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Practical implications of Ascaridia galli in free-range laying hens
Sub-Project No. 1.5.9

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Executive summary

Ascaridia galli (A. galli) is one of the most prevalent gastrointestinal parasite found in free range poultry. Infections with *A. galli* have been associated with reduced performance, health status, welfare, body weight gain and increased incidences of infectious diseases. While the current methods for controlling internal parasites in commercial layers include the use of anthelmintics and targeted management procedures, optimal use of such approaches require the development of reliable monitoring systems and awareness of critical threshold levels at which interventions are required. A pilot study was conducted to establish a reliable infection model with *(A. galli)* which then was used for the subsequent research work.

Two experiments were conducted to evaluate the impact of different levels of *Ascaridia galli* infection on the performance, egg quality and immune status of laying hens. In the first experiment, an artificial infection study was performed using 200 laying hens. Four treatment groups were orally inoculated with four levels of *A. galli* eggs- negative control (0 *A. galli* eggs), low (250 *A. galli* eggs), medium (1000 *A. galli* eggs) and high (2500 *A. galli* eggs). Levels of *A. galli* infection had no effect on excreta egg count, intestinal worm count, feed intake, body weight and feed conversion ratio (FCR). However, egg production ($P<0.01$) and egg mass ($P<0.01$) were significantly lower in low infected group hens as compared to control group hens. No impact of *A. galli* infection was observed on egg quality. Antibody titre detected in egg yolk using ELISA was significantly higher in medium and high infected hens compared to hens of the control group ($P<0.05$). However, the antibody titre in the hen serum was similar across all treatment groups ($P>0.05$)

In the second experiment, a natural infection study mimicked commercial conditions by allowing uninfected hens to access the ranges where the previous flock of infected hens from the artificial infection study used to range. The objective of this experiment was to examine how many hens will get infected on the subsequent batch and how high the impact of this infection would be. The impact of re-infection with *A. galli* was determined using production parameters and egg quality. A total of four treatment groups (negative control-dewormed hens; low exposure group – hens that ranged on an areas that previously housed hens infected with 250 *A.galli* eggs; medium exposure group – hens that ranged on an area that previously housed hens infected with 1000 *A.galli* eggs; and a positive control group) were subject to this study. Both excreta worm egg counts (EWEC) and total worm numbers were higher in hens of the medium group compared to the low infected group and hens of the negative control ($P<0.01$).

A. galli infection did not affect egg quality parameters and performance parameters except body weight which was lower in hens of the medium infected group ($P<0.001$) compared to hens of the low infected group but was similar to the positive control group.

Based on these results, we can conclude that *A. galli* causes no production loss within the first three months of the free range laying hen's production cycle. Since this project did not test the results over a full production cycle we cannot exclude any production loss during the later production cycle stages. Future evaluation of the need for treatment across the whole production cycle in advised, as producers may be unnecessarily incurring the costs of parasite control and reducing farm profits.

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BSA	Bovine serum albumin
ELISA	Enzyme linked immuno sorbent assay
FACS	Fluorescence- activated cell sorting
FCR	Feed conversion ration
HRP	Horseradish peroxidase
IgY	Immunoglobulin G
IgY	Immunoglobulin Y
PBS	Phosphate-buffered saline
pH	Pouvoir hydrogene
p.i.	Post infection
TMB	Tetramethylbenzidine
RPMI	Roswell Park Memorial Institute
EWEC	Ascarids worms in excreta/g wet weight

Introduction

Parasitic infections of the gastrointestinal tract can have a negative impact on health, welfare and productivity of laying hens (Ramadan and Abou Znada, 1991; Dahl et al., 2002; Gauly et al., 2007). *Ascaridia galli* (*A. galli*) is one of the most prevalent helminth parasite found in free range poultry (Permin et al., 1999; Gauly et al., 2007; Das et al., 2010). Infections with *A. galli* have been reported to decrease body weight and egg production of laying hens (Ikeme, 1971; Dahl et al., 2002), and increase the risk of secondary bacterial infections with *Pasteurella multocida* and *Escherichia coli* (Dahl et al., 2002; Permin et al., 2006). Rarely, ascarids migrate to the oviduct and become enshelled into the egg. If that occurs, the worms may be visible in the eggs (Reid et al., 1973) and possibly degrade the egg quality. However, the effect of different levels of *A. galli* infection on egg quality and a minimum threshold have not been documented, nor has the relationship between infection intensity and their impact on egg production been clear.

In Australia, free-range egg production is rapidly growing with an estimated market value share of 49% (AECL, 2016). About 58.6% of free- range egg producers in Australia noticed the presence of external or internal parasites in their flocks (unpublished Poultry CRC report, 2015). Investigations showed that most of the farmers rarely checked their flocks for parasite infection, and some farmers were not satisfied with the options available for preventing and treating parasites (unpublished Poultry CRC report, 2015). The examination of excreta samples randomly collected from free-range layer farms indicated that *A. galli* may be the most prevalent parasites infecting the Australian free range laying hens (unpublished Poultry CRC report, 2015). These findings are in general agreement with the findings in free-range enterprises in other countries (Sherwin et al., 2013; Yazwinski et al., 2013; Kaufmann, 2011; Jansson et al., 2010).

The direct impact of *A. galli* infection on the economy of the Australian egg industry is not known. While there are methods for controlling internal parasites in commercial egg producing hens including the use of anthelmintics, the optimal use of this approach requires the development of a reliable monitoring system and an awareness of the critical infection threshold at which interventions are required. Effective monitoring of parasitic loads enables farmers and farm managers to efficiently control parasite infections. Knowing the *A. galli* infection status of their flock will allow producers to deliver control strategies in a timely and cost effective manner. Rather

than using anthelmintics as a prophylactic treatment, which may increase selective pressure towards drug resistance, these drugs could be used in a more precise manner as therapeutic only when needed. This project was designed to investigate the impact of different levels of *A. galli* infection on health, production performance and egg quality of laying hens. In addition, the project aimed to examine and develop methods for determining treatment thresholds for *A. galli* control.

Objectives of the project

- To investigate the impact of different levels of *A. galli* infection on production performance, egg quality and immunological response of laying hens.
  (Low level: 250 sporulated *A. galli* eggs/hen, Medium level: 1000 sporulated *A. galli* eggs/hen, High level: 2500 sporulated *A. galli* eggs/hen)
- To determine the relationship between the levels of artificial infection with *A. galli* and the infection level of a subsequent flock of hens introduced to these ranges on production, egg quality and immunology of hens.
- To determine a threshold of parasite monitoring using practical and efficient detection methods such as antibodies against *A. galli* antigen in hen serum and egg yolk, as well as excreta worm egg counts).

Outline of the report

Chapter 1 describes a reliable infection model established for laying hens with *A. galli*, conducted in the Animal House isolator sheds at the University of New England, Armidale. It also describes the methodological development for detecting *A. galli* specific IgG antibodies, conducted at the CSIRO F.D. McMaster laboratories, Armidale. Chapter 2 describes the artificial infection study conducted at the UNE Laureldale Research Station, Armidale, to examine the impact of *A. galli* infection on production performance, egg quality and immune response in free-range laying hens. Chapter 3 describes the natural infection study using the infected ranges from the previous artificial infection trial. This experiment allowed for investigation of the relationship between the natural infections with the intestinal parasite *A. galli* and the impact of hen health and productivity. A general discussion in Chapter 4 summarises the results of the experimental work, and discuss the possible implications of this research for the egg industry.

CHAPTER 1: Establishing and validation of *A. galli* specific IgY ELISA

1.1 Establishing and standardising a method to detect *A. galli* specific IgY in serum of laying hens

1.1.1 Antigen preparation

Antigen required to coat plates in the ELISA assay was prepared by using frozen, female adult *A. galli* worms. The protein concentration of the *A. galli* antigen was determined using a Pierce BCA Protein Assay kit (ThermoFisher-Scientific^{TM}). The antigen was partitioned into aliquots and stored at -20°C for further use.

1.1.2 Assay development, optimization and establishment of positive and negative control samples

The following ELISA assay procedure was developed and validated as described (Norup et al., 2013) with slight modifications: 1) ELISA plates were coated with the *A. galli* antigen extract diluted in a carbonate buffer (pH 9.6) and incubated at 4°C overnight. 2) Wells of the ELISA plates were then blocked with blocking solution (Phosphate-buffered saline (PBS) with 0.5% Bovine serum albumin (BSA), pH 7.4) and incubated at room temperature (RT) for 2 hours. 3) Following washing, chicken serum samples and control samples (positive and negative) were serially diluted 10-fold across the plate in blocking solution and plates incubated for 1 hour at RT. 4) Following washing, goat anti chicken IgY conjugated to horse radish peroxidase (HRP) was added to wells and plates incubated for 1 hour at room temperature. Finally, plates were washed and 100 µl substrate solution (Tetramethylbenzidine: TMB) added. Plates were incubated in the dark for 10-12 minutes and colour development then stopped with the addition of 1M H_2SO_4. Colour development was quantified by reading the absorbance of individual wells at 450 nm with a 630 nm reference wave length.

In order to optimise the ELISA assay and for the use of internal positive and negative control standards, serum samples from infected (free range) and non-infected (caged) hens from commercial farms were harvested.

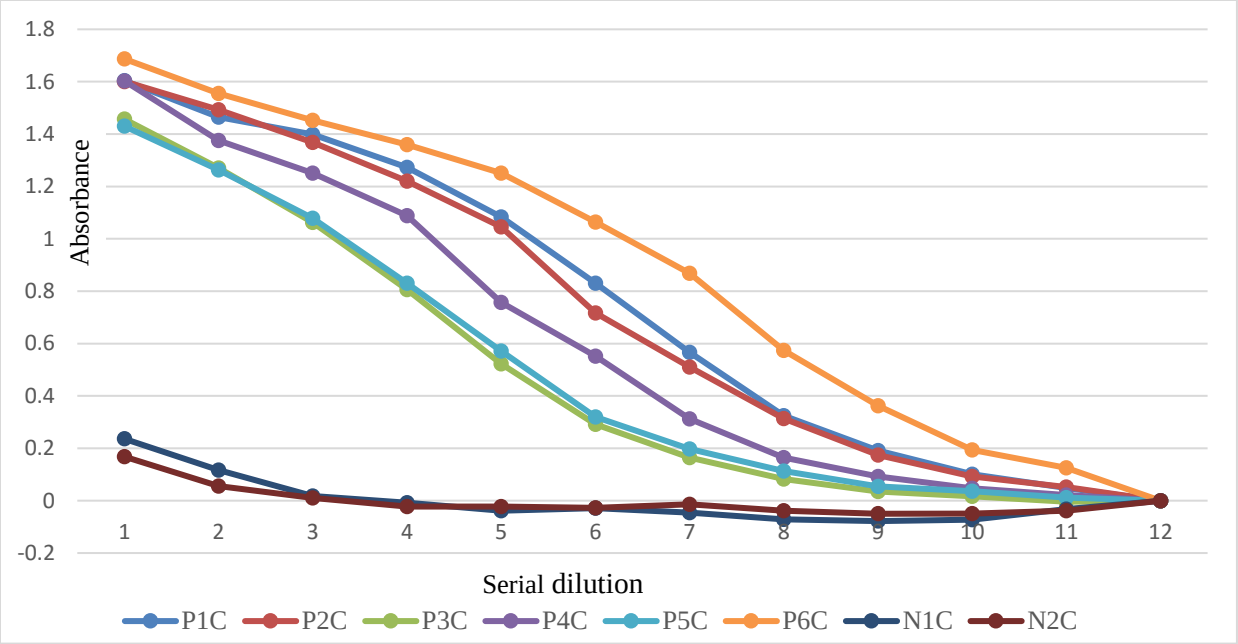

Figure 1: Absorbance of serially diluted positive and negative serum samples

A total of 8 serum samples, 6 strong positive samples (P1C, P2C, P3C, P4C, P5C, P6C) from hens with severe *A. galli* infestation and two negative samples (N1C, N2C) from hens never exposed to *A. galli* (caged birds) were used to optimize the ELISA assay. Optimal reagent concentrations were identified by assessing the positive sample absorbance: negative sample absorbance ratio. The combination of reagent concentrations yielding the highest ratio while having acceptable non-specific binding and background absorbance values was determined.

Various blocking solutions have been assessed for their ability to minimize non-specific binding. Once the optimal blocking solution (PBS with 0.5% BSA, pH 7.4) was identified and samples were serially diluted across ELISA plates to determine the optimal dilution range (Figure 1). Various coating antigen concentrations and conjugated antibody concentrations were assessed to determine the optimal concentrations for these reagents.

1.2 Establishing and validating an infection model with *A. galli* in laying hens

The research conducted was approved by the Animal Ethics Committee of the University of New England, Armidale, Australia (approval No AEC 14-089). Animals were individually housed in cages located at the University of New England facilities and treated in accordance with the Model Code of Practice for the Welfare of Animals, Australia (CSIRO, 2002).

In order to prepare embryonated *A. galli* eggs for artificial infection, mature *A. galli* nematodes were sampled from the intestine of naturally infected laying hens (Figure 2). The mature nematodes were washed in sterile phosphate-buffered saline (PBS) and transferred into Roswell Park Memorial Institute (RPMI) media at 37°C (with 0.1% 100 units/mL penicillin, 100 µg/mL of streptomycin, 250 ng/mL Amphotericin B) and cultured for three days which includes changing the media every 24 hours. *A. galli* eggs that were shed into the media by the adult worms were collected from the spent medium by centrifugation after each 24 hour period. The concentrated *A. galli* eggs were resuspended in 0.1 N H_2SO_4 and kept at 26 °C for up to six weeks. Embryonation of *A. galli* was judged to have occurred after 3 weeks of culture when fully formed nematodes were visible within the egg shell. For inoculation, the embryonated *A. galli* eggs were diluted into 0.05 M $NaHCO_3$ to neutralize the acid and then diluted in 0.05 M NaCl to generate the desired concentration of 500 or 1000 eggs/ml.

A total of 20 Lohmann brown laying hens were assigned to 4 treatment groups with 5 hens each. The individual hens were infected with *A. galli* eggs or adult worms at various stages. The infection models are shown in Table 1.

Two hens of each group were sacrificed 14 days post infection (p.i.) to evaluate the presence of immature parasites in the intestine. The remaining three hens were analysed for *A. galli* eggs in the excreta at 8 and 14 weeks post infection (p.i.). These three hens were sacrificed 16 weeks post infectionem (p.i.), their serum collected and their intestine examined for the presence of adult *A. galli* worms. The results (Table 1) indicate that only hens that were inoculated six times for the duration of two weeks (group 3) could be used for reliable detection of immature worms in the intestine at 14 days p.i. as well as mature worms at 16 weeks p.i..

Table 1: The effect of different infection models with *A. galli* on excreta egg counts, immature *A. galli* worms, mature *A. galli* worms and serum antibody titre at different times post infection

Details	**Group 1**	**Group 2**	**Group 3**	**Group 4**
Mode of inoculation	**oral**	**oral**	**oral**	**cloacal**
Frequency of inoculation (days/week)	3 times over the duration of 1 week	3 times over the duration of 1 week	3 times over the duration of 2 weeks	once
Number of embryonated eggs or adult worms inoculated	1000 eggs	1000 eggs	500 eggs	adult worms
Storage condition of eggs or adult worms	26˚C	Initial 4˚C, followed by 26˚C for the duration of 14 days	26˚C	Room temperature
Number of immature *A. galli* worms in hen intestine (14 days p.i.) / hen	0	0.33	12	0
Number of *A. galli* eggs in hen excreta (8 weeks p.i.) / hen	450	0	383	0
Number of *A. galli* eggs in hen excreta (14 weeks p.i.) / hen	1850	267	917	0
Number of mature *A. galli* worms in hen intestine (16 weeks p.i.) / hen	8	1.7	14.7	0
Serum antibody titre of *A. galli* specific IgY (14 weeks p.i.) / hen	1002	871	951	274
Total number of hens where mature or immature *A. galli* worms were present in the intestine	3	3	5	0

The results of this study suggest that the infection method applied to hens of group 1, as well as hens of group 3 are capable of infecting laying hens with *A. galli*. However, not all individuals were infected in these groups whereas 100% of hens in group 3 were diagnosed positive. Therefore, infection method 3 was the most reliable method tested and was applied the subsequent research.

CHAPTER 2: Artificial infection study to evaluate the impact of various levels of *A. galli* on health and performance of free-range laying hens

2.1 Introduction

Changes in consumer demand and an increased focus on animal welfare have resulted in alterations to the housing systems used for commercial laying hens. The traditional cages are being gradually replaced with floor husbandry and free range systems. These housing systems allow the birds to contact their excreta, the external environment and wild birds which in turn increases the chances of infection with parasites (Permin et al., 1999; Kaufmann et al., 2011). The prevalence of intestinal parasites such as nematodes can be up to 100% in free range systems (Sherwin et al., 2013; Yazwinski et al., 2012; Hoglund et al., 2012; Kaufmann et al., 2011; Martin and Pacho et al., 2005; Permin et al., 1999). Gastrointestinal parasites can have a negative impact on health, welfare and productivity of laying hens resulting in poor body weight gain, increased feed conversion ratio, reduced health conditions and overall reduced performance (Chadfield et al., 2001; Dahl et al., 2002; Gauly et al., 2007). Intestinal parasitic infections compromise the immune system of the affected host (Horning et al., 2003) and can increase the severity of concurrent diseases (Dahl et al., 2002; Permin et al., 2006). Among all gastrointestinal parasites, *A. galli* is the most prevalent and economically important nematode parasite worldwide. Ascarids may migrate to the oviduct and become enshelled into the hen's egg. This causes worms to be visible in eggs and reduce pigmentation of yolk (Reid et al., 1973) thereby degrading the egg quality. The following research was designed to investigate the impact of different intensities of *A. galli* infection on health, productivity performance and egg quality of laying hens. In addition, the project aimed to examine and develop methods for determining infection thresholds for optimised parasite control.

2.2 Objectives

- To determine the relationship between *A. galli* infection levels and productivity performance
- To determine effects of *A. galli* infection on egg quality and hen immunology

2.3 Materials and methods

2.3.1 Preparation of the housing facilities

The research conducted was approved by the Animal Ethics Committee of the University of New England, Armidale, Australia (approval No AEC 15-110). Hens were housed in accordance with the Model Code of Practice for the Welfare of Animals, Australia (CSIRO, 2002). Housing conditions were performed according to the breeder's recommendations (Lohmann, 2015). In detail, a total of 20 pens were equipped with feeder, drinker, nest boxes and perches. The floor area of each pen was designed of 8 m^2 slat area and 1 m^2 of solid floor covered with wood shavings. Range facilities were prepared according to the current minimum industry standard of 10,000 hens/ha as determined by the Australian Consumer Affairs Minister (CAF, 2016). Hens had access to the range from 9 am – 5 pm daily.

2.3.2 Sourcing of hens and parasites

Commercial laying hens (Lohmann brown) were obtained from a commercial pullet rearer at the age of 16 weeks. The total of 200 hens were housed in 20 pens, 10 hens per pen. Hens were individually numbered using leg band identification and were provided with *ad libitum* commercial feed and water in the shed. An adaption period of 2 weeks was allowed before hens were infected using method 3 as identified in milestone 1. In order to prepare the embryonated *A. galli* eggs for artificial infection, mature *A. galli* nematodes were sampled from the intestine of naturally infected laying hens (Figure 2). The mature nematodes were washed in sterile phosphate-buffered normal saline and transferred into RPMI media at 37°C (with 0.1% 100 units/mL penicillin, 100 µg/mL of streptomycin, 250 ng/mL Amphotericin B) and cultured for three days, changing the media every 24 hours. Eggs shed into the media were collected from the spent medium by centrifugation after each 24 hour period and concentrated eggs were resuspended in 0.1 N H_2SO_4 and kept at 26 °C for up to six weeks. Embryonation was judged to have occurred after 3 weeks of culture when fully formed nematodes were visible within the shell. For inoculation, the embryonated eggs were diluted in an equal volume of 0.05 M $NaHCO_3$ to neutralize the acid and then diluted in 0.05 M NaCl to the desired concentration.

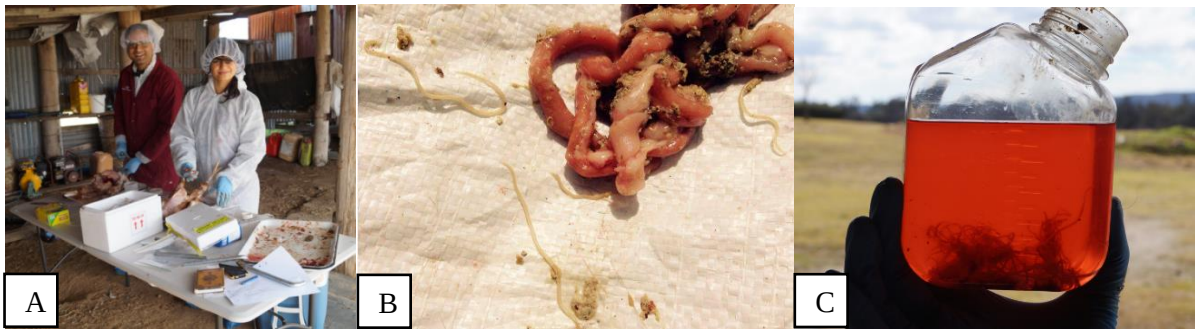

Figure 2: Various steps of harvesting mature *A. galli* for the production of infectious *A. galli* eggs. (A); Processing of laying hen intestine from spent hens (B); Mature *A. galli* worms migrating from the intestinal lumen (C); Mature *A. galli* worms harvested in RPMI media

2.3.3 Establishment of infection and scheduling of experiment

Hens were individually infections at low (250 *A. galli* eggs), medium (1000 *A. galli* eggs), and high (2500 *A. galli* eggs) levels and compared to a control group. Hens were orally inoculated with respective doses in 1 ml volume, administered in 6 applications over the duration of a two week time period. Hens of the control group were inoculated with the same transfer medium, but no *A. galli* eggs (sham infection). Hens of the control group were maintained by deworming in 4 week intervals with levamisole (4 mg/hen) administered through the drinking water. Each of the 4 treatment groups had 5 replicates consisting of 10 hens per pen.

The effect of the *A. galli* infection was investigated at 5 different time points: time point 0: the day prior to infection when hens were 19 weeks of age, time point 1: 5 weeks p.i. when hens were 25 weeks of age, time point 2: 10 weeks p.i. when hens were 30 weeks of age, time point 3: 15 weeks p.i. when hens were 35 weeks of age and time point 4: 20 weeks p.i. hens were 40 weeks of age.

In order to determine the impact of *A. galli* infection on hen production and health the hen's feed intake, body weight, egg weight and numbers of eggs were obtained at all time points. Hen house production (%) was calculated weekly taken into account the total number of eggs laid per hen/day (productivity (%) = number of eggs/number of hens*100). In order to determine the impact of the early and later stage of *A. galli* infection on internal and external egg quality, the hen's eggs were examined at timepoint 2 and 4. A total of 6 eggs/pen were analysed on two consecutive days. External characteristics such as egg weight, shell weight, shell thickness and reflectivity and

breaking shell strength and internal characteristics such as yolk colour, albumen height, and Haugh unit were measured.

In order to measure circulating antibodies against *A. galli* antigen, serum was collected from the same 4 individual hens per pen at each of the five time points (0, 1, 2, 3 and 4). The serum was stored at -80°C until further analysis.

Fresh samples of hen excreta were collected from each pen 8, 10, 15 and 20 weeks p.i. The number of *A. galli* eggs was evaluated using a modified McMaster flotation method. Briefly, 4 g of excreta was ground and placed in a 60 ml Mc Master Jar with 10 ml of water, and the samples were soaked for 30 minutes. Then, saturated NaCl solution was added to a total volume of 60 ml, stirred, and the suspension loaded onto McMaster egg counting chambers where *A. galli* eggs where counted by microscopic examination at 40x magnification (Stereo compound microscope Olympus CX31, Tokyo, Japan).

2.3.4 FACS (Fluorescence- Activated Cell Sorting)

For measuring intraepithelial lymphocytes (cytotoxic T-cells: $CD4^{-}CD8^{+}$; T-helper cells: $CD4^{+}CD8^{-}$; double positive cells: $CD4^{+}CD8^{+}$), intraepithelial lymphocytes were isolated from the small intestine of chickens according to the method used by Röhe (2014). Mouse anti-Chicken CD3-AF647, mouse anti-Chicken CD4-FITC, mouse anti-chicken CD8a-PE, and Mouse IgG1-PE, Mouse IgG1-AF647, and mouse IgG1-PE were purchased from Southern Biotech (Birmingham, Alabama, USA). The crude preparations of intraepithelial lymphocytes were resuspended in ice-cold Hanks balanced salt solution (HBSS) without calcium or magnesium at approximately 1×10^{6} cells per ml. The cells were incubated with the primary anti-CD4, anti-CD8 cocktail or isotype control antibody preparation for 30 min on ice and in the dark. The cells were centrifuged three times at 200g for 5 minutes at 4°C and resuspended in ice-cold PBS with 0.1% Bovine Serum Albumin (BSA). The centrifugation and wash steps were repeated twice. After the final centrifugation the cell pellets were resuspended in 200 µl of ice-cold PBS with 0.1% BSA and cell surface marker data acquired on a FlowSight flow cytometer (Amnis, USA). The flow cytometer was compensated using the AbC anti-Mouse Bead Kit conjugated to fluorophore isotype control antibodies (Thermoscientific, Carlsbad, USA).

2.3.5 Final sampling of hens at 40 weeks of age

At 40 weeks of age, the body weight and eviscerated carcass weight of all hens (n=199) were taken. In addition, intestines were collected from one hen per replicate (n=20) for Flourescence Activated Cell Scanning (FACS) analysis. Counts of adult *A. galli* were all undertaken on dissected fresh intestines from all hens (n=199). For three birds from each replicate, the small intestine (duodenum, jejunum and ileum), and the caecum and colon were collected to determine immature stages of *A. galli*, and the number of any other helminth parasites present in these birds (n=60). The content of the coprodeum was collected from each individual hen to count the number of *A. galli* eggs.

2.4 Statistical analysis

Statistical analysis of the data collected to was performed using GLM procedure of SAS software (Version 9.3, SAS Institute, Cary, NC, USA). Flow cytometric data were analysed using IDEAS application software version 6.0 (Amnis, USA). Populations of intact single cells were gated by analyzing forward scatter side scatter plots and confirmed by fluorescent micrograph analysis of cells from imaging flow cytometric data. Within these single cell populations, cells that were positive for CD4 or CD8 or double positive compared to isotype controls were gated and quantified. Statistical analysis of flow cytometric data were performed using Prism version 5 (GraphPad, USA). Two-way ANOVAs were performed with challenge and age as main effects. P-values < 0.05 were considered significant.

2.5 Results & discussion

2.5.1 The effect of *A. galli* infection on production parameters

The effects of *A. galli* infection on various production parameters are displayed in Table 2. No impact of *A. galli* on feed intake, body weight and FCR could be observed at any time point. Hen production was significantly different among the treatment groups/ infection levels. While hens of every infected group produced less eggs than the control group, only the productivity of hens in the low infection group was statistically significant (p=0.002). However, when comparing the control group to all infected hens without differentiation of the infection level, the overall productivity was statistically significantly different (p=0.05) (Table 3). Similarly, the statistical difference was close to significant (p=0.06) when comparing FCR of hens of the control group to all infected hens, regardless the infection level (Table 3). No effect of the infection levels on egg weight and egg mass could be observed. The total number of *A. galli* eggs in the excreta was significantly higher in the three treatment groups compared to the control group (p=0.0001), indicating the infection intensity used was higher than the background infection levels from the outdoor ranges. Anthelmintic treatments were used as an additional measure to keep infection rates in the control group at a minimum. There were no statistically significant differences in the number of *A. galli* eggs in the excreta amongst the various infection level treatments. This indicated that there is no impact of the infection levels tested on *A. galli* egg shedding and the groups are comparable infections. The egg counts increased from week 8 till week 11 in all infected groups, but then declined, possibly indicating an immunological or other physiological response that limited infections. Parasite burden as worm count at the final time point was analysed for correlations with FCR, feed intake, body weight, eviscerated body weight and liver weight at the time points measured on a pen-level basis (FCR and feed intake) or an individual bird basis (other measures). Also, parasite burden, expressed as eggs of Ascarids worms in excreta/g wet weight (EWEC) at the pen level was analysed for correlations with FCR and feed egg count within time points. Using linear regression, the highest R^2 value obtained, 0.22, was for EWEC on FCR during time point 4. Therefore there was little evidence for a linear relationship between parasite burden and bird performance parameters during the experiment. Because of the use of anthelmintics in the control group, data from the infected birds alone was used to investigate correlations.

Table 2: The effect of different levels of *A. galli* infection on feed intake, egg mass, egg weight, FCR, productivity, body weight and excreta egg counts of parasites in free range laying hens.

Parameters		Feed intake (g/hen/d)	Egg mass (g)	Egg weight (g)	FCR	Productivity (%)	Body weight (g)	WEC eggs/g (log_{10})
Treatments (Trt)	**Control (n = 50)**	121±4.26	52.3^{a}±1.38	63.4^{ab}±0.86	2.34±0.09	94.0^{a}±1.50	1971±0.02	0.39^{b}±0.18
	Low (n = 50)	121±5.08	48.1^{b}±2.63	63.2^{b}±0.82	2.67±0.18	85.3^{b}±4.00	2012±0.02	2.53^{a}±0.18
	Medium (n = 50)	128±5.36	51.7^{a}±1.88	64.1^{ab}±0.89	2.49±0.10	92.1^{a}±1.88	1984±0.02	2.94^{a}±0.11
	High (n = 50)	130±5.17	51.6^{a}±1.86	64.5^{a}±0.80	2.57±0.14	91.2^{ab}±1.86	2007±0.02	2.95^{a}±0.19
Timepoints (T)	**18 weeks**	116±5.15	39.6±1.85	58.1±0.39	3.03±0.17	78.0±3.53	1896±0.01	2.11±0.29
	25 weeks	124±3.53	53.5±0.56	64.5±0.28	2.32±0.07	94.9±1.03	2009±0.01	2.68±0.29
	35 weeks	133±6.27	53.5±0.61	65.4±0.28	2.50±0.11	92.9±1.05	2009±0.01	2.14±0.30
	40 weeks	128±4.04	57.1±0.58	67.1±0.33	2.23±0.07	96.7±1.1	2061±0.02	1.89±0.26
P-value	**Trt**	0.45	0.003	0.02	0.20	0.002	0.17	0.0001
	T	0.09	<0.001	<0.001	<0.0001	<0.001	<0.0001	<0.0001
	Trt x T	0.42	0.0004	0.90	0.36	<0.001	0.95	0.06

[a b] Means in each row for each factor with different superscripts differ significantly ($p < 0.05$)

Table 3: The effect of *A.galli* infection on laying hen performance (control vs. treatment)

	Feed intake/ hen/day (g)	Egg weight (g)	Egg mass/day (g)	FCR	Productivity (%)
Control (n=50)	121 ±4.27	63.4 ±0.86	52.3 ±1.39	2.34 ±0.09	94.0 ±1.50
Infected hens (n=150)	127 ±3.00	63.9 ±0.48	50.5 ±1.12	2.58 ±0.08	89.5 ±1.62
P-value	0.354	0.192	0.146	0.066	0.05

Control = non-infected hens, Treatment = all infection levels; [a b] Means in each row for each factor with different superscripts differ significantly ($p < 0.05$).

2.5.2 The effect of *A. galli* infection on internal and external egg quality

Egg quality parameters (egg weight, shell reflectivity, shell thickness, weight, percentage, thickness and breaking shell strength) were evaluated during early (5 weeks p.i – hens 25 weeks of age) and late (20 weeks p.i. – hens 40 weeks of age) stages of infection. Results are displayed in Table 4. No difference in any of the parameters could be observed among the treatment groups. The effect of time on egg weight, shell thickness, shell percentage, shell weight, albumen height and Haugh unit was expected, since those parameters vary with hen age. However, there was no statistically significant interaction between the duration of the infection and the infection level, indicating that infection with *A. galli* has a minor impact on the parameters investigated. Parasite burden as worm count at the final time point was analysed for correlations with egg quality and egg productivity measures at the time points measured on a pen-level basis. Also, parasite burden as EWEC at the pen level was analysed for correlations with egg quality and egg productivity measures within time points. Using linear regression, the highest R^2 value obtained, 0.26, was for EWEC on egg weight during time point 1. Therefore there was little evidence for a linear relationship between parasite burden and egg production or quality parameters across the experiment. Because of the use of anthelmintics in the control group, data from the infected birds alone was used to investigate correlations.

Table 4: The effect of various levels of *A. galli* infection in laying hens on egg weight, shell weight shell reflectivity, shell thickness, shell percentage and breaking shell strength, deformation, albumen height, Haugh unit, and yolk score

Parameters		Egg weight (g)	Shell weight (g)	Shell reflectivity (%)	Shell thickness	Shell percent	Breaking shell strength (N)	Deformation	Albumen height	Haugh Unit	Yolk score
Treatments (Trt)	**Control**	64.4±0.78	6.50±0.12	20.8±0.59	0.46±0.01	10.1±0.25	47.9^{ab}±1.27	0.31±0.01	9.25±0.37	94.4±1.97	10.6±0.09
	Low	64.7±0.80	6.55±0.12	20.4±0.63	0.45±0.01	10.1±0.11	49.9^{ab}±0.96	0.31±0.004	9.23±0.36	94.5±1.92	10.4±0.19
	Medium	65.6±1.03	6.50±0.14	21.6±0.57	0.44±0.01	9.96±0.33	48.2^{ab}±1.60	0.31±0.008	8.97±0.26	92.6±1.42	10.6±0.10
	High	64.9±1.06	6.30±0.13	20.9±0.48	0.44±0.01	9.71±0.23	44.4^{a}±1.52	0.34±0.014	9.43±0.36	95.1±1.99	10.4±0.15
Timepoints (T)	**25 weeks**	63.0^{b}±0.45	6.16^{b}±0.07	20.4±0.36	0.44^{b}±0.0	9.79^{b}±0.0	47.8±1.22	0.32±0.007	10.0^{a}±0.12	98.5^{a}±0.60	10.5±0.11
	40 weeks	66.8^{a}±0.51	6.77^{a}±0.05	21.5±0.41	0.45^{a}±0.0	10.17^{a}±0.0	47.5±0.82	0.30±0.007	8.43^{b}±0.18	89.8^{b}±0.96	10.5±0.08
P-value	**Trt**	0.67	0.19	0.47	0.21	0.15	0.06	0.17	0.51	0.46	0.75
	Time	<0.0001		0.06	0.02	0.01	0.84	0.10	<0.0001	<0.0001	0.86
	Trt*Time	0.65		0.32	0.80	0.59	0.79	0.79	0.36	0.37	0.77

[a b] Means in each row for each factor with different superscripts differ significantly ($p < 0.05$)

2.5.3 The effect of different levels of *A. galli* infection on body weight, carcass weight, intestinal parasite burden, parasite shedding, serum antibodies and intraepithelial lymphocytes response

Compared to uninfected control hens, infected hens had significantly higher numbers of adult *A. galli* worms in their intestines (Table 5). Furthermore, the total *A. galli* egg number in the coprodeum content was significantly higher. The total number of *A. galli* in the intestine was highest in hens that received a total of 1000 eggs during the oral inoculation (medium level of infection) (Figure 3). The number of *A. galli* eggs in the excreta was not statistically different among the various infection levels. No effect of parasite infection on body weight and eviscerated weight could be obtained. Liver weight was higher in the medium infected group ($p=0.02$) compared to other groups. The correlation between adult *A. galli* worm counts and *A. galli* egg counts in the excreta at the final time point was 0.70, with an R^2 of 0.50, indicating a relationship between the two measures of parasite burden as expected. Because of the use of anthelmintics in the control group, data from the infected birds alone was used to investigate correlations.. The intestinal intraepithelial lymphocytes (cytotoxic T-cells: $CD4^-CD8^+$; T-helper cells: $CD4^+CD8^-$; double positive cells: $CD4^+CD8^+$) were monitored at different levels of *A. galli* infection using flow cytometry (Table 6). There was no significant effect of *A. galli* infection when comparing the different levels of infection compared to the control ($p>0.05$). Also, there was no significant effect of *A. galli* infection when comparing the control group to all infected hens regardless of the infection level ($p>0.05$).

Table 5: The effect of *A. galli* infection on body weight, eviscerated weight, liver weight, worm counts in the intestine and worm egg counts in the coprodeum 20 weeks p.i.

Parameters		Body weight (kg)	Eviscerated body weight (kg)	Liver weight (g)	Adult Worm counts ($\sqrt[3]{}$)	Worm egg counts ($\sqrt[3]{}$)
Treatments (Trt)	**Control**	2.01±.02	1.54±0.01	41.7^b±0.68	0.85^b±0.82	1.68^b±89.3
	Low	2.07±0.02	1.58±0.02	42.4^b±0.93	1.41^a±1.17	6.64^a±123.1
	Medium	2.06±0.01	1.56±0.01	45.3^a±0.87	1.64^a±2.97	7.18^a±411.0
	High	2.08±0.02	1.59±0.01	43.95^b±0.84	1.37^a±2.23	5.63^a±147.0
P-value		0.124	0.124	0.02	<0.01	<0.01

[a b] Means in each row for each factor with different superscripts differ significantly ($p < 0.05$)

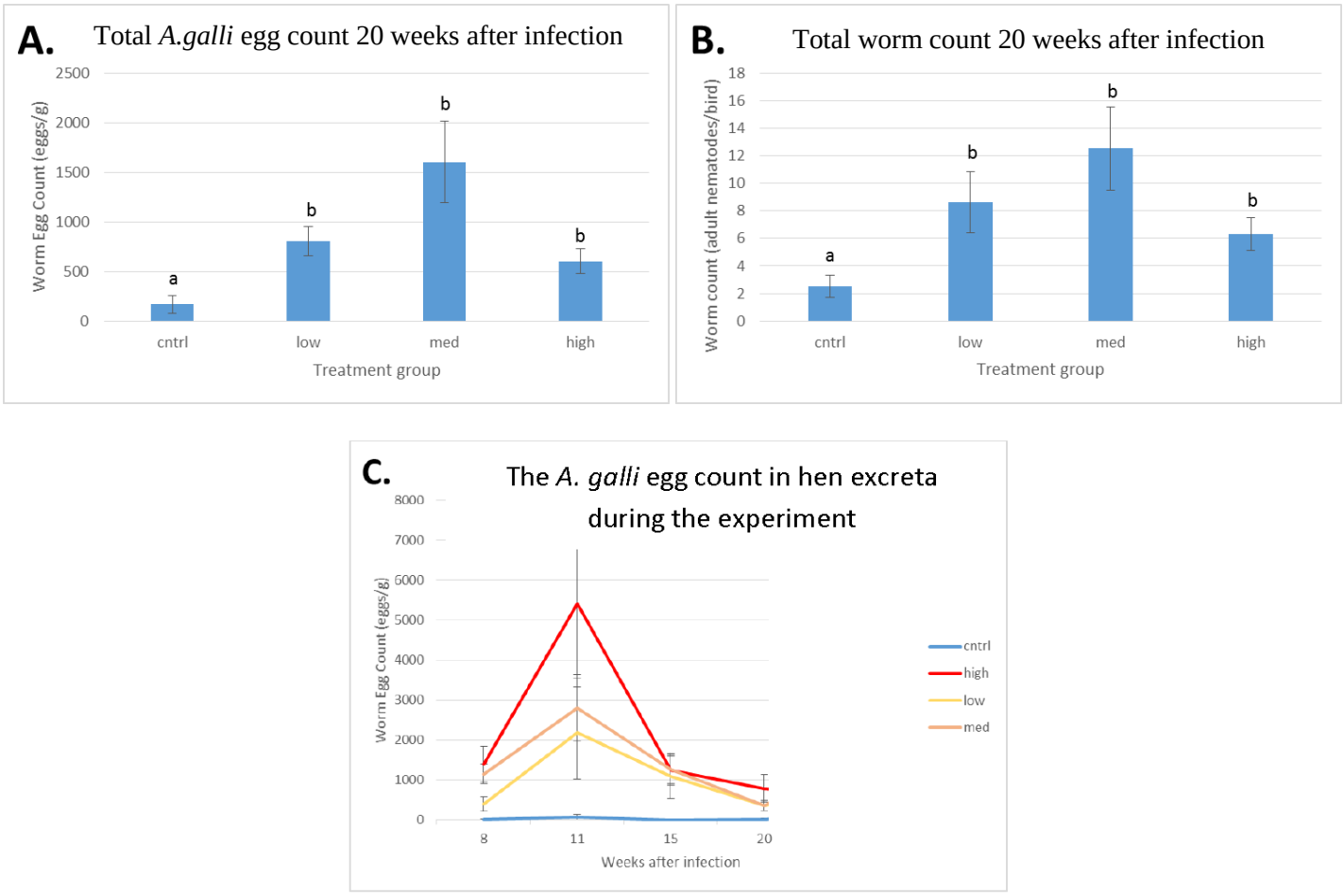

Figure 3: The outcome of *A. galli* infection at three infection levels, and the level of infection in the control pen birds. (A): *A. galli* egg counts assessed on individual birds at the final time point; (B): The count of *A. galli* adult worms from the small intestine of birds at the final time point (C): The pen level of A. galli eggs at each sampling point throughout the experiment Error bars indicate twice the standard error of the untransformed mean. Letters indicate statistical significance (treatment level $p<0.001$ in A, B & C). In all graphs the infection groups are not significantly different from one another but all infected groups are significantly greater than the control. Data were cube root transformed for the statistical analysis (ANOVA, Genstat).

Table 6: The effect of *A. galli* infection on intestinal intraepithelial lymphocytes

Parameters		**T – helper cells: $CD4^+CD8^-$**	**cytotoxic T-cells: $CD4^-CD8^+$**	**double positive cells: $CD4^+CD8^+$**
Treatments (Trt)	**Control**	13.0±1.0	6.52±3.2	3.17±1.4
	Low	16.6±1.7	10.9±3.2	6.77±1.8
	Medium	14.7±3.6	5.24±1.6	5.30±2.1
	High	15.9±3.0	7.37±3.3	5.46±1.6
P-value	**Trt**	0.47	0.57	0.28

2.5.4 The effect of different levels of *A. galli* infection on serum and yolk antibodies detected by ELISA

The same 4 individual hens from each pen (n=20) were bled to obtain serum at each sample timepoint 1, 2, 3 and 4. This serum was analysed for anti-*A. galli* antibodies, but no significant differences between treatment groups were observed over all or at any time point. (Figure 4). The mean antibody level for timepoint 0 pre-infection was used as a co-variate which showed no difference to the antibody levels at other timepoint. The factors which contributed to this were first, most birds had a positive test for anti-*A. galli* antibody at the beginning of the experiment, suggesting exposure to *A. galli* during the rearing phase (Figure 4). The effect of the starting antibody concentration was taken into account during the statistical analysis, but it did not alter the outcome except at time point 4. Secondly, many of the infected treatment group birds we analysed showed an increase in antibody followed by a decrease, but the timing of the peaks, even within treatment groups, was not synchronised. Thirdly, some birds from both the control and infected treatment groups exhibited a constant antibody level that did not change.

Six eggs per pen were collected at timepoint 2 and 4 of the experimental period and processed for measuring anti-*A. galli* antibodies (Figure 5). At timepoint 2, high and medium infected group had significantly higher antibody titre in yolk as compared to the control group but no difference to low infected group. And at timepoint 4, low, medium and high infected treatments groups had significantly higher yolk antibody titre compared to the control group but no difference was observed among the infected levels.

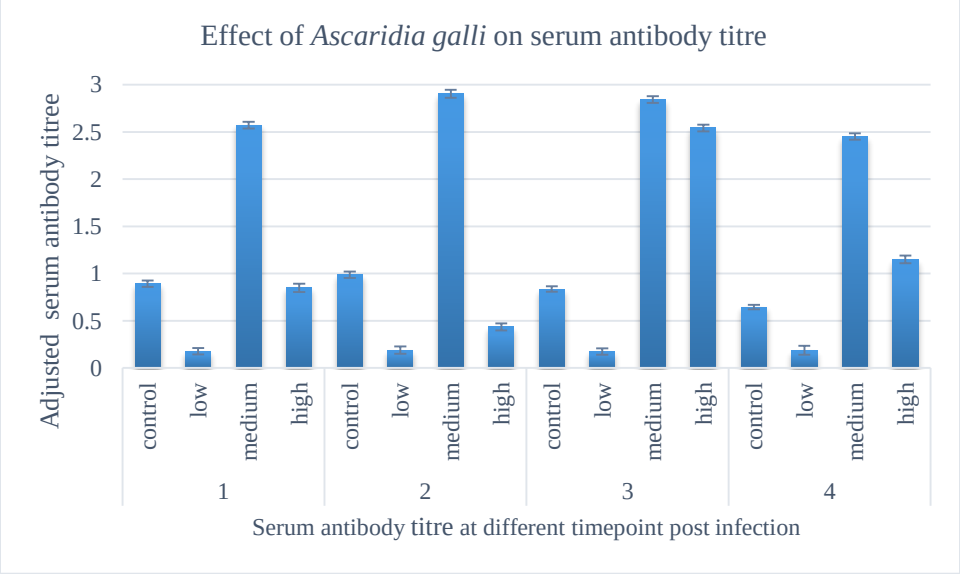

Figure 4: The effect of *A. galli* infection on the *A. galli* antibody level in in serum at each time point of the experiment

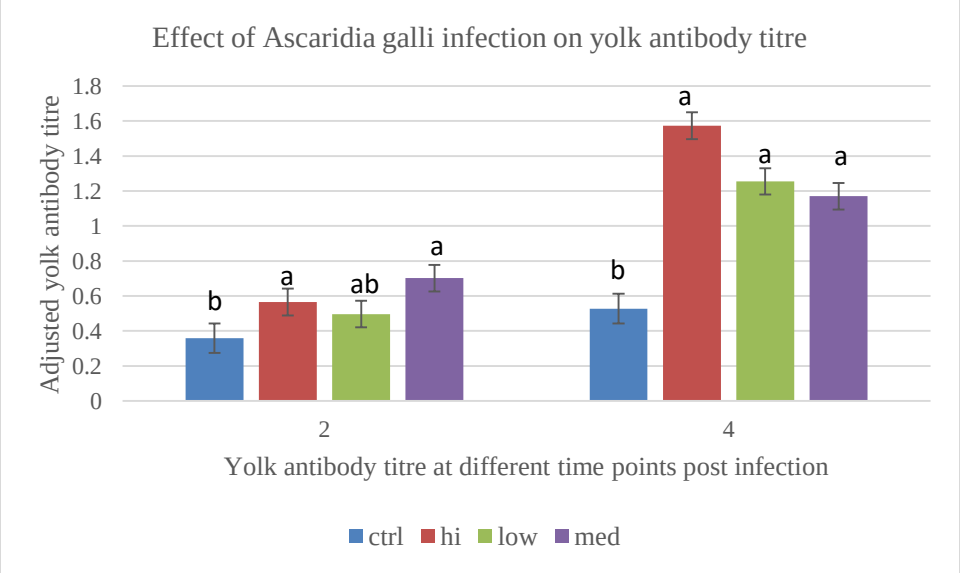

Figure 5: The effect of *A. galli* infection on the *A. galli* antibody level in egg yolk at each time point of the experiment

CHAPTER 3: Natural infection study to evaluate the impact of various levels of *A. galli* on performance and health of free-range laying hens

3.1 Introduction

Ascaridia galli infection in chickens occurs by ingestion of infective eggs containing the infective larvae stage either second stage larvae (L2) (Herd & McNaught, 1975) or third stage larvae (L3) (Ackert, 1923) from the contaminated areas. The *A. galli* eggs remain infective for years in deep litter systems. Occasionally, the eggs can be ingested by earthworms and transmitted to birds ingesting them (Permin and Hansen, 1998). There is a likelihood of infection for hens kept on ranges contaminated by earlier flocks of infected birds. Therefore, the aim of this study was to investigate infection intensity of natural *A. galli* uptake and the impact on hen performance and egg quality.

3.2 Objectives

- To determine the infection intensity of hens that were infected using ranges contaminated with *A. galli*.
- To determine the effects of infection intensity on hen performance, egg quality and specific *A. galli*- antibody production.

3.3 Materials and methods

3.3.1 Animal experiment

The animal experiment was approved by the Animal Ethics Committee of the University of New England, Armidale, Australia (approval No AEC 16-075). Hens were housed in accordance with the Model Code of Practice for the Welfare of Animals, Australia (CSIRO, 2002). Housing conditions were performed according to the breeder's recommendations (Lohmann, 2015). In detail, a total of 20 pens were equipped with feeder, drinker, nest boxes and perches. The floor area of each pen was designed of 8 m^2 slat area and 1 m^2 of solid floor covered with wood shavings. The stocking density of hens on the range followed the current minimum industry standard of 10,000 hens/ha (CAF, 2016). Hens had access to the range from 9 am – 5 pm daily.

A total of 200 of Lohmann brown layers were randomly assigned to 4 treatment groups with 5 replicates (10 hens per replicate). Hens of the negative control group was maintained by deworming in 4 week intervals with levamisole (4 mg/hen) through oral inoculation of individual hens. In order to eliminate and possibility of *A. galli* infection from the range, all ranges that were accessed by hens of the negative control group were paved with bitumen. Hens of the low infection group ranged on an area, where the previous flock of hens had been infected with 250 *A. galli* eggs/hen and had accessed the range for 20 weeks. Hens of the medium infection group ranged on an area, where the previous flock of hens had been infected with 1000 *A. galli* eggs/hen and had accessed the range for 20 weeks. Hens of the positive control group birds ranged on an area, where the previous flock of hens had been infected with 1000 *A. galli* eggs/hen and accessed the range for 20 weeks. In addition, hens of the positive control group were orally inoculated with 1000 *A. galli* eggs at the age of 18-19 weeks following the procedure described in Chapter 2.

3.3.2 Sample collection

Sample collection was performed at 4 different time points: time point 1: time prior to range access/infection when hens were 16 weeks of age, time point 2: 4 weeks after range access when hens were 20 weeks of age, time point 3: 9 weeks after range access when hens were 25 weeks of age, and time point 4: 14 weeks after range access when hens were 30 weeks of age.

To determine the impact of natural *A. galli* infection on hen production including the effect of infection on the onset of lay, the number of eggs and egg mass was performed continuously. Feed intake and body weight was obtained at 25 and 30 weeks of age. The impact of natural *A. galli* infection on internal and external egg quality, eggs were examined at the end of the experiment at time point 4. A 6 eggs/pen were analysed on two consecutive days. External characteristics included egg weight, shell weight, shell thickness and reflectivity and breaking shell strength. Internal characteristics included yolk colour, albumen height, and Haugh unit.

In order to measure circulating antibodies against *A. galli* antigen, serum was collected from all individual 200 hens at each of four time points (1, 2, 3 and 4). The serum was stored at -20°C until needed for analysis. To measure antibodies against *A. galli* in yolk, yolk extraction was performed at time points 2, 3 and 4.

Fresh samples of hen excreta were collected from each pen at 19, 21, 23 weeks of their age, followed by weekly collection until the end of the experiment. The number of *A. galli* eggs was evaluated using a modified McMaster flotation method outlined in chapter 2.

3.3.3. Final sample collection

At 30 weeks of age, the body weight and eviscerated carcass weight of all hens (n=200) was obtained. All hens were dissected and the total number of adult *A. galli* worms from the upper part of the intestine (duodenum, jejunum and ileum) were counted. The coprodeum content from each individual hen was also collected to count the number of *A. galli* eggs (n=200). Four hens per replicate were used to investigate immature stages of *A. galli* in the small intestine (duodenum, jejunum and ileum) and caecum (n=80).

3.4 Statistical analysis

Performance data were analysed following a 4×2 factorial arrangement using JMP statistical software version 8 (SAS Institute Inc, Cary, NC) to test the main effects of treatment, age and their interactions. Worm and egg count data were not normally distributed and thus were transformed to cube root before analysis. Data were subjected to two-way ANOVA with repeated measures, and means were separated by Student's t- test at a probability level of 0.05.

3.5 Results and discussion

3.5.1 The effect of natural infection with *A. galli* on hen performance

The effects of *A. galli* infection on the various production parameters are shown in Table 7. There was no significant effect of the infection level on hen performance (FI, FCR, egg production, egg mass, egg weight, etc) and egg quality. However hens of the medium infection group had lower body weight than hens of the low infection group and the negative control group while the body weight of medium infected group was comparable to the body weight of hens of the positive control group. No differences were observed among all the treatment levels (negative control, positive control, low and medium). The number of *A. galli* eggs in the hen excreta was higher in the medium infected group compared to the low infected group but was similar to the positive control group. No *A. galli* eggs were observed in the excreta of the birds from the negative control group at any time point.

Table 7: The effect of different levels of *A. galli* infection on feed intake, egg mass, egg weight, FCR, productivity, body weight and excreta egg counts of parasites in free range laying hens.

Parameters		Feed intake (g/hen/d)	Egg mass (g)	Egg weight (g)	FCR	Productivity (%)	Body weight (g)	WEC eggs/g (cube root)
Treatment (Trt)	**Negative control**	128	54.7	59.4	2.34	92.1	1886^{ab}	0.00^{c}
	Positive control	125	56.4	59.5	2.22	93.6	1864^{bc}	9.07^{ab}
	Low infection	129	55.5	59.6	2.32	93.1	1921^{a}	6.71^{b}
	Medium infection	133	56.9	60.3	2.34	94.4	1840^{c}	10.71^{a}
	SEM	2.66	1.01	0.30	0.05	1.33	13.12	1.16
Time point (T)	**25 weeks**	120^{b}	54.0^{b}	58.9^{b}	2.23^{b}	91.1^{b}	1848^{b}	6.52
	30 weeks	137^{a}	57.7^{a}	60.4^{a}	2.38^{a}	95.5^{a}	1908^{a}	6.73
	SEM	1.88	0.72	0.21	0.03	0.94	9.28	0.82
P-value	**Trt**	0.20	0.43	0.15	0.26	0.68	<0.01	<0.001
	T	<0.001	<0.001	<0.001	<0.01	<0.01	<0.001	0.86
	Trt x T	0.46	0.84	0.72	0.28	0.95	0.86	0.71

$^{a\,b}$ Means in each row for each factor with different superscripts differ significantly ($p < 0.05$).

Routine anthelmintic treatment by oral inoculation successfully maintained the negative control group non-infected throughout the whole experimental period. To investigate any possible effect of parasite worm burden on any of the performance parameters, a regression/correlation analysis was performed. Intestinal worm counts were correlated with feed intake, body weight, liver weight, FCR, egg mass, egg weight and productivity at each time points on a replicate - level. Also, parasite burden, expressed as eggs of *A. galli* worms in excreta/g wet weight basis (EWEC) was analysed for correlations with adult worm counts at the final time points. A strong linear relationship was obtained between intestinal worm counts and *A. galli* egg counts in the excreta ($R^{2=}0.80$). For other parameters investigated using linear regression, the highest R^2 value obtained, 0.13, was for WWEC on body weight during time point 4. There was no evidence for a linear relationship between WEC on the performance parameters.

3.5.2 The effect of natural infection *A. galli* on internal and external egg quality

The egg quality parameters (egg weight, shell reflectivity, shell weight, shell thickness, shell percentage, breaking shell strength, deformation, albumen height, Haugh unit and yolk score were evaluated at the end of experimental period when hens were 30 weeks of age. Results are displayed in Table 8. No difference in any parameters could be observed among the treatment groups, indicating that infection with *A. galli* has a negligible impact on the parameters investigated. Parasite burden expressed as total intestinal worm count was correlated with the egg equality parameters and egg production at the final time point. Also, parasite burden expressed as EWEC was correlated with egg quality and egg production. Using linear regression, the values obtained were 0.22, was for number of worms worm/ albumen height, 0.2 for number of worms/shell weight, 0.2 for worm count/Haugh unit. Therefore, there was a little evidence for a linear relationship between parasite burden and egg quality parameters during the experiment.

Table 8: The effect of various levels of *A. galli* infection in laying hens on egg weight, shell weight shell reflectivity, shell thickness, shell percentage and breaking shell strength, deformation, albumen height, Haugh unit, and yolk score

Parameters		**Egg weight (g)**	**Shell weight (g)**	**Shell reflectivity (%)**	**Shell thickness (mm)**	**Shell percent**	**Breaking shell strength (N)**	**Deformation**	**Albumen height (mm)**	**Haugh Unit**	**Yolk score**
Treatment	**Negative control**	59.7	6.07	19.1	0.44	10.2	45.1	0.28	10.07	99.9	10.1
	Positive control	60.8	6.24	19.5	0.43	10.3	45.5	0.28	10.67	102.2	10.1
	Low infection	60.1	6.29	19.8	0.44	10.5	45.7	0.30	10.27	100.5	10.0
	Medium infection	61.5	6.35	18.8	0.44	10.3	46.0	0.28	10.64	102.0	10.3
	SEM	0.56	0.07	0.50	0.004	0.15	1.09	0.01	0.17	0.75	0.19
P-value	**Treatment**	0.15	0.08	0.54	0.85	0.43	0.93	0.24	0.06	0.11	0.63

[a b] Means in each row for each factor with different superscripts differ significantly ($p < 0.05$).

3.5.3 The effect of different levels of *A. galli* infection on body weight, carcass weight, carcass yield, liver weight and yield, intestinal parasite burden and parasite eggs in the coprodeum of laying hens at 30 weeks of age

At the end of the experimental period, all hens were sacrificed to count adult worms in the intestine, content from the coprodeum was obtained for *A. galli* eggs, hen body weight, eviscerated body weight, liver weight and liver yield were also recorded. Results are displayed in Table 9. *A galli* infection did not affect body weight, eviscerated body weight as well as the liver weight and yield. However, there were significant differences among treatment groups regarding the total numbers of intestinal adult worms and the number of *A. galli* eggs in the coprodeum. Hens of the medium infected group had significantly higher in intestinal adult worm counts (P<0.001) and *A. galli* egg counts (P<0.001) compared to the low infection group (P<0.001) but was similar to the positive control group (P<0.001).

Table 9: The effect of *A. galli* infection on body weight, eviscerated weight, liver weight, worm counts in the intestine and worm egg counts in the coprodeum of laying hens at 30 weeks of age

Parameters		**Body weight (g)**	**Eviscerated body weight (g)**	**Eviscerated yield (%)**	**Liver weight (g)**	**Liver yield (%)[1]**	**Liver yield (%)[2]**	**Intestinal worm counts ($^3\sqrt{}$)**	**Coprodeum egg counts ($^3\sqrt{}$)**
Treatment (Trt)	**Negative control**	1899	1558	82.1	42.4	2.23	2.72	0.00^{c}	0.00^{c}
	Positive control	1848	1513	81.9	38.3	2.07	2.54	3.17^{ab}	13.77^{ab}
	Low infection	1911	1564	82.0	39.7	2.09	2.54	2.85^{b}	11.84^{b}
	Medium infection	1868	1530	81.9	38.4	2.06	2.53	3.46^{a}	14.96^{a}
	SEM	17.87	17.08	0.69	1.35	0.06	0.08	0.19	1.00
P-value	**Trt**	0.09	0.16	0.99	0.15	0.21	0.26	<0.001	<0.001

[a b] Means in each row for each factor with different superscripts differ significantly ($p < 0.05$)

[1]percentage of live body weight

[2] percentage of eviscerated weight

The regression analysis R^2 between the total number of adult *A. galli* worms the total number of *A. galli* eggs in the excreta was 0.80, indicating a strong linear relationship (Figure 6).

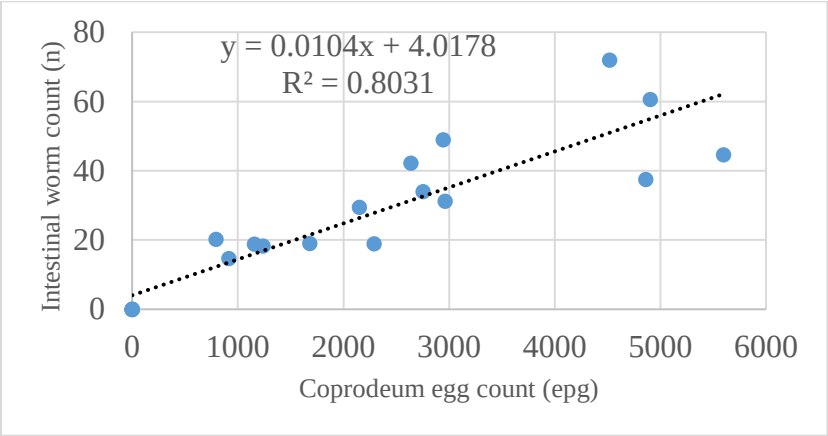

Figure 6: Correlation between the total number of intestinal worms and the total number of *A. galli* eggs obtained from the coprodeum.

3.5.4 The effect of different levels of *A. galli* infection on weekly egg production, weekly egg weight and weekly excreta egg counts

The egg production and egg weight were recorded throughout the whole experimental period to investigate if natural infection with *A. galli* delays laying onset and peak of production. Weekly egg weight and egg production is displayed in the Figure 7. Excreta from each pen was collected weekly and was analysed for the presence of *A. galli* eggs. No significant difference could be observed on weekly egg production, weekly egg weight among all the treatment levels, indicating no significant impact of *A. galli* infection on the production and egg weight. There was a significant effect of different infection levels on the excreta egg counts for *A. galli*. On week, 23 and 25, medium level of infection had higher *A. galli* egg shed in the excreta (P=0.04 and P=0.0009 respectively) compared to the positive control group but was similar to the low infection group. No *A. galli* eggs were detected in excreta of the negative control group. The total number of *A. galli* eggs were not statistically different among hens assigned to the positive control group, medium infection and low infection group but were significantly higher compared to hens of the negative control group.

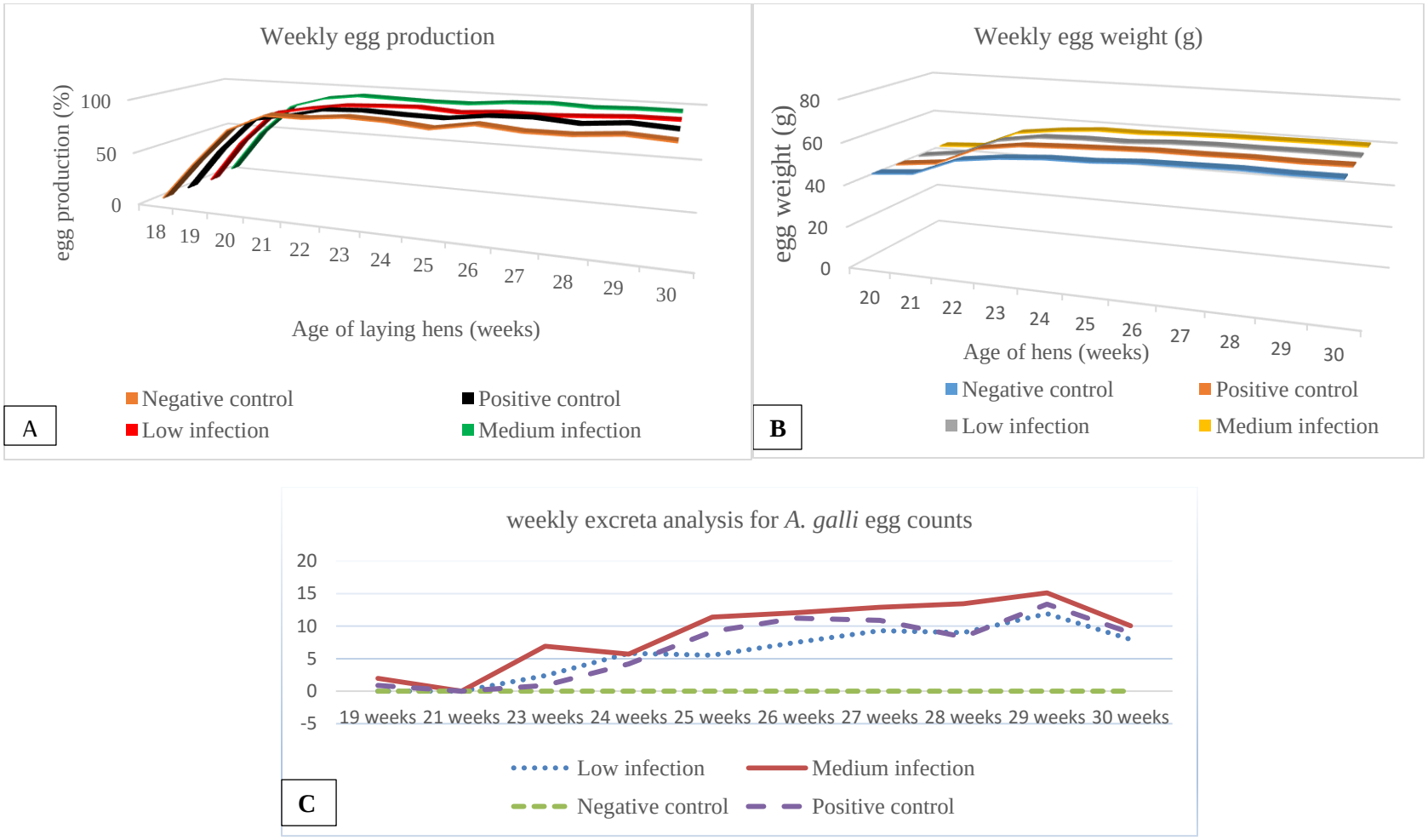

Figure 7: The effect of different levels of *A. galli* infection on (A): Weekly egg production, (B): Weekly egg weight and weekly (C): Total number of *A. galli* eggs in the excreta (excreta egg counts data are cube root transformed)

3.5.5 Prevalence of other gastrointestinal parasites

Besides *A. galli*, other nematode parasites such as *Capillaria*, *Trichostrongylus* and *Heterodera* are also common and may be present in free-range laying hens. In the present study, *Heterakis* and *Capillaria* could be observed in the excreta of hens (Table 10). None of these parasites were present on the negative control treatment group.

Table 10: Total numbers of gastrointestinal parasites/hen other than *A. galli*

Treatment	***Heterakis***	***Capillaria***	***Trichostrongylus***
Negative control	0	0	0
Positive control	0.8	0.75	0
Low infection	1.15	0.25	0
Medium infection	0.8	0	0
SEM	0.35	0.25	0
P-value	0.16	0.16	0

CHAPTER 4: Combined discussion of the results

4.1 Reviewing the objectives

A. galli is one of the most prevalent helminth parasites in free range hen egg production systems (Gauly et al., 2007; Das et al., 2010). The parasite can survive in the external environment such as a poultry range for years, which allows for transmission between several flocks despite an temporarily unused range. Infected hens can shed thousands of parasite oocysts within a short time period. There is a lack of information on the parasite infection threshold levels which might impact hen egg production and/or hen welfare. In the present project, we used artificial *A. galli* infection to investigate the effect of different levels of parasite burden (250 *A. galli* eggs/hen, 1000 *A. galli* eggs/hen, 2500 *A. galli* eggs) on the production parameters, bird immune status, health and egg quality. Furthermore, we investigated the effect of natural infection with *A. galli* that hens consumed from a contaminated range. Hens were allowed to range on the previously infected area and we investigated the quantity and time it took until an infection was established. Production parameters, infection intensity and egg quality parameters were analysed to measure the impact of natural infection. The key objectives of this research were:

1. To examine the impact of different levels (low: 250 *A. galli* eggs, medium: 1000 *A. galli* eggs, high: 2500 *A. galli* eggs) of *A. galli* infection on immune response, performance and egg quality
2. To examine the impact of natural *A. galli* infection on the subsequent flock of hens and to determine its effect on hen performance and egg quality
3. To determine acceptable threshold of parasites and efficient detection methods by measuring circulating antibodies against *A. galli* antigen in hen serum, antibodies against *A. galli* antigen in egg yolk, and *A. galli* eggs in hen excreta

4.2 Major observations

Development of a reliable infection model for *A. galli* in laying hens

The establishment and validation of an infection model with *A. galli* as described in Chapter 1, allows for a reliable infection method that can now be used in future research studies. Among four different types of method used for infecting laying hens, the method of using 500 *A. galli* eggs stored at 26°C was most successful to infect hens including elevated levels of serum antibodies and shedding more *A. galli* eggs in the hen excreta. This method of artificial infection was successfully used in our subsequent animal trials as outlined in chapter 2 and 3. Currently, the infection method relies on the harvest of mature female *A. galli* worms obtained from infected poultry. In the future, long-term storage of *A. galli* eggs may be investigated which will allow for long term storage of the infective material.

The impact of *A. galli* infection on performance of free range laying hens

The investigations described in chapter 2 regarding the impact of *A. galli* infection on hen performance indicate a minor disadvantage in infected hens. The impact of infection levels among hens given identical doses of embryonated *A. galli* eggs non-uniform. While the intestine of some hens housed up to >80 adult worms, the majority of hens housed <10 mature worms. This explains why the inoculation level of *A. galli* had no statistical effect on *A. galli* eggs in the hen. Furthermore, when comparing the different infection levels no statistical difference for effects on feed intake, hen body weight or FCR could be observed. However, egg production ($P=0.002$) and egg mass ($P=0.003$) were significantly lower in hens assigned to the low infection group compared to hens of the control group. When comparing hens of the control group to all infected hens without differentiation of the infection levels (combining all the infection levels together), overall hen day egg production was significantly lower in infected birds ($P<0.05$) and a trend for higher FCR in infected birds could be observed.

The impact of natural infection with *A. galli* on performance

The results described in chapter 3 of this report demonstrate that 100% of hens that accessed a contaminated range did get infected. Surprisingly, hens that ranged on the medium contaminated range were infected to a higher level with *A. galli* and shedded more *A. galli* eggs in the excreta compared to hens of the positive control group. However, no significant differences on hen performance throughout the experimental period could be observed. Importantly, the mean worm

infection levels from natural infection surpassed the infection levels in the first artificial challenge by a large degree. For example, artificially infected hens of the medium group (1000 eggs/hen) had a mean intestinal worm burden of 12.51 (±2.97) worms/hens, while naturally infected hens of the medium group had a mean intestinal worm burden of 43.92 (±5.71) worms/hen, despite a shorter ranging period of 20 weeks. Similarly, the intestinal worm burden on hens assigned to the low infection group was twice as high in natural infected hens compared to artificial infected hens. Therefore there is scope for the infection levels within a production enterprise to accumulate between flocks, and this should be taken into account when implementing control strategies.

Impact of *A. galli* infection on egg quality

Despite the differences in infection intensity, neither the hens that were artificial infected, nor the hens that were naturally infected with *A. galli,* demonstrated any effect on hen egg quality. Egg quality was investigated twice in the artificial infection study, at 30 and 40 weeks of age and once, at 30 weeks of age, in the natural infection study.

Control of worms by routine oral deworming

In the artificial infection study, hens were treated every 4 weeks with anthelmintic (levamisole) applied to the drinking water following guidelines of the manufacturer (4 mg/hen/day). However, *A. galli* eggs in the excreta of negative control hens could be frequently detected and occasionally mature worms could be found in the intestine of those hens. In contrast, individual inoculation with levamisole in hens of the negative control group of the natural infection trial resulted in negative test results throughout the study. Individual variation in the uptake of medicated drinking water and subsequently subtherapeutic administration the anthelmintic is the most likely explanation for these observations.

Prevalence of other parasites

Besides *A. galli*, other nematodes such as *Capillaria, Trichostrongylus* and *Heterodera* are known to be present in free-range laying hens. From both experiments (chapter 2 and chapter 3), the intestinal contents of four birds from each replicate (n=80) pen examined for the presence of mature and immature parasites other than *A. galli* (table 10). While hens used for the artificial infection study (chapter 2) suffered from concurrent *Capillaria* infection, hens used for the natural

infection study (chapter 3) were presented with both *Heterodera* and *Capillaria spp.*. *Trichostrongylus spp.* were not detected in any individual.

Implications

The results from this project have evaluated the impact of *A. galli* infection on hen production. In both trials, there was little evidence that the investigated *A. galli* infection levels cause production loss within the first three months of the production cycle. However, this project did not test the impact of infection on the full production cycle and the critical threshold to which the production level and/or egg quality is affected still needs to be determined.

In this project, oral inoculation of the anthelmintics was effective in controlling the worm infestation of the hens rather than drug administration through the drinking water. This is an aspect that should be examined further. The currently available products all recommend dosing in the drinking water, but the recommendations may have been based on experiments with caged-hens only. Therefore a re-evaluation of parasite control using anthelmintics in the free range egg production industry should be made in consultation with producers, manufacturers and the regulatory authority (APVMA).

Practical recommendations

1) Providing other health issues are not impacting on the birds, and nutrition is optimal, no treatment for *A. galli* appears to be necessary during the first three months of the free range hen egg production cycle. However, as the parasite burden on the range increases, early intervention is crucial to avoid a build-up.

2) We have no experimental evidence for the impact of the infection levels 20 weeks p.i.. As in other livestock: parasite systems, treatment should only be undertaken where there is evidence of infection.

3) We have confirmed the usefulness of excreta worm egg counts (EWEC) as a means of monitoring for the presence of *A. galli*. Further extension work to help producers undertake and/or interpret the results of WEC is needed, and further research is needed to establish treatment thresholds in the later parts of the production cycle.

4) We have developed an antibody detection method which works using both hen blood (serum) and egg yolk. Future research might be able to develop this test so that it can be used as a routine method for early detection of *A. galli* infections in laying hens.

5) In our experiments, the treatment of birds using anthelmintics added to the water supply proved to be inferior compared to individual bird treatments. This is an aspect that should be examined further. The currently available products all recommend dosing in the drinking water, and a departure from this advice would constitute an off-label product use, making eggs unsuitable for sale. Therefore a re-evaluation of parasite control using anthelmintics in the free range egg production industry should be made in consultation with producers, manufacturers and the regulatory authority (APVMA).

6) Future Research - Our ELISA test uses a crude *A. galli* extract as antigen. This contains multiple proteins some of which are naturally exposed to the immune system (surface and secreted/excreted antigens), and other hidden antigens. To develop an ELISA for routine use by diagnostic service providers a simpler, in vitro-produced antigen will be needed. As the first step in this process we will separate the *A. galli* extract antigen using one dimensional electrophoresis and use western

blotting to identify protein bands that are recognised robustly by immune sera collected from the birds in experiments one and two (ie, by the majority of birds over the majority of time points).

7) Future Research - Liver samples have been collected and stored from both natural and artificial infection study, it would be interesting to see if *A. galli* infection limits the ability of birds to store energy in liver lipid reserves, because this may be indicative of production loss in the latter part of the production cycle, which we could not assess in these experiments. We will analyse the lipid content of the collected livers in subsequent research.

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Plain English Compedium Summary

Project Title	Implications of *Ascaridia galli* in free-range laying hens
Poultry CRC Project No.	1.5.9
Researcher	Isabelle Ruhnke
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Project Overview	*Ascaridia galli* is one of the most prevalent gastrointestinal parasite in increasing free-range production systems. This parasite has been associated with the production losses and degrading egg quality. This study examined the effect of different levels of Ascaridia galli infection on the performance, egg quality and immune response of the free-range laying hens.
Research	Chapter 1 demonstrates the reliable method which can be used in future research. The production performance results described in Chapter 2 of the artificial infection study shows the little evidence of the impact of *A. galli* infection on the production. The results described in Chapter 3 of this report shows that hens are likely to become infected from previous flocks. Hens which ranged on the medium infection group of the previous study showed higher numbers of *A. galli* in their intestine as well as higher shedding of *A. galli* eggs in the excreta compared to the positive control Both artificial and natural infection with *A. galli* did not affect the external nor internal egg quality.
Project Progress	Project completed
Publications	Sharma, N., Hunt, P., Swick, R.A., Hine, B and Ruhnke, I. 2015. Development of a reliable infection model with *Ascaridia galli* in laying hens. Australian Poultry Science Symposium. Sharma, N., Hunt, P., Swick, R.A., Hine, B., Sharma, N.K., Normant, C., Iqbal, Z and Ruhnke, I. 2016. The effect of *Ascaridia galli* on performance and egg quality of free range laying hens. Australian Poultry Science Symposium