Helminth infections in laying chickens in Australia: prevalence, diagnosis, and improved methods of worm egg storage and multiplication

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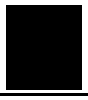
Declaration

The work presented in this thesis was conducted in the department of Animal Science, School of Environmental and Rural science at University of New England, Australia. I certify that the work in this thesis has not been submitted for any degree at this or any other institution to the best of my knowledge and belief. This thesis does not contain any materials previously published or submitted for publication by another author, except where due reference has been made. Any assistance received in this scientific work or in preparing this thesis and all sources used have been acknowledged in this thesis.

Anwar Shifaw Yesuf:  Date: 26/01/23

Dedication

I would like to dedicate this thesis to my parents, my wife Aisha Hussein Muhammed, and my children Tesnim, Selwa and Abdul-hafiz Anwar.

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List of publications from this thesis

Journal articles

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2. **Shifaw, A**., Feyera, T., Elliott, T., Sharpe, B., Walkden-Brown, S.W., Ruhnke, I., 2021. Comparison of the Modified McMaster and Mini-FLOTAC methods for the enumeration of nematode eggs in egg spiked and naturally infected chicken excreta. Veterinary Parasitology 299, p. 109582.
3. **Shifaw, A.,** Feyera, T., Elliott, T., Sharpe, B., Ruhnke, I. and Walkden-Brown, S.W., 2022. Method optimisation for prolonged laboratory storage of *Ascaridia galli* eggs. *Veterinary Parasitology*, *309*, p.109758.
4. **Shifaw, A.,** Ruhnke, I., Elliott, T., Sharpe, B., Feyera, T. and Walkden-Brown, S.W., 2022. Ascaridia galli eggs obtained from fresh excreta, worm uteri or worms cultured in artificial media differ in embryonation capacity and infectivity. *Veterinary Parasitology*, 310, p.109792.
5. **Shifaw, A.,** Feyera, T., Sharpe, B., Elliott, T., Walkden-Brown, S.W. and Ruhnke, I., 2022. Prevalence and magnitude of gastrointestinal helminth infections in cage-free laying chickens in Australia. *Veterinary Parasitology: Regional Studies and Reports*, 37, p.100819.

Conference presentations

1. **Shifaw, A.,** Feyera, T., Sharpe, B., Elliott, T., Walkden-Brown, S.W., Ruhnke, I., 2021. Comparison of the Mini-Flotac and modified McMaster methods for enumeration of Ascaridia galli eggs in chicken excreta. Proceedings of the 32nd Australian poultry Science symposium, Sydney, 9-10 February 2021.
2. 2. Feyera, T., Sharpe, B., Elliott, T., Ruhnke, I., **Shifaw, A.,** Walkden-Brown, S.W., 2021. No evidence of levamisole resistance in *Ascaridia galli* on a free-range egg farm in Australia. Proceedings of the 32nd Australian poultry Science Symposium, Sydney, 9-10 February 2021.

3. Feyera, T., Sharpe, B., Elliott, T., Ruhnke, I., **Shifaw, A**., Walkden-Brown, S.W., 2021. Anthelmintic efficacy against chicken nematodes following individual or group administration assessed by worm and worm egg count reduction. In 28th International Conference of the World Association for the Advancement of Veterinary Parasitology (WAAVP), Dublin, 19-22 July 2021.
4. Feyera, T., Elliott, T., Sharpe, B., Ruhnke, I., **Shifaw, A**., Walkden-Brown, S.W., 2022. Evaluation of *in vitro* methods of anthelmintic efficacy testing against the chicken round worm *Ascaridia galli*. 26th World's Poultry Congress, 2022.
5. Feyera T, **Shifaw A,** Ruhnke I, Sharpe B, Elliott T, Walkden-Brown SW (2021). Anthelmintic efficacy against chicken nematodes following individual or group administration assessed by worm and worm egg count reduction. Ascaridia galli. 26th World's Poultry Congress, 2022.
6. Walkden-Brown, S.W., Feyera, T., **Shifaw, A**., Elliott, T., Ruhnke, I., Sharpe, B., 2021. Improved understanding and control of worm infections in free-range layers. Australasian Veterinary Poultry Association Annual Scientific Meeting, November 2021.

Additional published works by the author relevant to the thesis but not forming part of it

1. Feyera, T., Elliott, T., Sharpe, B., Ruhnke, I., **Shifaw, A.,** Walkden-Brown, S.W., 2022. Evaluation of *in vitro* methods of anthelmintic efficacy testing against *Ascaridia galli.* Journal of Helminthology 96, e29: 1-12
2. Feyera, T., **Shifaw, A**., Sharpe, B., Elliott, T., Ruhnke, I., Walkden-Brown, S.W., 2022. Worm control practices on free-range egg farms in Australia and anthelmintic efficacy against nematodes in naturally infected layer chickens. Veterinary Parasitology: Regional Studies and Reports 30, p.100723
3. Feyera, T., Sharpe, B., Elliott, T., **Shifaw, A**., Ruhnke, I., Walkden-Brown, S.W., 2021. Anthelmintic efficacy evaluation against different developmental stages of *Ascaridia galli* following individual or group administration in artificially trickle-infected chickens. Veterinary Parasitology 301, p.109636.

4. Feyera, T., **Shifaw, A.,** Ruhnke, I., Sharpe, B., Elliott, T., Walkden-Brown, S.W., 2021. *Ascaridia galli* challenge model for worm propagation in young chickens with or without immunosuppression. Veterinary Parasitology 301, p.109624.
5. Feyera, T., Ruhnke, I., Sharpe, B., Elliott, T., **Shifaw, A**., Walkden-Brown, S.W., 2021. Comparative therapeutic efficacies of oral and in-water administered levamisole, piperazine and fenbendazole against experimental *Ascaridia galli* infection in chickens. Veterinary Parasitology 298, p.109514.

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List of abbreviations

Acronym	Abbreviation
AE	Australian Eggs
AEC	Animal ethics committee
AM	Arithmetic mean
ANOVA	Analysis of Variance
AS	Aerobic storage
AR	Anthelmintic resistance
BZ	Benzimidazole
CI	Confidence interval
CL	Confidence level
CV	Coefficients of variation
CRC	Cooperative Research Centre
DEC	Day of egg collection
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EEC	Excreta egg count
ELISA	Enzyme-linked immunosorbent assays
EPG	Eggs per gram
FAO	Food and Agriculture Organization
FCR	Feed conversion ratio
FEC	Faecal egg count
FS	Flotation solution
GIT	Gastrointestinal tract
GM	Geometric mean
H_2SO_4	Sulphuric acid
IgG	Immunoglobulin G
IL-4, 13	Interleukin-4, 13
ITS	Internal transcribed spacers
IP	Incubation period

L2	Second larval stage
L3	Third larval stage
LB	Lohman Brown
LSL	Lohmann Selected Leghorn (LSL)
DL	Danish Landrace
LSM	Least-squares means
MgSO4	Magnesium sulphate
MF	Mini-FLOTAC
MM	McMaster
mRNA	Messenger ribonucleic acid
NA	Not applicable
NaCl	Sodium chloride
NaOCl	Sodium hypochlorite
NaOH	Sodium hydroxide
NSW	New South Wales
PBS	Phosphate-buffered saline
PCR	Polymerase-chain-reaction
RPMI	Roswell Park Memorial Institute
SD	Standard deviation
SEM	Standard error of the mean
SG	Specific gravity
SM	Storage media
SP	Storage period
ST	Storage temperature
Th-1 and 2	T-helper 1 and 2
UNE	University of New England
USA	United States of America
WAAVP	World Association for the Advancement of Veterinary Parasitology
$ZnSO_4$	Zinc sulphate

Abstract

This thesis describes a series of studies designed to investigate important aspects of gastrointestinal helminth parasites in chicken. The studies aimed broadly to 1) provide compiled information about the status and trends of helminth infections in poultry operations worldwide and assess the prevalence and magnitude of helminth infections in commercial cage-free laying chickens in Australia; 2) Evaluate and optimise diagnostic tools for routine monitoring of nematode infections in chickens; 3) optimise prolonged laboratory storage methods for both undeveloped and embryonated stages of nematode eggs.; and 4) evaluate the embryonation and infectious capacity of *A. galli* eggs isolated from excreta, worm uteri or worms cultured in artificial media.

This thesis commences with Chapter 1 (General introduction) that contains an outline of the background, research problem, research aim, and propositions. The literature review chapter 2 provides an overview and summarises the key aspects of helminthiasis in chicken relevant to the research work and identify areas where knowledge is lacking.

The objective of the first study (Chapter 3) was to provide an overview of the published information regarding the epidemiology and the diagnostic approaches of chicken helminth infection. Six databases were searched for studies and a total of 2,985 articles published between 1942 and 2019 were identified and subsequently screened for eligibility using title/abstract and full text assessment, resulting in 191 publications used in the study. Post-mortem diagnostics (73.8%) and the flotation technique (28.8%) were commonly used to detect helminth infections with pooled prevalence of 79.4%. More than 30 helminth species in chicken populations were identified including *A. galli* (35.9%), *H. gallinarum* (28.5%), *Capillaria spp.* (5.90%) and *Raillietina spp.* (19.0%) being the most prevalent. The reported prevalence of helminth infection decreased over time in developing countries while it increased in the developed world. Chicken kept in back yard and free-range systems had a markedly higher pooled prevalence of helminth infection (82.6 and 84.8%, respectively) compared to those housed in cage production systems (63.6%).

The aim of the second study (Chapter 4) was to determine the prevalence and worm burdens of intestinal helminth infection in cage-free laying chickens in Australia. In an online survey of worm prevalence, a high proportion of respondents reported detection of *Ascaridia galli* (77%), followed by tapeworms (69%) and caecal worms (*Heterakis gallinarum*) (62%), whereas fewer respondents (23%) reported the presence of hair worms (*Capillaria spp.*) in their flocks. Total worm recovery from 407 laying hens on four farms found that 92.1% of hens harboured one or more helminth parasite with a prevalence of 73 to 100% across farms. Mixed infections were common with 79% of hens harbouring two or more helminth species. The prevalence of nematode species *H. gallinarum*, *A. galli* and *Capillaria spp*. was 87, 82 and 35% respectively, whereas the overall prevalence of the cestodes was 12%. The hens harboured an average of 71 worms with *H. gallinarum* having the highest mean burden (45.5 worms/hen) followed by *A. galli* (22.0 worms/hen), *Capillaria spp*. (2.7 worms/hen) and cestodes (0.8 worms/hen). When investigating intestinal excreta (n = 10) and caecal excreta (n = 10) of 16 flocks, all sampled flocks were egg count positive for ascarid infections, predominantly *A. galli* and *H. gallinarum,* respectively.

The aim of the third study (Chapter 5) was to assess and optimise laboratory and field sampling methods for routine monitoring of nematode infections in chickens by evaluating the sensitivity, accuracy, and precision of the Modified McMaster (MM) and Mini-FLOTAC (MF) methods using laying chicken excreta samples spiked with estimated true numbers of eggs (Experiment 1 = 5-1500 EPG (eggs/g); Experiment 2 = 5-500 EPG) without and with operator effects, respectively or using individual fresh excreta (n = 230) and fresh floor excreta (n = 42) from naturally infected free-range layer farms. The Coefficient of Variation was assessed within and between operators and the time spent on sample preparation and counting was also evaluated. MM was more accurate than MF, particularly at higher EPG levels, but slightly less precise and sensitive, particularly at low EPG levels, while taking less laboratory time per sample. Our observations indicate that the MM method is more appropriate for rapid diagnosis of chicken nematodes in the field. Pooled fresh floor excreta samples would be sufficient to indicate infection level in free range farms.

The aim of the fourth study (Chapter 6) was to determine ideal storage conditions for maximising the viability of *A. galli* eggs and maintaining viability for the longest

period. A 2 x 2 x 3 x 5 factorial experimental design was employed to investigate the effects of storage temperature (4˚C or 26˚C), storage condition (aerobic or anaerobic), storage medium (water, 0.1 N H_2SO_4 or 2% formalin) and storage period (4, 8, 12, 16 and 20 weeks). The viability of eggs was assessed after eggs in all treatments were held aerobically at 26˚C for 2 weeks after the storage period to test embryonation capacity. The maintenance of viability during storage at 4˚C was optimal under anaerobic conditions while at 26˚C it was optimal under aerobic conditions. Anaerobic conditions at 26˚C led to a rapid loss of viability while aerobic conditions at 4˚C had a less severe negative effect on maintenance of viability. Egg storage in 0.1 N H_2SO_4 resulted in a significantly higher viability overall (54.7%) than storage in 2% formalin (49.2%) or water (37.3%). Untreated water was the least favourable storage medium when eggs were stored at 26˚C while it was a medium of intermediate quality at 4˚C. The lowest rate of decline was seen with storage of eggs under anaerobic conditions at 4˚C or aerobic conditions at 26˚C in 0.1 N H_2SO4 with a decline rate of approximately 2% per week with no significant difference between the two. Therefore, this study has clearly revealed anaerobic conditions required for prolonged storage of *A. galli* eggs in the pre-embryonated state at 4˚C. It has also identified that 0.1 N H_2SO4 provides the best preservation against degradation during storage, particularly at 26˚C under aerobic conditions.

The aim of the final study (Chapter 7) was to compare the infectivity of *A. galli* eggs isolated from *A. galli egg* sources (worm uteri, excreta or eggs shed in vitro) under two infection regimens. A 3x2 factorial arrangement was employed to test the infectivity of *A. galli* eggs from the three sources and two modes of infection (single or trickle infection). One hundred and fifty-six Isa-Brown one day-old cockerels randomly assigned to the six treatment groups (n = 26) were orally infected with embryonated *A. galli* eggs obtained from the three *A. galli egg* sources (worm uteri, excreta or eggs shed in vitro) administered either as single dose of 300 eggs at one day-old or trickle infected with 3 doses of 100 eggs over the first week of life. Eggs obtained from cultured worms or excreta exhibited a higher embryonation capacity than eggs obtained from worm uteri. The findings showed that eggs shed by cultured worms or isolated from worm uteri had greater infective capacity than eggs harvested from excreta and that trickle rather than single infection resulted in higher worm establishment rate.

Thesis organization

This thesis is structured into 8 chapters: a general introduction, a literature review, five chapters based on published data synthesis (1) and critical experimentation (4) and a general discussion and conclusions. The thesis commences with an outline of the background, research problem, research aim, objectives and propositions in Chapter 1 (General introduction). The literature review chapter is written as stand-alone contribution in which the literature is reviewed to summarise the key aspects of helminthiasis in chicken relevant to the research work and identify areas where knowledge is lacking. The research chapters (Chapters 3-7) are written or documented as standalone contributions and structured in journal format. The general discussion and conclusions in Chapter 8 integrates the main findings of the thesis and discussed their implications. Limitations of the doctoral work and areas for future work are also identified.

Chapter 1. General introduction

1.1. Background

Poultry products have been one of the main food sources for human nutrition for thousands of years, providing meat and eggs that contain high-quality protein, essential vitamins and minerals (Farrell, 2013; Windhorst, 2017; Laca et al., 2021). The poultry industry is one of the most rapidly expanding and adapting livestock sectors. It has grown substantially in countries of all income levels over the last 15 years and continues to grow due to increasing demand (Mottet and Tempio, 2017; Yildiz, 2021). In most countries, poultry production contributes significantly to the agriculture sector generating substantial economic income (Ferdushy et al., 2016). In most parts of the world today, poultry are kept in both extensive and intensive production systems (Permin and Hansen, 1998; Ola-Fadunsin et al., 2019b). Cage-based egg production systems have, until recently, been the predominant commercial husbandry system in developed countries. Cage systems provide advantages such as efficient egg production under controlled conditions preventing the spread of infectious and parasitic diseases (Permin et al., 1999b; Kaufmann et al., 2011; Sharma et al., 2018). Specifically relevant to internal parasites, cage-based systems isolate the hen from their excreta, therefore breaking the transmission cycle of nematode parasites (Groves, 2021). In recent times, however, concerns about animal welfare, driven largely by consumer preferences and retailer commitment, have resulted in a shift towards non-caged based production, including barn and free-range production systems (Permin et al., 1999b; Berg, 2002; Kaufmann et al., 2011). The primary goals of barn and free-range production systems are to maintain laying performance and egg quality while promoting overall hen health and welfare (Tuyttens et al., 2008) despite the risk of parasite infection which appear to be re-emerging in these systems (Permin et al., 1999b; Berg, 2002; Jansson et al., 2010; Kaufmann et al., 2011b). The spread of nematode infection appears to be unavoidable in free-range and litter-based housing systems, since the bedding material and available range area enable direct contact of chickens with their excreta, which allows the parasite life cycle completion via the faecal-oral transmission route (Permin et al., 1999b; Kaufmann et al., 2011b; Wongrak et al., 2014).

Poultry are susceptible to a wide range of helminth parasites, including nematodes (roundworms), cestodes (tapeworms), and trematodes (flatworms), which are all of concern to the poultry production (Permin and Hansen, 1998; Macklin, 2013). Helminth infections are common worldwide (Thapa et al., 2015a) and are regarded as major constraints in both large commercial operations and small backyard flocks (Van et al., 2019), with more severe consequences in cage-free systems which are known for their high infection rates (Kaufmann and Gauly, 2009; Kaufmann et al., 2011b; Sherwin et al., 2013; Wongrak et al., 2014; Thapa et al., 2015a; Wuthijaree et al., 2017). In the backyard production system, parasite infection has been reported with up to 100% prevalence (Permin and Hansen, 1998). Helminthiasis can have negative impacts on poultry health and production performance by reducing feed conversion rate, decreasing weight gain or inducing weight loss, and reducing egg laying performance (Ackert and Herrick, 1928; Ikeme, 1971b; Ramadan and Znada, 1991; Kilpinen et al., 2005; Daş et al., 2011d; Sharma et al., 2018a; Permin, 2020). Furthermore, helminthiasis can increase host susceptibility to other infectious diseases and reduce the host immune response to immunization against infectious diseases (Hørning et al., 2003; Pleidrup et al., 2014; Dalgaard et al., 2015). Roundworms (nematodes) are the most important helminth parasites in poultry (Permin and Hansen, 1998; Ruff, 1999) with *Ascaridia galli, Heterakis gallinarum* and *Capillaria* species being the most prevalent (Permin et al., 1999b; Kaufmann et al., 2011b; Sherwin et al., 2013). Cestode infections are commonly encountered in poultry raised in barn, free-range, or backyard conditions. However, the majority of tapeworm species are either considered harmless or have mild pathogenicity of low economic importance (Permin and Hansen, 1998; Ruff, 1999).

Like in other high income countries, free-range egg production is becoming increasingly popular in Australia (Scott et al., 2017). This is due to the perception that products produced in less intensive systems are of higher welfare status compared to the intensive systems (Singh and Cowieson, 2013). Since 2012, the Australian retail production of free-range eggs has surpassed that of cage and barn eggs (Scott et al., 2017) with the latest percentages recorded in 2022 at 57, 31 and 11%, respectively. The free-range sector constitutes an even higher estimated grocery market value share of 62% (AEL, 2022) which is in line with the expansion of the free-range sector worldwide (Singh et al., 2017). However, unlike caged housing systems, free range

systems allow for faecal-oral pathogen cycling and exposure to other environmental conditions associated with higher health risks for hens. As a result of the industry trend shift towards cage-free husbandry systems, helminth infections have become more important in commercial poultry operations (Scott et al., 2009a; Ruhnke, 2015b; Scott et al., 2017). Given the expansion of cage-free egg production, a review of the literature reveals no recent evidence on the current status of gastrointestinal worm infection in the Australian poultry production systems. However, an old survey from several decades ago found an individual prevalence of infection with helminths of 92.7% in domestic chickens in Queensland (Broadbent, 1942). Since then, there appear to be no published epidemiological studies on the prevalence and magnitude of helminth infections in the sector. Therefore, a detailed prevalence study was conducted and is reported in chapter 4 to address this problem.

With the re-emergence of nematode infection in free-range chickens, there is growing interest in measuring nematode infections and their impact on hens housed in this system. Nematode infection levels must be diagnosed and monitored on a regular basis to ensure adequate monitoring of effective long-term nematode parasite management (Heckendorn et al., 2009). Accurate diagnosis of nematode infection is thus a critical component of parasite control. In poultry, post-mortem examination and worm enumeration allow for the accurate identification of the individually recovered parasites based on their morphology and is thus regarded as the gold standard recommended by the World Association for the Advancement of Veterinary Parasitology (WAAVP (Yazwinski et al., 2003). However, this method is not only relatively costly in terms of time, labour and sacrificed chickens, but the sacrifice of chickens to achieve this is ethically undesirable. Alternatively, gastrointestinal nematode infection can indirectly be estimated using the excreta egg count (EEC) technique, where eggs per g of excreta (EPG) are determined. The number of eggs in the excreta can then be used to estimate the actual worm burdens of the host animals. This method is non-invasive and cost effective without animal welfare concerns so would help overcome the above constraints with measuring actual worm burdens (Daş et al., 2011c; Daş and Gauly, 2014; Daş et al., 2017; Feyera et al., 2021b). However, one of the main limitation of EEC is that *A. galli* and *H. gallinarum* eggs cannot be reliably differentiated in mixed infections, whereas worms can be easily identified and counted based on location and morphology. EEC techniques, which are based on

microscopic identification and counting of the parasitic eggs in faecal specimens, are currently the most widely used methods for diagnosing intestinal parasites in both field and laboratory settings (Cringoli et al., 2004; Cringoli et al., 2021). EEC techniques are commonly used in epidemiological studies to estimate the prevalence and magnitude of gastrointestinal nematode infection in live birds (Wongrak et al., 2014; Thapa et al., 2015a; Wuthijaree et al., 2017), determining anthelmintic efficacy (Feyera et al., 2021a; Feyera et al., 2021c), and selecting parasite resistant chicken breeds (Permin and Ranvig, 2001; Gauly et al., 2001b; Schou et al., 2003). However, inherent biological factors such as excreta and egg shedding inconsistency and parasite and host factors (Michael and Bundy, 1989; Villanúa et al., 2006; Daş et al., 2011c), combined with the disparity in the accuracy, precision, and sensitivity of EEC techniques, may pose a practical concern of diagnostic technique in free-range systems (Paras et al., 2018). This necessitates the optimisation of the most practical, cost effective, accurate, sensitive, precise and rapid excreta-based diagnostic tools to facilitate the adoption of regular nematode parasite detection and monitoring in the poultry industry.

McMaster (MM) is one of the most commonly used EEC method and Mini-FLOTAC (MF) can be regarded as modifications of the MM principle. MF is a sensitive technique that has been proposed to replace MM (Das¸ et al., 2020), but it has not been thoroughly evaluated for nematode infections in chickens. Comparative studies of MF and MM techniques in several mammalian host-parasite studies indicated that the MF showed a higher sensitivity, accuracy, and precision (Godber et al., 2015; Cringoli et al., 2017; de Castro et al., 2017; Noel et al., 2017; Scare et al., 2017; Bosco et al., 2018; Paras et al., 2018; N´apravníkov´a et al., 2019; Amadesi et al., 2020). While MF has been evaluated in several mammalian farm animal species (Cringoli et al., 2017), information concerning its application for quantification of EECs in domestic or wild avian species is rare (Borrelli et al., 2015; Carrera-Játiva et al., 2018). Only two published studies (Bortoluzzi et al., 2018; Das¸ et al., 2020) compared the MF with the MM technique for detecting Eimeria oocysts and nematode eggs respectively which differ significantly in size and morphology. According to Paras et al. (2018), the accuracy of an EEC technique can vary depending on the host animal species. When infection intensities of different parasite species in the same host species are assessed, different EEC techniques can yield varying accuracies. In equine study, Nápravnková

et al. (2019) demonstrated that MM was more accurate than MF for recovering strongylid eggs, whereas MF was superior to MM for recovering ascarid eggs, implying the importance of host/parasite species-specific comparison of different EEC techniques. Therefore, a detailed comparison of MM and MF was undertaken for nematode infection in chicken and is reported in Chapter 5.

The outcomes of any *in vivo* or *in vitro* studies on chicken nematodes such as anthelmintic resistance testing or parasite propagation often depend on the ability of the parasite eggs to develop to the infective stage in the laboratory and be stored with minimal loss of infectivity until use. Fresh nematode eggs of the required numbers and nematode worms are not always readily available at the time of need. Laboratory storage methods for preserving viable eggs for an extended period of time are currently not clearly identified in the literature with many inefficient and ineffective methods cited and little standardisation of approach. Therefore, investigating conditions that optimise the embryonation capability of parasite eggs and maintain viable infective stages during laboratory storage, is of paramount importance for future research. The viability and infectivity of *A. galli* eggs under laboratory conditions mainly depends upon temperature, oxygen concentration, humidity and storage medium. Recent studies have shown that worm eggs can retain viability of up to 50 and 80% for up to 10 weeks when stored at 25˚C or 4˚C under aerobic (Tarbiat et al., 2018; Feyera et al., 2020) and anaerobic laboratory conditions (Tarbiat et al., 2018) respectively. These suggest that oxygen is an essential requirement for embryonation to occur and appears necessary to maintain optimal viability at embryonation temperatures with the right combination of storage media. More research is needed to optimise these methods and associated storage requirements, such as storage media, storage temperature, and oxygen availability, in order to maintain maximum viability of infective worm eggs for experimental purposes over a longer period of time. Therefore, a detailed factorial experiment was conducted and is reported in chapter 6 to address this issue.

Experimental infections of chickens with *A. galli* can result in low establishment rates and thus low worm burdens when compared to worm burdens observed in natural infections (Permin et al., 1997c; Marcos-Atxutegi et al., 2009; Daş et al., 2010b; Sharma et al., 2018c). There are currently not any well-defined *in vivo* experimental models for *A. galli* infection, so there is a room for improvement and standardisation.

The availability of characterised strains and clear guidelines on optimal methods of multiplication and maintenance would aid research into this parasite. The accurate detection of viable parasite stages, particularly eggs, is required for *in vivo* nematode bioassays. Artificial infection with chicken ascarids, for example, is dependent on the source of eggs, which must incubate and embryonate outside the host (Elliott, 1954). Due to the lack of an international library of parasite isolates that can serve as a source of standardised routine experimental infections, experimental studies have been conducted with eggs recovered directly from host excreta or mature worms (i.e. the worm's uteri or cultured worm into artificial media). Eggs recovered form worm uteri and worms cultured in artificial media have removed the risk of harvesting eggs of mixed nematode infections and reduced time and labour involved in the extraction of eggs compared with extraction from excreta. However, these approaches are not always feasible given the requirement of chicken necropsy to recover mature worms. Despite the aforementioned drawbacks, recovering eggs from excreta can be a more efficient method of stock multiplication than sacrifice and worm harvest (Feyera et al., 2021b). The protocols used to recover eggs from these sources can vary greatly and are mostly non-standardized, potentially resulting in substantial variation of embryonation and subsequently infectivity of embryonated eggs. Furthermore, embryonation media and temperature can affect the embryonation and infectivity of eggs from different sources and the effect may vary depending on egg sources (Permin et al., 1997b; Tiersch et al., 2013). To the best of our knowledge, no systematic study has been conducted on the comparative infective capacity of embryonated *A. galli* eggs in conjunction with various modes of infection. This issue was addressed by an experiment reported in chapter 7.

Given the research gaps on the aspects of chicken helminths discussed above, the overall goal of this doctoral thesis was to assess and optimise methods for improving the ability to investigate and manage gastrointestinal worms in commercial poultry. With this goal in mind, five specific objectives were set:

1. To provide compiled information about the status and trends of helminth infections in poultry operations worldwide
2. To assess the prevalence and magnitude of helminth infections in commercial cage-free layer chickens in Australia.

3. To evaluate and optimise EEC tools for routine monitoring of nematode infections in chickens.
4. To optimise prolonged laboratory storage methods for both undeveloped and embryonated stages of nematode eggs.
5. To evaluate the infectious capacity of *A. galli* eggs recovered from host excreta or the worm uteri or worms cultured into artificial media and subjected to different extraction procedures.

In light of these objectives, the following general propositions were tested.

i. A systematic analysis of global data on the prevalence of helminth infection in free-range chickens will indicate high prevalence and magnitude of different helminth species with a generally increasing trend over time.
ii. Cage-free production systems in Australia will have a high prevalence and magnitude of worm infection in laying chickens.
iii. The Mini-FLOTAC method would outperform the McMaster method for recovering chicken nematode eggs from egg spiked chicken excreta and field samples.
iv. Storage at 4˚C must be anaerobic to prolong survival in the unembryonated state and storage at 26˚C must be aerobic to prolong survival in the embryonated state but bacterial degradation of eggs must be prevented with a preservative.
v. Eggs isolated from excreta, worm uteri or worms cultured in artificial media may differ in their embryonation capacity as well as infectivity.

Chapter 2. Literature review

2.1. Egg production and production systems

2.1.1. The global egg industry

Poultry is the fastest growing agricultural sub-sector, as the global demand for eggs and meat rises due to an increasing population, higher incomes, and urbanisation (Mottet and Tempio, 2017; Yildiz, 2021). The global poultry market value was estimated USD 318.58 billion in 2021 and is expected to be worth USD 493.21 billion by 2026 at a compound annual growth rate of 8.9% (Yildiz, 2021). The poultry egg industry makes a significant contribution to the global protein supply (Mottet and Tempio, 2017; Windhorst, 2017; Nkukwana, 2018; Laca et al., 2021; Salami et al., 2022), with an annual production of over 93 million tonnes of egg (FAO, 2020; Yildiz, 2021). The global demand for eggs is expected to rise by 39% between 2005 and 2030 (MacLeod et al., 2013). Globally, 51.1 millions of tons of eggs were produced in 2005, and 76.7 millions of tons were produced in 2018, representing a 50.1% increase (FAO, 2020). Currently, China is the world's largest egg producer, which increased output by 67.8% between 1995 and 2005 (Clark, 2007) producing 466 billion eggs in 2018, accounting for 34% of the global market (Laca et al., 2021). Regarding the egg production system, traditional battery hen cages remain prevalent in the global market, despite the fact that the movement towards greener methods is gaining traction (da Silva Pires et al., 2021). This movement in favour of bettering animal welfare coincides with consumer market awareness, which demands that food be produced ethically and in accordance with good management practises.

2.1.2. The Australian egg industry

The Australian egg industry is characterised by intensive, modern and highly efficient production systems (Wiedemann and McGahan, 2011). In terms of management practises, housing characteristics, and range features, the Australian commercial poultry industry is comparable to that of other developed countries (Scott et al., 2017). In Australia, there are three main egg farming systems: free range, barn-laid, and cage

housing (Scott et al., 2009). In the 2021-22 financial year, Australian egg farmers produced 6.6 billion eggs from 21.9 million layer hens. Of these, the distribution of egg production between states was 35% in New South Wales, 30% in Queensland, 20% in Victoria, 5% in South Australia, 3% in Tasmania and 8% in Western Australia, and state flock percentages of 36, 31, 21, 5, 1 and 7% respectively was reported (AEL, 2022). Free-range egg production has grown significantly over the last 15 years and accounted for 57 and 62% of all grocery retail sales volume and value respectively. However, cage and barn-laid eggs remain in high demand as a low-cost source of high-quality protein (AEL, 2022). Mimicking the global situation, the increased public demand is also considered the driving force for the growth of poultry products produced in less confined systems (Berg, 2002; Singh et al., 2017). Free-range laying hen housing systems provide more opportunities for the expression of natural behaviour, because of higher environmental choices and exercise levels, resulting in potentially better hen welfare than controlled indoor systems (Campbell et al., 2020). Despite the potential welfare benefits, free-range systems bear higher risks of exposing hens to infectious diseases such as parasitic infections in comparison to cage housing systems (Berg, 2002; Singh et al., 2017; Campbell et al., 2020; Groves, 2021).

2.2. Poultry helminthiasis

Helminths are divided into two types: roundworms (nematodes) and platyhelminths/flatworms (cestodes and trematodes), which are a major impediment to socioeconomic development (Consortium, 2019). Helminths are common gastrointestinal parasites of poultry that have a negative impact on the animal's health, well-being, and production. Chickens are naturally infected with a wide range of parasitic helminths through oral ingestion of infective helminth stages. Gastrointestinal nematodes are the most numerous and economically significant helminths in poultry. Helminths primarily infect the digestive tract, but some have also been found in the lung, trachea, and eye (Soulsby, 1982; Permin and Hansen, 1998; Ruff, 1999; Macklin, 2013).

2.2.1. Gastrointestinal nematodes

Nematodes are members of the phylum Nemathelminths and the class Nematoda. Nematodes are the most common and important helminth species in poultry considering the number of pathogenic species, their health impact of infection on the host, and their economic importance (Ruff, 1999; Yazwinski and Tucker, 2008). More than 50 species have been described in poultry, with the majority of these causing pathological damage to the host (Permin and Hansen, 1998; McDougald, 2020). The morphological appearance of poultry nematodes includes unsegmented, elongated, and usually cylindrical body while the latter can vary greatly between nematode species. Nematode worms have an alimentary tract and the sexes are distinct. The life cycle can be direct or indirect, with or without an intermediate host (Soulsby, 1982). The genera Ascaridia (large roundworms), Heterakis (caecal worms), and Capillaria (hairworms or threadworms) are the most common nematode parasites encountered in poultry production (Soulsby, 1982; Permin and Hansen, 1998; Yazwinski and Tucker, 2008; McDougald, 2020).

2.2.1.1. *Ascarida galli*

A. galli is found worldwide in galliform birds of all ages, but is most common in birds under 12 weeks of age (Herrick, 1926; Ackert and Herrick, 1928; Ackert et al., 1935a). *A. galli* is a member of the superfamily Ascaroidea, which is part of the phylum Nematoda. The adult worms reside in the lumen of the host's intestine (Soulsby, 1982). The body of an adult *A. galli* is semitransparent, cylindrical and has a creamy-white colour with distinct sexual dimorphism (Ramadan and Abouznada, 1992). Females are longer than males, measuring 72-116 mm and having a straight posterior terminal, whereas males measure 51-76 mm and have a curved posterior terminal (Permin and Hansen, 1998). Ascaridia have a direct life cycle which includes two principal populations: the sexually active adult worms in the gastrointestinal tract of the host and a resistant free-living stage (eggs) in the environment. Sexually mature female adult worms lay eggs in the lumen of the intestine, and the eggs are excreted into the environment, where they serve as a source of infection for new hosts (Ackert, 1931; Permin and Hansen, 1998; Permin et al., 1998a). When being kept at ideal temperature (25-30˚C) and humidity (> 85%), eggs develop into their embryonated stage (L3) within 10-20 days (Ackert, 1931; Tugwell and Ackert, 1952; Reid, 1960). Infection occurs via the faecal-oral route when the host consumes infective parasite eggs (L3;

embryonated eggs) from the external environment (Permin and Hansen, 1998; Permin et al., 1998a; Jansson et al., 2010; Kaufmann et al., 2011b; Andersen et al., 2013; Wongrak et al., 2015a-a; Thapa et al., 2017a). After ingesting embryonated eggs, L3 larvae hatch within 24 hours in the jejunum and duodenum of the host's small intestine, then embed into the mucosa layer to initiate the tissue-associated (histotrophic) phase (Ackert, 1923; Ramadan and Abouznada, 1992). The duration of the histotrophic phase can range between 3 and 54 days depending on a variety of factors such as infection dose, host age or immunity, viability and virulence of infective eggs (Todd et al., 1952; Tugwell and Ackert, 1952; Ikeme, 1971a; Herd and McNaught, 1975; Permin and Hansen, 1998; Luna-Olivares et al., 2012). Following the histotrophic phase, the larvae (L4) return to the intestinal lumen and reach sexual maturity. The mature worms in the intestine lumen produce eggs, which are excreted into the external environment, where the life cycle continues. The life cycle is completed when a new host ingests infective parasite eggs (Ramadan and Abouznada, 1992; Tarbiat et al., 2015). The average pre-patent period for *A. galli* is 5-8 weeks after host infection (Ackert, 1931; Kerr, 1955; Ikeme, 1971a; Permin and Hansen, 1998).

The pathogenicity of *A. galli* depends upon the interaction of various host and parasite factors. The majority of pathological lesions are caused by larvae penetrating and migrating into the intestinal mucosa, resulting in adhesion and decreased length of intestinal villi due to proliferation of mucus-secreting cells (Ikeme, 1971c; Luna-Olivares et al., 2015). It was also reported that heavy infections with adult *A. galli* worms caused intestinal obstruction (Ikeme, 1971c; Ramadan and Znada, 1991; Torres et al., 2019). Worms can also cause enteritis by dilatation of the intestine and the formation of nodules, with the most visible lesions being haemorrhagic enteritis, extensive destruction of the glandular epithelium, and rare mucosal necrosis (Permin and Hansen, 1998; McDougald, 2020). Mortality may occur as a result of intestinal wall damage and blood loss caused by migrating larvae, which may be accompanied by secondary infection (Guberlet, 1924; Ackert and Herrick, 1928). Chickens harbouring large number of *A. galli* worms may show nonspecific signs such as loss of body weight and appetite, ruffled feathers, drooped wings, retarded muscular and osteological development, altered hormone levels, and depression (Ackert and Herrick, 1928; Ramadan and Znada, 1991; Gauly et al., 2007).

Weight loss in chickens has been found to be negatively related to an increase in worm burden indicating that *A. galli* infection can have a negative impact on chickens' performance (Ackert and Herrick, 1928; Reid and Carmon, 1958; Brewer and Edgar, 1971; Gauly et al., 2001a; Feyera et al., 2021b). According to these authors, *A. galli* infection can reduce weight gain in chickens and complications with secondary bacterial infections such as *Escherichia coli* (Permin et al., 2006) or *Pasteurella multocida* (Dahl et al., 2002) may greatly exacerbate this effect. Furthermore, the pathogenic effects of this parasite are associated with reduced nutrient absorption and utilisation and impaired growth and laying performance (Das¸ et al., 2011, 2012; Sharma et al., 2019). Infection with *A. galli* has also been associated with reductions in egg quantity and quality traits (Ikeme, 1971b; Stehr et al., 2019a; Tarbiat et al., 2020) which could be worsened by secondary bacterial infections (Dahl et al., 2002; Permin et al., 2006). Consequences of infection, especially the level of mortality have been shown to be associated with infection level (Hinrichsen et al., 2016). By contrast, some other studies reported that *A. galli* infection did not have effect on egg production and egg quality traits (Ackert and Herrick, 1928; Gauly et al., 2007; Sharma et al., 2018a; Sharma et al., 2018b). This variation is most likely due to differences in study design, infection dose, strain and age of experimental bird, and study population size.

2.2.1.2. Heterakis gallinarum

Heterakis gallinarum is a nematode parasite residing in the caeca of several avian species. The ring-necked pheasant is the most commonly observed host for *H. gallinarum*, followed by guinea fowl and chickens (Lund and Chute, 1972). The mature worms have a white appearance and are barely visible when caeca are opened during necropsy. Males measure 7–13 mm in length and have a straight tail with dissimilar spicules, whereas females measure 10–15 mm in length and have a long, narrow, and pointed tail (Permin and Hansen, 1998; Yazwinski and Tucker, 2008; McDougald, 2020). This nematode is a common parasite of chickens kept in free range systems (Yazwinski and Tucker, 2008; McDougald, 2020), with a prevalence ranging from 70 to 99% (Permin et al., 1999b; Kaufmann et al., 2011a; Kaufmann et al., 2011b; Wongrak et al., 2014; Thapa et al., 2015; Wongrak et al., 2015a; Wuthijaree et al., 2017).

H. gallinarum has a direct life cycle that does not require an intermediate host to complete development, and poultry raised at high density on litter are most vulnerable to this nematode infection (Cupo and Beckstead, 2019). Adult caecal worms in the host's caeca produce non-embryonated eggs, which are then excreted by the host. Under ideal environmental conditions, they develop into infective eggs after about 2 weeks. The optimal temperature for egg development is 17-20°C with a constant supply of oxygen with a relative humidity of > 85% (Saunders et al., 2000). While *H. gallinarum* has a direct life cycle, earthworms and houseflies can promote mechanical transport between hosts. After infective eggs are ingested by a susceptible host, the infective larvae hatch in the small intestine and migrate through the lumen of the intestine into the caeca within 24 hrs where they further develop into adult worms. Most of the adult worms are found in the blind ends of the caeca during necropsy. The prepatent period of *H. gallinarum* varies between 24 to 30 days (Permin and Hansen, 1998; Yazwinski and Tucker, 2008).

H. gallinarum is less pathogenic and rarely causes clinical disease in mono-infection (McDougald, 2020). However, in cases of severe infection, caecal wall inflammation and thickening, as well as nodule formation on the mucosa and submucosa and hepatic granulomas, have been observed (Kaushik and Deorani, 1969; Riddell and Gajadhar, 1988). However, the degree of damage caused by *H. gallinarum* may be exacerbated by *Histomonas meleagridis* coinfection (Tyzzer, 1934). This nematode parasite's economic significance stems from its role as a vector for the transmission of *H. meleagridis*, the causative agent of histomoniasis (blackhead disease) (Permin and Hansen, 1998; Yazwinski and Tucker, 2008; Hauck and Hafez, 2013; McDougald, 2020). The caecal worm, *H. gallinarum* is a primary reservoir of the *H. meleagridis*, which is transmitted via Heterakis eggs with varying efficiency (Hauck and Hafez, 2013). The protozoans invade the digestive tracts of the adult nematodes and travel to the reproductive tract where they become encapsulated in the egg (Gibbs, 1962; Ruff et al., 1970). Dormant caecal worm eggs can survive in the environment for three years while harbouring infective Histomonas (Farr, 1961). The presence of *H. gallinarum* is required for transmission of *H. meleagridis* within or between chicken flocks (McDougald, 2005). In contrast to turkeys, chickens do not experience direct transmission of *H. meleagridis* via the phenomenon of cloacal drinking (Hu et al., 2004). Infection with *H. meleagridis* causes morbidity in chickens, with a 10%

mortality rate. The symptoms are more severe in turkeys, with up to 100% mortality (McDougald, 2005; Hauck and Hafez, 2013).

2.2.1.3. *Capillaria*

Capillaria are also referred to as hairworms, or threadworms. Six important *Capillaria* spp. are thought to be found in poultry: *C. contorta, C. obsignata, C. anatis, C. annulata, C. coundinflata, and C. bursata* (Soulsby, 1982; Permin and Hansen, 1998). Predilection sites differ between species and are located throughout the gastrointestinal tract. *C. annulata* and *C. contorta* live in the crop and oesophagus, respectively, whereas *C. obsignata*, *C. coundinflata*, and *C. bursata* live in the small intestine and *C. anatis* lives in the caeca. Infection is most common in gallinaceous birds, which are found all over the world (Soulsby, 1982; Permin and Hansen, 1998; De Rosa and Shivaprasad, 1999). Birds kept in free-range and deep-litter systems are susceptible to capillaria infection. For example, Kaufmann et al. (2011b) reported 75.3% prevalence of *capillaria spp.* in free-range hens. *Capillaria spp.* can transmit via direct or indirect life cycle. The life cycle of *C. contorta, C. obsignata, and C. anatis* are direct, whereas *C. annulata, C. coundinflata, and C. bursata* have indirect life cycles that use earth worms, snails and other insect vectors as intermediate host (Rickard and Pohl, 1969; Permin and Hansen, 1998). Depending on the temperature, larval development in the egg takes 8–15 days and worms mature in 20–26 days after being consumed by the final host (Macklin, 2013).

Capillaria parasites, particularly a large number of *C. obsignata*, can cause haemorrhagic enteritis and thickening of the intestinal walls, resulting in poor feed absorption, as well as nonspecific clinical signs such as inactivity, depression, emaciation, diarrhoea, retarded growth, and even death (Levine, 1938b; Wakelin, 1965; De Rosa and Shivaprasad, 1999; Yazwinski and Tucker, 2008). *C. annulata* and *C. contorta* have also been linked to severe crop and oesophageal inflammation (Rickard and Pohl, 1969; De Rosa and Shivaprasad, 1999).

2.2.2. Cestodes (tapeworms)

Cestodes belong to the phylum Platyhelminthes and the class Cestoda. Cestodes are characterised by the absence of a digestive tract and hermaphroditic worms with flattened, ribbon-like segments that obtain nutrients from the host's gut contents (Yazwinski and Tucker, 2008; McDougald, 2020). Every cestode has a scolex (the head), a neck, and a body (strobila). The strobila is made up of segments known as proglottids. The number of segments varies between species, and cestodes can grow to be 30-50 cm long. The segments furthest away from the neck develop and separate from the body. Each segment/proglottid has one or more sets of reproductive organs that can become crowded with an egg mass as it matures into a gravid proglottid. These gravid segments contain numerous eggs which are released to the environment with the excreta (Permin and Hansen, 1998; McDougald, 2020).

Cestodes are commonly found in free-range or backyard flocks of poultry because all tapeworms have indirect life cycles and require intermediate hosts such as earthworms, beetles, flies, ants, snails, or grasshoppers depending on the species of tapeworms. These parasites are more common during the warmer seasons, when intermediate hosts are abundant. Tapeworms are acquired by birds through the ingestion of intermediate hosts, with a broader range of such hosts available in free-range production systems (Permin and Hansen, 1998; McDougald, 2020). Many tapeworm species were considered of rare occurrence in intensive poultry rearing regions because caged birds do not come into contact with intermediate hosts. Because intermediate hosts are required to sustain the life cycle, infections are also uncommon in indoor systems (Permin and Hansen, 1998; McDougald, 2020).

More than 1,400 tapeworm species have been described in domesticated poultry and wild birds. Most commonly diagnosed cestodes in the intestine of poultry include; *Raillietina* species (*R. echinobothrida*, *R. tetragona* and *R. cesticillus*), *Hymenolepis* species (*H. cantaniana* and *H. carioca*), *Davainea proglottina*, *Choanotaenia infundibulum* and *Amoebotaenia cuneate* (Permin and Hansen, 1998; Yazwinski and Tucker, 2008; McDougald, 2020). Most species of cestodes are either harmless or mildly pathogenic (Permin and Hansen, 1998; Ruff, 1999). However, few can cause moderate to severe infection in the host. For example, it was reported that the microscopic tapeworms *D. proglottina* and *R. tetragona* reduced weight gain and egg production following experimental infections (Levine, 1938a; Nadakal and

Vijayakumaran Nair, 1980). In severe cases, *D. proglottina* can cause haemorrhagic enteritis which can lead to death in young birds. Clinical signs generally include emaciation, dull plumage, slow movements, breathing difficulties, thickened mucosal membranes that produce haemorrhage, leg weakness, and paralysis (McDougald, 2020). *R. echinobothrida* is usually listed as one of the most pathogenic tapeworms because its presence has often been associated with nodular disease of chickens. Nadakal et al. (1973) reported parasitic granulomas approximately 1–6 mm in diameter at the sites of worm attachment 6 months after experimental infection with 200 cysticercoids. Catarrhal hyperplastic enteritis, as well as lymphocytic, polymorphonuclear, and eosinophilic infiltration, were all associated with this condition.

2.2.3. Epidemiology and risk factors of helminth infection in chickens

The prevalence of helminth infection in poultry appears to be significantly reduced in commercial cage poultry production systems due to improved management, housing conditions, biosecurity, and hygiene (Permin and Hansen, 1998; Permin et al., 1999). Despite the fact that studies on the prevalence of helminth infections in chickens have been published from several countries, we failed to find compiled global prevalence data to date. Prevalence report from caged layer flocks and broiler chickens are uncommon, most likely due to lower likelihood of helminth occurrence in hens housed in caged systems and broiler chickens with a short life span of around 35-42 days, which is often not much longer than the pre-patent period of the main helminth parasites (Permin et al., 1999b; Ruff, 1999). In contrast, helminth infections continue to be a major concern in cage-free production systems (Permin and Hansen, 1998; Permin et al., 1999b). Changes in consumer demand in several countries have resurrected alternative production systems in which animals frequently have free access to bedding and outdoor areas, resulting in helminth infections (Permin et al., 1999b). Thus, the prevalence of helminth infection in commercial free-range laying chickens has been reported to be as high as 50-100% (Permin et al., 1999b; Kaufmann and Gauly, 2009; Jansson et al., 2010; Kaufmann et al., 2011a; Kaufmann et al., 2011b; Sherwin et al., 2013; Wongrak et al., 2014; Thapa et al., 2015a; Wuthijaree et al., 2017). Similarly, in backyard chickens, helminth parasites are widely distributed

throughout the world, with up to 100% prevalence has been reported in several studies (Permin et al., 1997c; Magwisha et al., 2002; Permin et al., 2002; Rabbi et al., 2006; Mukaratirwa and Hove, 2009; Jinga et al., 2012; Ekpo et al., 2013; Ilyes et al., 2013; Ben Slimane, 2016; da Silva et al., 2016). The most commonly reported helminth parasites in poultry are *A. galli, H. gallinarum, and Capillaria spp* which are of leading importance in terms of prevalence, and economic impact and welfare consequences (Kaufmann et al., 2011b; Wongrak et al., 2014; Wuthijaree et al., 2017). Multiple helminth infections are also common in free-range husbandry systems, implying that the conditions under this system are favourable for the simultaneous development of different helminth species (McDougald, 2020).

Several factors influence the prevalence and severity of helminth infections, including the distribution of intermediate hosts and the number of infective parasite eggs in the environment. Host factors such as the bird's breed, age, and immune status can all influence helminth infections (section 2.4.3) (Magwisha et al., 2002; Abdelqader et al., 2008; Ben Slimane, 2016). Furthermore, the free-ranging management practices and climatic conditions (temperature and humidity) may alter the population dynamics of the parasites, resulting in substantial changes in the prevalence and intensity of helminth infections (Magwisha et al., 2002; Kaufmann et al., 2011b; Wuthijaree et al., 2017). This is because free-range scavenging chickens are in direct contact with parasite vectors, soil and excreta that allow helminth parasite to complete their life cycle (Abdelqader et al., 2008). Moreover, in this system, infective parasite stages such as eggs are difficult to remove and have a high risk of accumulation over time, acting as a continuing source of infection to newly placed birds (Thapa et al., 2017a). These factors may explain the wide range and distribution of nematode infections in poultry (Pandy et al., 1992; Permin et al., 1997c). While helminth infections with an indirect life cycle (e.g. cestodes) were common during the wet season due to the abundance of intermediate hosts such as insects and snails that maintain the helminth life cycle, helminths with a direct life cycle, such as *A. galli* and *H. gallinarum*, did not show a direct impact of seasonal variation on disease prevalence (Permin et al., 1997c; Kaufmann et al., 2011b). The epidemiology of these parasite infections depend upon the survivability of their eggs in the external environment. For example, *A. galli* eggs can survive and remain infective in the external environment (in the soil or litter) for at least a year due to their resistant egg shell that provides a high degree of protection

to the developing enclosed embryo or larva from adverse environmental factors (Ackert, 1931; Permin and Hansen, 1998; Katakam et al., 2014; Thapa et al., 2015b; Thapa et al., 2017a). Ackert (1931) and Christenson et al. (1942) described that *A. galli* eggs are protected by three important envelopes: the vitelline membrane which is the inner most highly permeable part, the outer thick resistant shell and a thin, albuminous layer (covering the outer shell). These layers (egg shells) are critical to its resistance to desiccation and long-term survival in the environment (Ackert, 1931).

2.3. Diagnosis of helminth infection in poultry

Accurate diagnosis of helminth infection in poultry is critical for implementing effective treatment and control strategies (Permin and Hansen, 1998; Macklin, 2013). A variety of methods have been used to detect adult helminth parasites, their eggs, and intermediate stages. Helminth parasites are commonly detected in chicken populations through post-mortem examination of the chicken's gastrointestinal tract or microscopic examination of excreta (Permin and Hansen, 1998). Clinical examinations of birds can occasionally reveal helminth infection, but due to the non-specificity of the clinical signs, their use for diagnosis is limited (Permin and Hansen, 1998). For research purposes, immunological and molecular methods can be used for detecting helminth infection in poultry (Macklin, 2013).

2.3.1. Post-mortem examination

Post-mortem examination (necropsy), which is well-known for its accurate and reliable species identification, is a widely used diagnostic method (Wilson et al., 1994; Yazwinski et al., 2003; Macklin, 2013). However, it may not always be the most economically viable method for routine diagnosis when sacrificing birds only for this purpose. This technique requires the complete inspection of the intestinal mucosa and contents from the oesophagus to the cloaca, including the caeca where recovered parasites can be isolated and identified by their key morphological characteristics and counted to determine the prevalence and intensity of infection (Wilson et al., 1994; Permin and Hansen, 1998; Yazwinski et al., 2003; Silver, 2011; Yazwinski et al., 2013).

2.3.2. Copromicroscopic techniques

Even in the era of genomics, metagenomics, proteomics, and bioinformatics, the diagnosis of gastro-intestinal nematodes still relies predominantly on coproscopy (i.e. microscopic examination of faeces) (Demeler et al., 2013). Copromicroscopic techniques are one of the most commonly used diagnostic methods in veterinary parasitology (Cringoli et al., 2004). It usually involves the concentration of parasitic eggs by either sedimentation or flotation in order to separate helminth eggs from excreta material. Flotation methods involve separating eggs from excreta debris using a variety of flotation solutions with specific gravities floating worm eggs to the surface of the suspension (Cringoli et al., 2004; Pereckienė et al., 2007; Ballweber et al., 2014; Lester and Matthews, 2014). Faecal sedimentation techniques with a liquid soap-in-water solution are the method of choice for the detection of fluke eggs (trematodes) in birds that do not float in commonly used flotation media (Grenier and Ritchie, 1996). Copromicroscopic diagnosis of helminth infections can be either qualitative to determine the presence or absence of helminth eggs or quantitative to estimate number of eggs per gram of excreta (EPG), the so called excreta egg counts (EECs) (Cringoli et al., 2004; Silver, 2011). Classifying a host qualitatively as infected or uninfected based on presence of eggs in a faecal sample is of crucial importance for diagnosis purpose. However, it provides only limited information as two different hosts either with only a fecund female worm or another one with thousands of worms are classified the same (Daş et al., 2020).

The principle of EEC remains a cornerstone in veterinary parasitology and this approach based on flotation principle remains a popular tool in avian parasitology (Permin and Hansen, 1998). EEC techniques which determine parasite EPG of excreta and use flotation are based on the microscopic examination of an aliquot of excreta suspension from a known volume of an excreta sample (Nicholls and Obendorf, 1994; Cringoli et al., 2004). The use of the flotation principle to detect nematode eggs in poultry excreta is a reliable method for confirming the presence of gastrointestinal nematodes (Macklin, 2013). Quantitative EEC techniques are useful tools to indirectly estimate worm burdens of a host animal as a positive relationships between worm

burdens and EEC have been demonstrated in mono-infections (Train and Hansen, 1968; Permin and Ranvig, 2001; Yazwinski et al., 2009; Silver, 2011; Daş et al., 2011c; Daş and Gauly, 2014; Thapa et al., 2015a; Daş et al., 2017). EEC techniques are generally used to estimate the prevalence and the magnitude of gastrointestinal nematode infection in live birds for epidemiological studies (Sherwin et al., 2013; Wongrak et al., 2014; Thapa et al., 2015a; Wuthijaree et al., 2017), selecting parasite resistant chicken breeds (Permin and Ranvig, 2001; Gauly et al., 2001a; Gauly et al., 2001b; Schou et al., 2003; Kaufmann et al., 2011a; Wongrak et al., 2015a-a), and for determining anthelmintic efficacy (Tarbiat et al., 2016; Acorda et al., 2019; Feyera et al., 2021a). Taken together, all of these are proposed for the control of gastro-intestinal infections in chickens (Gauly et al., 2001b; Tarbiat et al., 2017) and the use of EEC technique as parasite monitoring tools is becoming more popular, highlighting the importance of the egg count test.

Parasite egg counting techniques were described over 10 decades ago to recover and count parasite eggs in faecal samples using principle of egg flotation (Bass, 1909), and faecal egg counting remains essential in parasitology research and in clinical practice today (Nielsen, 2021). Traditional flotation techniques are classified into two types: test tubes and cover slips approach or, the counting chambers. The test tube and cover slip approach to faecal egg counting was first described in 1928, outlining a technique based on centrifugation of faecal matter suspended in flotation medium contained in centrifuge tubes with glass cover slips on top (Lane, 1928). This method was later adapted for counting *Haemonchus contortus* eggs in sheep faeces, and it is now known as the Stoll technique (Stoll, 1930). The widely used Wisconsin (Cox and Todd, 1962) and Cornell-Wisconsin (Egwang and Slocombe, 1982) methods are the modifications of the Stoll technique. A classic technique using an egg counting chamber is the McMaster (MM) technique, which was first described in the 1930s (Gordon and Whitlock, 1939), and is widely regarded as an industry standard today. Multiple modifications have been developed and several counting chamber based techniques such as the Moredun method (Jackson, 1974), FECPAK (Presland et al., 2005), FLOTAC (Cringoli, 2006; Cringoli et al., 2010) and Mini-FLOTAC (MF) (Cringoli et al., 2013; Cringoli et al., 2017) can be regarded as modifications of the MM principle. Given the recent influx of novel semi-automated and automated egg counting systems (Slusarewicz et al., 2016; Scare et al., 2017; Sukas et al., 2019; Cain et al., 2020;

Elghryani et al., 2020; Inácio et al., 2020; Nagamori et al., 2020; Cringoli et al., 2021), the existing systems such as MM and FLOTAC techniques, as well as MF and Fill-FLOTAC are widely used egg counting techniques in veterinary parasitology and need to be refined and tested in different host parasite species. Techniques to detect chicken nematode eggs in excreta are largely an extrapolation of methods used in other livestock species.

The MM technique, developed and improved at the McMaster laboratory of the University of Sydney (Gordon and Whitlock, 1939; Whitlock, 1948), is the most universally used quali-quantitative egg counting to enumerate parasite eggs in excreta samples (Rossanigo and Gruner, 1991; Nicholls and Obendorf, 1994; Rinaldi et al., 2007). Several modifications of MM method have been published for decades and their application have been described in the previous studies (MAFF, 1986; Coles et al., 1992; Nicholls and Obendorf, 1994; Ward et al., 1997; Roepstorff and Nansen, 1998; Pereckienė et al., 2007; Vadlejch et al., 2011; Ballweber et al., 2014). Given several modifications of MM method, most teaching and research parasitological laboratories use their own modifications to existing protocols to determine which method is best suited for the purpose at hand (Pereckienė et al., 2007). Most of these modifications used different flotation solution, sample dilutions, weight of excreta examined, slide areas or volume, number of chambers, floatation time, counting procedures and addition of a centrifugation step to achieve varying analytic sensitivities (Whitlock, 1948; MAFF, 1977; Dunn and Keymer, 1986; Cringoli et al., 2004; Pereckienė et al., 2007; Vadlejch et al., 2011). In assessing egg count, these modifications differ not only in diagnostic performance but also in technical performance (e.g., ease of use, cost, safety, timing, etc.) (Cringoli et al., 2004; Pereckienė et al., 2007).

In spite of its popularity in parasitology, the MM method beset with a number of technical limitations. MM lacks sensitivity and precision particularly at low egg counts (Mes, 2003; Daş et al., 2020). The variability in egg counts that not associated with laboratory error derived from the MM is entirely predictable from statistical theory. This variability is inevitable and arises from the random distribution of eggs within a well-mixed faecal suspension which conforms to a Poisson distribution (Torgerson et al., 2012; Torgerson et al., 2014). Further mixing of the suspension will not alleviate the issue. However, increasing the number of replicates of a single sample will result

in decreased variation. Alternatively, using a technique which does not have a high dilution factor or lowering the dilution factor of the MM method would reduce both variability as well as the associated confidence limits (Torgerson et al., 2012; Torgerson et al., 2014). Examining large amounts of excreta suspension (i.e. 1-1.5 ml) can also improve the diagnostic performance of MM method (Cringoli et al., 2004; Nápravníková et al., 2019).

FLOTAC and Mini-FLOTAC are two other chamber-based egg counting techniques that use a modified MM principle. The FLOTAC technique is a sensitive, accurate, and precise quali-quantitative analysis of protozoan and helminth infections in humans and animals (Cringoli et al., 2010; Cringoli et al., 2017). However, it requires centrifugation, and hence might be out of reach in resource-constrained settings. To overcome this bottleneck, a simplified device, the MF was developed in 2013 as a novel direct method for the diagnosis of intestinal parasitic infections (Barda et al., 2013; Cringoli et al., 2013; Cringoli et al., 2017). MF is a promising diagnostic tool with high sensitivity and precision useful in resource limited settings where intestinal parasitic infections are widespread (Cringoli et al., 2013; Cringoli et al., 2017). MF procedure does not require any centrifugation step or expensive equipment. However, the MF, like all other FEC techniques, necessitates specialised personnel and takes time, particularly when a large number of specimens are examined, as in large epidemiological surveys (Cringoli et al., 2013; Cringoli et al., 2017; Daş et al., 2020). MF is recommended to be used in combination with Fill-FLOTAC allows the collection, weighing, homogenization, filtration, and filling of the apparatus of faecal samples in the Fill-FLOTAC container for subsequent microscopic examination (Cringoli et al., 2013; Cringoli et al., 2017). The sensitivity of FLOTAC is 1 egg/gram (EPG), whereas the respective sensitivity of Mini-FLOTAC is 5 EPG. This is explained by a 5-fold-lower sample volume in the Mini-FLOTAC chambers (2 ml of excreta suspension) as compared with that of FLOTAC (10 ml excreta suspension).

2.3.2.1. Diagnostic performance of egg counting techniques

The usefulness of any EEC technique is influenced by the sensitivity, accuracy and precision of the method. Analytical sensitivity is defined as the lowest number of parasite elements (e.g. eggs or oocytes) that can be estimated or measured using a

given excreta egg counting technique. Sensitivity thus only provides qualitative information for the presence or absence of parasite egg in host excreta samples. Diagnostic sensitivity is a commonly used qualitative parameter describing the performance of diagnostic tests, but it only has implications at low egg count levels (Nielsen, 2021). The diagnostic sensitivity of the technique cannot predict quantitative performance whereas accuracy and precision can affect diagnostic sensitivity at low egg count levels (Cain et al., 2020; Nielsen, 2021). Thus, accuracy and precision are highly relevant for describing the performance of diagnostic tests (Paras et al., 2018; Daş et al., 2020; Nielsen, 2021). The term accuracy indicates how close a measured egg count value is to the true value providing quantitative information on the magnitude of parasite infection. Accuracy is also determined by the proportion of eggs lost during sample processing and flotation (Nielsen, 2021). Precision is a measure of the variation between repeated test results or counts between sample replicates, operators, laboratories, time, etc and is generally presented in terms of a coefficient of variation (standard deviation/mean) for various known sources of variation. The sources of egg count variation can be classified as biological or technical. The distribution of eggs within faecal matter and variation within and between samples taken from the same animal are examples of biological sources (Yu et al., 1998; Torgerson et al., 2012; Torgerson et al., 2014). Another biological source of variation is density-dependent fecundity, which appears to be caused by female worms suppressing each other's egg production via unknown mechanisms (Walker et al., 2009). Technical sources include loss of eggs during filtration and flotation, mixing and suspending samples, the flotation capacity of eggs present, and the training and experience of the analyst reading the sample (Cain et al., 2020; Cain et al., 2021; Nielsen, 2021). Furthermore, precision estimates will vary greatly depending on which hierarchical levels of variation (sample, subsample and slurry) that are considered in the study design (Cain et al., 2020).

The most commonly used EEC method validation approach in veterinary parasitology is samples spiked with known quantities of parasite eggs (de Castro et al., 2017; Noel et al., 2017; Bosco et al., 2018; Paras et al., 2018; Amadesi et al., 2020; Daş et al., 2020). Spiking a known number of parasite eggs within an egg-free excreta sample may represent a gold standard for evaluating true accuracy, but this method is not always superior to using samples from naturally infected animals. The difficulty in

determining accuracy stems from the fact that the true egg count is never known because samples spiked with known quantities of ova are plagued by a number of potential issues. The source of the eggs and the method used to obtain them are obviously critical. Egg isolation from excreta/faces involves a series of sieving and washing procedures followed by flotation-centrifugation procedures to concentrate eggs (Bosco et al., 2018; Paras et al., 2018; Das¸ et al., 2020), which can cause damage and reduce recovery rates in flotation-based systems. Furthermore, spiked eggs may not be fully distributed and integrated within the faecal matter. Spiked samples, as a whole, serve as a simulation of naturally infected samples, but they cannot completely replicate either the natural distribution of eggs throughout faecal material or their incorporation into the faecal matrix (Cain et al., 2020; Daş et al., 2020; Nielsen, 2021). The accuracy estimates are not reproducible across egg spiked studies using the same methods, EPG levels, and parasite egg type, demonstrating that this method may not be a superior alternative study design (Noel et al., 2017; Scare et al., 2017; Bosco et al., 2018; Napravnikova et al., 2019). Furthermore, comparative accuracy of MM and MF reported across different host parasites studies (Noel et al., 2017; Scare et al., 2017; Bortoluzzi et al., 2018; Bosco et al., 2018; Paras et al., 2018; Nápravníková et al., 2019; Amadesi et al., 2020; Daş et al., 2020), the mean cumulative and range accuracy of all of these studies and EPG levels for MM and MF are 48.5% (22-79%) and 53.5% (18-100%), respectively. The results of demonstrating accuracy are inconsistent, and there is no clear difference between the two methods based on the available literature data. The apparent lack of consistency between egg spiking studies is most likely due to differences in study design, egg source, sample size, egg recovery and sample spiking methodology, flotation medium and densities used, detection limit of the techniques, and true EPG/OPG levels. The two EEC methods are comparable within each study but not necessarily across studies for the reasons stated above. Therefore, study designs for accuracy studies clearly require standardisation, as well as clear guidelines and protocols and it is also important to properly validate an EEC method prior to its comparison with existing standard techniques.

Given the potential biases associated with the spiking approach, using samples from naturally infected animals and simply comparing egg count magnitudes determined by different techniques is an alternative to evaluating relative accuracy which can still be useful. Although this does not allow for an estimation of percentage accuracy of the

true egg count, it can be simply concluded that the technique yielding the highest egg counts is the most accurate, and this has been the approach in several studies (Slusarewicz et al., 2016; de Castro et al., 2017; Paras et al., 2018; Went et al., 2018; Nápravníková et al., 2019). Because the true EPG value of clinical samples is unknown and most field samples are only analysed once per sample, accuracy in estimating infection magnitude in terms of EPG counts is the most important diagnostic parameter in natural infection to make treatment decision (Paras et al., 2018).

The available egg counting techniques vary in sensitivity, precision and accuracy (Ballweber et al., 2014; Nielsen, 2021). Variation of technical performance such as loss of eggs during sample processing, type of flotation solution, eggs type and flotation capability and analyst training also play a critical role in determining the performance of egg counting techniques (Cain et al., 2021; Nielsen, 2021). Furthermore, physical, biological, or epidemiological parameters related to the parasite or host factor influence the diagnostic performance of egg counting techniques (Ballweber et al., 2014; Lester and Matthews, 2014). Given the significant variation in the performance of various egg counting techniques, no single technique is suitable for all purposes, and the technique of choice is determined by the intended objective. As demonstrated by several researchers (Paras et al., 2018; Nápravníková et al., 2019), the diagnostic performance of egg counting techniques can be host parasite species dependent, implying the need for method optimization in different host parasite species such as gastrointestinal nematodes of poultry.

2.3.3. Non-conventional diagnostic methods

Currently, molecular biology techniques are increasingly used to diagnose parasite structures in order to enhance the identification and characterization of parasites (Tavares et al., 2011). Molecular methods have also been used to detect and identify avian parasites, such as *A. galli* (Nadler et al., 2007; Katakam et al., 2010; Biswas et al., 2021; Tarbiat et al., 2021), *Heterakis spp.* (Bazh, 2013; Bobrek et al., 2019; Cupo and Beckstead, 2019*), Capillaria spp*. (Tamaru et al., 2015) and *cestodes spp*. (Jyrwa

et al., 2014; Butboonchoo et al., 2016; Butboonchoo and Wongsawad, 2017; Makwanise et al., 2020; Panich and Chontananarth, 2021), at various developmental stages. The polymerase chain reaction (PCR) has found broad applicability because its sensitivity permits enzymatic amplification of gene fragments from minute quantities of nucleic acids derived from limited amounts of parasite material (Gasser, 1999). PCR-based technologies such as PCR-RFLP (Restriction fragment length polymorphism), and random amplified polymorphic DNA (RAPD) (Gasser, 1999; Zarlenga and Higgins, 2001), single-sequence conformational polymorphism (SSCP)(Gasser and Monti, 1997; Gasser et al., 1998), real-time fluorescence-based PCR (Costa et al., 2000) and multiplex PCR (Zarlenga et al., 1999; Zarlenga et al., 2001) have been available for parasite identification and differentiation. PCR for species identification uses universal primers that amplify the partial cytochrome c oxidase subunit II (cox2) gene, a fragment of the rDNA gene comprising the internal transcribed spacers (ITS), and the partial nicotinamide adenine dinucleotide dehydrogenase subunit 1 gene (ND1). This is followed by sequencing of the PCR products used to characterize different worms (Macklin, 2013). Because parasite DNA remains unchanged throughout the many life-cycle stages of nematodes, molecular-based identification methods focusing on the parasite genome are generally performed at any developmental stage where DNA can be obtained (Zarlenga and Higgins, 2001). However, due to cost and time constraints, PCR may currently not be used as a routine diagnostic tool. Furthermore, there are few published reference sequences that must be validated before the tests can be used for routine diagnosis (Macklin, 2013).

Immunological methods such as Enzyme-linked immunosorbent assays have also been used in avian parasite diagnostics. Some studies reported that detecting *A. galli/H. gallinarum* infection in chickens using an ELISA system that measures anti-ascarid antibody in plasma and egg yolks of infected hosts (Martin-Pacho et al., 2005; Daş et al., 2017; Sharma et al., 2018c; Dao et al., 2019), but only provides qualitative information. An ELISA system based on *A. galli* antigens in chicken faeces was recently developed to detect and quantify infections with both *A. galli* and *H. gallinarum* (Oladosu et al., 2022). However, in this study, the correlation between faecal antigen concentration and all worm burden parameters was weak, providing less information about infection intensities. Despite this, the results demonstrated that a

non-invasive copro-antigen ELISA assay can provide a reliable and accurate qualitative diagnosis of the two most common intestinal nematodes in chickens.

2.3.4. Factors affecting EECs and correlation with worm load

2.3.4.1. Factors affecting EECs

Several factors influence the precision and representative ability of egg counts in relation to worm burden. Excreta consistency can skew EEC results; for example, diarrhoea increases excreta moisture, which dilutes the number of worm eggs (Le Jambre et al., 2007; Turner et al., 2010; Daş et al., 2011c). It has been reported that parasites egg excretion may vary from hour to hour (with in day) or day to day (between egg excretion days) due to endogenous or exogenous factors (Oju and Mpoame, 2006; Villanúa et al., 2006; Wongrak et al., 2015b). In most cases, parasite eggs are shed at a higher rate during the day, particularly late afternoon or early morning (Wongrak et al., 2015b). Egg excretion by chicken nematodes *A. galli* and *H. gallinarum,* for example, has been shown to follow a diurnal fluctuation pattern (Wongrak et al., 2015b), with significant variations within and between egg excretion days (Villanúa et al., 2006). When measuring precise excreta egg output, the total daily egg output should ideally be determined by collecting and weighing all excreta (Daş et al., 2011c). Because of the intermittent egg shedding nature of the nematodes parasites, single excreta droppings collected at random are less likely to provide a reliable quantification of parasite eggs, whereas samples collected over multiple days would only allow for reliable egg detection (Daş et al., 2011c; Daş et al., 2017). For example, the caecal nematode of poultry, *H. gallinarum*, is restricted to the caeca and the *H. gallinarum* eggs are released into caecal lumen (Fine, 1975), but the caecal excreta is passed to the external environment periodically once or twice a day in distinct caecal droppings rather than the much more frequent intestinal droppings (Clarke, 1979) and the eggs are not even homogenously distributed within the caecal excreta (Fine, 1975).

Excreta egg output may also be influenced by uneven distribution of eggs within excreta (i.e. clustering of eggs within parts of excreta) (Yu et al., 1998; Torgerson et

al., 2012; Torgerson et al., 2014), host diet (Ikeme, 1971a; Michael and Bundy, 1991), worm population dynamics (Ikeme, 1971a; Michael and Bundy, 1989; Permin et al., 1997a; Lester and Matthews, 2014), density dependent fecundity of female worms (EEC/number of female worm), age and immunity of animals (Michael and Bundy, 1989; Stear et al., 1996; Gauly et al., 2005). Quantification method and related factors such as type and density of flotation fluid (Cringoli et al., 2017), a low sensitivity of egg counting techniques (Michael and Bundy, 1989), and sample preservative (Rinaldi et al., 2011) and dilution factor (Cringoli et al., 2017) highly influence worm egg recovery and enumeration. Technical variables add additional variability to EEC analyses, such as egg distribution in, and subsampling of the excreta suspension; egg loss in the sample preparation process; and variation in EEC performance due to user error, fatigue, skill level and subjectivity (Vidyashankar et al., 2012).

2.3.4.2. Correlation of EECs and worm load

Estimations of gastrointestinal nematode infection intensity in living birds rely widely on EECs. EEC can be used for a wide range of purposes from diagnosis to breeding for parasite resistance (Gauly et al., 2001a). In general, EECs are assumed to correlate with the actual worm burdens of the host animals (Daş et al., 2011c). However, several factors affect the correlation between excreta egg output and worm load and thus pose a practical diagnostic problem for this method, including for anthelmintic resistance testing using a worm egg count reduction approach (Permin and Hansen, 1998; Tarbiat et al., 2017; Sharma et al., 2019). Accurate enumeration or interpretation of EEC is complicated in mixed infections. Worm species can differ in egg production per adult female, pathogenicity and periodicity of shedding. A recent observation was that in a mixed infection of *A. galli*, *H. gallinarum*, and *Capillaria spp*., the latter contributed only a very small number of eggs to the overall egg counts, and showed no significant diurnal fluctuation (Wongrak et al., 2015b). Furthermore all parasites of a host do not simultaneously contribute to the countable eggs in a single sample of excreta droppings. This applies particularly to parasites that reside in physically separated organs such as the caeca (Villanúa et al., 2006). The composition of the worm population is a key factor for EECs to strongly correlate with and reflect intestinal worm burden as immature lifecycle stages and male worms may cause damage to the host, but do not produce eggs (Tompkins and Hudson, 1999; Daş et al., 2014). Strong

associations between EEC and worm counts can be expected in the case of artificial mono-infection, resulting from a single inoculation or infection of similar age, where birds can be protected from re-infection, resulting a resident population of adult worms at the time of sampling (Daş et al., 2014). In natural infections, where continuous exposure to infection is normal and worm burdens of individual hosts consist of mixed species and different parasitic stages, a strong correlation between overall worm load and egg count cannot be expected (Daş et al., 2014; Sharma et al., 2018c). On the other hand, it has been suggested that estimation of EECs to assess worm load may be suitable during the late patent period as egg production and per capita fecundity is highly stable during this phase (Daş et al., 2014). For instance, experimental infections with *H. gallinarum* showed linearly decreasing worm counts up to 30 days post-infection. But thereafter, worm counts remained almost constant, implying stability of the patent infections resulting from a single inoculation (Tompkins and Hudson, 1999; Daş et al., 2014). Calculations for correlations between EPG and female worm counts imply that all the female worms of a bird are laying nearly similar numbers of eggs (similar fecundity) which may not always be true. Some others suggested that eggs excreted per day derived from daily excreta collection, instead of EPG may provide a more reliable information for the correlation between worm burden and egg count (Daş et al., 2011c). This is undoubtedly true, but for most purposes, conducting an EEC test in birds after the pre-patent period of the suspected parasites provides a simple and low-cost indication of the severity of nematodiasis in a chicken population.

2.4. Recovery, laboratory storage and *in vivo* maintenance of chicken nematode eggs: emphasis to *Ascaridia galli*

Nematode eggs recovery is useful not only for diagnostic purposes but also for research purposes since recovered eggs can be used for either *in vitro* or *in vivo* parasite assays such as artificial infection and anthelmintic efficacy testing (Feyera et al., 2021b; Feyera et al., 2022b). In ruminant helminthology, eggs for parasitic nematodes are traditionally recovered from host faeces using the flotation principle. This

technique usually involves a series of sieving and washing procedures followed by flotation-centrifugation procedures to concentrate eggs (Paras et al., 2018). After recovery, the eggs can either be used immediately for experiments or stored after hatching to larvae for further experimentation and strain maintenance. Prolonged nematode preservation protocols developed so far are mainly based on cryopreservation of infective larval (L3) stages (Coles et al., 1992; Chylinski et al., 2015) and proved that parasites can survive and remain infective for as long as 15 years in liquid nitrogen (Gill and Redwin, 1995; Chylinski et al., 2015). However, the possibility of cryopreserving nematode eggs for a prolonged period has been rarely attempted and not successful so far although some studies have shown short-term success for eggs of *Angiostrongylus cantonensis* (Uga et al., 1983*), Toxocara canis* (Chung et al., 2004) and *Parascaris equorum* (Koudela and Bodeček, 2006) at freezing temperatures down to -80°C. Likewise, storage conditions to maintain viability of chicken nematode infective stages for prolonged periods in the laboratory have not either been developed or optimized so far. *A. galli* is the most studied poultry parasite, with many studies involving artificial infection.

2.4.1. Sources and isolation of *A. galli* eggs for experimental purposes

Bioassays of ascarid parasites require sufficient number of eggs which must incubate and embryonate outside of the host (Elliott, 1954). For parasite assays involving *A. galli*, eggs are most commonly recovered from excreta (Rahimian et al., 2016; Daş et al., 2020) and from mature worms either by disruption of the worm's uterus (Permin et al., 1997a; Daş et al., 2010) or by in vitro culturing of female worms in artificial media and recovering eggs shed into the media (such as physiological saline and RPMI) (Feyera et al., 2020). Eggs recovered through physical disruption of worm uteri are known for their low embryonation rate and are not feasible alternatives for experiments requiring high number of eggs with optimum embryonation capacity (Rahimian et al., 2016). Recovery from female worms kept in artificial media is possible in terms of collecting mature eggs with high viability, but only a limited number of eggs can be isolated per female worm (Feyera et al., 2020). This is due to the fact that worms are subject to an artificial environment, and their survival as well as physiological activity, including egg laying, will decrease over the course of the

incubation period (Feyera et al., 2020). Furthermore, these two approaches are not always feasible given the requirement to euthanise chickens to recover mature worms. Excreta collected from chickens harbouring specific nematode infection can also serve as source of eggs. This involves more laboratory work, sequential sieving and washing through a series of sieves followed by flotation-centrifugation procedures (Rahimian et al., 2016; Tarbiat et al., 2018; Daş et al., 2020). Furthermore, the saturated salt solutions used to float eggs may cause significant distortion of eggs particularly at high specific gravities (Ballweber et al., 2014), thus affecting subsequent embryonation capacity. Protocols used to recover eggs from all these three sources are mostly not standardized and it is expected that there could be considerable variation which could affect embryonation and subsequent infectivity of embryonated eggs. Therefore, the comparative efficiency of the viability of various sources of worm egg needs to be systematically investigated.

2.4.2. Prolonged laboratory storage of viable *A. galli* eggs

The parasite eggs must go through an embryonation process to reach the infective stage (Permin and Hansen, 1998). The viability of *A. galli* eggs in the laboratory conditions primarily depend upon oxygen concentration, temperature and humidity or culture media, with embryonic development times ranging from 1 to 8 weeks (Ackert, 1931; Reid, 1960; Ramadan and Abouznada, 1992; Tarbiat et al., 2015). Embryonation is assessed by microscopic examination of eggs using the morphological classifications. The different stages of *A. galli* eggs were classified into five developmental stages (Ackert, 1931; Ramadan and Abouznada, 1992; Cruz et al., 2012). 1) Undeveloped stage: unembryonated or single-celled eggs with no visible cell division process. 2) Cleavage stage: During this stage, fertile eggs begin cell division into large blastomeres containing 2-16 cells, allowing them to progress to the early morula stage. This stage advances a morula or late-morula by further division into smaller blastomeres, eventually reaching the blastula stage. 3) Vermiform stage: the internal mass of the eggs began to differentiate and contained early vermiform embryos, which formed kidney-shaped larvae. 4) Embryonated stage: a slender, coiled, and motile larva develops, followed by the formation of the embryo's head and tail. 5) Infertile

eggs or dead eggs have poorly defined structures, a contracted, ruptured, or disrupted eggshell with an abnormal intra-capsular mass, or they are dark, completely translucent, and have a shrunken internal embryonic mass or an embryonic mass with lost integrity.

Different species of parasitic nematodes have evolved different strategies to ensure their survival in environmental extremes (Tarbiat et al., 2015). The epidemiology of nematode infection depends to a large extent upon the egg's ability to survive in different environmental variables (Katakam et al., 2014; Tarbiat et al., 2015; Thapa et al., 2017a). The egg-shell of nematodes has been referred to as one of the most resistant biological structures (Wharton, 1986)), which offers a high degree of protection to the developing embryo. The egg shell is impermeable to all substances except lipid solvents, gases and perhaps liquid water (Wharton, 1980). Because of these reasons, *A. galli* eggs can survive and remain infective in the external environment for up to one year (Thapa et al., 2015b). Given this, the following sections discuss the most common abiotic factors affecting parasite eggs in laboratory conditions:

2.4.2.1. Temperature

Temperature has a well-documented effect on the development and viability of nematode eggs (Ackert, 1931; Christenson et al., 1942; Reid, 1960; Kim et al., 2012; Katakam et al., 2014; Tarbiat et al., 2015). *A. galli* eggs can develop to their infective stage at temperatures ranging from 16±1°C to 34±1°C, with a temperature range of 25 to 30°C considered optimal (Ackert and Herrick, 1928; Reid, 1960; Tongson, 1967; Mero and Gazal, 2008; Tarbiat et al., 2015). In the above temperature ranges, increased percentage of embryonation (8-90%), with increasing temperature (16-30°C), and 30-35°C was considered the threshold or maximum temperature (Tarbiat et al., 2015). Ackert (1931) reported that incubating an *A. galli* egg takes about 16 days to reach the infective stage at 30°C, with development accelerated by raising the temperature to 33°C, and the eggs becoming infective in 10-12 days. According to the author, the "tadpole" stage (vermiform stage) occurs about eight days after incubation begins. According to infection experiments (Reid, 1960), egg development took as little as five days at 33°C and 32 days at 19°C, indicating that when embryonation temperature

rises within this limit, egg development time decreases. As a result, maximum larval viability can be achieved when eggs are embryonated at the optimal temperature range.

There have been a few studies published on the effects of low temperatures on *A. galli* eggs. The temperature at which cleavage begins in water cultures ranges between 10° C and 15° C (Ackert, 1931; Tarbiat et al., 2015). Low temperatures (4°C to 6°C) inhibit egg development, but that eggs moved at room temperature, development to the infective stage was accomplished. It is common practise to keep ascarid nematode eggs in the refrigerator at these temperatures, where they will remain viable without cell division (Gamboa, 2005; Kim et al., 2012; Tarbiat et al., 2015; Feyera et al., 2020). It appears that such eggs are not completely dormant and may undergo physiological changes. Ackert (1931) reported that non-embryonated eggs kept at 0°C for one month were unable to reach the infective stage when incubated at 30°C while still viable. Similarly, Levine (1937) found that the eggs were non-viable after 45 days at -1°C, and the mechanism of cold tolerance is still unknown to this day. A strategy for cold tolerance in *A. galli* is similar to that of other nematodes (Wharton and Allan, 1989).

When the storage temperature rises above 35°C, ascarid egg development ceases (Reid, 1960; Mero and Gazal, 2008; Tarbiat et al., 2015). Ackert (1931) reported that twelve hours at 43°C was lethal for eggs at all stages of development. Similarly, Itagaki (1927) found that *A. galli* incubated at 50-53°C was not lethal in 5 minutes, but was lethal at 54°C for all of the eggs, indicating that thermal death of *A. galli* eggs occurs above 54°C (Christenson et al., 1942). However, investigations on Swedish farms clearly showed that despite using high-pressure (50-80°C) and water steam cleaning, which did not effectively control the spread of *A. galli* infection (Höglund and Jansson, 2011).

2.4.2.2. Humidity

The development rate of nematode eggs is proportional to the percentage of humidity (Gaasenbeek and Borgsteede, 1998; Gamboa, 2005; Tarbiat et al., 2015). Embryonation of *A. galli* eggs requires > 85% relative humidity in the optimal temperature range (McRae, 1935; Hansen et al., 1953; Tarbiat et al., 2015). Relative humidity has a significant impact on the viability of *A. galli* eggs, which are extremely

susceptible to desiccation. At 22°C, the chicken ascarid eggs did not survive an average relative humidity of less than 80% (McRae, 1935). As a result, *A. galli* eggs should require a highly saturated environment before they can complete the development process at the optimal temperature range (Tarbiat et al., 2015). Similarly, the survival rate of *A. suum* eggs was 96 and 62% when kept at a relative humidity of 100% and 77.5% respectively after 12 weeks (Gaasenbeek and Borgsteede, 1998).

2.3.2.3. Oxygen

Low oxygen (i.e. insufficient oxygen) concentration may inhibit the development of nematode eggs by supressing their overall metabolic activity (Saunders et al., 2000; Tarbiat et al., 2015). This may maintain viability of eggs for long storage period (Ackert and Cauthen, 1931b). During low oxygen concentration conditions, nematode eggs may enter the dormant phase (anoxybiosis) in order to reduce their metabolic requirements with egg development resuming when exposed to oxygen (Gaasenbeek and Borgsteede, 1998; Saunders et al., 2000). For example, Tarbiat et al. (2015) reported that non-embryonated eggs survived for at least 16 days at 25ºC in anaerobic condition without development. Similarly, Saunders et al. (2000) found that undeveloped *H. gallinarum* eggs remained viable after 60 days of insufficient oxygen exposure. Gaasenbeek and Borgsteede (1998) also reported that *A. suum* eggs were also found to be viable when stored under anaerobic conditions at 15ºC for 12 weeks by suppressing egg development, and when exposed to oxygen, more than 80% of the eggs developed to the infective stage. Similarly, unembryonated eggs stored under anaerobic conditions at 4˚C, maintain optimal viability when incubated at 25˚C under aerobic condition, with a minimal decline rate over time (Tarbiat et al., 2018). Oxygen is thus an essential requirement for embryonation to occur and appears necessary to maintain optimal viability at embryonation temperatures (Hansen et al., 1953; Saunders et al., 2000; Tarbiat et al., 2015; Feyera et al., 2020). This indicates that the rate of embryonation or viability is influenced by the combined effect of oxygen and temperature.

2.3.2.4. Storage media

Culture media is one of the most important factors influencing embryonation and the subsequent infectivity of nematode eggs**.** The most widely used culture media for nematode egg embryonation are sulphuric acid (0.1N), formalin (0.5-2%) and potassium dichromate solution (0.1-2%) to prevent putrefaction of the parasite eggs and inhibit the fungus and bacterial growth (Ackert and Herrick, 1928; Ackert and Cauthen, 1931a; Ackert et al., 1935b; Levine, 1936; Permin et al., 1997a; Tiersch et al., 2013). Incubation media may also play a significant role to hasten egg viability and development rate. For instance, Tarbiat et al. (2018) found that eggs storage under either aerobic or anaerobic conditions at 4°C in excreta media had the highest viability compared to eggs stored in water. Similarly, embryonation rates of the *Capillaria obsignata* eggs kept in four culture media (0.5 % formalin, 2 % formalin, 0.1 % potassium dichromate and 0.1 N sulphuric acid), and 0.1 N sulphuric acid resulted in the highest embryonation rate (Tiersch et al., 2013). The authors conclude that 0.1 N sulphuric acid and 0.5% formalin can successfully be used as incubation media for *C. obsignata* eggs, whereas potassium dichromate impairs subsequent infectivity of the eggs. In addition, Oksanen et al. (1990) reported that when *Ascaris suum* eggs were allowed to embryonate in 0.1 N H_2SO_4 and 1% formalin, 0.1 N sulphuric acid provided higher percentage embryonated eggs and embryonated more quickly compared to 1% formalin. It was also reported that *A. galli* eggs from worm uteri incubated in 0.1 N H_2SO_4 maintained better viability and infectivity than those incubated in 2% formalin under similar conditions (Permin et al., 1997b). Incubation of *Ascaris suum* eggs with the three solutions (distilled water, 0.5% formalin and 0.1 N sulphuric acid) for 28 days, the highest percentage of viability was recorded with 0.1 N sulphuric acid (91.2 ± 0.6%), followed by distilled water (90.0 ± 3.7%) and 0.5% formalin solution (87.6 ± 0.5%) but not statistically significant (Amoah et al., 2017). Todd et al. (1952) concluded that the development of *A. galli* in chicken was influenced by the temperature and the egg-containing medium during embryonation to the infective stage. These findings from the literature suggest that the culture media may have a significant effect on the viability and development of nematode eggs.

Some incubation chemicals may result in inactivation of eggs (Gaspard et al., 1996) due to the damage of the egg shell of the parasites, which protect the egg and enhance the survival ability of eggs. For example, the observed decrease of egg viability when formalin is used as an incubation solution, might be due to the ability of formalin to

penetrate into the eggs by diffusion (Oksanen et al., 1990). The penetration of formalin and other chemicals into the eggs may be enhanced during hatching of the eggs, when an increase in permeability of the eggs occurs (Clarke and Perry, 1988; Quilès et al., 2006). A change in the permeability of the cell-wall layer of eggs may thus result in the loss of viability (de Souza et al., 2011). Although sulphuric acid had the best performance in percentage embryonation as incubation media for most ascarida nematode eggs, it is very corrosive and therefore poses health concern for laboratory personnel, especially when there is direct exposure to sufficient concentrations. Exposure to formalin may also result in irritation and corrosive effects (Pandey et al., 2000; Amoah et al., 2017).

2.3.2.5. Storage time

As outlined above, free-living stages of nematodes have to spend certain periods of time outside their host. Irrespective the influence of environmental factors (temperature, humidity and etc.) on survival of the parasite eggs, the age of the nematode eggs are considered to be a critical factor influencing their infectivity. For example, Ackert et al. (1947) reported that egg cultures of *A. galli* incubated in water at 30°C to 33°C for 36 days proved to be more viable than eggs of a 120-day-old cultures. Their criterion for judging viability was the average lengths of the worms recovered from experimental chickens after feeding eggs from the two types of cultures. Similarly, Elliott (1954) also observed that embryonated *A. galli* eggs stored aerobically in water for 36 weeks at 28°C was infective up to 2%. Todd et al. (1950) reported that the greatest host injury resulted from feeding chickens infective eggs 14-21 days old that the virulence of the parasite decreased as the infective eggs aged. These authors concluded that virulence was inversely proportional to aging of the infective *A. galli* eggs. Furthermore, Christenson et al. (1942) reported that cultures of embryonated *A. galli* eggs in 2% potassium dichromate solution remained viable for nearly 2 years as assessed by an in *ovo* larval motility testing method. However, the most reliable method to monitor infectivity of eggs is to infect chickens with fully developed eggs that may not necessarily be infective (Ackert et al., 1947; Todd et al., 1950; Geenen et al., 1999). So far, the interplay effect of the aforementioned factors has not been tested in a single factorial experiment to determine which combination of

laboratory conditions that influence the embryonation rate and the viability of chicken nematode eggs over long periods of time.

2.4.3. Induced infection with *A. galli* eggs and factors affecting infection outcome in chickens

Artificial infections of *A. galli* have been reported in several publications and most of the studies on the population dynamics of *A. galli* have focused on the effects of host genetics, age and nutrition on worm burden (Ackert et al., 1935a; Permin and Ranvig, 2001; Gauly et al., 2001a; Idi et al., 2004; Gauly et al., 2005; Daş et al., 2010). However, variation in experimental study outcomes have been commonly observed due to lack of defined experimental model for propagation and amplification of characterized *A. galli* strains in chickens. Most experimental infection models have been conducted with eggs isolated from worm uteri (Ackert and Herrick, 1928; Ackert and Cauthen, 1931b; Ackert et al., 1931a; Permin et al., 1997a; Permin et al., 1998b; Gauly et al., 2005; Daş et al., 2010) and some infection models have used eggs isolated from host excreta (Ferdushy et al., 2012; Luna-Olivares et al., 2012; Ferdushy et al., 2013; Ferdushy et al., 2014; Luna-Olivares et al., 2015) and worms cultured in artificial media (Feyera et al., 2020). The development of nematode infections is not only affected by abiotic environmental conditions that affect egg embryonation and viability, such as temperature, humidity and oxygen concentration but also by inner factors like host-parasite and parasite-parasite interactions, which may cause density dependent effects on worm populations (Paterson and Viney, 2002). The outcome of infection of *A. galli* in the chicken intestine is influenced by multitudes of host factors.

2.4.3.1. Effect of infection dose

The size of worm population, worm establishment rate and worm length can be affected by the infection dose. An inverse relationship between size of egg dose and worm establishment rate of *A. galli* infection has been demonstrated in previous studies where the percentage establishment and growth rate of the worms decreased as size of the infective egg doses increased (Ackert et al., 1931a; Ikeme, 1971a; Permin et al., 1997a; Permin and Ranvig, 2001; Gauly et al., 2001a). Moreover, lower number

of adult worms and egg excretion was observed in high infection dose (1, 000 eggs/day) compared to a low infection dose (10 eggs/day) (Ikeme, 1971a). This author stated that in chickens receiving 1,000 eggs/day, worm egg shedding was delayed or suppressed likely due to inhibition of larval development. From author's point of view, this could have been due to a density dependent phenomenon in the form of worm-to-worm interactions or alternatively being related to host immunity as also reported elsewhere. A similar view was reflected in other studies (Madsen, 1962a, 1962b) after noting bimodal growth curves in *A. galli* and *H. gallinarum* infections of chickens and also in the light of similar findings in mammalian trichostrongylids (Dunsmore, 1960). Similar observation was reported by Roberts (1937) who observed that despite large number of eggs fed, very few worms had survived and the majority of these were very small in size. Herd and McNaught (1975) reported that the association between high dose rates and arrested development simply reflects a greater antigenic stimulation and immune reaction in chickens following exposure to greater numbers of immature worms in tissue phase. The mucosal phase of *A. galli* larvae, in strict sense, cannot be considered to be part of the normal life cycle, but rather as a reaction to host resistance. The phenomenon of arrested development has been reported in the parasitic larval stages of at least 30 different nematode parasites (Michel, 1968). The precise point at which arrested development is characteristic of each species and by which the underlying mechanisms is still not clear (Herd and McNaught, 1975). The main factors influencing occurrence of the mucosal phase include age of chicken (Edgar *el al.*, 1957), viability and virulence of infective eggs (Todd et al., 1952) and dose of infection (Permin et al., 1997a).

2.4.3.2. Chicken breed

The natural resistance of hosts to helminth infections may be supported by genetic constitution of the host. Epidemiologic and experimental data both suggest that the number and the length of mature intestinal nematode worms were used as indicators criteria of host resistance (Ackert, 1942). Experimental evidence of chicken breed differences in resistance to *A. galli* infections reported by Ackert et al. (1935b) found significant different in natural resistance. For example, heavy body weight breeds, such as Rhode Island Reds, Plymouth Rocks and Barred Plymouth Rocks had significantly smaller *A. galli* worms than did the lighter weighing White Leghorn and

Minorca breeds. A strain of heavy White Minorca chickens in other studies proved to be more resistant to *A. galli* than a lighter strain of the same breed with different genetic constitution (Ackert and Wilmoth, 1934).

In addition, Gauly et al. (2001b) studied that groups of 20-week-old white Lohmann Selected Leghorn (LSL) and Lohmann Brown (LB) hens reared under helminth-free conditions were artificially infected with 250 embryonated *A. galli* eggs to determine genetic differences of *A. galli* eggs output between the two breeds. The authors observed that the mean EEC were significantly higher in white LSL hens than in LB hens. A similar study was conducted by Schou et al. (2003) who investigated the establishment and effect of *A. galli* infections in four different commercial layer-lines hens (ISA Brown, New Hampshire (NH), Skalborg (Sk) and a cross of NH and Skalborg (NHxSk)) infected with 500 embryonated *A. galli* eggs, thereby exploring the possible influence of the genetic background of chickens on helminth infections. The authors found that although no significant difference between any of the lines in total number of worms (adults and larvae) was observed, the chickens of the Sk line excreted significantly higher number of *A. galli* eggs throughout the study compared to the other lines indicating that the Sk line appeared to be more susceptible to *A. galli* infections than the three other lines. The chickens of the Sk line also tended to harbour numerically more adult worms than the other lines and both the male and the female worms recovered from the Sk line by weeks 6 and 9 post infection were longer than the worms from the other lines clearly support the indication that the Sk line was more susceptible.

In another study, conducted to compare resistance to *A. galli* infections in Lohman Brown (LB) and Danish Landrace (DL) chickens (Permin and Ranvig, 2001), the establishment rates were significantly higher for single and trickle infected DL groups compared to single and trickle infected LB groups. This agrees with the results of Sadun (1948) who concluded that at reinfection, the resistance induced by a previous infection was strong enough to provoke the discharge of most or all of the worms of the primary infection, regardless the size of the dose or the number of egg administrations (single or trickle infection). This self-cure phenomenon has not been well studied in relation to *A. galli* infections in chickens. It is, however, a well-known phenomenon in sheep in relation to *Haemonchus contortus* infections (Soulsby and

Stewart, 1960) who defined self-cure as the immunologic reaction of the host which results in the loss of a burden of adult parasites at the time of a challenge infection with infective stages.

Epidemiological evidence of breed differences in resistant to helminth infections was also reported by several researchers (Kaufmann et al., 2011a; Wongrak et al., 2015a-a). Kaufmann et al. (2011a) investigated the worm burden in two helminth-infected chicken genotypes, LSL and LB hens, in a free range system. At all monitoring dates, LSL hens had significantly higher EEC than LB hens. In contrast, LB had a significantly higher worm burden than LSL (192.3 vs. 94.3). Wongrak et al. (2015a-a) also reported that LB classic hens had numerically higher worm numbers than LB plus hens with 241and 184 worms, respectively.

2.4.3.3. Age and host immunity

Chicken age, which is directly related to the ability to develop immunity or tolerance, has the potential to affect infection establishment (Idi et al., 2004). Resistance to *A. galli* infection generally increases with age, which has been attributed to the host's ability to develop both intestinal and systemic cellular or humoral immune responses (Ackert et al., 1935a; Idi et al., 2004). Herrick (1926) observed a gradual increase of the host's resistance as birds became older and the resistance increased with age up to 103 days after which no further increase could be determined. Subsequent confirmations of these findings led to the studies of Ackert and Herrick (1928) who conducted research on the role of age in natural resistance to *A. galli* infection in chickens. The authors found that four months old birds were much more resistant to the nematode infections than the one month old chicks. Furthermore, Ackert et al. (1935a) observed a marked increase in the resistance of chickens to the length of mature *A. galli* with increasing age, started with chickens at 45 days of age, and significant differences in the lengths of the nematodes were noted up to 93 days of age, when the maximum impact of the host on *A. galli* length was observed. The authors suggested that, as a chicken grows older, its body develops more potent growth-inhibiting factors, which may suppress the development of *A. galli.* These findings are again supported by Riedel and Ackert (1951) who compared two different ages of chickens infected with 500 embryonated *A. galli* eggs and found that the age of

chickens affected both *A. galli* number and development. Similarly, Egerton and Hansen (1955) have found that chickens produce a humoral factor in response to an *A. galli* infection and that both an age-related immunity and tolerance in chickens toward *A. galli* exist. Tongson and McCraw (1967) also reported a decrease in total *A. galli* burden in male white Leghorn chicks infected with 1,000 infective *A. galli* eggs at 2, 4, 8, 12, or 16 weeks of age. Idi et al. (2004), however, reported that worm burden and female fecundity values were not significantly different between age groups indicating that the chickens' age only partially influences resistance to *A. galli* infection (Idi et al., 2004). These authors, after comparing ranges of parasitological variables in naïve chickens of 1 day, 1 month and 4 month of age concluded that the chickens' age had a restricted effect on *A. galli* artificial infection. According to these authors, total worm recoveries from the three age groups were not significantly different although egg excretion rates were reduced in 1 month-old chickens.

Resistance to *A. galli* infection in chicken can also develop due to older birds' ability to express a greater number of goblet cells dedicated to protective immunity against *A. galli* (Ackert et al., 1939). This can be manifested as lower establishment of incoming worms, inhibition of larval development, expulsion of adult worms and supressed egg excretion. Furthermore, immunological studies in growing birds have shown that infected chickens produce significant amounts of specific IgG antibodies against *A. galli* (Marcos-Atxutegi et al., 2009; Schwarz et al., 2011; Norup et al., 2013; Daş et al., 2018). Single-comb White Leghorn chickens infected with embryonated eggs had significantly fewer worms at 14 and 42 days after being challenged than control birds (Brewer and Edgar, 1971). These authors indicated that this immunity partially protected birds against worm numbers and the effect of worms on weight gain. On the other hand, Ackert et al. (1935a) and (Ikeme, 1973) reported that age-related resistance is more important than acquired immunity whereas Permin and Ranvig (2001) concluded that acquired immunity plays a major role as Danish Landrace and Lohman Brown chickens were able to expel the worms from a primary infection in response to a second infection, so-called self-cure. This agrees with the results of Sadun (1948) who reported that at reinfection the resistance induced by a previous infection was strong enough to provoke the discharge of most or all of the worms of the primary infection, regardless the size of the dose or the number of egg administrations (single or trickle infection). Another study indicated that in young

chickens trickle infected with 100 embryonated eggs twice weekly, the establishment rate was constant during the initial phase of infection, with larvae accumulated. However, 3-4 weeks after infection, the total number of established larvae decreased, indicating the onset of acquired resistance, which leads to a lower establishment rate of incoming infections as well as the expulsion of an already established infection (Ferdushy et al., 2014). According to these authors, continued infection exposure for 10 weeks resulted in smaller-sized worms, indicating immunologically mediated inhibition of larval development (Herd and McNaught, 1975). This is due to the prior exposure of the infective stages of the parasite to a hostile environment as result of acquired host immunity (Eysker, 1997) and crowding factor/dose dependent factors (Ikeme, 1971a; Fleming, 1988). Humoral and local immune responses were associated with worm expulsion (Stehr et al., 2018) and host immunity may generally influence worm population dynamics.

2.4.3.4. Nutrition status of the host

Diet and nutrition may also influence infection outcome. For instance, Ackert et al. (1931b) observed significantly more and longer *A. galli* worms in chickens fed a vitamin A-deficient diet than in chickens fed a vitamin A-rich diet. Similar observation were reported that the group of chickens supplied with sufficient vitamin had the fewest and shortest *A. galli* worms compared to those deficient with vitamins and proteins (Ackert and Beach, 1933). Ikeme (1971a) reported that chickens on low vitamin and mineral supplementation had higher worm egg output than birds with adequate vitamin and mineral supply, following infection with 10 *A. galli* eggs /day for 6 weeks. Lysine-deficient diets have also shown to be accompanied by longer *A. galli* worms and higher worm burdens in growing chickens (Cuca et al., 1968). Lysine deficiency compromises antibody response and cell-mediated immunity of chickens by limiting protein synthesis resulting in impairment of the immune response (Chen et al., 2003). Nutritional quality of diets is therefore a critical factor in reducing the impact of *A. galli* infection on poultry production, especially in ecological farming and free-range production systems where the prevalence of the parasites is high (Permin et al., 1998b).

2.4.3.5. Mode of infection

The mode of egg inoculation may also have an impact on the outcome of infection. Infections can be induced by inoculation of infective eggs either as a single high dose or repeated small doses. It has been reported that use of a single artificially high bolus of infective stages results in a robust immune response which drives rapid parasite expulsion (Sadun, 1948). In contrast, the split trickle infection regime, which mimics the kinetics of natural infection, develops the immune response slowly resulting in a gradual increase in worm burden followed by partial expulsion (Glover et al., 2019; Colombo and Grencis, 2020). However, trickle-infection over an extended period was reported to lower establishment rate and worm burden due to either impaired larval growth or partial worm expulsion (Ferdushy et al., 2014). This is due to the onset of acquired resistance, which results in both a lower rate of establishment of incoming infections and the expulsion of already established nematodes (Permin et al., 1997a; Permin and Ranvig, 2001; Ferdushy et al., 2014). Crowding or density dependency arrest in development in trickle-infected chickens could also result in small-sized worms especially when a high dose is used (Ikeme, 1971a; Ferdushy et al., 2014). It was also demonstrated that trickle infection with *A. galli* in chicken leads to impaired establishment and larval growth rather than a complete protection against re-infection (Ferdushy et al., 2014). It should be noted, however; that trickle infection over a short period with moderate doses of infective eggs will favour establishment rate and an accumulation of larvae as suggested previously (Sadun, 1948; Ferdushy et al., 2014).

2.5. Conclusion and research direction

Helminth infections have the potential to negatively impact poultry welfare, health, and production performance. For many years, the transmission of helminth infections in poultry was reduced by keeping birds in confinement with little to no contact with excreta material, resulting in lower infection in cage systems. In contrast, helminth infections continue to be a problem in free-range systems, sometimes with a prevalence rate of as high as 100%. Regular diagnosis of helminth infection is thus essential to monitor economically important helminth infection in free range systems. Post-mortem examination, in conjunction with the egg count technique, may provide reliable diagnostic information on helminth infection in poultry. Practically, diagnosis

in live birds is feasible only by EEC. However, EEC is affected by physical, epidemiological and biological factors, the result should be interpreted with caution. With the re-emergence of the chicken nematode, research interest on economically important parasites such as *A. galli* and *H. gallinarum* has increased. In the future, research studies will require the availability of defined methods for the recovery, laboratory storage, and in vivo maintenance of strains of these parasites with specific and documented characteristics. There is thus a need to optimise methods for the isolation, preservation and propagation of viable infective stages of important chicken nematodes for critical experimentation.

Chapter 3. Global and regional prevalence of helminth infection in chickens over time: a systematic review and meta-analysis

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3.1. Abstract

Gastrointestinal helminth parasites are a concern for the poultry industry world-wide as they can affect the health, welfare, and production performance. A systematic review of the prevalence over time in different countries may improve our understanding of gastrointestinal helminthiasis in chickens and subsequently lead to improved poultry health. The aim of this systematic review and meta-analysis was to provide an overview of the published information regarding the epidemiology and the diagnostic approaches of chicken helminth infection. Six databases were searched for studies and a total of 2,985 articles published between 1942 and 2019 were identified and subsequently screened for eligibility using title/abstract and full text assessment, resulting in 191 publications to be used in the study. Post-mortem diagnostics (73.8%) and the flotation technique (28.8%) were commonly used to detect helminth infections with pooled prevalence of 79.4% ranging from 4 to 100%. More than 30 helminth species in chicken populations were identified including *A. galli* (35.9%), *H. gallinarum* (28.5%), *Capillaria spp.* (5.90%) and *Raillietina spp.* (19.0%) being the most prevalent. The reported prevalence of helminth infection decreased over time in developing countries while it increased in the developed world. Chicken kept in back yard and free-range systems had a markedly higher pooled prevalence of helminth infection (82.6 and 84.8%, respectively) compared to those housed in cage production systems (63.6%). This may indicate the need for more rigorous control and prevention measures in free-range and back yard production systems using regular deworming coupled with access to early and accurate diagnosis allowing for early intervention.

3.2. Introduction

The increasing demand for poultry products for human consumption has resulted in substantial growth of extensively and intensively housed poultry over the last few

decades (Permin and Hansen, 1998; Ola-Fadunsin et al., 2019). As a result, poultry production is making a significant and increasing contribution to the national economy of most countries (Ferdushy et al., 2016). However, the production performance of poultry can be significantly reduced due to various intestinal helminth parasites (Permin et al., 1999b; Ruff, 1999; Van et al., 2019).

Gastrointestinal helminthiasis is caused by roundworms (nematodes), tapeworms (cestodes), and flukes (trematodes) (Ruff, 1999; Macklin, 2013; Ola-Fadunsin et al., 2019). With respect to the health impact of the infection, the abundance of pathogenic species and the economic importance, nematodes are the most important intestinal worms in the poultry industry (Ruff, 1999; Macklin, 2013; Ola-Fadunsin et al., 2019). Impacts associated with nematode infections include reduced health, welfare and production performance due to reduced feed conversion ratio, reduced growth rates or weight loss, reduced egg production and egg quality, intestinal damage and in severe cases, death (Ramadan and Znada, 1991; Daş et al., 2010; Sharma et al., 2019). Nematode infections can have direct adverse effects on the host, inducing the breakdown of the gastrointestinal barrier but indirect damage can also occur via increased susceptibility to secondary infectious diseases (Dahl et al., 2002; Eigaard et al., 2006; Permin et al., 2006) and reduced host immune response (Horning et al., 2013; Pleidrup et al., 2014; Dalgaarda et al., 2015).

A prevalence rate of as high as 100% has been reported in chickens housed in back yard (Rabbi et al., 2006) and free-range systems (Sherwin et al., 2013). The prevalence of helminth infections can be influenced by many factors such as the climatic conditions and agro-ecological zones, the accumulation of infective stages of larvae or eggs in the environment, the presence of intermediate hosts and the individual susceptibility of the final host (Magwisha et al., 2002). Temperature and humidity can be considered as determinants for the occurrence and the level of helminth infection by influencing transmission through survival in the environment and developmental success of the infective stage (Permin et al., 1997c; Magwisha et al., 2002; Dube et al., 2010; Ola-Fadunsin et al., 2019). Most poultry nematodes have a direct lifecycle and the faecal-oral route is the main route of infection contributing to the higher susceptibility of poultry in free range and floor poultry production systems due to in

close contact to their excreta and soil (Permin et al., 1999; Jansson et al., 2010; Kaufmann et al., 2011b; Wongrak et al., 2014, 2015a). As such, the ongoing global growth in poultry production coupled with the trend to move from caged to more extensive housing conditions (free-range, barn) will favour the parasite infection. Therefore, the objective of this review was to provide compiled information on the epidemiology of chicken helminthiasis, the change in housing systems over time, and the diagnostic methods used to obtain information on prevalence.

3.3. Materials and methods

3.3.1. Data Collection Strategy

A preliminary search of key databases (PubMed, Embase, ProQuest, Web of Science, Google Scholar and Scopus) was conducted to ensure the availability of sufficient and relevant published articles, to validate the rationality of this review objectives and to identify and refine key search terms. Based on these findings, the following combination of terms was used to search in the PubMed, EMBASE, ProQuest, Web of Science and Scopus databases for the main study: (Prevalen* OR Epidemiolog* OR Magnitude OR Occurrence) AND (Helminth* OR Gastrointestinal Worm* OR Gastrointestinal Nematode* OR Cestode*) AND (Poultry OR Chicken* OR Domestic fowl OR Hen*) by using title/abstract and years from 1942 to 2019 in the search engines. When searching the database of Google scholar “advanced search”, the following search for any of the following words or phrases being present anywhere in the articles was conducted: prevalence helminth chicken; epidemiology helminth poultry; prevalence gastrointestinal nematodes or cestodes; occurrence intestinal helminth hens or domestic fowl, and at least one of the following words anywhere in the article or in the title of an article: prevalence helminth nematode cestodes chicken. All searched articles from each database were imported into Endnote X9 (Clarivate Analytics, Philadelphia, PA, USA) in order to identify and delete duplicate articles.

3.3.2. Article Selection

Eligibility criteria were then applied to screen articles. These criteria were selected in line with the guidelines by the Preferred Reporting Items for Systematic Reviews and Meta-analyses statement (Moher et al., 2009). The following inclusion criteria were applied: 1) providing prevalence data on helminths in avian species 2) only relating to chicken or domestic fowl populations 3) English language only 4) published studies and original articles 5) reports on natural (not experimental) infection. Exclusion criteria were: 1) unrelated articles, 2) repeat publications using the same data, 3) unpublished articles, preceding papers, conference, books, case reports, review articles, systematic reviews, and articles without full text available, 4) experimental infection.

3.3.3. Data Extraction

Data were extracted manually and entered into a Microsoft excel sheet (2016, Microsoft Corporation, Redmond, Washington, USA). The extracted information included study details (such as authors, years, study regions, breed/strain, age, sex, production system, study design, sampling type, and sample size), diagnostic method, and helminth species and prevalence.

3.3.4. Quality Assessment

Quality assessment was carried out by two independent appraisers based on the Joanna Briggs Institute (JBI) critical appraisal tool for systematic reviews (Institute, 2017) using nine selection criteria and four rating variables (“yes”, “no”, “unclear”, and “not applicable”) for each criteria. Calculating the overall score was conducted by adding the values for all nine selection criteria where “yes” was valued as one while “no”, “unclear” and “not applicable” were valued as zero. The published articles were then categorized as poor, fair, and good studies using the same approach described by Tawfik et al. (2019) where a score mark was given for each article grouped as 1 to 3, 4 to 6, and 7 to 9 representing poor, fair, and good quality research, respectively.

3.3.5. Data Synthesis

The production systems of domestic chicken were categorised as village/back yard, free-range, deep litter/barn, and enriched cage/conventional cage production systems. Village or back yard chickens were defined as any domestic chicken kept extensively in the village as rural or scavenging in a back yard or traditional production system being allowed to roam freely at least during daylight hours. Free-range was considered to be a commercial husbandry farming system where chicken had access to a range area. Deep litter and barn production system studies included chicken reared on the ground with indoor housing whereas caged housing referred to chicken confined in single or group cages raised off the ground. The sample size was significantly varied across studies and mainly based on individual birds. Post-mortem examination was defined as an inspection and dissection of the intestine of a dead chickens to determine the presence of worms and magnitude of infection. Excretal examination was defined as a microscopic examination of the presence of parasite eggs in the excreta of chickens. The reported individual or flock prevalence of helminth infection was categorized as low (< 11%), moderate (12 to 30%), high (31 to 75%), or very high (76 to 100%). The median prevalence and interquartile range of helminth prevalence were calculated for each category of continents, production systems and helminth species.

3.3.6. Statistical Analysis

All statistical meta-analysis were performed using RevMan Review manager 5.4 (Cochran collaborations, 2020) while regression analysis were performed using JMP14 (SAS Institute Inc., Cary, NC, USA). Due to the anticipated heterogeneity of the prevalence studies, random effect meta-analysis was performed based on inverse variance model using the effect size of the total sample size, number of positive samples, and standard error. Pooled estimated prevalence presented at 95% confidence interval (DerSimonian and Laird, 1986; Field, 2001). The prevalence data (proportions) were transformed into logits (log odds) and analysed using logistic regression as described previously (Sutton et al., 1999; Bland and Altman, 2000; Sanchez et al., 2007; Barendregt et al., 2013). In brief, prevalence proportion odds ratio (POR) = $(P)/(1-P)$. Hence, logit (P) or log (POR) = $\ln(P/(1-P))$, where P is the proportion of prevalence value. Standard error (SE) of the log odds ratio (logits) was calculated as the square root of variance (Var) log odds ratio: SE (ln

(POR) = (Var(ln(POR))1/2. Var(ln(POR) was computed as the sum of reciprocal of the number of positive cases (N +event) and negative cases (N -event) for prevalence data. Var(ln(POR) = $\frac{1}{\text{N+event}} + \frac{1}{\text{N−event}}$. Therefore, SE of logit (P) = SE(ln(POR) = $(\frac{1}{\text{N event}} + \frac{1}{\text{N−event}})1/2$ is equivalent to SE (ln(POR) = $(\frac{1}{\text{N} \times \text{P} \times (1 - \text{P})})1/2$, where P is proportion of prevalence value and N is total number of cases (sample size). The 95% CI of logit proportion was defined as: logit (P) ±1.96 ×SE of logit (P). Thus, 95% CI = $\ln(\frac{\text{P}}{1 - \text{P}}) \pm 1.96 \times \left(\frac{1}{\text{N} \times \text{P} \times (1 - \text{P})}\right) 1/2$; (lower, upper limit). The transformed results of the prevalence estimate for the meta-analysis were back-transformed to obtain informative pooled prevalence and confidence intervals by using the following inverse logits formula as described in Roalfe et al. (2008). Logit^{-1} (P) = $\frac{\exp(P)}{1 + \exp(\text{P})}$

95% CI (back transformation) = $\frac{\exp[\text{logit(P)} \pm 1.96 \times \text{SE logit(P)}]}{1 + \exp[\text{logit(P)} \pm 1.96 \times \text{SE logit(P)}]}$

Heterogeneity between studies was tested by the Tau-squared, I-squared, Cochran's Q test or Chi-squared (P > 0.05) tests. The values of 25%, 50%, and 75% for I^2-statistics testing the degree of heterogeneity was considered as low, moderate, and high, respectively (Higgins and Thompson, 2002). Subgroup meta-analysis was performed to identify potential heterogeneity of prevalence estimates across study continents, years, and production systems and chicken types. Linear trends of helminth prevalence over time was investigated using linear regression analysis. A p-value < 0·05 was considered statistically significant for all analyses.

3.4. Results

3.4.1. Descriptive outcome of systematic studies

3.4.1.1. Description of the dataset

From a total of 2,985 published studies identified in the six search databases and the reference list of eligible studies, 191 published articles were retained after the screening process (Figure 3.1). Applying the JBI critical appraisal checklist, 30 of the

191 articles were deemed to be poor while the remaining 107 and 54 articles were classified as fair and good quality, respectively.

3.4.1.2. ***Data investigation***

A total of 191 studies published between 1942 and 2019 were selected for systematic review. The 191 included studies were conducted in 50 different countries and approximately 66,307 samples with the mean ± SD sample size of 347.2 ± 474.9 were analysed. The current review showed that the number of published studies has increased over time and varied across regions and production systems. Number of studies per continent, year, study design, diagnostic methods, and production systems are shown in Figure 3.2. The majority of studies (178; 93.2%) were conducted during the last two decades whereas the smallest number of studies were conducted prior to 2000. More than 86% of studies were conducted from developing countries of which 39/191 (20%) and 23/191 (12%) studies were conducted in Nigeria and India, respectively. The detail of the individual countries' prevalence study distribution are shown in Figure 3.3. Diagnosis was based on post-mortem examination in 141 studies (73.8%) whereas 55 (28.8%) used the egg flotation techniques (simple flotation, McMaster; or FLOTAC). The vast majority studies were conducted on back yard or village chicken production systems followed by barn/deep litter, cage, and free-range/organic production systems, respectively (Figure 3.2). Furthermore, the majority of studies were conducted on indigenous chicken breeds while a smaller number of studies were conducted on improved commercial layer and broiler chicken strains (Figure 3.2). Additionally, 45 and 31 studies were conducted on female and male chickens respectively while the remaining studies did not specify. Overall, the reported helminth prevalence varied from 4 to100%.

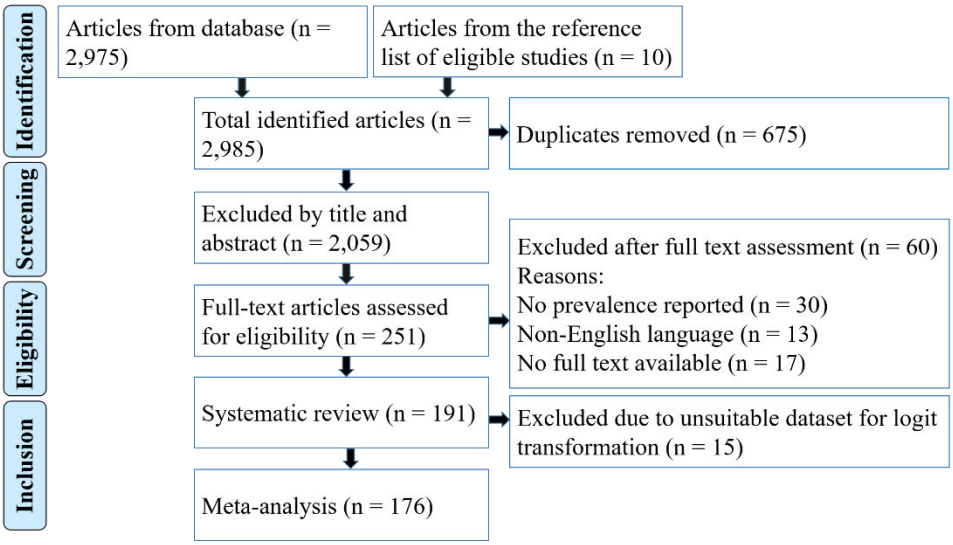


Figure 3.1. Adapted PRISMA article selection process. The figure provides details of the selection of publications used for the systematic review and the meta-analysis.

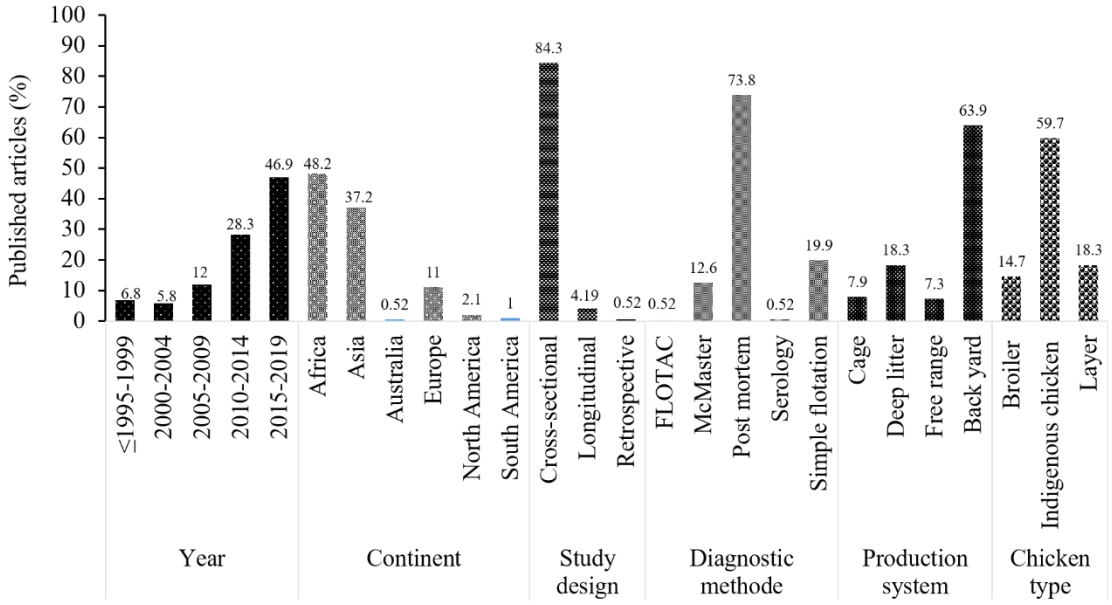


Figure 3.2. Distribution of the 191 research articles by year, region, study design, diagnostic methods, production system, and chicken type.

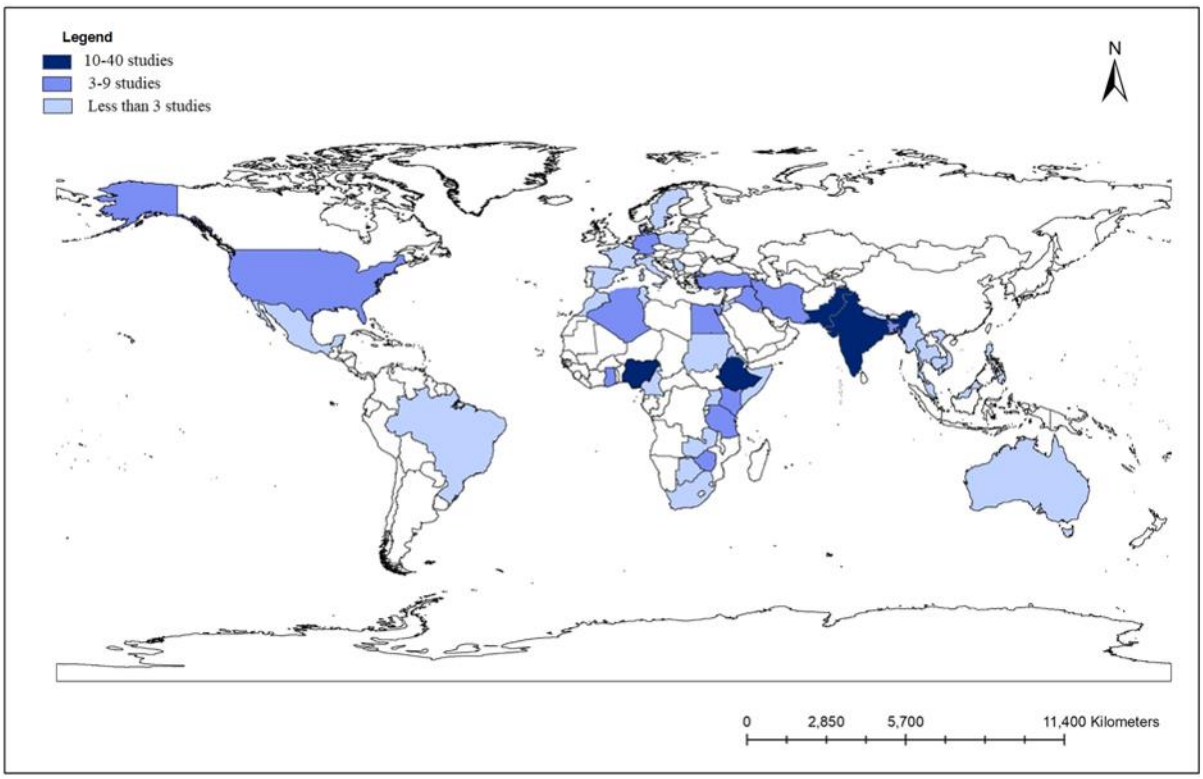


Figure 3.3. Individual countries where the prevalence studies were conducted.

3.4.1.3. Prevalence report by helminth species

More than 30 different helminth parasite species were identified in total, of which *A. galli* (n = 164), *Heterakis gallinarum* (n = 161), *Capillaria spp*. (n = 88), *Raillietina tetragona* (n = 83), *Raillietina echinobothrida* (n = 79) and *Raillietina cesticillius* (n = 70) were the most studied parasite species, respectively. In general, nematodes were the most studied helminth parasite (90%), followed by cestodes (66%) and trematodes (10%; Figure 3.4). The reported prevalence of helminth species varied among species and ranged from 0.30 to100% (Figure 3.5A and B).

3.4.1.4. Spacio-temporal distribution of helminth prevalence and its trend over time

The overall helminth prevalence reported across regions ranged between 4 and 100%. The details of reported helminth prevalence by regions are shown in Figure 3.6. Most studies that reported high prevalence range (31 to 100%) were conducted in Africa (n

= 83), Asia (n = 59), Europe (n = 16), and North America (n = 4). Among 191 studies, 14 studies reported a prevalence of 100% including Africa (11), Asia (2), and Europe (1). The prevalence range in Africa, Asia, and Europe was 11.9 to 100%, 4 to 100%, and 10.5 to 100%, respectively. Regression analysis indicated a decreasing trend in reported helminth prevalence in Africa and Asia whereas an increase helminth prevalence in Europe and North America has been seen recently (Figure 3.7). However, the relatively low R^2 value reflects considerable variation of the reported prevalence.

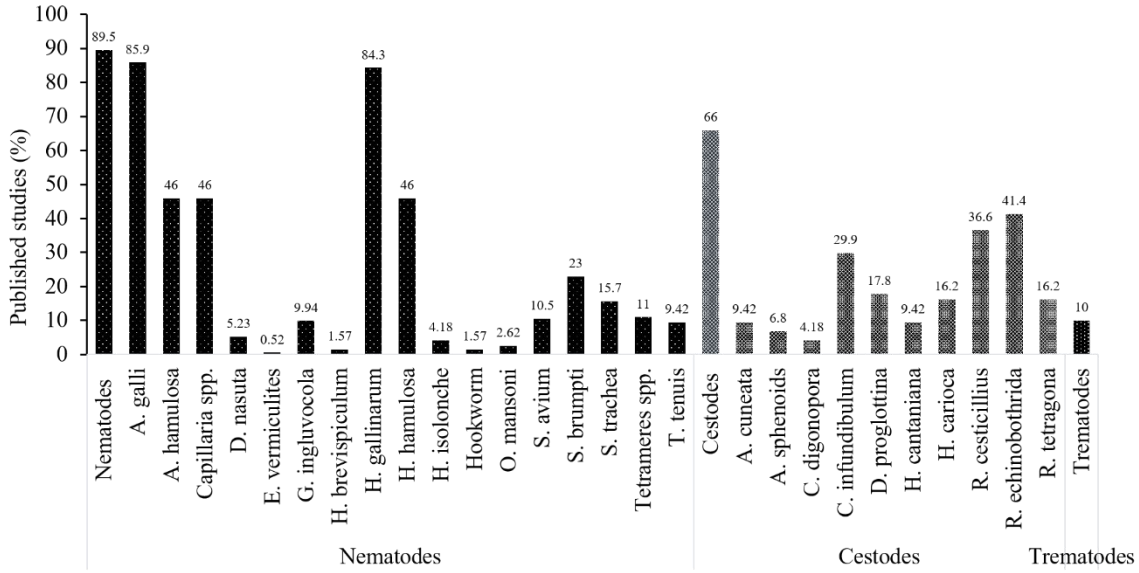
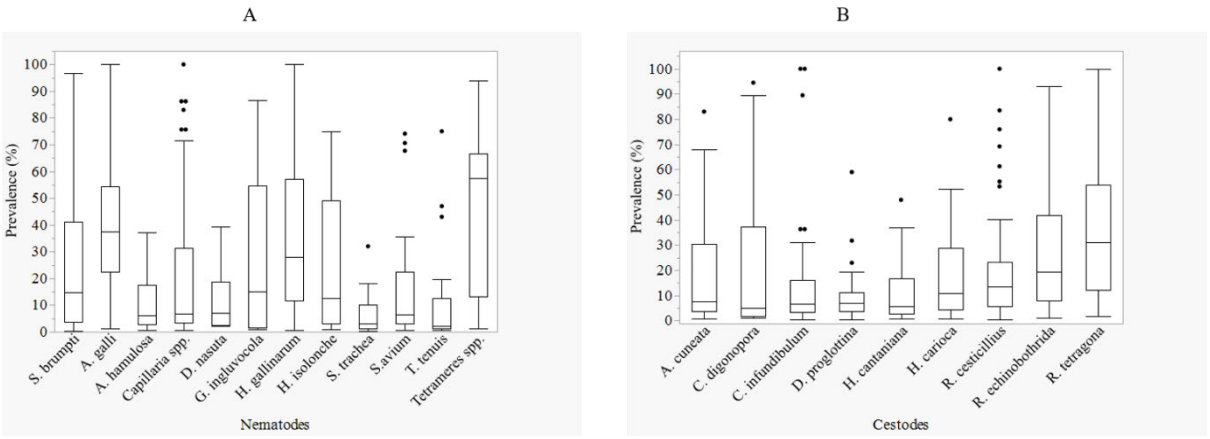


Figure 3.4. Distribution of number of published studies by nematodes, cestodes, and trematodes



species.

Figure 3.5. (A) Prevalence distribution by nematodes and (B) cestodes parasites. The box plots include the minimum and maximum prevalence, median prevalence (Q2), lower quartile (Q1), upper quartile (Q3), and outliers (indicated by dots).

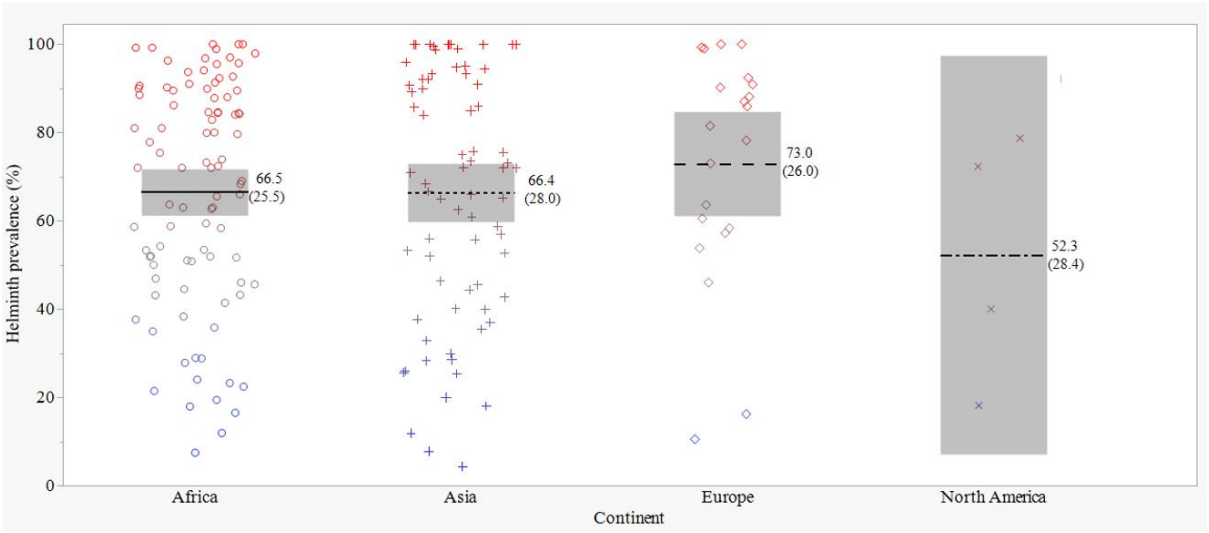


Figure 3.6. Descriptive presentation of the reported helminth prevalence by region. Different coloured symbols represent reported prevalence value for each prevalence study and show the pattern of prevalence distribution across each region. The dot or solid lines indicate the mean ± SD

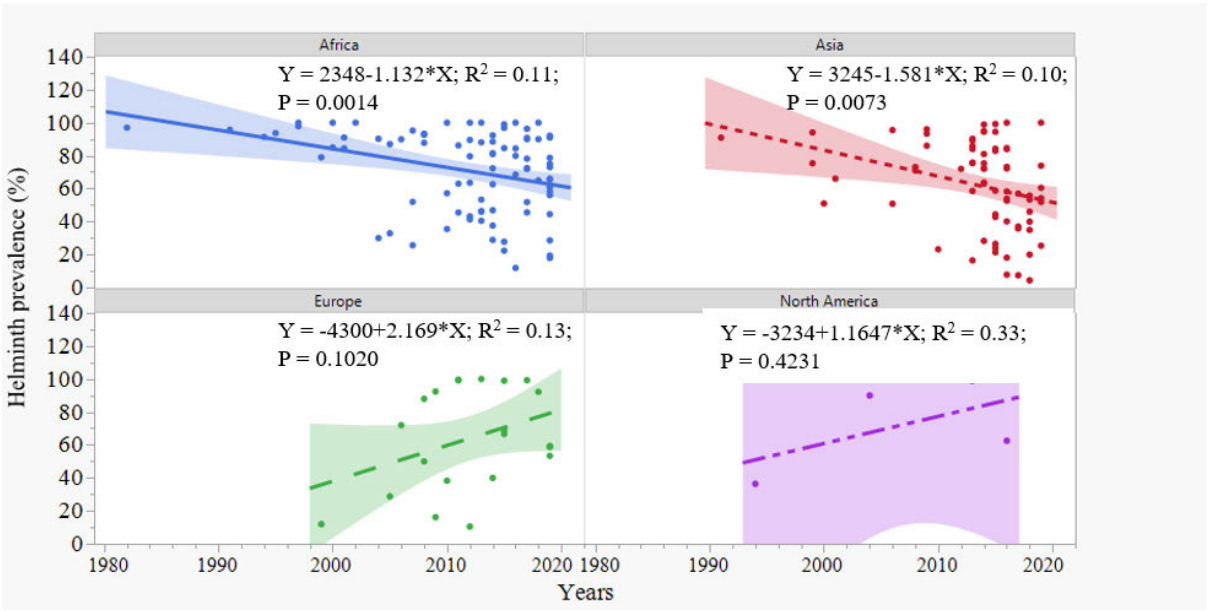


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Figure 3.7. Helminth prevalence reported by year across regions. Each symbol represents one research study. The trend lines were created based on the individual reported values (dots).

3.4.1.5. Reported prevalence by production system

Description of production systems, and chicken type across regions and the prevalence range are presentenced in Table 3.1. A total of 186 studies provided information about their production system. Most of studies that were conducted in back yard or village chickens were from Africa and Asia whereas all free range or organic systems were

conducted in Europe. The prevalence expressed in terms of range, mean ± SD, and quartile range. The prevalence range across production system was 0 to 100%. Most studies (80%) reported a prevalence of 31 to 100%, of which 46.6% of studies reported a prevalence of 76 to 100% and 66.6% of studies were conducted on back yard production with average prevalence of 73.6%. The 14 studies that reported a 100% prevalence were conducted in backyard/village chickens (n = 13) and the free range/organic (n = 1) production system. Furthermore, 35 and 28 studies were conducted on layer and broiler chickens with a prevalence range of 4 to 100% and 0 to 99%, respectively (see Table 3.1). Only 76 studies revealed the sex of their study population: 45 studies were conducted on female chickens while 31 studies conducted on male chickens. The helminth prevalence for males ranged from 11 to 92% with a median of 53% whereas the prevalence of female birds had a wider range (11 to 100%) and higher median (68%).

3.4.2. Meta-Analysis results of published studies

A total of 176 studies were included for meta-analyses with 15 studies excluded due to unsuitable dataset for logit transformation. The result of meta-analysis is summarised in Table 3.2. The overall pooled helminth prevalence estimate was 79.4% and heterogeneity between studies was significantly high ($P < 0.00001$). Therefore, sub-group meta-analysis was conducted using continents, years, production systems, and chicken type. Heterogeneity between subgroup studies ranged from 84 to 97%.

Table 3.1. Description of production systems, chicken types, diagnostic methods, number of studies across continents and their corresponding helminth prevalence rates

Study characteristics		Number of studies				Sample size		Prevalence distribution		
		Africa	Asia	Europe	North America	Mean±SD[1]	Range	Prevalence range (%)	Mean±SD[1]	Quartile (1st, 2nd, 3rd)
Production system	Back yard	71	47	4	0	268 ±368.8	75-3,773	4.39-100	73.6±24.0	58.7, 81.5, 92.5
	Free range	0	0	14	0	447±295.8	60-907	24-100	78.4±20.8	58.3, 81.5, 99.2
	Deep litter	19	9	5	2	457±802.5	45-3,100	1-98.9	43.4±25.9	25.4, 36.6, 58.7
	Cage	5	7	3	0	401±976.9	58-500	0-80	20.8±25.9	3, 11, 31.7
Chicken type	Broiler	11	9	5	3	509±832.1	90-3,542	0-98.9	39.1±32.7	12.1, 33.2, 63.5
	Layer	11	9	15	0	347±375.7	65-1,996	4-100	54.5±30.5	26.5, 57.7, 75.4
	Indigenous chicken	70	43	1	0	228±186.2	75-889	4.39-100	72.7±24.8	58.3, 81, 92.2
Diagnostic method	Post mortem examination	69	56	14	2	294±347.1	55-3,100	4-100	70.6±26.3	52.4, 75.6, 92.5
	Simple flotation technique	20	8	1	1	424±625.8	50-3,773	7.43-100	56.2±24.7	40.3, 58.3, 76.7
	McMaster technique	6	7	6	1	515±527.2	70-1,996	7.8-99.2	56.5±26.2	38.3, 56, 72.3

[1]SD = standard deviation

Table 3.2. Subgroup meta-analysis for pooled prevalence of helminth infection and test of heterogeneity across study period, continents, production systems, and chicken type

	Logit transformed		Logit back transformed		Tests of heterogeneity			
Study characteristics	Pooled proportion (%)	95% CI	Pooled prevalence (%)	95% CI[1]	Cochran's (Q)/ (chi ^{2}) test	I-squared (I^2)	DF[*]	P-value
Year								
< 1995-1999	2.22	1.44-3.43	90.2	27.9-100	358.1	97%	11	< 0.00001
2000-2004	1.63	1.14-2.34	83.6	25.7-99.9	34.9	80%	7	< 0.0001
2005-2009	1.66	1.28-2.15	84.0	27.0-99.8	243.1	91%	22	< 0.00001
20010-2014	1.39	1.22-1.59	80.1	26.9-98.9	475.7	89%	50	< 0.00001
2015-1019	1.15	1.04-1.27	76.0	29.1-97.4	1059	92%	80	< 0.00001
Continent								
Africa	1.49	1.33-1.66	81.6	24.7-99.1	926.9	91%	82	< 0.00001
Asia	1.23	1.10-1.38	77.4	28.4-98.1	921.3	92%	68	< 0.00001
Europe	1.28	1.02-1.61	78.9	29.9-99.1	235.9	92%	19	< 0.00001
North America	1.55	0.89-2.71	82.5	21.7-99.9	33.0	91%	3	< 0.00001
Production system								
Back yard	1.56	1.41-1.72	82.6	23.1-99.3	1059	90%	107	< 0.00001
Free range	1.72	1.36-2.17	84.8	22.6-99.8	76.9	84%	12	< 0.00001
Deep litter	0.91	0.8-1.02	71.3	34.1-94.8	287.3	88%	34	< 0.00001
Cage	0.56	0.38-0.83	63.6	42.9-91.7	62.4	84%	10	< 0.00001
Chicken type								
Layer	1.04	0.87-1.24	73.9	33.9-96.9	413.9	92 %	32	< 0.00001
Broiler	0.77	0.66-0.9	68.4	35.5-93.3	186.6	89%	21	< 0.00001
Indigenous	1.55	1.4-1.71	82.5	23.2-99.3	864.6	88%	102	< 0.00001
Overall effect estimates	1.35	1.26-1.46	79.4	24.6-98.5	2258.2	92%	175	< 0.00001

[1]CI = confidence interval

[*]DF = degree of freedom

3.5. Discussion

This review provides evidence on the prevalence of helminth parasites in chickens stratified by region, production type, helminth species, and diagnostic methods using 191 published studies across the globe from 1942 to 2019. The number of publications on chicken helminth infections increased markedly in the last two decades. Most of the studies were conducted in Africa (n = 92) and Asia (n = 72) followed by Europe (n = 21). The pooled prevalence of helminth infection was 79.4%, ranging across studies from 4 to 100%. The prevalence in Africa and Asia is decreasing significantly overtime while the prevalence in Europe and North America is increasing trend. The vast majority of the studies (73.8%) employed post-mortem examination to detect presence of worms whereas a considerable number of studies were based only egg flotation techniques. *A. galli* and *H. gallinarum* were the most commonly reported helminth parasites followed by *Capillaria* and *Raillietina spp.*

The number of publications on helminth prevalence increases from year to year. The number of publications on helminth prevalence increased exponentially in the last two decades indicating that 1) attention has been given to chicken health by researchers, stakeholders, producers, governmental, and non-governmental institutions at regional, national, and global levels, and 2) scientific evidence and awareness about the impact of helminth infection on chicken profitability and productivity is more frequently communicated. One possible reason might be that chicken health receives more attention due to the increased demand and volume of production as well as a relative shift in egg production in some regions. In addition, the economic impact of helminths on chicken production might have gained larger awareness and welfare concerns result in higher demands on chicken health. As a consequence, veterinary interest has expanded which might have supported not only diagnostic procedures but also initiate research interest on chicken health.

Most prevalence studies were conducted in Africa (n = 92), followed by Asia (n = 72), and Europe (n = 21). One reason for the high interest on helminths in Africa and Asia may be due to the fact that approximately 80% of meat and eggs produced in these continents are from back yard chickens and small-scale production system (Pym et al.,

2006; Dube et al., 2010) where not only the uncontrolled exposure to the soil, but also the warm and partially humid tropical climate provides ideal conditions for the presence of insect vectors and the helminths to develop. In contrast, Europe and North America housed their chicken traditionally in barns or cages with only recent shifts to free-range, also reflected in the recent increased number of publications in these countries.

Overall pooled prevalence of helminth infection in chicken was 79.4%, ranging across studies from 4 to 100%. Reasons for this variation might be due to the large variety of production systems, management procedures investigated, the broad range of the agro-ecological zones being investigated, climatic/environmental conditions, seasonal dynamics, the number and availability of intermediate hosts involved, the diagnostic and sampling methods used, and various host factors including susceptibility to genetic resistance of the host. While evidence is provided that a high prevalence (> 76%) of helminths can be obtained in every continent, most of the high prevalence studies were conducted in Africa (n = 83) with the overall pooled prevalence of 81.6%. The possible reasons for these may be associated with a relatively high proportion of studies being conducted in back yard production systems (Abebe et al., 1997; Nnadi and George, 2010; Abdullah and Mohammed, 2013). In addition to this, the tropical climatic condition is suitable/favorable for the propagation and development of the infective stage of helminth infection (Abebe et al., 1997; Ben Slimane, 2016). Adequate moisture and temperature of the environment in the tropics are indeed the most determinant factors for the development of the infective stage of helminth parasite and that may influence the epidemiology of parasite infection (Wuthijaree et al., 2019). Although a high pooled helminth prevalence was reported in developing countries associated with the backyard production system and environmental factors, the prevalence is decreasing overtime in these countries most likely due to increasing access to anthelmintic treatment over the last few decades. However, the current review showed that a high prevalence (78.9%) was also reported across European countries with increasing prevalence overtime. This is likely linked with the re-emerging of helminth infection due to shifting production systems into a free-range and organic production system (Jansson et al., 2010; Thapa et al., 2015). Moreover, anthelmintic application has been prohibited in the organic production systems in

Europe, further promoting helminths as a re-emerging disease (Kaufmann et al., 2011a; 2011b; Wongrak et al., 2015a).

Most studies were conducted on backyard and free range systems reported a relatively high prevalence rate. This is reflected in the fact that 14 back yard and free-range studies reported a 100% prevalence. These findings agreed with the report of Permin and Hansen (1998) and Nnadi and Georg (2010). Moreover, the overall pooled prevalence in back yard and free-range production systems was 82.6 and 84.8%, respectively. These results are not surprising and the most likely explanation would be due to scavenging activities and roaming habit of free-range and backyard chicken where the direct contact with excreta and soil, and the non-commercial approach of owners would most likely include poor management including a lack of anthelminthic drug application (Abdullah and Mohammed, 2013; Abdelqader et al., 2008). Furthermore, the free-range and back yard scavenging production system can play an important role in shedding helminth infections by contaminating the environment with parasite eggs and larvae (Mwale and Masika, 2011; Wamboi et al., 2020). In contrast, birds housed in caged systems off the ground are largely separated from their excreta, disrupting the direct life cycle of the parasite which is reflected in the significantly lower average prevalence compared to back yard and free range (Permin et al., 1999; Permin and Hansen, 1998). However, some caged flocks still experienced a prevalence of up to 80%, which is somewhat surprising and the authors suggested poor sanitation including cross contamination of feed with excreta and heavy accumulation of excreta and dust that may have facilitated the fast development and propagation of embryonated eggs and possibly insect vectors (Ponnundurai and Chellappa, 2001). Another possible reason may be that the breeder pullets have been reared on the floor and therefore became infected in the rearing facility prior to being caged. In addition, caged birds are more commonly laying hens and the longer life span of these hens (usually at least 70-80 weeks, sometimes multiple laying cycles), allows for a greater manifestation of helminth infection compared to broiler production where the life span may be as short as 32 days (McDougald, 2020).

Detection of the presence of helminth parasite in chicken population was mostly carried out using either post-mortem examination of chicken's gastrointestinal tract or excretal examination. Vast majority of the studies (73.8%) employed post-mortem

examination, and some egg flotation techniques (simple flotation, McMaster technique, and FLOTAC) were employed in studies subject to this review to detect presence worms in chickens. Our systematic review indicated that post mortem examination, known for its accurate and reliable diagnostic approach for helminth species identification was a widely used diagnostic method (Macklin, 2013) but may not be the most economically viable method for routine diagnosis when sacrificing birds only for this purpose. However, equipment such as a microscope and glass slides are required to use alternative, less invasive techniques such as the flotation technique based on excreta egg count analysis (Das et al., 2017). In addition, due to the intermittent egg shedding nature of the nematodes and allows only for reliable egg detection if samples were collected over multiple days (Das et al., 2011c; Das et al., 2017). With the McMaster techniques being known for its uncomplicated and cheap use, it was unsurprisingly the most commonly used technique (12.6%) whereas the relatively new FLOTAC method with improved sensitivity and precision was only used once due probably to the expense of equipment and time (Cringoli et al., 2010; Das et al., 2020).

Nematodes were the most studied helminth parasite (90%), followed by cestodes (66%) and trematodes (10%). The most commonly reported and prevalent nematodes species were A. *galli* (35.7%), *H. gallinarum* (29.5%), and *capillaria species* (5.90%) with an overall prevalence ranging from 0.30 to 100%. *A. galli* was the most prevalent helminth in all production systems and co-infections with *H. gallinarum* were common, which is not surprising given the fact that both parasites have the same life cycle and require same environmental conditions (Das et al., 2017). Cestodes were detected less often, with the highest prevalence of cestodes species being *Raillietina species*, including *R. tetragona* (30.6%), *R. echinobothrida* (20.0%) and *R. cesticillius* (13.0%), once again being highest in back yard and free-range chicken most likely due to the reasons discussed above. In addition, cestodes require an intermediate host such as houseflies or beetles for their transmission, their overall lower prevalence may have been linked to less opportunities of transmission (Dube et al., 2010). Likewise, trematodes require two to four intermediate hosts to complete their life cycle, and the eggs hatch only in water (Permin and Hansen, 1998; Abdelqader et al., 2008; Mcdougald, 2020). As a result, basic biosecurity that prevents chicken to access lake

or water bodies might have been sufficient to control the transmission of these trematodes.

Heterogeneity test indicated that substantial heterogeneity was found between prevalence studies. Source of heterogeneity between prevalence studies may arise due to methodological and clinical variation which contribute for the presence of statistical heterogeneity (variation of effect size between studies) (Higgins and Thompson, 2002). Barendregt et al. (2013) argued that the main concern for meta-analysis is heterogeneity; however, the aim of disease burden studies, such as prevalence study is to obtain best prevalence estimate based on the available data. Thus, a reported pooled prevalence estimate is considered to be valid as a measure of helminth prevalence. The potential source of variation between studies in the current meta-analysis may be due to variation in study design, sampling methods, level of exposure to parasite, physiological and genetic status of animal, sample size, study location, environmental condition, and diagnostic methods.

3.6. Conclusions

Helminths are globally common and highly prevalent in chicken with significant variation across the regions and production systems. The prevalence is decreasing overtime in developing countries due to increasing recognition and regular deworming over the last few decades. However, a higher helminth infection prevalence has been recently seen in a certain regions where a continuous shifting from cages towards the extensive systems (free-range, organic, backyard). The rapid growth of commercial free-range production has changed the dynamicity of helminth transmission; hence, epidemiological evidences on the existence, spreading, and the underlying factors are needed to understand transmission models and institute control strategies.Therefore, increased awareness amongst those producers combined with access to accurate and early diagnosis would be crucial for early intervention.

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STATEMENT OF AUTHORS' CONTRIBUTION

We, the Research PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

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Chapter 4. Prevalence and magnitude of gastrointestinal helminth infections in cage-free laying chickens in Australia

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4.1. Abstract

Helminth infections have been re-emerging with the growing popularity of free-range and floor-based chicken production systems. The aim of this study was to determine the prevalence and worm burdens of intestinal helminth infection in cage-free laying chickens in Australia. In an online survey of worm prevalence, a high proportion of respondents reported detection of *Ascaridia galli* (77%), followed by tapeworms (69%) and caecal worms (*Heterakis gallinarum*) (62%), whereas fewer respondents (23%) reported the presence of hair worms (*Capillaria spp.*) in their flocks. Total worm recovery from 407 laying hens on four farms found that 92.1% of hens harboured one or more helminth parasite with a prevalence of 73 to 100% across farms. Mixed infections were common with 79% of hens harbouring two or more helminth species. The prevalence of nematode species *H. gallinarum, A. galli* and *Capillaria spp*. was 87, 82 and 35% respectively. Five cestode species were found with a low individual chicken prevalence (*Raillietina tetragona* 4.7%, *Raillietina echinobothrida* 3.2%, *Raillietina cesticillus* 5.2%, *Choanotaenia infundibulum* 4.4%, *and Hymenolepis cantaniana* 4.4% were found. The hens harboured an average of 71 worms with *H. gallinarum* having the highest mean burden (45.5 worms/hen) followed by *A. galli* (22.0 worms/hen), *Capillaria spp*. (2.7 worms/hen) and cestodes (0.8 worms/hen). The sex ratio (female: male worms) was 1.38:1 for *A. galli*, and 1.77:1 for *H. gallinarum*. There was a strong positive correlation between *A. galli* female worm count and excreta egg count (EECs) (r_s= 0.94, P < 0.0001) and also between total nematode worm count and EEC (r_s = 0.82, P < 0.0001) in individual hens. When investigating intestinal excreta (n = 10) and caecal excreta (n = 10) of 16 chicken flocks the prevalence of infection with ascarid worms in intestinal and caecal excreta was 71 and 78% respectively and 27% prevalence of *Capillaria spp*. in intestinal excreta with mean EECs of 407, 404, and 18 eggs/gram of excreta (EPG), respectively. These results suggest that most chickens kept in free range or floor production systems are infected with one or more helminth parasite species. Heavy worm infections would likely affect the production performance and welfare of birds with adverse economic impact. Strategic or tactical anthelmintic treatment with effective anthelmintic could reduce this impact.

4.2. Introduction

In response to public concern for animal welfare, the adoption of non–cage production systems for chickens (e.g. free range, organic, and floor production) has increased significantly (Miao et al., 2005). However, such systems increase the risk of infection with helminth parasites, due to increased contact with infective eggs in faeces or intermediate hosts, allowing helminths to complete their lifecycle. As a result, helminth infections have been re-emerging in commercial free range egg production systems (Permin et al., 1999b; Kilpinen et al., 2005; Jansson et al., 2010; Kaufmann et al., 2011a; Kaufmann et al., 2011b; Wongrak et al., 2014; Thapa et al., 2015a; Wuthijaree et al., 2017; McDougald, 2020; Shifaw et al., 2021a) and is expected to increase further with economic and welfare implications (Scott et al., 2009b; Ruhnke, 2015a; Scott et al., 2017). Several studies outside of Australia have found that more than 50% of the free range laying hens harbour nematode infections with *Heterakis gallinarum*, *Ascaridia galli* and *Capillaria spp*. being the most prevalent and important in terms of animal welfare, health and economic consequences (Permin et al., 1999b; Kaufmann and Gauly, 2009; Kaufmann et al., 2011b; Wongrak et al., 2014; Thapa et al., 2015a; Wongrak et al., 2015a; Wuthijaree et al., 2017). The last prevalence survey conducted in Australia was in Queensland by Broadbent (1942) who reported individual bird infection prevalence of 76.7%, 39.6% and 6.9 % for *H. gallinarum*, *A. galli* and *Capillaria spp.* respectively.

Helminth infections can be associated with retarded growth, weight loss, and reduced egg production, poor absorption of nutrients, changed hormone levels, and adverse effect on behaviour patterns (Ackert and Herrick, 1928; Ikeme, 1971a; Kilpinen et al., 2005; Gauly et al., 2007; Daş et al., 2010; Daş et al., 2011d; Sharma et al., 2018a). Furthermore, heavy or severe *A. galli* infections may cause intestinal obstruction and haemorrhage, leading to paralysis of legs, diarrhoea, and death in severe cases (Ackert and Herrick, 1928; Ramadan and Znada, 1991; Macklin, 2013; Torres et al., 2019; McDougald, 2020). Additionally, *A. galli and H. gallinarum* may serve as animate vectors for bacterial (e.g. salmonellosis, *Pasteurella multocida* and *Escherichia coli)* and protozoan (e.g. histomoniasis) infections (Dahl et al., 2002; Chalvet-Monfray et al., 2004; Eigaard et al., 2006; Permin et al., 2006; McDougald, 2020). *A. galli* can also supress the host's immune system as revealed by impaired responses to Newcastle

disease vaccination (Hørning et al., 2003; Pleidrup et al., 2014; Dalgaard et al., 2015). This may increase susceptibility to secondary bacterial infection and mortality in organic free range laying chickens (Hinrichsen et al., 2016).

The pathogenicity of the majority of tapeworm species remains unknown and it has been suggested that most tapeworm species are either harmless or mildly pathogenic while few species such as *Raillietina echinobothrida* can cause enteritis and weight reduction in young chickens (Samad et al., 1986; Permin and Hansen, 1998). Helminth infections occur either when the host ingests the infective parasite eggs (embryonated eggs containing infective larvae) or by ingesting transport or intermediate hosts (Permin et al., 1999b; Jansson et al., 2010; Kaufmann et al., 2011b; Wongrak et al., 2015a). Preventing infection in the free-range system is difficult due to the close contact of chickens with their excreta and animate vectors as well as the durability of infective eggs in the environment. This favours parasite population dynamics as parasite eggs can accumulate and potentially develop infectivity of the soil, litter, pasture or animate vectors (Thapa et al., 2015a; Wongrak et al., 2015a). Especially in the outdoor environment, parasite eggs at infective stages cannot be removed easily, thus acting as a reservoir of infection due to environmental resistance of nematode eggs, particularly eggs of *A. galli and H. gallinarum* (Permin and Hansen, 1998; Thapa et al., 2017b).

The presence of helminth infections in poultry is mostly diagnosed by post-mortem examination of the intestinal tract to visualise the parasites or by microscopic examination of the excreta to visualise the helminth eggs. Post-mortem examination, coupled with processing of gut contents to recover parasites, enables accurate and reliable species identification, and total worm burden that indicates the intensity of infection (Macklin, 2013; Zloch et al., 2021; Shifaw et al., 2021a). However, this requires killing of birds which may not economically viable for routine diagnosis. Routine examination of culled or dead birds is useful and can provide some indication of flock infection, but it may not be representative of the population under consideration. The occurrence and magnitude of nematode infection in the living birds is also estimated by excreta egg counts (EEC) which are expressed in terms of eggs per gram of excreta (EPG) that are expected to correlate with the worm burden of the host animals (Thapa et al., 2015a; Daş et al., 2017). However, the relationship between

EEC and worm count is affected by several biological factors, including fluctuations in female egg production between and within day, the sex ratio of the worm population, excreta consistency, uneven distribution of eggs within the excreta, variation in the rate of production of host excreta, effects of host immunity and worm density of female worm fecundity, the sensitivity of excreta egg counting techniques, and the similarity of the eggs of *H. gallinarum* and *A. galli* (Permin et al., 1997a; Permin et al., 1998b; Daş et al., 2011c; Daş et al., 2017). Furthermore, *H. gallinarum* is located in the caeca and its eggs are shed irregularly to the external environment in caecal excreta passed only a couple of times per day in caecal droppings so reliable *H. gallinarum* egg detection requires excreta samples were collected over multiple days (Daş et al., 2011c; Daş et al., 2017). Prevalence data may also be estimated by survey questionnaires of farmers or veterinarians but this is considered less reliable than direct measurement using parasitological methods.

In order to design and implement appropriate and sustainable control measures against gastrointestinal helminth infection in cage-free production systems, it is important to determine the prevalence, spectrum and the magnitude of helminth infection in this system. We were not able to find any formal epidemiological information on gastrointestinal helminth infection in the Australian chickens since 1942. However, a recent survey of semi-intensive free-range egg producers conducted by Singh et al. (2017) revealed that 32% of producers noticed external or internal parasites in their flock while the remaining 68% could not see sign of parasite infestation or never/rarely checked their flocks for parasite infestation. Therefore, the aim of this study was to evaluate the importance, prevalence and magnitude of gastrointestinal helminth infections in the commercial cage-free egg production sector in Australia.

4.3. Materials and Methods

Three different studies were undertaken to achieve the objective. Firstly, an online worm prevalence survey was conducted using an on-line questionnaire to determine the attitudes of cage-free egg producers towards worm prevalence and their willingness to provide excreta samples for further studies. Secondly, excreta samples from 16 flocks were assessed for excreta egg counts to provide information on

prevalence of nematode infections and magnitude based on egg counts. Thirdly, an in-depth prevalence study based on total worm counts using end-of-lay hens was conducted on four free-range farms. This enabled detailed assessment of worm prevalence, the magnitude of infection and spectrum of helminth parasites. The use of animals for this research was approved by the Animal Ethics Committee of the University of New England (approval number AEC19-082).

4.3.1. Study 1. On-line worm prevalence survey

A descriptive cross-sectional survey using an anonymous internet-based questionnaire was employed to assess farmer attitudes, experiences and practices regarding helminth infections and their control. The survey comprised 37 questions which contained questions about participant demographics, farm and flock characteristics, perceived intestinal worm importance, infection monitoring, deworming and worm control practices, and other information, including willingness to participate in on-farm surveys of worm prevalence. A more detailed description of this survey and results pertaining to farm and flock characteristics, anthelmintic use and practices and other worm control measures has been published recently (Feyera et al., 2022a). This paper reports some aspects of farmer demographics and perceived importance and prevalence of worm infections. A link for the survey was emailed to cage-free egg producers from their industry body in December 2019. Invitations to participate containing a link to the on-line survey were sent by email to 203 cage-free egg farmers who were members of Australian Eggs Ltd (AEL). The target population for this survey was commercial cage-free egg farmers across Australia registered with the industry association AEL with the following pre-defined inclusion criteria: i) accredited as cage-free egg producers ii) have been engaged in cage free production for at least three years, and iii) housing more than 500 hens in their farm. Farmers were invited to participate in the survey via an email invitation from AEL containing a link to the online information sheet, consent form, and questionnaire. The survey and associated documentation was approved by the University of New England Human Ethics Committee (approval number HE19-207).

4.3.2. Study 2. Prevalence and egg count study based on excreta submission

Respondents to the on-line surveys who had voluntarily agreed to participate in further studies and provided contact information were requested to submit 20 fresh excreta samples comprising 10 intestinal excreta and 10 caecal excreta from each of two flocks on the farm (approx. 3 g each). Intestinal excreta are normally solid in consistency, contain white crystals of urates and are expected to contain mainly *A. galli* eggs whereas caecal excreta are brownish, pulpier and contain mainly eggs of *H. gallinarum* (Thapa et al., 2015a; Cupo and Beckstead, 2019). While there are some morphological differences between the eggs of the main ascarid parasites *A. galli* and *H. gallinarum* (Zloch et al., 2021), they cannot be easily differentiated morphologically so in this study the eggs are reported as ascarid eggs as described previously (Thapa et al., 2015a; Daş et al., 2020). The eggs of *Capillaria spp*. are distinctive and easily distinguished from the other species. Samples were submitted in a kit supplied to farmers including collection instructions, individual excreta sample vials, spatulas and labelled bags to separate intestinal from caecal samples, a lab submission form and pre-paid addressed return mail pouch to submit the samples. Samples from 16 flocks were submitted as detailed in Table 4.1. Of the 16 flocks, 10 (62.5%) were kept in barn systems whereas the remaining 6 (37.5 %) were from free range systems. The chicken strain was Hyline Brown and flock sizes ranged from 13,000 to 34,000 chickens per flock. Average age at the time of sampling for pullet and adult birds was 11±2 and 47±14 weeks (mean ± SD), respectively.

4.3.3. Study 3. Prevalence and worm count study

A cross-sectional study was conducted in four selected free-range farms across Australia. This was conducted just prior to scheduled depopulation and killing of the hens at the end of their productive life (75-104 weeks of age). Farms that met the Australian free-range egg production guidelines, as well as farmers willing to provide randomly selected hens for sacrifice during scheduled depopulation, were chosen for flock/farm recruitment. Details of the four selected farms are presented in Table 4.2. Of the 4 farms, two farms were organic free range systems whereas the remaining 2

farms were conventional free range systems. Organic free-rage systems do not use anthelmintic or other synthetic chemicals for bird treatment, whereas conventional free-rage systems may do so if necessary. The strain of chicken on farm 1 was Hyline Brown and was ISA Brown on the other farms. Flock sizes ranged from 1,500 to 8,000 hens per flock. Range of age at the time of sampling was between 75 and 104 weeks. A sample size of 100 hens per flock was used based on an assumed prevalence of 50% with a desired absolute precision of 10% and a 95% level of confidence (Thrustfield, 1995). The randomly selected hens (407 in total) were subject to detailed post-mortem examination for worm count in accordance with the guidelines of the World Association for the Advancement of Veterinary Parasitology (WAAVP) (Yazwinski et al., 2003).

Table 4.1. Details of participating egg farms in Study 2 (excreta submission).

Flock	State	Farm No	Production system*	Age of chickens (weeks)	Strain of chickens	Age of chickens at last deworming	Number of chickens/flock
1	Victoria	1	Con. free-range	34	Hyline Brown	8 weeks	NA
2	Victoria	1	Con. free-range	55	Hyline Brown	8 weeks	NA
3	Victoria	2	Barn	8	Hyline Brown	Never	27000
4	Victoria	2	Barn	16	Hyline Brown	Never	27000
5	Victoria	3	Barn	11	Hyline Brown	Never	34000
6	Victoria	3	Barn	10	Hyline Brown	Never	34000
7	Victoria	4	Barn	11	Hyline Brown	Never	13000
8	Victoria	4	Barn	9	Hyline Brown	Never	13000
9	Victoria	6	Barn	10	Hyline Brown	Never	27000
10	Victoria	6	Barn	11	Hyline Brown	Never	27000
11	Victoria	7	Barn	12	Hyline Brown	Never	17000
12	Victoria	7	Barn	14	Hyline Brown	Never	13000
13	Tasmania	5	Org. free-range	42	Hyline Brown	Never	15000
14	Tasmania	5	Org. free-range	53	Hyline Brown	Never	19000
15	Tasmania	5	Org. free-range	29	Hyline Brown	Never	19000
16	Tasmania	5	Org. free-range	69	Hyline Brown	Never	15000

* NA =not available; Con. = conventional; Org. = organic

Table 4.2. Details of participating farms in Study 3 (prevalence and worm count)

Farm information	Farm 1 (QLD)	Farm 2 (Costal NSW)	Farm 3 (Northern NSW)	Farm 4 (West NSW)
Chicken strain	Hyline Brown	ISA Brown	ISA Brown	ISA Brown
Sampling date	08/2020	10/2020	12/2020	12/2020
Hen age at sampling	80 weeks	78 weeks	104 weeks	75 weeks
Housing system	Org. free- range	Org. free-range	Con. free-range	Con. Free-range
Flock size	3416 hens	8000 hens	4000 hens	15000 hens
Indoor stocking density	9 hens/m^2	4 hens/m^2	NA	1 hen/m^2
Outdoor stocking density	900 hens/ha	615 hens/ha	1250 hens/ha	10000 hens/ha
Anthelmintic treatment used	Never	Never	Yes	Yes
Hen age at last anthelmintic treatment	Never	Never	94 weeks	13 weeks
Anthelmintic compound	None	None	Piperazine	Mebendazole

QLD = Queensland; NSW = New South Wales; NA= Not available; Con. = conventional; Org. = organic

4.3.4. Field and laboratory methods

4.3.4.1 Excreta egg count (EEC)

Excreta egg count (EEC) was determined by the McMaster technique employed following the procedure described by Shifaw et al. (2021b) providing a limit of detection of 40 EPG. In brief, 2.5g of each excreta sample was diluted in 47.5 ml of saturated salt solution (specific gravity = 1.20), thoroughly homogenised, sieved and a 0.5 ml aliquot loaded into a chamber on a Whitlock universal slide and examined under 40 x magnification power. Eggs counted were multiplied by 40 eggs/gram of excreta (EPG) to provide an EEC in EPG.

4.3.4.2. Helminth collection and identification

Birds were humanely killed by cervical dislocation on location at each farm. Immediately after killing, gastrointestinal tracts were removed from the carcass, packed in labelled sealable plastic bags, placed on ice and transported to the parasitology laboratory of the Department of Animal Science, University of New England. The samples were then either stored at 4°C for examination within 48h, or frozen at −20°C for later parasitological examination. For the examination, the gastrointestinal tracts were opened longitudinally from the oesophagus to the rectum, including both caecal tubes, using Mayo scissors. Contents of each section (crop, gizzard, duodenum, jejunum, ileum, large intestine and both caecal tubes) were sieved with a 100 μm mesh sieve, flushed with tap water and all visible worms retained on the sieve were collected and counted then the remaining sample and the scraped mucosa in a Petri dish containing saline were examined under a stereomicroscope (40x) to count immature and microscopic worms. Species differentiation was based on morphological characteristics (i.e. helminthological keys) as described elsewhere (Permin and Hansen, 1998; Yazwinski and Tucker, 2008; McDougald, 2020). Adults of the key ascarid worm species (i.e. *A. galli* and *H. gallinarum*) were sexed to determine sex ratio using helminthological keys: Female *A. galli* worms are longer than males having a straight posterior terminal, whereas males have a curved posterior terminal. Male *H. gallinarum* worms have a straight tail with dissimilar spicules, whereas females have a long, narrow, and pointed tail (Permin and Hansen, 1998;

Yazwinski and Tucker, 2008; McDougald, 2020). Harvesting of cestodes were done by submerging the intestine in water floating the worms to increase their visibility. The worms, including the scoleces were removed easily from mucosa with fine needles. Staining-destaining techniques with Harris' Haematoxylin dye were used to identify the morphology of key cestode organs (e.g. Scolex, rostellum, suckers, number of genital pores per segment, and eggs within an egg capsule in gravid proglottide) as described previously (Sepulveda and Kinsella, 2013). Scoleces were counted for worm burden determination. These data were used to calculate species-specific prevalence and worm burden per hen. During post-mortem sample collection, digesta samples (approximately 4g) were taken from the terminal end of the large intestine of each chicken for excreta egg count (EEC) determination.

4.3.5. Statistical analysis

All statistical analyses were performed using JMP®16 (SAS Institute Inc., Cary, NC, USA). For the online survey, responses were downloaded, checked for meaningful content and coded prior to statistical analysis. Free text statements grouped according to similarity where appropriate. For each variable, a descriptive analysis was carried out, producing frequencies for categorical variables and means and medians for continuous variables, by respondent characteristics of interest. The prevalence of individual helminths species was calculated as the proportion of the host population examined infected with a specific parasite during the study. Mean worm burden and EEC were tested for normality. Both worm count and EEC data had skewed distributions with most chickens having low burdens. Therefore, non-transformed means are presented with medians and ranges. Worm count and EEC were transformed by cube root for statistical analysis. One-way analysis of variance was used to analyse transformed worm count and EEC fitting the fixed effect of farms, flocks or bird age (adult > 20 weeks, pullet < 20 weeks). The Tukey HSD test was used to compare mean worm burden between farms or flocks. Contingency table analysis using the Chi-square test was used to test prevalence data for significant differences between farms. Spearman correlation (r_s) was used to determine the degree of association between parasitological measurements. A significance level of $P \leq 0.05$ was used for all analyses.

4.4. Results

4.4.1. Study 1. Online survey

A total of 16 participants responded to this survey which represented 8% of the 203 free-range egg farmers who were sent an email request to participate in the survey. The respondents were drawn from all states of Australia with highest representation from Victoria (5), New South Wales (4) and Queensland (3). The majority of the egg producers (38%) had 30–40 years of poultry farming experience, while a slightly lower proportion (31%) had less than 10 years of experience. Regular monitoring of hens for intestinal worm infection was practiced by 61% of respondents and occasional monitoring by 31% with only a single respondent (8%) indicated that they never monitor for worm infection. The monitoring frequency ranged widely from weekly or continuous investigation which included opening of culled/fresh dead hens (33%) to annually (8%). The most common method of monitoring parasites was by necropsy and physical detection of parasite in the intestine (83%) while 25% used excreta egg counts and 8% examined excreta for the presence of worms. Worm examinations were mostly performed by farm or company personnel (92%), with only 8% relying solely on laboratory diagnosis. Use of veterinarians or laboratories for monitoring was reported by 42% of respondents. A high proportion of respondents reported detection of *A. galli* (77%), with similar proportions reporting detection of tapeworms (69%) and caecal worm (*Heterakis spp.*) (62%) whereas small numbers of respondents reported the presence of hair worms (*Capillaria spp.*) (23%).

4.4.2. Study 2. Prevalence and egg counts based on submission of excreta

The estimated prevalence and EEC of chicken ascarid parasites (*A. galli/H. gallinarum)* and *Capillaria spp.* in caecal and intestinal excreta are presented in Table 4.3. Ascarid eggs were detected in both intestinal and caecal excreta, but *Capillaria* eggs were only detected in intestinal excreta. All flocks were infected with each of the

nematodes with ascarid (*A. galli/H. gallinarum)* having the highest mean prevalence (74.8%) followed by *Capillaria spp.* (27.3%). Prevalence (P = 0.0004) and EEC (P < 0.0001) of ascarid eggs differed significantly between flocks in intestinal but not caecal samples, and not for *Capillaria spp.* Mean ascarid egg counts were very similar in intestinal *(*407, range 0 – 6600) and caecal (404, range 0 – 6480) excreta. Due to the skewed nature of the data, median values were much lower being 120 and 200, respectively. *Capillaria* spp. had much lower egg counts (mean EEC = 18, range = 0 – 160) (Table 4.3). When EEC was analysed on the transformed scale, the 6 mature layer hen flocks had significantly higher ascarid burdens in caecal excreta than the 10 pullet flocks (untransformed means of 599 and 276 EPG respectively; P = 0.01). There was an opposite, but non-significant trend in intestinal excreta with higher counts in pullets (440 EPG) than hens (345 EPG) (P= 0.49; Table 4.4). As the older flocks came from two farms, and the younger flocks for 3 different farms, the effect of age cannot be clearly separated from a flock effect. EEC (P = 0.046) of ascarid eggs differed significantly between production systems in caecal samples but not intestinal excreta samples for ascarid and *Capillaria spp*. (Table 4.4).

4.4.3. Study 3. Prevalence and worm counts based on post mortem gut examination

The prevalence and worm counts of helminth parasites based on post-mortem examination are presented in Table 4.5. Out of 407 hens, 92.1% harboured one or more helminth parasites. The prevalence of helminth parasites ranged from 73 to 100% across farms. The most prevalent species of nematodes were *H. gallinarum* (86.7%), *A. galli* (81.6%) and *Capillaria spp*. (34.9%), with significant variation between farms (Table 4.5). The prevalence of cestodes was 12%. Identified cestodes spp. were *Raillietina tetragona (*4.70%), *Raillietina echinobothrida (*3.20%), *Raillietina cesticillus (*5.20%), *Choanotaenia infundibulum (*4.40%*), and Hymenolepis cantaniana (*4.40%*)* (Table 4.5). A large proportion of the hens, 44.2 and 31.2% were infected with two and three helminth species respectively, while 13.3% harboured one and 3.4% harboured four species (Table 4.6). The highest worm burden was for *H. gallinarum* with a mean of 45.5 worms per hen. Mean worm counts for *A. galli, Capillaria spp*. and cestodes were 22.0, 2.72 and 0.78 worms per hen respectively (Table 4.5). The hens harboured an average of 71.0 worms of which 70.0 were

nematodes. There was variation in prevalence, worm count and parasite composition across farms. The sex ratio (female: male worms) was 1.38:1 for *A. galli*, and 1.77:1 for *H. gallinarum* (Table 4.5).

4.4.3.1. *Correlation between major parasitological parameters*

The association between different parasitological measurements on individual chickens across all farms is shown in Table 4.7. There were strong positive associations between most variables. With regard to the association between nematode worm counts and EEC, the overall correlation was 0.82 ($P < 0.0001$). However, the association between *A. galli* female worm count and EEC was 0.94 ($P < 0.0001$). Associations of *H. gallinarum* and *Capillaria spp*. worm counts and EEC were much lower than observed for *A. galli*.

Table 4.3. Nematode worm egg counts from 16 flocks submitted by free-range and barn egg producers. If the nematode egg was detected in at least one sample, the flock was considered positive.

Flock	Farm	Intestinal excreta											Caecal excreta				
		Ascarid EEC (EPG)					*Capillaria* spp. EEC (EPG)						*Ascarid* EEC (EPG)				
		n/flock	Prevalence	Mean	Median	Range	n/flock	Prevalence	Mean	Median	Range	n/flock	Prevalence	Mean	Median	Range
1	1	10	80^{ab}	664^{abc}	160	0 - 5280	10	20	12	0	0 - 80	10	90	976	140	0 - 4280
2	1	10	80^{ab}	500^{abc}	240	0 - 2880	10	30	16	0	0 - 80	10	90	1068	220	0 - 6480
3	2	10	80^{ab}	120^{bc}	120	0 - 280	10	30	16	0	0 - 80	10	80	184	160	0 - 440
4	2	10	80^{ab}	172^{bc}	160	0 - 360	10	30	16	0	0 - 80	10	90	196	180	0 - 520
5	3	10	80^{ab}	504^{ab}	420	0 - 1360	10	50	32	20	0 - 80	10	90	404	300	0 - 1240
6	3	10	90^{a}	456^{ab}	460	0 - 1080	10	220	24	0	0 - 160	10	80	524	280	0 - 2080
7	4	10	90^{a}	316^{bc}	280	0 - 880	10	20	12	0	0 - 80	9	89	373	360	0 - 840
8	4	21	95.2^{a}	1503^{a}	840	0 - 6600	21	33	30	0	0 - 160	-	-	-	-	-
9	6	10	30^{c}	24^{c}	0	0 - 120	10	20	8	0	0 - 40	10	50	112	40	0 - 360
10	6	10	20^{c}	28^{c}	0	0 - 200	10	10	4	0	0 - 40	10	60	184	160	0 - 440
11	7	11	54.5^{bc}	55^{bc}	40	0 - 160	11	9.1	4	0	0 - 40	11	73	222	120	0 - 880
12	7	10	70^{b}	88^{bc}	80	0 - 280	10	30	12	0	0 - 40	10	80	296	260	0 - 840
13	5	10	60^{b}	76^{bc}	40	0 - 280	10	40	36	0	0 - 160	9	89	347	360	0 - 560
14	5	10	70^{b}	188^{bc}	100	0 - 960	10	30	16	0	0 - 80	10	70	428	260	0 - 1040
15	5	10	50^{bc}	116^{bc}	40	0 - 440	10	20	12	0	0 - 80	10	70	228	180	0 - 640
16	5	10	80^{ab}	528^{ab}	220	0 - 1520	10	40	24	0	0 - 80	10	70	524	500	0 - 1360
Overall		**172**	**71**	**407**	**120**	**0 - 6600**	**172**	**27**	**18**	**0**	**0 - 160**	**149**	**78**	**404**	**200**	**0 -6480**
P-value			**P = 0.001**	**P < 0.0001**				**P = 0.826**	**P = 0.781**				**P = 0.596**	**P = 0.318**		

n = number of hens; EEC = excreta egg count; EPG = egg/gram of excreta. Means not sharing a common letter in the superscript in the same column differ significantly ($P < 0.05$). P-value is based on cubic root transformed EEC data.

Table 4.4. Prevalence and EEC of nematode parasites in different age groups and production systems

Measured parameters		*Ascarid eggs*				*Capillaria spp.*	
		Prevalence (intestinal)	Prevalence (caecal)	EEC (EPG)(intestinal)	EEC (EPG)(caecal)	Prevalence	EEC (EPG)
Age group	Pullet	71.4	76.6	440	276[b]	26	17
	Adult	70	79.6	345	599[a]	30	19
	P-values	0.844	0.665	0.488	0.01	P = 0.566	P = 0.587
Production systems	Org. free-range	65	74.4	227	382.5[ab]	32	22
	Con. free-range	80	90	582	1022[a]	25	14
	Barn	71.4	76.6	440	275.5[b]	26	17
	P-values	0.465	0.305	0.245	0.046	P = 0.7072	P = 0.669

EEC = excreta egg count; EPG = egg/gram of excreta. Org = organic; Con. = conventional. P-value is based on cubic root transformed EEC data.

Table 4.5. Prevalence of helminth infection and individual chicken worm counts presented as arithmetic mean (WC), median and range on four free range farms (total number of hens = 407)

Parasites	Farm 1 (n= 107; QLD)				Farm 2 (n=100;Coastal NSW)				Farm 3 (n= 100; Northern NSW)				Farm 4 (n =100; West Sydney)				Sum of all farms	
	Prev.%	WC	Range	Median	Prev.%	WC	Range	Median	Prev.%	WC	Range	Median	Prev.%	WC	Range	Median	Total prev. %	Total WC
A. galli	71[b]	11.40[c]	0-102	5	98[a]	29.4[b]	0-220	21	58[b]	3.21[c]	0-13	1.5	100[a]	44.9[a]	4-353	31	81.6	22
Sex ratio (F:M)	-	1.36	0±3.5	1.33	-	1.34	0.38-3	1.29	-	1.58	0-4	1.6	-	1.4	0.66-2.5	1.23	-	1.38
H. gallinarum	81.3[b]	32.6[b]	0-215	20	99[a]	64.9[a]	0-412	41.5	67[c]	5.95[c]	0-23	4	100[a]	79.5[a]	12-287	60	86.7	45.5
Sex ratio (F:M)	-	2.27	1.5-5.3	2.0	-	1.50	0.72-4	1.4	-	2.16	0-5	1.8	-	1.37	0.7-2.75	1.33	-	1.77
Capillaria spp.	33.6[b]	1.06[b]	0-7	0	51[a]	3.76[a]	0-32	2	19[c]	1.00[b]	0-11	0	36[a]	5.18[a]	0-41	0	34.9	2.72
Total nematodes	93.5[a]	45.0[c]	0-234	35	100[a]	98.1[b]	3-476	71.5	73[b]	10.0[d]	0-36	8.5	100[a]	129.6[a]	21-541	102	91.6	70
Raillietina tetragona	2.80	0.09	0-4	0	0	0	0	0	0	0	0	0	16	0.60	0-6	0	4.70	0.17
Raillietina echinobothrida	2.80	0.06	0-3	0	0	0	0	0	0	0	0	0	10	0.36	0-5	0	3.19	0.11
Raillietina cesticillus	1.87	0.05	0-3	0	2.00	0.06	0-5	0	0	0	0	0	17.2	0.69	0-12	0	5.20	0.20
Choanotaenia infundibulum	0	0	0	0	0	0	0	0		0	0	0	18	0.89	0-11	0	4.40	0.22
Hymenolepis cantaniana	0	0	0	0	0	0	0	0	0	0	0	0	18	0.42	0-5	0	4.40	0.22
Total cestodes	7.5	0.20	0-4	0	2	0.06	0-5	0	0	0	0	0	39	2.96	0-21	0	12	0.78
All worms	95.3	45.2	0-234	35	100	98.1	3-476	71.5	73	10.0	0-36	8.5	100	132.6	21-541	105	92.1	71

Means not sharing a common letter along the row in the superscript differ significantly ($P < 0.05$). P-value is based on cubic root transformed worm count data.
QLD = Queensland; NSW =New South Wales; WC = worm count

Table 4.6. The number and percentage of hens harbouring different numbers of helminth species (total helminth infection (nematodes and cestodes), nematode species or cestode species) (n = 407)

Number of parasite species	The number and percentage of hens harbouring parasite infections		
	Total helminth infections	Nematode infections	Cestode infections
Negative for all	32 (7.8%)	34 (8.4%)	360 (88%)
Positive for one parasite species	54 (13.3%)	54 (13.3%)	20 (4.9%)
Positive for two parasite species	180 (44.2%)	196 (48.2%)	17 (4.2%)
Positive for three parasite species	127 (31.2%)	123 (30.2%)	11 (2.7%)
Positive for four parasite species	14 (3.4%)	-	-

Table 4.7. Correlation (r_s) between various parasitological parameters measured in individual free range laying hens (n = 407)

Parameter	Total *A. galli*	*A. galli* (♀)	*A. galli* (♂)	Total *H. gallinarum*	*H. gallinarum* (♂)	*H. gallinarum* (♀)	Total Nematodes	*Capillaria spp*.
Total *A. galli*	-	-	-	-	-	-	-	-
A. galli (♂)	0.99**	0.97**	-	-	-	-	-	-
A. galli (♀)	0.99**	-	-	-	-	-	-	-
Total *H. gallinarum*	0.66**	0.66**	0.67**	-	-	-	-	-
H. gallinarum (♂)	0.68**	0.68**	0.68**	0.99**	-	-	-	-
H. gallinarum (♀)	0.65**	0.65**	0.65**	0.99**	0.97**	-	-	-
Total nematodes	0.84**	0.84**	0.83**	0.94**	0.94**	0.94**	-	-
Capillaria spp.	0.25*	0.26*	0.25*	0.17*	0.17*	0.17*	0.29**	-
Nematode EEC	0.94**	0.95**	0.91	0.68**	0.66	0.70**	0.82**	0.28**

** = P < 0.0001; * = P < 0.05; EEC = excreta egg count.

4.5. Discussion

This study provides the first insight into the prevalence and magnitude of helminth infection in chickens in Australia since 1942. The overall prevalence of helminth infection in cage-free laying chickens based on post-mortem examination was 92.1%, with high prevalence of infection with *H. gallinarum* (86.7%) and *A. galli* (81.6%) and moderate prevalence of *Capillaria spp.* (34.9%) with significant variation between farms. Cestodes were the least prevalent helminth. Hens harboured an average of 71.0 worms, with *H. gallinarum* having the highest mean worm burden per hen (45.5) followed by *A. galli* (22.0), *Capillaria spp (*2.72) and cestodes (0.78). Prevalence based on excreta examination provided a very similar picture with high prevalence (74.8%) of ascarid infections and lower prevalence of infection with *Capillaria spp.*

(27.3%). Positive correlation between EEC and nematode worm burden was observed in the current study.

The high prevalence of the helminth infection observed in the current study is in close agreement with the prevalence reported in previous studies (Broadbent, 1942; Abebe et al., 1997; Kaufmann and Gauly, 2009; Kaufmann et al., 2011b; Ferdushy et al., 2016; Grafl et al., 2017; Wuthijaree et al., 2017; Zloch et al., 2021). The high reported prevalence of helminth infection in the current study and those of others in free range systems (Rabbi et al., 2006; Kaufmann and Gauly, 2009; Kaufmann et al., 2011b; Sherwin et al., 2013; Wongrak et al., 2014; Ferdushy et al., 2016; Wuthijaree et al., 2017) is not surprising, given this and the scavenging and roaming habits of free-range chickens allowing direct contact with excreta and soil harbouring infective eggs. In the Australian context, the prevalence of *H. gallinarum* in the current worm count study (86.7%) is consistent with the 76.7% prevalence reported by Broadbent (1942) in Queensland 70 years ago, but the prevalence of *A. galli* (39.6 %) and *Capillaria* spp. (7%) infections in that study were considerably lower than detected in the current study. The measured prevalence was also broadly consistent with the results of the online survey, in which 77 and 62% of respondents indicated that they had detected *A. galli* and *H. gallinarum* in the previous 12 months on their farms, respectively. This is despite the fact that the prevalence based on the online survey assessed prevalence at the farm rather than the individual bird level and results depended on the ability of farmers to accurately identify the parasite species. However, the current prevalence was higher than those reported globally for all production systems with median prevalence values of 36% for *A. galli* and 29% for *H. gallinarum*, respectively (Shifaw et al., 2021a). This is not surprising as the free-range focus of the present study would lead to higher worm prevalence than studies that included caged layers. Indeed, the meta-analysis of Shifaw et al. (2021a) found that the mean prevalence of helminth infection of 78 and 74% in free range and backyard flocks was close to that observed in the present study and considerably higher than the 43.4 and 20.8% prevalence for barn and caged systems, respectively.

Mixed infections were common, with 79% of chickens harbouring two or more helminth species. This result is consistent with the report of Kaufmann et al. (2011b) who reported that 77% chickens harboured two or three helminth species. Multiple

helminth infections in free-range husbandry systems imply that the conditions under this system are favourable for the simultaneous development of different helminth species (McDougald, 2020). The most commonly observed helminths, *H. gallinarum* and *A. galli* were usually found in the same host as co-infections in 74% of chickens. This is not surprising given the fact that both parasites have a similar direct life cycle and influenced by the same environmental conditions (Daş et al., 2017; Shifaw et al., 2021a) as well as being genetically closely related species (Nadler et al., 2007; Wang et al., 2016; Daş et al., 2017). In the current study, the mean worm burden of mixed nematode infection was 70 worms/hen ranging up to 540 worms/hen. Based on the findings of other studies, this is likely to have affected production performance. For instance, a significant reduction of laying performance, feed conversion ratio, egg weight and daily egg mass by 8, 4, 2 and 9% respectively was observed in laying hens infected with 13-136 worm/hen of mixed nematode infection (Stehr et al., 2019a). Tarbiat et al. (2020) observed that flocks of laying hens treated with targeted anthelmintic treatment had significantly higher egg laying performance, egg mass and feed conversion rate compared with untreated birds. Likewise, a significant 5% reduction of body weight was observed in growing birds infected with 8-27 nematode worms (Stehr et al., 2019b). Birds heavily infected with *A. galli* show leg weakness, ruffled feathers, drooping wing, emaciation, diarrhoea and intestinal obstruction (Ackert and Herrick, 1928; Ramadan and Znada, 1991; Torres et al., 2019). This is also associated with increased mortality and welfare problems in laying hens kept in non-cage housing systems (Hinrichsen, 2016).

A strong positive association between nematode worm burden and EEC was observed in the current study. Similar observation was demonstrated by other studies (Permin and Ranvig, 2001; Daş et al., 2011c; Daş and Gauly, 2014b; Thapa et al., 2015a; Daş et al., 2017; Feyera et al., 2021a; Feyera et al., 2021b; Shifaw et al., 2022b; Feyera et al., 2022a). EEC is the most used method of estimating the size of worm burdens in the living animal. This count has been used to indicate a parasitic infection, to describe worm burdens (more eggs indicating more worms), or to estimate the size of worm burdens, and to determine the egg production of various species. It also has been used to evaluate anthelminthic efficacy by relating a decrease in eggs after treatment to decreased worm burdens (Train and Hansen, 1968; Feyera et al., 2021b). The association between *A. galli* female worm count and EEC was higher than observed

for *H. gallinarum*. This is because *H. gallinarum* that resides in the caeca do not simultaneously contribute to the countable eggs in a single sample of excreta droppings and its eggs are shed irregularly to the external environment through the caecal excreta (Daş et al., 2011c). Furthermore, *A. galli* females are much bigger than *H gallinarum* females and produce a far greater number of eggs (Daş et al., 2014a; Wongrak et al., 2015b).

Ascarid eggs (*A. galli* /*H. gallinarum*) detected in excreta samples were found in all of the free range or deep litter flocks sampled. It can reasonably be inferred that the ascarid eggs observed in the intestinal excreta (71% prevalence) are predominantly those of *A. galli* while those in the caecal samples (78% prevalence) are predominantly of *H. gallinarum* (Daş and Gauly, 2014a; Cupo and Beckstead, 2019). The clear separation of the origins of these two excreta types is reinforced by the complete absence of Capillaria eggs in the submitted caecal samples. The high prevalence of these nematodes is with the findings of Sherwin et al. (2013) and Pennycott and Steel (2001) who reported that 100 and 96% respectively of free range flocks had *A. galli* and *H. gallinarum* eggs detected in the excreta. A study of infection dynamics of *A. galli* in non-caged laying hens in Sweden reported that all flocks became infected when placed in an environment containing infective eggs (Höglund and Jansson, 2011). This is because *A. galli* and *H. gallinarum* eggs are resistant to environmental conditions and are able survive there at least for one year (Permin and Hansen, 1998). The rearing or placement of birds in a previously infected environment (Höglund and Jansson, 2011) likely explains the high prevalence of infection in pullets reported in the current study. In contrast, Zloch et al. (2021) reported that no gastrointestinal helminth infections were found in pullet flocks in Austria with a mean age of 17 weeks. The method of pullet rearing in that study was not specified. According to Jansson (2011), the lack of a hygiene barrier at the entrance of the house or unit increased the risk of infection, implying that parasite eggs were introduced to the farms horizontally. This author also reported that the risk of infection increased with the age of the barn's equipment; for example, when compared to equipment used for one year, ≥7-years-old equipment increased the risk of ascarid infections by an odds ratio of 7.5. Birds kept either outdoors or in deep litter are likely to be exposed to ascarid infections, such as *A. galli* and *H. gallinarum* since the infection cycle runs full circle when the parasite eggs are consumed with excreta or infected litter/soil (Dänicke et al., 2009).

A high prevalence of tapeworm infection (39%) was only detected on farm 4. On farms 1 and 2, a low prevalence of tapeworms was found (7.5% and 2% respectively) while no tapeworms were found on Farm 4. Raillietina spp. were most common among tapeworm species identified with prevalence ranging from 1.9 to 17.2% on infected farms. On farm 4, two additional species were observed at moderate prevalence, *Choanotaenia infundibulum* and *Hymenolepis cantaniana* (both 18% prevalence). In the study of Broadbent (1942) in Queensland, the overall prevalence of tapeworm infection was not provided but prevalence of 7 different species ranged from 4.3 to 35.9%, the latter being for *Raillietina tetragona.* Thus, it appears that tapeworm infection had a significantly higher prevalence in this early study compared to the present study. The comparatively low prevalence of tapeworm infection in the present study also contrasts with the 69% of survey respondents who claim to have detected tapeworms in the past 12 months. The low overall cestode prevalence of 12% in the present study is consistent with the 15% prevalence reported by Zloch et al. (2021) and is perhaps not surprising as cestodes require an intermediate host such as earth worm, houseflies or beetles breed in contaminated litter or soil for their transmission (Permin and Hansen, 1998). Their overall lower prevalence and worm burden may have been linked to fewer opportunities of transmission (Shifaw et al., 2021a). Because of their complex lifecycle tapeworms are difficult to work with experimentally, as the eggs shed from the mature worms in chicken excreta are not directly infective for chickens as is the case with nematode eggs.

4.6. Conclusions

The vast majority of hens housed in non-cage production systems in this study were infected with at least one helminth parasite, with high mixed worm burden. The observed worm burdens are likely to have negative effect on production performance in the flocks with the heavier loads. There was a much higher prevalence and burden of nematodes than cestodes. Therefore, tactical or strategic anthelmintic treatment should form an important part of the management of free range chicken flocks.

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We, the Research PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

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Chapter 5. Comparison of the Modified McMaster and Mini-FLOTAC methods for the enumeration of nematode eggs in egg spiked and naturally infected chicken excreta

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5.1. Abstract

Excreta egg counting techniques are used for indirectly estimating the magnitude of gastrointestinal nematode infection in live animals. The aim of this study was to optimise laboratory and field sampling methods for routine monitoring of nematode infections in chickens by evaluating the sensitivity, accuracy, and precision of the Modified McMaster (MM) and Mini-FLOTAC (MF) methods using laying chicken excreta samples spiked with estimated true numbers of eggs (Experiment 1 = 5-1500 EPG (eggs/g); Experiment 2 = 5-500 EPG) without and with operator effects, respectively or using individual fresh excreta (n = 230) and fresh floor excreta (n = 42) from naturally infected free-range layer farms. The Coefficient of Variation was assessed within and between operators and the time spent on sample preparation and counting was also evaluated. MF was more sensitive than MM at ≤ 50 EPG level but not above this while MM had a significantly higher egg recovery rate than MF for ≥ 50 EPG levels (MM = 89.7% , MF = 68.2%; $P < 0.0001$). Operator factors did not have a significant effect ($P = 0.358$-0.998) on egg counts across methods and EPG levels. The CV between replicates of the MM and MF methods for ≥ 50 EPG was 43.4 and 36.5%, respectively. The inter-observer CV of the MM and MF methods for ≥ 50 EPG levels was 63.8 and 44.3% respectively. When the naturally infected free-range layers which were individual caged for excreta sampling, the proportion of samples positive for MM and MF were 91.7 and 96.5%, respectively ($P = 0.023$). MM resulted in significantly ($P = 0.029$) higher excreta egg counts (604) than MF (460) with the difference between methods greatest at higher EPG levels. Fresh floor excreta (pooled or individual) and individual caged chicken excreta did not have significant effect on egg counts ($P = 0.274$). The total time taken for sample preparation and egg counting was significantly lower using the MM method (4.3-5.7 min) than the MF method (16.9-23.8 min) ($P < 0.0001$). In conclusion, MM was more accurate than MF, particularly at higher EPG levels, but slightly less precise and sensitive, particularly at low EPG levels, while taking less than 25% of the laboratory time per sample. Our observations indicate that the MM method is more appropriate for rapid diagnosis of chicken nematodes in the field. Pooled fresh floor excreta samples would be sufficient to indicate infection level in free range farms.

5.2. Introduction

Nematode parasites are re-emerging in commercial poultry farms due to the expansion of free-range and floor egg production systems in response to consumer demand and welfare concerns with caged systems (Permin et al., 1999b; Jansson et al., 2010; Kaufmann et al., 2011a; Kaufmann et al., 2011b; Thapa et al., 2015a; Wuthijaree et al., 2017; Shifaw et al., 2021a). Gastrointestinal roundworms including *Ascaridia galli, Heterakis gallinarum,* and *Capillaria* species are the most prevalent among helminth parasites in free-range laying chickens (Permin et al., 1999b; Kaufmann and Gauly, 2009; Kaufmann et al., 2011b; Sherwin et al., 2013; Wongrak et al., 2014; Wuthijaree et al., 2017; Shifaw et al., 2021a).

Regular diagnosis and monitoring of nematode infection levels is essential for sustainable control strategies against nematode parasites (Heckendorn et al., 2009). Techniques for detecting or estimating nematode eggs in animal faecal samples range from direct smear (Beaver, 1950) to simple flotation and centrifugal flotation techniques (Gordon and Whitlock, 1939; MAFF, 1986; Cringoli et al., 2010; Cringoli et al., 2017) and recently an advanced automated egg-counting techniques (Mes et al., 2001; Mes et al., 2007). Excreta egg counting techniques which determine parasite eggs per gram of excreta (EPG) and use flotation are based on the microscopic examination of an aliquot of excreta suspension from a known volume of an excreta sample (Nicholls and Obendorf, 1994; Cringoli et al., 2004).

Quantitative egg counting techniques are useful to indirectly estimate worm burdens as positive relationships between worm burdens and excreta egg counts have been demonstrated (Daş et al., 2011c; Thapa et al., 2015a; Daş et al., 2017). Excreta egg counting techniques are generally used to estimate the prevalence and the magnitude of gastrointestinal nematode infection in live birds for epidemiological studies (Daş et al., 2011c; Daş et al., 2017; Daş et al., 2020), selecting parasite resistant chicken breeds (Permin and Ranvig, 2001; Gauly et al., 2001a; Gauly et al., 2001b; Schou et al., 2003; Kaufmann et al., 2011a; Wongrak et al., 2015a), and for determining anthelmintic efficacy (Tarbiat et al., 2016; Acorda et al., 2019; Feyera et al., 2021a). Due to the differences in sensitivity, accuracy and precision of excreta egg counting techniques (Daş et al., 2011c; Paras et al., 2018) and growing interest in measuring nematode

infection intensity in free-range layers, clear identification of the most practical, sensitive, accurate, reliable and precise excreta-based diagnostic tools is warranted to facilitate uptake of regular detection and monitoring of nematode parasite infection in the poultry industry.

Excreta egg counting techniques and their modifications have been in use since the 1930s to achieve authentic and reliable outcomes (Nápravníková et al., 2019). The McMaster method was developed and improved at the University of Sydney, McMaster laboratory (Gordon and Whitlock, 1939; Whitlock, 1948), and it is still the most widely used egg counting technique for the detection of parasite infection in animal species (Nicholls and Obendorf, 1994; Cringoli et al., 2004; Pereckienė et al., 2007; Ballweber et al., 2014; Nápravníková et al., 2019). As described by various authors (Pereckienė et al., 2007; Vadlejch et al., 2011; Ballweber et al., 2014; Nápravníková et al., 2019), several McMaster modifications published over time have varied in amounts of flotation solution used, density of the flotation solution, weight of the excreta and aliquot examined, flotation time, absence or presence of centrifugation, number of counting chambers, and multiplication factors. These all affect the sensitivity, accuracy, and precision of the technique (Dunn and Keymer, 1986; MAFF, 1986; Rehbein et al., 1999; Cringoli et al., 2004; Pereckienė et al., 2007; Kochanowski et al., 2013). While the McMaster technique is generally considered to have poor sensitivity and high variability at low egg counts (Mes et al., 2001; Daş et al., 2011c; Daş et al., 2020), it is the most popular method due to its simple protocol, re-use of equipment, reasonably cheap cost and short labour time (Daş et al., 2020).

The FLOTAC method and its derivative, the Mini-FLOTAC were developed in 2006 and 2013, respectively as an alternative to replace the McMaster technique for the diagnosis of gastrointestinal parasite infection in different mammalian species with improved accuracy, sensitivity and precision (Cringoli, 2006; Cringoli et al., 2010; Cringoli et al., 2013; Cringoli et al., 2017). The FLOTAC technique is an egg counting technique incorporating a centrifugal flotation system in a chambered device sensitive to one EPG (Cringoli, 2006; Cringoli et al., 2010). However, the main drawback of this technique is its complexity and the need of a special centrifuge device. In addition, the FLOTAC is time and labour-intensive as it requires several steps to process each

sample (Knopp et al., 2009; Cringoli et al., 2010; Barda et al., 2013; Lester and Matthews, 2014; Cringoli et al., 2017).

In contrast, the Mini-FLOTAC has been reported to be more user friendly and a simplified version of FLOTAC that does not require any centrifugation steps and can therefore be used at any laboratory or on-farm (Cringoli et al., 2017; Bosco et al., 2018). However, the average processing time of individual excreta samples using Mini-FLOTAC has reported to be around 12 min (Cringoli et al., 2017) which is twice as long as the sample processing time reported for the McMaster method (Noel et al., 2017; Daş et al., 2020).

The diagnostic performance of the Mini-FLOTAC has been assessed and compared with the McMaster technique in several mammalian host-parasite studies indicating that the Mini-FLOTAC showed a higher sensitivity, accuracy, and precision (Godber et al., 2015; Cringoli et al., 2017; de Castro et al., 2017; Noel et al., 2017; Scare et al., 2017; Bosco et al., 2018; Paras et al., 2018; Nápravníková et al., 2019; Amadesi et al., 2020). However, there are only two published studies compared the Mini-FLOTAC with the McMaster technique in avian species, one for detecting *Eimeria* oocysts (Bortoluzzi et al., 2018) and the other for nematode eggs (Daş et al., 2020).

Egg counts from pooled faecal samples may have significant advantage over multiple individual faecal samples in terms of saving time and also providing promising egg count results. Faecal egg count has been generally accepted for monitoring sheep flock nematode control programs (Nicholls and Obendorf, 1994; Rinaldi et al., 2014).

This study expands on the investigation of Daş et al. (2020) by evaluating the diagnostic performance (sensitivity, accuracy and precision) of the MM and the MF methods in spiked samples containing a wider range of EPG without and with using multiple operators. Additional analyses were conducted using excreta samples from naturally infected chickens. The overall objective was to optimise laboratory and field sampling methods for routine monitoring of nematode infections in laying chickens. Under this broad objective we evaluated two specific propositions:

1. That the predicted advantages of the MF method over the MM method would be sufficient to make it the preferred method for monitoring of nematode infections
2. That pooled fresh floor excreta samples will be the most suitable sample to collect from free range layer flocks to indicate the infection level.

5.3. Materials and Methods

To meet the aims of this study, three related experiments were conducted. Experiments 1 (section 5.3.1) and 2 (section 5.3.2) compared the MM and MF using excreta samples containing estimated true number of eggs without and with operator effects, respectively. Experiment 3 (section 5.3.4) evaluated the two methods using excreta samples obtained from naturally infected free-range laying chicken farms. Flow chart representation of study design and methodology of different phases of experiments are shown in Fig. 5.1. This research was approved by the Animal Ethics Committee of the University of New England with approval number AEC19-082.

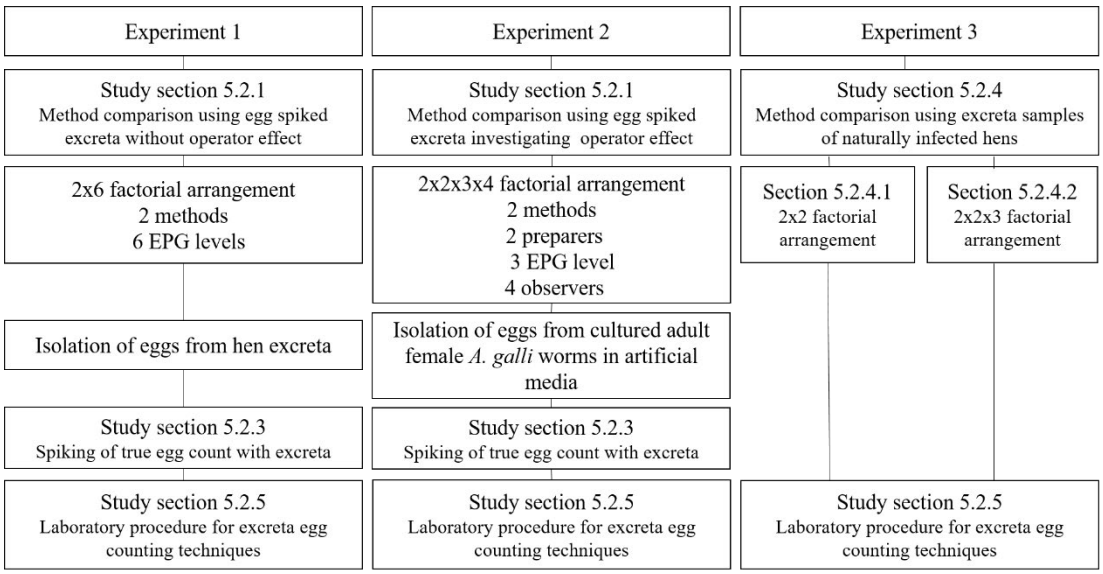

Figure 5.1. Flow chart representation of study design and methodology of different phases of experiments.

5.3.1. Experiment 1. Method comparison using egg spiked excreta samples without operator effect

MM and MF were compared for the enumeration of *Ascaridia galli* eggs in chicken excreta samples spiked with estimated true numbers of *A. galli* eggs. Excreta was collected from caged chickens free of gastrointestinal nematode infection confirmed by necropsy. A 2 x 6 factorial arrangement was employed to test two excreta counting techniques (MM and MF), and six EPG levels (5, 50, 200, 500, 1000, and 1500 EPG) with 12 replicates of each combination. Sample processing and reading of slides were performed by one operator with time spent on sample processing (sample preparation from the beginning of excreta weighing to slide reading and egg counting) for each sample replicate was recorded. Eggs for spiking the excreta samples were obtained from excreta of chickens with artificial mono-specific *A. galli* infection as described previously (Rahimian et al., 2016; Daş et al., 2020). In brief, the excreta slurry was flushed under tap water and passed through a series of 6 sieves (pluriSelect Life Science, Leipzig, Germany) with decreasing mesh apertures of 750, 500, 200, 150, 90 and 75 μm in order to separate the eggs from the larger particles, and A. *galli* eggs were then collected on a 30-μm sieve. Eggs retained on the sieve were rinsed into a 50 mL tube using tap water. This tube was centrifuged at 1620 x g for 1 min allowing for concentration of the parasite eggs at the bottom of the tube. Supernatant was then removed leaving a volume of 5 mL in the tube. A 45 mL of saturated sodium chloride with specific gravity (S.G. = 1.2 was then added and the tubes again centrifuged at 1620 x g for 1 min. After centrifugation, the supernatant containing the parasite eggs were collected on a 30-μm sieve and rinsed with a large amount of tap water. The eggs on the sieve were then recovered by washing off the screen and stored in water at 4°C for 12 hrs before being used to spike the egg free excreta as detailed in section 4.3.3. The estimated egg losses during isolation process was approximately 0.6% of the total estimated eggs.

5.3.2. Experiment 2. Method comparison with egg spiked excreta investigating operator effect

The effect of the operator on the diagnostic performance of the MM and MF methods was assessed using chicken excreta spiked with *A. galli* eggs in a 2 x 2 x 3 x 4 factorial arrangement testing the effects of egg counting techniques (MM and MF), person preparing the samples (preparer A and B), EPG levels (5, 50, 500 EPG) and persons counting the eggs (observers 1, 2, 3, 4) with each combination replicated 3 times for the whole procedure resulting in a total of 144 counts. The time spent for sample preparation and egg counting were recorded separately. For this experiment, *A. galli* eggs were harvested using cultured adult female *A. galli* worms in artificial media as described previously (Ruhnke et al., 2017; Sharma et al., 2017; Feyera et al., 2020). The collected eggs stored in water at 4°C for 28 days before being used to spike the egg free excreta as outlined in section 5.3.3.

5.3.3. Spiking of estimated true EPG count with excreta samples

To prepare excreta samples of estimated EPG concentration, estimated true numbers of *A. galli* eggs were added into chicken excreta samples. Egg count per ml of the stock solution was determined using the MM technique based on the arithmetic mean counts of 3 aliquots of a 0.15 ml subsample. The required number of spiked eggs and volume was then calculated for each level of egg concentration (i.e. EPG level). The appropriate amounts of egg solution ranging from 0.45 to 2 ml was then added to the weighed (50-100 g) parasite eggs free excreta for each EPG level and thoroughly mixed with a spatula to achieve the estimated EPG counts (Experiment 1 = 5, 50, 200, 500, 1000, and 1500 EPG; Experiment 2 = 5, 50 and 500 EPG). The prepared samples were then subject to excreta egg counts (EEC) using the MM and MF methods on the same day.

5.3.4. Experiment 3. Method comparison using excreta samples of naturally infected hens

The final experiment compared the two egg counting techniques (MM and MF) for assessing EPG in excreta samples obtained from commercial free-range layer flocks harbouring natural nematode infections from the field. The prevalence of nematode based on post-mortem examination (i.e. 100 birds/flock) was 100% for both farm A and farm B (non-published data).

5.3.4.1. Individual fresh chicken excreta samples

This experiment employed a 2x2 factorial arrangement to test the effect of farms (A and B) and two egg counting techniques (MM and MF). One hundred and ten and 120 randomly selected free-range layers from farm A and B, respectively were transported to the University of New England research facilities and placed into individual cages with ad libitum feed and water supply. After a one-week adaptation period, fresh individual excreta samples were collected on paper sheets placed beneath each cage within an hour of paper placement. The individual caged chicken excreta samples were mixed by stirring manually with a spatula and two sub-samples (2.5 g for MM and 5 g for MF) were taken for EEC analysis, resulting a total of 230 counts by each method.

5.3.4.2. Comparison of EEC between excreta sample types

To assess and compare the EEC from different sample types, a 2x2x3 factorial arrangement was employed to test the effect of two egg counting techniques (MM and MF), farms (A and B) and sample types (n = 3). The fresh floor excreta samples collected from the poultry shed floor (individual = 20; pooled = 1 per farm), and individual caged fresh chicken excreta (n = 110-120 per farm) described the details in section 5.3.4.1. Pooled samples were obtained by combining a single sample from each of the five different indoor and outdoor locations. All samples were transported to the lab in a chilled cool box and stored at 4°C for 48 hrs until analysis. Individual fresh floor excreta samples were analysed as same procedure as section 5.3.4.1. Pooled excreta samples were analysed in five replicates for each method.

5.3.5. Laboratory procedure for excreta egg counting techniques

MF was performed as described by Cringoli et al. (2017) providing a limit of detection of 5 EPG. The protocol requires two purchased items of equipment, the Fill-FLOTAC sample preparation container and the reading chamber. The Fill-FLOTAC is a 50 ml plastic container allowing for excreta collection, homogenization and filtration through a 250 μm filter located under the lid of the Fill-FLOTAC. In brief, 5 g of excreta was weighed into the Fill-FLOTAC container, 45 mL of saturated sodium chloride salt solution (S.G. = 1.20) added, then homogenised, filtered and two 1 ml aliquots loaded into the Mini-FLOTAC chambers until a meniscus was formed. MF slides were then allowed to sit for 10 minutes and read under 40 x magnification power with eggs counts multiplied by 5 to provide the EEC in EPG units. The MM technique employed followed the basic principle described by Whitlock (1948) providing a limit of detection of 40 EPG. In brief, 2.5 g of each excreta sample was diluted in 47.5 ml of saturated salt solution (S.G. = 1.20), thoroughly homogenised, sieved and a 0.5 ml aliquot loaded into a chamber on a Whitlock universal slide (JA Whitlock and CO, Eastwood, NSW, Australia) and examined under 40 x magnification power. Eggs counted were multiplied by 40 to provide an EEC in EPG units.

5.3.6. Statistical analysis

All statistical analyses were performed using JMP 14 (SAS Institute Inc., Cary, NC, USA). EEC values were tested for normality and not-normally distributed EECs were transformed by cube root prior to data analysis to better meet the assumptions of analysis of variance. The cube root transformed and back-transformed EECs were presented with standard error and 95% confidence intervals, respectively. For experiments 1 and 2, up to four-way full factorial analysis of variance (ANOVA) in the linear model platform of JMP was used to analyse EECs and sample processing time fitting the fixed effects of preparer (n = 2), egg counting techniques (MM, MF), spiked EPG level, and counter (n = 4) and their interactions in the model. For experiment 3, EECs were subjected to analysis of variance fitting up to three effects, these being farm, sample type and egg counting technique, and interactions up to 3-way. Tukey Kramer pairwise multiple comparisons were employed to test for

significant differences between levels of a given factor in the analysis. Additionally, student t-test was employed to test significance difference between the excreta egg count of two farms and methods irrespective of other factors. A spearman rank correlation (rho), i.e. non-parametric test, was used to assess any association between estimated true and observed egg counts. Linear regression analysis was also used to evaluate linear associations between measured variables in naturally infected sample data.

The sensitivity of MM and MF techniques detecting the presence of infection was determined within each EPG level, and calculated as: Sensitivity (%) = [True Positives/(True Positives +False Negatives)]∗100. Fisher's exact test was used to compare the significance difference between the sensitivity of the two methods. Precision of MM and MF was compared using the coefficients of variation (CV %). Inter-replicate and inter-observer coefficient of variation were calculated as follows: Inter-replicate CV (mean replicate CV) = [(standard deviation/mean)*100] for EPG count of all sets of replicates within each EPG level, method and operator, whereas inter-observer CV (mean observer CV) calculated as = [(standard deviation/mean)*100] for each observer mean EPG count for each EPG level and method. All CV present with 95% confidence interval as described previously (Abdi, 2010). Percentage accuracy was assessed directly with recovery rate of the spiked eggs (Amadesi et al., 2020; Daş et al., 2020). Percentage of egg recovery rate was calculated the formula described previously (Bosco et al., 2018). Recovery rate = 100-[(true EEC-observed EEC) /true EEC) ∗ 100]. Statistical significance was considered at $P < 0.05$ for all analyses.

5.4. Results

5.4.1. Experiment 1: Sensitivity, accuracy, and Coefficient of Variation of MM and MF

The average sensitivity of MM and MF at detecting positive samples across all six EPG levels was 79.2 and 93.1%, respectively ($P = 0.014$; Table 5.1). The differences in sensitivity were greatest at the 5 EPG level and there was no significant difference

in the sensitivity between MM and MF for ≥ 50 EPG level (Table 5.1). The mean egg counts of MM and MF across the six egg spiked EPG levels are presented in Table 5.1 and the results of the complete analysis of the data are presented in Table 5.2. The mean egg counts obtained by the MM method were 21% higher ($P = 0.035$) than by the MF method (Table 5.1). The mean egg counts also differed significantly between spiked EPG levels ($P < 0.0001$). In addition, there was a highly significant ($P < 0.0001$) interaction between the egg counting methods and the EPG level as shown in Table 5.2. This revealed that both methods underestimated the true EPG counts, but as EPG level increased the MF method under-estimated the true EPG level to a greater extent (30%) compared to the MM method (8%) as shown in Table 5.1. Because of this, MM was significantly more accurate for ≥ 50 EPG levels (89.7%) than MF (68.2%) ($P < 0.0001$) as well as in overall recovery rate ($P = 0.035$; Table 5.1). Spearman's rank correlation revealed a significant ($P < 0.0001$) positive relationship between true EPG values and observed EPG values for MM (Rho = 0.98) and MF (Rho = 0.99) (data not shown). The variation between replicates for each EPG level and method is presented in Table 5.1. The CV tended to decrease with increasing EPG level. The overall CV of MM and MF between replicates for ≥ 50 EPG-levels was 43.4 and 36.5% respectively (Table 5.1).

Table 5.1. Mean egg counts, sensitivity, recovery rate and coefficient of variation (CV %) between replicates (n = 12) for EEC determined by MM and MF methods in excreta samples containing different estimated number of *A. galli* eggs.

Test attribute	ECT	Estimated true number of eggs (EPG levels) or range included in overall estimates							
		5 (n = 12)	50 (n = 12)	200 (n = 12)	500 (n = 12)	1000 (n = 12)	1500 (n = 12)	5-1500 (n = 72)	≥ 50 (n = 60)
Arithmetic mean EEC[1]	MM	0^{k}	30^{i}	157^{g}	453^{e}	897^{c}	1377^{a}	486^{a}	583^{a}
	MF	3^{j}	35^{h}	131^{g}	337^{f}	664^{d}	1050^{b}	370^{b}	443^{b}
Sensitivity %	MM	0^{b}	75^{b}	100	100	100	100	79.2^{b}	95^{a}
	MF	58^{a}	100^{a}	100	100	100	100	93.1^{a}	100^{a}
Recovery rate %	MM	0	60	78.5	90.6	89.7	91.8	89.6^{a}	89.7^{a}
	MF	60	70	65.5	67.4	66.4	70	68.5^{b}	68.2^{b}
CV (95% CI)	MM	NA	60.3 (33.2-87.4)	7.71 (4.25-11.2)	3.69 (2.03-5.35)	3.63 ((1.99-5.26)	1.88 (1.04-2.72)	65.4 (54.7-76.1)	43.4 (35.6-51.2)
	MF	88.2 (48.6-127.8)	5.15 (2.83-7.46)	2.54 (1.39-3.68)	2.28 (1.27-3.30)	1.81 (0.99-2.62)	0.62 (0.34-0.89)	54.2 (45.3-63.0)	36.5 (29.9-43.0)

Abbreviations: CV = coefficient of variation; NA = not applicable; MM = Modified McMaster; MF = Mini-FLOTAC; EPG = eggs per gram of excreta; EEC = excreta egg count; ECT = excreta egg counting technique. Different superscripts indicate a significant difference ($P < 0.05$). Method comparisons were done based on cube root transformed EECs. [1] Multiple comparison (a-j) were done to demonstrate significant interaction between the effect of methods and EPG levels.

Table 5.2. Summary of analysis of variance of excreta egg counts (EECs), time spent for sample preparation and egg counting in for each method, EPG-level (5-1500) and significant interaction

Experiment/factor/level	Cube Root LSM EEC ±SEM	Back transformed LSM EEC (95% CI)	Arithmetic mean EEC	Preparation time (sec ±SEM)	Counting time (sec ±SEM)	Total time (sec ±SEM)
Egg spiking experiment 1						
Overall mean	5.9±0.1	212 (202-222)	428	489±0.4	147±0.4	635±0.6
Methods	P = 0.035			P < 0.0001	P < 0.0001	P < 0.0001
MM	6.0±0.1[a]	223 (207-236)	486	187±0.6[b]	70±0.6[b]	257±0.8[b]
MF	5.9±0.1[b]	201 (188-215)	370	791±0.6[a]	223.3±0.6[a]	1014±0.8[a]
EPG Level	P < 0.0001			P = 0.143	P < 0.0001	P < 0.0001
5	0.5±0.1[f]	0.1 (0.02-0.3)	1.5	488±1.0[a]	121±1.0[f]	607±1.4[f]
50	2.9±0.1[e]	25 (19-31)	34	489±1.0[a]	127±1.0[e]	616±1.4[e]
200	5.2±0.1[d]	142 (125-161)	144	491±1.0[a]	136±1.0[d]	627±1.4[d]
500	7.3±0.1[c]	391 (356-428)	395	489±1.0[a]	163±1.0[c]	652±1.4[c]
1000	9.2±0.1[b]	773 (718-831)	780	488±1.0[a]	153±1.0[b]	641±1.4[b]
1500	10.6±0.1[a]	1205 (1131-1283)	1213	487±1.0[a]	180±1.0[a]	667±1.4[a]
Significant interaction (P-values)						
Method*EPG Level	P < 0.0001	-	-	P = 0.03	P < 0.0001	P < 0.0001

Abbreviations: LSM = least square means; SEM = standard error means; CI = confidence interval; EEC = excreta egg count; MM = Modified McMaster; MF = Mini-FLOTAC; Different superscripts (a-f) indicate a significant difference ($p < 0.05$) for each factor. Preparation and counting time were presented in seconds.

5.4.2. Experiment 2. Effect of operator factors on sensitivity, accuracy and precision of MM and MF

Sensitivity of MM and MF with operator factors are presented in Table 5.3. MF had higher overall sensitivity than MM (79.2 and 59.7%, respectively, $P < 0.009$). Analyses of excreta egg count and factors affecting it are shown in Table 5.3. The mean egg count using MM and MF with 4 operators at three EPG levels (5, 50, and 500) tended to be higher for MM (117 EPG) than MF (71 EPG) but the difference was not significant ($P = 0.365$). However, there was significant interaction between the effects of the egg counting technique and EPG level ($P = 0.011$) due to significantly higher EPG count of MM at 500 EPG level. The operator factors did not have a significant effect overall or interactions with other effects ($P > 0.05$ for all). When the recovery rate of eggs relative to true EPG was considered, MM was more accurate for all EPG levels (63.2%) than MF (38.4%; Table 5.4). Both methods underestimated true egg count in the hands of 4 observers. Coefficient of variation of MM and MF based on observer counts are presented in Table 5.4. Inter-observer CV declined with increasing EPG level for both methods where the overall CV for ≥ 50 EPG levels was 63.8 and 44.3% for MF and MM, respectively. Intra-observer was a major source of variation, particularly at low EPG levels and therefore no statistical significant difference was detected between the methods.

5.4.3. Effect of excreta egg counting technique and operator on sample preparation and reading time

The results of the analysis of time spent on sample preparation and counting in experiments 1 and 2 is presented in Table 5.2 and 5.3, respectively. The MF method took significantly longer in both preparation time and counting time per sample in both experiments, resulting in total time per sample that was 3.94 and 4.43-fold higher in experiments 1 and 2, respectively (Table 5.2 and 5.3). The time taken for egg counting steps was also significantly affected by EPG level (both experiments) and the operator doing the counts (Experiment 2).

Table 5.3. Summary of analysis of variance of excreta egg counts (EECs), time spent for sample preparation and egg counting in for each method, EPG-level (5-500), operator factor and significant interaction.

Experiment/factor/level	Cube Root LSM EEC ±SEM	Back transformed LSM EEC (95% CI)	Arithmetic mean EEC	Preparation time (sec ±SEM)	Counting time (sec ±SEM)	Total time (sec ±SEM)
Egg spiking experiment 2						
Overall mean ±SE	3.0±0.1	27 (22-33)	94	552±4.1	248±8.2	800±7.9
Methods	P = 0.365			P < 0.0001	P < 0.0001	P < 0.0001
MM	3.1±0.2^{a}	30 (22-40)	117	204±5.8^{b}	91±11.6^{b}	295±10.9^{b}
MF	2.9±0.2^{a}	25 (18-35)	71	900±5.8^{a}	405±11.6^{a}	1306±10.9^{a}
EPG level	P < 0.0001			P < 0.0001	P < 0.0001	P < 0.0001
5	0.8±0.2^{c}	0.5 (0.1-2)	7	537^{b}	206^{c}	743±13.4^{c}
50	1.9±0.2^{b}	8 (4-13)	18	539^{b}	246^{b}	784±13.4^{b}
500	6.2±0.2^{a}	244 (203-290)	258	581^{a}	292^{a}	873±13.4^{a}
Preparer	P = 0.358			P < 0.0001	P = 0.01	P = 0.236
A	3.1±0.2^{a}	30 (22-40)	107	579^{a}	230^{b}	809±10.9^{a}
B	2.9±0.2^{a}	25 (18-33)	82	525^{b}	265^{a}	790±10.9^{a}
Observer	P = 0.673				P < 0.0001	P < 0.0001
1	3.1±0.2^{a}	30 (19-44)	99	-	336^{a}	886±15.4^{a}
2	2.8±0.2^{a}	22 (14-34)	90	-	172^{c}	724±15.4^{b}
3	3.2±0.2^{a}	32 (21-45)	98	-	190^{c}	743±15.4^{b}
4	2.9±0.2^{a}	26 (17-38)	89	-	293^{b}	848±15.4^{a}
Significant interaction (P-values)						
Methods*EPG Level	P = 0.011	-	-	P = 0.023	P = 0.01	P = 0.546
Methods*Observer	P = 0.735	-	-	-	P < 0.0001	P < 0.0001

Abbreviations: LSM = least square means; SEM = standard error means; CI = confidence interval; EEC = excreta egg count; MM = Modified McMaster; MF = Mini-FLOTAC; Different superscripts (a-c) indicate a significant difference (P < 0.05) for each factor. Preparation and counting time were presented in seconds.

Table 5.4. Mean excreta egg counts, sensitivity, recovery rate, inter-observer and intra-observer variation of MM and MF based on observer counts for each EPG level.

Test attribute	ECT	Estimated true number of eggs (EPG levels) or overall estimates				
		5	50	500	All EPG levels	≥ 50 EPG levels
Arithmetic mean EEC[1]		n = 24	n = 24	n = 24	n = 72	n = 48
	MM	10^{c}	22^{b}	318^{a}	117^{a}	170^{a}
	MF	2^{c}	14^{bc}	198^{a}	71^{a}	106^{a}
Sensitivity %		n = 24	n = 24	n = 24	n = 72	n = 48
	MM	33.3^{a}	45.8^{b}	100^{a}	59.7^{b}	73^{b}
	MF	37.5^{a}	100^{a}	100^{a}	79.2^{a}	100^{a}
Recovery rate %	MM	200	44	63.6	63.2^{a}	61.8^{a}
	MF	40	28	39.6	38.4^{a}	38.4^{a}
		n = 8	n = 8	n = 8	n = 24	n = 16
Inter-observer variation (95 % CI)	MM	37.6 (15.3-59.9)	38.1(15.5-60.7)	6.6 (2.68-10.5)	75.9 (53.2-98.6)	63.8 (39.8-87.8)
	MF	52.5 (21.4-83.6)	11.5 (4.68-18.3)	2.8 (1.14-4.46)	76.6 (53.7-99.5)	44.3 (27.6-60.9)
Intra-observer variation (95 % CI)		n = 6	n = 6	n = 6	n = 18	n = 12
1	MM	77.5 (20.0-134.9)	109.5 (28.3-190.7)	16.2 (4.20-28.2)	77.9 (50.5-105.3)	75.6 (41.7-109.5)
	MF	156.4 (40.4-272.4)	19.1(4.92-33.3)	3.70 (0.95-6.44)	77.2 (50.1-104.3)	43.5 (23.9-63.0)
2	MM	109.5 (28.3-190.7)	156.3 (40.3-272.3)	16.9 (4.40-29.4)	92.4 (59.9-124.9)	80.6 (44.4-116.8)
	MF	154.9 (39.9-269.8)	19.8 (5.10-34.5)	4.92 (1.26-8.57)	82.5 (53.5-111.5)	49.8 (27.4-72.2)
3	MM	154.9 (39.9-269.8)	109.5 (28.3-190.7)	11.0 (2.84-19.2)	93.2 (60.4-125.9)	70.2 (38.7-101.7)
	MF	109.5 (28.3-190.7)	8.82 (2.27-15.4)	5.17 (1.33-9.00)	71.9 (46.6-97.2)	42.1 (23.2-61.0)
4	MM	154.9 (39.9-269.8	110.9 (28.62-193.2)	17.8 (4.59-31.0)	92.2 (59.8-124.6)	69.4 (38.2-100.6)
	MF	244.9 (63.2-426.6)	13.5 (3.48-23.5)	7.23 (1.86-12.6)	85.2 (52.2-115.2)	45.6 (25.1-66.1)

Abbreviations: CV = coefficient of variation; EEC = excreta egg count; EPG = eggs per gram of excreta; MM = Modified McMaster; MF = Mini-FLOTAC; ECT = excreta egg counting technique. The different superscripts (a, b, c) indicate the significant difference ($P < 0.05$). Comparison of methods were based on cube root transformed EECs. [1] Multiple comparison (a-c) were done to demonstrate significant interaction between the effect of methods and EPG levels.

5.4.4. Experiment 3. Method comparison using in individual caged and fresh floor excreta from naturally infected laying chicken

The results of analysis of variance for factors affecting excreta egg count in Experiment 3 is presented in Table 5.5. Of the 230 fresh individual excreta samples analysed, 91.7 and 96.5% were positively identified for nematode infection for MM and MF respectively (P = 0.023). The analysis of EEC data from these samples revealed significant effects of farm and egg counting method but no significant interactions between the two parameters. Although the MM method resulted in significantly higher EPG counts (604) than MF (460; P = 0.029), there was a very strong linear association between the two measurements with MF estimating 0.83% of the EPG counted by MM (Fig. 5.2). The comparison analysis of excreta samples collected from the poultry shade floor and individual caged chicken revealed significant effects of farm, egg counting method, and sample type. Interactions between these effects were only significant for farm x sample type (P = 0.0012) (Table 5.5, Part B). The MM method resulted in higher egg counts than MF (687 and 503 EPG respectively). There were no significant differences between fresh floor excreta (pooled or individual) and individual caged fresh chicken excreta, indicating that fresh floor excreta (pooled or individual) may predict the true population of EECs.

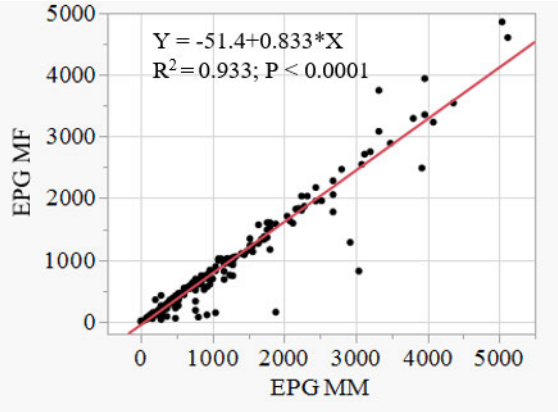

Figure 5.1. Linear regression showing the linear association between EEC determined by the Modified Mc Master (MM) and Mini-FLOTAC (MF) methods in naturally infected individual fresh excreta samples at increasing EPG (egg per gram of excreta)

values for each egg counting technique. Each dot point represents an excreta sample from a single chicken (n = 230).

Table 5.5. Least Square Means of excreta egg count and prevalence of infection determined by MM and MF in naturally infected individually caged hens excreta and pooled or individual fresh floor excreta obtained from commercial free-range farms.

Experiment/factor/level	Cube root LSM EEC±SEM	Back transformed LSM EEC (95% CI)	Arithmetic mean EEC	Prevalence %
A) Individual caged excreta sample				
Overall mean	8.1±0.2	529 (467-596)	850	94.1
Farms	P = 0.002			P = 0.5640
A	8.6±0.2[a]	638 (538-750)	1001	94.0[a]
B	7.5±0.2[b]	433 (359-516)	699	94.2[a]
Methods	P = 0.029			P = 0.0227
MM	8.5±0.2[a]	604 (509-709)	955	91.7[b]
MF	7.7±0.2[b]	460 (382-549)	745	
B) Comparison of EEC between excreta sample types				96.5[a]
Overall mean	8.4±0.3	591 (476-719)	774	94.5
Farm	P = 0.480			P = 0.2827
A	8.2±0.4[a]	548 (401-728)	766	93.7[a]
B	8.6±0.4[a]	635 (472-833)	781	95.2[a]
Method	P = 0.136			P = 0.0315
MM	8.8±0.4[a]	687 (515-895)	886	92.5[b]
MF	7.9±0.4[a]	503 (365-674)	662	96.4[a]
Sample types	P = 0.274			P = 0.2983
Pooled fresh floor excreta	9.2±0.8[a]	787 (460-1242)	795	100[a]
Individual caged bird excreta	8.1±0.2[a]	529 (469-593)	850	94.1[a]
Individual fresh floor excreta	7.8±0.4[a]	484 (357-637)	677	95[a]
Significant interactions (P-value)				
Farm*Sample type	P = 0.0012	-	-	-

Abbreviations: LSM = Least square means; SEM = standard error means; CI = confidence interval; EPG = egg per gram of excreta; MM = Modified McMaster; MF = Mini-FLOTAC. The superscripts (Haidich) indicate the significant difference ($P < 0.05$). LSM comparison was based on cube root transformed EECs.

5.5. Discussion

The most important finding of this study is that while both methods, the MM and MF underestimated the estimated true EEC, the underestimation by MF was greater and increased with increasing EEC. This underestimation was not only observed in both spiking studies but also in experiment 3 with samples of unknown EPG where the EECs for the MF method were significantly lower than those detected by the MM method. This low recovery rate by the MF method in the EEC range coupled with the significantly greater time required to prepare and count samples make it less suitable than the MM method for field diagnostic application. However, the greater sensitivity

of the MF method at very low EEC makes it also a more appropriate test when determining the freedom from infection is required. When investigating the preferred sample type for submission from the field, our data showed that fresh excreta samples collected from the shed floor either individual or pooled, had the best prediction of the true flock EEC as determined by individual counts on 110-120 caged chickens obtained from Farm A and B.

Our first proposition that the predicted advantages of the MF method over the MM method would be sufficient to make it the preferred method for monitoring of nematode infections was not supported by the egg count findings in the egg spiking and natural infection experiments. The egg spiking results showed that MM had recovered more nematode eggs > 50 EPG level compared to MF with the divergence in recovery rate increasing with increasing EPG. In naturally infected chickens, the arithmetic mean egg counts were 1001 and 699 EPG on Farms A and B based on individual counts of 110 and 120 birds, respectively, which were within the range the MF method demonstrated significant underestimation. The recovery rate of MM methods increased as EPG or infection levels were increasing whereas MF continued to underestimate the true population of EEC and similar findings were reported in different host–parasite studies (Noel et al., 2017; Bortoluzzi et al., 2018; Nápravníková et al., 2019; Daş et al., 2020). In contrast, Bosco et al. (2018) and Godber et al. (2015) reported a 100% recovery rate of MF in gastrointestinal nematode eggs spiked in sheep faeces.

The mean EEC in naturally infected chickens from the two free range farms was 850 EPG which is much higher than the minimum detection limit of 40 EPG employed in the present study. Similarly, other researchers observed that mean EPG in naturally infected laying chickens were at least 10-fold higher than the minimum detection limit of 50 EPG (Thapa et al., 2015a; Daş et al., 2020). Additionally, the MM method detected 91.7% of the naturally infected chickens, indicating that MM can accurately and sufficiently detect most chicken nematode infections in field samples. Significantly more time required to process samples using the MF method compared to the MM method is also a major drawback of the MF method as observed in previous studies (Noel et al., 2017; Daş et al., 2020). For these reasons, MM is the preferred method for field evaluation of EEC in poultry flocks where treatment decisions are

required. However, the greater sensitivity of MF at very low EPG levels which is a feature of this method and has also been reported by other studies (Noel et al., 2017; Scare et al., 2017; Bosco et al., 2018; Amadesi et al., 2020; Daş et al., 2020). This allows MF to be the preferred method for diagnosing very low nematode burdens, or testing to certify freedom from infection. Accuracy is the most important diagnostic parameter for estimating the magnitude of nematodes infection in natural infections as the true EPG value of clinical samples are unknown and most often field samples are analysed once per sample (Paras et al., 2018). While multiple replicate counts are known to increase the accuracy, it may be difficult to get sufficient excreta samples from naturally infected individual birds.

The reduced recovery rate of MF relative to MM as infection level increased may be due to the lower multiplication factor of MF and the nature of host excreta. As described by Ballweber et al. (2014), egg counting techniques with lower analytical sensitivity can result in poor parasite egg recovery rate, and Bortoluzzi et al. (2018) suggested that the larger quantity of debris in chicken excreta may impair homogenization efficiency, and partially hinder the filtration and filling process of Fill-FLOTAC system, and result in a reduction of eggs entering the MF counting chambers.

The possible reasons for underestimated egg recovery rate in egg spiked study using MM and MF are likely due to parasite egg isolation or spiking procedures and duration of egg storage (i.e., experiment 2) may lead to egg losses and affect egg flotation process and recovery rate of egg counting techniques (Cringoli et al., 2017; Norris et al., 2019). Operators did not have a significant effect on mean egg counts in the present study due to high within operator variation. This may suggest that the reliability and the accuracy of all egg counting techniques relies on having experienced and skilful operators (Ballweber et al., 2014). In this study, different amount of excreta used for both methods may contribute a potential cofounding effect in the comparison of egg recovery rate. However, multiplication factor may be expected to offset this negative effect.

With regard to test sensitivity, MF was significantly more sensitive than MM only at 50 EPG or below which was expected as the two egg counting techniques differ in minimum detection limit or analytical sensitivity by design (Lester and Matthews,

2014; Daş et al., 2020). The sensitivity of egg counting techniques highly rely on the amount of excreta sample to be examined, the true EPG level (concentration or density of eggs) and the multiplication factor of the egg counting methods (Ballweber et al., 2014; Lester and Matthews, 2014; Levecke et al., 2015; Daş et al., 2020) and thus the sensitivity of the MM method can be increased by counting more chambers. As noted above, the better sensitivity of the MF method makes it more appropriate than the MM test in situations where detection of very low EEC is important.

In this study MF had a relatively lower coefficient of variation resulting in a higher overall precision than MM. This is likely due to the combined effect of the Fill-FLOTAC homogenising and filtering device, and the two counting chambers allowing for the examination of a larger amount of excreta material (2 ml) and its greater analytical sensitivity (Cringoli et al., 2017; de Castro et al., 2017; Went et al., 2018). Our data showed that egg count variability measured by coefficient of variation was particularly pronounced at lower EPG level for both techniques but the overall variation was greater for the MM than the MF. The precision and sensitivity of egg counting techniques can be generally improved by examining larger volumes of excreta suspension by increasing the number of chambers counted, performing multiple excreta egg counts, or repeating of slide reads from the same chambers (Cringoli et al., 2004; Vidyashankar et al., 2012; Ballweber et al., 2014; Lester and Matthews, 2014; Daş et al., 2020). The simplicity and speed of the MM method make these attractive alternative means of improving test sensitivity and precision without recourse to an alternative test.

Our second proposition that pooled fresh floor excreta samples will be the most suitable sample to collect from a free range flock to indicate infection level was partially supported by the findings of experiment 3. The analysis of excreta samples collected from the shed floor (i.e. pooled or individual) and individual caged birds did not have significant difference on EECs revealed that pooled fresh floor excreta may predict the true population of EECs. This allows for representative sample submission, where pooling excreta samples collected from five different locations in poultry shed and it may not only reduce work load for the farm management for sample collection and shipment but also time saving for laboratory analysis.

5.6. Conclusions

The higher accuracy and the much shorter sample processing time of the MM method makes this method more suitable for field evaluation of EEC in laying chicken flocks compared to the MF method. On the other hand, the greater sensitivity and precision of the MF method, particularly at low EPG counts may have application in situations where freedom from nematode infection is being investigated or very low EEC are anticipated. Our findings in this regard are in agreement with a recent study (Daş et al., 2020) who concluded that MM is faster and relatively more accurate but less precise than MF. For submission of samples from the field for diagnostic purposes, 20 fresh excreta samples collected from the floor of the poultry house and counted individually, or one pooled fresh excreta collected from five different location in poultry shed provided an adequate estimate of the flock EEC and this would be sufficient for routine monitoring of chicken flock nematode infection.

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STATEMENT OF AUTHORS' CONTRIBUTION

We, the Research PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

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STATEMENT OF ORIGINALITY

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We, the Research PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

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Chapter 6. Method optimization for prolonged laboratory storage of *Ascaridia galli* eggs

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*Statement of authors' contribution and statement of originality are at the end of chapter

6.1. Abstract

Eggs in the infective stage of the chicken nematode *Ascaridia galli* are often required for in *vivo* and *in vitro* studies on this parasite. The reliability of any artificial *A. galli* infection depends on the viability and embryonation capacity of *A. galli* eggs. The aim of this study was to determine ideal storage conditions for maximising the viability of *A. galli* eggs and maintaining viability for the longest period. A 2 x 2 x 3 x 5 factorial experimental design was employed to investigate the effects of storage temperature (4˚C or 26˚C), storage condition (aerobic or anaerobic), storage medium (water, 0.1 N H_2SO_4 or 2% formalin) and storage period (4, 8, 12, 16 and 20 weeks). The viability of eggs was assessed after eggs in all treatments were held aerobically at 26˚C for 2 weeks after the storage period to test embryonation capacity. Based on morphological characteristics, they were categorised as undeveloped, developing, vermiform, embryonated or dead. The maintenance of viability during storage at 4˚C was optimal under anaerobic conditions while at 26˚C it was optimal under aerobic conditions. Anaerobic conditions at 26˚C led to a rapid loss of viability while aerobic conditions at 4˚C had a less severe negative effect on maintenance of viability. Egg storage in 0.1 N H_2SO_4 resulted in a significantly higher viability overall (54.7%) than storage in 2% formalin (49.2%) or water (37.3%) ($P < 0.0001$). Untreated water was the least favourable storage medium when eggs were stored at 26˚C while it was a medium of intermediate quality at 4˚C. The viability of *A. galli* eggs decreased significantly with storage time ($P < 0.0001$) depending on the other factors. The lowest rate of decline was seen with storage of eggs under anaerobic conditions at 4˚C or aerobic conditions at 26˚C in 0.1 N H_2SO4. Eggs in these treatments retained up to 72% of overall viability at 20 weeks with a decline rate of approximately 2% per week with no significant difference between the two. Therefore, this study has clearly revealed opposing aerobic conditions required for prolonged storage of *A. galli* eggs in the pre-embryonated state at 4˚C. It has also identified that 0.1 N H_2SO4 provides the best preservation against degradation during storage, particularly at 26˚C under aerobic conditions. Achieving strictly anaerobic conditions can be difficult to achieve so storage aerobically at 26˚C may be preferred for simplicity.

6.2. Introduction

There has been a revived and growing research interest in the chicken nematode *Ascaridia galli* due to its re-emergence in the poultry industry (Tarbiat et al., 2018; Shifaw et al., 2021a). Increasing use of floor based and free-range egg production systems allow chickens direct contact with their excreta facilitating the completion of the life cycle of *A. galli* via the faecal-oral transmission route (Permin et al., 1999b; Jansson et al., 2010; Kaufmann et al., 2011a; Kaufmann et al., 2011b; Wongrak et al., 2014; Shifaw et al., 2021a). Several experimental studies have been conducted in recent years to better understand various aspects of this parasite. Artificial infection of ascarids depends on the source of eggs, which must incubate and embryonate outside the chicken host (Ackert and Herrick, 1928; Elliott, 1954).

The viability and infectivity of *A. galli* eggs chiefly depends upon temperature, oxygen concentration, humidity and storage medium influencing the survival or longevity and development of *A. galli* eggs under laboratory conditions (Ackert, 1931; Ackert et al., 1947; Todd et al., 1952; Reid, 1960; Tarbiat et al., 2015; Tarbiat et al., 2018; Feyera et al., 2020). The optimum incubation temperature for *A. galli* eggs has been reported to be between 25 and 30˚ C to allow a constant development of eggs (Ackert and Herrick, 1928; Ackert, 1931; Ackert et al., 1940; Ackert et al., 1947; Tarbiat et al., 2015). Availability of oxygen is an essential requirement not only for embryonation to occur but also to maintain optimal viability at embryonation temperatures (Hansen et al., 1953; Saunders et al., 2000; Tarbiat et al., 2015; Feyera et al., 2020). At lower temperatures (4-6 ˚C), egg development is limited due to low metabolic activity and thus oxygen is not required to maintain viability. Instead, strict anaerobic conditions are required to keep eggs in a metabolically inactive state (Saunders et al., 2000; Tarbiat et al., 2015; Tarbiat et al., 2018). Similarly, embryonation of *A. galli* eggs requires > 85% relative humidity at optimum temperature (Tarbiat et al., 2015). Culture and storage media may also have significant effect on the viability and development of parasite eggs. Sulphuric acid (0.1N) and formalin (2%) are the most commonly used culture media for *A. galli* eggs. These are important to prevent putrefaction of the parasite eggs and inhibit fungal and bacterial growth (Ackert and

Herrick, 1928; Ackert et al., 1935b; Permin et al., 1997a; Tiersch et al., 2013). Among the various developmental stages of *A. galli* eggs, the undeveloped stages are the most resistant to non-freezing low temperatures (0-6 ˚C) (Ackert and Cauthen, 1931b), and thus temperatures ranging from 4 - 6°C are a suitable preservative temperature for nematode egg storage in the laboratory (Gamboa, 2005; Kim et al., 2012; Tarbiat et al., 2015; Feyera et al., 2020).

Preserving *A. galli* eggs under laboratory conditions facilitates the maintenance and propagation of characterised strains for research purposes, for example, those with different anthelmintic resistance profiles (Feyera et al., 2021b). The reliability of any artificial *A. galli* infection depends on the viability or embryonation capacity of *A. galli* eggs (Rahimian et al., 2016). *A. galli* eggs are most commonly obtained from mature worms either by disruption of the worm's uterus (Ackert, 1931; Ackert et al., 1931a; Ackert et al., 1947; Permin et al., 1997a; Gauly et al., 2001a; Gauly et al., 2001b; Daş et al., 2010), by in vitro culturing of female worms in artificial media (Ruhnke et al., 2017; Feyera et al., 2020; Feyera et al., 2021a; Feyera et al., 2021b; Feyera et al., 2021c) or by recovering *A. galli* eggs from poultry excreta (Ferdushy et al., 2012; Tarbiat et al., 2015; Rahimian et al., 2016; Shifaw et al., 2021b). The availability of parasite egg isolate banks for artificial infection would allow for the use of defined strains of *A. galli* within and between laboratories. However, parasite egg banks for different *A. galli* isolates have not been established to date due to the lack of optimized methods allowing for prolonged storage of viable eggs (Feyera et al., 2020). Optimizing prolonged storage of *A. galli* eggs will also reduce the necessity for frequently recovering eggs from female worms or fresh excreta from chickens harbouring *A. galli* specific infections.

Given the clear evidence that unembryonated eggs stored at 4˚C maintain the highest viability under anaerobic conditions, but that eggs require oxygen for embryonation to occur and appear to maintain high viability under aerobic conditions, it remains unclear as to which combination of oxygen availability and temperature will allow the preservation of eggs for the longest period. The current study was designed to address this issue in a single experiment for the first time by evaluating the viability and embryonation capacity of *A. galli* eggs kept in 0.1 N H_2SO_4, 2% formalin and water media under aerobic and anaerobic storage conditions at both 4 and 26°C for 20 weeks

in a factorial arrangement. Under this objective we tested the following basic hypotheses 1) maintenance of viability during storage at 4˚C will be optimal under anaerobic conditions; 2) maintenance of viability during storage at 26˚C will be optimal under aerobic conditions; 3) 0.1 N H_2SO_4 and 2% formalin will be required to achieve maintenance of viability at 26˚C, but not 4˚C; and 4) a combination of aerobic storage at 26˚C with 0.1 N H_2SO_4 and 2% formalin will maintain viability of stored eggs for at least 20 weeks.

6.3. Materials and Methods

6.3.1. Ascaridia galli eggs

Ascaridia galli eggs were extracted from excreta of laying chickens subject to mono-specific *A. galli* infections as described previously (Rahimian et al., 2016; Tarbiat et al., 2018; Shifaw et al., 2021b). Briefly, the prepared excreta slurry was flushed with tap water and passed through a series of 6 sieves (pluriSelect Life Science, Leipzig, Germany) with mesh aperture sizes of 750, 500, 250, 90, 75 and 63 µm, and the eggs were then collected on a 30 µm sieve. To isolate eggs from the material retained on the 30 µm sieve, it was washed off and transferred to 50 ml conical centrifuge tubes. The tubes were then centrifuged at 1620 x g for 1 min and the supernatant was discarded leaving approximately 5 ml of the content in the tube. Then, 45 mL of saturated salt solution (specific gravity = 1.2) was added and the tubes centrifuged again at 1620 x g for 1 min. After centrifugation, the supernatant containing eggs was passed through the 30-µm sieve and rinsed with a large amount of deionized water before being washed off the sieve into 50 ml falcon tubes with the eggs stored in water in these tubes at 4 °C for less than 24 hrs before being used for storage treatments outlined below.

6.3.2. Experimental design and setup

A 2 x 2 x 3 x5 factorial experimental design was used to test the effects of storage temperature (4˚C or 26˚C), aerobic condition (aerobic or anaerobic), storage medium (water, 0.1 N H_2SO_4 or 2% formalin) and storage period (4, 8, 12, 16 and 20 weeks)

with each combination replicated 3 times for the whole procedure, resulting in a total of 180 samples (Fig. 6.1). Briefly, 0.2 ml of water containing 500 *A. galli* eggs was added to 1.7 ml of storage medium in Eppendorf tubes which were then subjected to storage conditions noted above. After the relevant treatment periods, eggs were monitored for viability and development as described below (section 6.3.3). To ensure aerobic conditions, a small pore of 2 mm diameter was created in the lids of the relevant Eppendorf tubes, allowing for a constant exchange of air. To create anaerobic conditions, Eppendorf tube lids were also perforated with a 2 mm diameter hole and after the lid was tightly closed the tube was placed in a rack inside an impermeable vacuum-pack plastic bag (VAC bags, iKON pack, Australia), air extracted from the bag and vacuum sealed in a large research vacuum sealer (MULTIVAC, D-87787 Wolfertschwenden, Germany). All experimental samples were then stored at either temperature for the defined storage period. For storage at 26˚C, samples were kept in a temperature-controlled incubator, whereas samples stored at 4 were kept in refrigerator set at 4˚C. Temperatures were monitored regularly throughout.

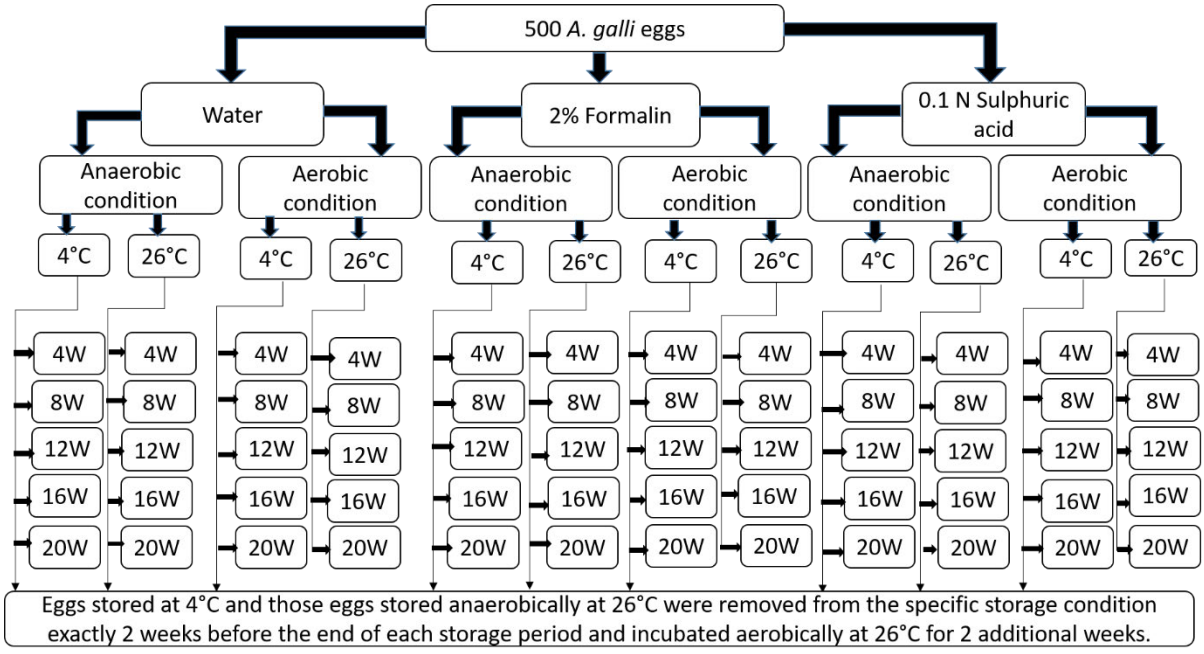

Figure 6.1. Experimental design. The *A. galli* eggs were exposed to different conditions in three storage media; water, 0.1 N H_2SO_4 and 2% formalin under aerobic and anaerobic condition at 4˚C or 26˚C in triplicates procedure. Eggs aerobically stored at 26°C were directly assessed for viability without further incubation at the end of each storage period and eggs stored at 4°C and those stored anaerobically at

26°C were removed from the specific storage condition exactly 2 weeks before the end of each storage period and incubated aerobically at 26°C for 2 additional weeks. After the final incubation, for each replicate samples, around 100 eggs were examined microscopically.

6.3.3. Monitoring of egg viability and development

Eggs monitoring for viability was performed using the following approaches: 1) samples aerobically stored at 26°C were directly assessed for viability without further incubation at the end of each storage period. 2) Samples stored at 4°C and those stored anaerobically at 26°C were removed from the specific storage condition exactly 2 weeks before the end of each storage period and incubated aerobically at 26°C for 2 additional weeks. This allowed for embryonation and therefore statistical analysis so that the viability of egg samples stored at both temperatures under different conditions could be directly compared by fitting a full factorial model.

After the incubation period, egg viability was evaluated by microscopically examining morphological characteristics under a compound binocular microscope equipped with a digital Nikon H550S camera (Nikon Corporation, Tokyo, Japan). For each replicate sample, 100 eggs were examined and the proportion of eggs at different stages of development (unembryonated, early development, vermiform and embryonated) or dead were recorded for each category as described previously (Tarbiat et al., 2015; Thapa et al., 2017a; Feyera et al., 2020) and shown in Fig. 6.2. Undeveloped eggs (Fig. 6.2a) contained a single cell, which almost completely filled the eggshell with a granulated appearance. Eggs undergoing mitosis (two cells and above) without signs of differentiation were classified as early development stages (Fig. 6.2b, c). The vermiform stage (Fig. 6.2d) was characterized by a tadpole-like or kidney shape embryo which almost filled the entire capsular space with high terminal opacity. Embryonated eggs that had completed development contained coiled slender motile larvae (Fig. 6.2e, f). Eggs with an abnormal intra-capsular mass, disrupted eggshell and a shrunken internal embryonic mass were considered dead (Fig. 6.2g, h).

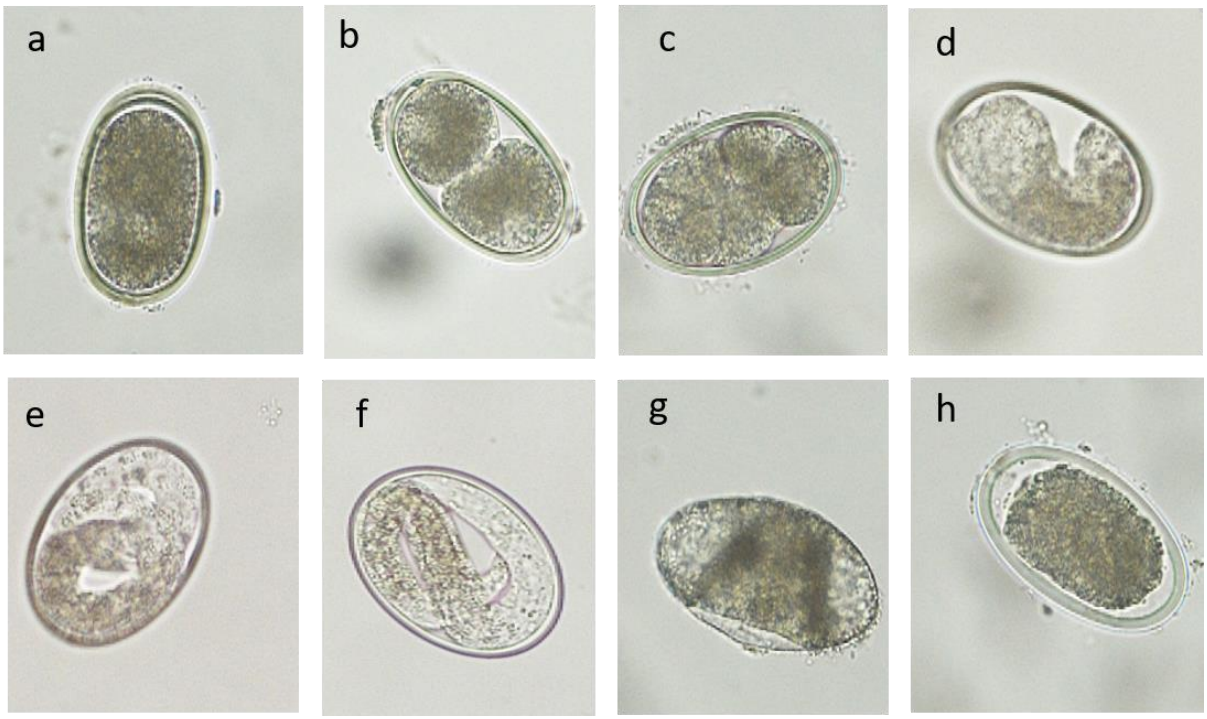

Figure 6.2. Morphological characteristics of different developmental stage of *A. galli* eggs: undeveloped (a); early developing without differentiations (b and c); vermiform (d); embryonated (e and f); dead (g and h).

6.3.4. Statistical analysis

Data analysis were performed using appropriate models in JMP® software version 14.3.0 (SAS Institute Inc., Cary, NC, USA). Data complied with the assumptions of analysis of variance (ANOVA) and transformation of data was not required. A full factorial analysis of variance (ANOVA) in the linear model platform of JMP was used to analyse treatment effects on the percentage of eggs in the different egg developmental stages (undeveloped, developed, vermiform, embryonated stages) fitting the fixed effects of storage medium (water, 2% formalin, or 0.1 N H_2SO_4), aerobic conditions (aerobic or anaerobic), storage temperature (4 or 26), storage period (4, 8, 12, 16, or 20 weeks) and their interactions in the model. Overall percentage of egg viability was defined as the proportion of eggs in developmental stages other than dead. Percentages were analysed in triplicate counts of 100 eggs, thus a total of 300 eggs were evaluated per treatment combination. Data are presented as least-squares means (LSM) and standard errors (SEM). Statistical comparisons between means within main effects (> 2 levels) were performed using Tukey's HSD test. Linear

regression was used to test the association between egg viability and embryonation and to determine the rate of decline in viability with storage time by regressing viability level against week within treatment groups. $P < 0.05$ was considered significant difference in all analysis.

6.4. Results

The percentage of morphologically intact or undamaged eggs at the time of initial storage was 99.3% and the viability percentage of these eggs following incubation at 26°C aerobically for 14 days was 90.5% indicating a satisfactory level of initial viability of the eggs used in the study. The experimental factors and their interactions on the percentage of the different developmental stages of *A. galli* eggs is presented in Table 6.1. There was a strong linear association between the percentage of embryonation and viability at the end of each treatment period ($R^2 = 0.99$; Fig. 6.3), indicating that most viable eggs embryonated within 2 weeks of exposure to air at 26˚C. Thus, the percentage of eggs that were embryonated can be taken as the marker of viability. The analysis of storage data revealed that all main effects in the statistical model and most of interactions were statistically significant ($P < 0.05$; Table 6.1), indicating complex relationships between the factors tested. The significant 4-way interactions are best illustrated in Fig. 6.3 and the two and three-way interactions are presented in Table 6.2.

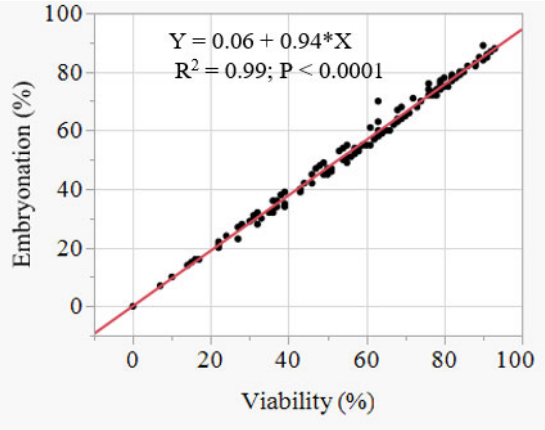

Figure 6.3. Linear association between the percentage of eggs embryonated and the percentage of viable eggs (embryonated plus other viable stages) at the end of each

treatment period. Each dot represents the mean of 3 replicate counts of 100 eggs with all treatments combined.

6.4.1. Effect of storage period on egg viability and embryonation

Egg viability and embryonation decreased significantly with storage period ($P < 0.0001$; Table 6.1). Overall, there was a linear weekly decline in overall egg viability of 3.30% ($y = 86.7-3.30x$) and in overall embryonation percentage of 3.10% ($y = 81.2-3.10x$). However, these rates of decline were greatly affected by other treatment factors as shown by the many significant interactions in Table 6.1 and Fig. 6.4. These differences are discussed in the sections below.

6.4.2. Effect of storage medium on egg viability and embryonation

Eggs stored in 0.1 N H_2SO_4 had a significantly higher overall viability (54.7%) compared with eggs stored in 2% formalin (49.2%) which in turn had a significantly higher viability than those stored in water (37.3%) ($P < 0.0001$). The significant three-way interaction between storage medium, storage temperature and storage condition ($P < 0.05$) was due to a marked reduction in embryonation levels in eggs stored under anaerobic condition at 26°C particularly in water. Eggs stored in water had a significantly higher overall percentage of dead eggs (62.7%) than those stored in 0.1 N H_2SO_4 (45.3%) or 2% formalin (50.8%) ($P < 0.0001$; Table 6.1), indicating that water without a preservative is a poor medium for long term egg storage, particularly at the 26˚C temperature (Table 6.2; Fig. 6.4). Storage of eggs in 0.1 N H_2SO_4 resulted in the highest level of maintenance of embryonation capacity at both at 26˚C and 4˚C but under aerobic and anaerobic conditions respectively (Table 6.2). These treatments resulted in viability of 47and 56% respectively and embryonation capacities of 47 and 52% after 20 weeks of storage (Fig. 6.4). These treatments provided the minimum decline rate in viability of approximately 2%/week. At this rate, viability may be completely lost by around 45 weeks of storage (Table 6.2). Egg storage under aerobic conditions in 2% formalin provided viability and embryonation rates not significantly

different from storage in 0.1 N H_2SO_4 at 26˚C, but at 4˚C it resulted in the fastest loss of viability by 20 weeks of storage (Table 6.2 and Fig. 6.4).

6.4.3. Effect of aerobic storage condition on egg viability and embryonation

The effect of aerobic storage condition was dependent on the storage temperature (AC x ST interaction $P < 0.0001$, Table 6.1). Storing eggs under aerobic conditions resulted in significantly higher embryonation and viability rates at 26˚C, but the reverse was true for unembryonated eggs stored at 4˚C for which anerobic condition were more successful (Table 6.2 and Figure 6.4). The adverse effects of anaerobic storage at 26˚C were dramatic with no viability retained by 12 weeks in any storage medium and by 4 weeks when stored in water at this temperature (Fig. 6.4). These adverse effects were greater than the adverse effects of aerobic conditions at 4˚C. Under aerobic conditions, eggs stored at 26˚C had significantly higher embryonation and viability than those stored aerobically at 4˚C in both 0.1 N H_2SO_4 and 2% formalin (Table 6.2; Fig 6.4). This was not the case for aerobic storage in water which produced intermediate level of maintenance of viability at both 26˚c and 4˚C.

6.4.4. Effect of storage temperature on percentage of viability and embryonation

Over all treatments and storage times eggs stored at 4°C had a significantly higher percentage of total viable eggs (57.3%) than those stored at 26°C (36.9%) ($P < 0.0001$; Table 6.1). However, this effect was strongly influenced by aerobic storage condition and storage media as indicated by significant interaction between these effects (Table 6.1). High maintenance of viability could be attained at both temperatures under different aerobic conditions and storage media. At 4˚C, anaerobic storage in 0.1 N H_2SO_4 resulted in 52% embryonation capacity retained after 20 weeks of storage with minimal loss of embryonation capacity during storage (1.81% / week). At this rate of loss, a complete loss of embryonation would occur after 50 weeks of storage. Maintenance of viability under anaerobic conditions at 4˚C was numerically, but not statistically lower for the other storage media at 20 weeks. Storage at 26˚C also provide

good maintenance of viability, but only under aerobic conditions. Aerobic conditions at this temperature are almost mandatory if a reasonable duration of maintenance of viability is to be obtained. Aerobic storage at 26˚C in 0.1 N H_2SO_4 resulted in 47% embryonation capacity retained at 20 weeks with a rate of loss of embryonated eggs of 1.99 % per week and complete loss of embryonated eggs predicted after 45 weeks of storage. Having a preservative to inhibit microbial growth appears to be essential with a rapid decline in viability to complete loss by 20 weeks occurring when eggs were stored at 26˚C under aerobic condition in water alone. Egg storage at 26˚C under anaerobic conditions resulted in total loss of viability when the storage medium was water and retention of viability to less than 12 weeks if either preservative was included in the medium.

Table 6.1. Result of analysis of variance. Percentages (LSM±SEM) of different *A. galli* egg developmental stages following storage in different storage media, aerobically or anaerobically at either at 4°C or 26°C for 4 weekly intervals up to 20 weeks.

Parameter	Undeveloped	Early development	Vermiform	Embryonated	Total viable	Non-viable
Overall mean ±SEM	0.55±0.51	1.27±0.07	0.76±0.05	44.5±0.54	47.1±0.55	52.9±0.55
Effect and level						
Storage media (SM)	P = 0.2529	P = 0.9225	P = 0.1245	P < 0.0001	P < 0.0001	P < 0.0001
0.1% NH_2SO_4	0.61±0.08^{a}	1.31±0.12^{a}	0.80±0.01^{a}	52.0±0.94^{a}	54.7±0.96^{a}	45.3±0.96^{c}
2% Formalin	0.61±0.08^{a}	1.25±0.12^{a}	0.88±0.01^{a}	46.5±0.94^{b}	49.2±0.96^{b}	50.8±0.96^{b}
Water	0.43±0.08^{a}	1.26±0.12^{a}	0.60±0.01^{a}	35.0±0.94^{c}	37.3±0.96^{c}	62.7±0.96^{a}
Aerobic condition (AC)	P = 0.0008	P = 0.7531	P = 0.5019	P < 0.0001	P < 0.0001	P < 0.0001
Aerobic	0.73±0.07^{a}	1.30±0.09^{a}	0.80±0.08^{a}	50.4±0.76^{a}	53.2±0.79^{a}	46.8±0.79^{b}
Anaerobic	0.37±0.07^{b}	1.25±0.09^{a}	0.72±0.08^{a}	38.6±0.76^{b}	40.9±0.79^{b}	59.1±0.79^{a}
Storage temperature (ST)	P < 0.0001	P < 0.0001	P = 0.0001	P < 0.0001	P < 0.0001	P < 0.0001
4°C	0.78±0.07^{a}	1.80±0.09^{a}	0.98±0.08^{a}	53.8±0.77^{a}	57.3±0.79^{a}	42.7±0.79^{b}
26°C	0.32±0.07^{b}	0.75±0.09^{b}	0.53±0.08^{b}	35.3±0.77^{b}	36.9±0.79^{b}	63.1±0.79^{a}
Storage period (SP)	P < 0.0001	P < 0.0001	P < 0.0001	P < 0.0001	P < 0.0001	P < 0.0001
4 weeks	1.27±0.11^{a}	2.19±0.15^{a}	0.94±0.12^{b}	70.8±1.21^{a}	75.2±1.24^{a}	20.8±1.24^{e}
8 weeks	0.77±0.11^{b}	2.02±0.15^{a}	1.50±0.12^{a}	55.0±1.21^{b}	59.3±1.24^{b}	40.7±1.24^{d}
12 weeks	0.30±0.11^{c}	1.05±0.15^{b}	0.66±0.12^{bc}	43.2±1.21^{c}	44.9±1.24^{c}	55.0±1.24^{c}
16 weeks	0.27±0.11^{c}	0.61±0.15^{b}	0.38±0.12^{c}	33.0±1.21^{d}	34.3±1.24^{d}	65.7±1.24^{b}
20 weeks	0.11±0.11^{c}	0.50±0.15^{b}	0.30±0.12^{c}	20.6±1.21^{e}	21.6±1.24^{e}	78.4±1.24^{a}
Interactions (P-values)						
SP*AC	P = 0.2205	P < 0.0001	P = 0.0119	P < 0.0001	P < 0.0001	P < 0.0001
SP*ST	P = 0.7943	P = 0.0377	P = 0.2387	P = 0.0068	P = 0.0050	P = 0.0050
AC*ST	P = 0.0017	P < 0.0001	P = 0.0455	P < 0.0001	P < 0.0001	P < 0.0001
SP*SM	P = 0.3955	P = 0.7230	P = 0.8195	P = 0.5254	P = 0.2750	P = 0.2750
AC*SM	P = 0.3096	P = 0.7661	P = 0.5395	P = 0.8199	P = 0.7777	P = 0.7777
ST*SM	P = 0.7338	P = 0.1768	P = 0.1060	P < 0.0001	P < 0.0001	P < 0.0001
SP*AC*ST	P = 0.3778	P = 0.1121	P = 0.0524	P < 0.0001	P < 0.0001	P < 0.0001
SP*AC*SM	P = 0.5824	P = 0.9775	P = 0.5790	P < 0.0001	P < 0.0001	P < 0.0001
SP*ST*SM	P = 0.6423	P = 0.3389	P = 0.6390	P = 0.0248	P = 0.0176	P = 0.0176
AC*ST*SM	P = 0.2311	P = 0.1278	P = 0.1782	P = 0.0035	P = 0.0128	P = 0.0128
SP*AC*ST*SM	P = 0.4120	P = 0.1058	P = 0.9676	P < 0.0001	P < 0.0001	P < 0.0001

^{abc} Means within effects not sharing a common letter in the superscript differ significantly (P <0.05).

Table 6.2. Egg embryonation and viability percentages (LSM±SEM) together with rate weekly rate of loss of viability for significant 2- and 3-way interactions between treatment effects.

Significant interactions*	Embryonation ±SEM	Viability ±SEM	Decline rate per week (%)	
			Embryonation	Viability
Aerobic condition*temperature				
Anaerobic, 4°C	63.2±1.1[a]	67.4±1.1[a]	2.34	2.43
Aerobic, 26°C	57.2±1.1[b]	59.3±1.1[b]	2.99	3.33
Aerobic, 4°C	44.3±1.1[c]	47.1±1.1[c]	4.11	4.46
Anaerobic, 26°C	13.3±1.1[d]	14.5±1.1[d]	2.78	3.01
Storage medium*aerobic condition				
0.1 N H_2SO_4, aerobic	58.8±1.3[a]	61.3±1.4[a]	2.77	3.11
2% Formalin, aerobic	52.4±1.3[b]	54.9±1.4[b]	3.46	3.82
Water, aerobic	41.1±1.3[c]	43.5±1.4[c]	4.41	4.75
0.1 N H_2SO_4, anaerobic	45.3±1.3[c]	48.0±1.4[c]	3.15	3.35
2% Formalin, anaerobic	40.5±1.3[c]	43.5±1.4[c]	3.08	3.31
Water, anaerobic	20.9±1.3[d]	31.2±1.4[d]	1.44	1.49
Storage medium*temperature				
0.1 N H_2SO_4, 4°C	59.5±1.3[a]	62.8±1.4[a]	2.69	2.87
2% Formalin, 4°C	51.0±1.3[b]	54.8±1.4[b]	3.51	3.75
Water, 4°C	50.7±1.3[b]	54.4±1.4[b]	3.48	3.71
0.1 N H_2SO_4, 26°C	44.6±1.3[c]	46.5±1.4[c]	3.25	3.59
2% Formalin, 26°C	41.8±1.3[c]	43.8±1.4[c]	3.05	3.38
Water, 26°C	19.3±1.3[d]	20.3±1.4[d]	2.36	2.54
Aerobic condition*temperature*media				
Anaerobic, 4°C, 0.1 N H_2SO_4	68.9±1.9[a]	72.3±4.3[a]	1.81	1.83
Aerobic, 26°C, 0.1 N H_2SO_4	67.4±1.9[a]	69.3±4.3[ab]	1.98	2.30
Aerobic, 26°C, 2% formalin	63.6±1.9[ab]	65.8±4.3[ab]	2.27	2.60
Anaerobic, 4°C, 2% formalin	62.9±1.9[ab]	65.2±4.3[ab]	2.35	2.21
Anaerobic, 4°C, water	57.9±1.9[bc]	62.5±4.3[b]	2.88	2.99
Aerobic, 4°C, 0.1 N H_2SO_4	50.2±1.9[cd]	53.3±4.3[c]	3.58	3.92
Aerobic, 4°C, water	43.5±1.9[de]	46.3±4.3[cd]	4.08	4.43
Aerobic, 4°C, 2% formalin	39.2±1.9[e]	42.0±4.3[d]	4.67	5.04
Aerobic, 26°C, water	38.1±1.9[e]	40.7±4.3[d]	4.73	5.10
Anaerobic, 26°C, 0.1 N H_2SO_4	21.9±1.9[f]	23.7±4.3d[e]	6.33	6.83
Anaerobic, 26°C, 2% formalin	18.1±1.9[f]	19.8±4.3[e]	5.33	5.83
Anaerobic, 26°C, water [1]	0.00[g]	0.00[f]	≥25[#]	≥25[#]

* P values for the interactions can be obtained from Table 1.

[abc] Means within effects not sharing a common letter in the superscript differ significantly ($P<0.05$)

[#]0% viability detected at first check after 4 weeks of storage

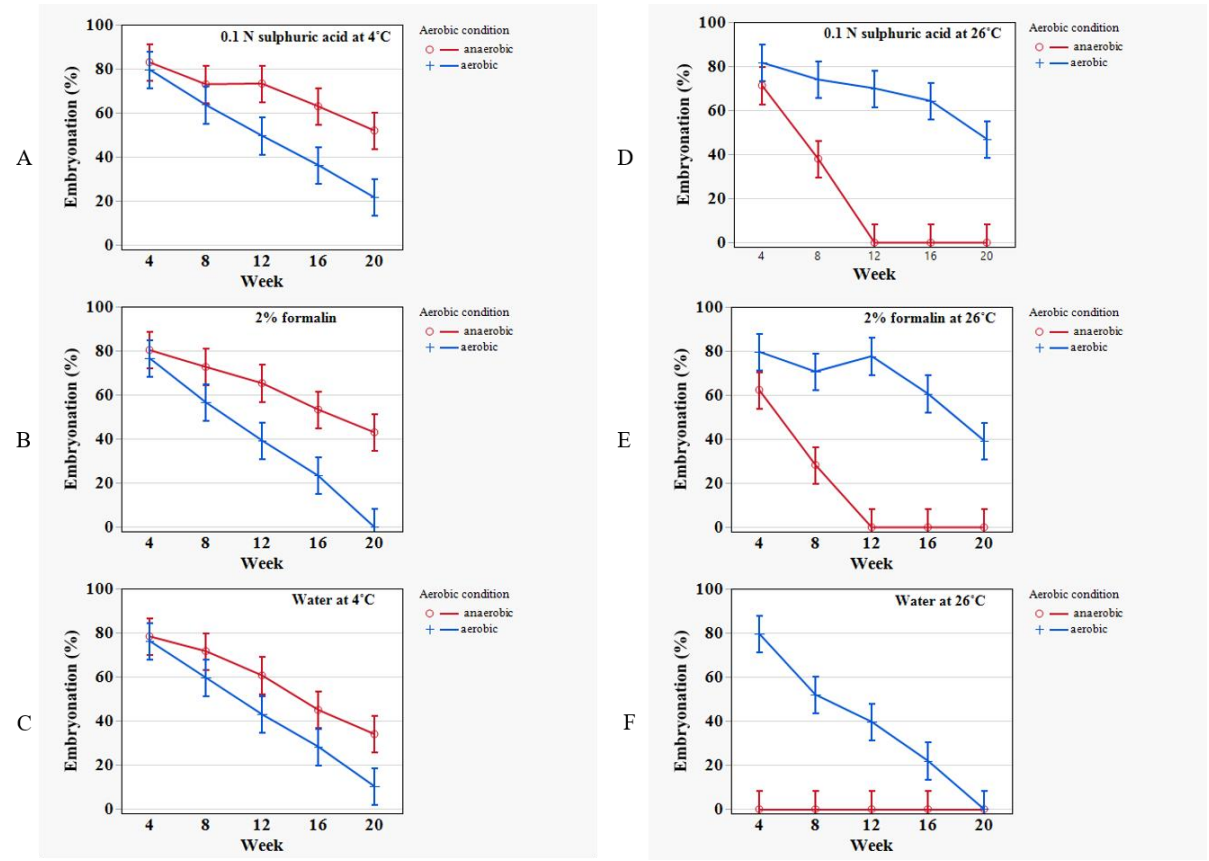

Figure 6.4. Interaction plots (A-F) showing the highly significant 4-way interaction between the effects of aerobic conditions (aerobic, anaerobic), storage temperature (4˚C, A-C; 26˚C D-F) and storage medium (0.1N H_2S0_4 A, D; 2% formalin B, E; water C, F) on embryonation of eggs following each storage period (SP) in weeks.

6.5. Discussion

The main findings of this study are that optimum storage of eggs requires anaerobic conditions at 4˚C and aerobic conditions at 26˚C and that prolonged storage can be achieved at either temperature under the right aerobic storage conditions and media. When stored in 0.1 N H_2SO_4 eggs maintained anaerobically at 4˚C and aerobically at 26˚C retained a high percentage of overall viability (56 and 47 % respectively) and embryonation (52 and 47 % respectively) after 20 weeks of storage. This equated to a decline rate in embryonation capacity of approximately 2% per week. At this rate of decline, 51% of embryonation capacity would be retained at 20 weeks storage, 11% by 40 weeks of storage and complete loss of viability by 45 weeks of storage. Eggs stored at 26˚C in 0.1 N H_2SO_4 maintained the highest percentage of viability followed

by 2% formalin, while water was the poorest medium. The adverse effects of having the inappropriate aerobic conditions were far greater at 26˚C where anaerobic conditions led to rapid loss of viability of embryonated eggs, whereas at 4˚C aerobic conditions reduced viability rates of unembryonated eggs, but not to the same extent.

The first hypothesis that maintenance of viability during storage at 4˚C will be optimal under anaerobic conditions and the second hypothesis that maintenance of viability during storage at 26˚C will be optimal under aerobic conditions were both unequivocally supported by the data and clearly shown for the first time in a single experiment. For many nematode species, egg development can be inhibited by lack of oxygen, and it appears that storage at 4˚C under anaerobic condition maintains the viability of unembryonated eggs for up to 45 weeks, most likely by slowing down metabolic processes. Similar observations have been reported for *A. galli* eggs (Tarbiat et al., 2018), equine strongyle eggs (Sengupta et al., 2016) and sheep strongyle eggs (Rinaldi et al., 2011) when eggs were stored at 4 °C under anaerobic conditions for shorter periods of 72, 28 and 21 days, respectively than in the present experiment. Gaasenbeek and Borgsteede (1998) reported that anaerobic storage of *Ascaris suum* at 15°C resulted in 80% of the eggs being viable after a 12-week storage period. Tarbiat et al. (2015) reported that *A. galli* eggs incubated under anaerobic condition at room temperature for the durations of 8 and 2 weeks maintained 87% of viability. Further development does require oxygen and our results and those of others suggest that not only is it a requirement to enable embryonation, it is also required to maintain the viability of the egg in the embryonated state. Several studies demonstrated that unembryonated nematode eggs stored under anaerobic conditions were able to undergo embryonation when provided with sufficient oxygen at room temperature (Saunders et al., 2000; Tarbiat et al., 2015; Tarbiat et al., 2018). Oxygen thus appears to be essential for the development of nematode eggs and maintenance of embryonated eggs meaning that the aerobic and temperature conditions for storing eggs in the undeveloped state, differ markedly from those required for storing eggs in the embryonated state.

The complete loss of viability observed during anaerobic storage in water at 26°C after 4 weeks of storage was in agreement with a previous study (Tarbiat et al., 2018) who observed that *A. galli* eggs stored anaerobically at 25°C in water or excreta were not

viable after 14 and 42 days, respectively. The authors hypothesised that the loss of viability under anaerobic conditions occurred due to oxygen depletion as a result of the metabolic activity when undergoing developmental processes. It was thus speculated that insufficient oxygen supply in metabolically active eggs can be lethal (Tarbiat et al., 2018). In the present study anaerobic storage in 2 % formalin and 0.1 N H_2SO_4 extended the period before complete loss of viability between 8 and 12 weeks perhaps by slowing the rate of embryonic metabolism, but more likely due inactivation of aerobic microbes that would have depleted any residual oxygen present. However, it is clear that embryonated eggs stored at 26˚C should not be maintained under anaerobic conditions. In contrast, when storing unembryonated eggs at 4°C aerobic conditions are less critical with storage under the less desirable aerobic conditions resulting only in a loss of viability of 4.5% per week with 10% viability after 20 weeks of storage. Similar rates of decline in embryonation capacity have been demonstrated for *A. galli* eggs stored at 4°C under aerobic conditions with approximately 6.0% reported by (Feyera et al., 2020) and 4.4% per week by (Tarbiat et al., 2018). In summary, our results and those of others agreed that the decline rate of viability at 4°C under aerobic conditions was significantly greater than during anaerobic storage. These suggest a significant adverse effect of oxygen on unembryonated eggs stored at 4˚C. Conversely our results confirm the absolute requirement of embryonated eggs for aerobic conditions for prolonged storage at embryonation temperatures in the mid 20˚C range.

Our third hypothesis that 0.1 N H_2SO_4 and 2% formalin will be required to achieve maintenance of viability at 26˚C, but not 4˚C was only partially accepted. At 26˚C these storage media had significant positive effects by extending egg viability under both aerobic and anaerobic conditions, presumably by inhibiting microbial growth and egg spoilage. At 26˚C under aerobic condition 0.1 N H_2SO_4 provided greater egg viability at 20 weeks than 2% formalin but this was not statistically significant. This observation is in line with Amoah et al. (2017) who observed that the viability of *Ascaris suum* eggs stored for 28 days in 0.1 N H_2SO_4 or 2% formalin did not differ significantly. However, aerobic storage of uterine *A. galli* eggs for five weeks at 18°C in 0.1 N H_2SO_4 maintained higher percentage of viability (44%) than eggs stored in 2% formalin (26%) (Permin et al., 1997b). This was consistent with Tiersch et al. (2013) who suggested that storage in 0.1 N H_2SO_4 was preferred to 2% formalin and

0.1% potassium dichromate for the embryonation of *Capillaria obsignata* eggs. Under anaerobic conditions at 26˚C in the present study both 0.1 N H_2SO_4 and 2% formalin increased the duration of viability relative to water but did not allow any eggs to maintain viability after 12 weeks. However, at 4˚C, aerobic storage in 0.1 N H_2SO_4, but not 2% formalin, maintained viability significantly better than storage in water. It has been suggested that prolonged exposure to formalin may result in penetration of this chemical into the eggs by diffusion, resulting in toxicity (Nelson and Darby, 2001; Amoah et al., 2017).

The fourth hypothesis that a combination of aerobic storage at 26˚C with 0.1 N H_2SO_4 and 2% formalin will maintain the longest duration of viability of stored eggs was only partly supported by our results. While high maintenance of viability was obtained at 26˚C with aerobic storage in 0.1 N H_2SO_4, a numerically, but not statistically higher maintenance of viability was obtained by anaerobic storage at 4˚C in the same medium. Both resulted in a decline rate of embryonation of only approximately 2% per week. In either storage media, the decline in viability of eggs under aerobic storage at 26°C was almost equivalent to that observed in a previous study (Feyera et al., 2020) who reported that the decline rate of egg viability at 26°C under aerobic conditions in 0.1 N H_2SO_4 was 2.0% at the end of incubation periods of 2, 4 and 6 weeks. Therefore, storage of eggs at 26°C under aerobic conditions and at 4°C under anaerobic conditions in either 0.1 N H_2SO_4 or 2% formalin would provide at least 40-45 weeks of duration of viability. This is in broad agreement with previous studies on *A. galli* (Ackert, 1931; Elliott, 1954) who observed that eggs stored aerobically at room temperature remained viable and infective for 26 and 36 weeks respectively. However, longer viable storage periods have been also reported. Eggs of *A. galli* and *H. gallinarum* were found to survive in a 2% potassium dichromate solution at room temperature for 95 and 90 weeks, respectively as assessed by an *in ovo* larval motility testing method (Christenson et al., 1942). Tarbiat et al. (2018) demonstrated that in eggs stored in excreta under anaerobic conditions at 4°C the embryonation capacity declined by only about 0.15% per week over a 72-day storage period. They also observed virtually no loss of viability over the same period in samples stored anaerobically in water. The reasons for the differences in persistence of viability between experiments is not clear, but it may be associated with the ability to achieve completely anaerobic conditions or methods of assessing egg viability. Many researchers have demonstrated that the

infectivity of *A. galli* eggs diminished with storage time (Ackert et al., 1947; Todd et al., 1950; Todd et al., 1952; Elliott, 1954) but even morphologically viable embryonated eggs may not necessarily reflect *in vivo* infectivity. The most reliable method to assess infectivity is to infect chickens (Ackert and Herrick, 1928; Elliott, 1954; Thapa et al., 2017a).

6.6. Conclusions

This study has shown that long term storage of *A. galli* eggs is best achieved using 0.1 N H_2SO_4 as the storage medium and either anaerobic conditions at 4˚C which will maintain the eggs in the unembryonated state, or aerobic conditions at 26˚C for which will enable embryonation and maintenance of the eggs in the embryonated state. These conditions allow the storage of the eggs until approximately 40 weeks of age before use, when viability could be expected to be around 11%. While storage at 4˚C may seem ideal, achieving strict anaerobic conditions is difficult and a 2 week embryonation period post storage is required before infectivity is attained, so storage at 26˚C or room temperature aerobically may be preferred for simplicity.

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STATEMENT OF AUTHORS' CONTRIBUTION

We, the Research PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

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STATEMENT OF ORIGINALITY

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Chapter 7. *Ascaridia galli* eggs obtained from fresh excreta, worm uteri or worms cultured in artificial media differ in embryonation capacity and infectivity

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7.1. Abstract

Ascaridia galli infection models use eggs isolated from chicken excreta, worm uteri and worms cultured in artificial media. The aim of this study was to compare the infectivity of *A. galli* eggs isolated from these sources under two infection regimens. A 3x2 factorial arrangement was employed to test the infectivity of *A. galli* eggs from the three sources and two modes of infection (single or trickle infection). One hundred and fifty-six Isa-Brown one day-old cockerels randomly assigned to the six treatment groups (n = 26) were orally infected with embryonated *A. galli* eggs obtained from the three *A. galli egg* sources (worm uteri, excreta or eggs shed in vitro) administered either as single dose of 300 eggs at one day-old or trickle infected with 3 doses of 100 eggs over the first week of life. Twenty-two negative control birds remained uninfected. Eggs obtained from cultured worms or excreta exhibited a higher embryonation capacity ($P = 0.003$) than eggs obtained from worm uteri. There were higher worm establishment (infectivity) rates from embryonated eggs originating from cultured worms and worm uteri compared with eggs obtained from fresh excreta ($P < 0.0001$). Trickle infection resulted in a significantly higher total worm burden ($P = 0.002$), establishment rate ($P = 0.002$) and excreta egg counts (EEC, $P = 0.025$) than single infection. Worm length was greater in birds infected with embryonated eggs from excreta than from uteri or cultured worms ($P < 0.0001$). However, mode of infection did not affect worm length ($P = 0.719$) and weight ($P = 0.945$). A strong significant positive linear correlation was observed between EECs and female worm counts at 12 weeks of post infection sampling ($r = 0.75$; $P < 0.0001$). Body weight of birds was negatively correlated with both worm burden ($r = -0.21$; $P < 0.01$) and EEC ($r = -0.20$; $P < 0.05$) at 12 weeks post infection. In conclusion, our results show that eggs shed by cultured worms or isolated from worm uteri had greater infective capacity than eggs harvested from excreta and that trickle rather than bolus infection resulted in higher worm establishment. These factors should be taken into account when considering artificial infection protocols for *A. galli*.

7.2. Introduction

Ascaridia galli is the most prevalent gastrointestinal helminth in backyard, floor-based and free-range chicken production systems (Permin and Hansen, 1998; Permin et al., 1999a; Kaufmann et al., 2011b; Shifaw et al., 2021a) due to its direct life cycle coupled with the long survivability of the resistant eggs in the external environment (Permin and Hansen, 1998; Tarbiat et al., 2015; Thapa et al., 2017a). The growing importance of this parasite in commercial egg production systems is due to the expansion of the cage-free egg production systems (Permin et al., 1999a; Gauly et al., 2001a; Kaufmann and Gauly, 2009; Jansson et al., 2010; Kaufmann et al., 2011a; Kaufmann et al., 2011b; Andersen et al., 2013; Wongrak et al., 2014).

There has been renewed research interest on *A. galli* in the past couple of decades to investigate different aspects of this parasite. Experimental infections under controlled conditions are important to understand the biology and host-parasite interaction (Ackert, 1923, 1931; Roberts, 1937; Tugwell and Ackert, 1952; Egerton and Hansen, 1955; Madsen, 1962b; Brewer and Edgar, 1971; Herd and McNaught, 1975; Paterson and Viney, 2002; Colditz, 2008; McRae et al., 2015), the factors regulating parasite population dynamics (e.g., worm establishment, growth, and fecundity) (Ackert et al., 1931a; Ikeme, 1971a; Permin et al., 1997a; Gauly et al., 2005), host resistance to the parasite (Ackert et al., 1935b; Permin and Ranvig, 2001; Gauly et al., 2001b; Schou et al., 2003; Abdelqader et al., 2007) and the anthelmintic resistance status of this nematode (Tarbiat et al., 2016; Feyera et al., 2021a; Feyera et al., 2021c; Feyera et al., 2022a). However, experimental *A. galli* infections have often produced low establishment rates and lower worm burdens than those observed with natural infections and there is no standardised and consistently used *A. galli* experimental infection model that optimises parasitological parameters of interest for these purposes (Permin et al., 1997a; Gauly et al., 2001a; Marcos-Atxutegi et al., 2009; Daş et al., 2010; Sharma et al., 2018c; Feyera et al., 2021b). Some of the issues with poor establishment rates may be due to deficiencies in the viability of eggs used for artificial infection which could be affected by egg isolation procedures (Rahimian et al., 2016) or the laboratory storage conditions following egg isolation (Tiersch et al., 2013; Tarbiat et al., 2015; Tarbiat et al., 2018; Feyera et al., 2020). Some of this variation

could also be due to worm expulsion over time as a result of the immune response of the chicken host (Stehr et al., 2018).

Experimental infections are also important to study the effect of the parasite on the host and to propagate defined strains with particular characteristics. For instance, pure nematode isolates of defined anthelmintic resistance status can only be maintained by a cycle of laboratory storage of the parasite eggs followed by a passage through the susceptible host for amplification and subsequent use in the animal experiments (Feyera et al., 2021a; Feyera et al., 2021c). Parasite propagation studies aim ultimately at multiplying stocks and recovering infective stages for maintenance of characterized strains. Thus, the number of female nematode worms and their reproductive capacity as measured by *per capita* fecundity are determining factors to be considered for *in vivo* parasite propagation (Feyera et al., 2021b). *A. galli* experimental infection models use eggs isolated from worm uteri (Ackert, 1923; Ackert and Herrick, 1928; Ackert, 1931; Ackert et al., 1947; Permin et al., 1997a; Gauly et al., 2001a; Gauly et al., 2001b; Daş et al., 2010), host excreta (Ferdushy et al., 2012; Luna-Olivares et al., 2012; Katakam et al., 2014; Tarbiat et al., 2015; Rahimian et al., 2016) or worms cultured in artificial media (i.e. *in vitro* culture of female worms and recovering eggs shed into the media) (Ruhnke et al., 2017; Sharma et al., 2018a; Sharma et al., 2018b; Feyera et al., 2020). Protocols used to recover eggs from these sources are mostly not standardized and it is expected that there could be considerable variation which could affect embryonation and subsequent infectivity of embryonated eggs. It is well established that damaged nematode eggs have no potential to initiate embryonic development (Johnston and Dennis, 2012). Female nematodes lay only mature eggs (Kim et al., 2012) and not all eggs isolated from worm uteri are mature or able to complete embryonation (Tiersch et al., 2013). Only the eggs isolated in the portion of the worm uterus proximal to the vagina contains a high percentage (> 80%) of fertile eggs (Ackert, 1931). Eggs isolated from fresh excreta have a higher embryonation ability than uterine eggs probably due to the maturation differences noted above (Rahimian et al., 2016). However, to our knowledge no investigations have been published to compare the infective capacity of embryonated eggs from these sources (i.e. eggs from excreta, worm uteri and worms cultured in artificial media).

The propagation of ascarids is based upon infection with embryonated eggs which must incubate outside of the host to attain the infective embryonated stage (Ackert and Herrick, 1928; Elliott, 1954). This is true for *A. galli* for which fully embryonated eggs appear morphologically as a mature coiled embryo within each egg which then hatches and maintains itself in the host (Ackert and Herrick, 1928; Ackert, 1931). Once embryonated, eggs are expected to have similar high levels of infectivity, but the mere morphological presence of an embryo may not always necessarily indicate a fully infective egg (Christenson et al., 1942; Geenen et al., 1999). The later authors noticed that although *Ascaris suum* motile larvae developed with in the eggs after approximately four weeks of incubation at room temperature, the eggs did not become infective to pig until the cultivation was continued for a further 2-6 weeks. This also applies to some nematode parasites of chickens such as *A. galli* and *Heterakis gallinarum* (Riley and James, 1921; Alicata, 1934; Christenson et al., 1942). The degree of infectivity of embryonated egg may also vary depending on other factors, such as the embryonation media, temperature and age of embryonated eggs (Todd et al., 1952; Oksanen et al., 1990; Permin et al., 1997b; Tiersch et al., 2013). Furthermore, the infectivity of embryonated eggs may be affected by egg source. As described by Permin et al. (1997b), birds infected with 500 embryonated eggs isolated from worm uteri had higher *A. galli* establishment rates and worm burdens than birds infected with 500 embryonated eggs extracted from excreta.

Mode of inoculation may also influence nematode infection outcome. While most artificial *A. galli* studies have used a single inoculation approach, some studies have employed trickle infections with variable infection outcomes (Permin et al., 1997a; Permin and Ranvig, 2001; Gauly et al., 2001b; Idi et al., 2004; Daş et al., 2010; Ferdushy et al., 2013; Sharma et al., 2017; Feyera et al., 2021b). There is a need to systematically investigate the extent to which relevant parasitological parameters such as infection establishment rate, worm load and worm egg output are affected by mode of infection. In principle, trickle infection attempts to mimic natural parasite exposure by infecting animals with multiple low-doses of a specific parasite egg rather than a single high-dose, and is expected to yield a higher establishment rate than a single high dose, which appears to be the norm for low establishment rate (Yazwinski et al., 2003). Trickle infection results in the slow acquisition of immunity, as evidenced by a gradual increase in worm burden followed by partial expulsion (Glover et al., 2019). In

contrast, experimental infections with an artificially high single bolus result in rapid expulsion of the primary infection and strong immunity to subsequent challenges (Colombo and Grencis, 2020).

In light of the above, the aim of this study was to compare the infective potential and consequences on host growth *A. galli* eggs from worm uteri, host excreta or shed by cultured worms in artificial media administered to day old chickens in a single dose or as a trickle infection. Under this aim we tested the following basic propositions: 1) Eggs isolated from fresh excreta and worms cultured in artificial media will have a higher embryonation potential than eggs recovered from worm uteri; 2) The infective capacity of embryonated eggs will vary with the source of eggs 3) Trickle infection will result in higher establishment rate, worm burdens and EEC than bolus infection with the same total dose; 4) The negative effect of worm infection on body weight will increase with the established worm burden in individual chickens.

7.3. Materials and methods

7.3.1. Chickens and their management

A total of 178 day old commercial cockerel layer chickens (Isa Brown) were purchased from a commercial hatchery. On arrival (day 0), chicks were allocated to treatment groups and placed in separate floor pens (n = 26) to commence infections on the next day. Each pen had a floor space of 0.9 m² with wood shavings as bedding material and were located in an indoor climate-controlled research facility. Temperature was gradually decreased from 35°C on the first day to 24°C in the 4th week. Light was reduced in this time period from 24 to 12h/day and subsequently maintained on 12 h/day. At 7 weeks-of-age, the birds were moved and housed individually in enriched layer cages with wire flooring area of 0.37 m² in order to prevent faecal-oral infection. Water was provided using cup supported nipple drinkers and feed (standard commercial layer starter and grower rations) (Riverina Pty Ltd, Queensland, Australia) was offered using feeder troughs. Birds were closely monitored daily for ill health, and sick birds were treated or humanely euthanised if deemed appropriate. This research

was approved by the Animal Ethics Committee of the University of New England with approval number AEC20-084.

7.3.2. Experimental design and application of treatments

This experiment employed a 3x2 factorial arrangement to test three sources of *A. galli* eggs (isolated from female worm uteri, excreta or cultured worm eggs shed in vitro) and two modes of infection (single or trickle infection with the same total number of eggs). This experiment used one hundred and fifty-six cockerel chickens randomly assigned to the six infection treatment groups (n = 26) and 22 birds as negative controls. Infection commenced on the day after arrival (day 1). One day-old chicks were orally infected with either a single dose of 300 embryonated *A. galli* eggs using a 5-cm oral gavage needle, or trickle infected with 100 embryonated eggs on days 1, 4, and 7 with eggs were obtained from three sources (female worm uteri, excreta or cultured worm eggs shed in vitro). The animals were carefully restrained during inoculation of infective eggs. Uninfected negative controls (n = 22) received water.

7.3.3. Source and preparation of *A. galli* eggs for experimental infections

A. galli eggs were acquired from artificially infected chickens in a separate research study (Feyera et al., 2021c). In brief, day-old layer cockerel chicks were trickle infected orally with 100 embryonated *A. galli* eggs on three occasions over the first or third weeks of life (total dose of 300 eggs). At 6 weeks post infection (WPI), the birds were moved to enriched individual layer cages and at 9 WPI, fresh individual excreta samples were collected from each bird separately (n = 16) and excreta egg counts (EEC) determined as described in section 7.3.4.2. Immediately after excreta collection, the birds were killed and dissected for worm collection as outlined in section 7.3.4.3. Birds that were either free from worms or only positive for male worms were excluded from both the faecal and worm collection, thus the eggs isolated from excreta and adult female worms (i.e. from worm uteri and adult worms cultured in artificial media) can be assumed to have originated from the same worm infra-populations.

7.3.3.1. Egg isolation from fresh excreta, worm uteri (uterine eggs) and worms cultured in artificial media

A. galli eggs were extracted from the pooled experimental excreta as described previously (Rahimian et al., 2016; Tarbiat et al., 2018; Shifaw et al., 2021b; Shifaw et al., 2022a). Briefly, the prepared excreta slurry was flushed with tap water and passed through a series of 7 sieves (pluriSelect Life Science, Leipzig, Germany) with mesh aperture sizes of 750, 500, 250, 90, 75, 63 and 30 µm, with eggs retained on the final sieve. The eggs were washed off the 30 µm sieve and transferred to 50 ml conical centrifuge tubes. The tubes were then centrifuged at 1620 x g for 1 min and the supernatant was discarded leaving approximately 5 ml of the residue containing the eggs in the tube. Then, 45 mL of saturated salt solution (specific gravity = 1.2) was added and the tubes centrifuged again at 1620 x g for 1 min. After centrifugation, the supernatant containing eggs was passed through a 30-µm sieve to retain the eggs which were rinsed with a large volume of deionized water before being washed into 50 ml falcon tubes. There eggs were then incubated in 0.1 N H_2SO_4 aerobically in these tubes at 26 °C for 5 weeks before being used for inoculation.

Uterine eggs were harvested from the female adult worms according to a previously established procedure (Daş et al., 2010; Rahimian et al., 2016). Female worms freshly recovered from the intestine were opened longitudinally with scissors and placed on a 63 µm sieve over a Petri dish. Opened worms were then squeezed gently using a pestle rubbing on a sieve in a Petri dish. The eggs were separated from the worm tissues by sieving and flushing with a little water before being placed in 50 ml falcon tubes. The harvested eggs were then incubated in 0.1 N H_2SO_4 at 26 °C for 5 weeks before being used for inoculation.

A. galli eggs shed by cultured adult female *A. galli* worms in artificial media were obtained as described previously (Feyera et al., 2020; Feyera et al., 2021a; Feyera et al., 2021b). In brief, during post-mortem examination (as outlined in section 7.3.4.3), mature female *A. galli* worms were collected from the intestines of artificially infected chickens. The freshly collected adult female *A. galli* worms were then placed in Petri

dishes and washed in sterile phosphate-buffered saline (PBS). The clean worms were then together placed into Roswell Park Memorial Institute (RPMI) medium containing (0.1% 100 units/mL penicillin, 100 µg/mL of streptomycin, 250 ng/ mL amphotericin B) in a glass jar to a volume that covered the worms, and cultured at 37° C for 24 hours. The medium containing parasite eggs was then collected in to 50 ml screw cap falcon tubes and centrifuged at 1620 x g for 1 min. The concentrated eggs at the bottom of the falcon tube were then incubated in 0.1 N H_2SO_4 at 26°C for 5 weeks before being used for inoculation.

7.3.3.2. Determination of embryonation percentage and preparation of samples for inoculation

After five weeks of incubation, the embryonation percentage of eggs were determined by microscopically examining morphological characteristics under a compound binocular microscope equipped with a digital Nikon H550S camera (Nikon Corporation, Tokyo, Japan). For each egg source, 100 eggs replicated 3 times were examined and eggs that had completed development containing coiled slender larvae were recorded as fully embryonated eggs as described previously (Feyera et al., 2020; Shifaw et al., 2022). To prepare eggs for inoculation, fully embryonated egg count per ml of the stock solution was determined based on the arithmetic mean counts of 3 aliquots of a 0.15 mL subsample from each egg source. The required number of inoculated eggs and volume was then calculated for each egg source, treatment group and bird. The individual infection dose comprised either a single dose of 300 fully embryonated eggs in suspension or a trickle dose of 100 fully embryonated eggs in suspension. All doses were adjusted to a volume of 0.4 ml of per bird.

7.3.4. Sampling and measurements

7.3.4.1. Body weight measurement and health observation

From the infection day (day 1) onwards, individual bird weights were recorded every second WPI until 12 weeks (i.e. 2, 4, 6, 8, 10, 12 WPI). Chickens were observed twice daily for general health until 7 WPI and then once daily thereafter.

7.3.4.2. Excreta collection and egg count analyses

Individual excreta samples were collected at 8, 9, 10, 11 and 12 week post initial infection from all birds, by the placement of paper sheets beneath each cage for 1-hour during the mid-morning, when egg concentrations in excreta are likely to be close to maximal (Wongrak et al., 2015). EECs were determined on individual samples using the Modified McMaster technique with analytical sensitivity of 40 eggs/gram of excreta (EPG) as described by (Shifaw et al., 2021b).

7.3.4.3. Post-mortem parasitological examination

All birds that survived at the end of experiment (n = 171) were sacrificed at 12 WPI after the last sampling using electrical stunning followed by cervical dislocation for post-mortem parasitological examination following the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines (Yazwinski et al., 2003). For each bird, immediately after euthanasia, the gastrointestinal tract was removed, the small intestine opened longitudinally using Mayo scissors and contents flushed with tap water through a 100 μm mesh sieve. The contents on the sieve were then transferred to a Petri dish containing physiological saline. All visible adult *A. galli* worms were then collected for determining sex, body length and weight and the remaining sample examined under a microscope to enumerate luminal larvae. Histotrophic or tissue stage larvae were not investigated. Length was measured with a ruler and worms/bird were weighed on an electronic scale to the nearest 0.01 g.

7.3.5. Statistical analysis

All statistical analyses were conducted using appropriate models of JMP®16 (SAS Institute Inc., Cary, NC, USA). EEC and worm counts (worm establishment rate = worm recovery per bird/infection dose x100, female and male numbers, and total worm burden) were transformed by cube root prior to data analysis to better meet the assumptions of analysis of variance (ANOVA). Body weight of chickens, worm length and weight and percentage embryonated did not require transformation. Embryonation

percentage was analysed by one way ANOVA fitting the fixed effect of egg source. Body weight and excreta egg count measurements over time were subjected to repeated measures of analysis of variance fitting individual bird as a random effect in the restricted maximum likelihood model and fitting up to three fixed effects (source of *A. galli* eggs, mode of infection and week post infection) and their interactions in the model. Non-repeated measurements of continuous variables, such as worm counts were analysed by 2-way full factorial ANOVA fitting the fixed effects of egg source (uteri, culture or excreta), mode of infection (single or trickle) and their interactions in the model. Mean worm length and weight in individual chickens was analysed by 3-way full factorial ANOVA fitting the fixed effects of egg source (uteri, culture or excreta), mode of infection (single or trickle), worm sex and their interactions with total worm count fitted as a covariate. Tukey HSD were employed to test for significant differences between levels of a given factor in the analysis. The Chi square test of independence was also used to compare the prevalence of infected birds between treatment groups. Linear regression analysis and correlation was used to evaluate the association between measured parasitological variables in individual chickens. Control bird weights were not included in the association between body weight and parasitological variables. Statistical significance was considered at $P < 0.05$ for all analyses.

7.4. Results

7.4.1. Egg embryonation percentage

The percentage of embryonation of *A. galli* eggs isolated differed significantly with egg source ($P = 0.003$). Eggs obtained from excreta and cultured worms had almost double the embryonation rate of those extracted from worm uteri (79.3±5.4, 81.6 ±5.4 and 41.7±5.4 %, respectively).

7.4.2. Clinical signs and body weight

A total of seven birds died during the experiment (1 from each of the excreta trickle and uterine single infection groups, 2 from the cultured single group and 3 from the

cultured trickle group). All deaths occurred during the first seven weeks, prior to being placed in individual cages and these chickens are excluded from all analyses. Anecdotal observation revealed that a small number of infected birds manifested occasional mild diarrhoea by observation of excreta consistency during excreta collection. No other clinical signs were observed in any of the birds. There was no overall effect of infection on body weight and no significant interaction with time.

7.4.3. Excreta egg counts

EEC was determined for all birds from 8 to 12 weeks post infection and the egg shedding pattern during this period was significantly influenced by both treatment effects in the experiment. Fig. 7.1 illustrates differences in egg count patterns over time for each egg source (Fig. 7.1 A) and the mode of infection (Fig. 7.1 B). Overall, a lower excreta egg count was found in birds infected with the same doses of embryonated eggs isolated from excreta than from worm uteri or cultured worm eggs ($P < 0.0001$; Table 7.1). Birds given trickle infection had a higher excreta egg count than those subject to single infection ($P = 0.025$; Table 7.1). Excreta egg count increased over time in all groups (Fig. 7.1).

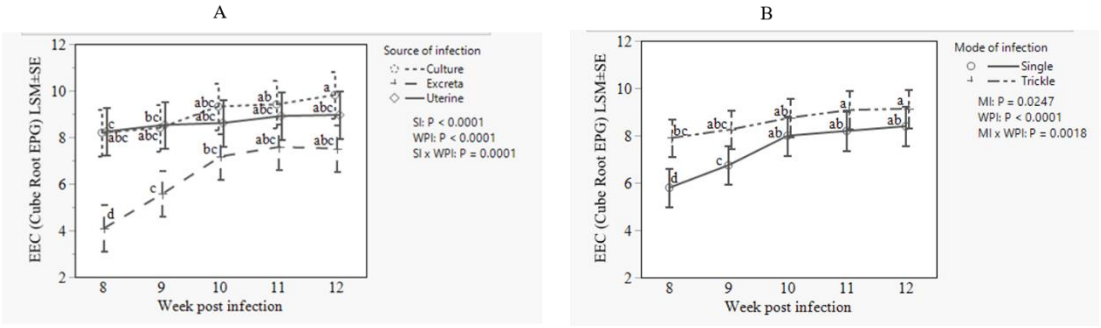

Figure 7.1. Analysis of excreta egg counts showing the significant interaction between; A) week post infection (WPI) and source of infection (SI) ($P < 0.0001$); and B) week post infection and mode of infection (MI) ($P = 0.0018$). All data are Least Square Mean (LSM) ±standard error (SE). Means at each time point not sharing a common letter in the superscript differ significantly ($P < 0.05$).

7.4.4. Prevalence, mean worm count and worm establishment rate

Consistent with the infection protocol, immature stages of *A. galli* were not detected, confirming the absence of re-infection during the experiment. Uninfected control birds were free of *A. galli* infection as confirmed by macroscopic and microscopic examination of the contents of the small intestines. As shown in Table 7.1, there was no significance of the prevalence of infection in birds infected with embryonated eggs acquired from worms cultured in artificial media compared with birds infected with eggs isolated from worm uteri or excreta. Egg source had a significant effect on cube root transformed worm counts, worm establishment rates and EECs ($P < 0.0001$, Table 7.1). Birds infected with embryonated eggs originating from excreta had significantly lower total worm counts, worm establishment rates, numbers of female and male worms compared with birds infected with the same dose of embryonated eggs originating from worm uteri or worms cultured in artificial media (Table 7.1). Trickle infection resulted in significantly higher total worm burden and establishment rates than single infection ($P = 0.002$; Table 7.1). There was no significant interaction between source and mode of infection on worm count ($P = 0.32$).

With regard to measurements made on the worms (Table 7.2), birds infected with the same dose of embryonated eggs originating from worm uteri and worms cultured in artificial media had lower female worm length and weight and male worm length and weight compared with birds infected with eggs originating from excreta ($P < 0.0001$). This effect was independent of total worm count which was fitted as a covariate in the model. Mode of infection did not significantly affect either worm length ($P = 0.719$) or weight ($P = 0.945$). The average weight and length of female worms was significantly higher than that of males (Table 7.2).

Table 7.1. Summary of treatment effects and significance on prevalence of infection at necropsy, mean worm count and worm establishment rate at 12 weeks post infection.

Parameter	Prevalence (%)*	EEC (EPG)			Total worm count (worms/bird)			Worm establishment rate (%)		
		Mean[1]	Median	Range	Mean[1]	Median	Range	Mean[1]	Median	Range
Overall	95.3	828.6	720	0-3560	7.7	7	0-21	2.6	2.3	0-7
Effect and level										
Infection source	P = 0.066	P < 0.0001			P < 0.0001			P < 0.0001		
Excreta egg	90	522.5[b]	400	0-2800	4.7[b]	4	0-14	1.6[b]	1.3	0-4.3
Uterine eggs	96	969.3[a]	840	0-3360	8.5[a]	8	0-21	2.8[a]	2.7	0-7
Cultured eggs	100	994.1[a]	960	0-3560	9.9[a]	10	0-21	3.3[a]	3.3	0-7
Mode of infection	P = 0.658	P = 0.0247			P = 0.002			P = 0.002		
Single infection	94.5	723.1[b]	560	0-3480	6.2[b]	5	0-20	2.1[b]	1.7	0-6.7
Trickle infection	96.0	934.1[a]	880	0-3560	9.2[a]	9	0-21	3.1[a]	3	0-7
WPI	-	P < 0.0001			-	-	-	-	-	-
Week 8		677.8[c]	360	0-3560	-	-	-	-	-	-
Week 9		763.9[b]	600	0-3200						
Week 10		843.4[a]	720	0-2760		-	-	-	-	-
Week 11		912.7[a]	880	0-2800						
Week 12		945.3[a]	840	0-3200	-	-	-	-	-	-
Interactions (P-values)										
SI*MI	-	P = 0.120			P = 0.315	-	-	P = 0.315	-	-
WPI*SI		P < 0.0001			-	-	-	-	-	-
WPI*MI	-	P = 0.0018			-	-	-	-	-	-
SI*MI*WPI	-	P = 0.479			-	-	-	-	-	-

* = *A. galli* positive individual birds at necropsy.

Abbreviations: EEC = excreta egg count; EPG = eggs/gram of excreta; SI = infection source; MI = mode of infection; WPI = week post infection; LSM = least square means; SE = standard error

Means within effects not sharing a common letter in the superscript differ significantly (P < 0.05).

[1]Values presented are untransformed means but statistical analysis was performed using cube root transformed data.

Table 7.2. Summary of treatment effects and significance on worm measurements at the time of worm recovery at 12 weeks post infection.

Parameter	Length (cm/worm)			Weight (g/worm)		
	LSM±SE	Median	Range	LSM±SE	Median	Range
Overall	6.1	5.9	3-9.5	0.09±0.002	0.06	0.02-0.17
Effect and level						
ırce of infection	P < 0.0001	-	-	P < 0.0001	-	-
Excreta egg	6.4±0.6[a]	6.4	4.2-9.4	0.09±0.002[a]	0.09	0.02-0.17
Uterine egg	5.8±0.6[b]	5.3	3.0-9.5	0.08±0.002 [b]	0.05	0.02-0.17
Cultured egg	5.9±0.6[b]	5.7	3.5-8.6	0.08±0.002 [b]	0.08	0.03-0.17
ıde of infection	P = 0.719			P = 0.945		
Single infection	6.1±0.05	5.9	3.5-9.5	0.086	0.06	0.02-0.17
Trickle infection	6.1±0.05	5.6	3.0-8.6	0.086	0.07	0.02-0.17
ırm sex	P < 0.0001			P < 0.0001		
Female	7.5±0.05[a]	7.5	5.2-9.5	0.13±0.001[a]	0.13	0.08-0.17
Male	4.7±0.05[b]	4.7	3.0-6.5	0.05±0.001[b]	0.05	0.02-0.06
nificant interactions (p-values)						
SI*MI	P = 0.004	-	-	P = 0.001	-	P = 0.8859
ariate (P-values)						
Total worm count	P = 0.6935	-	-	P = 0.0503	-	-

EEC = excreta egg counts; EPG = eggs /gram of excreta; WPI = week post infection; cm = centimetre; mg = milligram; g = gram
SI = source of infection; MI = mode of infection: LSM = least square means; SE = standard error
Means within effects not sharing a common letter in the superscript with in the same column differ significantly (P < 0.05).
[1]Values represent untransformed mean but statistical analysis was performed using cube root transformed data.

7.4.5. Association between measured variables

Correlations between parasitological parameters are presented in Table 7.3. Body weight of birds was negatively correlated with both worm burden (r = -0.21; P < 0.01) and excreta egg counts (r = -0.20; P < 0.05) at 12 WPI (Table 7.3). The linear regression equation indicates a 4 ±1 g reduction in bodyweight at 12 weeks for every 100 EPG in excreta and a reduction in bodyweight of 4.6±1.8 g for every *A. galli* worm present (Fig. 7.2 A and B). The correlation between the numbers of female worms and the number of male worms was 0.58 (P < 0.01). Linear regression analysis revealed a strong positive association between EEC and total and female worm counts at 12 WPI sampling (P < 0.0001; Fig. 7.2 C and D). Worm length and weight were negatively correlated with worm counts (P < 0.01; Table 7.3).

Table 7.3. Correlation coefficients (r) between parasitological parameters determined at 12 weeks post infection in chickens artificially infected with *A. galli* eggs (n = 150 infected chickens).

Parameters	EEC[1]	TWC	FWC	FWL	FWW	MWC	MWL	MWW	WER
Fecundity[1]	*0.18*	-	-	-	-	-	-	-	-
TWC	***0.69***	-	-	-	-	-	-	-	-
FWC	***0.75***	***0.86***	-	-	-	-	-	-	-
FWL	-0.13	*-0.20*	*-0.23*	-	-	-	-	-	-
FWW	-0.13	**-0.35**	***-0.35***	***0.72***	-	-	-	-	-
MWC	***0.52***	***0.91***	***0.58***	-0.14	*-0.27*	-	-	-	-
MWL	0.02	-0.12	-0.02	***0.49***	*0.28*	*-0.18*	-	-	-
MWW	0.04	-0.06	0.02	***0.45***	***0.42***	-0.10	***0.66***	-	-
WER	***0.69***	1.00	***0.86***	*-0.15*	***-0.34***	***0.92***	-0.12	-0.06	-
BW[1]	*-0.20*	*-0.21*	*-0.23*	-0.22	*0.21*	-0.15	0.12	0.27	*-0.21*

[1] = at 12 week post infection; P < 0.0001 = bold and italic; P < 0.05 = italic; not significant = normal font. Abbreviations: TWC = total worm counts; FWC = female worm counts; FWL = female worm length; FWW = female worm weight; MWC = male worm counts; MWL = male worm length; MWW = male worm weight; WER = worm establishment rate. Control bird weights were not included in the association between body weight and parasitological variables.

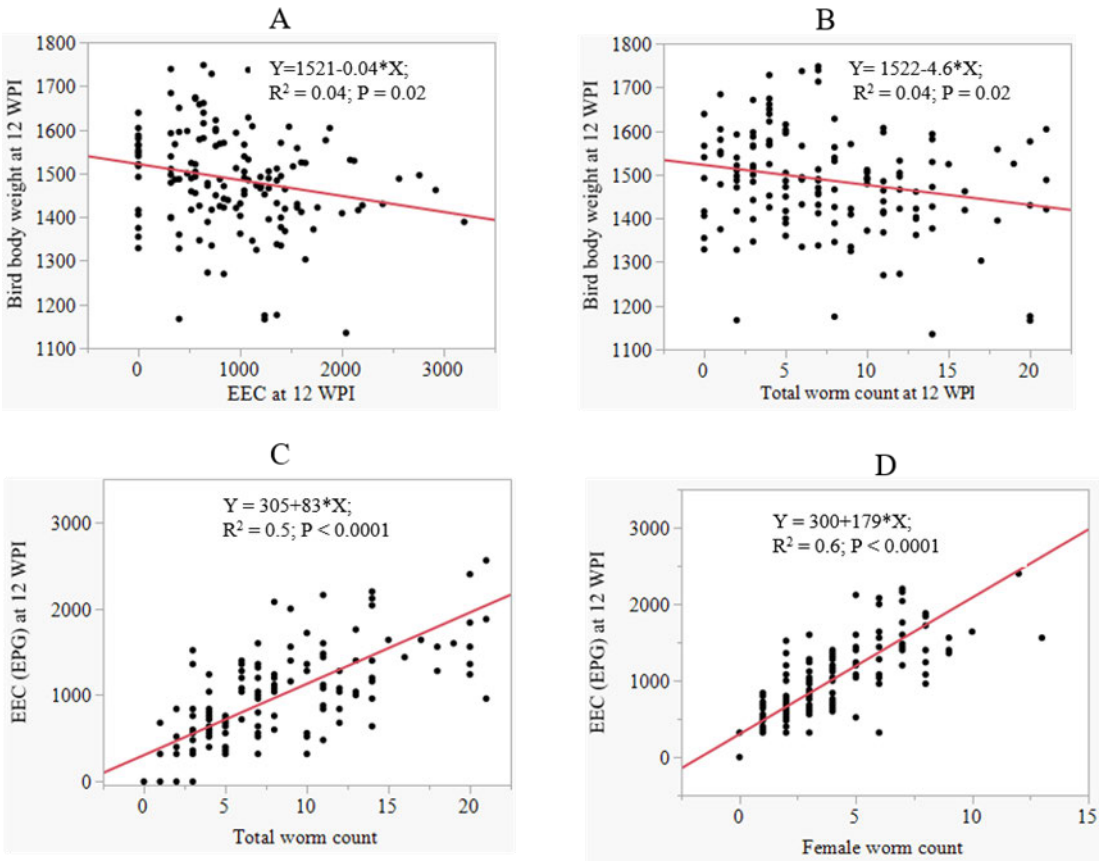

Figure 7.2. Linear regression plots showing the association of excreta egg count (EEC) and total worm count with bird bodyweight (A, B) and of total worm count and female worm count with EEC (C, D) at 12 weeks post infection (WPI) (n = 149).

7.5. Discussion

A major effect of *A. galli* egg source on egg quality in this study was on embryonation capacity, with eggs isolated from worms cultured in artificial media and excreta having almost double the embryonation capacity of eggs extracted from worm uteri. However, numerous post-embryonation effects on infectivity were observed following inoculation of birds using the same doses of embryonated eggs obtained from each source. Inoculation with embryonated eggs originating from worm uteri and worms cultured in artificial media resulted in significantly higher worm counts, worm establishment rates and EECs than seen in birds infected with embryonated eggs originating from excreta despite similar prevalence of infection. Trickle infection with 3 split doses resulted in significantly higher total worm burden and establishment rate than single infection. Source of infection and mode of infection did not affect chicken body weight. However, body weight of individual birds had a significant negative linear association with individual worm burden and excreta egg counts at 12 weeks post infection. These indicated a 4 g reduction in bodyweight at 12 weeks for every 100 eggs per gram of excreta and a 4.6 g reduction in bodyweight for every *A. galli* worm present. A significant positive linear association between worm burden and egg count was observed at 12 WPI sampling.

The first proposition that eggs isolated from fresh excreta and eggs shed by cultured worms in artificial media will have a higher embryonation percentage than uterine eggs was supported by the data. In the present study, almost double the embryonation rate was obtained from eggs isolated from worms cultured in artificial media and excreta than eggs obtained from worm uteri. This is in agreement with previous findings (Rahimian et al., 2016) who demonstrated that approximately 90% of excreta eggs are able to complete embryonation, whereas approximately 40% of uterine eggs completed embryonation when eggs were incubated in a potassium dichromate (0.1%) medium at 25°C for 4 weeks. In contrast, 95% embryonation was reported for eggs isolated in both excreta and worm uteri and subsequently incubated in vermiculite at 18°C for five weeks (Permin et al., 1997b). These authors also reported 41% embryonation when using eggs isolated from worm uteri and subsequently incubated in 0.1 N H_2SO_4 at 18°C for five weeks. This finding is also consistent with the

embryonation of eggs from worm uteri in the present study and indicative of the importance of the medium used for embryonation. On the other hand, when using *A. suum* eggs isolated from faeces and incubated in 0.1 N sulphuric acid, 1% formalin or water at 25°C for 5 weeks, 88, 85 and 91% developed to infective stages, respectively. However, eggs isolated from the distal 2.5-4 cm of the worm uterus and incubated in 0.1 N sulphuric acid, 1% formalin or water also showed a high development capacity with 95, 88 and 94% developing into infective stages, respectively (Oksanen et al., 1990). These results indicate that while harvesting of eggs from worm uteri may result in reduced development capacity, this is not necessarily the case and success may be influenced by the egg isolation procedure, embryonation media and temperature. Indeed, most experimental infection models with the large roundworm *A. galli* are performed on the basis of eggs recovered from the worms' uterus. This is the simplest procedure for obtaining large numbers of eggs in a clean suspension. As demonstrated by Ackert (1931) and Tiersch et al. (2013), eggs extracted from fully matured adult female worms or the first two-thirds of their uterus (proximal to the vagina) were found to be > 80% embryonated eggs. The very detailed study of Ackert (1931) found embryonation percentages of 0 %, 3 %, 65 %, 90 %, 98 % and 98 % of eggs recovered from the narrow part of the uterus distal to the vagina, or 10, 20, 30, 40 and 45 (at vagina) mm closer to the vagina respectively. However, the embryonation rate and infectivity of uterine eggs obtained is also significantly influenced by the embryonation media used (Permin et al., 1997b; Tiersch et al., 2013).

Female worms cultured in artificial media are also commonly used as a source of eggs as these are shed in large numbers in the first 2-3 days of culture (Feyera et al., 2020). In the present study, eggs oviposited *in vitro* had a similar embryonation ability to fully matured eggs in excreta as has been found in a previous study (Rahimian et al., 2016). Although using *A. galli* eggs recovered from worms cultured in artificial media and worm uteri is not always feasible given the absolute requirement of chicken necropsy, these approaches have other advantages including removing the risk of harvesting eggs of mixed helminth infections and reduced time and labour involved in the extraction of eggs, compared with extraction from excreta. Eggs isolated from worms cultured in artificial media, in particular have high morphological quality and embryonation capacity (Feyera et al., 2020; Feyera et al., 2021b) which were shown in the present study also have high infectivity. The final method of obtaining *A. galli* eggs is by

extraction from the excreta of infected chickens followed by cleaning and concentrating to obtain highly concentrated and refined egg suspensions. The main drawbacks of this procedure are the laborious extraction, washing and concentrating steps and the unsuitability of excreta material isolated from naturally infected animals due to the likelihood of mixed helminth infections. For example, the eggs of *H. gallinarum* are difficult to differentiate morphologically from those of *A. galli* eggs (Thienpont et al., 1986*)*. Despite these drawbacks, it can be a more efficient method of stock multiplication than sacrifice and worm harvest (Feyera et al., 2021b). For example, as described by Feyera et al. (2020), *in vitro* egg production per adult female *A. galli* harvested was 6,044 depending on worm size and maturity level. In the present worm count data, the total mean female worm count per bird infected with 300 eggs was 3.6 (table 3). The total egg yield at bird sacrifice at 12 WPI would thus be 21,758 eggs/bird or a 73-fold increase on the initial infecting dose. On the other hand, as to collection from excreta towards the end of the experiment, the birds would have been producing approximately140 g excreta per day (Daş et al., 2017). The average EEC from the 300 egg dose in trickle infected day old chickens at 12 weeks post-infection was 934 EPG. Total collection of excreta for a single day with an egg recovery rate with sugar solution of 68% (Feyera et al., 2022b) would be 88,917 eggs in a single day or a 296-fold recovery of the original infective dose daily.

The second hypothesis that infective capacity of embryonated eggs will vary with the source of eggs was supported by the data. Birds infected with equivalent doses of embryonated eggs from worm uteri and cultured worms in artificial media had significantly higher worm and egg counts compared with birds infected with eggs isolated from excreta. This finding is in agreement with a previous study (Permin et al., 1997b) which compared the infectivity of eggs isolated from excreta and worm uteri incubated in different embryonation media. These authors reported that eggs isolated from worm uteri and subsequently incubated in 0.1 N H_2SO_4, vermiculite, and 2% formalin at 18°C for five weeks respectively provided higher worm burdens compared to eggs isolated from excreta and embryonated in either formalin or vermiculite. The authors also highlighted that high embryonation rate may not ensure infectivity of *A. galli* eggs. This indicates that the conditions experienced by embryonating *A. galli* eggs can influence their ability to infect chickens even though the same dose of fully embryonated eggs was administered from each source. In

another study involving experimental inoculation of pigs with equal doses of fully embryonated *A. suum* eggs isolated from worm uteri or pig faeces, embryonated eggs originating from worm uteri provided numerically higher counts of white spots in the livers and larvae in the lungs 7 days post infection than eggs of faecal origin (Oksanen et al., 1990). These studies are consistent with the high infectivity of embryonated eggs originating from worm uteri in the current experiment. However, in critical experiments where each animal needs to be infected with a defined number of infective ascarid eggs, the eggs in some cultures are clumped in masses making accurate counting of them impossible (Riedel, 1947). This is supported by Jørgensen (1978) who stated that eggs isolated from worm uteri are very sticky and they tend to gather in clusters or the uterine tissue present in the suspension that may influence the precision of dose determinations.

The differences in infectivity of embryonated eggs from different sources highlights the established principle that a fully developed embryonated egg with an active embryo may not necessarily indicate the infectivity of the larva contained in it and the most reliable method to determine infectivity is to infect chickens (Ackert and Herrick, 1928; Elliott, 1954; Thapa et al., 2017a). Rahimian et al. (2016) observed that majority of excreta eggs attain full embryonation at least one week ahead of uterine eggs. If this occurred in the present experiment, it means that fully embryonated excreta eggs contained older larvae than those from worm uteri when inoculated. We have previously found that *in ovo* larval viability declines by a rate of 3.4% per week at incubation temperatures of 26˚C (Feyera et al., 2020). Hansen et al. (1956) indicated that exposure of eggs to reagents such sodium hydroxide (NaOH) and sodium hypochlorite (NaOCl) which are known de-shelling reagents (Elliott, 1954; Dick et al., 1973; Feyera et al., 2020) that remove the hard eggshell decreasing the infectivity of embryonated eggs by 70% compared with untreated embryonated eggs. It is possible that the salt solution used to recover eggs from excreta might have affected the integrity of *A. galli egg* shell in similar way and reduce infectivity. Indeed, sucrose rather than salt solutions for flotation and extraction of eggs are recommended for optimal infectivity (Gibbons et al., 2014).

The third hypothesis that trickle infection would result in higher establishment rate, worm burdens and EEC than bolus infection with the same total dose was supported

by the findings. Trickle infection increased total worm burden and establishment rate by 38% and EEC by 36% compared with single infection. Yazwinski et al. (2003) recommended that repeated low infection doses enhance infectivity compared to a single high-level dose because trickle-infected hosts develop immunity more gradually, resulting in reduced worm expulsion. In contrast, single high dose infected host may acquire strong immunity, resulting most worm expulsion. Likewise, Feyera et al. (2021b) demonstrated that establishment rate tends to be dose-dependent and is higher at low trickle doses compared with high doses. Indeed, administration of multiple dose for extended periods may diminish worm establishment rate due to either acquired immunity or induction of arrested development (Ferdushy et al., 2014). However, our results using trickle infection over a 1-week period indicate that it is likely better to induce artificial infection than a single dose by reducing the impact of dosing error at any individual dosing event.

Although a total of 95% of infected chickens harboured adult worms irrespective of the infective egg source, the overall worm establishment rate was 2.6%, similar to that of previous studies of Feyera et al. (2021b), Permin and Ranvig (2001) and Permin et al. (1997a) who reported establishment rates of 2.2, 2.4 and 2.9 % for infection doses of 300, 500 and 500 embryonated eggs, respectively. However, it was lower than the establishment rate of 14.2 % for an infection dose of 100 embryonated eggs reported by Permin et al. (1997a). As described by different researchers, *A. galli* establishment rate in chickens is infection dose dependent with a negative association. This may be explained by competition among larvae establishing themselves in the same predilection site (Ikeme, 1971a; Paterson and Viney, 2002) or by immune interactions between host and parasite (Sadun, 1949). For example, Ackert et al. (1931a) studied infections with doses of *A. galli* (syn. *A. lineata*) ranging from 25 to 500 eggs and found a gradually increasing establishment rate as the doses decreased, indicating a reverse dose dependency. Permin et al. (1997a) and (Feyera et al., 2021b) also demonstrated that establishment rate is inversely related to egg dose. In the latter study this was the case in naïve chickens infected in the first week of life indicating that parasite factors or host innate immune responses rather than acquired immunity are likely responsible for this effect. The comparatively low establishment rate observed in the current study could be attributed to the fact that the infected birds were necropsied only once at 12 WPI in the current study, at which point many worms may

have been expelled by the developing host immune response. Although histotrophic larvae in the tissue phase were not assessed at necropsy, it is unlikely that there would be many of these 12 weeks after infection and so not measuring them is unlikely to have had a bearing on the measured establishment rate. More accurate estimation of establishment rate would require earlier necropsy and worm and larval counts.

Birds infected with eggs isolated from excreta had significantly larger sized worms compared with other egg sources even after correcting for total worm count. The reasons for this are not clear. The average worm length of the infected birds in this study was in the range observed by others (Permin et al., 1997a; Gauly et al., 2005; Gauly et al., 2007; Daş et al., 2011d). Ackert et al. (1931a), Permin et al. (1997a) and Gauly et al. (2001a) reported that a tendency toward increased length of worms following chickens infected with the smallest doses of eggs. Similarly, Roberts (1937) observed that birds fed large number of eggs resulted in very few worms and the majority of these were very small in size. These results indicate that worm size has an inverse dose dependency.

EEC was positively correlated with worm burden at 12 WPI consistent with other studies finding an association between these measurements (Train and Hansen, 1968; Permin and Ranvig, 2001; Daş et al., 2011c; Daş et al., 2014; Thapa et al., 2015a; Daş et al., 2017; Feyera et al., 2021a; Feyera et al., 2021b). Given that eggs are only shed by mature female worms, EEC will most closely correlate with total worm count in studies such as the present one in which only a single species and age cohort of parasites (mature adults) are present at the time of sampling.

The fourth hypothesis that negative effect of worm infection on body weight will increase with the established worm burden in individual chickens was supported by the current findings. Body weight at 12 WPI was negatively associated with both worm burden and EEC at the same age. These effects were not large, but were highly significant and indicated a 4 g reduction in bodyweight at 12 weeks for every 100 EPG in excreta and a 4.6 g reduction in bodyweight for every *A. galli* worm present. The mean bird weight at this age was 1521 g so an EEC value of 1500 EPG could be expected to reduce weight by 60 g or just 3.9%. A negative association between EEC or worm burden and weigh has been found in some previous studies (Reid and

Carmon, 1958; Brewer and Edgar, 1971; Gauly et al., 2001a; Feyera et al., 2021b), but not others (Gauly et al., 2007; Sharma et al., 2018a). Dose-dependent reduction in growth rate with about 5% reduction at 10 WPI compared to uninfected birds was observed in a recent study by Feyera et al. (2021b). An early study indicated that in birds infected with 500-2000 embryonated *A. galli* eggs, there was a reduction in weight gain of 12-91g/week between 2 and 9 weeks post infection (Ackert and Herrick, 1928). Reid and Carmon (1958) reported that every worm caused a 4.6 g body weight reduction at 3 WPI a similar sized reduction to that seen in the present study after 12 weeks. Studies have shown that chickens infected with *A. galli* lost weight due to elevated nutrient requirement for health related defence reactions of the birds in the critical post-infectious days starting at week 4 post-infection (Daş et al., 2010). This is because infection with *A. galli* stimulates a strong immune response in chickens and develops histotrophic stages of the parasite during this period (Marcos-Atxutegi et al., 2009; Daş et al., 2017). Other recent studies have shown that immunosuppression reduced dose-dependent effects on body weight (Feyera et al., 2021b) potentially implicating the immune response in the pathogenesis of weight loss, as has been reported in ruminant species.

7.6. Conclusions

This study found that eggs isolated from worms cultured in artificial media and excreta had almost double the embryonation capacity of eggs extracted from worm uteri. However, following embryonation, eggs isolated from worms cultured in artificial media and worm uteri had greater infectivity capacity compared with eggs obtained from excreta. Thus, our results confirm that high embryonation rate may not necessarily translate into high infectivity of eggs. Trickle infection resulted in higher worm establishment rates than single infection. Combining the effects on embryonation and infectivity of embryonated eggs in this experiment, 100 eggs isolated from cultured worms, worm uteri or excreta could be expected to result in establishment of 2.7, 1.17 and 1.27 worms respectively using this infection model. These factors should be taken into account when considering artificial infection protocols for *A. galli*.

Higher Degree Research Thesis by Publication
University of New England

STATEMENT OF AUTHORS' CONTRIBUTION

We, the Research PhD candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated in the *Statement of Originality*.

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STATEMENT OF ORIGINALITY

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We, the Research PhD candidate and the candidate's Principal Supervisor, certify that the following text, figures and diagrams are the candidate's original work.

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Chapter 8. General discussion

Helminth infections are more common with the ongoing global increase in poultry production and the trend towards more extensive production systems. The main objective of the studies conducted in this thesis were to 1) provide compiled information about the status and trends of helminth infections in poultry operations worldwide 2) assess the prevalence and magnitude of helminth infection in commercial cage-free layer chickens in Australia 3) evaluate and optimise diagnostic tools for routine monitoring of nematode infections in chickens and; 4) optimise prolonged laboratory storage methods for both undeveloped and embryonated stages of nematode eggs and 5) evaluate the infectious capacity of *A. galli* eggs recovered from chicken excreta or mature the worm's uteri or worms cultured into artificial media and subjected to different extraction procedures.

In light of these objectives, the following general propositions were tested.

i. A systematic analysis of global data on the prevalence of helminth infection in free-range chickens will indicate high prevalence and magnitude of different helminth species with a generally increasing trend overtime.
ii. The cage-free production systems in Australia will have a high prevalence and magnitude of worm infection in laying chickens.
iii. The Mini-FLOTAC method would outperform the McMaster method for recovering chicken nematode eggs from egg spiked chicken excreta and field samples.
iv. *A. galli* egg storage at 4°C must be anaerobic to prolong survival in the unembryonated state and storage at 26°C must be aerobic to prolong survival in the embryonated state but bacterial degradation of eggs must be prevented with a preservative.
v. *A. galli* eggs isolated from excreta, worm uteri or worms cultured in artificial media may differ in their embryonation capacity as well as infectivity.

The results of the previous chapters provide evidence that the objectives were successfully met. The findings contribute to a better understanding of gastrointestinal helminths in chickens and their relevance to current production systems.

8.1. Helminth infections in chickens: Global and Australian perspective

Poultry are susceptible to a wide spectrum of gastrointestinal helminth parasites (Permin and Hansen, 1998). There is a growing relevance of these parasites due to the increased use of barn, free-range and organic egg production systems which facilitate the transmission cycle. Because of the continuous exposure to the excreta in which nematode eggs are shed and intermediate hosts where eggs are not infective stage (e.g. cestodes), cage-free layers housed are more susceptible to gastrointestinal worm infections, with a near-100% prevalence (Kaufmann et al., 2011b; Wuthijaree et al., 2017). Several studies have been published on the prevalence of helminth infections in chickens. However, to the best of our knowledge, no compiled worldwide prevalence data on helminth infection in chickens under different production systems is available to date. Despite the ongoing trend of cage-free egg production in Australia, the status of gastrointestinal worm infection in Australian poultry production systems was last investigated more than 80 years ago.

8.1.1. The status and trends of helminth infections in poultry operations worldwide

The first general proposition of this thesis that a systematic analysis of global data on the prevalence of helminth infection in free-range chickens would indicate a relatively high prevalence with a generally increasing trend overtime was supported by the findings in Chapter 3. Our systematic review revealed that chicken kept in backyard and free-range systems had a markedly higher pooled prevalence of helminth infection rates (82.6 and 84.8%, respectively) than those housed in cage production systems (63.6%). The reported prevalence of helminth infection decreased over time in developing countries while it increased in the developed world. Although developing

countries had a high pooled helminth prevalence associated with the backyard production system and environmental factors, the prevalence was decreasing over time in these countries. One of the potential reasons for this might be that developing countries may have gained increased access to anthelmintic treatment over the last few decades. Another possible reason is that these countries' intensive production systems have recently expanded with improved management systems, which may reduce helminth infection. However, the current review found a high prevalence (78.9 %) across European countries. This might be most likely associated with increased free-range and organic production systems resulting re-emerging helminth infections (Jansson et al., 2010; Thapa et al., 2015). Furthermore, anthelmintic use has been prohibited in organic production systems in Europe, further promoting the occurrence of helminth infections (Kaufmann et al., 2011a, 2011b; Wongrak et al., 2015a).

8.1.2. Prevalence, spectrum, and magnitude of helminth infection on Australian cage- free layer farms

The second general proposition of this thesis that the cage-free production systems in Australia will have a high prevalence and magnitude of worm infection in laying chickens was supported by the findings in chapter 4. The findings of chapter 4 showed that overall the 4 cage-free egg farms in this study experienced a high prevalence (92.1%) and magnitude of worm infection (71 worms/hen) based on post-mortem examination. The prevalence of helminth infections with *H. gallinarum*, *A. galli*, *Capillaria spp.* and *cestode spp.* was 88, 82, 35 and 12% respectively with an overall prevalence ranging from 73 to 100% across farms. The highest mean worm burden per hen was *H. gallinarum* (45.5) followed by *A. galli* (22.0), and *Capillaria spp.* (2.72). This is in close agreement with the prevalence in chickens in Queensland in 1942 (Broadbent, 1942) and the reported prevalence in other international studies (Abebe et al., 1997; Kaufmann and Gauly, 2009; Kaufmann et al., 2011b; Ferdushy et al., 2016; Wuthijaree et al., 2017). Furthermore, prevalence based on excreta examination provided a very similar picture with high prevalence (74.8%) of ascarid infections and lower prevalence of infection with *Capillaria spp*. (27.3%). Ascarid eggs (*A. galli* /*H. gallinarum*) were found in all of the flocks examined. It can be inferred that the ascarid eggs observed in the intestinal excreta (71%) are predominantly those of *A. galli* while

those in the caecal samples (78%) are predominantly of *H. gallinarum* (Das¸ and Gauly, 2014a; Cupo and Beckstead, 2019). The complete absence of capillaria eggs in the submitted caecal samples supports the clear separation of the origins of these two excreta types. Further validation of the separate analysis of intestinal and caecal samples to identify and quantify infection with *A. galli* and *H. gallinarum* respectively would be useful. The possible explanation of the high prevalence and intensity of nematode infections in the present study is due not only to the fact that cage-free-production systems have poor biosecurity capabilities, but also to the unique properties of these production systems, which appear to provide favourable conditions for helminth infections (Permin et al., 1999). For example, free-range chickens are in direct contact with excreta and intermediate host, allowing the parasite to complete their life cycles. Furthermore, parasite eggs are able to remain viable for several months in external environment and thus hens are at high risk of infection (Thapa et al., 2017a).

A high worm burden is likely to cause direct and indirect production loss. The mean worm burden of nematode infection (e.g. *H. gallinarum*, *A. galli,* and *Capillaria spp.*) in the current study was 70 worms/bird ranging up to 540 worms/hen. This is likely to have had a negative impact on production performance based on the findings of other studies. For example, a significant reduction of laying performance, feed conversion ratio, egg weight and daily egg mass by 8, 4, 2 and 9% respectively was observed in laying hens infected with 13–136 worm/hen of mixed nematode infection (Stehr et al., 2019a). Layer hens infected with *A. galli* (10 worms/bird) lost an average weight 94 g compared with control birds during 7-10 week post infection but this effect was diminished later (Kilpinen et al., 2005). Furthermore, worm burden and body weight gain are negatively correlated, as demonstrated by the findings of chapter 7, which indicated a 4.6 g reduction in bodyweight for every *A. galli* worm present. This is consistent with the earlier study reported that every worm caused a 4.6 g body weight reduction at 3 week post infection (Reid and Carmon, 1958). Other study reported that non-treated chickens (mean burden of 40 worms/hen) showed a 3% lower body weight gain when compared to levamisole treated chickens (Permin, 2020). However, other studies have found associations between reduced weight and the number of adult worms in the intestine (Ikeme, 1971b) with the magnitude of growth depression ranging from zero to significant reduction (Chubb and Wakelin, 1963). Helminth

infections in chicken are also costly, due to expenses for treatment and other direct or indirect losses caused by reduced performance and death of the hen in severe infections (Ruhnke, 2015b). Effective registered anthelminthic are few in Australia and internal parasites represent a challenge to the welfare of free-range flocks (Groves, 2021). This is even more problematic in organic production systems, as none of the effective treatments can be used. Induction of resistance in the resident ascarid population could be expected in farms where these anthelmintic have been used extensively. However, a recent study found no anthelminthic resistance on five free-range layer farms in Australia (Feyera et al., 2022a; 2021a; 2021c), but this is insufficient to draw this conclusion. Although targeted anthelmintic treatment may reduce the negative impact of parasite infections, accurate and practical field diagnostic tests are required to effectively monitor and control worm infection.

Given the high infection pressure in free-range systems, adequately control of helminth can only be achieved through a combination of different strategies. Thus, regular diagnosis and anthelmintic treatment in combination with other preventive measures e.g., biosecurity, disinfection and segregation of birds by age groups or quarantine the birds for the concerned parasite prepatent period and then examine multiple faecal samples, are the fundamental principles of the effective control of roundworms in poultry (Tarbiat et al., 2016). Different management strategies have been suggested to enhance parasite control in poultry farms mainly by targeting free-living stages to interrupt the parasite life cycle. This could be achieved by implementation of strategies that may reduce bird contact with the intermediate host or mechanical vector, or decrease contact with the source of contamination, such as excreta (Ruff, 1999). Placing chickens on slatted wire or plastic material above the ground allowing the excreta to pass through is a highly effective husbandry method for limiting or eliminating helminth infections by preventing faecal oral cycling (Permin and Hansen, 1998; Ruff, 1999; Jansson et al., 2010; Höglund and Jansson, 2011). Control of intermediate hosts by application of approved insecticides to soil and litter when premises are unoccupied can interrupt the life cycle of some cestodes (Macklin, 2013). Ability for total confinement in cage systems means that better cleaning and disinfection procedures can apparently be achieved in these systems as opposed to the free-range scenario (Permin et al., 1999; Jansson et al., 2010).

The current study's findings were based on a prevalence data of physical survey of 4 egg farms and, worm or egg counts from 16 farms/flocks and may not accurately reflect the general field prevalence. Therefore, extensive prevalence surveys based on systematic sampling of representative farms are needed on a wider range of poultry flocks across Australia.

8.2. The performance of MM and MF methods for the diagnosis of chicken nematode infections

Quantitative EEC techniques are useful tools for estimating the intensity of nematode infection in live birds to guide treatment decisions or for epidemiological survey.
The third general proposition of this thesis that the MF method would outperform the MM method for recovering of chicken nematode eggs from egg spiked chicken excreta and field samples was not supported by the findings in chapter 5. The finding of this chapter demonstrated that MM had recovered a greater number of nematode eggs overall compared to MF. The egg recovery rate MM is linearly increasing with increasing EPG levels or infection levels. However, the MF method not only underestimated EEC overall, but the underestimation also increased as either EPG levels of infection levels increased. This is consistent with the earlier studies (de Castro et al., 2017; Bortoluzzi et al., 2018; Nápravníková et al., 2019; Daş et al., 2020). However, MF was more sensitive than MM at low EPG levels (< 50 EPG). This is not surprising given the differences in minimum detection limit of the two EEC techniques. Sensitivity is directly related to the minimum detection level of an EEC technique, and is of particular importance for diagnostic purposes when very low number of eggs is present in excreta sample, e.g. to certify animals as free of infection. This is similar to the interpretation of Nielsen (2021) who stated that sensitivity only has implications at low egg count levels. The MF had also greater overall precision than the MM method; however, both methods showed egg count variability, particularly at lower EPG levels. Our observation is consistent with the earlier studies that the estimation of precision tends to depend on the EEC level with lower precision demonstrated at lower counts (N´apravníkov´a et al., 2019; Das¸ et al., 2020; Cain et al., 2020, 2021). Precision is especially important in faecal egg count reduction testing;

however, a highly sensitive or precise EEC technique does not guarantee high accuracy, which in this study differed markedly between the two methods.

The majority of chickens in cage-free systems are highly infected with nematodes, particularly, *A. galli* and *H. gallinarum* coexist in the same host as reported in chapter 4 and other studies (Kaufmann et al., 2011a; Thapa et al., 2015; Wongrak et al., 2015a, b). Thus, the most difficult challenge is not identifying infected animals, which can be done using either serological methods (Daş et al., 2017; Sharma et al., 2018; Stehr et al., 2019), or simple qualitative coprological methods, but accurately estimating the intensity of infection using EEC method. For example, mean EEC of ascarid infection in cage-free systems reported in chapter 4 was approximately 400 EPG that is ten-fold higher than the minimum detection limit of MM method (40 EPG) employed in both chapter 4 and 5. This implies that a less sensitive EEC method can even detect most of the infected chickens accurately. Thus, rather than EEC techniques with high sensitivity but low accuracy, EEC techniques with high accuracy should be used to quantify nematode infections in chickens. In addition, the sensitivity and the precision of MM can be improved by increasing the amount of excreta suspension examined up to 1.5 mL (Cringoli et al., 2004). This is in line with the interpretations of literature data by Lester and Matthews (2014), Ballweber et al. (2014) and Das¸ et al. (2020) that performance of an EEC technique will increase as the amount of faecal material or the volume of suspension examined increases.

Both EEC methods were also tested with pooled and individual naturally infected excreta samples to determine which samples from farmers should be submitted to a laboratory. The finding showed that the MM method resulted in significantly higher EPG counts than MF. No significant differences in EEC were seen between pooled and individual fresh excreta samples indicating that both samples could be used as a representative for sample submission but pooled fresh excreta is more convenient sample submission in terms labour, cost and ease of sample collection or transportation to the laboratory from farmers. Thus, pooled fresh floor excreta samples would be sufficient to indicate infection level in free range farms. However, Both MM and MF underestimated the true EEC overall so automated egg counting method need to be tested for chicken nematode eggs. However, identifying *A. galli* and *H. gallinarum* eggs in the same host excreta using the EEC method to accurately estimate infection

intensity remains a challenge because their eggs are morphologically similar, making error more likely. Therefore, non-invasive advanced molecular assay should be required and recently, Tarbiat et al. (2021) have developed a novel duplex Droplet Digital™ PCR (ddPCR) assay to overcome this challenge although this technology comes at formidable cost per test. However, analysing caecal and intestinal samples separately can reduce the likelihood of errors in distinguishing *A. galli* and *H. gallinarum* eggs in the same host and provide an accurate estimate of infection intensity. Overall, the finding of this thesis suggest that the higher accuracy and the much shorter sample processing time of the MM method makes this method more suitable for routine and rapid diagnosis of chicken nematodes in the field, and thus this method was used for the remainder of this thesis experimental study.

8.3. Worm egg recovery, storage and propagation in chicken

For *A. galli* infection models, eggs are most commonly recovered from excreta (Rahimian et al., 2016; Daş et al., 2020), worm uteri (Permin et al., 1997a; Daş et al., 2010) or worms cultured in artificial media (Ruhnke et al., 2017; Sharma et al., 2018a, 2018b; Feyera et al., 2020). It is not known whether eggs from these different sources have the same infective capacity. Laboratory storage techniques that maximise infectivity potential and the period that samples can be stored while retaining infection are extremely important to minimise the labour and financial costs associated with obtaining parasite stages for routine experimental purposes. It is also useful for assisting in the preservation of reference parasite strains with specific characteristics. In *vivo* and *in vitro* studies on the chicken nematode *A. galli* frequently require the use of eggs in the infective stage. The viability and embryonation capacity of *A. galli* eggs determine the reliability of any artificial *A. galli* infection that requires the incubation and development of sufficient number of eggs outside of the chicken host (Ackert and Herrick, 1928; Elliott, 1954). The findings of this thesis also demonstrated that eggs of chicken nematodes, *A. galli*, for experimental purposes can be isolated effectively from the three main sources including excreta (Chapter 5, 6, 7), worm uteri (Chapter 7) and worms cultured in artificial media (Chapter 4, 7).

8.3.1. The combined effect of aeration, storage temperature and media for prolonged eggs storage prior to *in vivo* and *in vitro* experiments

The fourth general proposition of this thesis that *A. galli* egg storage at 4°C must be anaerobic to prolong survival in the unembryonated state and storage at 26°C must be aerobic to prolong survival in the embryonated state but bacterial degradation of eggs must be prevented with a preservative was supported by the findings in chapter 6. The findings of this chapter demonstrated that viability was best maintained during storage of eggs in 0.1 N H_2SO_4 or 2% formalin as the storage medium at either 4°C under anaerobic conditions which maintained the eggs in the unembryonated state, or at 26°C under aerobic conditions which allowed embryonation and maintenance of the eggs in the embryonated state. Lack of oxygen can inhibit egg development in many nematode species, and it appears that storage at 4°C under anaerobic conditions maintains the viability of unembryonated eggs for up to 45 weeks. This is most likely by slowing down metabolic processes that maintains viability of eggs for long storage period (Ackert and Cauthen, 1931b). Our findings are consistent with previous reports of different host parasite species (Rinaldi et al., 2011; Sengupta et al., 2016; Tarbiat et al., 2018) that when eggs were stored at 4°C under anaerobic conditions for shorter periods of time than in the current experiment. Under anoxic conditions, nematode eggs may enter the dormant phase (anoxybiosis) in order to reduce their metabolic requirements while remaining viable by suppressing their development, with egg development resuming when exposed to oxygen (Gaasenbeek and Borgsteede, 1998; Saunders et al., 2000). Oxygen is thus an essential requirement for embryonation to occur and appears necessary to maintain optimal viability at embryonation temperatures. The combination of 0.1 N H_2SO_4 or 2% formalin with anaerobic conditions at 4°C or aerobic conditions at 26°C retained up to 72% overall viability at 20 weeks storage time, with a decline rate of approximately 2% per week. At this rate of decline, eggs appear to be viable for up to 40-45 weeks when stored at 26°C under aerobic conditions and at 4°C under anaerobic conditions. Our observation is consistent with reports from earlier studies that *A. galli* eggs stored aerobically in water for 36 weeks at 28°C were viable and infective to chickens (Elliott, 1954). The 2% mortality rate/week under aerobic storage at 26°C in either 2% formalin or 0.1 N H_2SO_4 was consistent with the previous study that the mortality rate of egg viability at 26°C

under aerobic conditions in 0.1 N H_2SO_4 was 2.0% at the end of incubation periods of 2, 4 and 6 weeks (Feyera et al., 2020). In contrast, other studies demonstrated that eggs stored in either of excreta or water under anaerobic conditions at 4°C the embryonation capacity lost by only about 0.15% per week over a 72-day storage period (Tarbiat et al., 2018). The reasons for the differences in persistence of viability between experiments may be associated with the ability to achieve completely anaerobic conditions or methods of assessing egg viability. However, the combination of anaerobic storage at 26°C with any storage medium did not retain viability by 12 weeks and by 4 weeks when stored in water at this combination. This finding agreed with previous research that *A. galli* eggs stored anaerobically at 25°C in water or excreta were not viable after 14 and 42 days, respectively (Tarbiat et al., 2018). Possible reason for this observation could be that *A. galli* eggs kept at room temperature have active metabolism which requires oxygen to proceed with the embryonation process. Deprivation of oxygen in metabolically active eggs may speculatively interfere with embryonation and can be lethal.

The data from chapter 6 showed that the viability of stored eggs declined at different rates after storage of unembryonated eggs at 4°C under anaerobic or aerobic conditions with a 2.4 and 4.5% rate of loss of viability observed during storage period, respectively. Storage of fresh unembryonated eggs at 4°C under aerobic condition caused high rate of loss in viability as a function of the refrigeration period. The main adverse effect of such storage was the death of the eggs prior to development. Therefore, when storing unembryonated *A. galli* eggs at low temperature (4°C), aerobic conditions are unfavourable resulting a loss of viability of 4.5% per week with 10% viability retained after 20 weeks of storage. Similar rates of decline in embryonation capacity have been demonstrated for *A. galli* eggs stored at 4°C under aerobic conditions with approximately 6.0% reported by Feyera et al. (2020) and 4.4% per week by Tarbiat et al. (2018). The combined results of the present study and those of others suggest that under anaerobic conditions, storage of unembryonated state at 4°C maximises the duration of viability, whereas storage at 4°C under aerobic conditions significantly reduces viability duration at least by two-fold that of anaerobic storage. In contrast, the current results confirm the absolute requirement of embryonated eggs for aerobic conditions for prolonged storage at embryonation temperatures at 26°C but preservatives are required to prevent putrefaction of the

parasite eggs and inhibit the fungus and bacterial growth. While storage at 4˚C may seem ideal, achieving strict anaerobic conditions is difficult and a 2 week embryonation period post storage is required before infectivity is attained, so storage at 26˚C or room temperature aerobically may be preferred for simplicity. Further study is merited to determine if morphologically viable embryonated *A. galli* eggs stored aerobically at room temperature for prolonged period retain infectivity to chicken, or whether viability can be prolonged by allowing embryonation at 26˚C then storing at a somewhat lower temperature ≤ 20˚C.

8.3.2. The effect of egg source on embryonation and infection establishment

The final general proposition of this thesis that *A. galli* eggs recovered from excreta, worm uteri or shed by worms cultured in artificial media may differ in their embryonation capacity as well as infectivity was supported by the findings in chapter 7. The results of this chapter showed that eggs obtained from cultured worms or excreta exhibited a higher embryonation capacity than eggs obtained from worm uteri. This is not surprising as eggs from the former two sources represent mature eggs shed by worms whereas the latter source would include eggs at different stages of development, including immature eggs. Our observation is consistent with reports from earlier studies that eggs obtained by physical disruption of worm uteri are known for their relatively low embryonation potential due to maturity differences (Rahimian et al., 2016). The high level of embryonation of eggs from excreta in the present study is also broadly agreement with findings of these authors. Another study reported that eggs originated from worm uteri incubated in 0.1 N H_2SO_4 at room temperature for five weeks yielded a relatively low (41%) embryonation (Permin et al. 1997b). This finding is equivalent with the embryonation of eggs from worm uteri in the current study. However, these authors also reported that the only 26% of eggs isolated from worm uterine embryonated after being incubated in 2% formalin indicating the influence of embryonation media. Our finding that a high proportion of eggs derived from excreta and worms cultured in artificial media embryonated after five week storage and incubation periods (approximately 80%) supports the observation that mature female worms lay mature eggs capable of completing development (Kim et al., 2012). Regarding stock multiplication, periodic total excreta collection and egg

separation from the excreta would be more efficient given the high fecundity of the *A. galli* parasite (Wongrak et al., 2015b). Separation of eggs from the excreta may not be required prior to storage as the study of Tarbiat et al. (2018), and the findings of chapter 6 showed that the viability of eggs can be retained for at least 45 week at 4˚C under strictly anaerobic conditions in 0.1 N H_2SO_4. This indicated that the combination of 0.1 N H_2SO_4 and excreta at 4˚C under restrict anaerobic conditions will effectively maintain the viability of eggs for the longest time in the laboratory.

The general proposition was also supported by the findings regarding infectivity of eggs originating from different sources. Birds infected with similar doses of embryonated eggs from worm uteri and cultured worms in artificial media had significantly higher worm and egg counts compared with birds infected with embryonated eggs originating from excreta. The current finding is in agreement with a previous study (Permin et al., 1997b) that reported that eggs originated from worm uteri and subsequently incubated in 0.1 N H_2SO_4, vermiculite, and 2 % formalin at 18˚C for five weeks respectively provided higher worm burdens compared to eggs originated from excreta. Similarly, in experimental ascariasis in pigs involving eggs of faecal and uterine origin, a higher rate of infection was observed using eggs of uterine origin (Oksanen et al., 1990). These studies appear to highlight that not only embryonation media, but also isolation procedure and incubation temperature, play a considerable role in the embryonation and infectivity of uterine eggs. It has also been stated that a high embryonation rate does not guarantee nematode egg infectivity, implying that the conditions undergone by embryonating eggs can influence their ability to infect chickens even when the same dose of fully embryonated eggs was administered from each source (Permin et al., 1997b; Tiersch et al., 2013). Furthermore, the saturated salt solutions used to float eggs originated from excreta may cause significant distortion of eggs particularly at high specific gravities (David and Lindquist, 1982; Ballweber et al., 2014), thus affecting the infectivity capacity of excreta eggs. The differences in infectivity of embryonated eggs from different sources thus highlight the established principle that a fully developed embryonated egg with an active embryo does not always indicate the infectivity of the larva contained within it, and that infecting chickens is the most reliable method of determining infectivity (Ackert and Herrick, 1928; Elliott, 1954; Thapa et al., 2017). Eggs recovered form worm uteri and worms cultured in artificial media have removed the risk of harvesting

eggs of mixed nematode infections and reduced time and labour involved in the extraction of eggs compared with extraction from excreta. Eggs recovered by physical disruption of worm uteri are known for their relatively low embryonation potential due to maturity differences. Recovery of egg from female worms maintained in artificial media is feasible in terms of collecting mature eggs of high viability but only a moderate number of eggs could be isolated per female worm (Feyera et al., 2020). However, these two approaches are not always feasible given the requirement of chicken necropsy to recover mature worms. On the other hand, egg extraction from excreta collected from chickens harbouring specific nematode infection can also serve as source of eggs. This technique involves laborious egg extraction procedure and laboratory work. Furthermore, the saturated salt solutions used to float eggs may cause eggs damage (David and Lindquist, 1982; Ballweber et al., 2014), thus affecting subsequent embryonation capacity. Despite these drawbacks, recovering eggs from excreta can be a more efficient method of stock multiplication than sacrifice and worm harvest.

The percentage of administered or ingested eggs that turn in to worms (i.e. overall worm establishment rate; 2.6%) was similar to that of previous studies of Feyera et al. (2021b), Permin and Ranvig (2001) and Permin et al. (1997a) who reported establishment rates of 2.2, 2.4 and 2.9% for infection doses of 300, 500 and 500 embryonated eggs, respectively. However, it should be noted that the levels of infection observed in this study (7.7 *A. galli* worms/bird) in birds infected with an infection dose of 300 embryonated eggs tended to be lower than those seen in naturally infected free-range hens in chapter 4 (22 *A. galli* worms/hen). It is suggested that naturally infected birds with low continuous infection levels develop immunity more gradually, resulting in partial or less worm expulsion (Sadun, 1948). In contrast, in induced infections with infective eggs are artificially concentrated, incubated, stored, or inoculated. It is unknown whether these artificial manipulations have an impact on subsequent establishment rate and worm development when compared to natural infection. Nonetheless, several studies indicated that two host factors appear to have an impact on *A. galli* infection levels in chickens. One difference is that young chickens less than 12 weeks old are more susceptible to infection than adult chickens (Magwisha et al., 2002; McDougald, 2020), as the worms establish and mature more rapidly (Ackert et al., 1935; Kerr, 1955). Second, even though it is generally agreed

that naiive chickens older than 12 weeks of age develop a stronger resistance against *A. galli* (Ackert and Herrick, 1928; Ackert et al., 1935a; Kerr, 1955), laying hens are more susceptible to infection especially during the period of lay when host protective immunity might be weaker (Daş et al., 2018). This may in part explain the higher numbers of *A. galli* worms in naturally acquired infections in laying hens in addition to continuous exposure to small infection doses which would enhance establishment rate (Yazwinski et al., 2003). Furthermore, in the current study, the birds were kept in cages separated from their excreta, reducing the possibility of re-infection and thus low worm burden compared to the situation in natural infection. Therefore, the absence of continuous re-infection in the current infection model makes it suitable for worm propagation studies. In the current study, trickle infection resulted in a higher worm burden when compared to single infection at similar doses, indicating that trickle infection mimicked the kinetics of natural infections and can be considered an artificial infection model for *A. galli.*

Taken as a whole, this thesis will contribute significant scientific knowledge in the field of poultry parasitology study. The outcomes of chapters 3 and 4 are, for example, important for policymakers, poultry farmers, epidemiologists, and other beneficiaries in developing strategically sound poultry health interventions and control. According to the findings of Chapter 5, MF appears to be unimportant in field diagnosis where birds are predicted to have a large worm burden and thus EPG, and it is also labour expensive, whereas the MM approach is more ideal for rapid detection of chicken nematodes in the field. However, MF is more significant when the severity of worm infection is considered to be low, as well as for post-treatment worm screening during anthelmintic effectiveness trials. The results of chapters 6 and 7 will be useful for future poultry parasitology research, particularly *in vivo* or *intro* experimental studies focusing on the biology and population dynamic of nematodes, and anthelmintic resistance test that will contribute scientific knowledge in the understanding and in the tackling of helminth infection in poultry.

8.4. Conclusions and practical implications

The main conclusions and implications of the work of this thesis are summarized below:

1. The pooled prevalence of helminth infection in chicken kept in backyard and free-range systems was considerably high with the prevalence decreasing over time in developing countries while increasing in developed countries.
    1.1. This could point to the need for more stringent control and prevention measures in free-range and backyard production systems, such as regular deworming and access to early and accurate diagnosis, which would allow for early intervention.
2. In Australia, laying chicken in cage-free systems are infected with a high prevalence and magnitude of nematode infections that are likely to have a negative effect on production performance and welfare.
3. A strong association between EEC and adult *A. galli* worm burdens was found in this study.
    3.1. The association was weaker when mixed infections with other species were found. EECs can be a useful measure of worm burdens when the distinctive eggs of *Capillaria* spp. are always counted separately from ascarid eggs (eggs of *A. galli* and *H. gallinarum* are difficult to differentiate).
    3.2. Analysing caecal and intestinal samples separately would enable to provide a clearer picture over the infection dynamics and epidemiology of the two nematode parasites *(A. galli* and *H. gallinarum).*
4. MM appears to be more appropriate than MF for routine and rapid diagnosis of chicken nematodes in the field.
    4.1. MM has a relatively higher egg recovery capacity and requires significantly less time to process samples than the MF method.
    4.2. MM is the preferred method for evaluating EEC in the field in poultry flocks where treatment decisions are required.
    4.3. MF to be the preferred method for diagnosing very low nematode burdens or certifying infection freedom.
5. Prolonged storage of *A. galli* eggs depends not only on the combination of oxygen availability and temperature but also on storage media and the egg development stage targeted.

5.1. The maintenance of viability during storage at 4°C was optimal under anaerobic conditions while at 26°C it was optimal under aerobic conditions.

5.2. Long term storage of *A. galli* eggs is best achieved using 0.1 N H_2SO_4 as the storage medium and either anaerobic conditions at 4°C which will maintain the eggs in the unembryonated state, or aerobic conditions at 26°C for which will enable embryonation and maintenance of the eggs in the embryonated state.

5.3. Loss of viability of approximately 2%/week for 20 weeks can be expected during storage in 0.1 N H_2SO_4 under anaerobic conditions at 4°C or aerobic conditions at 26°C and if this linear rate of decline is maintained, storage for up to 45 weeks (11% remaining viability) can be expected, prior to needing to passage the eggs back through chickens.

5.4. Anaerobic conditions at 26°C led to a rapid loss of viability while aerobic conditions at 4°C had a less severe negative effect on maintenance of viability.

5.5. Storage of *A. galli* eggs in their unembryonated state under suboptimal conditions in water drastically reduces viability and developmental ability over time.

5.5.1. Eggs can only be stored in the unembryonated state at 4°C under anaerobic conditions are required to maximise viability.

5.6. Storage at 26°C or room temperature may be preferable but eggs will be in an embryonated state which cannot be used for assays requiring unembryonated eggs.

5.6.1. Morphologically normal embryonated eggs may not always bear viable larvae inside.

5.6.2. Prolonged storage under this condition my result in loss of infectivity over time

5.6.3. Preservatives such as sulphuric acid (0.1N) and formalin (2%) are needed to prevent fungal and bacterial growth.

5.6.4. A constant supply of oxygen and a relative humidity > 85% or storage in aqueous media should be maintained.

6. The embryonation and infective capacity of *A. galli* eggs varied depending on the source of the eggs isolated.

6.1. *A. galli* eggs isolated from worms cultured in artificial media and excreta had almost double the embryonation capacity of eggs extracted from worm uteri.
6.2. Following embryonation, eggs isolated from worms cultured in artificial media and worm uteri had greater infectivity compared with eggs obtained from excreta.
6.3. High embryonation rate may not necessarily translate into high infectivity of eggs.

7. Trickle infection resulted in higher worm establishment rates than single infection.

8.5. Limitations of the study and suggestions for future research

8.5.1. Limitation of the study

This study, like most experimental studies of this type, has limitations. The main limitations are summarised below:

1. The physical prevalence survey was assessed on only 4 farms which limits the implication of the findings for the whole sector. Travel restrictions during the COVID-19 pandemic severely restricted the number of farms able to be accessed in this study.
2. The low response rate to the online farmer survey reduced the value of the findings as a reflection of perceptions and practices in the wider industry.
3. Because larval recovery methods were not used in the physical prevalence study likely due to time and resource availability prevalence and worm burdens in relevant studies might have been partly underestimated.
4. Because of the similar anatomical structures of the tapeworms, accurate quantification of worm numbers in a given host may not always be accurate.
5. In chapter 5, different amounts of excreta used in both methods may have contributed a potential cofounding effect in the comparison of egg recovery rate between MF and MM methods.
6. In chapter 7, the infected birds were necropsied only once at 12 WPI, at which point many worms may have been expelled by the developing host immune response. Histotrophic larvae in the tissue phase were not assessed at an earlier necropsy that would allow for a more accurate estimation of establishment rate.

Serial necropsies are usually needed to have a more accurate pictures of infection dynamics in relation to time dependency.

7. The amount of excreta in 24 hours was not quantified for the EEC and worm burden correlation. The number of eggs shed per day may be a better predictor of worm burdens than EPG at a given time point, which is likely to be broadly reflective of total egg production over a given period.
8. Old papers usually do not have full text through online databases, thus they were not heavily included in the review paper on prevalence of chicken helminths. Therefore the literature review might have been partly biased toward recent papers.
9.

8.5.2. Future research

1. Extensive prevalence surveys possibly including molecular identification of various nematode and cestode spp. circulating in the sector are needed on a wider range of poultry flocks across Australia
2. Determine the effect of chicken excreta sample preservation on the accuracy of EEC techniques
3. Evaluate the effects of different flotation fluids on infectivity and viability of parasite egg.
4. Studies that clearly define worm count and EEC counts where productivity is compromised and treatment intervention is indicated.
5. Determining the extent to which morphologically viable embryonated *A. galli* eggs stored aerobically at room temperature in appropriate preservative media retain their infectivity for chickens over time.
6. Determine if storage of embryonated eggs can be extended by intermediate temperatures (10-20˚C) which would not be fatal to the embryo, but slow its metabolic rate.
7. Research is needed in chickens infected with only *A. galli* or *H. gallinarum* to determine the proportion of eggs shed in intestinal or caecal excreta by each species.
8. Research is needed to assess the diagnostic performance of the two methods (i.e. MF and MM) in anthelmintic efficacy test in poultry.

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