2180	23	0.999
Abdominal fat pad (g)	122	126	128	4	0.839
Abdominal fat pad /BWT (%)	5.55	5.71	5.84	0.16	0.763
AME intake (kcal/BW $^{0.75}$/d)	169 ^{c}	172 ^{bc}	186 ^{ab}	3	0.040

[1] Data are means of 20 hens per each dietary treatment. ($P < 0.05$) by one-way ANOVA.
[2] Abbreviations: BWT (g), the average body weight of two birds which were killed for fat pad weight measurements; Abdominal fat pad (g), abdominal fat pad weight; Abdominal fat pad/BWT (%), the ratio of abdominal fat pad to the correspondent body weight.

6.3.2. mRNA gene expressions and Mitochondrial counts

The PPARG expression was higher in the jejunum of MR layers compared to LR birds ($P < 0.05$) (Table 6-5). Dietary NE/AME content did not alter the expression of the genes PRKAG2D, ND2, SDHA, UQCRFS1, COXIII, ATP15W, avANTP and ACACA ($P > 0{\cdot}05$). Similarly, dietary treatments did not change the mitochondrial count per cell ($P > 0.05$).

Table 6-5 The effect of different treatments on mRNA gene expressions [1].

Gene [2]	PPARG	PRKAG2D	ND2	SDHA	UQCRFS1	COXIII	ATP15W	avANTP	ACACA	mt/cell
Treatment										
Low NE/AME	0.894 [c]	1.093	1.047	1.063	1.104	1.072	0.982	1.006	0.971	93.3
Medium NE/AME	1.139 [ab]	1.016	0.990	1.002	1.018	1.012	1.077	1.041	1.101	80.3
High NE/AME	1.045 [bc]	1.572	1.017	0.984	0.995	0.995	0.981	1.056	1.006	98.4
SEM	0.034	0.176	0.031	0.032	0.052	0.040	0.023	0.040	0.034	4.5
P value	0.009	0.385	0.770	0.572	0.666	0.718	0.164	0.874	0.278	0.257

[1] Data are means of 15 hens per each dietary treatment. ($P < 0.05$) by one-way ANOVA.

[2] Abbreviations: PPARG, Peroxisome proliferator-activated receptor gamma; PRKAG2, Protein kinase AMP-activated non-catalytic subunit gamma 2; ND2, NADH dehydrogenase subunit 2; SDHA, Succinate dehydrogenase complex flavoprotein subunit A; UQCRFS1, ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1; COX III, Cytochrome c oxidase subunit III; ATP5A1W, ATP synthase subunit alpha; avANT, ATP/ADP antiporter; ACACA, Acetyl-CoA carboxylase alpha; mt/cell, mitochondrial count per cell .

6.3.3. Discussion

The MR diet upregulated the PPARG expression in the current study; in addition, the HR diet also increased the PPARG expression numerically. It was observed that birds fed MR and HR diets had higher AME intake and this may indicate that expression of PPARG is related to AME intake and higher AME intake may lead to increased expression of PPARG in layer hens. It has been reported that feed restriction and low energy intake reduced PPAR-γ2 mRNA levels in rats, mice, and humans (Vidal-Puig et al., 1996; Vidal-Puig et al., 1997; Arai et al., 2004). This agrees with our finding of the relation between AME intake and PPARG expression level. As MR and HR diets were higher in EE, it is likely that the dietary fat content may contribute to the expression level of PPARG. Dietary fats are important modulators of PPARG and this may relate to the regulation of energy balance (Cecil et al., 2006). Kliewer et al. (1997) suggested that PPAR α and γ are physiological sensors for lipid homeostasis which can be triggered by dietary fatty acids. Sato et al. (2004) reported that PPARγ expression was higher when chickens fed linoleic compared to those fed oleic acid; further, the level of PPARγ mRNA increased in the liver compared to adipose tissue during the laying period which might be because of more demands for lipogenesis and fat deposition in developing oocysts. The same researchers also observed that the body fat deposition as a depo tissue can be affected by PPARG function in the body.

The AMPK is the fundamental regulator of energy balance and food intake within the cell of the animal body (Minokoshi et al., 2004). The AMPK is stimulated by increased AMP/ATP ratio and enhances energy production by stimulating mitochondrial biogenesis (Hardie et al., 2003). As an immediate action to restore cellular energy charge, AMPK enhances the availability of carbohydrates and fats as fuels for mitochondrial oxidation to produce ATP. In the current study, dietary treatments did not result in the change of AMPK expression in the jejunum. The level of AME/NE difference between diets might not be big enough to provoke any effect on PRKAG2 gene regulation or the effect may not be produced in the jejunum. Cho et al. (2017) observed that PRKAG2 in the muscle and cell-free plasma did not differ by feeding ducks the diets with low and medium levels of AME (2300 and 2900 kcal/kg diet); on the other hand, high AME level (3300 kcal/kg diet) (with higher dietary fat) upregulated PRKAG2 in those tissues possibly to maintain energy homeostasis.

Almost 90% of ATP production occurs in mitochondria via ETC (Lehninger et al., 1993). The genes encoding proteins complexes involving oxidative phosphorylation might be affected by

dietary compositions. In the present study, however, gene expression data did not show such effect in the jejunum. It might be possible that oxidative phosphorylation in layers is not sensitive to relatively subtle difference present in diets. Lemieux et al. (2008) reported that long-term feeding diets with different fat and fatty acid profile resources were not able to change the mitochondrial respiration rate at ETC complex I, II or IV in rats heart. Further, the mRNA expression of avANT and COX III did not differ in broilers with the different genetic line (Ojano-Dirain et al., 2007).

The ratio of dietary NE/AME corresponds to the amount of heat increment of feed. This heat dissipation can be used by chickens for body thermoregulation. Internal heat production which applies for body thermoregulation is accompanied by the uncoupling of aerobic metabolism in oxidative phosphorylation. NE/AME treatment applied in the current study had no effect on avANT expression. The calorimetry measurement of the same birds from a previous study (Barzegar et al., 2018) showed close values for HI of feed produced per g of feed intake as 38, 39, and 40 kcal/g feed for HR, MR, and LR laying hens. As the diet-induced thermogenesis was very similar so that HP variation due to diet content may not be detectable thus an indifferent expression of avANT in the jejunum. Previous research showed that both fasted and cold-acclimated chickens increased avANT expression in skeletal muscle to produce heat for body thermoregulation (Toyomizu et al., 2002; Toyomizu et al., 2006). Addition of long chain fatty acid esters in rat ration inhibited the *in vitro* ANT activity in liver (Lerner et al., 1972). Although the diets used in the current study contain different fatty acid level, the treatments had no effect on avANT expression. Mujahid et al. (2009) confirmed that supplementing the high level of olive oil (6.7%) in diet had no effect on avANT expression in muscle mitochondria of chicken.

6.4. Conclusion

In conclusion, the results of this study suggest that dietary NE/AME ratio regulate at least one gene involved lipid uptake and adipogenesis in the jejunum. However, other genes were not responsive meaning the dietary treatment only affects key genes in the ETC pathway to regulate the energy expenditure at least in the small intestine where digestion and absorption occur. The effect of dietary NE/AME and EE content on the lipogenic genes expressions should be investigated in other tissues of layers such as liver or uterus which are the main sites for energy metabolism and lipogenesis in laying period.

Acknowledgment

Authors acknowledge Poultry CRC and the Australian Egg Corporation Limited for supporting this study.

Chapter 7. General conclusion

An energy evaluating system should be able to assess the animals energy requirements and also be capable of calculating the energy required to achieve the genetic performance potential of the animal. Therefore, each energy system consists of at least two sets of databases; one set reflecting energy requirements and the other set the energy values of feedstuffs. Consequently, inaccurate prediction of feed ingredients values results in lower performance of animals and increases diet cost. This is important as feed represents 65-75% of the total production cost.

Feed formulation using the AMEn system will be affected by different feed compositions, different birds strain and the different age range of the birds. Most available tabulated data are from experiments using adult cockerels or growing broilers. Specific values were rarely available for laying hens. The bioassay experiment in the current study revealed that the measured AMEn values of ingredients using laying hens were close to those calculated from standard tabulated data using adult cockerels. Also the AME, AMEn, and AMEs values of ingredients using regression based on the inclusion rate of dietary ingredients in diets where comparable to those obtained by the reference diet substitution method. Energy efficiency and net energy prediction of feed in laying hens can be predicted precisely from dietary AME and nutrients contents. Calorimetry measurements provided a possibility to calculate the energy retention and relevant efficiencies of dietary AME intake either as EE or protein or also as retained energy in egg and body in the current study. Dietary protein and amino acid contents were enough to meet the layers protein requirements for maintenance and production purposes while body fat reserves mobilized to meet layers energy requirements for both maintenance and production. Equations enabled the prediction of NE based on positive correlation with AME and EE and negative correlation of CP content in diets of the current experiment. Utilization of NE for AME revealed that using the AME system specifically underestimated the available energy of high-protein content ingredients (SBM) compared to cereals and oil. Validation of NE-based diets formulation confirmed that formulating diets with higher NE/AMEn (more fat inclusion) improved the egg mass, FCR and egg quality parameters of hens in different ages. Furthermore, fat supplemented layers with higher NE/AMEn dietary content improved the feeds energy utilization and energy retention. This study also confirmed that dietary fat and fatty acid profile stimulate the genes encoding the lipid uptake and adipogenesis in mitochondria.

The findings suggest that using NE-based formulation system provides an opportunity for poultry nutritionists and layers industry to evaluate the available feeds energy contents with some improvements in performance parameters and lowering the costs. Further investigations on different ingredients and dietary compositions by other chicken categories will be warranted the application of NE database for layers.

References

Akter, Y., S. Greenhalgh, M. R. Islam, C. Hutchison, and C. J. O'Shea. 2018. Hens ranked as highly feed efficient have an improved albumen quality profile and increased polyunsaturated fatty acids in the yolk. J. Anim. Sci. 96:3482-3490.

Annison, E., and R. White. 1961. Glucose utilization in sheep. Biochem. J. 80:162.

Annison, G., R. Hughes, and M. Choct. 1996. Effects of enzyme supplementation on the nutritive value of dehulled lupins. Br. Poult. Sci. 37:157-172.

AOAC. 2016. Official Methods of Analysis of AOAC International. 20th ed.

Aoun, M., C. Feillet-Coudray, G. Fouret, B. Chabi, D. Crouzier, C. Ferreri, C. Chatgilialoglu, C. Wrutniak-Cabello, J. P. Cristol, and M.-A. Carbonneau. 2012. Rat liver mitochondrial membrane characteristics and mitochondrial functions are more profoundly altered by dietary lipid quantity than by dietary lipid quality: effect of different nutritional lipid patterns. Br. J. Nutr. 107:647-659.

Applegate, T. 2005. The nutritional value of dehulled-degermed corn for broiler chickens and its impact on nutrient excretion. Poult. Sci. 84:742-747.

Arieli, A., A. Meltzer, and A. Berman. 1980. The thermoneutral temperature zone and seasonal acclimatisation in the hen. Br. Poult. Sci. 21:471-478.

Armsby, H. P., and J. A. Fries. 1915. Net energy values of feeding stuffs for cattle. J. Agric. Res. 3:435-491.

Atteh, J., and S. Leeson. 1983. Effects of dietary fatty acids and calcium levels on performance and mineral metabolism of broiler chickens. Poult. Sci. 62:2412-2419.

Atteh, J., and S. Leeson. 1984. Effects of dietary saturated or unsaturated fatty acids and calcium levels on performance and mineral metabolism of broiler chicks. Poult. Sci. 63:2252-2260.

Balnave, D. 2004. Challenges of accurately defining the nutrient requirements of heat-stressed poultry. Poult. Sci. 83:5-14.

Barekatain, M., J. Noblet, S. Wu, P. Iji, M. Choct, and R. Swick. 2014. Effect of sorghum distillers dried grains with solubles and microbial enzymes on metabolizable and net energy values of broiler diets. Poult. Sci. 93:2793-2801.

Barzegar, S., S. B. Wu, and R. A. Swick. 2017. Metabolizable energy of ingredients in peak layers Aust. Poult. Sci. Symp. 28:200.

Barzegar, S., S. B. Wu, and R. A. Swick. 2018. Implementation of net energy evaluating system in layer hens: Validation by performance and egg quality Poult. Sci. 97 (E-suppl. 1):47.

Begin, J. 1967. The relation of breed and sex of chickens to the utilization of energy. Poult. Sci. 46:379-383.

Berg, J., J. Tymoczko, and L. Stryer. 2002. Section 22.5, Acetyl Coenzyme A carboxylase plays a key role in controlling fatty acid metabolism in Biochemistry.W.H. Freeman, New York, USA.

Bikker, P. 1994. Protein and lipid accretion in body components of growing pigs: effects of body weight and nutrient intake . PhD thesis, Wageningen Agricultural University, Netherlands.

Birkett, S., and K. de Lange. 2001. Limitations of conventional models and a conceptual framework for a nutrient flow representation of energy utilization by animals. Br. J. Nutr. 86:647-659.

Blaxter, K. 1989. Energy Metabolism in Animals and Man.Cambridge university press, Cambridge, UK.

Boekholt, H., P. Van Der Grinten, V. Schreurs, M. Los, and C. Leffering. 1994. Effect of dietary energy restriction on retention of protein, fat and energy in broiler chickens. Br. Poult. Sci. 35:603-614.

Bottje, W., and B.-W. Kong. 2013. Cell Biology Symposium: feed efficiency: mitochondrial function to global gene expression. J. Anim. Sci. 91:1582-1593.

Bourdillon, A., B. Carré, L. Conan, J. Duperray, G. Huyghebaert, B. Leclercq, M. Lessire, J. McNab, and J. Wiseman. 1990a. European reference method for the in vivo determination of metabolisable energy with adult cockerels: reproducibility, effect of food intake and comparison with individual laboratory methods. Br. Poult. Sci. 31:557-565.

Bourdillon, A., B. Carré, L. Conan, M. Francesch, M. Fuentes, G. Huyghebaert, W. Janssen, B. Leclercq, M. Lessire, and J. McNab. 1990b. European reference method of in vivo determination of metabolisable energy in poultry: reproducibility, effect of age, comparison with predicted values. Br. Poult. Sci. 31:567-576.

Brouwer, E. 1965. Report of sub-committee on constants and factors Pages 441-443 in Energy metabolism. K. L. Blaxter ed. Academic Press, London, UK.

Caldas, J., K. Hilton, N. Boonsinchai, J. England, A. Mauromoustakos, and C. Coon. 2018. Dynamics of nutrient utilization, heat production, and body composition in broiler breeder hens during egg production. Poult. Sci. 97:2845-2853.

Carré, B., J. Gomez, and A. Chagneau. 1995. Contribution of oligosaccharide and polysaccharide digestion, and excreta losses of lactic acid and short chain fatty acids, to dietary metabolisable energy values in broiler chickens and adult cockerels. Br. Poult. Sci. 36:611-630.

Carre, B., and H. Juin. 2015. Partition of metabolizable energy, and prediction of growth performance and lipid deposition in broiler chickens. Poult. Sci. 94:1287-1297.

Carré, B., M. Lessire, and H. Juin. 2002. Development of the net energy system for broilers. Proc. East. Nutr. Conf. 38:140-149.

Carré, B., M. Lessire, and H. Juin. 2013. Prediction of metabolisable energy value of broiler diets and water excretion from dietary chemical analyses. Animal. 7:1246-1258.

Carré, B., M. Lessire, and H. Juin. 2014. Prediction of the net energy value of broiler diets. Animal. 8:1395-1401.

Cecil, J. E., P. Watt, C. N. Palmer, and M. Hetherington. 2006. Energy balance and food intake: the role of PPARγ gene polymorphisms. Physiol. Behav. 88:227-233.

Chepete, H. J., H. Xin, M. C. Puma, and R. S. Gates. 2004. Heat and moisture production of poultry and their housing systems: Pullets and layers. ASHRAE Transactions 110:286.

Cho, J., J. Jeong, Y. Jeong, J. M. Heo, and I. Choi. 2017. Gene expression patterns in Korean native ducks (Anas platyrhynchos) with different apparent metabolisable energy (AME) levels. Livest. Sci. 202:67-73.

Choct, M. 2004. The net energy value of commonly used plant ingredients for poultry in Australia.RIRDC Publication, Australia.

Chudy, A., W. Souffrant, S. Kuhla, and H. Peters. 2003. Energy and protein (AA) metabolism of high productive laying hens in dependence on exogenous factors. Publ. Eur. Assoc. Anim. Prod. 109:339-344.

Chwalibog, A., A. H. Tauson, and G. Thorbek. 2004. Energy metabolism and substrate oxidation in pigs during feeding, starvation and re-feeding. J. Anim. Physiol. Anim. Nutr. 88:101-112.

Close, W., M. Verstegen, and L. Mount. 1973. The energy costs of maintenance and production in the growing pig. Proc. Nutr. Soc. 32:72A.

Cozannet, P., M. Lessire, C. Gady, J. Metayer, Y. Primot, F. Skiba, and J. Noblet. 2010a. Energy value of wheat dried distillers grains with solubles in roosters, broilers, layers, and turkeys. Poult. Sci. 89:2230-2241.

Cozannet, P., Y. Primot, C. Gady, J. Métayer, M. Lessire, F. Skiba, and J. Noblet. 2010b. Energy value of wheat distillers grains with solubles for growing pigs and adult sows. J. Anim. Sci. 88:2382-2392.

Dale, N. 2000. Soy products as protein sources in poultry diets Pages 283-288 in Soy in Animal Nutrition, Savoy, IL.

Damme, K., F. Pirchner, H. Willeke, and H. Eichinger. 1987. Fasting Metabolic Rate in Hens 1. Effects of Body Weight, Feather Loss, and Activity. Poult. Sci. 66:881-890.

Davi, E. 2000. Fat (total, saturated, and unsaturated) in foods: Method 99606 of AOAC International Gaithersburg, USA.

De Groote, G. 1974. A comparison of a new net energy system with the metabolisable energy system in broiler diet formulation, performance and profitability Br. Poult. Sci. 15:75-95.

De Lange, C., and H. Birkett. 2005. Characterization of useful energy content in swine and poultry feed ingredients. Can. J. Anim. Sci. 85:269-280.

Denbow, D. M. 2015. Gastrointestinal anatomy and physiology. Pages 337-366 in Sturkie's Avian Physiology Elsevier, California, USA.

DSM. 2008. Yolk color fan.DSM, Basel, Switzerland.

El-Senousey, H., A. Fouad, J. Yao, Z. Zhang, and Q. Shen. 2013. Dietary alpha lipoic acid improves body composition, meat quality and decreases collagen content in muscle of broiler chickens. Asian-Australas. J. Anim. Sci. 26:394.

Emmans, G. 1994. Effective energy: a concept of energy utilization applied across species. Br. J. Nutr. 71:801-821.

Englyst, H., and G. Hudson. 1987a. Colorimetric method for routine measurement of dietary fibre as non-starch polysaccharides. A comparison with gas-liquid chromatography. Food. Chem 24:63-76.

Englyst, H., and G. Hudson. 1987b. Colorimetric method for routine measurement of dietary fibre as non-starch polysaccharides. A comparison with gas-liquid chromatography. Food. Chem. 24:63-76.

Farrell, D. 1972. An indirect closed circuit respiration chamber suitable for fowl. Poult. Sci. 51:683-688.

Farrell, D. 1974. General principles and assumptions of calorimetry. Energy requirements of poultry. Br. Poult. Sci. LTD:1-24.

Farrell, D. 1975. A comparison of the energy metabolism of two breeds of hens and their gross using respiration calorimetry. Br. Poult. Sci. 16:103-113.

Farrell, D. 1976. Influence of protein and amino acid balance in the diet of chickens on efficiency of utilization of dietary energy. Proc. Symp. Energy. 7:97-100. Vichy, France.

Farrell, D. 1999. In vivo and in vitro techniques for the assessment of the energy content of feed grains for poultry: a review. Aust. J. Agric. Res. 50:881-888.

Farrell, D., A. Smulders, P. Mannion, M. Smith, and J. Priest. 1997. The effective energy of six poultry diets measured in young and adult birds. Proc. Symp. Energy. Metab. 14:371-374.

Fraps, G. S. 1946. Composition and productive energy of poultry feeds and rations. Bull. No. 678. Texas Agricultural Experiment Station, College Station, USA.

Fuller, H., N. Dale, and C. Smith. 1983. Comparison of heat production of chickens measured by energy balance and by gaseous exchange. J. Nutr. 113:1403-1408.

Garnsworthy, P., J. Wiseman, and K. Fegeros. 2000. Prediction of chemical, nutritive and agronomic characteristics of wheat by near infrared spectroscopy. J. Agric. Sci. 135:409-417.

Geraert, P., M. MacLeod, M. Larbier, and B. Leclercq. 1990. Nitrogen metabolism in genetically fat and lean chickens. Poult. Sci. 69:1911-1921.

Geraert, P., M. MacLeod, and B. Leclercq. 1988. Energy metabolism in genetically fat and lean chickens: diet-and cold-induced thermogenesis. J. Nutr. 118:1232-1239.

Grobas, S., J. Mendez, C. De Blas, and G. Mateos. 1999,. Laying hen productivity as affected by energy, supplemental fat, and linoleic acid concentration of the diet. Poult. Sci 78:1542-1551.

Hardie, D. G., S. A. Hawley, and J. W. Scott. 2006. AMP-activated protein kinase–development of the energy sensor concept. J. Physiol. 574:7-15.

Hardie, D. G., J. W. Scott, D. A. Pan, and E. R. Hudson. 2003. Management of cellular energy by the AMP-activated protein kinase system. FEBS Lett. 546:113-120.

Hätel, H. 1986. Influence of food input and procedure of determination on metabolisable energy and digestibility of a diet measured with young and adult birds. Br. Poult. Sci. 27:11-39.

Hill, F., and D. L. Anderson. 1958. Comparison of metabolizable energy and productive energy determinations with growing chicks. J. Nutr. 64:587-603.

Hilliar, M., S. Wu, J. Roberts, and R. Swick. 2016. Indirect calorimetry measured by open and closed systems in laying hens. Honours thesis. University of New England., Australia.

Hudson, N., W. Bottje, R. Hawken, B. Kong, R. Okimoto, and A. Reverter. 2017. Mitochondrial metabolism: a driver of energy utilisation and product quality. Anim. Prod. Sci. 57:2204-2215.

Hy-Line. 2016. Hy-Line Brown commercial layers 2016 - Management Guide, http://www.specialisedbreeders.com.au/wp-content/uploads/2016/04/BRN-COM-AUS.pdf.

Jadhao, S., C. Tiwari, and M. Khan. 1999. Energy requirement of Rhode Island red hens for maintenance by slaughter technique. Asian-Australas. J. Anim. Sci. 12:1085-1089.

Janssen, W. M. M. A. 1989. European table of energy values for poultry feedstuffs. 3rd ed., Beekbergen, Netherlands: Spelderholt Center for Poultry Research and Information Services.

Just, A. 1982. The net energy value of balanced diets for growing pigs. Livest. Prod. Sci. 8:541-555.

Karakas, P., H. Versteegh, Y. Van Der Honing, J. Kogut, and A. Jongbloed. 2001. Nutritive value of the meat and bone meals from cattle or pigs in broiler diets. Poult. Sci. 80:1180-1189.

Kielanowski, J. 1965. Estimates of the energy cost of protein deposition in growing animals. Symp. Energy. Metab. 3:13-20.

King, J. 1957. Comments on the theory of indirect calorimetry as applied to birds. Northwest. Sci. 31:155-169.

Klandorf, H., P. J. Sharp, and M. G. Macleod. 1981. The relationship between heat production and concentrations of plasma thyroid hormones in the domestic hen. Gen. Comp. Endocrinol. 45:513-520.

Kleiber, M. 1947. Body size and metabolic rate. Physiol. Rev. 27:511-541.

Krogdahl, Å. 1985. Digestion and absorption of lipids in poultry. J. Nutr. 115:675-685.

Kuchipudi, S. V., M. Tellabati, R. K. Nelli, G. A. White, B. B. Perez, S. Sebastian, M. J. Slomka, S. M. Brookes, I. H. Brown, and S. P. Dunham. 2012. 18S rRNA is a reliable normalisation gene for real time PCR based on influenza virus infected cells. Virol. J. 9:230.

Leeson, S., and J. Summers. 1997. Feeding programs for broilers. Pages 207-298 in Commercial Poultry Nutrition University Books, Guelph, ON, Canada.

Leeson, S., and J. Summers. 2009. Commercial Poultry Nutrition. Nottingham University Press, UK.

Lehninger, A., D. Nelson, and M. Cox. 1993. Principles of Biochemistry.Worth Publishers, New York, USA.

Lei, L., and Z. Lixian. 2012. Effect of 24 h fasting on gene expression of AMPK, appetite regulation peptides and lipometabolism related factors in the hypothalamus of broiler chicks. Asian-Australas. J. Anim. Sci. 25:1300.

Lemieux, H., P. Blier, and J.-C. Tardif. 2008. Does membrane fatty acid composition modulate mitochondrial functions and their thermal sensitivities? Comp. Biochem. Physiol. 149:20-29.

Li, K., C. K. Warner, J. Hodge, S. Minoshima, J. Kudoh, R. Fukuyama, M. Maekawa, Y. Shimizu, N. Shimizu, and D. Wallace. 1989. A human muscle adenine nucleotide translocator gene has four exons, is located on chromosome 4, and is differentially expressed. J. Biol. Chem. 264:13998-14004.

Lin, H., H. Jiao, J. Buyse, and E. Decuypere. 2006. Strategies for preventing heat stress in poultry. World's. Poult. Sci. J. 62:71-86.

Liu, W., C. H. Lin, Z. K. Wu, G. H. Liu, H. J. Yan, H. M. Yang, and H. Y. Cai. 2017. Estimation of the net energy requirement for maintenance in broilers. Asian-Australas. J. Anim. Sci. 30:849.

Lopez, G., and S. Leeson. 2005. Utilization of metabolizable energy by young broilers and birds of intermediate growth rate. Poult. Sci. 84:1069-1076.

Lopez, G., and S. Leeson. 2008a. Assessment of the nitrogen correction factor in evaluating metabolizable energy of corn and soybean meal in diets for broilers. Poult. Sci. 87:298-306.

Lopez, G., and S. Leeson. 2008b. Aspects of energy metabolism and energy partitioning in broiler chickens. Pages 339-352 in Mathematical Modelling in Animal Nutrition. CABI, Wallingford, UK.

Luiting, P. 1990. Genetic variation of energy partitioning in laying hens: causes of variation in residual feed consumption. World's. Poult. Sci. J. 46:133-152.

MacLeod, M. 1990. Energy and nitrogen intake, expenditure and retention at 20 in growing fowl given diets with a wide range of energy and protein contents. Br. J. Nutr. 64:625-637.

MacLeod, M., S. Tullett, and T. Jewitt. 1979. Effects of food intake regulation on the energy metabolism of hens and cockerels of a layer strain. Br. Poult. Sci. 20:521-531.

Mateos, G. G., and J. L. Sell. 1980. Influence of graded levels of fat on utilization of pure carbohydrate by the laying hen. J. Nutr. 110:1894-1903.

May, J. 1978. Effect of fasting on T3 and T4 concentrations in chicken serum. Gen. Comp. Endocrinol. 34:323-327.

McDonald, P., R. A. Edwards, J. F. D. Greenhalgh, C. A. Morgan, and R. G. Wilkinson. 2011. Evaluation of foods: energy content of foods and energy partition within the animal Pages 254-280 in Animal Nutrition.Pearson, U.S.A.

McLean, J. 1972. On the calculation of heat production from open-circuit calorimetric measurements. Br. J. Nutr. 27:597-600.

McLean, J., J. A. MacLean, G. Tobin, and J. McLean. 1987. Animal and human calorimetry. Cambridge University Press, UK.

McNab, J., and C. Fisher. 1981. The choice between apparent and true metabolizable energy systems-recent evidence. Symp. Poult. Nutr. 3:26-29.

Minokoshi, Y., T. Alquier, N. Furukawa, Y.-B. Kim, A. Lee, B. Xue, J. Mu, F. Foufelle, P. Ferré, and M. J. Birnbaum. 2004. AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. Nature 428:569.

Miranda, J. M., X. Anton, C. Redondo-Valbuena, P. Roca-Saavedra, J. A. Rodriguez, A. Lamas, C. M. Franco, and A. Cepeda. 2015. Egg and egg-derived foods: effects on human health and use as functional foods. Nutrients. 7:706-729.

Mollah, Y., W. Bryden, I. Wallis, D. Balnave, and E. Annison. 1983. Sudies on low metabolisable energy wheats for poultry using conventional and rapid assay procedures and the effects of processing. Br. Poult. Sci. 24:81-89.

Mujahid, A., Y. Akiba, and M. Toyomizu. 2009. Olive oil-supplemented diet alleviates acute heat stress-induced mitochondrial ROS production in chicken skeletal muscle. Am. J. Physiol. 297:690-698.

Musharaf, N. A., and J. Latshaw. 1999. Heat increment as affected by protein and amino acid nutrition. World's. Poult. Sci. J. 55:233-240.

NHMRC. 2013. Australian code of practice for the care and use of animals for scientific purposes. Australian Government Publ. Service.

Ning, D., Y. Guo, Y. Wang, and Y. Peng. 2013. Earlier Metabolizable Energy Intake Level Influences Heat Production during a Following 3-Day Fast in Laying Hens. Asian-Australas. J. Anim. Sci. 26:558.

Ning, D., J. Yuan, Y. Wang, Y. Peng, and Y. Guo. 2014. The net energy values of corn, dried distillers grains with solubles and wheat bran for laying hens using indirect calorimetry method. Asian-Australas. J. Anim. Sci. 27:209.

Nisoli, E., E. Clementi, C. Paolucci, V. Cozzi, C. Tonello, C. Sciorati, R. Bracale, A. Valerio, M. Francolini, and S. Moncada. 2003. Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. Science. 299:896-899.

Nitsan, Z., A. Dvorin, Z. Zoref, and S. Mokady. 1997. Effect of added soyabean oil and dietary energy on metabolisable and net energy of broiler diets. Br. Poult. Sci. 38:101-106.

Noblet, J., S. Dubois, E. Labussiere, B. Carré, and J. Van Milgen. 2010a. Metabolic utilization of energy in monogastric animals and its implementation in net energy systems. 3rd EAAP International Symposium on Energy and Protein Metabolism and Nutrition, Parma, Italy:573-582.

Noblet, J., S. Dubois, J. Lasnier, M. Warpechowski, P. Dimon, B. Carré, J. Van Milgen, and E. Labussière. 2015a. Fasting heat production and metabolic BW in group-housed broilers. Animal. 9:1138-1144.

Noblet, J., H. Fortune, X. Shi, and S. Dubois. 1994. Prediction of net energy value of feeds for growing pigs. J. Anim. Sci. 72:344-354.

Noblet, J., C. Karege, S. Dubois, and J. van Milgen. 1999. Metabolic utilization of energy and maintenance requirements in growing pigs: effects of sex and genotype. J. Anim. Sci. 77:1208-1216.

Noblet, J., and J. van Milgen. 2013. Chapter 2: Energy and energy metabolism in swine. Pages 23-57 in Sustainable Swine Nutrition., Iowa, USA.

Noblet, J., J. van Milgen, B. Carré, P. Dimon, S. Dubois, M. Rademacher, and S. Van Cauwenberghe. 2003. Effect of body weight and dietary crude protein on energy utilisation in growing pigs and broilers. European. Assoc. Anim. Prod. 109:205-208.

Noblet, J., J. van Milgen, and S. Dubois. 2010b. Utilisation of metabolisable energy of feeds in pigs and poultry: interest of net energy systems. Proc. Aust. Poult. Sci. Symp. 21:26-35.

O'Neill, S., and N. Jackson. 1974. The heat production of hens and cockerels maintained for an extended period of time at a constant environmental temperature of 23 C. J. Agric. Sci. 82:549-552.

Odabaşi, A., R. Miles, M. Balaban, and K. Portier. 2007. Changes in brown eggshell color as the hen ages. Poult. Sci. 86:356-363.

Ohtani, S., and S. Leeson. 2000. The effect of intermittent lighting on metabolizable energy intake and heat production of male broilers. Poult. Sci. 79:167-171.

Pastore, S. M., P. C. Gomes, G. da Silva Viana, E. A. da Silva, W. P. de Oliveira, L. V. S. Barbosa, A. Z. Fraga, and W. J. Alves. 2018. Standardized ileal digestible lysine requirement of white commercial layers in peak egg production. Biosci. J. 34:186-193.

Peguri, A., and C. Coon. 1993. Effect of feather coverage and temperature on layer performance. Poult. Sci. 72:1318-1329.

Pérez-Bonilla, A., S. Novoa, J. García, M. Mohiti-Asli, M. Frikha, and G. Mateos. 2012. Effects of energy concentration of the diet on productive performance and egg quality of brown egg-laying hens differing in initial body weight. Poult. Sci. 91:3156-3166.

Pérez, J.-M., and D. Sauvant. 2004. Tables of composition and nutritional value of feed materials. INRA.

Petersen, C. B. 1970. Efficiency of protein and fat deposition in growing chickens determined by respiration experiments. Europ. Assoc. Anim. Prod. 13:205-208.

Pirgozliev, V., and S. Rose. 1999. Net energy systems for poultry feeds: a quantitative review. World's. Poult. Sci. J. 55:23-36.

Puigserver, P., and B. M. Spiegelman. 2003. Peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α): transcriptional coactivator and metabolic regulator. Endocr. Rev. 24:78-90.

Pym, R., and D. Farrell. 1977. A comparison of the energy and nitrogen metabolism of broilers selected for increased growth rate, food consumption and conversion of food to gain. Br. Poult. Sci. 18:411-426.

Rabello, C. 2001. Equações de predição das exigências de energia e proteína para aves reprodutoras pesadas na fase de produção. Jaboticabal: Universidade Estadual Paulista.

Reid, B., M. Valencia, and P. M. Maiorino. 1978. Energy Utilization by Laying Hens I. Energetic Efficiencies of Maintenance and Production. Poult. Sci. 57:461-465.

Richards, M., and M. Proszkowiec-Weglarz. 2007. Mechanisms regulating feed intake, energy expenditure, and body weight in poultry. Poult. Sci. 86:1478-1490.

Rivera-Torres, V., J. Noblet, S. Dubois, and J. van Milgen. 2010. Energy partitioning in male growing turkeys. Poult. Sci. 89:530-538.

Robblee, N. M., and M. Clandinin. 1984. Effect of dietary fat level and polyunsaturated fatty acid content on the phospholipid composition of rat cardiac mitochondrial membranes and mitochondrial ATPase activity. J. Nutr. 114:263-269.

Roberts, J. R. 2004. Factors affecting egg internal quality and egg shell quality in laying hens. Poult. Sci. 41:161-177.

Rostagno, H. R., L. F. T. Albino, J. L. Donzele, P. C. Gomes, R. F. d. Olveira, D. C. Lopes, A. S. Firiera, and S. L. Barreto. 2011. Brazilian tables for poultry and swine: composition of feedstuffs and nutritional requirements. Universidade Federal de Viçosa, Departamento de Zootecnia.

Sakomura, N. K. 2004. Modeling energy utilization in broiler breeders, laying hens and broilers. Rev. Bras. Cienc. Avic. 6:1-11.

Sakomura, N., F. Longo, E. Oviedo-Rondon, C. Boa-Viagem, and A. Ferraudo. 2005b. Modeling energy utilization and growth parameter description for broiler chickens. Poult. Sci. 84:1363-1369.

Sakomura, N., K. Resende, J. Fernandes, C. Rabelo, F. Longo, and R. Neme. 2005a. Net energy requirement models for broiler breeders, laying hens and broilers. Proc. Eur. Symp. Poult. Nutr. Hungary. 15:459-461.

Salas, C., R. Ekmay, J. England, S. Cerrate, and C. Coon. 2016. Mechanisms of lipid mobilization towards egg formation in broiler breeder hens using stable isotopes. Poult. Sci. 96:383-387.

Samiullah, S., A. S. Omar, J. Roberts, and K. Chousalkar. 2016. Effect of production system and flock age on eggshell and egg internal quality measurements. Poult. Sci. 96:246-258.

Samiullah, S., J. Roberts, and S.-B. Wu. 2017. Downregulation of ALAS1 by nicarbazin treatment underlies the reduced synthesis of protoporphyrin IX in shell gland of laying hens. Sci. Rep. 7:6253.

SAS. 2010. SAS user's guide: statisticsSAS Institute, Cary, NC (EUA).

Sauvant, D. J., M. Pérez and G. Tran. 2004. Tables of Composition and Nutritional Value and Feed Materials. Pages 17-24. Wageningen Academic Publishers, Netherlands.

Schiemann, R., Nehring, K., Hoffmann, L., Jentsch, W. and Chudy, A. 1972. Energetische Futterbevertung und Energienormen. VEB Deutscher Landwirtschatsverlag. Berlin.

Sell, J., C. Angel, and F. Escribano. 1987. Influence of supplemental fat on weights of eggs and yolks during early egg production. Poult. Sci. 66:1807-1812.

Sibbald, I. 1979. The gross energy of avian eggs. Poult. Sci. 58:404-409.

Skulachev, V. P. 1991. Fatty acid circuit as a physiological mechanism of uncoupling of oxidative phosphorylation. FEBS Lett. 294:158-162.

Slinger, S., I. Sibbald, and W. Pepper. 1964. The relative abilities of two breeds of chickens and two varieties of turkeys to metabolize dietary energy and dietary nitrogen. Poult, Sci. 43:329-333.

Smith, W., and J. Pourezza. 1989. Supplemental lipids and sulphur amino acid utilization in laying hens. Br. Poult. Sci. 30:977-978.

Sorensen, P., J. Morgan, and D. Lewis. 1962. Nutrition of Pigs and Poultry, London: Butterworths press.

Spratt, R., H. Bayley, B. McBride, and S. Leeson. 1990. Energy metabolism of broiler breeder hens. 1. The partition of dietary energy intake. Poult. Sci. 69:1339-1347.

Summers, J. D., and S. Leeson. 1983. Factors influencing early egg size. Poult. Sci. 62:1155-1159.

Svihus, B. 2014. Function of the digestive system. J. Appl. Poult. Res. 23:306-314.

Svihus, B., and M. Gullord. 2002. Effect of chemical content and physical characteristics on nutritional value of wheat, barley and oats for poultry. Anim. Feed. Sci. Technol. 102:71-92.

Swennen, Q., G. Janssens, E. Decuypere, and J. Buyse. 2004. Effects of substitution between fat and protein on feed intake and its regulatory mechanisms in broiler chickens: energy and protein metabolism and diet-induced thermogenesis. Poult. Sci. 83:1997-2004.

Swick, R. A., S.-B. Wu, J. Zuo, N. Rodgers, M. R. Barekatain, and M. Choct. 2013. Implications and development of a net energy system for broilers. Anim. Prod. Sci. 53:1231-1237.

Theander, O., and E. Westerlund. 1993. Determination of individual components of dietary fiber. Pages 77-98 in Dietary Fiber in Human Nutrition. G. A. Spiller ed. CRC Press, Boca Raton, FL.

Tontonoz, P., E. Hu, and B. M. Spiegelman. 1995. Regulation of adipocyte gene expression and differentiation by peroxisome proliferator activated receptor γ. Curr. Opin. Genet. Dev. 5:571-576.

Toyomizu, M., D. Kirihara, M. Tanaka, K. Hayashi, and Y. Tomita. 1992. Dietary protein level alters oxidative phosphorylation in heart and liver mitochondria of chicks. Br. J. Nutr. 68:89-99.

van Milgen, J., J. Noblet, S. Dubois, and J.-F. Bernier. 1997. Dynamic aspects of oxygen consumption and carbon dioxide production in swine. Br. J. Nutr. 78:397-410.

van Milgen, J., J. Noblet, S. Dubois, B. Carr, and H. Juin. 2001. Utilization of metabolizable energy in broilers. Poult. Sci. 80:170.

van Ouverkerk, E., and S. Pedersen. 1994. Application of the carbon dioxide mass balance method to evaluate ventilation rates in livestock buildings. Proc. World. Agric. Engin. 12:516-529.

Vidal-Puig, A., M. Jimenez-Liñan, B. B. Lowell, A. Hamann, E. Hu, B. Spiegelman, J. S. Flier, and D. E. Moller. 1996. Regulation of PPAR gamma gene expression by nutrition and obesity in rodents. J. Clin. Invest. 97:2553-2561.

Vohra, P., W. Wilson, and T. Siopes. 1975. Meeting the energy needs of poultry. Proc. Nutr. Soc. 34:13-19.

Walsberg, G., and B. Wolf. 1995. Variation in the respiratory quotient of birds and implications for indirect calorimetry using measurements of carbon dioxide production. J. Exp. Biol. 198:213-219.

Wang, Y., Y. Mu, H. Li, N. Ding, Q. Wang, Y. Wang, S. Wang, and N. Wang. 2008. Peroxisome proliferator-activated receptor-γ gene: a key regulator of adipocyte differentiation in chickens. Poult. Sci. 87:226-232.

Waring, J., and W. Brown. 1967. Calorimetric studies on the utilization of dietary energy by the laying White Leghorn hen in relation to plane of nutrition and environmental temperature. J. Agric. Sci. 68:149-155.

Whitehead, C. C. 1995. Plasma oestrogen and the regulation of egg weight in laying hens by dietary fats. Anim. Feed. Sci. Technol. 53:91-98.

WHO. 1985. Energy and protein requirements: AO/WHO/UNU Expert Consultation. Pages 1-67. WHO, Geneva, Switzerland.

Wiseman, J., D. Cole, F. Perry, B. Vernon, and B. Cooke. 1986. Apparent metabolisable energy values of fats for broiler chicks. Br. Poult. Sci. 27:561-576.

Wu, S., Y. Huaming, B. Zhibin, Y. Xiaogang, and Z. Yumin. 2016. Heat production estimated from fasting layer hens at peak lay. World's. Poult. Congr. 25:197.

Wu, S., R. A. Swick, J. Noblet, N. Rodgers, D. Cadogan, and M. Choct. 2019. Net energy prediction and energy efficiency of feed for broiler chickens. Poult. Sci. in press 0:1-13, pey442.

Yang, H., Z. Wang, and J. Lu. 2009. Study on the relationship between eggshell colors and egg quality as well as shell ultrastructure in Yangzhou chicken. Afr. J. Biotech. 8:2898-2902.

Yin, R., X. Liu, C. Liu, Z. Ding, X. Zhang, F. Tian, W. Liu, J. Yu, L. Li, and M. H. de Angelis. 2011. Systematic selection of housekeeping genes for gene expression normalization in chicken embryo fibroblasts infected with Newcastle disease virus. Biochem. Biophys. Res. Commun. 413:537-540.