# Effect of insoluble fiber supplementation applied at different ages on digestive organ weight and digestive enzymes of layer-strain poultry

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**ABSTRACT** Two experiments were conducted to study effects of dietary insoluble fiber (IF) on digestive enzyme function in layer poultry. In Experiment 1, 8 wk old pullets were fed a control diet (Group C) or a diet (Group IF) supplemented with 1% IF (Arbocel RC). After 5 wk, 6 pullets per group were killed and organ samples collected. The remaining pullets in Group C were divided into two groups: half were fed the control diet (Group C) and half were given the IF diet (Group C-IF). Similarly, half the pullets in Group IF continued on the IF diet (Group IF) and half on the control diet (Group IF-C). At 10 wk, organ samples were collected. BW at wk 5 (IF, 1364.8g; C, 1342.9g) and 10 wk (IF, 1678.1g; IF-C, 1630.5g; C-IF, 1617.1g; C, 1580.4g) were not different. At wk 5, the relative proventricular weight (0.41 g/100g BW) and activities of pepsin (75.3 pepsin units/g proventriculus/min) and pancreatic general proteolytic activity (GP) (122.9 $\mu$mol tyrosine produced/g tissue) were greater ($P < 0.05$) than those of Group C (proventricular relative weight, 0.36; pepsin activity, 70.6; GP activity, 94.3). At wk 10, relative weights of liver and gizzard of Group IF were heavier ($P < 0.05$) than other treatments; activities of pepsin, GP, trypsin and chymotrypsin of IF pullets were significantly greater than other treatments as was mRNA expression for pepsinogens A (25.9 vs. 22.9) and C (13.1 vs. 10.8). In Experiment 2, 19 wk old hens were fed a control diet or a diet containing 0.8% IF (Arbocel RC) for 12 wk. Final BW after 12 wk was not different (IF, 1919.4 g; C, 1902.1 g). Pancreatic GP activity was greater ($P < 0.05$) in Group IF hens than Group C at wk 12 (122.2 vs. 97.0 $\mu$mol tyrosine released/min/g tissue)) as was relative gizzard weight (1.32 vs 1.10 g/100 g BW). The significantly improved digestive organ weights and enzyme activities in IF pullets may contribute to an improvement in feed utilization.

**Key words:** layer-strain, pullet, insoluble fiber, digestive enzyme activity, pepsinogen gene expression

## INTRODUCTION

Growth and development in layer pullets during rearing and early lay are important to the commercial poultry industry because of their influence on long-term production efficiency (Summers and Leeson, 1994). Incharoen and Maneechote (2013) showed that dietary insoluble fiber (IF) such as whole rice hulls (WRH) at 60 g/kg diet can be used to enhance growth and uniformity of pullets and improve egg production of laying hens. The inclusion in the diet of IF from oat hulls, at levels between 2 to 3% improves the growth performance of broilers fed low-fiber diets (Mateos et al., 2012) and dietary inclusion of 3% inulin or cellulose (Arbocel FD00) as an IF source has been shown to reduce feed intake and improved egg production of broiler breeder hens from 43 to 55 wk of age (Mohiti-Asli et al., 2012).

The potential benefits of fiber are dependent to a great extent on the physicochemical characteristics of the fiber source. The inclusion of various IF products as feed additives in the diets of growing or productive broiler- and layer-strains of poultry have been shown to induce positive changes on digestive physiology (e.g., Hetland et al., 2004, 2005; Mateos et al., 2012; Lim et al., 2013). Transit time (retention time) of ingested food from the crop to the gizzard and gizzard activity can be increased by IF and these physiological effects can improve mixing of feed particles with digestive secretions and hence improve digestion and absorption of nutrients (Mateos et al., 2012).

Digestive enzymes secreted by specific organs and regions of the digestive tract such as proventriculus, pancreas, and small intestines are responsible for the hydrolysis of dietary nutrients and play vital roles in the efficient absorption of the products of digestion. Adding IF in the form of oat hulls and wood shavings to broiler and layer diets has been shown to benefit nutrient digestion (Hetland et al., 2003, 2005) possibly as a result of an increased amylase concentration in the chyme of the jejunum (Hetland et al., 2003). The majority of

experiments examining the effects of IF on the functioning of digestive enzymes have examined the effects on pancreatic or intestinal lipases, amylases and peptidases (Hetland et al., 2003; Taylor and Jones, 2004; Sarikhan et al., 2009; Svihus, 2011). There has been very little systematic work on the effects of IF on proteolytic enzymes of the different supply organs of the digestive tract (proventriculus, pancreas and small intestine) of layer-strain poultry. Enhanced activity of such enzymes may improve the supply of amino acids for body and reproductive organ growth, which continue after supply organs have developed, and may thus contribute to improve egg production.

Despite pepsin digestion being the first stage in the enzymatic digestion of protein, there has been very little investigation into dietary stimulation of pepsin function or enhancement of synthesis of pepsinogens in poultry. Pepsinogens are the zymogen precursors of the active enzyme pepsin, and they are synthesized in poultry in the oxyntic cells of the proventriculus that also secrete hydrochloric acid (Hirschowitz, 1991). In adult poultry, two pepsinogens have been detected, pepsinogen A (cPgA) and pepsinogen C (cPgC). Pepsinogen A is secreted in greater amounts than cPgC (Donta and Van Vunakis, 1970; Sakamoto et al., 1998).

The first experiment was designed to determine whether the addition of a commercial IF product (Arbocel RC, JRS Co. Inc., Rosenberg, Germany) in the diets of young layer-strain pullets between the ages of 8 and 18 wk influenced supply organ growth and proteolytic enzyme activities in the proventriculus, pancreas, and small intestine. In addition, the experiment was designed to determine whether there was a residual effect of IF on the supply organs and enzyme activities 5 wk after it was removed from the diet. The effect of starting the IF feed at a later age, 13 wk, was also determined. Concentration of pepsin in proventricular tissue can be affected by hormones such as neurotensin and food in the gut (Degolier et al., 1997). On the other hand, pepsinogen mRNA activity is unlikely to be affected by short-term effects on pepsinogen secretion from oxyntic cells. We therefore wished to determine whether there was a correlation between pepsin activity and mRNA in proventricular tissue so as to provide an alternate means of measuring the effects of dietary changes on digestive function.

The second experiment was to determine the effect of the commercial IF product Arbocel RC on enzyme activities of layer hens kept under commercial conditions from point of lay at 18 to 19 wk of age through early egg production to 31 wk of age.

## MATERIALS AND METHODS

### Birds, Diets and Husbandry

In Experiment 1, 8-week-old Hy-Line Brown pullets were obtained from a commercial supplier in Melbourne, Victoria. All pullets were kept in the one house containing 12 pens (0.9 × 1.8 × 1.8 m, w × l × h) with slatted floors, Two experimental diets were used: a control diet (Table 1, Ridley AgriProducts Pty Ltd., Pakenham, 3810, Australia) without additives (Diet C) and the IF diet consisting of the control diet plus 1% IF (Arbocel RC Fine, JRS Co. Inc., Rosenberg, Germany which contains (manufacturer's analyses) 65% crude fiber and 20% acid detergent lignin). The analyzed concentrations of crude fiber in the two diets were 4.3% and 5.0% for Diet C and diet IF, respectively (FeedTest, Agrifood Technology Pty Ltd, Werribee, Vic., Australia). Feed and water were provided ad libitum.

In Experiment 2, Hy-Line Brown hens between the ages of 22 and 31 wk old were obtained from a commercial egg producer (Country Lane Poultry Farm, Tynong North, Victoria Australia). The company was testing, for their own information, whether addition of 0.8% Arbocel RC to the feed they normally used, would increase egg productivity, decrease cannibalism and decrease water content of the manure. They agreed to let us collect hens from their feeding trial. Two diets were formulated for the company by Ridley AgriProducts Pty Ltd. The Control diet (Table 1) was fed to half the hens in the layer house (Group C) and the other diet containing 0.8% Arbocel was fed to hens in the other half of the layer house (Group IF). The concentrations of crude fiber in the two treatments diets were analyzed by the company and were 3.3% for the Control diet and 4.1% for the IF diet.

The layer house held 50,000 hens and had fully automated temperature and humidity control with temperature sensors placed throughout the house to monitor conditions and adjust ventilation accordingly. There were 208 cages per tier of the 3-tier A-frame units; hens were housed five per cage and cage floor area was 500 cm$^2$ per bird. Hens were placed in the house at 18 to 19 wk of age. The Control diet was fed to hens in one half of the house and the IF diet was fed to hens in the other half of the house. Feed and water was provided at all times.

### Design of Experiments and Sample Collection

**Experiment 1** On the day of arrival leg-bands were placed on all pullets and they were weighed. Forty-eight pullets were allocated randomly to 12 pens, four birds per pen. Pullets in six pens were fed the control diet (Group C) for the first 5 wk and those in the remaining six pens were fed the 1% IF diet (Group IF). Pen treatments were randomized within the house. After 5 wk, when the pullets were 13 wk of age, six pullets (one per pen) were randomly selected from each treatment and killed with an intravenous overdose of pentobarbitone sodium (Lethabarb, Virbac Animal Health, Milperra, NSW, Australia). Within 5 min of the absence of a detectable palpebral reflex, the liver, gizzard, proventriculus,

**Table 1.** Nutrient content (g/100 g) on an as-fed basis of the control diets for grower diet (Experiment 1) and layer ration (Experiment 2) from manufacturer's data.[1]

| Grower diet (Experiment 1) | % | Layer diet (Experiment 2) | % |
|---|---|---|---|
| Wheat Fine | 57.97 | Wheat (11.5%) | 53.73 |
| Oats Fine | 10.57 | Soya (47.5%) | 16.1 |
| Peas Fine | 12.00 | Limestone (38%) | 5.57 |
| Meat Meal | 3.83 | Canola Exp (36/9) | 5.0 |
| Blood Meal | 2.00 | Peas | 5.0 |
| Canola Meal | 5.00 | Meat M (50%) | 4.83 |
| Soybean Meal | 2.90 | Oats (7%) | 3.33 |
| Millrun | 3.27 | Lime Grit (38-Ca) | 3.33 |
| Limestone Fine | 1.00 | Acid Oil-Coater | 1.33 |
| Supplements | 1.46 | Acid Oil | 0.90 |
|  |  | Farm Pride Layer supplement | 0.83 |
|  |  | Rovabio Excel (75%) | 0.03 |
|  |  | Choline CHL (75%) | 0.02 |
| Chemical composition (%) Data supplied by manufacturer | | | |
| Dry mater | 89.39 | Crude protein | 19.57 |
| Moisture | 10.61 | Lysine | 0.944 |
| Protein | 18.01 | Methionine | 0.469 |
| Fat | 3.04 | Crude Fat | 4.626 |
| Fiber[2] | 4.36 | Crude Fiber[3] | 3.450 |
| Calcium | 1.00 | Ash | 13.277 |
| Phosphorus | 0.56 | Calcium | 4.099 |
| Available phosphorus | 0.40 | Available phosphorus | 0.450 |
| ME kcal/kg | 2796.6 | ME Kcal/kg | 2800 |

[1]Ridley AgriProducts Pty Ltd., Pakenham, 3810, Australia.

[2]Analyzed CF - Diet C, 4.3%, and Diet IF 5.0% (FeedTest, Agrifood Technology Pty Ltd, Werribee, Vic., Australia).

[3]Analyzed CF - Control Diet, 3.3% and IF Diet, 4.1% (provided by Ridley AgriProducts Pty Ltd.).

pancreas, small intestine and ceca were removed. The gizzard was cleaned of contents and both it and the liver weighed. Whole proventriculus and pancreas were rinsed with cold 0.9% saline, blotted dry, weighed and stored at –80°C for later analysis of enzyme activities. The contents of the small intestines (jejunum and ileum) were washed out with 0.9% saline then the tract was divided into three equal lengths (proximal, medial and distal sections) and 1 cm segments from the middle of each section were collected, weighed separately and stored at –80°C for later analysis of enzyme activities. The contents of the ceca were washed out with saline.

At 5 wk the remaining 16 pullets in Group C were divided so that eight pullets continued on the control diet (Group C) and eight were given the IF diet (Group C-IF). Eight of the pullets from Group IF continued to be fed the IF diet (Group IF) and eight were given the control diet (Group IF-C). The pullets were housed in three pens per treatment with two pens of three and one of two pullets. After a further 5 wk (10 wk after the start of the experiment) when the pullets were 18 wk of age, they were killed and samples collected as before. In addition, tissues samples from the proventriculi of pullets in Groups C and IF were taken and stored in RNA*later*® (Cat. No. R0901, Sigma-Aldrich, Castle Hill, NSW, Australia) at –20°C for later analysis of pepsinogen gene expression. The experiment was conducted in accordance with the principles and specific guidelines of La Trobe University

Animal Ethics Committee (LTU-AEC) project number AEC12–12.

**Experiment 2** Because we had no control of the experimental layout of the layer house with hens in one half of the house being fed the Control ration (Group C), and hens in the other half of the house being fed the ration containing 0.8% IF (Group IF), it was decided that hens from the middle tiers of the two central rows in the house would be taken for sample collection. By doing so, samples would be taken from hens that were kept under as similar environmental conditions (but not feed and water delivery) as possible. At 3, 6, 9, and 12 wk after the start of the feeding trial, eight hens from Group C and eight from Group IF were randomly collected, one hen per cage. The hens were collected from cages along the length of a row of 208 cages. The hens were then transported by road (approximately 2 h) to the University campus and weighed, killed, and tissue samples (excluding small intestines) were collected as in Experiment 1. The experiment was conducted in accordance with the principles and specific guidelines of LTU-AEC, project number AEC11–42.

## Measurement of Enzyme Activities

**Proventricular Pepsin Activity** The assay for pepsin activity was based on the method of Anson (1938) as modified by Susbilla et al. (2003) and Delia (2008). The whole proventriculus was thawed and

homogenized on ice with a Polytron homogenizer (Kinematica AG, Lucerne, Switzerland) in a 0.01 M sodium buffer, pH 7. The homogenized samples were centrifuged and an aliquot of diluted supernatant was added to a test tube containing 2.5% acidified bovine hemoglobin (Cat. No. H2625, Sigma-Aldrich) substrate, pH 2.5. The samples were incubated at 41°C in a shaking water bath (Julabo SW, West Germany) for 10 min and the reaction stopped with 5% trichloroacetic acid (TCA) solution. The sample was filtered and neutralized with 0.5 M sodium bicarbonate solution. Free tyrosine released through hydrolysis of hemoglobin was measured with Folin-Ciocalteu's phenol reagent (Cat. No. F9252, Sigma-Aldrich) and absorbance measured at 750 nm in a spectrophotometer (Model U-1100 Spectrophotometer, Hitachi, Japan). A tyrosine standard curve (Cat. No. T3754, Sigma-Aldrich) was prepared from standard solutions ranging in concentration from 0 to 1mM.

Porcine stomach mucosal pepsin (pepsin A, EC 3.4.23.1, Cat. No. P7012, Sigma-Aldrich) was used to convert tyrosine released to pepsin units. Pepsin was dissolved in 0.01 M sodium phosphate buffer, pH 7 and standards of 0 to 350 $\mu$g/mL produced. Tyrosine released from hemoglobin by pepsin was measured as for the proventricular tissue. One unit of pepsin activity was defined as 1 $\mu$mol of tyrosine released/min/g proventricular tissue.

### Pancreatic General Proteolytic (GP) Activity

The method of Susbilla et al. (2003) with slight modification by Delia (2008) was used to determine the general proteolytic activity of the pancreas. Whole pancreas was homogenized in Ringer's solution, pH 7.4. The tissue extract was activated for 2 h on ice, with enterokinase (from porcine intestine, Cat. No. E0632, Sigma-Aldrich) in Tris-HCl solution, pH 7.4. The substrate (2% casein, Cat. No. C8654, Sigma-Aldrich in a 0.2 $M$ sodium phosphate buffer, pH 8) was then added to a test tube containing tissue extract. After incubating in a shaking water bath at 41°C for 20 min, the reaction was stopped with 5% TCA (wt/vol) and the solution filtered through Whatman No. 1 filter paper. After addition of 0.5 $M$ Na$_2$CO$_3$, free tyrosine in the solution was measured with Folin-Ciocalteu's solution. The absorbance was read at 750 nm and amount of tyrosine released from casein was determined from a tyrosine standard curve in 0.2 $M$ sodium phosphate buffer, pH 8. The GP activity was expressed as $\mu$mol of free tyrosine released from casein/min/g pancreatic tissue.

### Pancreatic Trypsin and Chymotrypsin Activities

These were measured by the method of Erlanger et al. (1961) as modified by Caviedes-Vidal and Karasov (2001). Pancreatic homogenate plus enterokinase solution was incubated for 1 h in a shaking water bath at 25°C. Trypsin activity was then determined with a 1mM N$\alpha$-benzoyl-DL-arginine 4-nitroanilide hydrochloride (DL-BAPNA, Cat. No. B4875, Sigma-Aldrich) solution, pH 8.2 and chymotrypsin activity

with 1 m$M$ N–glutaryl-L-phenylalanine 4-nitroanilide (GPNA, Cat. No. G2505, Sigma-Aldrich) solution, pH 7.6. Samples for trypsin and chymotrypsin determinations were incubated in a shaking water bath at 40°C for 20 min. The reaction was stopped with 30% acetic acid and the absorbance was read at 410 nm. The amount of 4-nitroaniline released from a substrate was obtained from a standard curve of 4-nitroaniline (Cat. No. N2128, Sigma-Aldrich) in 0.05 $M$ Tris-HCl buffer, pH 8.2, for trypsin activity and pH 7.6 for chymotrypsin activity.

Trypsin (EC 3.4.21.4) from bovine pancreas, (T1426, Sigma-Aldrich) was used to convert 4-nitroanaline concentration to trypsin units (Delia, 2008). Standard concentrations of trypsin were incubated with 1m$M$ DL-BAPNA solution, pH 8.2 and 4-nitroaniline measured as for the pancreatic tissue. One unit of trypsin activity was defined as 1 $\mu$mol of 4-nitroanaline released/min/g pancreatic tissue. To convert 4-nitroanaline concentration to chymotrypsin units, standards of $\alpha$-chymotrypsin (EC 3.4.21.1, Type II from bovine pancreas, Cat. No. C4129, Sigma-Aldrich) were prepared in a 1m$M$ HCl and incubated with 1m$M$ GPNA solution, pH 7.6, as for pancreatic tissue. One unit of chymotrypsin activity was defined as 1 $\mu$mol of 4-nitroanaline released/min/g pancreatic tissue.

### Small Intestinal Dipeptidase and Aminopeptidase Activities

The dipeptidase activity in each of the three intestinal segments (proximal, medial, and distal) was measured according to the method described by Delia (2008). Homogenates of each segment were incubated for 10 min with the substrate, 10 m$M$ glycyl-L-leucine (EC 212.785.9, Cat. No. G2002, Sigma-Aldrich) in Ringer's solution, pH 7.4, in a shaking water bath at 41°C. After filtering through Whatman No. 1 paper, the absorbance was measured at 220nm. The amount of glycyl-L-leucine hydrolyzed by the activity of dipeptidase in the small intestine was determined by calculating the decrease in absorbance. Dipeptidase activity per g intestinal tissue (nmol of glycyl-L-leucine hydrolyzed per g of jejunal plus ileal tissue/min) was calculated from the sum of the average activities determined for proximal, medial and distal sections.

The method of Caviedes-Vidal and Karasov (2001) was used to determine aminopeptidase-N activity. Homogenates of each segment were incubated with 2 m$M$ L-alanine-4-nitroanilide hydrochloride (Cat. No. A9325, Sigma-Aldrich) in a 0.2 $M$ sodium phosphate buffer, pH 7, at 40°C for 10 min in a shaking water bath. The absorbance was read at 384 nm and concentration of 4-nitroaniline formed was calculated from a 4-nitroaniline standard curve in 0.2 $M$ sodium phosphate buffer, pH 7, at concentrations ranging from 0 to 0.3 m$M$. The activity of aminopeptidase in the small intestine (jejunum and ileum) was calculated as for dipeptidase activity and was expressed as $\mu$mol of free 4-nitroaniline released from L-alanine-4-nitroanilide per g jejunal plus ileal tissue/min.

## Measurement of Tissue Protein

The protein concentrations in the homogenate extracts of the tissues were measured using the method of Lowry et al. (1951). Bovine serum albumen (Cat. No. A2153, Sigma-Aldrich) was used as standard. For the proventriculus, albumen was prepared in 0.01 $M$ phosphate buffer, pH 7.0, while for pancreas and small intestine it was prepared in Ringer's solution, pH 7.4. After incubation, tyrosine concentration was measured using Folin-Ciocalteu's solution.

## mRNA Expression of Pepsinogens A and C

Total mRNA was extracted from proventricular tissues (100 mg) using 1 mL of TRIzol reagent (Invitrogen, Life Technologies, Australia) according to the manufacturer's instructions. A Nano Drop ND-1000 spectrophotometer (ThermoFisher Scientific, Scoresby Vic., Australia) was used to quantify the extracted mRNA. Only samples with an absorbance A260/280 ratio of 1.9 to 2.0 were used. Extracted mRNA was further purified with Ambion 10x TURBO DNase Buffer (Cat. No. AM 1097, Life Technologies) according to the manufacture's instructions and stored at –80°C. The synthesis of cDNA from extracted mRNA was carried out with a DyNAmo™ cDNA synthesis kit (Cat. No. F-470L, ThermoFisher Scientific, Australia) as recommended by the manufacturer. A thermal cycler (Stratagene Mx3000P QPCR System, Agilent Technologies, Santa Clara, CA), was used to convert the mRNA into cDNA.

Primers for target and reference genes were, pepsinogen A (AB025283) forward GGGTGCCCTCTATC-TATTGC, reverse CAGTGTCATAGCCCAGGATG; pepsinogen C (AB025284) forward GGTGTCCTACT-GTGCCTGTG, reverse CCTGGTTTGTGATGG AGATG; $\beta$-Actin (NM˙205518) forward ATGGCTC-CGGTATGTGCAA, reverse TGTCTTTCTGGCC-CATACCAA (Grommen et al., 2008); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward GCCATCACAGCCACACAGA and reverse TTTCC-CCACAGCCTTAGCA (Azzam et al., 2011).

Simplex real-time qPCR analysis (RT-qPCR) was conducted using SensiMix SYBR Low-ROX kit (Cat. No. QT625–02, Bioline Australia Pty Ltd) in a Stratagene Mx3000P QPCR System (Agilent Technologies, Santa Clara, CA). Each well of a 96-well plate contained the same volume of both forward and reverse primer of either a target or a housekeeping gene along with template cDNA (or DNase-free water for no template controls) and reagents according to the manufacturer's instructions for the kit. For each sample a mixture of all reagents was added to a sterile 1.5-mL microcentrifuge tube and mixed well. The first two wells of a 96-well plate contained buffer instead of the cDNA template and were used as a control. From each 1.5-mL microcentrifuge tube that contained a 50-μL reaction mixture a 25-μL was aliquot added to each well (2 replicates per sample).

Agarose gel (2% agarose powder (Cat. No. BIO-41025, Bioline (Aust) Pty. Ltd., Alexandria, NSW, Australia) electrophoresis was carried out to confirm the purity and size of the bands of the PCR products. The PCR reaction composed of *Taq* buffer, dNTPs, forward and reverse primers, Taq DNA Polymerase, cDNA template and nuclease-free water (NFW) according to the manufacturer's instructions (QIAGEN-Australia, Doncaster, Australia).

**Calculation of Gene Expression** Primer efficiency of the target genes was tested using five dilutions of cDNA in duplicate. Complementary deoxyribonucleic acid (cDNA) was used and diluted as neat (no dilution), 1:5, 1:50, 1:500 and 1:5000 in NFW. Efficiency of PCR was determined for both the target and endogenous control genes from the standard curves generated with different dilutions of the cDNA synthesized from the chicken proventriculus using the formula PCR Efficiency = 10 $(-1/\text{slope})$. The slope values were calculated from standard curves and used in the following formula (Harrison et al., 2007; Gopinath et al., 2011) to obtain the corrected cycle time (Ct):

$$\text{Corrected Ct} = \text{Ct} + (\text{Nt} - \text{Ct}') \times (\text{S}/\text{Ś})$$

where Ct is the mean cycle time of the target gene Ct, Nt is the mean Ct of housekeeping genes of all the experimental samples, Ct$'$ is the mean Ct of housekeeping gene of the particular sample, S is the target gene slope, and Ś is the endogenous control slope.

## Statistical Analyses

Homogeneity of variances and normality of data were all test using IBM SPSS Statistics Version 23.0 for Windows (IBM Corporation, Armonk, NY) before analysis of significance between treatments was carried out. For Experiment 1, significance between treatments of pullets sampled 5 wk after the start of the experiment were analyzed with an independent t-test (SPSS 23.0 for Windows) with six pen replicates. For samples collected 10 wk after the start, the three pen replicates per treatment were analyzed by one-way ANOVA; when a significant difference between means was observed, post hoc significance between treatment means was determined using the Bonferroni test. Linear regression of pepsinogen A and pepsin activity was determined with SPSS. For Experiment 2, each hen was an experimental unit as no cage of five hens was sampled more than once. Differences between means were analyzed with an independent t-test. A value of $P < 0.05$ was considered statistically significant.

**Table 2.** Experiment 1 Live body weights (g) and relative weights (g/100 g live-weight) of internal organs of 8 wk old Hy-Line Brown layer strain pullets given a control diet[†] (C) or a diet supplemented with 1%, insoluble fiber[†] (IF) for 5 wk then either diet C or diet IF for a further 5 wk (Mean, pooled SEM).

| Time fed diet (Age) wk | 5 (13) | | | 10 (18) | | | | |
|---|---|---|---|---|---|---|---|---|
| Treatment | C[1] | IF[2] | SEM | C[1] | C-IF[3] | IF-C[4] | IF[2] | SEM |
| Body weight[‡] | 1342.9 | 1364.8 | 14.04 | 1580.4 | 1617.1 | 1630.5 | 1678.1 | 13.8 |
| Liver | 2.26 | 2.49 | 0.12 | 2.35[b] | 2.04[b] | 2.18[b] | 2.82[a] | 0.10 |
| Gizzard | 2.75 | 2.84 | 0.16 | 2.29[b] | 2.21[b] | 2.32[b] | 2.93[a] | 0.10 |
| Proventriculus | 0.36 | 0.41 | 0.02* | 0.34 | 0.34 | 0.35 | 0.39 | 0.01 |
| Pancreas | 0.25 | 0.26 | 0.02 | 0.21 | 0.20 | 0.20 | 0.24 | 0.01 |
| Small Intestine | 2.02 | 2.13 | 0.10 | 1.92[a,b] | 1.69[b] | 1.91[a,b] | 2.13[a] | 0.05 |
| Cecum | 0.66 | 0.60 | 0.06 | 0.67 | 0.69 | 0.64 | 0.76 | 0.02 |

[†]Control diet - Ridley AgriProducts Pty Ltd., Pakenham, 3810, Australia, insoluble fiber – Arbocel RC Fine, JRS Co. Inc., Rosenberg, Germany.

[‡]Weight ranges for Hy-Line Brown layer-strain, 13 wk 1126 – 1196g; 18 wk 1470 – 1570g (Hy-Line International, 2014) n = 6 (pen replicate) at 5 wk, n = 3 (pen replicate) at 10 wk.

*Means at 5 wk within the same row are significantly different ($P < 0.05$).

[a,b]Means at 10 wk within the same row with different superscript letters differ, $P < 0.05$.

[1]C = control diet (no added IF) from 0 to 10 wk.

[2]IF = insoluble fiber (IF) diet containing 1% Arbocel RC Fine, from 0 to 10 wk.

[3]C-IF = control diet from 0 to 5 wk; IF diet from 5 to 10 wk.

[4]IF-CO = IF diet from 0 to 5 wk; diet C from 5 to 10 wk.

**Table 3.** Experiment 2 Live body weight (g) and relative weights (g/100 g live-weight) of internal organs (g) of 19 wk old Hy-Line Brown laying hens fed a commercial diet (C) or the commercial diet plus 0.8% insoluble fiber[†] (IF) for 13 wk (Means, pooled SEM, n = 8).

| Time fed diet (Age) wk | 3 (22) | | | 6 (25) | | | 9 (28) | | | 12 (31) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treatments | C | IF | SEM | C | IF | SEM | C | IF | SEM | C | IF | SEM |
| Body weight[‡] | 1773.9 | 1743.9 | 53.23 | 1793.1 | 1822.1 | 66.78 | 1888.3 | 1882.7 | 66.83 | 1902.1 | 1919.4 | 54.95 |
| Liver | 1.91 | 2.21* | 0.11 | 2.10 | 2.34* | 0.08 | 2.23 | 2.40* | 0.07 | 2.10 | 2.27 | 0.15 |
| Gizzard | 1.19 | 1.25 | 0.05 | 1.18 | 1.21 | 0.06 | 1.14 | 1.23 | 0.08 | 1.10 | 1.32* | 0.05 |
| Pancreas | 0.17 | 0.17 | 0.01 | 0.18 | 0.18 | 0.01 | 0.18 | 0.18 | 0.01 | 0.19 | 0.21 | 0.01 |
| Proventriculus | 0.25 | 0.27 | 0.02 | 0.27 | 0.29 | 0.02 | 0.28 | 0.30 | 0.02 | 0.29 | 0.31 | 0.02 |

[†]IF – insoluble fiber as Arbocel RC Fine, JRS Co. Inc., Rosenberg, Germany.

[‡]Normal weight ranges for Hy-Line Brown layer-strain, 22 wk 1,720–1,820 g; 25 wk 1,790 – 1,910 g; 28 wk 1,830 – 1,950 g; 31 wk 1,840 – 1,960 g (Hy-Line International, 2014).

*Means within the same row at same time point differ ($P < 0.05$).

## RESULTS AND DISCUSSION

### *BW and Relative Weights of Organs*

The presence of 1% IF in the diet of pullets for five (Groups C-IF and IF-C) or 10 wk (Group IF) resulted in heavier BW than controls (Group C, Table 2) but there was no significant difference among the treatments. In Experiment 2, the BW of hens in Group IF were also not significantly different from control hens (Table 3). The inclusion of IF in the diets had no detrimental effect on BW of the pullets or laying hens as, in all treatments in Experiment 1 (Table 2), mean BW was heavier than the normal ranges for Hy-line Brown pullets and in Experiment 2 the BW of both treatment groups (Table 3) was within the normal ranges (Hy-Line International, 2014). Others have shown that IF can improve BW gain in both layer-strain and broiler birds, and it is possible that weight gain may be affected by age or developmental age at which IF is given or by the strain of poultry used. Incharoen and Manee-

chote (2013) showed that IF supplied as whole rice hulls to younger pullets and of a different strain than used here, 4- to 8-week-old HN Nick Brown layer-strain pullets, resulted in a significant increase in BW. González-Alvarado et al. (2007) showed that BW gain in broiler chicks was significantly improved by increased fiber content of the diet from 14- to 21-day-old but was not affected in younger birds and Bogusławska-Tryk (2005) showed that feeding broiler chickens from 0 to 42 d of age with Arbocel BWW-40 at four different levels (the final amounts added to the finisher ration being 0 (control), 0.45%, 0.7%, and 0.95%) led to significantly increased BW at the lowest and highest levels when compared with controls.

Feeding the IF diet for 5 or 10 wk in Experiment 1 did result in significant increases in the relative weights of some organs (Table 2). After being fed the IF diet for 5 wk the relative weight of the proventriculus in 13 wk old pullets (Experiment 1, Table 2) was significantly greater than that of the pullets on the control

diet. Feeding the IF diet for a further 5 wk to 18 wk of age resulted in significantly greater relative weights of the liver and gizzard (but not proventriculus) compared to the weights in the control (Group C) and other two treatments in which IF was fed for a shorter period (Table 2). A significant difference among relative weights of the small intestines was seen between those of Group IF and Group C-IF pullets (Table 2) although those from Group IF were heavier than small intestines in the other two groups (Groups C and IF-C). No other significant differences were found among relative weights of organs in Experiment 1.

Insoluble fiber has been shown to affect the weights of supply organs and can affect functioning of the GIT, digestibility of feed and feed conversion of poultry (e.g., Hetland et al., 2004, 2005; Mateos et al., 2012). Addition of pea fiber, wheat bran and oat bran either at low (18 g/kg) or high levels (375 g/kg) in broiler diets was shown by Jørgensen et al. (1996) to cause a significant increase in the weight of the GIT tract which is similar to the results of Experiment 1 in pullets given the IF diet for 10 wk. The effects of IF on gizzard and proventricular weights shown here are similar to those in an experiment by González-Alvarado et al. (2007), in which IF was included in the diets of broiler chicks from 1 to 21 d of age: the IF was included either as 3% oat hulls or 3% soy hulls, in low-fiber diets (crude fiber 2.5%). Oat hulls caused a significant increase in gizzard weight: proventricular weight was only significantly increased with soy hulls (González-Alvarado et al., 2007). Thus source of IF (and therefore proportions of the different fiber components) can influence organ development differently. Similar to the experiment reported here using Arbocel RC, Bogusławska-Tryk (2005) also reported no effect of the IF source, Arbocel BWW-40, on pancreas weight. Adding IF to poultry feeds has been shown to change the weight and length of the GIT (Siri, 1992; Viveros et al., 1994; Jørgensen et al., 1996; Smits et al., 1997) which supports the results obtained in Experiment 1. Taylor and Jones (2004) suggested that increased gizzard size could be a reason for the reduction in weight of the small intestine they found and may reflect an adaptation of the gut to increased availability of nutrients as a result of feeding a diet containing increased fiber concentration. A well-developed gizzard can positively enhance digestion as food is retained for a longer time in the upper digestive tract (proventriculus and gizzard) and this may activate digestive secretions from specific organs and result in more efficient nutrient assimilation (Jones and Taylor, 2001). González-Alvarado et al. (2007) showed that fiber improved total tract apparent retention of feed and feed conversion and it also caused a decrease in the pH in the gizzard. Fewer experiments using IF have been carried out on layer-strain poultry than broilers; however, Freitas et al. (2014) showed that 7 wk old Hy-Line Brown pullets given increased dietary neutral detergent fiber (NDF) for 5 wk had significantly increased

liver and intestinal weights but gizzard weight, unlike that in Experiment 1, was reduced.

In the adult hens in Experiment 2 only the gizzard at 12 wk and liver at 3, 6, and 9 wk after the start of the trial showed significantly higher relative weights. Others have reported on the effects of IF on organ weights in adult hens: the weights of gizzard and pancreas in 29-week-old Leghorn hens (Hetland et al., 2003) were similar to those of the Hy-Line Brown hens in Experiment 2 and the authors reported an increase in relative gizzard weight with increased IF intake. The authors (Hetland et al., 2003) also showed there to be a significant increase in the relative weights of the gizzards in 29-week-old Leghorn pullets supplemented with IF as wood shavings for 14 wk when compared with unsupplemented controls.

The effect of IF on concentrations of protein in proventriculus and pancreas differed between the two experiments reported here. The only significant difference in protein contents of the proventriculus were seen between Groups IF and C after 5 wk in Experiment 1 (Table 4). However in the laying hens in Experiment 2 (Table 5) significantly higher protein contents were seen in pancreatic tissue at 3, 6, and 9 wk after the start of the experiment in Group IF hens compared with those in Group C.

Overall, the results of the experiments reported here show that organ weights can be increased by the addition of IF to diets of growing pullets and in hens after the start of egg-laying. Such effects may help to contribute to improved feed utilization as has been reported by others.

## Enzyme Activities and mRNA Expression of Pepsinogen A and C

Besides the effects of IF on live-weights and supply organ weights the experiments reported here show significant effects of IF supplementation on the activities of proteases required at the different stages for digestion of dietary protein. Five wk after the start of Experiment 1, proventricular pepsin and pancreatic GP and chymotrypsin activities (Table 4), were higher ($P < 0.05$) in Group IF pullets than those in Group C. After 10 wk on those diets (Table 4), activities of pepsin, pancreatic GP, trypsin and chymotrypsin were significantly higher in Group IF than Group C pullets. This may suggest that a longer time of feeding IF is required to induce a significant response. Feeding the IF diet to the older pullets (13 to 18 wk of age) for 5 wk (Group C-IF) had no significant effect (compared to controls) on activities of pepsin or the three pancreatic enzymes measured (Table 4). Although activities of pepsin and pancreatic GP, trypsin and chymotrypsin in pullets fed first the IF and then the control diets from 13 to 18 wk of age (Group IF-C) were always higher than those of controls (Table 4) significant differences in enzyme activities in the pancreatic enzymes were only

**Table 4.** Experiment 1 Enzyme activities (per min/ g tissue) and protein content (mg/ g tissue) of 8 wk old Hy-Line Brown layer-strain pullets given a control diet[†] (C) and the control diet supplemented with 1% insoluble fiber[†] (IF) for 5 wk then either diet C or diet IF for a further 5 wk (Mean, pooled SEM).

| Time fed diet (Age) wk | 5 (13) | | | 10 (18) | | | | |
|---|---|---|---|---|---|---|---|---|
| Treatments | C | IF | SEM | C | C-IF | IF-C | IF | SEM |
| Pepsin[1] | 70.6 | 75.3* | 1.8 | 85.7$^b$ | 82.5$^b$ | 92.6$^b$ | 104.5$^a$ | 2.7 |
| GP-pancreas[2] | 94.3 | 122.9* | 4.6 | 96.6$^c$ | 101.4$^{b,c}$ | 107.6$^b$ | 125.8$^a$ | 3.4 |
| Trypsin[4] | 198.2 | 207.9 | 8.0 | 258.3$^c$ | 247.8$^c$ | 369.4$^b$ | 402.5$^a$ | 20.47 |
| Chymotrypsin[3] | 4.0 | 4.3* | 0.1 | 3.80$^b$ | 3.94$^b$ | 4.06$^b$ | 4.62$^a$ | 0.10 |
| Dipeptidase[5] | 14.1 | 14.7 | 0.7 | 12.74 | 12.38 | 10.94 | 12.57 | 0.39 |
| Aminopeptidase[6] | 79.2 | 87.2 | 3.6 | 55.73 | 59.95 | 58.62 | 59.53 | 1.95 |
| Protein-Proventriculus | 104.3 | 107.3* | 5.8 | 96.7 | 96.2 | 99.4 | 112.8 | 3.4 |
| Protein-Pancreas | 334.8 | 337.1 | 25.2 | 275.9 | 315.3 | 309.0 | 350.0 | 12.4 |

[†]Control diet - Ridley AgriProducts Pty Ltd., Pakenham, 3810, Australia, insoluble fiber – Arbocel RC Fine, JRS Co. Inc., Rosenberg, Germany.

n = 6 (pen replicate) at 5 wk, n = 3 (pen replicate) at 10 wk.

*Means after 5 wk on experimental diets within the same row are significantly different ($P < 0.05$).

[a–c]Means at 10 wk within the same row with different superscript letters differ $P < 0.05$.

For definitions of C, IF, C-IF and IF-C, see Table 2.

[1]Pepsin = pepsin activity in proventriculus, pepsin units $1\mu$mol tyrosine produced/ min/g proventricular tissue.

[2]GP-pancreas = general proteolytic activity, $\mu$mol free tyrosine released from casein substrate/ min/g pancreas.

[3]Chymotrypsin = chymotrypsin units of 4-nitroaniline released from N–glutaryl-L- phenylalanine 4-nitroanilide (GPNA)/ min/g pancreas.

[4]Trypsin = trypsin units of 4-nitroaniline released from $N\alpha$-benzoyl-DL-arginine 4-nitroanilide (DL-BAPNA)/min/g pancreas.

[5]Dipeptidase = $\mu$mol glycyl-L-leucine hydrolyzed/min/g tissue.

[6]Aminopeptidase = nmol 4-nitroaniline released L alanine-$p$-nitroanalide/min/g tissue.

**Table 5.** Experiment 2 Enzyme activities (per min/ g tissue) and organ protein content (mg/ g tissue) of 19 wk old Hy-Line Brown laying hens fed a commercial diet (C) or the commercial diet plus 0.8% insoluble fiber[†] (IF) for 13 wk (Means, pooled SEM, n = 8).

| Time fed diet (Age) wk | 3 (22) | | | 6 (25) | | | 9 (28) | | | 12 (31) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treatments | C | IF | SEM | C | IF | SEM | C | IF | SEM | C | IF | SEM |
| Pepsin | 80.0 | 89.1* | 2.18 | 87.0 | 89.2 | 2.33 | 90.0 | 95.5* | 2.66 | 87.9 | 93.1 | 3.90 |
| GP-pancreas | 116.4 | 139.0* | 4.63 | 93.8 | 107.8* | 4.17 | 59.2 | 81.6* | 5.77 | 97.0 | 122.2* | 4.14 |
| Trypsin | 229.2 | 217.3 | 6.98 | 266.9 | 278.7 | 12.74 | 210.8 | 237.8* | 8.64 | 208.8 | 220.0 | 9.94 |
| Chymotrypsin | 5.2 | 5.2 | 0.13 | 5.1 | 5.3 | 0.11 | 5.8 | 6.1* | 0.13 | 5.0 | 5.5* | 0.18 |
| Protein-Proventriculus | 171.7 | 165.8 | 4.04 | 175.0 | 179.3 | 7.83 | 179.1 | 182.6 | 8.11 | 174.6 | 178.1 | 7.51 |
| Protein-Pancreas | 352.0 | 399.5* | 4.78 | 468.9 | 493.8* | 9.72 | 423.5 | 445.2* | 10.09 | 397.4 | 412.4 | 7.23 |

[†]IF – insoluble fiber as Arbocel RC Fine, JRS Co. Inc., Rosenberg, Germany.

For units of activity and abbreviations see Table 4.

seen for GP activity when compared with that of Group C but trypsin activity was significantly greater than activities in Groups C and C-IF (Table 4).

For small intestinal dipeptidase and aminopeptidase activities in Experiment 1 no significant differences were observed among treatments after 5 or 10 wk on the experimental diets (Table 4).

In the laying hens (Experiment 2), the significant effects of IF were less than in the younger pullets in Experiment 1, and this could have been due the lower concentration of IF used in the second experiment (0.8 versus 1.0%). Pepsin activities (Table 5) increased gradually with age from 19 to 31 wk of age in both treatment groups however pepsin activity in Group IF hens was significantly higher after 9 wk compared with those in the control group. Pancreatic GP activity in Group C hens (Table 5) appeared to decrease with age while at all collection times (3, 6, 9, and 12 wk) GP activities were significantly higher in in Group IF hens than those of hens in Group C. For trypsin activities of the pancreas (Table 5) the only significant difference between treatments was seen at 12 wk, with Group IF having higher activities. Chymotrypsin activities (Table 5) in pancreatic tissues were significantly higher in Group IF compared with those of the control group at nine and 12 wk after the start of the feeding trial.

The mRNA expression in proventricular tissues of 18-week-old pullets was greater for cPgA than for cPgC in both treatment groups. Pullets in Group IF had significantly greater expression of both pepsinogen A

(corrected Ct 25.9 ± 0.5) and C (Corrected Ct 13.1 ± 0.5) compared to those in Group C where corrected Ct for cPgA mRNA was 22.9 ± 1.1 and that for cPgC, 10.8 ± 0.5. There was a positive correlation ($P < 0.05$) between cPgA mRNA activity and pepsin activity (y = 2.339x + 38.072, $R^2$ = 0.314, n = 16).

Because of the significant increase of both proventricular pepsin activity and mRNA pepsinogen expression in IF pullets (Experiment 1), it is unlikely that the higher levels of pepsin activity that were measured here were due to reduced secretion of pepsin (Gabriel et al., 2003) into the proventricular lumen. Decreased pepsinogen release would contribute to a higher retention of pepsinogen in the tissues (Gabriel et al., 2003).

The significantly greater mRNA expression of pepsinogens A and C in the pullets given IF reflects the higher pepsin activities determined in proventricular homogenates and probably reflects an increase in production of the zymogens of pepsin enzymes. The similarity in increase between mRNA of pepsinogen A and that of pepsinogen C suggests that the mechanism of stimulation of the two zymogens was similar and could have been caused by physical effects on the oxyntic (or oxynticopeptic) cells of the proventriculus or by one or more of the following that have been shown to influence pepsinogen production: increased production of cholecystokinin (CCK), decreased pH in the proventriculus and increased reflux of bile from the small intestine via the gizzard (Sommer and Kasper, 1981; Duke, 1982; Li and Owyang, 1993; Sacranie et al., 2005; Jiménez-Moreno et al., 2009; Svihus, 2011; Sacranie et al., 2012). Pepsin activity is stimulated by low pH and possibly through an increase in solubility of mineral salts (Incharoen and Maneechote, 2013) and others have reported on the decrease in proventricular and gizzard pH caused by IF (e.g., Hetland et al., 2003; Svihus, 2011).

The results obtained here on increased trypsin and chymotrypsin activities in the Group IF pullets after 10 wk on IF (Experiment 1), confirm those of Bogusławska-Tryk (2005) who showed that trypsin and chymotrypsin activities were increased relative to controls in 21-day-old broilers fed IF as Arbocel BWW-40. Regulation of the production of pancreatic enzymes is through hormones such as CCK, secretin and gastrin produced in the digestive tract (Sommer and Kasper, 1981; Li and Owyang, 1993; Owyang and Logsdon, 2004; Wang and Cui, 2007). Hormone production in the intestinal mucosa of pullets fed IF could have been increased through increased intestinal tissue production (Table 2), and this in turn could lead to increased production of zymogens of the pancreatic enzymes and thus to measurement of an increased activity of the enzymes. Significant increases in pancreatic GP and chymotrypsin activities were seen in both experiments reported here when IF was added to diets of layer-strain pullets or hens but trypsin activity was only significantly increased in pullets in Experiment 1. These different responses could have been due to the stage of maturity of the birds or to the differences in fiber concentration of the diets fed to the poultry in the two different experiments.

The effects of increased enzyme activity in IF supplemented poultry may have contributed to the observed increase in weights of supply organs in both experiments but it is not possible to identify the specific components of the IF that caused such effects. The commercial product, Arbocel RC, used in these experiments contained 65% crude fiber with 20% lignin (manufacturer's data) and is probably very different in composition from sources of IF used by other research workers.

The results of these experiments showing increased proteolytic digestive enzyme activities in the proventriculus and pancreas and in growth of supply organs may explain in part the improvements in protein digestibility reported by others following IF supplementation in poultry (e.g., Jaroni et al., 1999; Jiménez-Moreno et al., 2009). However, in order to be able to understand mechanisms by which IF can alter enzyme activities and or organ growth and to be able to understand why different IF sources produce different effects, it will be necessary to identify more accurately the different types and proportions of the constituents making up the IF sources used in poultry feeds.

## ACKNOWLEDGMENTS

## REFERENCES

Anson, M. L. 1938. The estimation of pepsin, trypsin, papain, and cathepsin with haemoglobin. J. Gen. Physiol. 22:79–89.

Azzam, M., X. Zou, X. Dong, and P. Xie. 2011. Effect of supplemental L-threonine on mucin 2 gene expression and intestine mucosal immune and digestive enzymes activities of laying hens in environments with high temperature and humidity. Poult. Sci. 90:2251–2256.

Bogusławska-Tryk, M. 2005. Effect of different levels of cellulose in the diet on the proteolytic activity of the pancreas in broiler chickens. Folia. Biologica. (Kraków.) 53:19–23.

Caviedes-Vidal, E., and W. H. Karasov. 2001. Developmental changes in digestive physiology of nestling house sparrows, Passer domesticus. Physiol. Biochem. Zool. 74:769–782.

Degolier, T. F., G. E. Duke, and R. E. Carraway 1997. Neurotensin decreases pepsin output and gastrointestinal motility in chickens. Poult. Sci. 76:1435–1493.

Delia, D. 2008. Protein requirements, digestion and taste preferences of two lorikeet species. PhD Thesis, School of Life Sciences, La Trobe University, Melbourne, Victoria, Australia. DOM 636.68653 D353p.

Donta, S. T., and H. Van Vunakis. 1970. Chicken pepsinogens and pepsins. Their isolation and properties. Biochem. 9:2791–2797.

Duke, G. E. 1982. Gastrointestinal motility and its regulation. Poult. Sci. 61:1245–1256.

Erlanger, B. F., N. Kokowsky, and W. Cohen. 1961. The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys. 95:271–278.

Freitas, E. R., Braz, P. H. Watanabe, C. E. B. Cruz, G. A. J. Nascimento, and R. M. Bezerra. 2014. Fiber level for laying hens during the growing phase. Ciência. e Agrotecnol. 38:188–198.

Gabriel, I., S. Mallet, and M. Leconte. 2003. Differences in the digestive tract characteristics of broiler chickens fed on complete pelleted diet or on whole wheat added to pelleted protein concentrate. Br. Poult. Sci. 44:283–290.

González-Alvarado, J., E. Jiménez-Moreno, R. Lázaro, and G. Mateos. 2007. Effect of type of cereal, heat processing of the cereal, and inclusion of fiber in the diet on productive performance and digestive traits of broilers. Poult. Sci. 86:1705–1715.

Gopinath, V., G. D. Raj, A. Raja, K. Kumanan, and S. Elankumaran. 2011. Rapid detection of Newcastle disease virus replication in embryonated chicken eggs using quantitative real time polymerase chain reaction. J. Virol. Methods. 171: 98–101.

Grommen, S. V., S. Taniuchi, V. M. Darras, S. Takahashi, S. Takeuchi, and B. De Groef. 2008. Identification of unique thyrotropin receptor (TSHR) splice variants in the chicken: The chicken TSHR gene revisited. Gen. Comp. Endocrinol. 156: 460–463.

Harrison, S. M., I. Tarpey, L. Rothwell, P. Kaiser, and J. A. Hiscox. 2007. Lithium chloride inhibits the coronavirus infectious bronchitis virus in cell culture. Avian. Pathol. 36:109–114.

Hetland, H., B. Svihus, and Å. Krogdahl. 2003. Effects of oat hulls and wood shavings on digestion in broilers and layers fed diets based on whole or ground wheat. Br. Poult. Sci. 44: 275–282.

Hetland, H., B. Svihus, and M. Choct. 2005. Role of insoluble fiber on gizzard activity in layers. J. Appl. Poult. Res. 14:38–46.

Hetland, H., M. Choct, and B. Svihus. 2004. Role of insoluble nonstarch polysaccharides in poultry nutrition. World's Poult. Sci. J. 60:415–422.

Hy-Line International. 2014. Hy-Line Brown management guide – Commercial layers. Hy-Line Inernational, http://www.hyline.com.au/wp-content/uploads/2014/03/HL-Brown-Commercial-Manual-BRN-COM-ENG.pdf.

Hirschowitz, B. I. 1991. Control of pepsinogen secretion. Curr. Opin. Gastroenterol. 7:863–869.

Incharoen, T., and P. Maneechote. 2013. The effects of dietary whole rice hull as insoluble fiber on the flock uniformity of pullets and on the egg performance and intestinal mucosa of laying hens. Am. J. Agric. Biol. Sci. 8:323–329.

Jaroni, D., S. Scheideler, M. Beck, and C. Wyatt. 1999. The effect of dietary wheat middlings and enzyme supplementation II: Apparent nutrient digestibility, digestive tract size, gut viscosity, and gut morphology in two strains of leghorn hens. Poult. Sci. J. 78:1664–1674.

Jiménez-Moreno, E., J. González-Alvarado, A. de Coca-Sinova, R. Lázaro, and G. Mateos. 2009. Effects of source of fibre on the development and pH of the gastrointestinal tract of broilers. Anim. Feed Sci. Technol. 154:93–101.

Jones, G., and R. Taylor. 2001. The incorporation of whole grain into pelleted broiler chicken diets: production and physiological responses. Br. Poult. Sci. 42:477–483.

Jørgensen, H., X. Q. Zhao, K. E. B. Knudsen, and B. O. Eggum. 1996. The influence of dietary fiber source and level on the development of the gastrointestinal tract, digestibility and energy metabolism in broiler chickens. Br. J. Nutr. 75:379–395.

Li, Y., and C. Owyang. 1993. Vagal afferent pathway mediates physiological action of cholecystokinin on pancreatic enzyme secretion. J. Clin. Invest. 92:418–424.

Lim, V. P., Jr., J. J. Juan, O. F. Celestino, J. V. San Andres, and E. A. Martin. 2013. Beneficial effects of insoluble raw fiber concentrate addition to layer diet. Philipp. J. Vet. Anim. Sci. 39:43–52.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Appl. Biol. Chem. 193:265–275.

Mateos, G., E. Jiménez-Moreno, M. Serrano, and R. Lázaro. 2012. Poultry response to high levels of dietary fiber sources varying in physical and chemical characteristics1. J. Appl. Poult. Res. 21:156–174.

Mohiti-Asli, M., M. Shivazad, M. Zaghari, M. Rezaian, S. Aminzadeh, and G. Mateos. 2012. Effects of feeding regimen, fiber inclusion, and crude protein content of the diet on performance and egg quality and hatchability of eggs of broiler breeder hens. Poult. Sci. 91:3097–3106.

Owyang, C., and C. D. Logsdon. 2004. New insights into neurohormonal regulation of pancreatic secretion. J. Gastroenterol. 127:957–969.

Sacranie, A., B. Svihus, V. Denstadli, B. Moen, P. Iji, and M. Choct. 2012. The effect of insoluble fiber and intermittent feeding on gizzard development, gut motility, and performance of broiler chickens. Poult. Sci. 91:693–700.

Sacranie, A., P. Iji, M. Choct, and T. Scott. 2005. Reflux of digesta and its implications for nutrient digestion and bird health. Proc. 17th Australian Poultry Science Symposium.

Sakamoto, N., H. Saiga, and S. Yasugi. 1998. Analysis of temporal expression pattern and cis-regulatory sequences of chicken pepsinogen A and C. Biochem. Biophys. Res. Commun. 250: 420–424.

Sarikhan, M., H. Shahryar, K. Nazer-Adl, B. Gholizadeh, and B. Behesht. 2009. Effects of insoluble fiber on serum biochemical characteristics in broiler. Int. J. Agric. Biol. 11:73–76.

Siri, S. 1992. Effects of dietary cellulose level on nutrient utilization in chickens. Asian-Aus. J. Anim. Sci. 5:741–746.

Smits, C. H. M., A. Veldman, M. W. A. Verstegen, and A. C. Beynen. 1997. Dietary carboxymethylcellulose with high instead of low viscosity reduces macronutrient digestion in broiler chickens. J. Nutr. Sci. 127:483.

Sommer, H., and H. Kasper. 1981. Effect of acetylcholine, gastrin, and glucagon alone and in combination with secretin and cholecystokinin on the secretion of the isolated perfused rat pancreas. Res. Exp. Med. (Berl.) 179:239–247.

Summers, J. D., and S. Leeson. 1994. Laying hen performance as influenced by protein intake to sixteen weeks of age and body weight at point of lay. Poult. Sci. 73:495–501.

Susbilla, J. P., I. Tarvid, C. B. Gow, and T. L. Frankel. 2003. Quantitative feed restriction or meal-feeding of broiler chicks alter functional development of enzymes for protein digestion. Br. Poult. Sci. 44:698–709.

Svihus, B. 2011. The gizzard: function, influence of diet structure and effects on nutrient availability. Worlds. Poult. Sci. J 67: 207–224.

Taylor, R. D., and G. Jones. 2004. The incorporation of whole grain into pelleted broiler chicken diets. II. Gastrointestinal and digesta characteristics. Br. Poult. Sci. 45:237–246.

Viveros, A., A. Brenes, M. Pizarro, and M. Castano. 1994. Effect of enzyme supplementation of a diet based on barley, and autoclave treatment, on apparent digestibility, growth performance and gut morphology of broilers. Anim. Feed. Sci. Technol. 48:237–251.

Wang, B. J., and Z. J. Cui. 2007. How does cholecystokinin stimulate exocrine pancreatic secretion? From birds, rodents, to humans. Am. J. Physiol. Regul. Integr. Comp. Physiol. 292:666–678.