Final Report

Project code: 18-425

Prepared by: Rebecca Forder

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Dietary inclusion of DV XPC® yeast metabolite to alleviate potential gut dysbiosis in a female broiler breeder line and its effect on hen and progeny performance.

Milestone 5. Final report on the potential for utilisation of DV XPC® as a supplement, detailing the production benefits both for breeders and progeny.

Dietary inclusion of DV XPC® yeast metabolite to alleviate potential gut dysbiosis in a female broiler breeder line and its effect on hen and progeny performance.

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In submitting this report, the researcher has agreed to Poultry Hub Australia publishing this material in an edited form.

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Project Summary

Project Title	***Dietary inclusion of DV XPC® yeast metabolite to alleviate potential gut dysbiosis in a female broiler breeder line and its effect on hen and progeny performance.***
Project No.	18-425
Date	Start: 14/11/18 End: 14/1/2020
Project Leader(s)	Rebecca Forder
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Project Aim	To alleviate potential gut dysbiosis in GGP broiler breeders, using a novel dietary yeast metabolite supplement, Diamond V XPC®. To determine if dietary inclusion of Diamond V XPC® to breeder rations would have a positive effect on progeny growth and performance.
Background	HiChick Breeding Company identified a great grandparent chicken meat line that consistently produces poorly formed, wet droppings during lay. Compared to other breeder lines, these birds also exhibit reduced egg production and higher mortality. Growing evidence linking maternal gut health with stress-induced disorders as well as the transgenerational effects on progeny development, led us to investigate the impact of this trait on both the hen as well as early-life programming of their progeny, with focus on physiological stress and production performance.
Research Outcome	The results indicate that stress accompanied by chronic intestinal inflammation may be causation for the increased intestinal permeability in hens from the poor performing line. Dietary inclusion of DV XPC did not influence intestinal permeability, WBC differentials and IL-6 or corticosterone concentration in either line. However, XPC supplementation did reduce mortality and improve egg production and hatchability in the poor performing line. Inclusion of XPC into breeder diets did not influence performance, yolk, plasma or feather hormone concentrations in progeny hatched from 32 week old hens.
Impacts and Outcomes	The potential financial gains from an industry perspective through improvements in litter quality, breeder performance and progeny growth are considerable, making investigating potential means to generate such gains worthwhile. Future investigation into the physiology and behaviour around point of lay is required to find novel strategies to alleviate stress and in turn potentially improve welfare and production outcomes at all tiers of the chicken meat breeder pyramid.
Publications	We aim to publish 3 papers, the first, focusing on the breeder hen data, the second on progeny performance and hormonal assay data and the third on the progeny sequencing data.

Executive Summary

HiChick Breeding Company identified a great grandparent chicken meat line that consistently produces poorly formed, wet droppings during lay. Compared to other breeder lines, these birds also exhibit reduced egg production and higher mortality. Growing evidence linking maternal gut health with stress-induced disorders as well as the transgenerational effects on progeny development, led us to investigate the impact of this trait on both the hen as well as early-life programming of their progeny, with focus on physiological stress and production performance. In addition, we aimed to alleviate this potential disturbed gastrointestinal homeostasis, using a novel dietary yeast metabolite supplement, Diamond V XPC®.

Broiler breeders (n=240) from two genetic lines (Line A. high performing & Line B, low performing, wet litter) were separated into four experimental groups based on line (A or B) and diet (control or *SC* (1000 ppm) at 23 weeks of age. Blood samples for analysis of stress, gastrointestinal permeability and general inflammatory biomarkers were collected at 23, 26, 32 and 37 weeks of age. Standard breeder production parameters such as mortality, egg production and hatchability were collected for the entire duration of the trail (47 weeks).

At 32 weeks, 82 eggs/treatment were collected and incubated. A sub-sample of eggs (n=7/treatment) were analysed for yolk corticosterone and testosterone. At hatch, 160 viable chicks were separated based on maternal treatment. Body weight (Bwt) and FCR were measured weekly. At 42d of age, eight birds/treatment were dual-energy x-ray absorptiometry (DEXA) scanned for body composition (BC). Plasma corticosterone concentration was analysed at d22, 37 and 42. In addition, RNA-Seq analysis was carried out on total RNA extracted from the hypothalamus and liver of male (n=6) and female (n=6) progeny from both Line A and B hens at two targeted time points: embryonic day 15 (hypothalamus) day 22 post-hatch (liver).

Dietary inclusion of DV XPC® reduced mortality and improved egg production and hatchability but only in the poor performing, wet litter line (Line B). DV XPC did not influence litter moisture, intestinal permeability, inflammatory biomarkers or corticosterone concentration in either line. Compared to Line A, the poor performing line (Line B) had a significant increase in intestinal permeability and litter moisture at 26 wks (onset of lay). Line B heterophil counts were increased markedly at wk 26 before declining. At wks 26, 32 and 37 there were also significant increases in monocytes. An increase in plasma corticosterone was also observed. No significant differences in H:L ratios, feather corticosterone or IL-6 was observed between lines.

Yolk CORT concentrations were higher in eggs from the poor performing line with plasma corticosterone consistently elevated in progeny from the poor performing line. No significant differences feather corticosterone was observed between lines. Progeny from the poor performing line tended to be lighter in bodyweight. FCR did not differ between lines or with dietary inclusion of DV XPC, any stage of the 42 day grow out period. Female progeny from hens from the poor performing line hens had a greater fat% and reduced lean% compared to male progeny from the same line. Dietary inclusion of DV XPC® to breeder diets did not positively influence progeny bodyweight, gain, FCR or body composition throughout the grow-out trial.

Comparison between males and females within lines were largely sex driven, most differentially expressed genes were located on the Z chromosome. Functional analysis of the DE genes did not identify pathways of significance between male and females within each line and between lines in embryonic brain. Functional analysis of DE genes in liver from day 22 post-hatch progeny, found four significant pathways identified in males and females between lines involved in fatty acid metabolism and oxidative phosphorylation.

The results indicate that stress accompanied by chronic intestinal inflammation may be causation for the increased intestinal permeability in the poor performing line. Future investigation into the physiology and behaviour around point of lay is required to find novel strategies to alleviate this stress and in turn potentially improve welfare and production outcomes.

This study was unable to provide links to any possible transgenerational effects (i.e. stress) to endocrine pathways in the brain or liver**.** Pathway analysis of liver samples provided some promising avenues to further explore the phenotypic differences in body composition, particularly fat %, observed between the two lines. Further work is required tease out mechanisms of potential transgenerational effects of stress in broiler breeders, both in F1 generations and beyond.

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Introduction

The chicken meat industry has made tremendous production gains through advanced utilisation of genetic selection and nutritional understanding to ensure that chicken meat remains a low cost, desirable product that consumers continuously demand (Zuidhof *et al.* 2015). The industry now requires new approaches to further advance efficiency and production, with developmental programming at the forefront of industry development (Hynd *et al.* 2016). Developmental programming is defined as "alterations in the *in ovo* environment, induced by the maternal environment, resulting in developmental adaptations that permanently change the structure, physiology, metabolism, health and production of the offspring". Such adaptations can be induced by several factors, including maternal nutrition and stress, which are both significant factors when considering current broiler breeder practices. Recent evidence suggests that the microbial community populating the gastrointestinal tract is capable of modulating neural and endocrine pathways, and the immune response as well as behaviour and growth, otherwise known as the gut-brain microbiota axis (Farzi *et al.* 2019). Gut dysbiosis occurs when there is a disruption in the gut-brain axis, and consequent disruption to the symbiotic relationship between gut microbial population and the intestinal mucosa. It has been speculated that intestinal inflammation develops as the result of an imbalance in the maintenance of homeostasis between intestinal commensals and immunity to pathogens, with an impairment of intestinal barrier function and increased intestinal permeability (Demaude *et al.* 2006). The gut microbiota is proposed to be modulated by the stress response, which, via the brain-gut-microbiota axis, feeds back and influences the inflammatory response through microbial metabolite regulation of cytokine production and influence on enteric gastrointestinal barrier function (de Punder and Pruimboom 2015; Schirmer *et al.* 2016). In mammalian literature, a disruption to the brain-gut-microbiota axis has been linked to low-grade chronic inflammation and consequent decreased reproductive performance and foetal development (Xie *et al.* 2016).

Additionally, progeny exposure to maternal stress during early development can change an organism's microbiota composition, and that the microbiota can alter the organism's ability to respond to stress after birth (Jasarevic *et al.* 2017; Hechler *et al.* 2019). Recent findings from our group have demonstrated that maternal stress in broilers can have significant negative effects on progeny body weight, stress-linked behaviour and immune response (Bowling *et al.* 2018). The extent to which alterations to the intestinal environment, including microbiota, affects the programming of gut-brain signalling pathways, of both the hen and her progeny, is an area of great interest. Considering commercial chicken meat birds now spend ~40% of their life *in-ovo*, the influence breeder hen rearing practices on progeny development, especially through maternal stress, may provide a pathway to improve production aspects through nutritional manipulations of already well-established industry protocols. Additionally, by optimising breeder hen practices, there is substantial opportunity to improve breeder hen health, reproductive capacity and welfare standards, all of which improve industry profitability, as well as community perception of the chicken meat industry.

The use of dietary supplements, such as pre and probiotics to alleviate various digestive disorders is not a new concept, and is a common occurrence in clinical practise (Gareau *et al.* 2010; Carding *et al.* 2015; Wang *et al.* 2015). However, the influence of these supplements on maternal reproductive performance and progeny phenotype is. Diamond V (DV) XPC® is a yeast metabolite extract from *Saccharomyces cerevisiae* and was originally utilised as an

alternative to in-feed antimicrobials (Gao *et al.* 2008). Price *et al.* 2018 recently investigated whether addition of DV XPC® to broiler diets reduced notable stress identifiers such as heterophil/lymphocyte ratio and plasma corticosterone concentration. (Al-Mansour *et al.* 2011) also reported a reduction in H/L ratio, and documented a trend of increased bodyweight over the six week trial period, although not significant. In other studies, bird growth rate has been significantly increased with dietary addition of XPC from day 21 onwards (Gao *et al.* 2008; Gao *et al.* 2009). The addition of XPC to broiler diets removed previously identified growth reductions after subjection to live-coccidiosis vaccines (Roto *et al.* 2017), although no noted shift in microbial populations has been documented in relation to XPC supplementation (Park *et al.* 2017). Gastrointestinal maturation is reportedly accelerated in turkey poults subjected to yeast metabolite supplementation, where goblet cell populations, crypt depth, villus height and surface area increase following supplementation (de los Santos *et al.* 2007). Thus it appears that dietary inclusion of XPC has numerous benefits on bird health and production. However, the use of XPC in breeder diets and its effects on hen performance has not been extensively studied and may provide a plausible means to introduce a cost effective way to enhance commercial progeny production, whilst improving hen health and reproductive output.

Studies investigating the effects maternal XPC supplementation on progeny performance are limited. XPC supplemented to sows during gestation tended to increase progeny birth weights, weaning weights and body weight gain, reduced still birth % and increased weaning rate % (Carroll *et al.* 2011; Song *et al.* 2017). Two separate studies feeding hydrolysed yeast (Araujo *et al.* 2018) and *Saccharomyces cerevisiae* (XPC) (Kidd *et al.* 2013) to broiler breeder hens, observed a significant increase in egg hatchability, progeny feed conversion ratio (FCR) as well as progeny breast muscle yield %. These findings suggest the dietary inclusion of XPC to breeder hens has the ability to influence progeny performance, although the precise mechanisms remain unclear. Additionally, both aforementioned studies using breeder hens were conducted in Brazil and the USA respectively, thus whether such supplementation would prove effective under Australian rearing practices requires further investigation.

HiChick Breeding Company had identified a great grandparent chicken meat line that consistently produces poorly formed, wet droppings during lay. Compared to other breeder lines, these birds also exhibit reduced egg production and higher mortality. Growing evidence linking maternal gut health, particularly gut dysbiosis, with stress-induced disorders as well as the transgenerational effects on progeny development, has led us to investigate the impact of this trait on both the hen as well as early-life programming of their progeny, with focus on physiological stress and production performance. In addition, we aimed to alleviate this potential gut dysbiosis, using a novel dietary yeast metabolite supplement, Diamond V XPC®.

Objectives

We aimed to test the following hypotheses:

1. The wet litter observed in this grandparent line is a result of gut dysbiosis, which is negatively impacting reproductive performance and progeny development
2. XPC will improve reproductive performance and faecal consistency of HiChick's problem grandparent line, through beneficial modulation of the intestinal environment, reducing physiological stress and inflammation, and
3. Dietary inclusion of XPC in breeder diets will have positive effects on progeny growth and performance, though alterations in endocrine signalling pathways.

Specifically, we aimed to investigate if dietary supplementation of XPC will improve breeder health and performance, as well as positively influence progeny health, development, and production performance.

Part A: Breeders

Methodology

GGP Broiler Breeders

Two Hundred and Forty Cobb 500 GGP broiler breeder hens from two different genetics lines (wet litter line; n=120 and high performing line which acted as our control; n=120), were hatched and reared under standard commercial conditions. At twenty three weeks of age, birds from each line were separated into two treatment groups (control vs DV XPC®; fed either a standard broiler breeder diet or a standard breeder diet supplemented with DV XPC (1000 ppm). Thus four groups were: line 1 control (n=60), line 1 XPC (n=60), line 2 control and line 2 XPC. The birds were housed in a commercial shed, in pens, consisting of ten hens and one cockerel, with six replicates per treatment. Birds had restricted access to feed and water and were fed once daily, with total quantities determined the day before dependent on average bird body weight and reproductive status. Ventilation, lighting and shed temperature were all maintained to industry specification and in accordance with the industry partners commercial conditions. Mortality, egg production and hatchability were recorded over the course of the trial.

Sample Collection

At 23, 26, 31 and 37 weeks of age forty birds (n=10 per treatment) were orally administered a Fluorescein isothiocyanate-dextran (FITC-d) solution (4.16 mg/kg Bwt). Blood samples, for both plasma and serum, were collected via vena puncture of the wing vein two and a half hours after FITC-d administration.

One drop of whole blood from each hen sample was placed on a glass microscope slide and a blood smear generated using the two slide wedge technique. The remaining blood samples were centrifuged (2000 g for 10 min). Plasma and serum was separated and stored at −20°C.

Faecal samples were collected from forty birds (n=10 per treatment) and 23, 26, 31 and 37 weeks of age by cornering of a section of the pen and lining the litter with plastic. Birds were

then placed in the cordoned section and left until faecal excretion occurred. Once a faecal sample was collected birds were returned to the pen and the process repeated. Faecal samples were stored on ice during collection and then frozen at -20ºC. These samples were banked, if the opportunity for microbial analyses arise.

Litter samples were also collected from each pen at 23, 26, 31 and 37 weeks of age. This consisted of using a bucket and obtaining litter from the front/middle/back of the pen and mixing it together before a 500 mL sub sample was collected in a sealed container and stored at 4°C. Litter samples were then analysed for moisture content percentage (MC%) using a moisture content analyser (Mettler Toledo MJ33). A total of 1.2-1.3 g of litter was analysed per pen with per pen samples run in triplicate at all collection time points.

FITC-d analysis (intestinal permeability)

Serum samples were diluted 1:5 in PBS pH 7.4, 50 µL was pipetted onto black 96-well fluorescent plates (Corning®, Sigma-Aldrich, St Louis MO, USA) A standard curve was adapted for every plate using 8, two- fold serial dilutions from the highest value 6,400 ng/mL until it reached 100 ng/mL. Non-FITC-d sera was also diluted 1:5 with PBS and used as a blank.

FITC-d levels of diluted sera were measured at excitation wavelength of 485 nm and emission wavelength of 528 nm, gain 70 (Synergy HT, Multi-mode microplate reader, BioTek Instruments, Inc., VT, USA). Fluorescence measurements were then compared to the standard curve.

Heterophil:Lymphocyte (H:L) counts

Smears were air-dried and fixed in methanol for 20 minutes following the protocol outlined by (Ballard and Cheek 2016). This was later complemented with staining using a Wright-Giemsa stain in an automated slide stainer (Siemens Hematek, Siemens, South Australia) and cover slipped. Leucocytes, including granular cells (heterophils, eosinophils, basophils) and non-granular cells (lymphocytes, monocytes), were counted at ×1000 (oil immersion lens) until a total of 100 cells per side was achieved. One slide was examined for each bird at 23, 26, 32 and 37weeks of age. H/L ratios were determined by dividing the number of heterophils by that of lymphocytes for each slide. For this study, we also focus predominately on comparing the number of monocytes, lymphocytes and heterophils.

Interleukin-6 (IL-6) Assay

A 24-well chicken IL-6 ELISA test kit (Cusabio; CSB-E08549Ch) was run to the manufacturer's specifications to determine the optimal dilution factor required for the plasma samples. Initial test samples were assayed in duplicate at concentrations of 'neat', 1:2 and 1:5 dilutions and the optimal sample dilution was determined to be 1:2. The 96-well chicken Interleukin-6 ELISA kit was then run to the manufacturer's specifications using 1:2 dilutions of wk 26 samples from Line A (n = 20) and Line B (n = 20), assayed in duplicate. Standard solutions of IL-6 at 15.6, 31.2 62.5, 125, 250, 500, and 1000 pg/mL were used. The absorbance of each assay was read at a wavelength of 450nm with a plate reader (BIO-RAD, Benchmark Plus Microplate Reader, South Australia)

Plasma Corticosterone

A commercially available enzyme-linked immunosorbent assay (ELISA; corticosterone ELISA kit ADI-900–097, Enzo Life Sciences, Farmingdale, NY) was used to measure corticosterone concentration in plasma samples collected at 32 and 37 weeks, following the manufacturer's protocol for small plasma samples. Steroid displacement reagent (10 µL at 1:100) was added to 10 µL of each sample. This was vortexed and left to stand for five minutes before 380 µL of ELISA buffer was added to make a final 1:40 dilution. All samples, standards, blanks and positive and negative blanks were assayed in duplicate on a 96 well plate, and the average of each duplicate was used to calculate the final CORT concentration. Standard solutions of CORT at 32, 160, 800, 4,000, and 20,000 pg/mL were used. The absorbance of each assay was read at a wavelength of 405 nm with a plate reader (BIO-RAD, Benchmark Plus Microplate Reader, South Australia)

Feather Corticosterone

Corticosterone was extracted from feathers using a methanol-based extraction technique previously described by Bortolotti *et al.* (2008). Briefly, whole feathers were weighed, and the calamus removed and re-weighed. The remaining feather was cut with scissors into ≤1 cm and pulverized using a QIAGEN Tissuelyser (Hilden, Germany), at a frequency of 30/s in three separate 30-second intervals to avoid overheating.

Feather samples were weighed (30 mg) and placed into low binding 15 mL glass borosilicate tubes. Ten mL HPLC-grade methanol was added to each sample and shaken in a tube shaker (Ratek, Orbital Tube Shaker, AdeLab, South Australia) for 30 min at room temperature, followed by overnight incubation in an orbital shaker (Ratek, Orbital Shaker Incubator, Adelab, South Australia) at 50°C at 300 rpm. Feathers were separated from the methanol using a vacuum filtration system (Grace™ Alltech 12-Port Vacuum Manifold, AdeLab, South Australia), using a frit disk (a porous disk made of plastic, to filter out solid particulate matter while allowing liquids to pass) and Whatman #1 filter paper placed on top of the disk. The feather remnants, original extraction tube and filtration tube were washed twice with 1 mL of additional methanol, which was added to the extractant. The methanol extracts were placed in a 50°C water bath and evaporated under a nitrogen stream. Once evaporated extract residues were resuspended in 500 µL of PBS (pH 7.6) and split into two 250 µL aliquots. Reconstituted samples were frozen at -20°C until analysed.

The amount of CORT in samples was measured using the same ELISA kit and standard protocol as the plasma samples, however these samples were run through the ELISA assay undiluted with assay buffer.

Statistical Analyses

Analysis of experimental data was performed by linear mixed model analysis for independent factors, line and diet, following the procedures of IBM®, SPSS® Statistics 25 program (Armonk, NY, USA). The data were checked for normality by the Shapiro–Wilk test. Non-normalised data was analysed using nonparametric tests including Mann-Whitney U and Kruskal-Wallis. For egg production data, a General Linear Model (GLM) procedure for repeated measures were used. A probability level of less than 5% ($P < 0.05$) was deemed as statistically significant.

Discussion of Results

Mortality

The addition of DV XPC reduced total mortality. However, the difference was only statistically significant within the poor performing, wet litter line (Control, 18.3%; XPC, 5%, *P=0.02*). No differences in mortality between lines were observed nor was there an interaction between line and diet.

Egg production

Feeding hens DV XPC did not increase egg production in the high performing line at any time point. At week 37, egg production started to decline in both lines. Interestingly, in the poorer performing line, although not statistically significant, inclusion of DV XPC tended to increase egg production between 26 and 37 weeks of age (Green box, Figure 1). Which in total, contributed to a 4.7% increase in egg production over this period (mean eggs/bird, control, 4.98 ± 0.14 vs DV XPC, 5.16 ± 0.11)

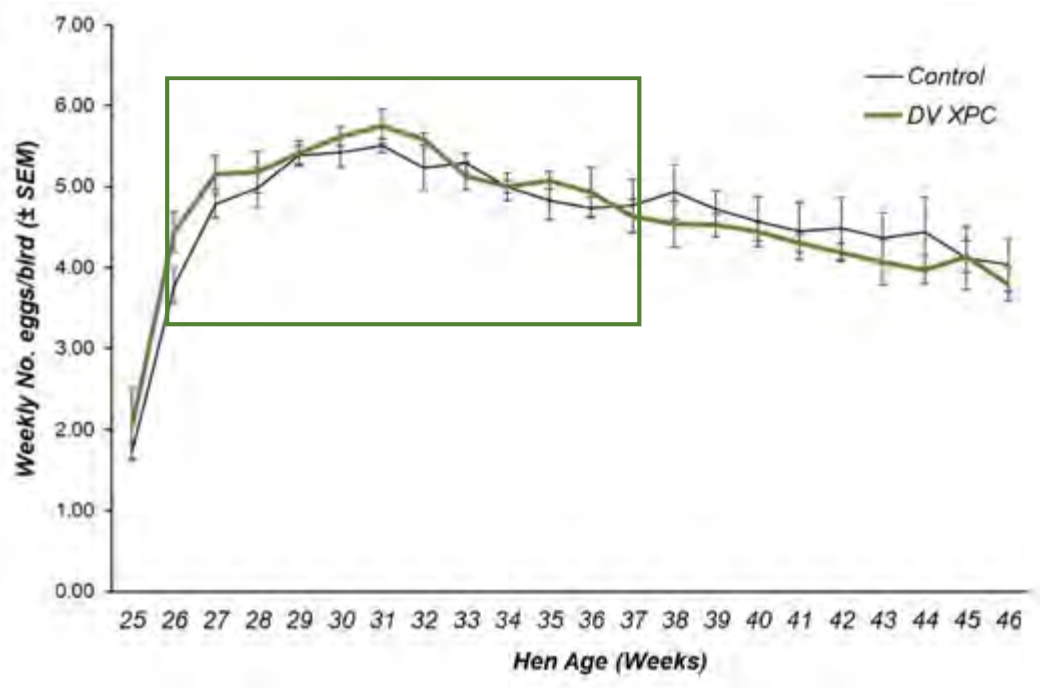

Figure 1. *Egg production in hens from the poor performing line (Line B) fed with and without DV XPC. Values are means ± SE.*

Hatchability

Hatchability in the poorer performing line was increased with the addition of DV XPC, but was only statistically significant when eggs were hatched from 35 week old hens (Figure 2). Eggs collected (n=300) at 32 weeks for the progeny grow out trial had an overall hatchability of 66.63% (Line 2, control = 64%; Line 2, XPC = 70.6%; Line 1, control = 69.3 %; Line 1, XPC = 62.6%), again although not statistically significant eggs from 58 hens that received XPC tended to have higher hatchability.

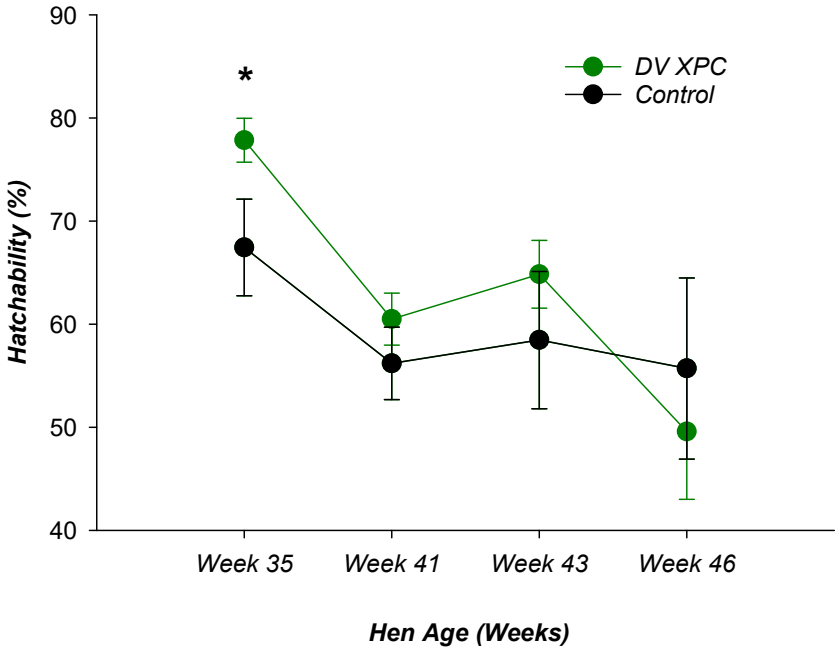

Figure 2. *Percentage hatch of eggs from hens from the poor performing line (Line B) fed diets with and without DV XPC. Values are means ± SE. Week 35 =1045 eggs set; week 41 =538 eggs set; week 42 =1387 eggs set and week 46 = 862 eggs set. *P = 0.03*

Summary

- ***Dietary inclusion of DV XPC® reduced mortality and improved egg production and hatchability but only in the poor performing, wet litter line (Line B)***
- ***DV XPC® did not influence any production parameters in the high performing line (Line A)***

Litter Moisture

Litter moisture increased in both lines from week 23 to onset of lay (week 26). The poor performing line (Line B) consistently had higher litter moisture from 26 weeks onwards compared to the high performing line (Line A). In line A, litter moisture continued to reduce after 26 weeks. The addition of DV XPC reduced litter moisture in the high performing line A at week 26 only (*line x diet interaction*, $P = 0.043$), this was not observed in the poor performing line at any time point (Figure 3).

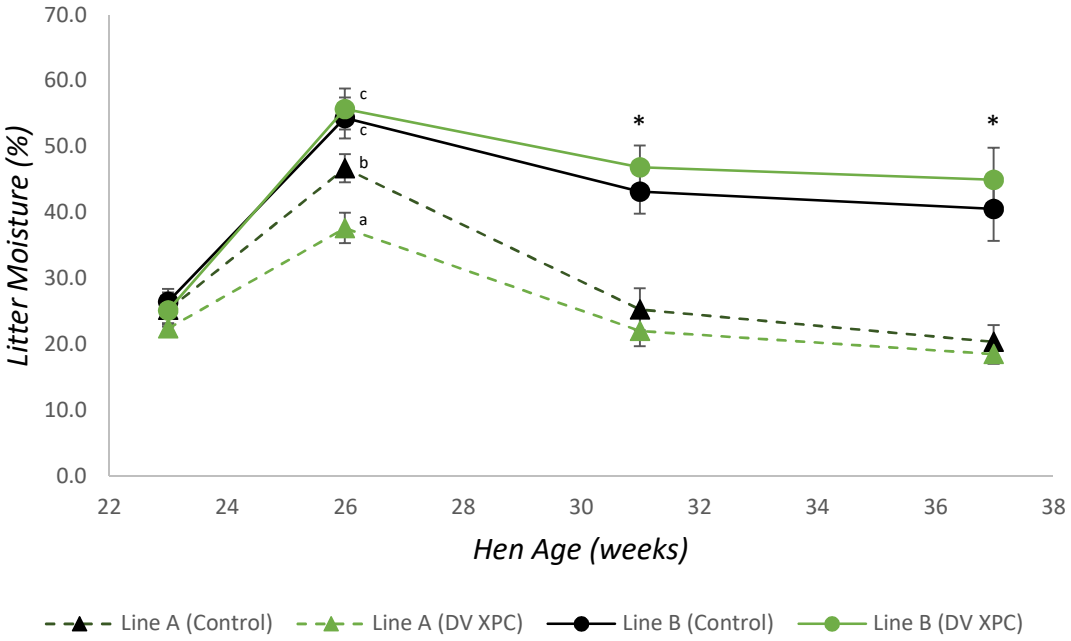

Figure 3. *Litter moisture content (%) from of pens containing both breeder lines fed with and without DV XPC. Values are means ± SE. Week 26, different letters denote a significant difference p < 0.05. *denotes significance (p < 0.05) between lines only.*

Intestinal permeability (FITC-dextran)

Dietary inclusion of DV XPC® fed at 23 weeks did not affect intestinal permeability in either breeder line at 26, 32 or 37 weeks. FITC-d results from 26 week old birds displayed a significant sharp increase in intestinal permeability in line B compared to line A (1058.89 ± 134,04 vs 769.21 ± 20.83), which then plateaued. (Figure 4; P = 0.04). Line B also displayed an increase in intestinal permeability at week 32 (peak lay) and week 37, which were similar to values obtained from line B.

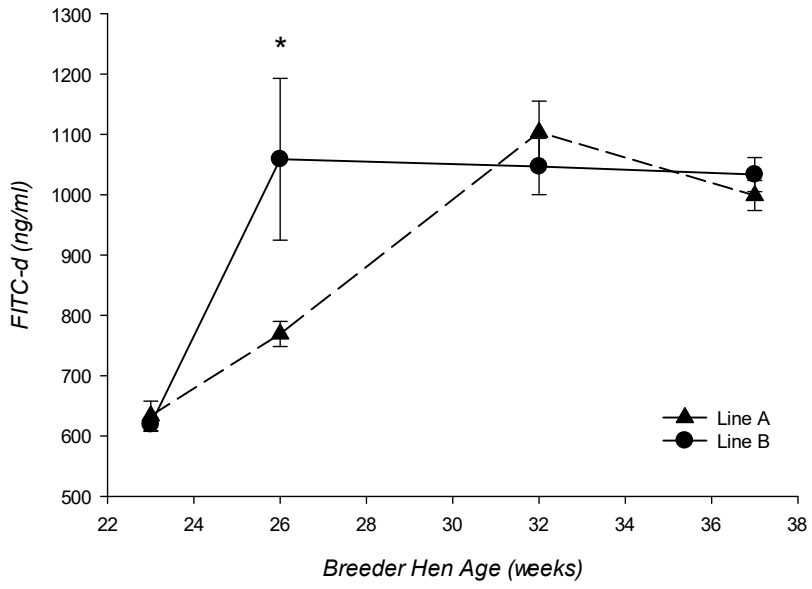

Figure 4. *Detection of serum FITC-d (ng/ml) as a marker of intestinal permeability in breeder hens from two genetic lines (12 and 58), at 23, 26 and 32 weeks of age. Values are mean ± SE.*

Summary

- ***Dietary inclusion of DV XPC did not influence litter moisture or intestinal permeability in either breeder line***
- ***The poor performing line (Line B) did display an increase in intestinal permeability at 26 weeks (on set of lay) of age which reflected the increase in litter moisture***

WBC differentials

Heterophil:Lymphocyte (H:L) ratio

Dietary inclusion of DV XPC fed at 23 weeks did not reduce H:L ratios at 26, 32 or 37 weeks of age. There were no significant differences in H:L ratios between lines at any time point. However, the ratio was slightly higher in line B birds at week 32, but was not significant due to the large variation within time points (Red box, Figure 5). By week 37, this trend was lost, with large variability between treatment groups. There was no relationship found between week 37 plasma corticosterone concentration and week 37 H:L ratio (r = 0.05; p = 0.076).

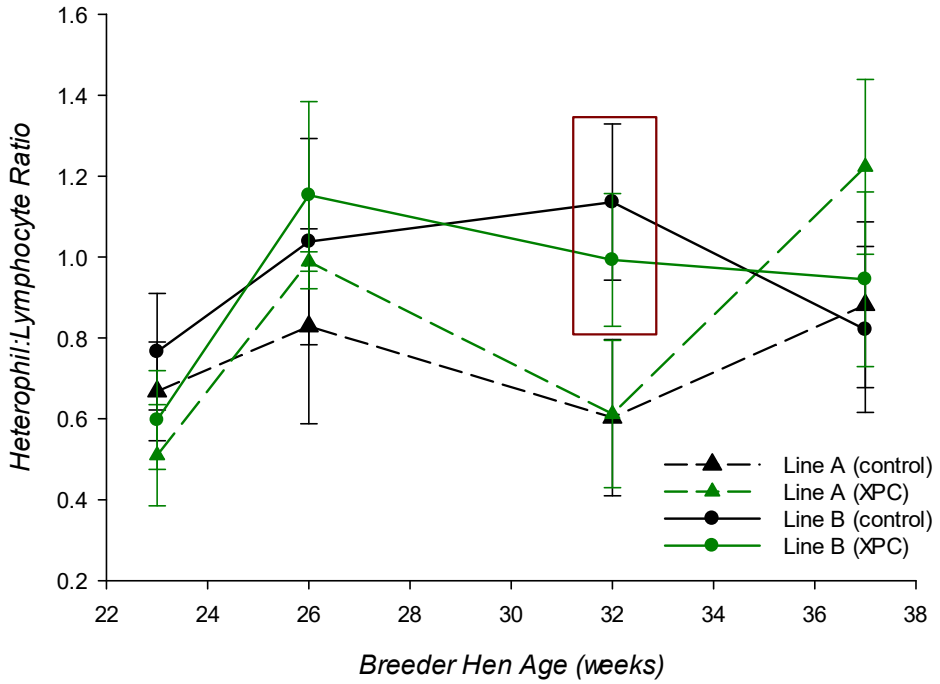

Figure 5. *Mean heterophil/lymphocyte ratios of breeder hens from two genetic lines (A and B), with and without dietary inclusion of DV XPC® at 23, 26, 32 and 37 weeks of age.*

Heterophil Count (%)

Although not significant (P = 0.152), and highly variable, Figure 6 heterophil % of Line A increased markedly from 28.40 ± 3.36 at wk 23 (pre-lay) to 39.55 ± 3.63 at wk 26 (onset of lay), which was higher than Line B (31.50 ± 4.10) and corresponds to the FITC-d data observed in Figure 4. Line A heterophil % then decreased at wk 32 and wk 37. Line B heterophil % continued to increase gradually to a maximum at wk 32 (36.44 ± 3.47) before plateauing. No significant diet x line interactions or significant diet effects were found at any time point for heterophil counts.

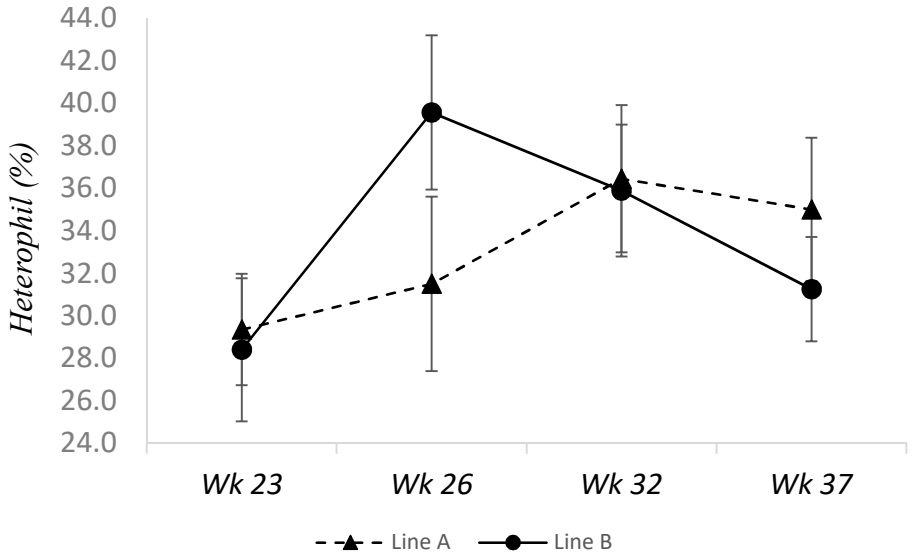

Figure 6. *Heterophil (%) in plasma as a marker of inflammation in breeder hens from two genetic lines; Line A (n = 20) and Line B (n = 20), at 23, 26, 32 and 37 wks of age. Values are mean ± SEM.*

Monocyte Count (%)

At wk 23, monocytes (%) in Line A (3.53 ± 0.87) and Line B (2.53 ± 0.78) were similar. Line A monocytes gradually increased at each time point, as did Line B but to a lesser extent after reducing slightly at wk 26. Notably, at wk 26 (P = 0.031), 32 (P = 0.029), and 37 (P = 0.035) there were significant increases in monocytes in Line A compared to Line B (Figure 7). Line A also displayed greater variation, most notably at wk 37 (SEM = 1.84) compared to Line B (SEM = 0.76). No significant diet x line interactions or significant diet effects were found at any time point for Monocyte counts.

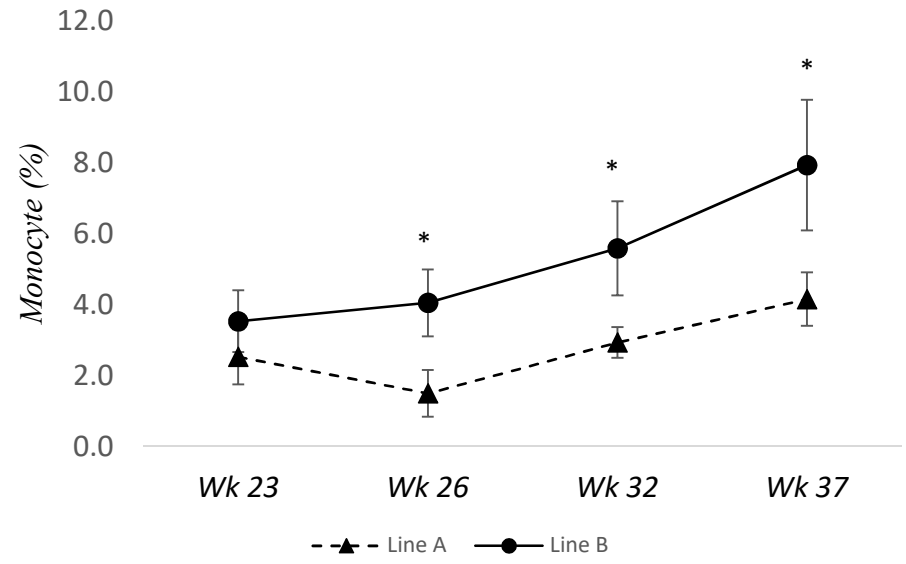

Figure 7. *Monocyte (%) as a marker of inflammation in breeder hens from two genetic lines; Line A (n = 20) and Line B (n = 20), at 23, 26, 32 and 37 wks of age. Values are mean ± SEM.*

Lymphocyte Count (%)

There were no significant differences between lymphocyte (%) in the two broiler lines, both displayed decreasing numbers over the four time points. Line A lymphocytes reduced from 56.80 ± 4.00 at wk 23 to 41.06 ± 2.95 at wk 37. Similarly, Line B lymphocytes decreased from 56.65 ± 3.52 at wk 23 to 49.30 ± 4.37 at wk 26, spiking at wk 32 (51.63 ± 3.84) before once again declining to 39.20 ± 3.79 at wk 37 (Figure 8). Both lines were highly variable. No significant diet x line interactions or significant diet effects were found at any time point for Lymphocyte counts.

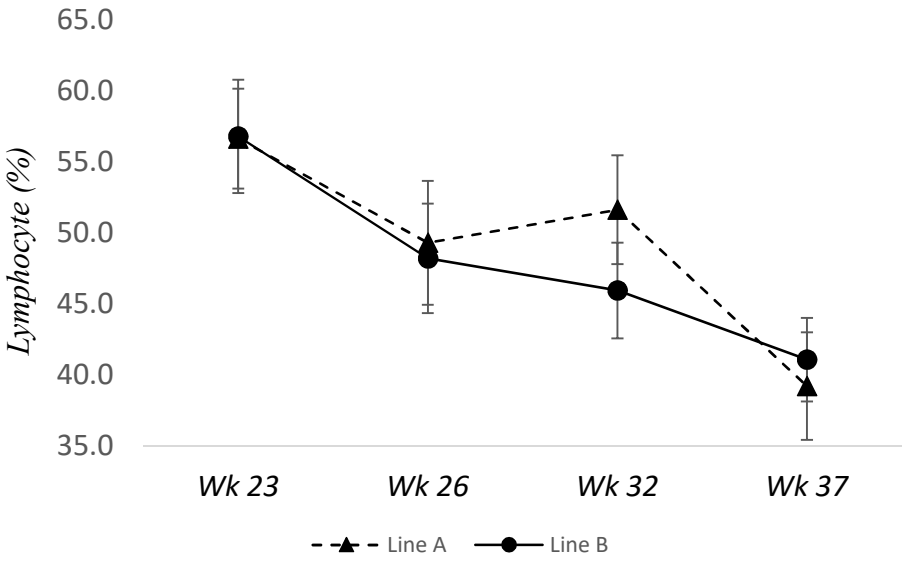

Figure 8. *Lymphocyte (%) as a marker of inflammation in breeder hens from two genetic lines; Line A (n = 20) and Line B (n = 20), at 23, 26, 32 and 37 wks of age. Values are mean ± SEM.*

Interleukin-6 (IL-6) Assay

Interleukin-6 (IL-6) levels were minimal to undetectable in 30% of samples from line A and 16% in Line B (>3.9 pg/ml). IL-6 results were highly variable ranging from 3.9 to 698 pg/ml. No significant differences were observed between breeder line or dietary supplementation with DV XPC. The changes in plasma IL-6 activity showed a strong correlation with the blood monocyte levels only in birds from line B ($R^2 = 0.742$), this was largely due to too an outlier in the line B birds (698 pg/ml), when removed the correlation was lost.

Summary

- ***The poor performing, wet litter line (Line B) may be experiencing chronic intestinal inflammation from 26 weeks (on set of lay) of age and onwards as indicated by monocyte %, and to a lesser extent H:L ratios, but was not supported by Il-6 data.***
- ***Dietary inclusion of DV XPC did not affect WBC differentials or IL-6 concentrations in either breeder line.***

Plasma Corticosterone

Large variation in week 37 plasma corticosterone were observed within Line B for both control and XPC groups. Line B had higher plasma corticosterone compared to Line A (5.05 ± 0.85 vs 2.98 ± 0.47 ng/ml; *P=0.04*). The addition of dietary XPC® had no effect on plasma corticosterone concentration (Figure 9). No interaction between line and diet was observed.

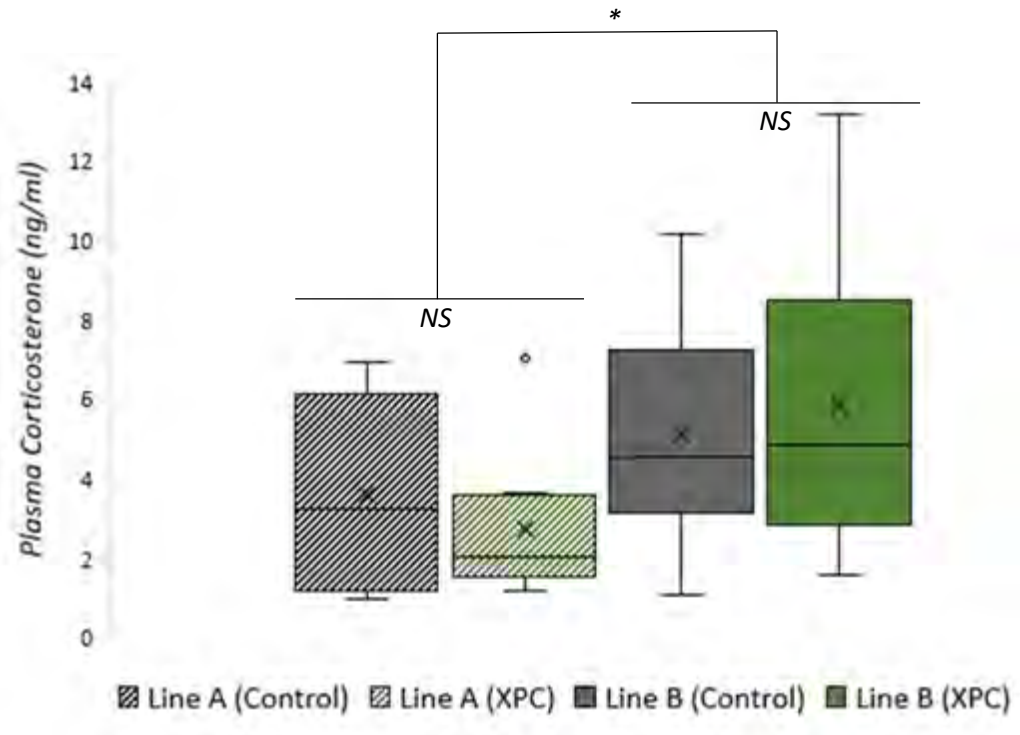

Figure 9. *Plasma corticosterone concentrations of 37 week old breeder hens from line A and B, +/- dietary inclusion of DV XPC®. x indicates mean values. * = P < 0.05; NS = Non significant*

Feather Corticosterone

Week 37 feather corticosterone had a large amount of variation between breeder hen line and dietary treatment groups (0.3 ng/ml to < 20 ng/ml; Figure 3), especially in the poor performing line (line B) fed the control diet. There were no significant differences in feather corticosterone concentration between lines. The addition of XPC® tended to lower corticosterone in comparison to control in both lines (XPC® (7.91 ±1.29) vs Control (9.82 ±1.30)) but was not statistically significant (p=0.558; Figure 10). There was no significant interaction observed between breeder line and dietary inclusion of DV XPC®.

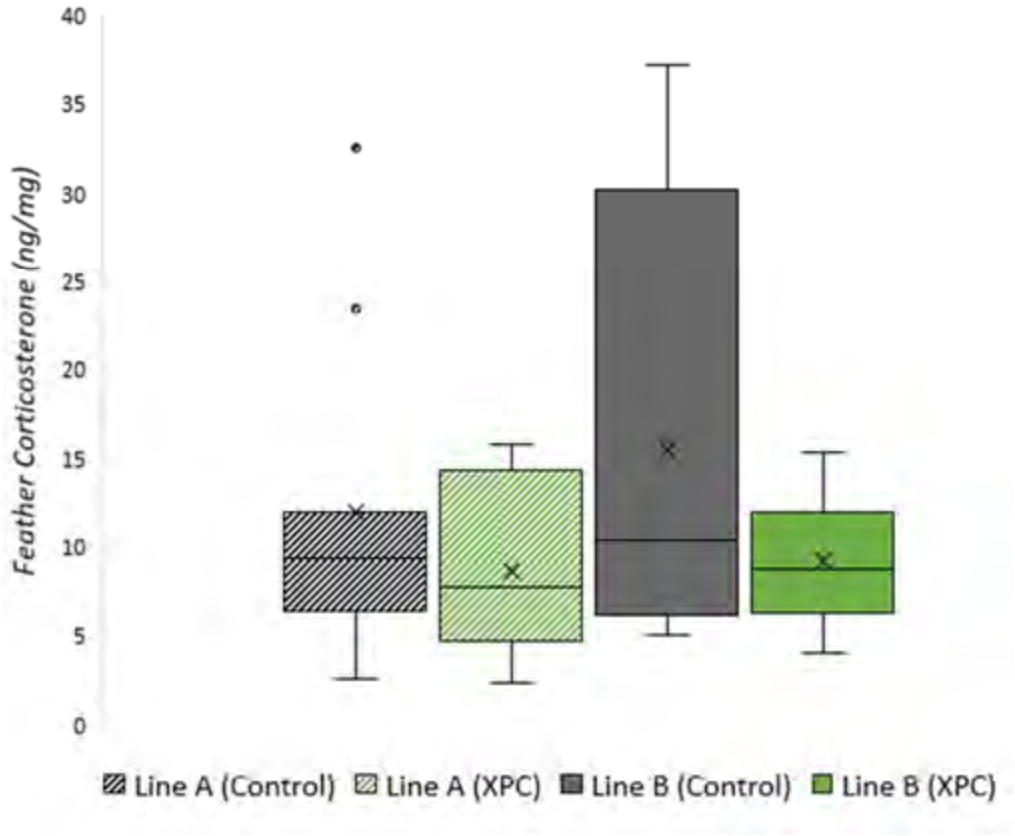

Figure 10. *Feather corticosterone concentrations of breeder hens from line A and B, +/- dietary inclusion of DV XPC®. x indicates mean values.*

Summary

- ***The poor performing line (Line B) had higher plasma corticosterone concentration compared to Line A.***
- ***No differences in feather corticosterone between lines was observed.***
- ***Dietary inclusion of DV XPC did not influence plasma or feather corticosterone concentrations. Results from feather corticosterone concentration does look promising but requires more, in depth investigation.***

Summary

The current study hypothesised that DV XPC® would improve reproductive performance and faecal consistency of HiChick's problem great grandparent line, through beneficial modulation of the intestinal environment, reducing physiological stress and inflammation. It was also hypothesised that the wet litter observed in this grandparent line is a result of gut dysbiosis, negatively impacting reproductive performance and progeny development.

DV XPC® fed to the poor performing line, Line B, may be beneficial in regards to egg production and hatchability during early-mid lay, but requires further investigation given the design of the experiment, number of birds and only one cockerel/pen. Improved hatch percentage was similar to that see by Kidd *et al.* (2013), who observed an increase hatch from parent hens at 32 and 39 weeks of age when hens were fed DV XPC.

The addition of dietary DV XPC (1,000 ppm) to breeder hen diets at 23 weeks of age did not affect gut permeability and did not improve the wet litter issue produced by Line B birds. The actually mechanisms of XPC still needs to be explored. Dietary inclusion of XPC has shown to promote digestive health, improve immune function and gut morphology (de los Santos *et al.* 2007). The addition of XPC to breeder diets in this instance, may purely be nutritional, given breeder hens are severely feed restricted, an additional 15% protein source at 1,000 ppm may be behind the improvement in egg production and hatchability during early-mid lay, and thus may not act by modulating the gastrointestinal environment. In order to determine if XPC is indeed have a modulating effect on the gastrointestinal tract, feeding XPC birds from hatch and studied throughout the life of the flock may be beneficial. There is early evidence to suggest at-hatch administration of probiotics can significantly alter gut microbial community structure and concurrent immune development compared to later dietary interventions, including at lay (Baldwin *et al.* 2018; Willson *et al.* 2019).

Intestinal permeability increased in both lines as they came into lay, however line A did not display the dramatic increase in litter moisture produced by line B birds. There is evidence to suggest that permeability does increase with acute and chronic stress (Söderholm and Perdue 2001) which may explain the increase in intestinal permeability during peak-lay in line A, although studies in birds are limited, The FITC-d levels appear to decrease from wk 32, which if continued, could correlate with resolution of an acute response that caused a rapid but relatively short-lived increase in intestinal permeability. The sudden increase in intestinal permeability at 26 weeks as Line B birds were coming into lay may have long term and permanent effects on this line. In human studies, there is evidence to suggest that leaky gut allows passage if immune triggers and illicit innate immune response, (Tersigni *et al.* 2018), if such a response exists in poultry, this could explain their faecal output and potentially overall egg production.

One such immune trigger is Interleukin-6 (IL-6), a proinflammatory cytokine released by monocytes elevated in response to many inflammatory conditions of the gastrointestinal tract in humans (Powell *et al.* 2017). In mammalian species, it is produced by many other cells including fibroblasts, endothelial cells and immune cells such as neutrophils, macrophages and T cells (Al-Asmakh *et al.* 2012). The cytokine is synthesised and released when Toll-like receptors are stimulated by bacterial lipopolysaccharide, or, in response to other inflammatory cytokines produced by activated myeloid cells, including interleukin-1 (IL-1) and tumour necrosis factor alpha (TNF-α), creating a positive feed-forward loop (Kelly *et al.* 2016b). IL-6 has also been shown to have an important role in the brain-gut-microbiota axis causing activation of the hypothalamic-pituitary-adrenal (HPA) axis, and thus a potential stress

response (El Aidy *et al.* 2014). Il-6 not only influences the immune system but it has been shown to affect intestinal epithelial tight junctions by increasing intestinal tissue expression of transmembrane TJ protein claudin-2, allowing a size dependent increase in flux of small molecules (Kelly *et al.* 2016a).

IL-6 plasma concentration in commercial poultry differs according to breed. In meat birds, both control Ross (52 day old) and Cobb (4 weeks old) birds reported ~70-75pg/ml (Mosleh *et al.* 2016; Rajput *et al.* 2019). In laying hens (Hyline Brown, 90 day old) it was reported to be > 20 pg/ml (Zhuang *et al.* 2019). In breeder hens, there is little information regarding total circulating plasma IL-6 concentrations, as many papers have used IL-6 as marker for inflammation using real-time quantitative PCR to measure gene expression in specific tissues. (Koppenol *et al.* 2015a; Chen *et al.* 2017; Jiang *et al.* 2018). As we were unable to collect tissues samples for these hens we could not investigate IL-6 gene expression in the gastrointestinal tract. It would be worth attempting such analyses in future studies to determine firstly, if IL-6 is elevated in hens from the poor performing line and secondly, whether IL-6 and inflammation are localised to gastrointestinal tissues.

From the results in the current study, it could not be determined whether inflammation alone is responsible for Line B's wet excreta and reduced performance. There are indications however, particularly from the heterophil counts, that acute inflammation may be present in Line B most severely at wk 26 (onset of lay), with increased monocyte, and lesser extent reduced lymphocyte data suggesting a subsequent shift to chronic inflammation, which has been observed in mammals, including humans (Murray *et al.* 2001; Maydych *et al.* 2017) and birds (Shini *et al.* 2010; Nazar and Marin 2011). Thus, additional data at further time points, and from a greater number of birds, is recommended. Further recommendations include quantification of a larger profile of immune biomarkers, T and B lymphocyte differentiation and presence or absence of osmotic imbalances or ion channel disruptions.

Increases in intestinal permeability and wet litter may be an indirect result of chronic stress. Heterophil:lymphocyte (H:L) ratios and plasma corticosterone were highly variable between birds and treatments, therefore no definitive links with intestinal permeability and production parameters could be made. H:L ratios for both lines were higher when compared to previous broiler studies (Price *et al.* 2018; Weimer *et al.* 2018) so our breeder hens may be experiencing stress, especially during lay. It has been reported that corticosterone deposition in feathers parallels modulation of the hypothalamo-pituitary-adrenal axis (endocrine regulation of the stress response) (Bortolotti *et al.* 2008). Birds may down-regulate the adrenocortical response to stress during the lay period as a strategy to maximize reproductive success (Wingfield and Sapolsky 2003; Bortolotti *et al.* 2008). The increase in plasma corticosterone, and to a lesser extent feather corticosterone in the poor performing, wet litter line, may be attributing to their poorer reproductive performance, as they may be unable to effectively modulate their stress response.

The use of the term 'stress' remains difficult to define as it may either indicate environmental constraints that decrease the performance of individuals or flock (stressor), or it may refer to an individual responding to the stressor (stress response). We cannot definitively state that Line B hens are more "stressed" than line A hens, however our results do suggest that as a flock, the huge variation observed for both plasma corticosterone, H:L ratios and intestinal permeability may account for the poorer performance of the A flock as a whole. A larger scale trial, sampling a greater number of birds, would be required to determine if this is indeed the case.

No correlation between intestinal permeability, H:L counts and inflammatory markers could be obtained, thus ***results from this study cannot definitively conclude that hens from the poor performing line are experiencing gut dysbiosis.*** Dysbiosis or dysbacteriosis has been characterised in broiler chickens. Clinical signs are similar to those observed in line B which includes increased water intake, wet, greasy droppings, dirty feathers, decreased feed intake and subsequent reduced feed efficiency (Teirlynck *et al.* 2011). Non-infectious factors have been attributed to this dysbacteriosis, including nutritional and management stressors (Teirlynck *et al.* 2011). In future, it would be beneficial to necropsy culled hens for histopathology to examine potential localised gastrointestinal inflammation, to definitively diagnose dysbiosis.

Concurrently, in-depth microbial analyses is also required to determine if the transition from pre to early-lay causes permanent alteration in intestinal microbiota and whether such alterations are responsible for increase in intestinal permeability and wet litter. The gut microbiota is proposed to be modulated by the stress response, which, via the brain-gut-microbiota axis, feeds back and influences the inflammatory response through microbial metabolite regulation of cytokine production and influence on enteric gastrointestinal barrier function (Rubio and Huang 1992; Powell *et al.* 2017). However, the order of these events is not entirely determined; whether there is a link with gut dysbiosis and low-grade chronic inflammation in broiler breeder hens requires further investigation.

The mechanisms by which wet excreta, and consequently wet litter, is occurring in these hens and whether it is indeed linked to gut dysbiosis warrants further study. Future, in depth, investigation of such on farm issues, will ultimately optimise breeder health and performance at all breeder tiers not just GGPs, and thus improve overall farm and industry productivity.

Part B: Progeny

Methodology

Embryonic sample collection

At 32 weeks of age 82 eggs per treatment were collected from the farm (n=328) and transported to the University of Adelaide, Roseworthy campus where they were incubated. Incubator settings were as follows: day 0-18, temperature: 37.6ºC, humidity: 55% and day 18-21, temperature: 36.7ºC, humidity: 60%. At embryonic day (ED) 15, 10 eggs per treatment (n=40) were randomly selected, the embryo was removed from the egg and humanely euthanized via decapitation. Yolk and embryonic tissue (liver, muscle, jejunum and brain) were collected. All samples were weighed and snap frozen in liquid nitrogen and stored at -80ºC. Additionally, ED15 bodyweights were recorded along with egg quality parameters including egg weight, egg length and egg width.

Grow-out experimental design

160 viable chicks were hatched and placed in rearing pens which housed 10 birds, with 4 replicates per treatment and raised under standard commercial conditions. Birds were subjected to a lighting schedule of 23:1 light: dark from day 0-4, after which a standard 16:8 light: dark schedule was implemented. Birds were fed *ab libitum* and provided a standard commercial starter diet (Laucke meat bird starter diet) from 0-21 days of age, from 21-42 days birds were fed a commercial finisher diet (Laucke meat bird finisher diet). Birds had unlimited access to water and were housed in a temperature and ventilation controlled environment, which were set to industry standard guidelines. Individual bird bodyweights and total pen feed intake were recorded weekly.

Post-hatch sample collection

At days 22 and 42, 10 birds per treatment (n=40) were humanely euthanised via cervical dislocation for tissue sampling. Samples collected included liver, brain, muscle and jejunum which were weighed before being snap frozen in liquid nitrogen and stored at -80ºC. Breast muscle, both left and right, was removed and weighed from 42 day old birds, after which a small section was removed and snap frozen in liquid nitrogen. a sub-sample of birds, 8 per treatment (n=32) were frozen for dual-energy x-ray absorptiometry scan (DEXA) to determine overall body composition, with the procedure performed in conjunction with the South Australian Research and Development Institute (SARDI) (Gilles Plains, SA, AUS).

Blood samples were taken from all euthanised birds at days 22 and 42, via cardiac puncture into the left ventricle. At 37d, blood samples n=40 were collected from live birds via the jugular vein.

Primary feathers 4 and 6 from the left wings were plucked from all birds after euthanasia at 42d and stored at room temperature for corticosterone analysis.

Blood, Feather & Yolk Hormonal Analyses

Blood samples were centrifuged at 2000 *g* for 10 minutes before plasma was collected and stored at -20ºC. Seven yolk samples per treatment (n=28) collected from 32 week old hens were homogenised in 1 mL of phosphate buffer saline solution (1x PBS, pH 7.4) per/g of egg yolk. Samples were divided into 1 mL aliquots and frozen at -80°C. Yolk and plasma samples were sent to the University of Western Australia for corticosterone (plasma and yolk) and testosterone (yolk only) extraction and analysis utilising a validated chicken radioimmunoassay.

Feather corticosterone was extracted and analysed as per described for breeder hens.

RNA Extraction

Total RNA was extracted using an RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany). Approximately 80 mg of frozen (-80ºC) d 22 liver tissues and ~200 mg of ED 15 hypothalamic tissues were homogenised in 2 mL of Qiazol reagent (Qiagen, Hilden, Germany). Aliquots (1 mL) of the Qiazol homogenate were combined with 100 µL of gDNA Eliminator Solution and shaken vigorously for 15 s. An additional 180 µL of chloroform was added, shaken vigorously for 15 s and the suspension held at room temperature for 2-3 minutes, then centrifuged for 15 min at 4ºC (12, 000 *g)*. The upper aqueous phase (300 µL) was collected and combined with an equal volume of 70% ethanol and transferred onto RNeasy Mini spin columns. The remaining collection and wash steps were performed according to the manufacturer's instructions. RNA was eluted in 100 µL of RNA-free water. Purity and concentration was determined using UV spectrophotometry (Nanodrop 1000; Thermo Scienfic, Wilmington, DE). RNA integrity was determined using an RNA tape-station (Agilent technologies 2200, Santa Clara, CA)

RNA-Seq Library Construction and Sequencing

RNA-Seq was carried out by the Deakin Genomics Centre (Deakin University, VIC). RNA samples were quantified with both the Qubit Broad-range and High-sensitivity assays (Invitrogen, USA) using a Qubit 3.0 Fluorometer (Invitrogen, USA). For each sample, 1 µg of RNA (in a volume of 50 µL) was used to prepare the library using the NuGen Universal Plus mRNA-Seq (San Carlos, CA, USA) according to the manufacturer's protocol. The PCR enrichment of adaptor-ligated DNA cycling conditions, denaturation, and annealing/extension cycle steps were repeated with a total of 12 cycles. Quantification and size estimation of the libraries was performed on both the Qubit 3.0 Fluorometer and 4200 TapeStation System (Agilent, Santa Clara, CA).

A 2 µL volume of each library was pooled into a new microfuge tube and enzymatically treated with Illumina Free Adapter Blocking Reagent (Illumina, San Diego, CA). The pooled library was pre-sequenced on the MiniSeq Sequencer (2 x 150 bp paired-end reads) (Illumina, San Diego, CA) to obtain the read distribution of each sample. Each library was then re-pooled to equal molar concentrations, enzymatic treated, denatured and normalized to 2 nM. Finally, the pooled library was sequenced on the NovaSeq 6000 Sequencer (2 x 150 bp paired-end reads) (Illumina, San Diego, CA) at the Deakin University Genomics Centre.

RNA-Seq Analysis

Reads were returned in fastq format and analysed as per (Willson *et al.* 2018). FastQC and adaptor sequences were trimmed from the 3' end of reads with Cutadapt (Martin 2011). Hisat2 (Pertea *et al.* 2016) was used to map reads to the reference genome Galgal6.0 (ftp://ftp.ncbi.nlm.nih.gov/genomes/Gallus_gallus). Duplicate reads were then removed. Stringtie (Pertea *et al.* 2016) was used to define the transcripts from the read mappings for each sample, and to merge the transcript definitions for all samples. Transcripts were cleaned up using in-house scripts. The number of raw read counts were calculated for each transcript and sample using the function featureCounts of the R package Rsubread (Liao *et al.* 2013). Another R package, edgeR (Robinson *et al.* 2010) was used to analyse differential gene expression using normalised counts per million transcripts (CPM) to correct for varying depth of sequence among samples. Transcript data were aggregated by gene. Genes where the maximum CPM was <1 were removed. Gross transcriptome relationships between the three types of bird were analysed by multidimensional scaling of the CPMs.

Functional Annotation Analysis and Statistical Analysis

Functional enrichment of the DE genes between line B female vs B male, line A female vs A male, Line B female vs A female and Line B male vs A male were conducted for gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways using (rick what script) and the web based tools in DAVID (Huang da *et al.* 2009b, 2009a). Only GO terms and KEGG pathways with $P < 0.05$ were taken into account as significantly enriched among the DE genes. Phenotypic data, including bodyweight, bodyweight gain and liver weights (normalised and actual), were analysed by a one-way ANOVA using SPSS (IBM SPSS Statistics 22). Gene expression levels were correlated with individual bodyweights using a Pearson's correlation in SPSS (IBM SPSS Statistics 22).

General Statistical Analyses

Analysis of performance and hormone data were performed by linear mixed model and general linear model analyses for treatment interactions, as well as breeder line and diet independently, following the procedures of IBM®, SPSS® Statistics 25 program (Armonk, NY, USA). The data were checked for normality by the Shapiro–Wilk test. Non-normalised data was analysed using nonparametric tests including Mann-Whitney U and Kruskal-Wallis. A probability level of less than 5% ($P < 0.05$) was deemed as statistically significant.

Discussion of Results

Egg Quality Measures

Embryonic day 0

Eggs collected at 26 and 32 weeks of age for egg quality measures found no significant differences in egg quality measures between lines or with maternal supplementation of XPC®. The only exception was shell weight and thickness, where eggs collected from 26 week old hens had thicker shells and increased shell weight when supplemented with XPC, with line B having thinner shells than line A. Eggs collected from 32 week old hens, again showed similar

differences in shell thickness and weight between lines. There was not effect of XPC® supplementation not any interaction between line and diet (Appendices A).

Embryonic day 15

Maternal dietary XPC supplementation did not influence external egg quality measures (egg weight, length and width, Table 1). Yolk weight (g) was lighter in eggs collected from XPC supplemented hens (22.89 ± 3.16 vs control, 24.84 ± 2.92). Eggs collected from Line A hens had heavier yolks that line B hens (25.49 ± 2.53 vs 22.06 ± 2.84). No interactions were observed between line, maternal diet or sex.

Table 1. Embryonic day 15 egg quality measures from two lines of breeder hens fed either a control (Con) or DV *XPC* supplemented diet. Values are means ± SE. Statistical significance was determined at the 95% confidence interval (P < 0.05).

Line	*Diet*	*Egg weight (g)*	*Egg width (mm)*	*Egg Length (mm)*	*Yolk Weight (% egg weight)*
Line A	Con	54.01 ± 0.66	43.15 ± 0.32	55.85 ± 0.61	26.37 ± 0.71[b,x]
Line B	Con	55.18 ± 1.09	43.17 ± 0.26	56.22 ± 0.83	23.13 ± 0.90[a,x]
Line A	*XPC*	52.86 ± 1.10	42.70 ± 0.33	55.90 ± 0.55	24.61 ± 0.82[b,y]
Line B	*XPC*	55.58 ± 1.22	43.20 ± 0.20	56.40 ± 0.70	20.99 ± 0.90[a,y]
Source of Variation					
Line		*NS*	*NS*	*NS*	*< 0.001*
Mat Diet		*NS*	*NS*	*NS*	*0.023*
Sex		*NS*	*NS*	*NS*	*NS*

[a-b] Mean values with different superscripts within a column are different dependent on line.
[x-y] Mean values with different superscripts within a column are different dependent on diet.

Yolk Hormonal Composition

Corticosterone concentration in yolk tended to be elevated in eggs collected from Line B hens compared to that of Line A (Table 2; $p = 0.069$). Additionally, no significant effect of maternal diet was identified in relation to yolk corticosterone or testosterone concentrations, neither was an interaction between line and diet identified.

Table 2. Day 0 yolk corticosterone and testosterone concentration from two lines of breeder hens fed either a control or DV XPC supplemented diet. Values are means ± SE. Statistical significance was determined at the 95% confidence interval (P < 0.05).

	Yolk Corticosterone (ng/g)	*Yolk Testosterone (ng/g)*
Breeder Line		
Line A	130.73 ± 8.04	10.12 ± 1.11
Line B	148.70 ± 5.01	10.66 ± 0.89
p-value	***0.069***	***0.709***
Maternal Diet		
Control	143.18 ± 7.54	11.06 ± 1.17
DV XPC	136.24 ± 6.60	9.72 ± 0.79
p-value	***0.475***	***0.349***

Summary

- ***Eggs collected from higher performing line (Line A) hens had thicker shells compared to eggs collected from the poor performing line (Line B)***
- ***Embryos from Line B had less yolk compared embryos from Line A.***
- ***Embryos from line B hens tended to have elevated yolk corticosterone concentration compared to embryos from line A hens.***
- ***Dietary inclusion of DV XPC® to breeder diets did not positively influence egg quality measures (except for shell thickness and weight from 26 week old hens), nor did it affect yolk hormone concentrations (corticosterone and testosterone).***

Growth & Performance

Line did not influence progeny sex ratio significantly, with 51.3% of progeny produced by Line A birds identified as male, compared to 48.7% female. Subsequently 46.7% of Line B offspring were male, with the remaining 53.3% identified as female. Furthermore, maternal supplementation with DV XPC® did not influence offspring sex ratio, although DV XPC® supplementation resulted in 53.2% of eggs hatching as females, whilst 46.8% hatched males. Offspring sex ratio was again not significantly influenced when line and maternal diet were analysed together.

Body Weight and Gain

A *line x sex* interaction was observed for body weights of ED15 embryos, where by line B female progeny were lighter compared to Line A female progeny (*p=0.030; Figure 11A*) no differences in male progeny body weight were observed. A *line x diet* interaction was also observed for ED15, where progeny from line A had lower bodyweights from hens that received XPC compared to controls (*p=0.024; Figure 11B*). There were no differences observed in Line B progeny. By hatch (D0), no interactions were observed, and all chicks has similar body weights, regardless of line, maternal diet or sex (Males 42.44 ± 3.05g; females 42.08 ± 3.16g)

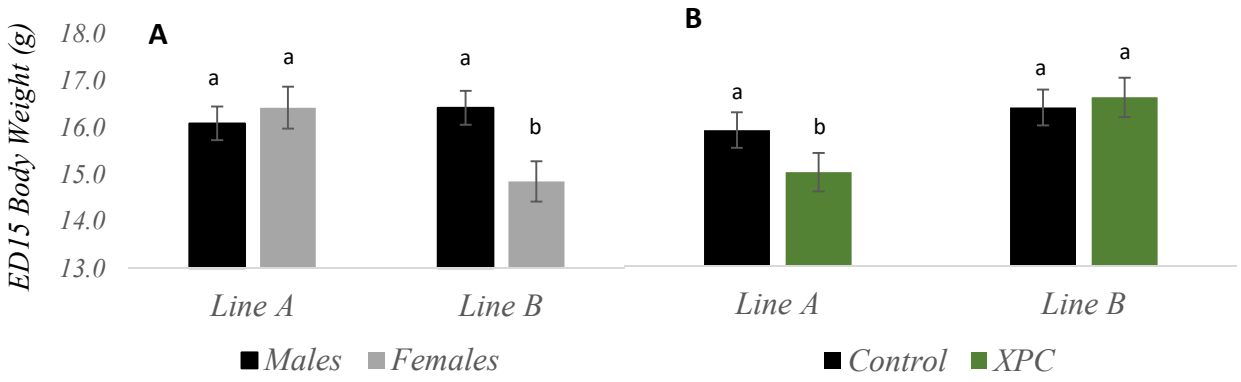

Figure 11. *Embryonic day 15 body weights from progeny two lines of breeder hens fed either a control or DV XPC supplemented diet. Values are means ± SE.* ***(A)*** *Differences between male and female progeny body weights between breeder lines.* ***(B)*** *Differences between line A and B progeny from hens fed -/+ DV XPC. Mean values with different letters are deemed statistically significant (p <0.05).*

At day 7, a *line x diet* interaction was again observed (*p =0.005*), with progeny from line A hens that received DV XPC® again lighter compared to progeny from hens that received the control diet. No differences in bodyweight were observed in progeny from line B hens, with or without supplementation with DV XPC® (Figure 12). Progeny from line B hens were lighter compared to progeny from Line A, irrespective of sex. Not surprising, body weight gain was greater in Line A progeny compared to Line B progeny from 0-7 days (96.97 ± 1.79 vs 83.10 ± 1.97g).

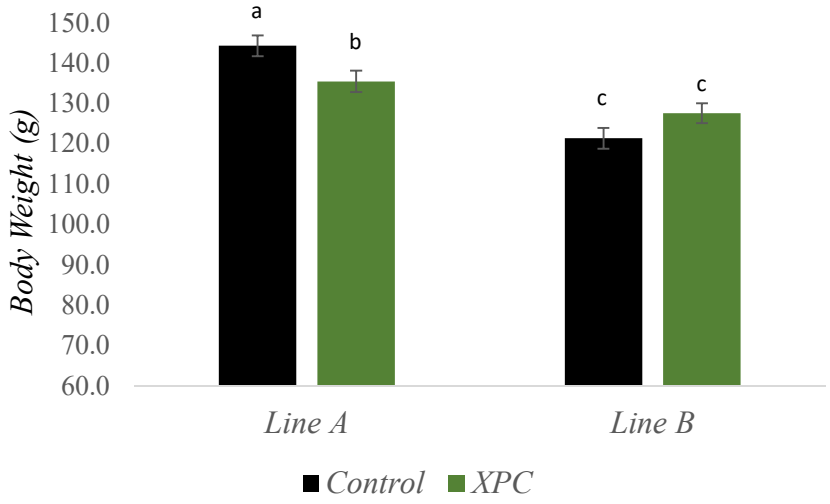

Figure 12. *Day 7 post-hatch body weights of progeny from two lines of breeder hens (A and B) fed either a control or DV XPC supplemented diet. Values are means ± SE. Mean values with different letters are deemed statistically significant (p <0.05).*

By day 14 post-hatch, significant differences in body weight and gains between male and female birds were observed, which continued throughout the trial *(p < 0.001*). From day 14 to 35 post-hatch, progeny from Line A hens were significantly heavier compared to progeny from line B (*p <0.001;* Figure 13), this also reflected in their increase in bodyweight gain (p <0.001). Maternal supplementation with DV XPC® did not influence body weight or gain at any of these time points. No interactions were observed for either line, maternal diet or sex in regards to body weight.

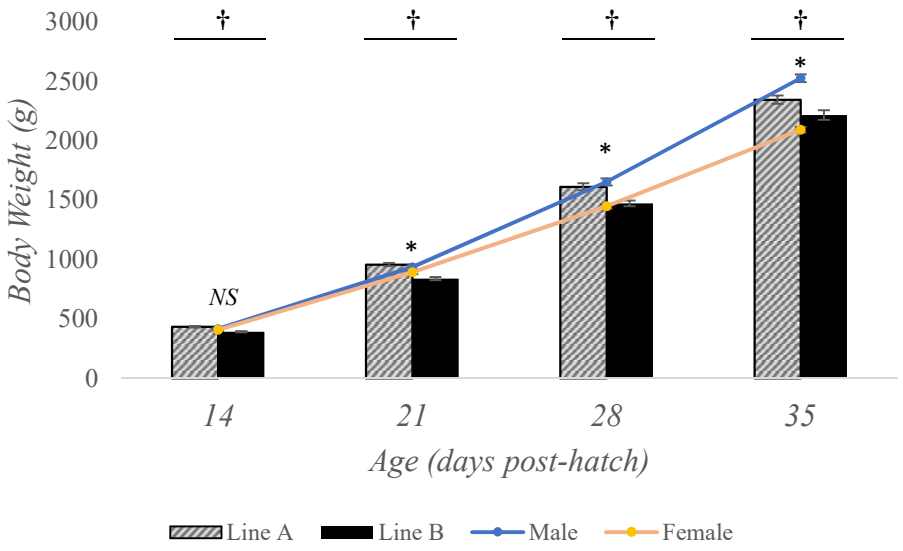

Figure 13. *Day 14-35 post-hatch body weights of male and female progeny from two genetic lines of breeder hens (A and B). Values are means ± SE. *denotes statistical significance between males and females at each time point (p <0.05).†denotes statistical significance between lines at each time point (p <0.05).*

Body weight gain from day 14-21 post-hatch, was the only time point that was affected by diet where by maternal supplementation with XPC decreased gain both between lines and males and females. Although statistically significant (p <0.001), these interactions were not observed for any other time point.

By day 42, a *line x sex* interaction was observed (*p <0.001*), whereby female progeny from Line B were lighter compared to Line A females. Male progeny bodyweight did not differ between lines (Figure 14). No other interactions, including maternal diet were observed.

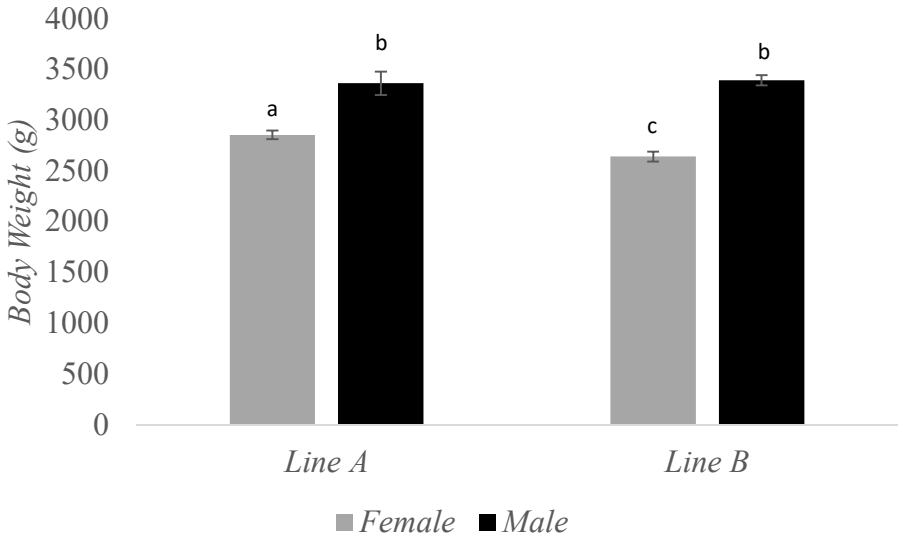

Figure 14. *Day 42 post-hatch body weights of male and female progeny from two genetic lines of breeder hens (A and B). Values are means ± SE. Mean values with different letters are deemed statistically significant (p <0.05).*

Again, body weight gain was greater in Line A progeny compared to Line B progeny from 35-42 days (754.51 ± 32.61 vs 727.53 ± 33.79 g, *p = 0.011*). With males having significant greater gains than females. Maternal supplementation with DV XPC, did not affect gain from d35-42.

Feed Conversion Ratio (FCR)

FCR did not differ between lines or with dietary inclusion of DV XPC, any stage of the 42 day grow out period. Total pen FCR for the entire trial ranged from 1.39-1.44 (Table 3).

Table 3. Weekly pen FCR from d 28-42, and total FCR for chicken meat birds hatched from two lines of breeder hens fed either a control (Con) or diet supplemented with DV XPC®. Values are means ± SE.

Line	***Diet***	***D 28-35***	***D 35-42***	***Total FCR***
Line A	Con	1.61 ± 0.05	1.78 ± 0.07	1.39 ± 0.04
Line B	Con	1.46 ± 0.08	1.66 ± 0.04	1.43 ± 0.04
Line A	*+XPC*	1.64 ± 0.03	1.73 ± 0.14	1.42 ± 0.05
Line B	*+XPC*	1.56 ± 0.07	1.79 ± 0.06	1.44 ± 0.02
Source of Variation				
Line		*NS*	*NS*	*NS*
Maternal Diet		*NS*	*NS*	*NS*
*Line*Maternal Diet*		*NS*	*NS*	*NS*

Body Composition

Dietary inclusion of DV XPC® to breeder diets did not influence progeny body composition from either line. There was a *line x sex* interaction for both fat and lean %, where by female progeny from line B hens had a greater fat % and reduced lean % compared to male progeny from the same line. Interestingly, this was not observed in progeny from Line A where both males and females had the same body composition (Table 4). Breast muscle yield however did not follow the same pattern, with Line B progeny having a higher BM yield than line Line A progeny, with females having a greater yield than males (Table 4).

Table 4. Day 42 body composition of both male and female chicken meat birds hatched from two lines of breeder hens (Line A and Line B). Hens were fed either a control or *XPC* supplemented diet. Body composition was analysed via DEXA scan. Breat muscle (BM) yield was measured from a subset of birds at d42, with both left and right BM's removed. Values are means (% bwt) ± SE.

Line	**Sex**	**N**	**BMC % Bwt**	**Fat % Bwt**	**Lean % Bwt**	**BM % Bwt**	**N (BM only)**
Line A	Male	14	1.26 ± 0.04^{a}	8.60 ± 0.87^{a}	90.13 ± 0.80^{a}	21.00 ± 0.79	12
	Female	18	1.12 ± 0.03^{ab}	8.78 ± 0.78^{a}	90.10 ± 0.66^{a}	22.77 ± 0.41*	8
Line B	Male	16	1.08 ± 0.04^{b}	6.16 ± 0.86^{b}	92.76 ± 0.90^{ab}	23.67 ± 0.42	11
	Female	16	1.15 ± 0.05^{a}	9.90 ± 0.63^{c}	88.95 ± 0.66^{b}	25.17 ± 0.39*	9
Source of Variation							
Line			-	-	-	< 0.001	
Mat Diet			NS	NS	NS	NS	
Sex			-	-	-	0.005	
Line x Sex			0.007	0.036	0.032	NS	

Different superscripts within a column are deemed statistically significant (p <0.05).
**denotes significance between males and females within line*

Summary

- ***Progeny from the poor performing line (Line B) were lighter compared to progeny from the higher performing line (Line A). Only female progeny were lighter at day 42 from line B.***
- ***FCR did not differ between lines or with dietary inclusion of DV XPC, any stage of the 42 day grow out period.***
- ***Female progeny from line B hens had a greater fat% and reduced lean% compared to male progeny from the same line.***
- ***Dietary inclusion of DV XPC® to breeder diets did not positively influence progeny bodyweight, gain, FCR or body composition throughout the grow-out trial.***

Plasma & Feather Corticosterone Concentration

Plasma corticosterone concentrations was significantly higher in progeny from Line B at 22, 37 and 42 days of age, irrespective of sex (Figure 15). Plasma corticosterone concentration decreased from 22 to 37 days of age, then increased at 42 days of age. XPC did not influence progeny plasma corticosterone concentrations at any age, whilst no significant interaction between maternal diet and breed was identified in relation to offspring plasma corticosterone concentrations at any age.

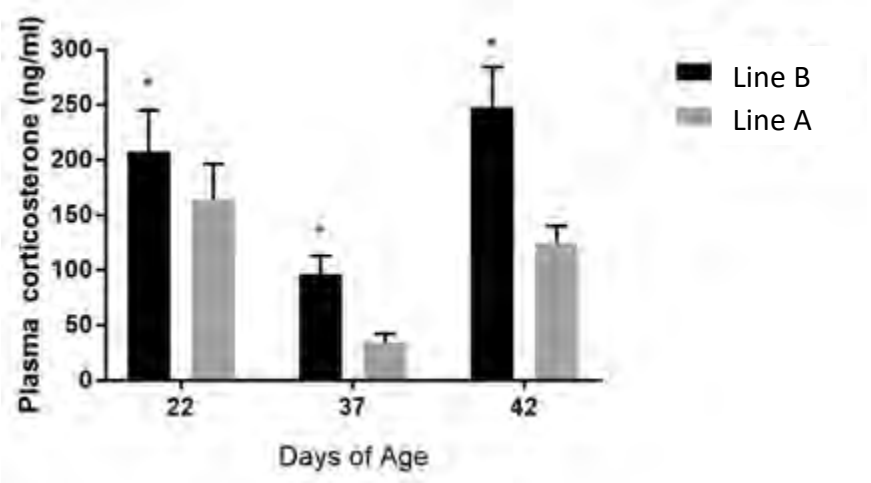

Figure 15. Plasma corticosterone concentration from 22, 37 and 42 day-old meat birds hatched from two breeder hen lines (A and B). Data is mean ± SE. * denotes statistical significance *(p <0.05).*

Feather corticosterone concentration did not differ between progeny from Line A or B hens, irrespective of sex. Maternal DV XPC® supplementation, did show an increase in feather corticosterone in progeny from both lines, however this was not statistically significant *(p = 0.560;* Figure 16) No interaction between line, maternal diet or sex was observed.

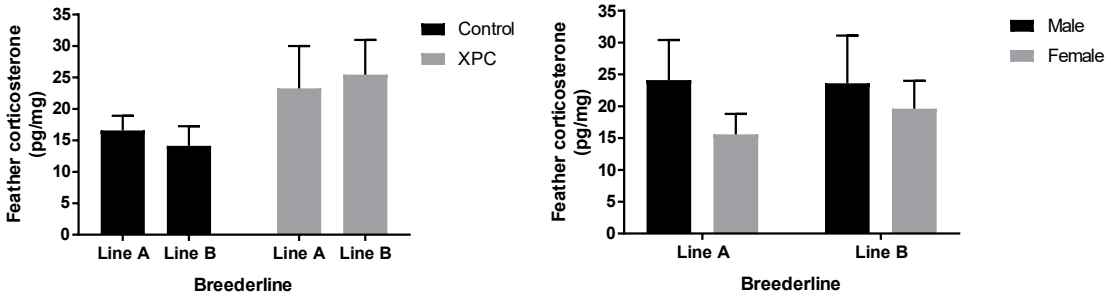

Figure 16. Feather corticosterone concentration from 42 day-old meat birds hatched from two breeder hen lines (A and B), fed with and without dietary DV XPC®. Data is mean ± SE.

Summary

- ***Progeny from the poor performing line (Line B) had higher plasma corticosterone concentration compared to Line A.***
- ***No differences in progeny feather corticosterone between lines was observed.***
- ***Maternal dietary DV XPC® did not influence progeny plasma or feather corticosterone concentrations.***

RNA-Seq

Identification of expressed transcripts and gross transcriptional relationships

RNA-Seq generated between ~25,000,000 and 35,000,000 raw 2 × 150 paired end reads per sample. A high number of duplicates were detected; 24.57% for brain and 38.98% for liver. In addition, unassigned ambiguity, multi-mapping, no feature and unmapped reads accounted for a combined total of 32.04% of brain and 23.84% of liver reads. The remaining assigned reads averaged (± SEM) 11, 903, 763 ± 128, 494 for brain and 10, 269, 002 ± 142,580 for liver. After filtering the low quality reads, 99.89% of reads were retained for brain and 99.91 % of reads were retained for liver. Clean reads for each subgroup are presented in Table 5.

Table 5. Number of sequences retained for each sample sub-category for brain and liver following low quality read filtering.

	Brain[1]	# of samples	Liver[1]	# of samples
Line A Female	11,732,332 ± 238,959	6	9,568,915 ± 519,183	4
Line A Male	12,051,124 ± 256,733	7	10,258,399 ± 145,502	7
Line B Female	12,175,539 ±261,342	6	10,699,080 ± 206,758	7
Line B Male	11,515,613 ± 229,132	5	10,246,337 ± 260,979	6

[1]values are mean ± SEM

A total of 24,356 genes were identified among the chicken libraries. After removal of genes with no or low counts in all samples (< 1 CPM), 15,323 and 13,6174 genes (both known and novel) remained for analysis for brain and liver samples respectively. Gross transcriptional analysis was undertaken using multidimensional scaling to determine how similar the transcriptomes were for male and females of Line A and Line B. Non-distinct clusters of Line A and B were seen in the brain, with separation between males and females evident only (Figure 17a). In the liver, a far more pronounced separation of the transcriptomes was observed, with distinct non-overlapping clusters for male and female of both Line A and Line B (Figure 17b)

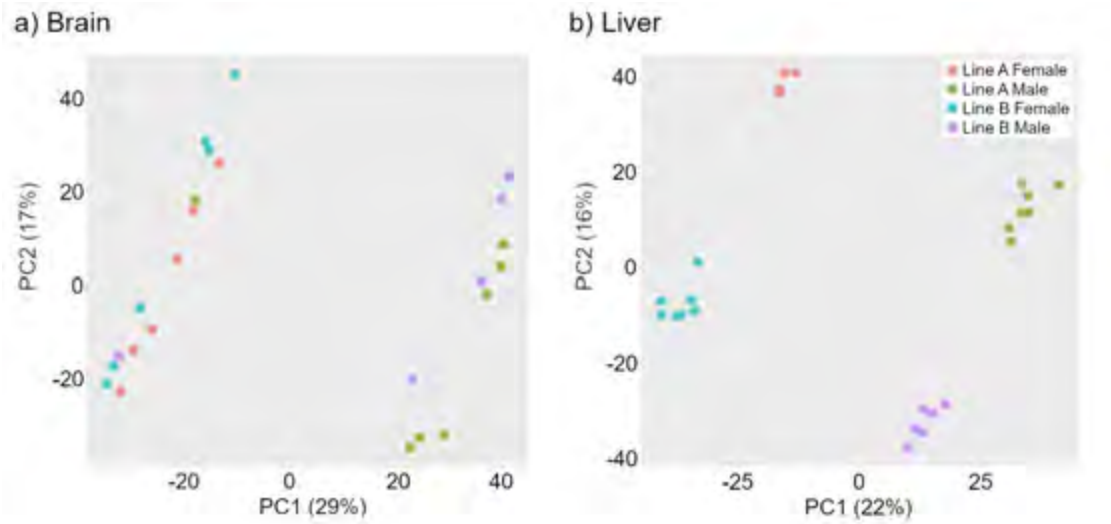

Figure 17. Principle component 1 vs principle component 2 analysis of Line A Female (red), Line A Male (green), Line B Female (blue) and Line B Male (purple) for a) Brain and b) Liver.

Embryonic Brain

Differentially Expressed (DE) genes between male and female progeny

A 2.9% transcriptome difference was detected between male and females within Line A. Of the 15.029 genes analysed, 443 were found to be DE. Comparisons of male and female progeny from Line B yielded similar results, with 341 of 14,618 genes DE (2.3% of the transcriptome). The DE genes were largely associated with the sex chromosomes, predominately appearing on the Z chromosome. The number of DE genes excluding those on the sex chromosomes reduced to 39 genes DE between male and females in Line A, and 14 genes DE between male and females in Line B, Figure 18a. Of the top five DE genes for Line A and Line B, there was one novel gene in common, LOC426626, alternatively known as AN1-type zinc finger protein 5-like. Three other genes were found to be novel. The top five genes for each comparison are presented in Table 6. Functional analysis of the DE genes using GO terms and Gene Set Enrichment Analysis (GSEA), including Hallmark, KEGG and Wikipathways did not identify pathways of significance between male and females within each line.

Differentially Expressed (DE) genes between progeny from Line A and B

Comparisons were made between females from Line A and Line B, and males from Line A and Line B. There was a 4.3% difference in the transcriptomes of females between the two lines (640 of 15,019 genes DE) and a 3% difference in the transcriptome of males between the two lines (444 of 15,002 genes DE). Unlike the male and female comparisons, distribution of the DE genes were not largely limited to the ZW chromosomes. Exclusion of the ZW chromosomes from analysis resulted in 628 DE genes for females, and 432 DE genes for males between Line A and Line B, Figure 18b. Five of the 10 DE genes were novel and uncharacterised, see Table 6. Functional analysis of the DE genes using GO terms and GSEA, including Hallmark, KEGG and Wikipathways, did not identify pathways of significance for male and females between each line.

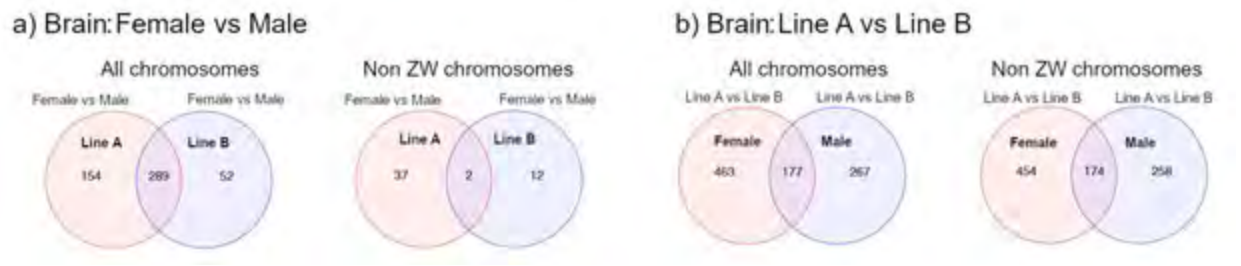

Figure 18. Differentially expressed (DE) genes comparing a) Sex and b) Line A and B, for genes expressed on all chromosomes, and non ZW chromosomes.

Table 6. Top five non ZW differentially expressed (DE) genes in embryonic brain tissue between male and females within Line A and B, and between Line A and B.

Ensembl reference	Ensembl gene ID	Description	FDR (<0.05)	NCBI Accession Number	Chromosome #	[1]↑↓
DE genes between male and female progeny						
Line A Female vs Line A Male						
ENSGALG00000014589	LOC426626 (Novel)	AN1-type zinc finger protein 5-like	5.93E-05	XM_015299879	28	↑
ENSGALG00000047516	*CCDC107* (Novel)	Coiled-coil domain containing 107	9.98E-06	XM_429533	1	↑
ENSGALG00000053073	*NTPCR*	Nucleoside-triphosphatase, cancer-related	4.59E-05	XM_426145	3	↑
ENSGALG00000008431	*SMARCA1*	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 1	1.81E-05	XM_420329	4	↑
ENSGALG00000028363	*SPG7*	Paraplegin matrix AAA peptidase subunit	3.91E-06	XM_414204	11	↑
Line B Female vs Line B Male						
ENSGALG00000009360	*CAPN2*	Calpain 2	1.02E-02	NM_205080	3	↑
ENSGALG00000014589	LOC426626 (Novel)	AN1-type zinc finger protein 5-like	4.13E-03	XM_015299879	28	↑
ENSGALG00000029238	*TOR1AIP1*	Torsin A interacting protein 1	7.81E-04	XM_015290502	8	↓
ENSGALG00000044278	*C3H1ORF131*	Chromosome 3 open reading frame, human C1orf131	5.33E-04	XM_015284382	3	↓
ENSGALG00000037597	*YOD1*	YOD1 deubiquitinase	3.07E-03	XM_425825	26	↑
DE genes between progeny from Line A and Line B						
Line A Female vs Line B Female						
ENSGALG00000007168	*C12H3ORF67*	Predicted Chromosome 12 open reading frame, human	2.63E-16	XM_015293305	12	↑
ENSGALG00000015836	*CEP162*	Centrosomal protein 162	3.82E-16	XM_004940400	3	↑
ENSGALG00000026161	Novel	Uncharacterised	7.61E-19	n/a	28	↑
ENSGALG00000034813	Novel	Uncharacterised	6.59E-20	n/a	MT	↑
ENSGALG00000001945	*RFT1*	RFT1 homolog	6.57E-21	XM_001233828	12	↓
Line A Male v Line B Male						
ENSGALG00000049492	*ARL2BP*	ADP ribosylation factor like GTPase 2 binding protein	2.07E-47	NM_001142872	11	↑
ENSGALG00000026161	Novel	Uncharacterised	1.78E-19	n/a	28	↑
ENSGALG00000044472	Novel	Uncharacterised	1.19E-19	n/a	16	↑
ENSGALG00000048880	Novel	Uncharacterised	5.74E-15	n/a	16	↓
ENSGALG00000028142	*HACD4*	3-hydroxyacyl-CoA dehydratase 4	2.04E-53	XM_424816	Z	↑

[1]↓ Gene down regulated; ↑ Gene up regulated: Regulation direction listed for first category i.e. Line A Female vs Line A Male, regulation direction listed for Line A Female.

Liver

Differentially Expressed (DE) genes between male and female progeny

Comparisons between male and females within Line A revealed a 4.4% difference in the transcriptomes, with 448 of the 13,144 genes DE. Similar results were found between male and female progeny from Line B, with 430 of the 13,285 expressed genes DE (3.2%). A large number of genes were again located on the Z chromosome. Exclusion of the ZW chromosomes from analysis reduced the DE genes to 186 between males and females in Line A and to 90 between males and females in Line B, Figure 19a. Of the top five non ZW DE genes, five were novel, with the gene AN1-type zinc finger protein 5-like again DE in each comparison, Table 7. Functional analysis of the DE genes using GO terms and GSEA, including Hallmark, KEGG and Wikipathways, did not identify pathways of significance between male and females within each line.

Differentially Expressed (DE) genes between progeny from Line A and B

Of the 13,149 expressed genes 1,074 were DE (8.2%) in female progeny comparing Line A and Line B, and 1,057 of 13,290 expressed genes were DE (8.0%) in the male livers between the two lines. Unlike the male and female comparisons within lines, distribution of the genes DE were not predominately found on the ZW chromosomes, with exclusion resulting in 1,031 DE genes in female and 1004 DE genes in male comparisons, Figure 19b. The top five DE genes non inclusive of the ZW chromosomes shared two DE genes in common for both sexes between lines. The gene *BLB2*, descriptively known as Major histocompatibility complex class II beta chain BLB2, (similar to HLA class II, D beta chain), was upregulated in Line A compared to Line B for each sex. Conversely, the gene *OSBPL6*, descriptively known as oxysterol binding protein like 6, was significantly downregulated in Line A compared to Line B for both sexes. An additional four DE genes were novel and/or uncharacterised, Table 7.

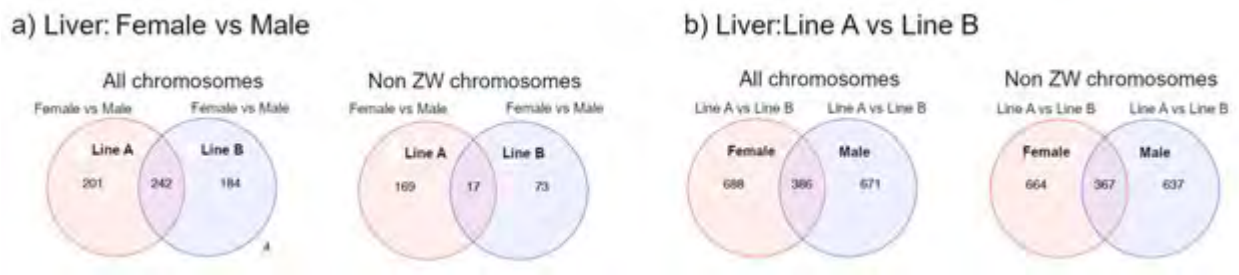

Figure 19. Differentially expressed (DE) genes comparing a) Sex and b) Line A and B, for genes expressed on all chromosomes, and non ZW chromosomes.

Functional analysis of the DE genes were conducted using GO terms and GSEA, including Hallmark, KEGG and Wikipathways. There were four significant pathways identified in females between Line A and Line B by Hallmark GSEA networks; TNFα signalling via NFkβ (P = 0.031), E2F targets (P = 0.002), Fatty acid Metabolism (P = 1.86E-04) and Oxidative Phosphorylation (P = 9.25E-05). KEGG GSEA was also significant for Oxidative Phosphorylation (P = 0.008). In the male comparisons of Line A and Line B, Hallmark GSEA networks were significant for Fatty acid Metabolism (P = 0.023), Oxidative Phosphorylation (P = 2.41E-04) and MYC targets V2 (P = 0.007), while KEGG GSEA was significant for Ribosome (P = 0.006).

Summary

- ***Comparison between males and females with lines were largely sex driven, most differentially expressed (DE) genes located on the Z chromosome***
- ***Functional analysis of the DE genes using GO terms and GSEA, did not identify pathways of significance between male and females within each line and between lines in embryonic brain.***
- ***Functional analysis of DE genes in liver from day 22 post-hatch progeny, found four significant pathways identified in males and females between lines involved in fatty acid metabolism and oxidative phosphorylation.***

Table 7. Top five non ZW differentially expressed (DE) genes in day 22 post-hatch liver tissue between male and females within Line A and B, and between Line A and B.

Ensembl reference	Ensembl gene ID	Description	FDR (<0.05)	NCBI Accession Number	Chromosome #	[1]↑↓
DE genes between male and female progeny						
Line A Female vs Line A Male						
ENSGALG00000027757	*ARL4A*	ADP ribosylation factor like GTPase 4A	7.20E-10	XM_025147366	2	↑
ENSGALG00000008233	*BUB1*	BUB1 mitotic checkpoint serine/threonine kinase	1.21E-19	XM_419301	3	↑
ENSGALG00000014589	LOC426626 (Novel)	AN1-type zinc finger protein 5-like	2.04E-28	XM_015299879	28	↑
ENSGALG00000042168	LOC100859636 (Novel)	Ring finger protein 170-like	2.25E-09	XM_025153133	8	↑
ENSGALG00000010763	*TRIP11*	PREDICTED: Thyroid hormone receptor interactor 11	1.85E-14	XM_421324	5	↑
Line B Female vs Line B Male						
ENSGALG00000011124	*ARHGAP24*	Rho GTPase activating protein 24	2.20E-08	XM_015276490	4	↓
ENSGALG00000014589	LOC426626 (Novel)	AN1-type zinc finger protein 5-like	3.51E-37	XM_015299879	28	↑
ENSGALG00000026776	*TIGAR*	TP53 induced glycolysis regulatory phosphatase	1.02E-07	XM_025142507	1	↑
ENSGALG00000004230	*LIPC*	PREDICTED: Gallus gallus lipase C, hepatic type (LIPC)	1.66E-05	XM_025154018	10	↓
ENSGALG00000035819	*TMEM51*	PREDICTED: Gallus gallus transmembrane protein 51	3.63E-06	XM_015297187	21	↑
DE genes between progeny from Line A and Line B						
Line A Female vs Line B Female						
ENSGALG00000030940	*BLB2*	Major histocompatibility complex class II beta chain BLB2, (similar to HLA class II, D beta chain)	2.50E-65	XM_015295014	16	↑
ENSGALG00000050315	*CLC2DL3*	Gallus gallus C-type lectin domain family 2 member D-like 3	1.00E-39	XM_423496	1	↑
ENSGALG00000050319	Novel	Uncharacterised	5.23E-29	n/a	2	↓
ENSGALG00000053446	LOC415662 (Novel)	C-factor-like	6.17E-27	XM_015279161	11	↓
ENSGALG00000009172	*OSBPL6*	PREDICTED: Oxysterol binding protein like 6	1.40E-30	XM_003641569	7	↓
Line A Male v Line B Male						
ENSGALG00000030940	*BLB2*	Major histocompatibility complex class II beta chain BLB2, (similar to HLA class II, D beta chain)	1.82E-29	XM_015295014	16	↑
ENSGALG00000039964	*KIFC1L*	kinesin-like protein KIFC1-like	3.48E-38	XM_015295019	16	↑
ENSGALG00000050319	Novel	Uncharacterised	3.49E-25	n/a	2	↓
ENSGALG00000050367	Novel	Uncharacterised	5.01E-25	n/a	2	↓
ENSGALG00000009172	*OSBPL6*	PREDICTED: Oxysterol binding protein like 6	9.86E-42	XM_003641569	7	↓

[1]↓ Gene down regulated; ↑ Gene up regulated: Regulation direction listed for first category i.e. Line A Female vs Line A Male, regulation direction listed for Line A Female.

Summary

The final hypothesis of this study was that dietary inclusion of DV XPC® in breeder diets would have positive effects on progeny growth and performance, though alterations in endocrine signalling pathways**.** The addition of dietary DV XPC® in breeder diets at 23 weeks of age ***did not have transgenerational effects on progeny growth and performance.*** Maternal supplementation with DV XPC® has previously been shown to improve production characteristics in broiler progeny produced from DV XPC® supplemented hens (Kidd *et al.* 2013). The findings from this study however appeared to be age dependent, with no progeny effects identified in birds hatched from 32 week old hens in relation to production traits. Conversely, Kidd *et al.*, 2013, also observed that progeny produced from 39 week old hens exhibited improved FCR and enhanced breast muscle yield. We collected, incubated and hatched eggs from 32 week old hens, results from this grow-out trial are consistent with findings by Kidd *et al*. 2013. Due to logistical reasons and facility bookings, we were unable to incubate hatch and rear birds from 39 week old hens.

Previous work comparing breeder age and post-hatch progeny performance parameters has yielded variable results (Koppenol *et al.* 2015b), with many factors contributing to chick quality and performance (Peebles *et al.* 1999). However, alterations to the *in-ovo* environment, particularly the hormonal composition of the egg are of particular intrigue, as previous work has identified an age effect in relation to egg composition (Sudo *et al.* 2004; Veiga-Fernandes and Pachnis 2017). Additionally, the age of breeder flock has been associated with variations in progeny performance and carcass composition, and may potentially result from variations in the composition of the *in-ovo* environment (Ding *et al.* 2017). ***The contributing mechanisms influencing the interaction between age of breeder hen and DV XPC® supplementation remain unclear***. Dietary supplementation of XPC®, may be able to optimise progeny performance from older hens, whether this is through improved egg quality, such shell thickness or other mechanisms such as yolk composition and utilization or embryonic development *in ovo*, requires further investigation.

In our study, our hatch rate was lower than expected so starting numbers were low, resulting in reduced replication and reduced number of birds/pen. In addition, our FCR data was based on mixed-sex pen feed intake. The minimal to no observed differences in performance parameters is likely a result of the overall power of the trial, and as mentioned previously, larger scale, single sex trials are required for more accurate and robust performance data analyses.

Considering the addition of DV XPC® to breeder hen diets failed to significantly influence plasma corticosterone concentrations, it is unsurprising that *in-ovo* concentrations of both yolk corticosterone and testosterone were not affected by DV XPC® supplementation. Interestingly, progeny from line B were consistently lighter in bodyweight than progeny from line A. Also, plasma and yolk corticosterone concentrations were higher in line B compared to line A. ***There is evidence linking elevated plasma corticosterone to lower bodyweights in birds*** (Post *et al.* 2003; Fairhurst *et al.* 2013). Correlations of bodyweight and plasma corticosterone on our birds at day 21 and 42 were performed, a significant moderate, negative correlations were observed for all birds at day 21 (-0.4, *P=0.027*), but not at day 42, although the relationship was still negative. There were no significant correlations within lines. ***Differences between lines***

observed in this study may be purely genetic, however a larger scale trial, sampling a greater number of birds, would be required to determine if this is indeed the case.

Recent literature exploring the metabolism of maternally derived hormones within yolk have suggested that only a fraction of yolk corticosterone ends up in the embryo, as is metabolised by enzymes in the extra embryonic membranes of the egg (Kumar *et al.* 2019), this has been suggested as a protective mechanism to improve fitness and survival (Carter *et al.* 2018). However what is not entirely clear, is under what conditions and what concentration of both maternal and *de novo* embryonic corticosterone are required to alter or "reprogram" embryonic development (Vassallo *et al.* 2019). Another crucial factor is at what time point during embryonic development this programming occurs in order to permanently alter phenotype? Such alterations may include the progeny's ability to cope with stress and/or changes to body weight and composition which was observed in this trial.

Using RNA-seq, we were unable to tease out these differences, only subtle phenotypic differences between lines accompanied by a lack of depth of reads after removal of duplicates, likely reduced the experimental capacity to detect differential expression and genes/pathways of interest. The major differences detected at ED15 in the brain were largely sex driven and did not provide ***links to any possible transgenerational effects (i.e. stress) to endocrine pathways in the brain and liver.*** Pathway analysis of liver samples provided some promising avenues to further explore the phenotypic differences in body composition, particularly fat %, observed between the two lines. These findings we be explored in more detail in our sequencing manuscript, which is currently under preparation.

Overall, the progeny grow out trial was disappointing, but it allowed us to address new questions and create better experimental designs for future trials. It would be beneficial to work with parent flocks to conduct grow out trials of broiler progeny, as "broilerising" GP progeny is not as effective nor does it truly represent commercial meat bird grow-out trials.

Further work investigating these factors, accompanied by in-depth behavioural analysis is required to tease out mechanisms of potential transgenerational effects of stress in broiler breeders, both in F1 generations and beyond.

Implications

Implications that arise from this study indicated that management strategies should be tailor made for specific lines of broiler breeders, and that blanket nutritional approaches may not be the most effective in optimising production performance of each line.

The study also highlights the importance of understanding stress associated with lay in broiler breeders. Regardless of breeder line, the impact of the onset of lay on the bird has been completely overlooked and investigation into the physiology and behaviour around point of lay is required to find means to alleviate this stress and in turn potentially improve welfare and production outcomes.

Recommendations

It has been recommended to HiChick to add DV XPC® to diets of their wet litter line to improve overall reproductive performance of their flocks. Although XPC did not alleviate the wet litter issue, we have recommended that the product be included in starter diets, so that is available from the time of hatch, in order to determine if XPC is indeed have a modulating effect on the gastrointestinal tract, feeding XPC birds from hatch and studied throughout the life of the flock may be beneficial.

It would also be beneficial to visit the farms which rear the GP flocks to assess litter quality. If there are transgenerational, line specific effects of wet droppings and litter, then by targeting the issue at the GGP level, management at the GP and potentially P level could be minimised. Even if industry can effectively manage the issue, by minimising or even removing the wet litter problem, you can ultimately improve efficiency on farm.

In depth, microbiota analyses, should accompany such studies where wet droppings and wet litter are an issue. Again, detecting a permanent shift in microbiota at significant physiological time points, such as point of lay, may enable a targeted approach in regards to nutritional or other supplement interventions. Optimising gut health, can positively influence all facets of production, from the hen itself to overall flock management.

Acknowledgments

The investigators would like to thanks HiChick Breeding Company and all their staff for access to their facilities and invaluable advice and support during the animal trials. We want to thank Dr Nicky-Lee Willson for her expertise and input into this study. We thank Dr Rick Tearle for Bioinformatic analyses of sequencing data.

Media and Publications

We aim to publish 3 papers, the first, focusing on the breeder hen data, the second on progeny performance and hormonal assay data and the third on the progeny sequencing data.

Intellectual Property Arising

N/A

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Appendices

Appendices A: Egg quality measurements from eggs collected from two lines of breeder birds (Line A and Line B) at 26 and 32 weeks of age. Hens were fed either a control or XPC supplemented diet (1000ppm). Values are mean (% bwt) ± SEM.

Breed	Diet	N	Egg Weight (g)	Egg Width (mm)	Egg Length (mm)	Yolk Weight (g)	Albumen Weight (g)	Dry Shell Weight (g)	Shell Thickness (µm)	Yolk Colour
26 weeks of Age										
Line A	Control	8	50.52 ± 1.17	37.25 ± 0.25	50.63 ± 0.73	12.78 ± 0.44	30.15 ± 1.07	5.02 ± 0.12 [a]	0.27 ± 0.009 [a, x]	2.63 ± 0.18
Line A	XPC	7	46.55 ± 1.70	36.14 ± 0.63	49.43 ± 0.72	11.40 ± 0.50	27.67 ± 1.28	4.94 ± 0.17 [a]	0.30 ± 0.006 [a, y]	3.14 ± 0.40
Line B	Control	5	46.59 ± 2.17	36.20 ± 0.66	49.60 ± 1.36	11.84 ± 0.37	28.01 ± 2.26	4.09 ± 0.11 [b]	0.22 ± 0.097 [b, x]	2.20 ± 0.37
Line B	XPC	6	46.20 ± 1.44	36.70 ± 0.33	48.17 ± 0.95	12.27 ± 0.57	27.17 ± 1.02	4.47 ± 0.22 [b]	0.25 ± 0.013 [b, y]	2.83 ± 0.31
32 Weeks of Age										
Line A	Control	7	63.47 ± 1.28	43.86 ± 0.34	57.79 ± 0.94	17.28 ± 0.55	37.07 ± 0.52	6.04 ± 0.18 [a]	0.28 ± 0.006 [a]	1.43 ± 0.20
Line A	XPC	7	61.19 ± 2.33	43.71 ± 0.57	56.71 ± 1.02	17.10 ± 0.33	35.89 ± 1.75	5.86 ± 0.23 [a]	0.28 ± 0.010 [a]	1.29 ± 0.18
Line B	Control	7	59.12 ± 0.96	42.71 ± 0.18	57.04 ± 0.48	16.68 ± 0.34	34.56 ± 0.68	5.41 ± 0.11 [b]	0.26 ± 0.007 [b]	1.57 ± 0.20
Line B	XPC	7	59.77 ± 1.38	43.29 ± 1.02	57.42 ± 0.75	17.44 ± 0.19	34.48 ± 1.20	5.15 ± 0.15 [b]	0.23 ± 0.010 [b]	1.43 ± 0.20
Source of Variation										
26 weeks of age										
Line			NS	NS	NS	NS	NS	< 0.001	< 0.001	NS
Mat. Diet			NS	NS	NS	NS	NS	NS	0.013	NS
Line x Diet			NS	NS	NS	NS	NS	NS	NS	NS
32 weeks of age										
Line			NS	NS	NS	NS	NS	0.001	0.002	NS
Mat. Diet			NS	NS	NS	NS	NS	NS	NS	NS
Line x Diet			NS	NS	NS	NS	NS	NS	NS	NS

[a-b] Mean values with different superscripts within a column are different dependent on line.
[x-y] Mean values with different superscripts within a column are different dependent on diet.